

The Rational Design and Use of Novel Small-Molecule Ice Recrystallization Inhibitors for the Cryopreservation of Hematopoietic Stem Cells and Red Blood Cells

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Dedicated to my love, Cédrick

*“What lies behind us and what lies ahead of us
are tiny matters compared to what lies within us”*

-Ralph Waldo Emerson

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Abstract

Over the past few decades, there has been an increase in the development of new cellular therapies used for the treatment of various conditions. For example, red blood cell (RBC) transfusions are used to treat patients with anemias, low oxygen carrying capacities resulting from extreme loss of blood (traumas or surgeries), and certain diseases and infections such as sickle cell disease and malaria. Hematopoietic stem cell (HSC) transplantations are used for the treatment of conditions such as leukemias, lymphomas, hematological diseases, anemias, and immunodeficiencies. Thus, the rapid development of therapies for the clinical treatment of diseases, particularly transfusion and transplantation, has resulted in an increasing need to preserve these cellular therapy products.

Cryopreservation is the only currently used method for the long-term storage of cells and tissues. At cryopreservation storage temperatures (typically between -80 °C and -196 °C), samples can be stored for extended periods of time when suspended in cryoprotectant solutions. Currently, the most commonly used cryoprotectants are 10% dimethyl sulfoxide (DMSO) for HSCs and 40% glycerol for RBCs. Unfortunately, DMSO fails to protect the functionality of HSCs after cryopreservation and as a result, up to 20% of HSC transplantations fail to engraft. The glycerol in thawed RBC units must be removed to <1% prior to transfusion to prevent intravascular hemolysis which is a time-consuming process and requires the use of specialized equipment. As such, there is an urgent need to develop improved cryoprotectants for HSCs and RBCs.

While DMSO and glycerol reduce cryo-injury and improve post-thaw recovery by diluting intracellular solutes and preserving cell membrane volumes, they are unable to control ice growth and recrystallization which is a major source of cellular injury during cryopreservation. Therefore, compounds with the ability to inhibit ice recrystallization could represent a new class of cryoprotectant with a novel mechanism of action. The development of new cryoprotectants that are non-toxic and capable of controlling ice recrystallization needs to be further investigated as they could dramatically improve current cryopreservation protocols.

This thesis focuses on the rational design of small-molecule ice recrystallization inhibitors. In particular, the key structural attributes required for ice recrystallization inhibition (IRI) activity are investigated. Furthermore, small-molecule IRIs are rationally designed to be less cytotoxic than previously discovered IRIs. This thesis also explores the use of small-molecule IRIs to improve the cryopreservation of HSCs and RBCs.

Previous structure-function analyses demonstrated that a particular balance between hydrophobicity and hydrophilicity was required for IRI activity; however, this amphiphilic balance had not been quantified in a meaningful way. This thesis explores the amphiphilic balance required for IRI activity. While this study determined that compounds with long alkyl chain lengths were highly IRI active, the surfactant nature of these molecules impeded their use in cellular applications. Therefore, this thesis also explores the impact of IRI activity upon replacement of the hydrophobic alkyl chain with aromatic rings. As a result, several very highly IRI active molecules were discovered. Importantly, these molecules were less cytotoxic than previously discovered IRI active small molecules.

Finally, this thesis explores the use of several IRI active small-molecules to improve the cryopreservation of various cell types. A number of *N*-aryl-aldonamides improved the post-thaw functionality of HSCs. The supplementation of the current cryoprotectant solution (10% DMSO) with small molecule IRIs resulted in an increase in CFU recovery and frequency of multipotent progenitors in UCB HSCs. This is significant because this would reduce the percentage of engraftment failure and allow for a larger proportion of cord blood banks' inventory to provide an adequate dose for patients requiring transplants. Several small molecule IRIs were also found to be effective cryoprotectants for RBCs with greatly reduced amounts of glycerol. This could dramatically reduce the deglycerolization time for RBCs post-thaw. These results demonstrate the potential of small-molecule IRIs to improve the current cryopreservation procedures for important cellular therapy products, such as HSCs and RBCs.

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List of Abbreviations

¹ H	Proton
¹³ C	Carbon
2,3-DPG	2,3-Diphosphoglycerate
7-AAD	7-aminoactinomycin D
Ac	Acetyl
ACN	Acetonitrile
ACP	Automated cell processor
AFGP(s)	Antifreeze glycoprotein(s)
AFP(s)	Antifreeze protein(s)
Ala	Alanine
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
APC	Allophycocyanin
AS-3	Additive solution – 3
ATP	Adenosine triphosphate
BA(s)	Biological antifreeze(s)
BFU-E	Blast-forming unit-erythroid
BM	Bone marrow
br	Broad
C-AFGP	C-linked antifreeze glycoprotein
CAM	Calcein acetoxymethyl
CBS	Canadian Blood Services
CD	Cluster of differentiation
CF	Carboxyfluorescein
CFU	Colony-forming unit
CFU-G	Colony-forming unit-granulocyte
CFU-GEMM	Colony-forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte
CFU-GM	Colony-forming unit-granulocyte, monocyte
CFU-M	Colony-forming unit-monocyte

CoMFA	Comparative molecular field analysis
CPA	Cryoprotective agent
CuAAC	Copper(I)-catalyzed Huisgen azide-alkyne cycloaddition
d	Doublet
dd	Doublet of doublets
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DRS	Domain recognition software
dt	Doublet of triplets
EI	Elongation index
ESI	Electrospray ionization
ESP	Electrostatic potential
EtOAc	Ethyl acetate
EtOH	Ethanol
Et ₂ O	Diethyl ether
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
GA	Genetic algorithm
Gal	Galactose
Glc	Glucose
GLUT(s)	Glucose transporter(s)
Glyc	Glycerol
GRIND	Grid-independent descriptors
GVHD	Graft-versus-host disease
$\hat{H}$	Hamiltonian operator
HBSS	Hank's balanced salt solution

Hct	Hematocrit
HCTU	1H-benzotriazolium 1-[bis(dimethyl-amino)methylene]-5-chloro-hexafluorophosphate (1-),3-oxide
HES	Hydroxyethyl starch
Hgb	Hemoglobin
HI	Hydration index
HLA	Human leukocyte antigen
HSC(s)	Hematopoietic stem cell(s)
HUVEC(s)	Human umbilical vein epithelial cell(s)
IC ₅₀	Half maximal inhibitor concentration
I _h	Hexagonal ice
IIF	Intracellular ice formation
IMDM	Iscoe's modified Dulbecco's medium
I/R	Ischemia/reperfusion
IRI	Ice recrystallization inhibition
ISHAGE	International Society of Hematotherapy and Graft Engineering
kDa	Kilo Daltons
kg	Kilogram
LC	Leukocyte concentrate
LDA	Limiting dilution analysis
Log <i>P</i>	Partition coefficient
LORCA	Laser-assisted optical rotation cell analyzer
LRMS	Low resolution mass spectrometry
LTC-IC	Long-term culture initiating cell
m	Multiplet
MCV	Mean cell volume
MEM	Minimum essential media
MeOH	Methanol
MetHgb	Methemoglobin
MGS	Mean grain size

MHz	Mega Hertz
MMFF	Merck Molecular Force Field
mm Hg	Millimeter of mercury
MNC	mononuclear cell
mOsm	Milliosmoles
MP	Microparticle
MSA	Molecular surface area
MSC(s)	Mesenchymal stem cell(s)
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
m/z	Mass-to-charge ratio
NADH	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
p50	Partial pressure of oxygen when Hgb is 50% saturated
PB	Peripheral blood
PBMNC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PI	Propidium iodide
PLSR	Partial least squares regression
PO ₂	Partial pressure of oxygen
PS	Phosphatidylserine
PSA	Polar surface area
PVP	Polyvinyl pyrrolidone
q	Quartet
QC	Quality control
QLL	Quasi-liquid layer
QSAR	Quantitative structure activity relationship
quint	Quintet
RBC(s)	Red blood cell(s)
RI	Refractive index

ROS	Reactive oxygen species
RPMI	Rosewell Park Memorial Institute
s	Singlet
SAGM	Sodium, adenine, glucose, mannitol
SAR	Structure activity relationship
SD	Saline/dextrose
SEM	Standard error of the mean
t	Triplet
td	Triplet of doublets
TH	Thermal hysteresis
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TNC	Total nuclear cell
TW	Transient warming
TWE(s)	Transient warming event(s)
UCB	Umbilical cord blood
VEGF	Vascular endothelial growth factor
v/v	Volume/volume

Chapter 1: Cryopreservation and its shortcomings

1.1 Cell therapies and bio-banking

Cell therapy involves the injection or transplantation of cellular material into a patient in an attempt to prevent or treat an illness.¹ There are two main principles by which cellular material can facilitate therapeutic action. Firstly, injected cells can produce a therapeutic effect through the release of molecules such as cytokines, chemokines and growth factors which facilitate self-healing.¹⁻⁴ These therapeutic molecules are either secreted by the injected cells themselves or the injected cells stimulate surrounding cells to produce and secrete them.¹⁻⁴ For example, mesenchymal stem cells (MSCs) were shown to improve myocardial function after ischemia/reperfusion (I/R) injury.² This was attributed to the release of growth factors including vascular endothelial growth factor (VEGF).²

The second principle by which a therapeutic action can be induced by the injection of cells is through the replacement of damaged cells and tissues.^{1,5-7} One of the first examples of this type of cellular therapy includes the transfusion of red blood cells (RBCs).⁸ Transfusions of RBCs are used to treat patients with anemias, low oxygen carrying capacities resulting from extreme loss of blood (traumas or surgeries), and certain diseases and infections such as sickle cell disease and malaria.⁸ Other examples include injection of cardiomyocytes to replace damaged heart tissue after myocardial infarction^{6,7} and bone marrow transplants for the treatment of damaged knee cartilage.⁹ Stem cell therapy is also employed to regenerate and replace damaged tissue. For example, the use of hematopoietic stem cells (HSCs) has increased over the past 20 years for regenerative medicine therapies.¹⁰ Transplantation of HSCs has been used to treat many different conditions including leukemias, lymphomas, hematological diseases, anemias and immunodeficiencies.¹⁰

The increase in the development of new cellular therapies over the past few decades has resulted in an increasing need for methods to store cellular therapy products. A major challenge in this field is finding suitable, safe and ethical sources of cells and tissues for research and therapy.¹¹ The development

of biobanks - facilities that collect and store cells and tissues - has enabled access to cells and tissues for research and therapy but requires that many of these products be cryopreserved.¹¹

1.2 Cryopreservation

1.2.1 Clinical importance of cryopreservation

Over the past few decades, there has been an increase in the development of new cellular therapies used for the treatment of various conditions such as sickle cell disease, malaria, myocardial infarction and various cancers.^{6-8,10} Cellular therapy products include cellular immunotherapies and autologous (obtained from the same individual) or allogenic (obtained from a donor) cells, such as blood products or stem cells, for certain therapeutic indications.^{10,12} As described above, examples include RBC transfusions to treat patients with anemias, low oxygen carrying capacities resulting from extreme loss of blood (traumas or surgeries), and certain diseases and infections such as sickle cell disease and malaria as well as HSC transplantations for the treatment of conditions such as leukemias, lymphomas, hematological diseases, anemias and immunodeficiencies.^{8,10} Therefore, there has been an increasing need for methods to safely store cell therapy products like RBCs and HSCs. The ability to bank cells and tissues used in cellular therapy would allow sufficient time to perform human leukocyte antigen (HLA) typing and matching of recipients and donors, facilitate the transport of products between different transplant centers and hospitals, and screen for transmissible diseases.¹³ Thus, the rapid development of therapies for the clinical treatment of diseases, particularly transfusion and transplantation, has resulted in an increasing need to preserve these cellular therapy products. Maintaining cells and tissues in a resting state for future use has centered on three methods of preservation: (1) hypothermic storage, (2) cryopreservation and (3) vitrification.

1.2.1.1 Hypothermic storage

Hypothermic storage involves preserving cells and tissues at chilled temperatures ranging between 4 °C and 10 °C and is used for short term storage (days to weeks). It is commonly used when

shipment of a patient biopsy is required. Storing cells and tissues at hypothermic storage temperatures results in a suppression of biological activity reducing the usage of cellular metabolites.^{8,14,15}

Unfortunately, for many cell types, protocols for hypothermic storage have not been optimized. The reduction in cell viability and functionality after hypothermic storage is correlated to storage time and therefore it is better to use hypothermically stored materials as soon as possible.^{8,16–18} Thus, hypothermic storage is limiting due to the short shelf-life.

1.2.1.2 Cryopreservation

To overcome the limiting short shelf-life associated with hypothermic storage, cells and tissues can be stored in a frozen state. Cryopreservation is the storage of cells and tissues at low sub-zero temperatures, typically between -80 °C and -196 °C. At these temperatures, biological activity is effectively stopped and samples can be stored for extended periods of time (months to years). Cryopreservation protocols have been extensively studied and optimized depending on cell type. Cooling and warming rates and the cryoprotectant type and concentration are very important.^{8,15,19,20} During cryopreservation, cryo-injury (described in 1.2.2) is experienced as a result of the cooling, storage and thawing of the sample. Cooling and warming rates and cryoprotectants are exploited to minimize this damage. Unfortunately, current clinically-used cryoprotectants exhibit cytotoxicity. Furthermore, they do not control ice growth or recrystallization, which is a major cause of cryo-injury. The main mechanisms of cryo-injury, including ice recrystallization, are discussed in Section 1.2.2.

Cryopreservation typically involves the use of low sub-zero temperatures (-80 °C to -196 °C), however another method involves the use of high sub-zero temperatures (-5 °C to -30 °C), referred to as “high-temperature cryopreservation”.²¹ This cryopreservation technique was inspired by the survival of organisms, such as frogs, brine shrimp, bacteria and yeast, during extreme weather conditions, such as freezing and dehydration.^{22–25} These organisms survive sub-zero temperatures due to their ability to accumulate large amounts of intracellular and extracellular sugars, such as trehalose, sucrose, and/or glucose.^{22–25} Trehalose has been shown to stabilize and preserve cell structures during freezing and drying

due to its protective interaction with lipid membranes.^{26–31} As such, it has been used in preservation of various cells such as RBCs, platelets, oocytes and pancreatic islets.^{32–40} Several groups have introduced methods to internalize trehalose since mammalian cell membranes are practically impermeable to sugars.^{41–45} These include electroporation⁴⁶, microinjection³⁴, and osmotic and thermal shock⁴⁷. Eroglu *et al.* studied the survival of mouse oocytes after high-temperature cryopreservation with trehalose.²¹ They found that in the absence of intracellular and extracellular trehalose, only 9% of oocytes survived cooling to -15 °C.²¹ The presence of extracellular trehalose (0.5 M) improved the survival to 60% and remarkably, 90% survival after cooling to -15 °C was achieved with intracellular and extracellular trehalose.²¹ The major downfall for high-temperature cryopreservation is the need to internalize trehalose. Many methods to internalize trehalose result in uncontrolled introduction of trehalose which can cause excessive cellular damage.⁴⁰

1.2.1.3 Vitrification

Vitrification is a form of cryopreservation that avoids ice growth and recrystallization during the cooling process by employing ultra-fast cooling rates and high concentrations of cryoprotectants.^{15,48–52} This method requires sophisticated equipment in order to obtain sufficiently high cooling rates and results in patient side effects due to the high concentrations of cytotoxic cryoprotectants used. Furthermore, devitrification, or ice crystal formation, during warming is a significant problem.⁵³

Cryopreservation is the only method approved for the storage of cells and tissues allowing for long-term preservation, even with the limitations briefly described. Therefore, there is an urgent need to improve upon the current cryopreservation protocols.

1.2.2 Mechanisms of cryoinjury

There are three main steps in the cryopreservation process: (1) addition of a cryoprotective agent (CPA); (2) cooling the sample to sub-zero temperatures (-80 °C or -196 °C); (3) warming the sample; and in some instances a fourth step includes removal of the CPA.¹³ Cellular injury resulting in reduced

viability and/or functionality can occur at any of these steps. The length of time between the addition of the CPA and cooling of the sample is critical and is minimized due to the toxicity of the CPA. After thawing and prior to transfusion or transplantation, the CPA is removed in most cases in order to minimize the cytotoxic effects upon administration of the product. The process of CPA removal often involves a series of washing and centrifugation steps which result in a reduction in cell recovery.⁵⁴ Specific CPAs and their mechanisms of action are described in Section 1.2.3. The cellular injury that occurs during the freezing and thawing steps of cryopreservation is referred to as cryo-injury and is best explained by Mazur's two factor hypothesis.^{13,55}

1.2.2.1 Cryo-injury during cooling

As cells are cooled to -5 °C, the extracellular solution and cells are supercooled but remain unfrozen.^{13,55,56} Ice nucleates in the extracellular solution at some point between -5 °C and -15 °C, while the intracellular solution remains unfrozen. As extracellular ice forms, solutes are excluded from the ice. As a result, the solute concentration increases in the extracellular space causing water to flow out of the cell as the system attempts to attain equilibrium. The cryoinjury experienced subsequently depends on the freezing rate employed and is described by Mazur's two factor hypothesis.^{13,55} If the cells are cooled too quickly, there is not enough time for sufficient loss of intracellular water. As the temperature decreases rapidly, the intracellular water freezes and intracellular ice formation (IIF) occurs.⁵⁷ Cells inflicted with IIF often experience membrane damage.⁵⁸ Furthermore, recrystallization of intracellular ice during thawing can cause mechanical damage to the cells.⁵⁸ However, intracellular ice can be innocuous^{59,60} depending on the amount of ice formed⁶¹, the location of the intracellular ice⁶²⁻⁶⁴ and the mechanism of formation⁵⁹. Conversely, if cells are cooled too slowly, the cells become severely dehydrated. This severe loss of volume not only disrupts the membrane but exposes the cell to very high solute concentrations causing cell injury (termed "solution effects").^{55,65-67} Thus, cooling must be sufficiently slow in order to prevent IIF while fast enough to avoid excessive cell shrinkage and concentration of the intracellular solutes (Figure 1.1).

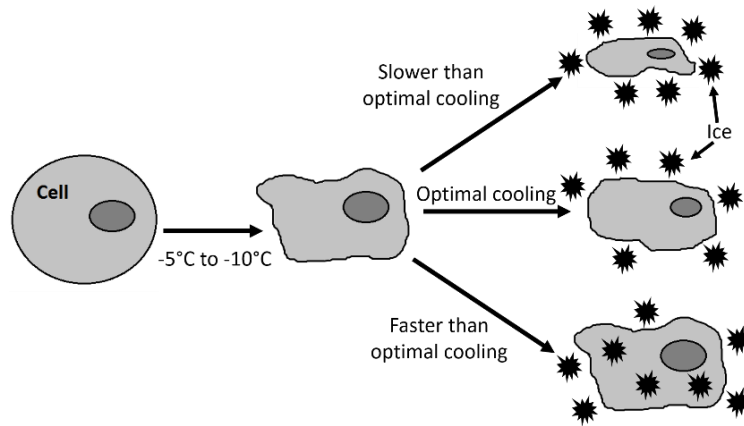


Figure 1.1. Schematic drawing of cells during freezing.¹³

There is an optimal cooling rate for each cell type which depends on the cells' membrane permeability to water and solutes. This was experimentally confirmed by Mazur in 1984 and the results are presented in Figure 1.2 as inverted "U-shape curves".⁶⁸ Cell survival as a function of cooling rate is plotted depicting decreased cell survival when cooling rates are faster or slower than optimal as a result of IIF or solution effects respectively.

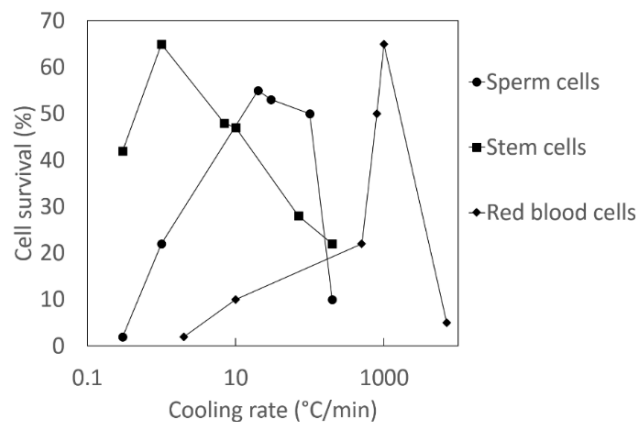


Figure 1.2. Survival of different cells at various cooling rates after freeze-thaw.⁶⁸

1.2.2.2 Cryo-injury during warming

The cryo-injury experienced during warming and thawing is a result of the recrystallization of ice.¹³ If the thawing rate is too slow, intra- and/or extracellular ice crystals will recrystallize into larger ones causing cell membranes to rupture and resulting in mechanical damage to the cells.^{13,69} Therefore, cells are typically subjected to very rapid thawing conditions in order to minimize this ice

recrystallization.⁶⁹ However, it is not possible to fully eliminate ice recrystallization and this type of cryo-injury has been correlated to reduced post-thaw viabilities in many cell types.^{61,62,68,70,71}

1.2.3 Cryoprotectants and their mechanisms of action

While reducing cryo-injury by employing optimal freezing and thawing rates is necessary, post-thaw cell viabilities are still reduced. As seen in Figure 1.2, even when using optimal freezing rates, cell survival is <70%.⁶⁸ Furthermore, optimal freezing rates may be impractical in a clinical setting; for example, the optimal freezing rate for RBCs is >1000 °C/min. To circumvent this, cryoprotective agents (CPAs) are utilized.

There are two classes of CPAs: permeating and non-permeating. Permeating cryoprotectants, such as dimethyl sulfoxide (DMSO) and glycerol, readily cross the cell membrane and protect cells from cryoinjury associated with slow cooling rates by preserving cell volumes and reducing “solution effects”.^{68,72,73} During slow cooling, cells experience lethally high solute concentrations. Penetrating CPAs effectively dilute these solutes and therefore reduce the temperature at which the critical salt concentration is reached. At the same time, penetrating CPAs prevent excessive cell shrinkage by replacing water within the cell.

Non-permeating cryoprotectants do not penetrate the cell membrane and function by stabilizing cell membranes.^{32,74–76} Examples of non-permeating cryoprotectants include hydroxyethyl starch (HES), polyvinyl pyrrolidone (PVP), polyethylene glycol (PEG), trehalose, sucrose and dextran. They are typically used when fast cooling rates are utilized because they increase the osmolality of the extracellular solution and accelerate dehydration.^{27,68,73}

1.2.4 Cryopreservation of hematopoietic stem cells

Hematopoietic stem cells (HSCs) are routinely used for regenerative therapies. HSC transplantation offers life-saving treatment for patients suffering from hematological cancers (leukemias and lymphomas), hematological diseases, anemias and immunodeficiencies.^{77,78} HSCs are identified as CD34-positive

(CD34+). CD34 is a stage-specific glycoprotein and identifies cells that are in the early stages of hematopoietic differentiation.⁷⁹ There are several sources of CD34+ cells including bone marrow (BM), peripheral blood (PB) and umbilical cord blood (UCB). PB contains the lowest percentage of HSCs with an average of 0.15% CD34+ cells.⁷⁹ UCB is considered an enriched source of HSCs, containing on average 8-10 times more CD34+ cells than PB.⁷⁹ BM contains the highest percentage of CD34+ cells (1.7%).⁷⁹ HSC transplantation from UCB offers advantages over treatments using BM and PB including reduced risk of graft-versus-host disease (GVHD), reduced risk of blood-borne infections, absence of risk to donor, and less stringent human leukocyte antigen (HLA) matching requirements.⁸⁰

In select situations, fresh stem cells can be used for allogenic transplants if the cells are transplanted within 72 hours of harvest.⁸¹ However, virtually all autologous and allogenic transplants require that the cells are cryopreserved.⁸¹ Cryopreservation is important for all stem cell types but is critical for the storage of HSCs from UCB because these cells are harvested from the donor at birth and required to be stored for an indeterminate period of time. Since the success of HSC transplantation is directly correlated to both the number and quality of cells transplanted, optimization of cryopreservation protocols for HSCs is an important issue for UCB banks.⁸²⁻⁸⁹

Clinical protocols for the cryopreservation of UCB HSCs use 10% dimethyl sulfoxide (DMSO) as the cryoprotectant solution.⁹⁰ While a 10% DMSO solution is very effective and maintains high levels of cell viability, there are significant drawbacks associated with the use of DMSO as a cryoprotectant. The most important is toxicity.^{81,91} DMSO has been shown to have adverse effects on the gastrointestinal, renal, hepatic, central nervous, cardiovascular and respiratory systems.⁹¹⁻⁹³ As a result, several side effects have been associated with the transfusion of DMSO into patients including headache, nausea, vomiting, hypertension, anaphylactic shock, and many others.⁹¹⁻⁹⁴ The toxic effects of DMSO also necessitate precautions during the cryopreservation process. For instance, DMSO must be introduced to the HSCs at 4 °C for no longer than 10 minutes prior to freezing to avoid toxicity to the cells being cryopreserved.^{90,95} In addition, the cryopreserved sample must be thawed quickly and transfused immediately to avoid incubation of the cells with DMSO.^{96,97} It has been found that the degree of toxicity is directly related to

the amount of DMSO transfused into the patient. Therefore, efforts to reduce toxicity include the removal of DMSO prior to transfusion.^{98–100} This process is expensive, time consuming, and results in cell loss.¹⁰¹ Yang *et al.* demonstrated that removing DMSO by centrifugation leads to a decrease in viability and recovery of CD34+ cells in cryopreserved UCB.⁵⁴

Although the use of DMSO during cryopreservation permits nearly 100% survival of HSCs, there have been reports of high levels of apoptotic CD34+ cells (approaching 30% in some cases) following cryopreservation, which has been correlated with an impaired capacity to engraft.^{54,102,103} Approximately 20% of patients receiving a UCB transplant will fail to engraft, in part due to the inadequate potency of the unit as a result of cryopreservation.¹⁰⁴ Additionally, DMSO fails to protect the functionality of HSCs post-thaw.^{104,105} Sasnoor *et al.* have found that cryopreservation impairs growth factor responsiveness and this correlated to a reduced ability to proliferate and differentiate post-thaw.^{106–108} Despite all of the problems associated with DMSO as a cryoprotectant, it remains the “gold standard” cryoprotectant for many cell types, including HSCs.

1.2.5 Hypothermic storage and cryopreservation of red blood cells

The most commonly transfused blood product is red blood cells (RBCs). Transfusions of RBCs are used to treat patients with anemias, low oxygen carrying capacities resulting from extreme loss of blood (traumas or surgeries), and certain diseases and infections such as sickle cell disease and malaria.⁸ Hypothermic storage is the most widely used storage method for RBCs. The blood is collected into an anticoagulant solution and leukoreduced to remove plasma and leukocytes.¹⁰⁹ The resulting red cell concentrate is stored in a stabilizing solution, such as Additive Solution 3 (AS-3) or SAGM (sodium, adenine, glucose and mannitol), at +4 °C. RBCs can be stored for up to 42 days using this protocol.¹⁰⁹ This relatively short storage period can result in shortages of blood supplies during emergencies when tens to hundreds of people require blood transfusions.^{18,110} Hospitals and blood banks keep RBC units on hand in anticipation of a potential need and therefore many RBC units remain unused and are wasted when expired. This method also relies on routine blood donations from the public to keep a consistent

stock. In addition, hypothermic storage does not fully suppress biological activity and as a result, hypothermic storage lesion occurs over the storage period.^{8,18,109,111–114} Elements of storage lesion include morphological changes, slowed metabolism, acidosis, loss of function in ion channels, apoptotic changes, and membrane vesiculation.^{109,115,116} Some of these changes occur within hours, but many occur over the course of several days or weeks.¹¹⁶ Hypothermic storage lesions compromise the safety and efficacy of the blood product and have been correlated to a reduced oxygen carrying capacity and increased toxicity.^{8,18,112,113,117}

Cryopreservation of RBCs would extend the storage life up to 10 years; however, it is not yet a routine practice.^{8,118,119} This is due to the current cryopreservation protocols used for RBCs. In North America and most of Europe, clinical cryopreservation protocols utilize “High Glycerol/Slow Freeze” preservation conditions employing slow cooling rates (1 °C/min) and storage at -80 °C using high concentrations of glycerol (40% v/v) as a cryoprotectant.⁸ Consequently, complicated and time-consuming deglycerolization procedures are required post-thaw to prevent intravascular hemolysis.^{8,117,118,120} It takes approximately one hour to adequately remove the glycerol from one unit of RBCs (400-500 mL) which is problematic in emergency scenarios when immediate transfusions are required.

In Europe, clinical protocols utilize “Low Glycerol/Rapid Freeze” preservation conditions. These conditions, optimized by Art Rowe *et al.*, utilize lower concentrations of glycerol (20% v/v), reducing the deglycerolization time.^{121–124} Another “Rapid Freeze” approach involves the use of extracellular additives, such as hydroxyethyl starch (HES). RBCs experience osmotic stress during the addition and removal of glycerol which can be evaded by extracellular additives, like HES, that do not permeate the cell membrane. Spettek *et al.* reported successful transfusion of RBCs cryopreserved with HES (11.5% w/w) without post-thaw CPA removal.^{125,126} However to ensure high recovery of RBCs, ultra-fast cooling rates and extremely low storage temperatures (-165 °C or -195 °C) are necessary for all low-glycerol procedures, requiring the use of liquid nitrogen for freezing and storage.^{121–126}

1.2.6 Limitations to conventional cryopreservation practices

The ability to store important cells and tissues such as hematopoietic stem cells and red blood cells is becoming increasingly important with the development of new cellular therapies used for the treatment of various diseases.^{8,10,78} Cryopreservation is the only known method for the long term storage of biological materials. However, current cryopreservation protocols are inadequate and result in impaired recovery and functionality post-thaw. Conventional cryoprotectants are significantly cytotoxic both *in vitro* and in a clinical setting.^{127–129} Removal of DMSO from HSCs post-thaw results in a decrease in cell viability and recovery^{54,101} and consequently the thawed product is often directly transfused resulting in the patient experiencing negative side effects. The removal of glycerol post-thaw in cryopreserved RBCs is required to prevent intravascular hemolysis and this process is time-consuming and requires specialized equipment.

While CPAs reduce cryo-injury and improve post-thaw recovery by diluting intracellular solutes, preserving cell membrane volumes, and stabilizing cell membranes, they are unable to control ice growth and recrystallization which is a major source of cryo-injury. Therefore, cells are typically subjected to very rapid thawing conditions in order to minimize this ice recrystallization.⁶⁹ However, it is not possible to fully eliminate ice recrystallization by employing rapid thawing conditions and this type of cryo-injury has been correlated to reduced post-thaw viabilities in many cell types.^{61,62,68,70,71} Recently, compounds with the ability to inhibit ice recrystallization were shown to be effective cryoprotectants for RBCs using greatly reduced amounts of glycerol.¹³⁰ These compounds represent a new class of cryoprotectant with a novel mechanism of action. Therefore, the development of new CPAs which are non-toxic and capable of controlling ice recrystallization needs to be further investigated as they could dramatically improve current cryopreservation protocols.

1.3 Ice and recrystallization of ice

1.3.1 Structure of ice

In order to understand ice recrystallization, the basic structure of ice must first be understood. Many different forms of ice exist depending on several conditions. Temperature, pressure, and presence of solutes affect the type of ice that will form.^{131–133} However, only one form of ice is relevant to cryopreservation and therefore other forms will not be discussed. Ice found at ambient pressures and temperatures possesses a hexagonal shape, termed hexagonal ice (I_h).¹³⁴ The crystalline structure results from a specific hydrogen bonding network. A water molecule forms two hydrogen bonds between the lone pairs on its oxygen atom and two hydrogen atoms from adjacent water molecules. Thus, each water molecule participates in four hydrogen bonds with neighbouring water molecules to form hexagonal plates (Figure 1.3). The hexagonal plate contains six equivalent prism faces and two equivalent basal faces possessing four axes: a_1 , a_2 , a_3 and c .^{134–137} At 0 °C and atmospheric pressure, ice grows as sheets of hexagonal-shaped crystals by addition of water molecules to the a -axes.^{134,135,137,138}

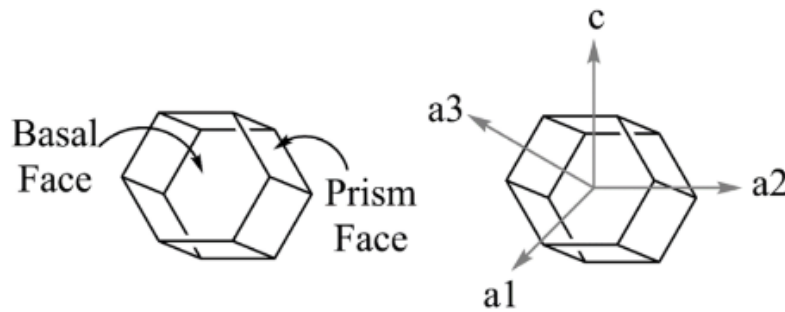


Figure 1.3. Hexagonal structure of ice (I_h), depicting basal and prism faces and a_1 , a_2 , a_3 , and c axes.

While ice appears to be one solid structure, it is in fact comprised of many ice crystals in very close proximity, separated by a layer of liquid water. There is a transition layer of semi-ordered water molecules between ice crystals and liquid water referred to as the quasi-liquid layer (QLL) (Figure 1.4).^{136,139–146} The thickness of the QLL increases as the temperature approaches the melting point.^{139,142,143}

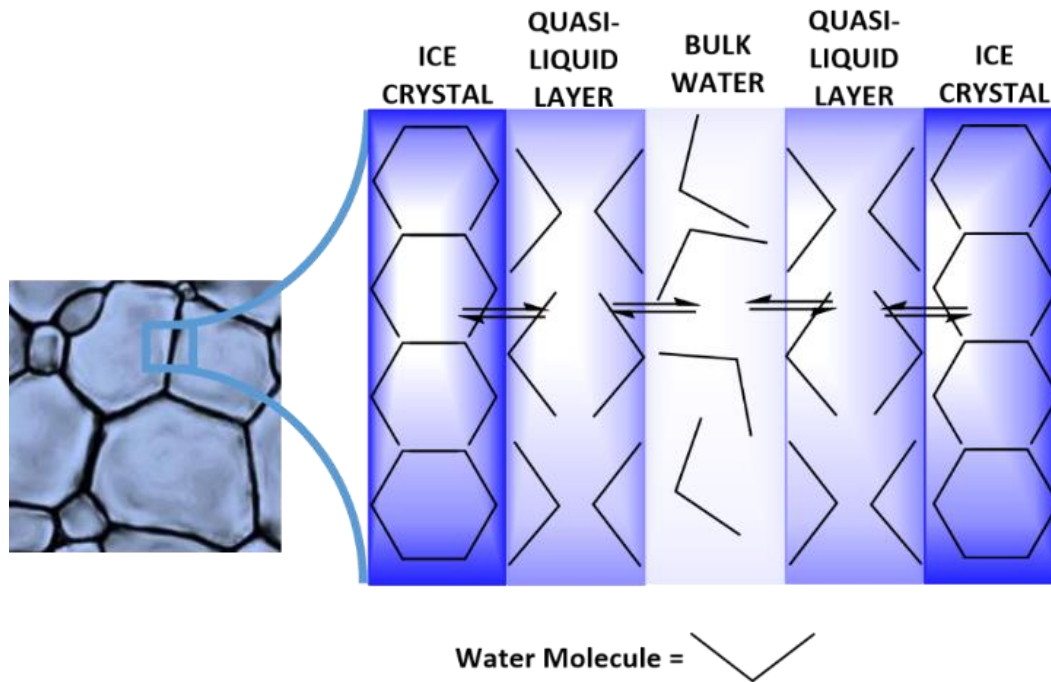


Figure 1.4. Schematic representation of the quasi-liquid layer (QLL) that exists between two ice crystals.

1.3.2 Mechanism of ice recrystallization

Ice recrystallization is defined as the growth of larger ice crystals (or grains) at the expense of smaller ones. It is a thermodynamically-driven process as a result of the reduction in free energy associated with ice crystal growth.¹⁴⁷ Ice recrystallization is believed to occur through grain boundary migration^{148,149} or Ostwald ripening.¹⁵⁰ Grain boundary migration is explained by examination of the curvature of large ice crystals compared to smaller ones. An ice grain (or crystal) possesses a hexagonal shape (I_h) in which water molecules participate in specific hydrogen bonding interactions as described in Section 1.3.1. Therefore, the water molecules at the edge of an ice crystal have a specific orientation. Grain boundaries are therefore defined as the interfaces between different oriented ice grains.^{148,149} In small ice grains, the boundaries are more curved and have a higher amount of surface energy in comparison to larger ice grains. Grain boundaries therefore migrate to reduce the degree of curvature in attempts to reduce surface energy.^{151,152}

The grain boundary migration theory for ice recrystallization ignores the presence of a QLL (Section 1.3.1). It assumes water molecules are directly transferred from one smaller ice grain to a larger one. The Ostwald ripening theory of ice recrystallization considers the entire system of ice crystals and liquid water and therefore is consistent with the presence of the QLL.¹⁵⁰ Water molecules at the boundary of an ice crystal are not able to form the optimal number of hydrogen bonds and therefore are higher in energy than water molecules inside the ice crystal.^{147,153} Since smaller ice crystals have a higher surface area to volume ratio than larger ones, they are thermodynamically less stable. Therefore, there is a thermodynamic driving force for water molecules to migrate from smaller ice crystals to larger ice crystals through the QLL resulting in a lower surface area to volume ratio.^{147,153} It is important to understand that in Ostwald ripening, the total ice volume remains constant while the average ice crystal size increases.¹⁵⁴

1.4 Natural cryoprotectants: biological antifreezes (BAs)

1.4.1 *Antifreeze (glyco)proteins and their antifreeze activities*

In the 1950s, Scholander and colleagues observed that marine teleost fish did not freeze despite living in water with temperatures below the freezing point of their blood (-1.9 °C).^{155,156} Certain proteins and glycoproteins in the blood of the teleost fish were attributed to their survival^{157–159}, later termed biological antifreezes (BAs). There are two classes of BAs: antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs).

AFPs are not only found in fish, but are also found in many insects. They are categorized as type I^{137,160–169}, II^{170–174}, III^{170–180} or IV^{181–183} depending on their size and secondary structure. AFGPs are found in Antarctic notothenioids and Arctic cod and are separated into eight classes, AFGP 1-8, based on their size ranging from 2.6 – 33.7 kDa. AFGPs consist of a tripeptide repeat of Ala-Ala-Thr where the secondary hydroxyl group of threonine is glycosylated with a β -D-galactosyl-(1,3)- α -N-acetyl-D-galactosamine disaccharide (Figure 1.5).^{137,158,159,184–188}

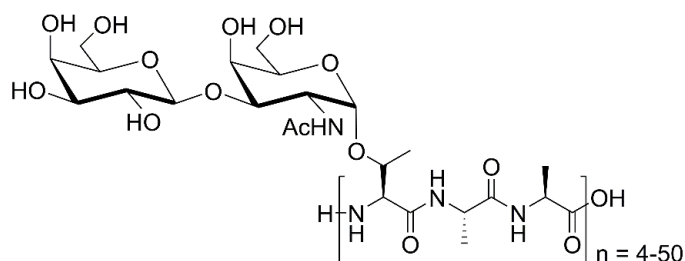


Figure 1.5. General structure of AFGPs.

AF(G)Ps exhibit two distinct antifreeze activities: ice recrystallization inhibition (IRI) and thermal hysteresis (TH). As mentioned in Section 1.3.2, ice recrystallization is the growth of larger ice crystals at the expense of smaller ones. A compound which inhibits this growth is referred to as an ice recrystallization inhibitor. IRI activity is typically measured using the “splat cooling assay”.^{189–191} Briefly, this assay involves dissolving the compound of interest in a phosphate buffered saline (PBS) solution. A 10 μ L aliquot is dropped from a height of two meters onto a pre-cooled ($-80\text{ }^{\circ}\text{C}$) aluminum plate to form a frozen wafer that is carefully transferred to a Peltier unit and held at $-6.4\text{ }^{\circ}\text{C}$ for 30 minutes. During this time, ice recrystallization occurs. The change in ice crystal size is observed through a camera fitted to a microscope and the average ice crystal size is quantified using a domain recognition software. Average ice crystal sizes are compared to a positive PBS control for ice recrystallization to obtain a percent mean grain size (% MGS).^{190,191} A smaller percentage thus corresponds to increased IRI activity. Previously, the Ben laboratory categorized molecules as inactive, moderately active or potent inhibitors of ice recrystallization depending on %MGS. The cut-off for classification was arbitrarily chosen such that molecules with a $\text{MGS} > 80\%$ were deemed inactive, molecules with a $\text{MGS} < 30\%$ were deemed potentially active and molecules with an MGS between 30–80% were considered moderately active. Recently, the Ben laboratory has implemented a more accurate way of representing IRI activity based on IC_{50} values.¹⁹² However, molecules that were previously assessed for IRI activity using %MGS will be represented as either inactive, moderately active or potentially active. Representative images of ice crystal sizes are shown in Figure 1.6. At $5.5\text{ }\mu\text{M}$, AFGP-8 is capable of inhibiting ice recrystallization, forming

ice crystals approximately 10% the size of those formed in PBS after this 30-minute recrystallization period.¹⁹³

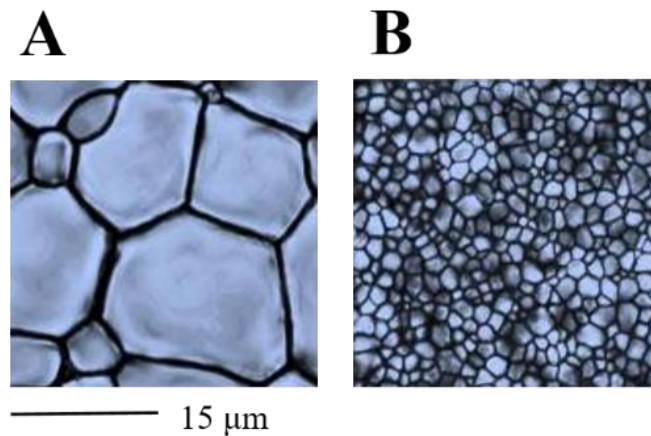


Figure 1.6. Photographs of ice crystal sizes obtained following the splat cooling assay. (A) No inhibition of ice recrystallization. (B) Inhibition of ice recrystallization.

The second type of antifreeze activity exhibited by biological antifreezes is thermal hysteresis (TH). Pure water has a melting point (T_M) equal to the freezing point (T_F) of 0 °C. In a solution of sodium chloride (NaCl), the freezing point is depressed and is equal to the melting point. In a solution containing a biological antifreeze, the localized freezing point depression relative to the melting point resulting in a temperature range during which ice crystal growth is arrested and is referred to as the TH gap (Figure 1.7).^{194–196} The TH gap typically spans from 1–1.5 °C and is the result of the irreversible binding of the biological antifreeze (BA) to the surface of ice.^{194,196–198} Within the TH gap, an ice crystal does not increase in size when the temperature of the solution is lowered below the melting point. However, when temperatures are below the TH gap, the ice crystal resumes growth, often uncontrollably, into long spicules.¹⁹⁹ This is the reason why BAs are not amenable for cryopreservation applications, which involve temperatures far below the TH gap. BAs actually increase cell death during cryopreservation which is attributed to their TH activity.^{97,200–202}

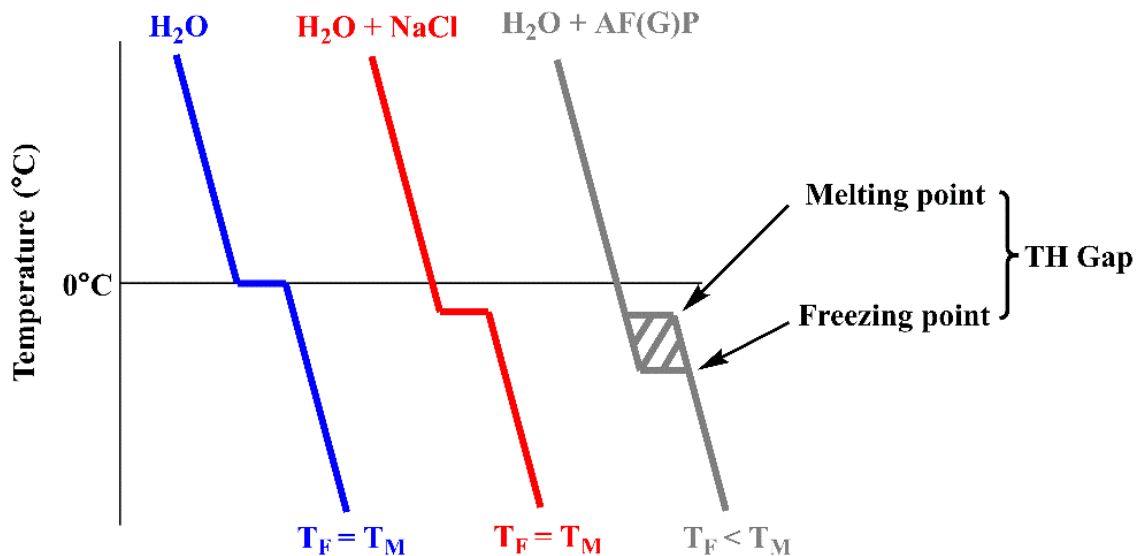


Figure 1.7. Diagram showing thermal hysteresis (TH). T_M represents the melting point and T_F represents the freezing point.

1.4.2 AFGP analogues

BAs are potent inhibitors of ice recrystallization. However, their associated TH activity prevents their use as cryoprotectants because the temperatures associated with cryopreservation are well below the TH gap. This results in an increase in cellular injury because ice growth resumes uncontrollably into damaging ice spicules at temperatures below the TH gap.^{97,200–202} Therefore, there has been an effort to design custom AFGP analogues which possess IRI activity but do not contain the detrimental TH activity for use in cryopreservation applications.

To date, a number of structure-function studies have generated glycopeptide analogues of AFGPs. The Sewald group demonstrated that replacement of the disaccharide in the native AFGP (shown in Figure 1.5) with an *N*-acetyl-D-galactosamine residue and (Ala-Ala-Thr)_n tripeptide repeats of three, four or five resulted in potent inhibitors of ice recrystallization.²⁰³ However, these analogues exhibited hexagonal ice crystal shaping indicating interactions with the ice crystal lattice. Other analogues investigated the effect of proline residues in the polypeptide backbone.²⁰³ These analogues were designed to cause the protein to adopt a distinct secondary structure in solution however these analogues were only

slightly IRI active indicating flexibility of the peptide backbone is important for IRI activity.²⁰³ Triazole-containing analogues were also investigated due to their ease of synthesis by employing “click” chemistry via the copper(I)-catalyzed Huisgen azide-alkyne cycloaddition (CuAAC).^{193,204–206} This strategy was used either to conjugate amino acid residues in the polypeptide backbone or to conjugate monosaccharide moieties to the polypeptide backbone. All of these analogues were weakly IRI active or inactive indicating that replacement of the amide bond in the side chain or in the polypeptide backbone with a triazole ring was detrimental to IRI activity.^{193,204–206}

The first AFGP analogues reported to have IRI activity while lacking TH activity were analogues **101** and **102** (Figure 1.8).^{191,207} Analogue **101** is a carbon-linked (C-linked) serine-based derivative which possesses four tripeptide repeating units with a C-linked galactosyl moiety. Analogue **102** also contains a galactosyl moiety coupled to the γ -amine of ornithine in the tripeptide repeat. Both analogues possess potent IRI activity at micromolar concentrations similar to AFGP-8, but unlike AFGP-8, they did not possess any significant TH activity.^{191,207} Since their discovery, additional studies have revealed that substitution of the galactose monosaccharide with glucose, mannose or talose monosaccharides resulted in a decrease in IRI activity.¹⁹¹ In comparison to galactose-containing analogue **102**, substitution of galactose with glucose to give analogue **103** resulted in only moderate IRI activity and substitution of galactose with either mannose (analogue **104**) or talose (analogue **105**) resulted in weak IRI activity (Figure 1.8). These differences in activities as a result of changing the monosaccharide residue were thought to be correlated to the hydration of the carbohydrate residue.¹⁹¹ Therefore, **102** containing the more highly hydrated galactose residues are able to disrupt the ordering of water molecules in the QLL or bulk-water to a greater extent than **105** containing talose residues which is a less hydrated monosaccharide. This was the first example of how very small structural changes in molecule can drastically influence the IRI activity of the molecule.

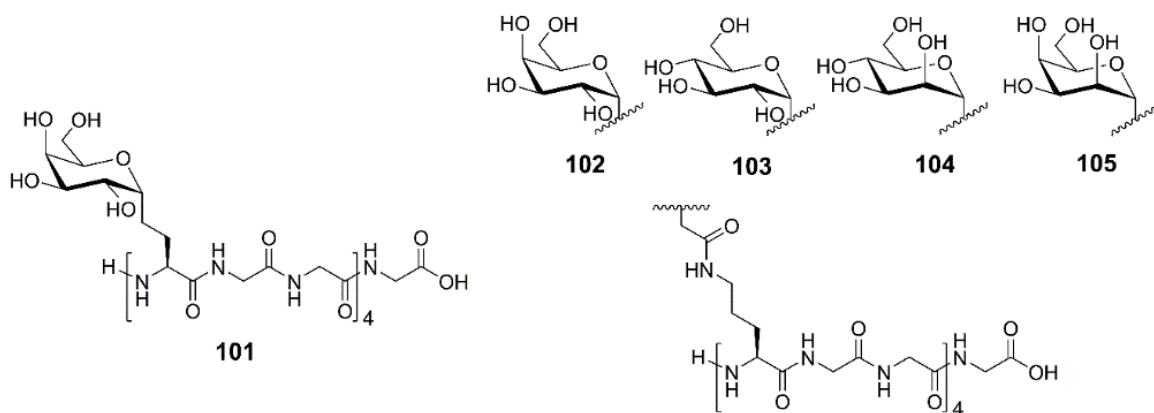
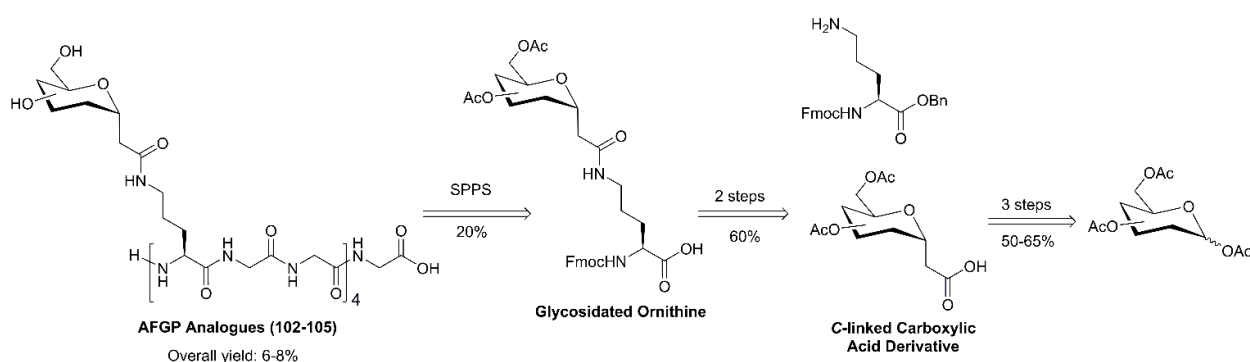


Figure 1.8. Structures of C-linked AFGP analogues **101-105**.^{191,207}

Overall, AFGP analogue structure-function studies have elucidated specific structural requirements necessary for IRI activity. The polypeptide backbone and number of tripeptide repeats is important, along with the nature of the carbohydrate residues and the side chain conjugating the carbohydrate to the peptide backbone.^{193,203–207} While some of these analogues are potent inhibitors of ice recrystallization and do not possess TH activity, these compounds are not amenable to the large-scale synthesis required to produce sufficient quantities for applications in cryopreservation. For example, the synthesis of analogues **102-105** involves first manipulation of the monosaccharide residue to obtain a C-linked carboxylic acid followed by glycosidation to the ϵ -amine of ornithine and subsequent solid phase peptide synthesis to obtain the final glycopeptide (Scheme 1.1).^{191,208} The glycosidated ornithine building block is obtained in



Scheme 1.1. Retrosynthetic scheme for synthesis of AFGP analogues **102-105**.^{191,208}

yields ranging from 30-40% and the solid phase peptide synthesis provides a yield of approximately 20%. Therefore, analogues **102-105** are obtained in overall yields of 6-8%. Due to the low overall yield and lengthy synthesis, these molecules are not amenable for large-scale synthesis. Subsequently, interest arose in the development of small molecule IRIs.

1.5 Small molecule ice recrystallization inhibitors

1.5.1 Simple monosaccharides and disaccharides

The use of small molecule IRIs would be more ideally suited to cryopreservation applications than large AFGP analogues. As mentioned in Section 1.3.2, substitution of the galactose moiety in analogue **102** with glucose, mannose, or talose resulted in a reduction in IRI activity. It was hypothesized that these differences in IRI activity was correlated to carbohydrate hydration and thus the AFGP analogue which possessed a galactose moiety (**102**) was more IRI active than the analogue possessing talose (**105**) because galactose is a more highly hydrated monosaccharide.^{191,209} Consequently, the IRI activities of simple monosaccharides and disaccharides with known hydration parameters were assessed. These were the first small molecules investigated for IRI activity. Mono- and disaccharide structures and their hydration numbers are shown in Table 1.1.

Of the monosaccharides assessed, D-galactose was found to be moderately IRI active, D-glucose and D-mannose were weakly active and D-talose was inactive.^{191,209} A linear correlation between hydration number and IRI activity of the monosaccharides was established (Figure 1.9A). The IRI activity of the disaccharides melibiose, lactose, trehalose and maltose also was correlated to hydration number but was not part of the same linear trend observed for the monosaccharides (Figure 1.9A).^{191,209}

Disaccharides typically have twice the number of water molecules tightly bound and thus the hydration numbers for disaccharides are roughly double those of monosaccharides. However, disaccharides are not twice as IRI active as monosaccharides. This result was attributed to the difference in total volume between mono- and disaccharides.²⁰⁹ The ratio between hydration number and partial

molar volume of the carbohydrate was defined as the hydration index (HI) and by using this metric, a linear correlation between all mono- and disaccharides assessed was observed between IRI activity and HI (Figure 1.9B).²⁰⁹

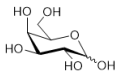
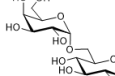
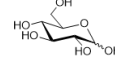
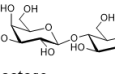
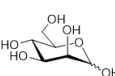
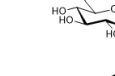
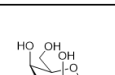
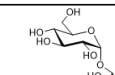
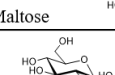
Carbohydrate	Hydration Number	Carbohydrate	Hydration Number
 D-Galactose	8.7 ± 0.2	 Melibiose	15.5 ± 0.3
 D-Glucose	8.4 ± 0.2	 Lactose	15.3 ± 0.3
 D-Mannose	8.1 ± 0.2	 Trehalose	15.3 ± 0.3
 D-Talose	7.7 ± 0.2	 Maltose	14.5 ± 0.3
		 Sucrose	13.9 ± 0.3

Table 1.1 Structures and hydration numbers of various mono- and disaccharides.^{209,210}

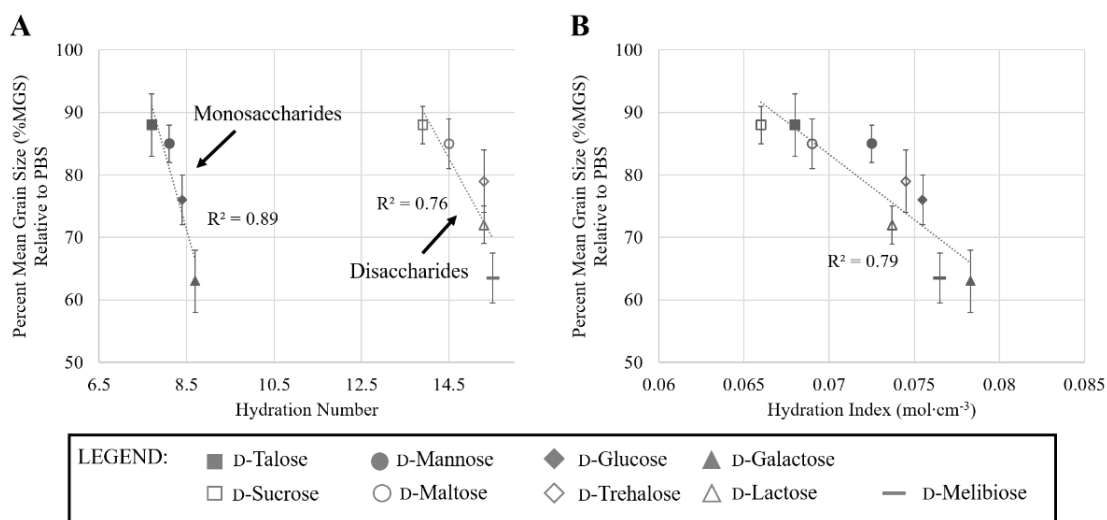


Figure 1.9. (A) Linear correlation between IRI activity (%MGS) and hydration number for monosaccharides and disaccharides.²⁰⁹ (B) Linear correlation between IRI activity (%MGS) and hydration index for all mono- and disaccharides assessed.²⁰⁹

The HI of a carbohydrate depends on both the hydration number as well as partial molar volume of the molecule.^{209–212} The hydration number is the number of tightly bound water molecules that surround the carbohydrate in an aqueous solution. This hydration number varies depending on the orientation of hydroxyl groups in the carbohydrate. For example, there are fewer water molecules surrounding D-talose compared to D-galactose in solution. Since more water molecules are tightly bound to D-galactose, there is a greater degree of disruption of the normal hydrogen bonding in water molecules in solution and thus the transition of water molecules to the QLL and eventually the ice crystal is also disrupted. It is this disruption that is hypothesized to be the reason D-galactose is a more IRI active monosaccharide than D-talose.

While this provided insight into a potential mechanism of inhibition, hydration numbers are not easily obtained for many compounds. Therefore, another metric correlating IRI activity to a more readily obtained chemical property is required.

1.5.2 Monosaccharide and disaccharide analogues

Since the discovery that simple carbohydrates exhibit moderate IRI activity, other monosaccharide and disaccharide derivatives were synthesized and assessed for IRI activity (Figure 1.10). The effect of modifications at the C1 position was investigated. An α - or β -C-linked allyl group was added at the C1 position resulting in moderate IRI activity (**106–111**).²⁰⁹ The fluorine-containing analogue **112** and its derivatives (**113–115**) were also only moderately active.²¹³ O-allyl groups (**116** and **117**) also did not increase IRI activity.²¹⁴

The structural features of the disaccharide in native AFGPs necessary for IRI activity were also investigated (Figure 1.11). Regioisomers of the β -D-galactosyl-(1,3)- α -D-N-acetyl-galactosamine, **118–120**, were synthesized and assessed for IRI activity.^{209,214} Additionally, the effect of removing the N-acetyl group (compound **121**) was investigated. Unfortunately, all of these derivatives were found to only be moderately IRI active.

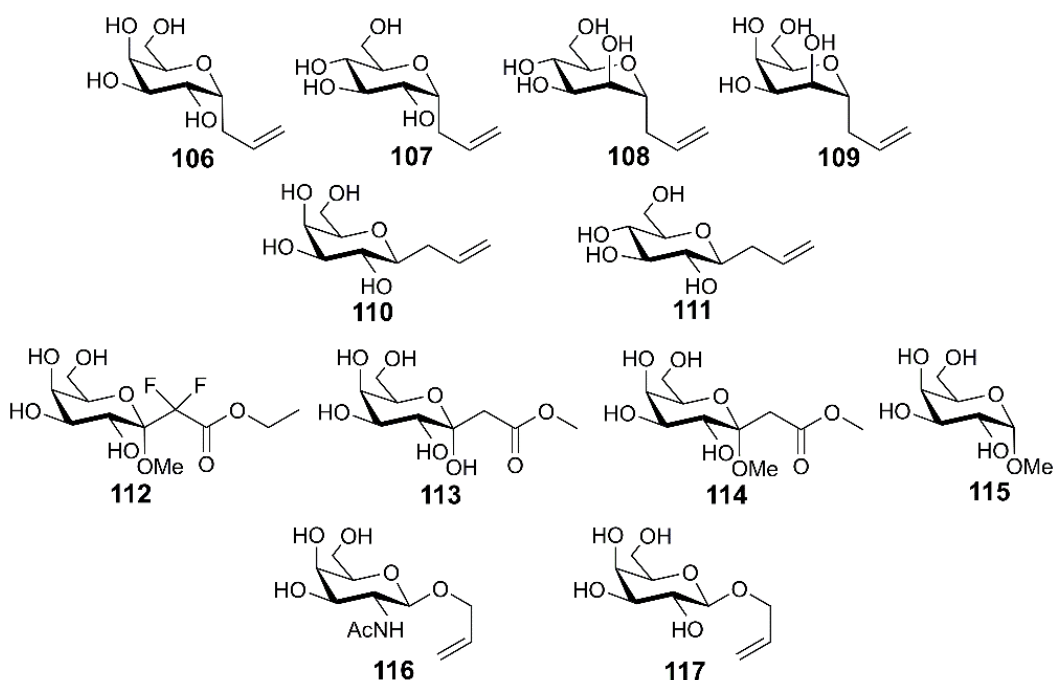


Figure 1.10. Structures of monosaccharide derivatives (**106-117**) assessed for IRI activity.

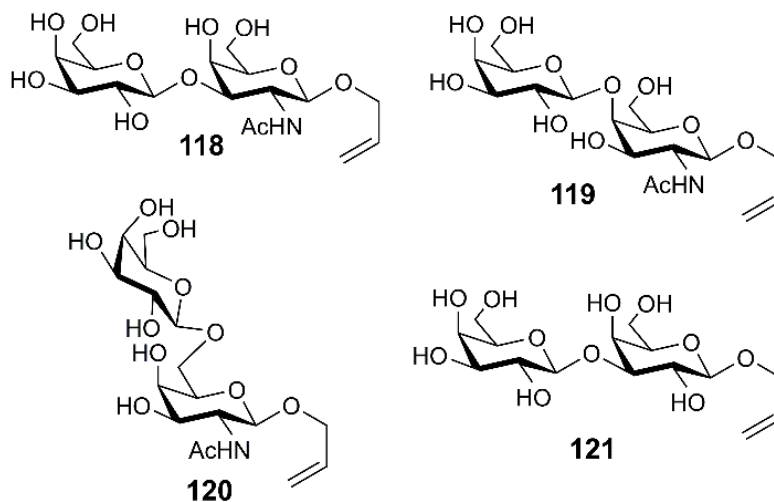


Figure 1.11. Structures of disaccharide derivatives (**118-121**) assessed for IRI activity.

1.5.3 Truncated AF(G)P analogues

At this point in time, AFGP analogues **101** and **102** were the only compounds possessing potent IRI activity. Therefore, analogue **102** was truncated to determine the smallest subunit that can possess potent IRI activity.²¹⁵ These analogues are depicted in Figure 1.12 and contain varying alkyl chain lengths

(**122-135**), as well as terminal amine, alcohol and carboxylic acid groups (**136-138**). Branched alkyl chains (**139-142**), unsaturations (**143-147**) and cycloalkyl groups (**148-149**) were also investigated.

These analogues were assessed for IRI activity and it was discovered that the analogues possessing longer alkyl chain lengths were more IRI active.²¹⁵ Derivatives with seven and eight carbon atoms (**129** and **130**), exhibited very potent IRI activity at 22 mM. Derivatives with alkyl chain lengths of nine and ten carbon atoms (**131** and **132**) were even more IRI active than **129** and **130** and were tested at much lower concentrations (5.5 mM) due to solubility issues. Lengthening the alkyl chain further limited their solubility and these compounds were only moderately active (similar to D-galactose at 22 mM). The presence of a terminal ionizable group (**136** and **137**) did not improve IRI activity. A terminal alcohol group (**138**) improved activity; however, it was less active than **129** and **130** at 22 mM. Analogues with a terminal alkene or alkyne (**143** and **144**) were more IRI active than the fully saturated form **125**.

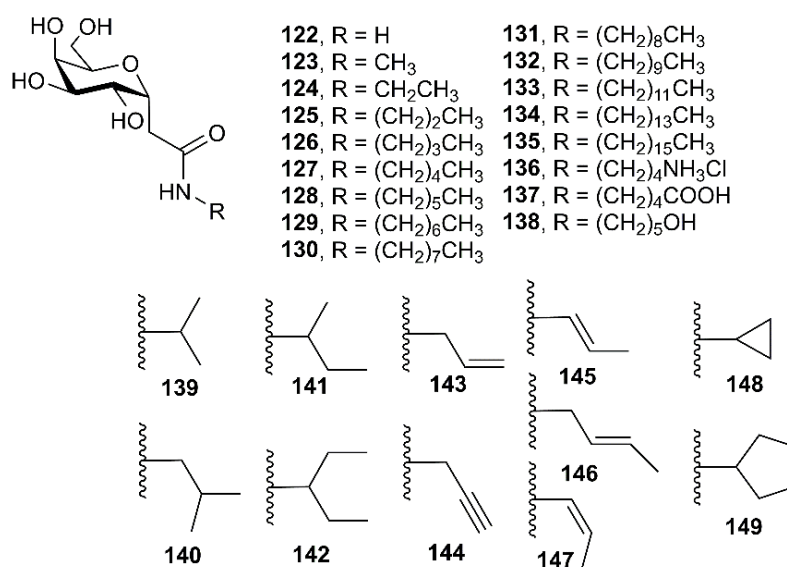


Figure 1.12. Structures of truncated C-linked AFGP analogues **122-149**.

Collectively, these data suggested that it was possible to develop small molecules that are highly active inhibitors of ice recrystallization. It was evident that long alkyl chains are important for IRI activity. This was the first indication that the balance between hydrophilic and hydrophobic moieties is important for IRI activity.

1.5.4 Carbohydrate-based hydrogelators and surfactants as IRIs

As seen in Section 1.4.1, IRI activity is correlated to the hydration of the molecule, which is attributed to the ability of the molecule to disrupt the hydrogen bonding network of bulk water. Therefore, other molecules which are capable of disrupting bulk water, such as surfactants and hydrogelators, were investigated for their ability to inhibit ice recrystallization. Hydrogelators are small molecules that self-assemble in water to form dynamic three dimensional networks of polymer chains that trap water molecules *via* surface forces and hydrogen bonding interactions.^{216–218} The first identified carbohydrate-based hydrogels were *N*-alkyl-gluconamides.^{216,219} Gluconamides (Figure 1.13A) are open-chain sugar amphiphiles composed of a hydrophilic head (linear form of glucose) and a hydrophobic tail (alkyl chain of varying lengths) connected through an amide bond. *N*-Alkyl-gluconamides and *N*-alkyl-erythronamides (Figure 1.13B) were assessed for IRI activity. *N*-ethyl-, *N*-propyl- and *N*-butyl-gluconamide (**150–152**) did not possess IRI activity while *N*-octyl- and *N*-hexyl-gluconamide (**153** and **154**) were found to be potent inhibitors of ice recrystallization. As expected, longer alkyl chain lengths was an important characteristic of IRI-active molecules. This was hypothesized to be because longer alkyl chain lengths are better able to disrupt the normal hydrogen bonding in bulk water compared to shorter alkyl chain lengths. However, all of the erythronamide derivatives (**155–158**) possessed moderate to weak IRI activity, regardless of the length of the alkyl chain. In contrast to the gluconamides, the erythronamides consist of a hydrophilic head that is shorter by two carbons. Clearly, a delicate balance

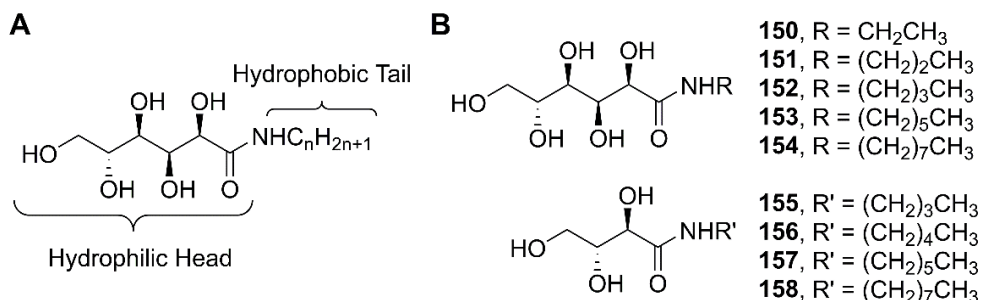


Figure 1.13. (A) Generic structure of *N*-alkyl-gluconamides²¹⁹, (B) *N*-alkyl-gluconamides and -erythronamides assessed for IRI activity.

between hydrophobic and hydrophilic moieties is important for IRI activity, which is consistent with the findings from the truncated AFGP analogues (Section 1.4.3). This realization was crucial to the rational design of the aryl glycoside small-molecule IRIs (Section 1.5.5) and ultimately led to the discovery of several aryl glycosides with potent IRI activity that were also effective cryoprotectants for RBCs.¹³⁰

N-Octyl-gluconamide (**154**) was the most potent IRI discovered up to this point.²²⁰ Therefore, the effect of several modifications to *N*-octyl-gluconamide on IRI activity was assessed.²²⁰ Additionally, the pyranose forms **163** and **164** were also assessed. The structures of these “*N*-octyl-gluconamide-inspired” molecules are shown in Figure 1.14. All modifications to *N*-octyl-gluconamide, including substitution of glucose for galactose (**159**), methylation of the amine (**160**), displacement of the carbonyl (**161**), and replacement of the amide bond with an ether linkage (**162**) resulted in dramatic decreases in IRI activity. The glucose-derived non-ionic surfactant β -octyl-D-glucopyranoside (**163**) possessed moderate activity similar to D-glucose alone. β -Octyl-D-galactopyranoside (**164**) possessed potent IRI activity and was significantly more active than D-galactose alone, potentially suggesting a synergistic effect between the high hydration number associated with galactose and the long hydrophobic alkyl chain.

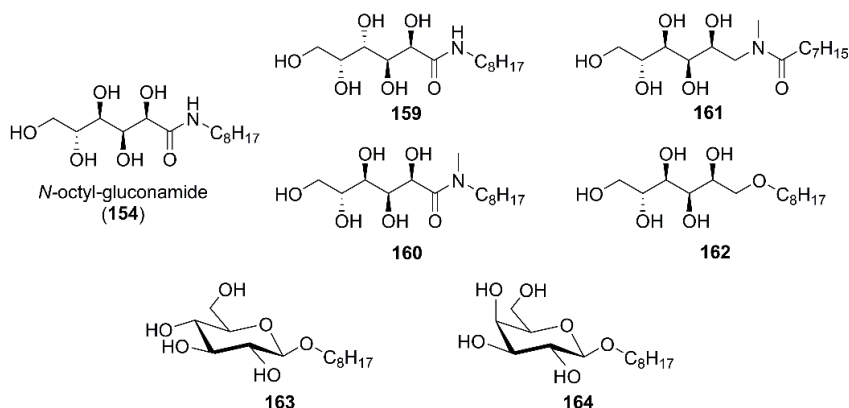


Figure 1.14. Structures of carbohydrate-based surfactants and hydrogelators.²²⁰

1.5.5 Aryl glycosides as IRIs

The most potent IRIs discovered to date are surfactant-like molecules possessing a hydrophilic carbohydrate-based head with a long hydrophobic alkyl chain tail. It was determined that a delicate

balance between hydrophobic and hydrophilic moieties was important for IRI activity, although this balance had not yet been quantified. While this was interesting structure-function information, these molecules are not useful for cellular applications due to their ability to interact with and solubilize cell membranes.^{221,222} Therefore, the alkyl chain was replaced with an aromatic ring. The aromatic ring was chosen as it is still hydrophobic and possesses six carbon atoms. Furthermore, access to potentially hundreds of derivatives was possible by incorporating different aromatic substituents which could be placed at different positions around the ring.

The first aryl-containing carbohydrate derivatives assessed were the aryl glycosides due to their relative ease of synthesis. These derivatives are easily accessed by reacting D-glucose pentaacetate with various phenol derivatives in the presence of a Lewis acid and subsequent acetate deprotection. A few of the aryl glycosides assessed are shown in Figure 1.15.¹³⁰ The aryl glycoside that does not possess any aryl substituents (Ph-Glc, **165**) had moderate IRI activity. *Para*-substitution of a hydroxyl, methyl or nitro group (**167-169**) did not improve IRI activity. However, *para*-substitution of a methoxy (β -PMP-Glc, **166**), fluorine (β -pFPh-Glc, **170**), or bromine (β -pBrPh-Glc, **171**) on the aryl ring resulted in a dramatic increase in IRI activity, with comparable activity to the surfactant-like molecules in Section 1.4.4. Amazingly, very small structural changes were shown to dramatically effect IRI activity. For example,

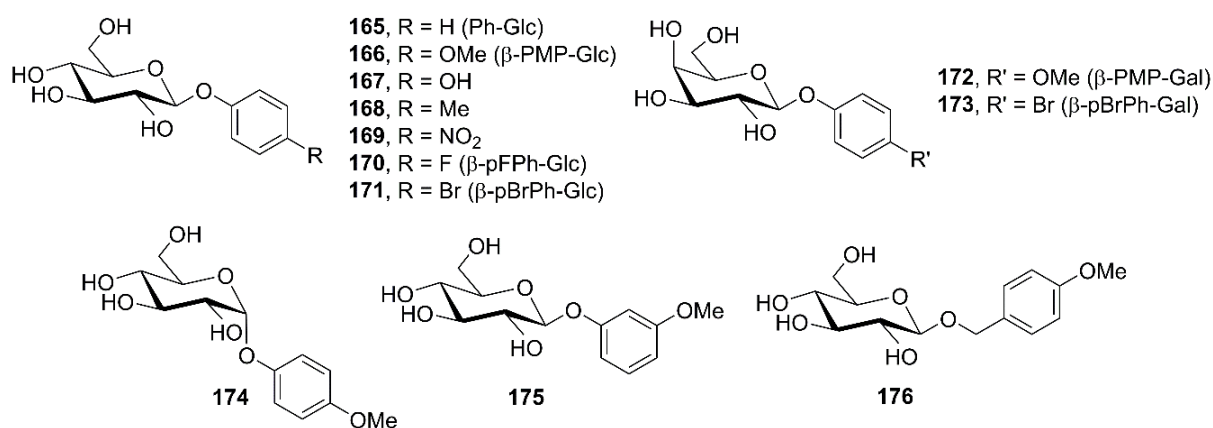


Figure 1.15. Structures of aryl glycosides assessed for IRI activity.¹³⁰

the galactose derivative β -PMP-Gal (**172**) is significantly less IRI active than β -PMP-Glc (**166**). However, the galactose derivative β -pBrPh-Gal (**173**) has similar IRI activity to the glucose form (**171**).

Regioselectivity of the aromatic substituent is also important. Moving the methoxy functional group from the *para* position (**166**) to the *meta* position (**175**) resulted in a decrease in IRI activity. A decrease in IRI activity was also seen when changing the linkage of the *para*-methoxy-phenyl group from β (**166**) to α (**174**), indicating the importance of the stereochemistry at C1. Furthermore, a carbon spacer between the aromatic ring and anomeric oxygen (seen in derivative **176**) is not tolerated.

1.5.6 Conclusions of current small molecule IRIs

Many years of structure-function analysis revealed that a balance is necessary between hydrophobic and hydrophilic moieties for IRI activity. While longer alkyl chains resulted in increased IRI activity, the surfactant-like nature of these molecules impaired their ability to function as novel cryoprotective agents. However, the realization that small molecules could possess potent IRI activity was instrumental in the subsequent discovery of the aryl glycosides, which are significantly less toxic than their alkyl-chain containing counterparts.

Overall, this section summarizes the discovery of several small carbohydrate-based molecules that have very potent IRI activity. The development of potent ice recrystallization inhibitors is important to the field of cryopreservation because uncontrolled ice growth and recrystallization is a major cause of cell injury during cryopreservation. The identification of non-toxic small molecules with potent IRI activity that are easily synthesized in large enough quantities for cryopreservation applications, such as the aryl glycosides, has greatly advanced the field. It was recently shown that several IRI active aryl glycosides were effective cryoprotectants for RBCs as they allowed for the use of greatly reduced concentrations of glycerol, ultimately reducing the deglycerolization time.¹³⁰ This is a clinically-relevant discovery as it would allow frozen RBC units to be more readily available in hospitals. These molecules could represent a new class of cryoprotectants capable of controlling ice growth and recrystallization.

Studies like this highlight the need for further investigation and optimization of the use of small molecules for the cryopreservation of RBCs and other valuable cellular therapy products like HSCs.

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Chapter 2: Goals and objectives

2.1 Introduction

The increase in the development of new cellular therapies over the past few decades has resulted in an increasing need for methods to store biological products. A major challenge in this field is finding suitable, safe, and ethical sources of cells and tissues for research and therapy.¹ The development of biobanks (facilities that collect and store cells and tissues) has enabled access to cells and tissues for research and therapy but requires that many of these products be cryopreserved.¹

Cryopreservation, the storage of cells and tissues at low sub-zero temperatures (between -80 °C and -196 °C), allows cellular therapy products such as hematopoietic stem cells (HSCs) and red blood cells (RBCs) to be stored for extended periods of time. While cryopreservation protocols have been extensively studied and optimized depending on cell type, there are significant drawbacks associated with cryopreservation. During cryopreservation, cryo-injury (described in Chapter 1, Section 1.1.2) is experienced as a result of the cooling, storage, and thawing of the sample. Cryoprotectants are exploited to minimize this damage. However, conventional cryoprotectants (dimethyl sulfoxide and glycerol) are significantly cytotoxic both *in vitro* and in a clinical setting.²⁻⁴ Removal of dimethyl sulfoxide (DMSO) from HSCs post-thaw results in a decrease in cell viability and recovery,^{5,6} and consequently the thawed product is often directly transfused resulting in the patient experiencing negative side effects. The removal of glycerol post-thaw in cryopreserved RBCs is required to prevent intravascular hemolysis, which is a time-consuming process and requires specialized equipment.

In addition to being cytotoxic, conventional cryoprotectants do not control the growth of large ice crystals during the thawing process, which is referred to as ice recrystallization and is a major cause of cryo-injury. Therefore, cells are typically subjected to very rapid thawing conditions in order to minimize this ice recrystallization.⁷ However, it is not possible to fully eliminate ice recrystallization by employing rapid thawing conditions and this type of cryo-injury has been correlated to reduced post-thaw viabilities

in many cell types.⁸⁻¹² Recently, compounds with the ability to inhibit ice recrystallization were shown to be effective cryoprotectants for RBCs using greatly reduced amounts of glycerol.¹³ These compounds represent a new class of cryoprotectant with a novel mechanism of action. Therefore, the development of new cryoprotectants which are non-toxic and capable of controlling ice recrystallization needs to be further investigated as they could dramatically improve current cryopreservation protocols.

To date, only a few novel compounds have been identified as potent ice recrystallization inhibitors (IRIs). Furthermore, the structural features necessary for potent IRI activity have not been definitively identified. Many previously discovered potent IRIs are surfactants and therefore were not amenable to cryopreservation applications due to their ability to interact with and solubilize cell membranes.^{14,15} Therefore, the goal of this thesis is to identify compounds which are not surfactants and which exhibit IRI activity. The structural features of these molecules that are required for IRI activity will be investigated in order to facilitate the rational design of future IRIs. The ability of these novel IRIs to improve the cryopreservation of HSCs and RBCs will also be explored. Therefore, the objectives of this thesis are as follows:

2.2 Objective 1 – Determining the influence of aryl moieties on IRI activity in truncated C-AFGP analogues

One of the first antifreeze glycoprotein (AFGP) analogues reported to be highly IRI active while lacking thermal hysteresis (TH) activity was OGG-Gal (Chapter 1, analogue **102**).^{16,17} OGG-Gal is a carbon-linked (C-linked) derivative containing a galactosyl moiety coupled to the γ -amine of ornithine in the tripeptide repeat. Since OGG-Gal is not amenable to the large-scale synthesis required to produce sufficient quantities for applications in cryopreservation, it was subsequently truncated to determine the smallest possible subunit exhibiting potent IRI activity.¹⁵ During this study, several very potent inhibitors were discovered that possess long alkyl chains (Figure 2.1A).¹⁵ These were some of the first small molecules discovered that exhibited potent IRI activity. However, their surfactant-like nature impaired

their use in cellular applications. Subsequently, several aryl glycosides (Figure 2.1B) were identified as potent inhibitors of ice recrystallization. Some of these compounds have also proven to be effective cryoprotectants for RBCs, allowing reduced glycerol concentrations.¹³

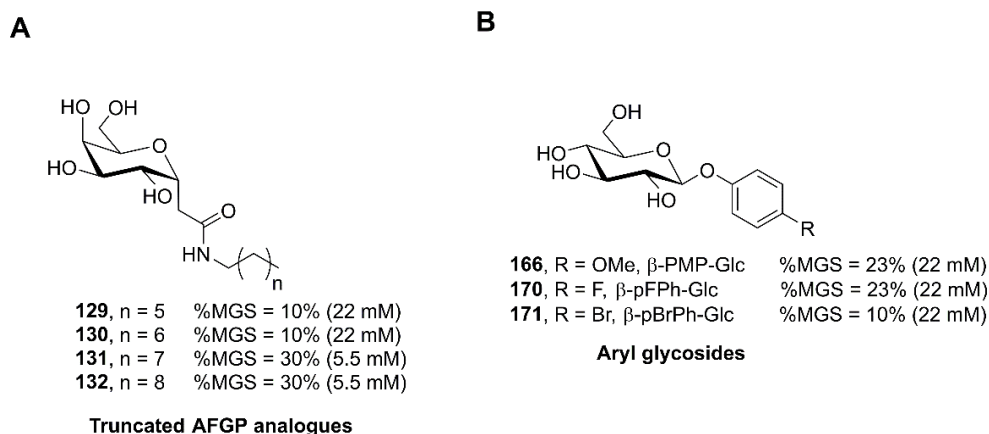


Figure 2.1. Structures of previously synthesized small molecules and their IRI activities.^{13,15–17}

Therefore, it is hypothesized that the replacement of the long alkyl chains in the truncated AFGP analogues with aromatic rings could result in the discovery of other IRI active derivatives. This new class of compounds, collectively referred to as *N*-aryl-D-pyranosyl-acetamides, are shown in Figure 2.2. These molecules will be synthesized and assessed for their ability to inhibit ice recrystallization.

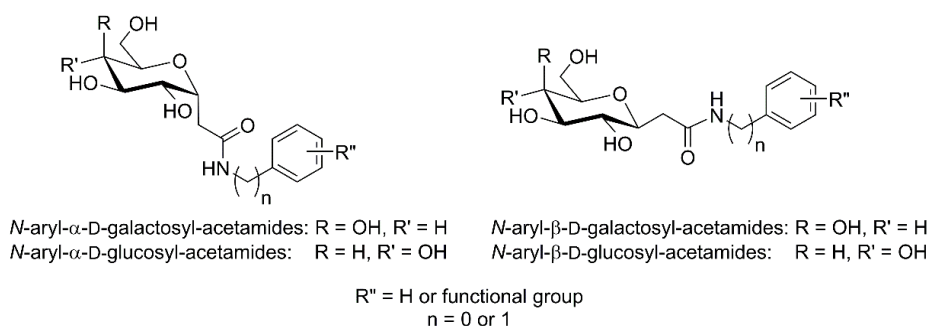


Figure 2.2. General structure of *N*-aryl-D-pyranosyl-acetamides.

2.3 Objective 2 – Quantifying the importance of amphiphilicity in small-molecule IRIs

Recently, two classes of small molecules were discovered by the Ben laboratory: the truncated AFGP analogues (Figure 2.3A) and the *N*-alkyl- and *N*-cycloalkyl-aldonamides (Figure 2.3B & C).^{14,15,18,19} The

truncated AFGP analogues possess varying degrees of IRI activity depending on the length of the alkyl chain.¹⁵ Truncated AFGP analogues with longer alkyl chains are more IRI active than analogues with shorter chains. The second class, *N*-(cyclo)alkyl-aldonamides (Figure 2.3B & C), also possess varying degrees of IRI activity depending on the length of the alkyl chain.

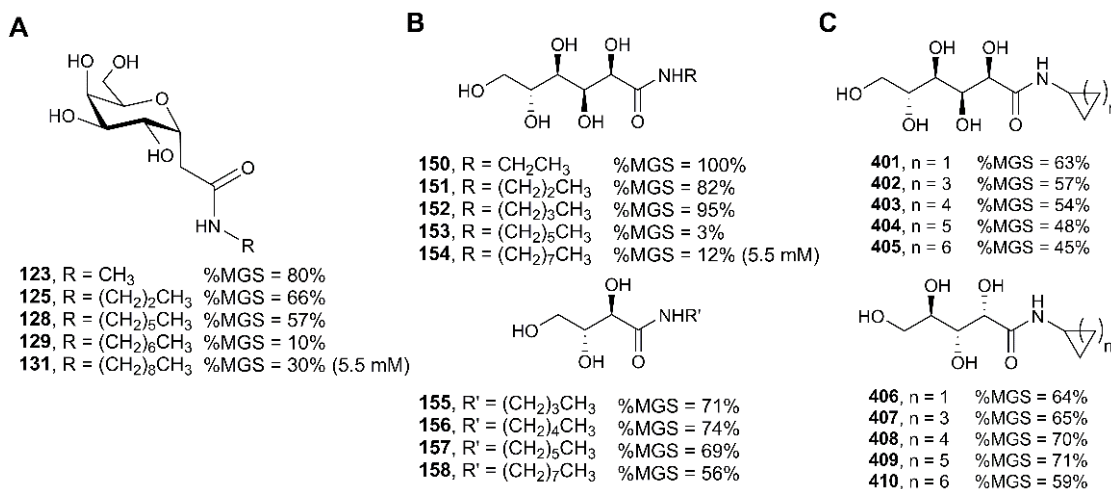


Figure 2.3. Structures of (A) truncated AFGP analogues¹⁵, (B) *N*-alkyl-aldonamides¹⁸, and (C) *N*-cycloalkyl-aldonamides previously assessed for IRI activity (at 22 mM unless otherwise indicated).

The length of the hydrophilic moiety is also important for the IRI activity of the *N*-(cyclo)alkyl-aldonamides. For example, *N*-hexyl-gluconamide (**153**) is a very potent inhibitor of ice recrystallization whereas *N*-hexyl-erythronamide (**157**) is inactive and only differs from **153** by two hydroxyl group units. Furthermore, *N*-cycloheptyl-arabonamide (**409**) is significantly less active than *N*-cycloheptyl-gluconamide (**404**) which differs by only one fewer hydroxyl group.

It was previously determined that IRI activity was not correlated to micelle formation.¹⁴ The important structural features required for IRI activity have not been elucidated and this has impeded the rational design of new small molecules with potent IRI activity. It is evident that a certain balance between hydrophobicity and hydrophilicity is required for IRI activity. However, this balance has yet to be quantified in a meaningful way. Therefore, objective 2 will seek to establish a correlation between IRI activity and amphiphilicity of the previously discovered surfactant small-molecules.

2.4 Objective 3 – Determining the influence of aryl moieties on IRI activity in gluconamides

Some of the first highly active small molecule ice recrystallization inhibitors discovered include the pyranose-based surfactant β -octyl-Gal (**164**) and the aldonamide-based hydrogelators *N*-octyl-gluconamide (**154**) and *N*-hexyl-gluconamide (**153**) shown in Figure 2.4.¹⁴ β -Octyl-Gal (**164**) and *N*-hexyl-gluconamide (**153**) exhibited potent IRI activity at 22 mM (21% and 3% MGS respectively) and *N*-octyl-gluconamide was highly IRI active at a significantly lower concentration of 0.5 mM (12% MGS).¹⁴ The *N*-alkyl-gluconamide derivatives with long alkyl chain lengths were shown to be more IRI active than derivatives with shorter alkyl chain lengths.

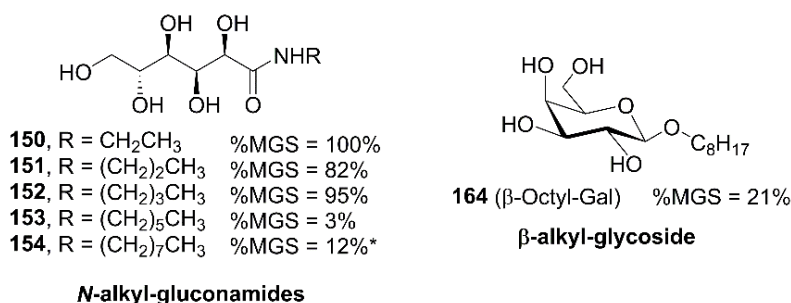


Figure 2.4. Structures of *N*-alkyl-gluconamides and β -alkyl-glycoside **164** previously assessed for IRI activity at 22 mM (asterisks indicates IRI activity assessed at 5.5 mM).¹⁴

However, due to the surfactant nature of these molecules, they were not amenable to cryopreservation applications.^{20,21} Subsequently, the aryl glycoside class of IRIs were explored and their structures are shown in Figure 2.5.^{13,18} These molecules are β -alkyl-glycoside analogues in which the long alkyl chain has been replaced with an aromatic ring. The aryl glycosides maintain amphiphilicity while being less likely to interact with or solubilize cell membranes. Many derivatives were explored with different aromatic substituents at various positions on the ring. This study resulted in the discovery of several potent inhibitors of ice recrystallization and these molecules are significantly less toxic than the pyranose-based surfactant β -octyl-Gal (**164**) and the aldonamide-based hydrogelators *N*-octyl-gluconamide (**154**)

and *N*-hexyl-gluconamide (**153**). Furthermore, some of these molecules were shown to be effective cryoprotectants for RBCs.¹³

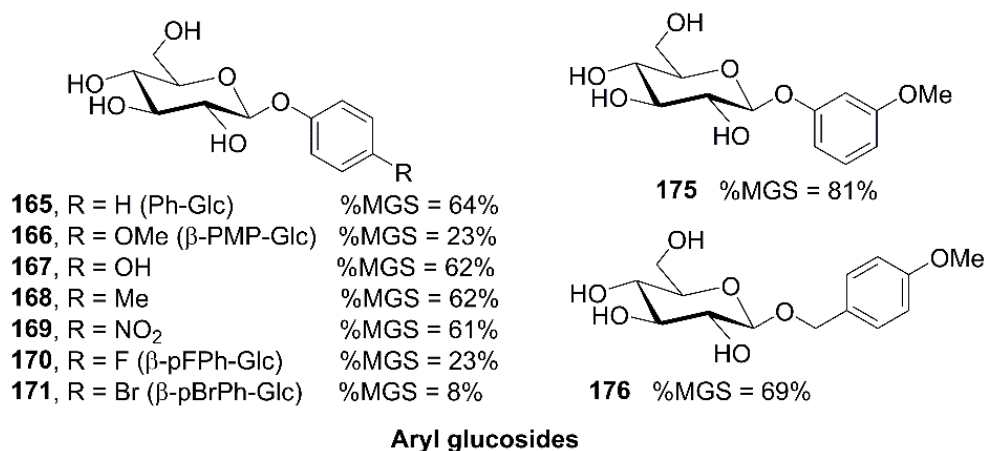


Figure 2.5. Structures of phenolic glucosides previously assessed for IRI activity.¹⁸

Since the replacement of the alkyl chain with an aromatic ring in the β -alkyl-glycosides resulted in the discovery of potent IRIs, it is hypothesized that the same replacement in the *N*-alkyl-gluconamides could also result in the discovery of potent IRIs. This new class of compounds is referred to as *N*-aryl-gluconamides (Figure 2.6).

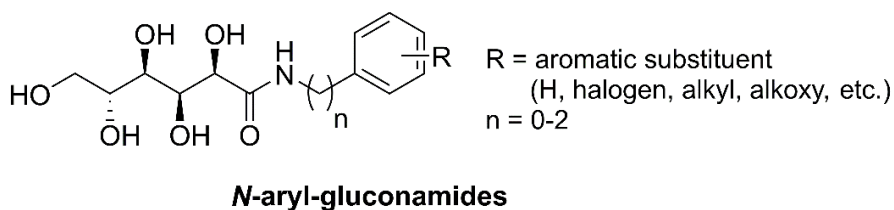


Figure 2.6. General structure of *N*-aryl-gluconamides.

Structure-function analyses of the aryl glucosides revealed that the identity of the aromatic substituent had a dramatic effect on the IRI activity of the molecule (Figure 2.5).¹⁸ The location of the aromatic substituent also impacted IRI activity. Therefore, *N*-aryl-gluconamides with various aryl substituents at different positions on the aromatic ring will be investigated. Furthermore, the effect of adding carbon or nitrogen spacers between the amide and aromatic ring will be assessed. The proposed structures to be synthesized and assessed for IRI activity are shown in Figure 2.7.

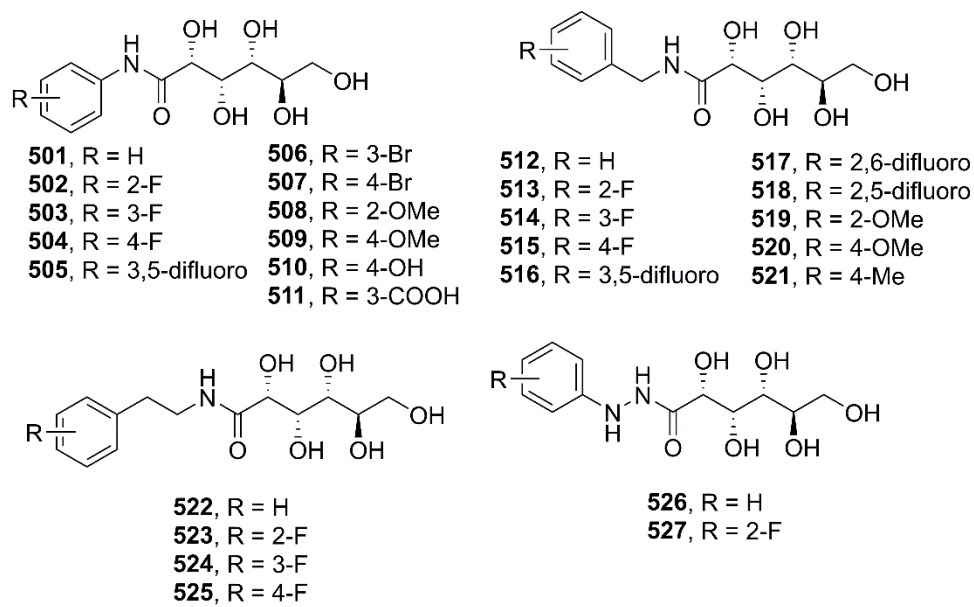


Figure 2.7. Structures of target *N*-aryl-gluconamides to be synthesized and assessed for IRI activity.

2.5 Objective 4 – Assessing the potential of IRIs to improve the cryopreservation of HSCs

Hematopoietic stem cells (HSCs) are routinely used for regenerative medicine therapies and HSC transplantation offers life-saving treatment for patients suffering from hematological cancers (leukemias and lymphomas), hematological diseases, anemias, and immunodeficiencies.^{22,23} In select situations, fresh stem cells can be used for allogenic transplants if the cells are transplanted within 72 hours of harvest.²⁴ However, virtually all autologous and allogenic transplants require that the cells are cryopreserved.²⁴ Since the success of HSC transplantation is directly correlated to both the number and quality of cells transplanted, optimization of cryopreservation protocols for HSCs is an important issue for umbilical cord blood (UCB) banks.

Cryopreservation is particularly important for UCB HSCs because these cells are harvested at birth for use at a later, undetermined date. Clinical protocols for the cryopreservation of UCB HSCs employ the use of 10% dimethyl sulfoxide (DMSO) as the cryoprotectant solution.²⁵ While a 10% DMSO solution is very effective and maintains high levels of cell viability, there are significant drawbacks associated with the use of DMSO as a cryoprotectant. These drawbacks are explained in detail in Chapter 1 (Section

1.2.4). Firstly, DMSO is toxic resulting in several side effects after transfusion of DMSO into patients including headache, nausea, vomiting, hypertension, anaphylactic shock, and many others.^{26–29} Efforts to reduce toxicity include the removal of DMSO prior to transfusion which is expensive, time consuming, and results in cell loss.^{30–32} Secondly, DMSO as a cryoprotectant fails to protect the functionality of HSCs post-thaw.^{6,33–36} Approximately 20% of patients receiving a UCB transplant will fail to engraft, in part due to the inadequate potency of the unit as a result of cryopreservation.³⁵ Despite all of the problems associated with DMSO as a cryoprotectant, it remains the “gold standard” cryoprotectant for many cell types, including HSCs.

Due to the inadequacies of DMSO as a cryoprotectant, efforts have been made to improve the cryopreservation of HSCs. These efforts have centered on alternate freezing and storage conditions, or improved cryoprotectant solution formulations. Woods *et al.* have demonstrated that lower concentrations of DMSO (5%) can be used if alternate freezing conditions are used (freezing of 4 °C/min to -44 °C before plunging in liquid nitrogen).³⁷ Other studies suggest that DMSO can be eliminated by the storage of cells by alginate encapsulation.^{38–40} However, the majority of efforts to improve the cryopreservation of HSCs involves the incorporation of additives to the conventional 10% DMSO cryoprotectant solution, or the elimination or reduction of DMSO content using alternative cryoprotectants.

The main mechanisms of cryoinjury, which are described in detail in Chapter 1 (Section 1.2.2), can be divided into three categories: (1) osmotic imbalances arising from disruption in solute concentration during ice formation and melting, (2) cell membrane damage, and (3) ice recrystallization injury. DMSO functions as a permeating cryoprotectant by replacing water within the cell, diluting high intracellular solutes and maintaining cell volume.⁴¹ However, DMSO is not capable of controlling ice growth or recrystallization.

Previous work from our laboratory has assessed the ice recrystallization inhibition (IRI) activity of several mono- and disaccharides.⁴² These sugars were assessed for their ability to function as cryoprotectants for CD34⁺ cells derived from UCB.⁴³ Interestingly, the IRI activity of the mono- and

disaccharides was found to correlate with post-thaw CD34⁺ cell viability.⁴³ All carbohydrates assessed were found to have equal or less cytotoxicity compared to 2.5 or 5% DMSO. However, it was found that disaccharides that were superior at cryopreservation, were also those that were more cytotoxic.⁴³ For example, lactose, sucrose, or trehalose (220 mM) resulted in high post-thaw cell viabilities; however, these were among the most cytotoxic carbohydrates examined. D-Galactose (220 mM) exhibited the best balance between cytotoxicity and cryopreservation ability.⁴³ Interestingly, D-galactose was the most potent IRI monosaccharide of those examined in the study with an MGS of 63%.⁴³

Since this study, many more highly active small-molecule IRIs were discovered. We hypothesized that non-toxic compounds possessing IRI activity could represent a new class of cryoprotectants capable of improving the cryopreservation of HSCs from UCB.

2.6 Objective 5 – Assessing the potential of IRIs to improve the cryopreservation of RBCs

The most commonly transfused blood product is red blood cells (RBCs). Transfusions of RBCs are used to treat patients with anemias, low oxygen carrying capacities resulting from extreme loss of blood (traumas or surgeries), and certain diseases and infections such as sickle cell disease and malaria.⁴⁴ The most widely used method for the storing of RBCs is by hypothermic storage at 4 °C. The major drawback associated with the hypothermic storage of RBCs is that the relatively short storage period (42 days) can result in shortages of blood supplies during emergencies.^{45,46}

Cryopreservation of RBCs would extend the storage life up to 10 years; however, it is not yet a routine practice because either large amounts of glycerol or ultra-fast cooling rates are required.^{44,47,48} Research on improving RBC cryopreservation has focused on methods to improve deglycerolization procedures to either allow faster access to RBC units post-thaw or increase the storage time after thawing and subsequent deglycerolization. Additionally, alternative additive solutions and cryosolutions have been explored. Previous work from the Ben laboratory determined β -PMP-Glc (**166**) and β -pBrPh-Glc (**171**) (Figure 2.8) to be effective cryoprotectants for RBCs using reduced amounts of glycerol (15% Glyc).^{13,18} Remarkably, double post-thaw RBC integrities resulted when **166** (110 mM) or **171** (55 mM) were added

to the 15% glycerol cryosolution (49% and 51% intact RBCs with **166** and **171**, respectively, compared to 25% intact RBCs with 15% glycerol control). This was significant because prior to this result, low glycerol cryosolutions under slow cooling conditions resulted in negligible RBC recoveries. However, deglycerolization of the RBCs post-thaw was problematic and resulted in high levels of hemolysis.

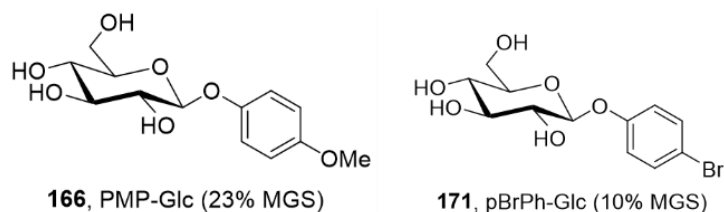


Figure 2.8. Chemical structure and IRI activity of aryl glycosides **166** and **171**.

Therefore, objective 5 seeks to improve the deglycerolization of RBCs frozen with aryl glycosides. The ability of newly discovered IRI active *N*-aryl-glycosides and *N*-aryl-alidonamides to effectively cryopreserve RBCs will also be assessed. Furthermore, the influence of these molecules on several RBC functions will be investigated.

2.7 Summary of goals and objectives

The goal of these studies is to elucidate the structural features required for IRI activity in order to rationally design potent inhibitors of ice recrystallization that can be utilized as novel cryoprotectants for important cellular therapy products such as hematopoietic stem cells (HSCs) and red blood cells (RBCs). Therefore, the overall objectives are as follows:

1. To design novel IRI active molecules that are less toxic which feature aromatic rings instead of alkyl chains.
2. To determine the relationship between IRI activity and amphiphilicity.
3. To explore the cryopreservation potential of IRI active molecules for HSCs and RBCs.

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Chapter 3: Determining the influence of aryl moieties on IRI activity in truncated C-AFGP analogues

3.1 Introduction

One of the first antifreeze glycoprotein (AFGP) analogues reported to have IRI activity while lacking TH activity was OGG-Gal (Chapter 1, analogue **102**) (Figure 3.1A).^{1,2} OGG-Gal is a carbon-linked (C-linked) derivative containing a galactosyl moiety coupled to the γ -amine of ornithine in the tripeptide repeat. OGG-Gal (**102**) possesses high IRI activity at micromolar concentrations similar to the native AFGP-8, but unlike AFGP-8, it does not exhibit any significant TH activity.^{1,2} Since OGG-Gal is not amenable to the large-scale synthesis required to produce sufficient quantities for applications in cryopreservation, it was subsequently truncated to determine the smallest possible subunit exhibiting potent IRI activity.³ During this study, several very active inhibitors were discovered that possess long alkyl chains (Figure 3.1B).³ These were some of the first small highly IRI active molecules discovered. However, their surfactant-like nature impaired their use in cellular applications. The IRI activities of these molecules, along with OGG-Gal (**102**) and several aryl glycosides, are presented in Figure 3.1. IRI activity is represented as a percent mean grain size (%MGS) relative to a phosphate buffered saline (PBS) positive control for ice recrystallization. Therefore, smaller percentages represent a more potent inhibitor of ice recrystallization.

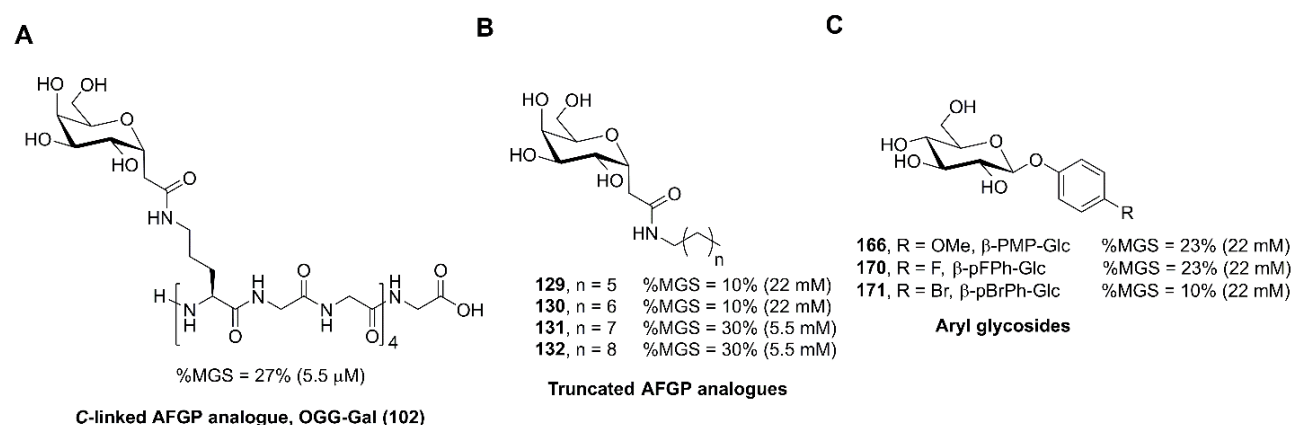


Figure 3.1. Structures of previously discovered potent inhibitors of ice recrystallization and their IRI activities.¹⁻⁴

Recently, several aryl glycosides (Figure 3.1C) have been identified as IRI active molecules. Some of these compounds have also proven to be effective cryoprotectants for RBCs, allowing reduced glycerol concentrations.⁴ Subsequently, replacement of the long alkyl chains in the truncated AFGP analogues with aromatic rings could result in the discovery of other highly active derivatives. This new class of compounds are collectively referred to as *N*-aryl-D-pyranosyl-acetamides and can possess either an α -C-linkage or a β -C-linkage (Figure 3.2). Galactose and glucose derivatives are referred to as *N*-aryl-D-galactosyl-acetamides and *N*-aryl-D-glucosyl-acetamides, respectively.

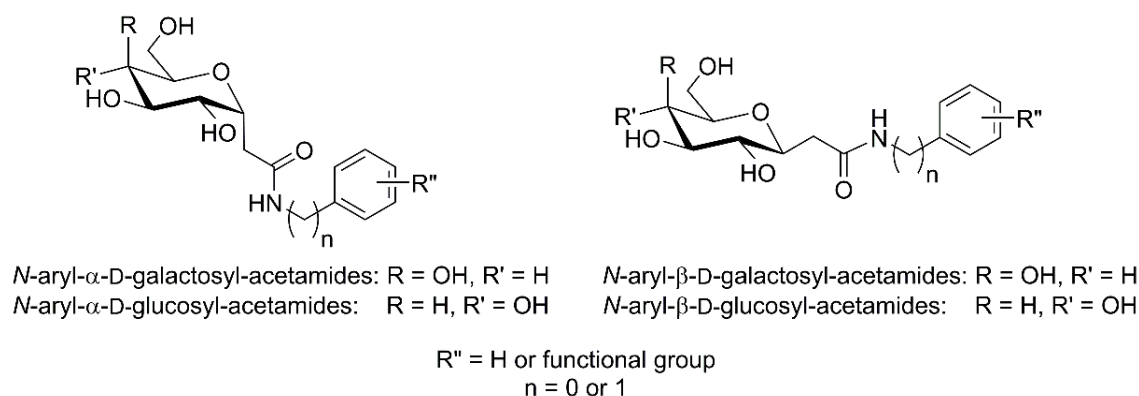


Figure 3.2. General structure of *N*-aryl-D-pyranosyl-acetamides.

In OGG-Gal (**102**), the stereochemistry at C1 was important: an α -linkage was required for IRI activity. In contrast, structure-function analyses revealed that a β -glycosidic bond was more favourable than an α -linkage in the aryl glycoside class.⁵ Furthermore, the location and identity of a substituent on the aromatic ring in the aryl glycoside class had a dramatic effect on IRI activity.^{4,5} For example, a *para*-methoxy substituent resulted in a potent inhibitor (23% MGS) while a *meta*-methoxy group resulted in an inactive molecule (80% MGS). Replacement of the *para*-methoxy substituent with a *para*-hydroxyl functional group resulted in a loss of activity (62% MGS).⁵ Dr. John Trant⁶ and Dr. Chantelle Capicciotti⁵ performed many structure-function analyses on these molecules in attempts to correlate the aromatic substituent with IRI activity. Their results of the *para*-substituted aryl-glycosides indicated that the IRI activity of these derivatives is sensitive to the electronic nature of the substituent at this position. For

example, a *para*-nitro group resulted in a weakly active compound whereas a *para*-methoxy group resulted in a highly IRI active molecule.⁵ Therefore, a Hammett plot was constructed to elucidate the relationship between IRI activity and its sensitivity to changes in electronic effects of the *para*-substituent. The Hammett equation is a linear free energy relationship that relates changes in reaction rates or equilibrium constants to changes in the electron donating/withdrawing ability of substituents.^{7–10} No correlation was found between IRI activity and aryl ring substituent electronic effects. Thus, extensive structure-function analyses on these molecules failed to identify a correlation to IRI activity.⁵

It was our hypothesis that the new class of compounds (*N*-aryl-D-pyranosyl-acetamides) will assess the effect of various substituents at different locations on the aromatic ring. Furthermore, the effect of the stereochemistry at C1 (α vs β) on IRI activity will be investigated. Figure 3.3 depicts the targets for this study. The synthesis of these targets was performed with the help of an undergraduate honours student, Julia Meyer.

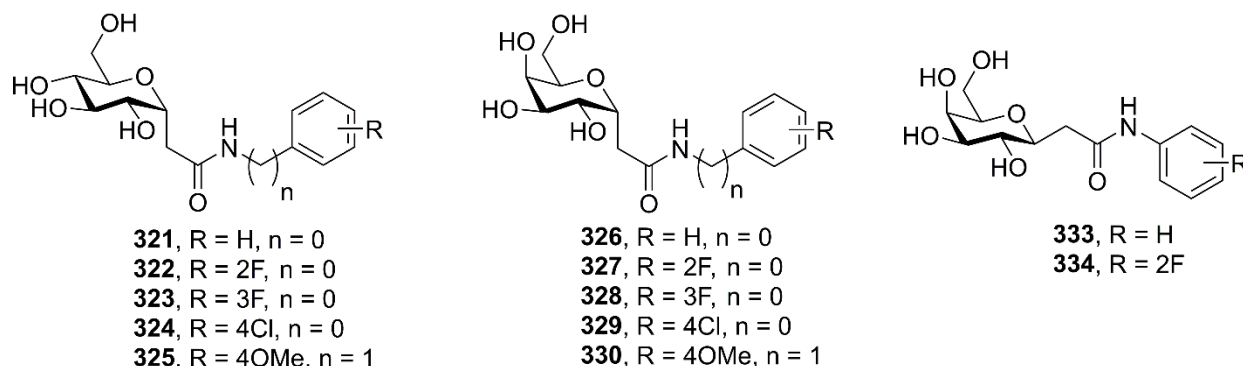
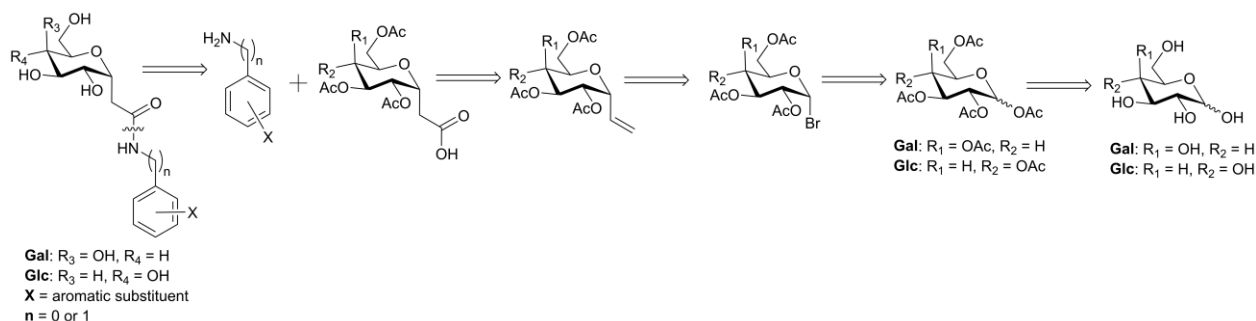


Figure 3.3. Target *N*-aryl-D-pyranosyl-acetamides for IRI assessment.

3.2 Retrosynthetic strategies for installation of C-linked carboxylic acid group at C1

The retrosynthetic route for the α -linked glucose and galactose derivatives, *N*-aryl- α -D-pyranosyl-acetamides, is depicted in Scheme 3.1. The synthetic route envisioned for these derivatives was based on the original synthesis of the (2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranosyl)acetic acid used in the synthesis of C-linked AFGP analogue OGG-Gal (**102**).^{1,2} Briefly, peracetylated D-glucose and D-galactose were converted to the corresponding acetobromo- α -D-pyranose before being subjected to Keck radical

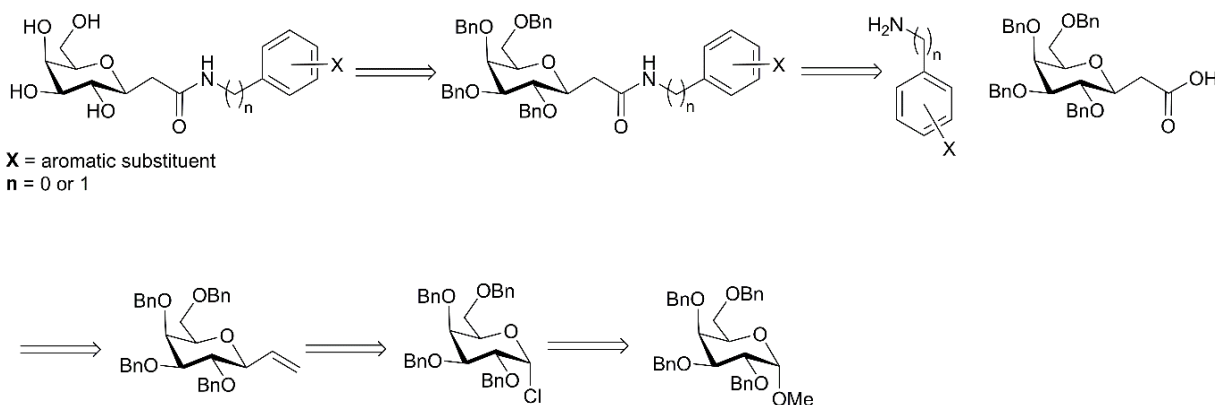
allylation to yield the allyl 2,3,4,6-tetra-*O*-acetyl- α -D-pyranoside. This *C*-linked allyl derivative was oxidatively cleaved to the corresponding carboxylic acid *via* a ruthenium-catalyzed oxidation. The (2,3,4,6-tetra-*O*-acetyl- α -D-pyranosyl)acetic acid derivatives was then coupled to the desired aryl amines using standard carboxylic acid-amine coupling protocols, followed by subsequent acetate deprotection to furnish the desired *N*-aryl- α -D-pyranosyl-acetamides.



Scheme 3.1. Retrosynthetic scheme for the synthesis of *N*-aryl- α -D-pyranosyl-acetamides.

For the β -linked derivatives, a different synthetic route was required in order to obtain the proper stereochemical configuration at the C1 position. The retrosynthetic route for the *N*-aryl- β -D-galactosyl-acetamides is depicted in Scheme 3.2. Briefly, 2,3,4,6-tetra-*O*-benzyl- α -methyl-galacto-D-pyranoside was converted to 2,3,4,6-tetra-*O*-benzyl- α -D-glucopyranosyl chloride using oxalyl chloride. A Grignard reaction was used for the installation of the allyl group *via* S_N2 displacement of the chlorine by allyl magnesium bromide. The ruthenium-catalyzed oxidation used in the synthetic route for the α -linked derivatives (*N*-aryl- α -D-pyranosyl-acetamides) could not be used because the ruthenium catalyst was found to be incompatible with the benzyl protecting groups. Oxidation of β -*C*-linked allyl group using ruthenium-catalyzed conditions on the substrate containing benzyl protecting groups resulted in degradation of the substrate. Therefore, the β -*C*-linked allyl group was oxidized to the carboxylic acid by conversion to an aldehyde using Lemieux-Johnson oxidation conditions, followed by a Pinnick oxidation. The (2,3,4,6-tetra-*O*-benzyl- β -D-galactopyranosyl)acetic acid derivatives were then coupled to the desired

aryl amines using standard amide coupling protocols, followed by subsequent debenzoylation to furnish the desired *N*-aryl- β -D-pyranosyl-acetamides.

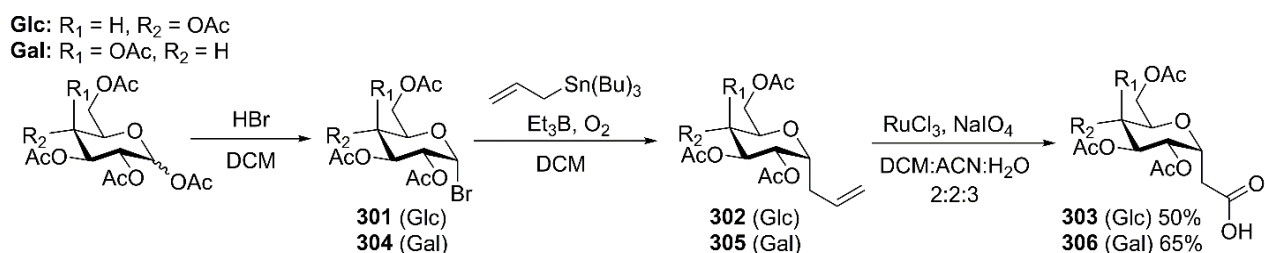


Scheme 3.2. Retrosynthetic scheme for the synthesis of *N*-aryl- β -D-galactosyl-acetamides.

3.3 Synthesis

3.3.1 Synthesis of (2,3,4,6-tetra-*O*-acetyl- α -D-pyranosyl)acetic acid

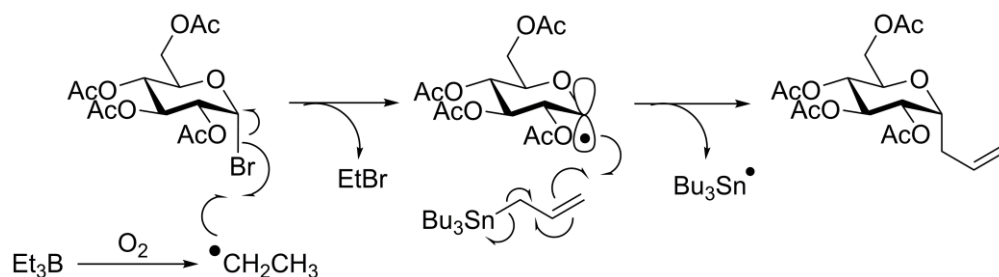
The synthesis of the α -linked carboxylic acid building blocks was previously optimized and is outlined in Scheme 3.3.^{1,2}



Scheme 3.3. Synthetic route for synthesis of (2,3,4,6-tetra-*O*-acetyl- α -D-pyranosyl)acetic acid.

The key step in the synthesis was the stereoselective addition of the allyl group at the C1 position. This reaction occurs *via* a Keck radical allylation (Scheme 3.4).^{11,12} The radical initiator used, triethyl borane, readily forms an ethyl radical in the presence of oxygen.^{12,13} The ethyl radical subsequently abstracts the halide from the alkyl halide to form the key radical intermediate.^{11,14} The radical is stabilized in the α -position as a result of the anomeric effect.¹⁴ Therefore, the allyl group was added

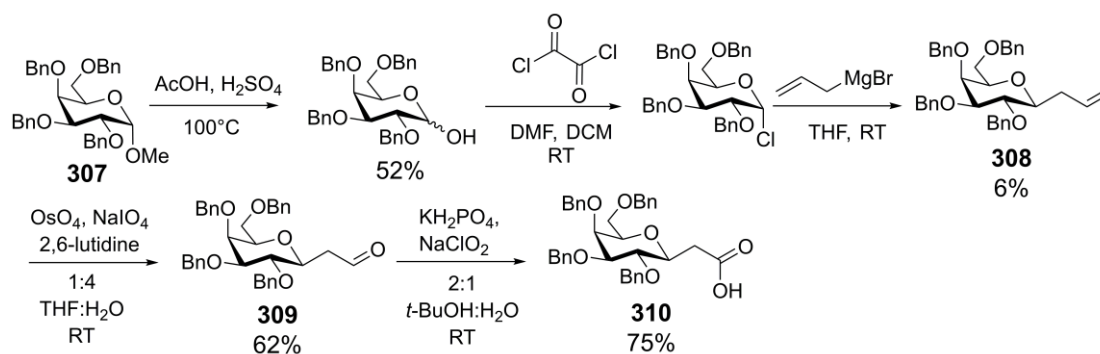
stereoselectively to the α -position at the C1 carbon. The allyl group was then oxidatively cleaved to the corresponding (2,3,4,6-tetra-*O*-acetyl- α -D-pyranosyl)acetic acid using ruthenium trichloride and sodium periodate. While the key Keck radical allylation proves extremely useful for the selective formation of the α -linked carboxylic acids, it required that a different synthetic pathway be determined for the formation of the β -anomers.



Scheme 3.4. Keck radical allylation reaction stereoselectively adds allyl group to the α -position at C1.^{11–14}

3.3.2 Synthesis of (2,3,4,6-tetra-*O*-benzyl- β -D-galactopyranosyl)acetic acid

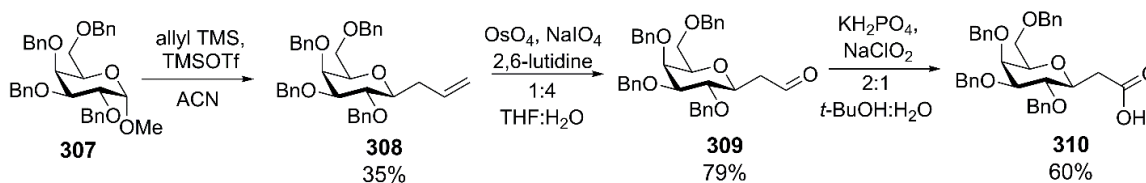
The synthesis of the β -linked carboxylic building block was performed as previously described, outlined in Scheme 3.5.¹⁵ The key step in the synthesis was again the stereoselective β -addition of the allyl group at the C1 position. The *C*-allyl group was added in the β -position through an S_N2 displacement of an α -substituted chlorine atom at C1. The chlorine was displaced using allylmagnesium bromide resulting in addition of the *C*-allyl group to the β -position at C1. Benzyl protecting groups were used instead of acetate protecting groups to avoid deprotection at this key step. The use of benzyl protecting groups required the use of alternate oxidation conditions because oxidation of the β -*C*-linked allyl group using ruthenium-catalyzed conditions on the substrate containing benzyl protecting groups resulted in degradation of the substrate. Therefore, Lemieux-Johnson oxidation conditions were used to oxidize the allyl group to the corresponding aldehyde followed by further oxidation to the carboxylic acid employing the Pinnick oxidation. Unfortunately, this synthetic route resulted in a low overall yield (1%), so a new pathway was investigated. The first two steps were modified because this was where the greatest reduction in yield was experienced (Scheme 3.5).



Scheme 3.5. Synthetic route for synthesis of (2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)acetic acid.

3.3.3 Revised synthesis of (2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)acetic acid

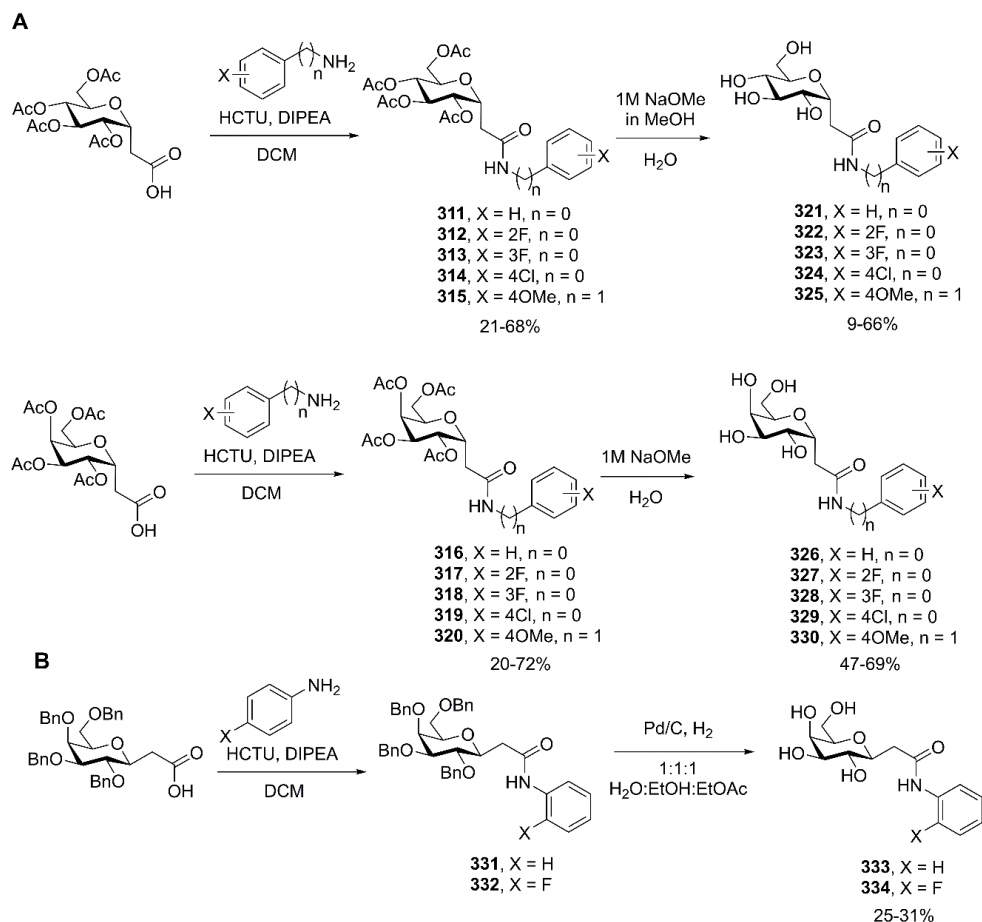
The low overall yield resulting from the original synthesis necessitated a revision to the synthesis of the (2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)acetic acid. In the previous route, the first two steps resulted in the largest reduction in yield. Therefore, the revised synthesis, depicted in Scheme 3.6, was designed to modify or avoid these two steps. A report by Miller *et al.* showed that the allyl 2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranoside can be produced directly from the starting material **307** via a Hosomi-Sakurai reaction.¹⁶ Stereoselectivity can be controlled by choice of solvent. By performing the reaction in DCM, a 1:19 β:α product distribution is obtained.¹⁷ β-selectivity can be obtained by performing the reaction in acetonitrile rather than in DCM, resulting in a 9:1 β:α product ratio in 35% yield. The same oxidative cleavage conditions as described in the previous synthesis (Lemieux-Johnson followed by Pinnick) were utilized for an overall yield of 17%.



Scheme 3.6. Revised synthetic route for the synthesis of (2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)acetic acid.

Once the *C*-linked carboxylic acids were obtained, they were coupled to various aryl amines using 1H-benzotriazolium 1-[bis(dimethyl-amino)methylene]-5-chloro-hexafluorophosphate (1-),3-oxide

(HCTU) as a coupling reagent. Subsequent alcohol deprotection furnished the desired *N*-aryl- α -D-pyranosyl-acetamides. The aryl amine coupling and deprotection conditions are depicted in Scheme 3.7. Aryl amines with halogen substituents were chosen because aryl-glucosides with halogen substituents were very IRI active.⁵ β -PMP-Glc (**166**) was also highly IRI active⁵ and therefore the *para*-methoxy substituent was also assessed in the *N*-aryl- α -D-pyranosyl-acetamides.



Scheme 3.7. (A) Coupling of (2,3,4,6-tetra-*O*-acetyl- α -D-pyranosyl)acetic acid to aryl amines, (B) Coupling of (2,3,4,6-tetra-*O*-benzyl- β -D-galactopyranosyl)acetic acid to aryl amines.

3.4 Ice recrystallization inhibition activity of *N*-aryl- α -D-pyranosyl-acetamides

N-aryl- α -D-pyranosyl-acetamides (**321-330**, **333** and **334**) were assessed for their ability to inhibit ice recrystallization using the splat cooling assay.^{1,18,19} The IRI activities of the *N*-aryl- α -D-glucosyl-acetamide derivatives are shown in Figure 3.4. The IRI activity of D-galactose, a moderate inhibitor of ice

recrystallization (64% MGS), is included for reference. The *N*-aryl- α -D-glucosyl-acetamide derivatives were moderate to weak inhibitors of ice recrystallization with activities ranging from 58 to 85% MGS. Aryl substituents (halogen and methoxy functional groups) were chosen due to the high IRI activity in the corresponding aryl glycosides.^{4,5}

It is interesting to note that the activity of the truncated AFGP analogue containing an alkyl chain length of six carbons had a moderate IRI activity of 58%, similar to D-galactose.³ A truncated AFGP analogue with an alkyl chain length of seven carbons showed dramatically increased IRI activity to 10%.³ This suggested that the size of the hydrophobic region was important for IRI activity. Since derivatives **321-324** contain only six carbons in the form of an aromatic ring, the effect of increasing the number of carbons to seven by incorporating a benzylic carbon was evaluated in compound **325**. Compound **325** also possessed the *para*-methoxy substituent. This substituent was found to be effective in aryl glycoside, β -PMP-Glc (**166**, 23% MGS).^{4,5} Unfortunately, this did not improve the IRI activity and **325** was actually less active ($p < 0.05$) than several derivatives without the extra carbon spacer.

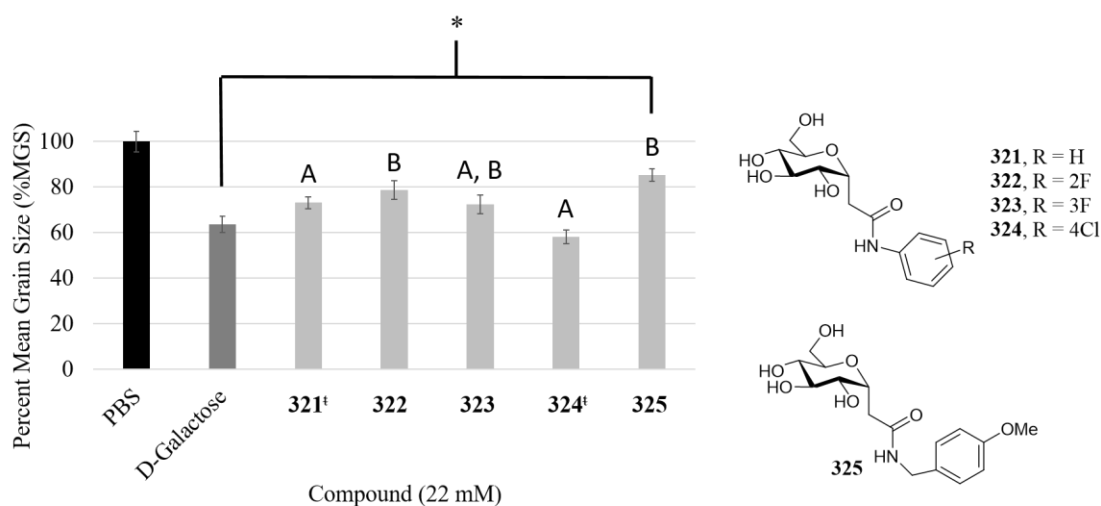


Figure 3.4. IRI activity of *N*-aryl- α -D-glucosyl-acetamide derivatives **321-325**. Compounds were assessed at 22 mM ([†]indicates assessed at 11 mM). Error bars represent standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed by the unpaired Student's T-test. Asterisks indicate significant difference compared to control. Statistical significance ($p < 0.05$) between compounds is indicated by different letters above bars in the graph.

The glucose derivatives possessed only moderate to weak IRI activity. Since D-galactose was the most IRI active monosaccharide that has been assessed by our lab^{1,15}, the D-galactose versions of these derivatives, *N*-aryl- α -D-galactosyl-acetamides (**326-330**) were assessed for IRI activity (Figure 3.5). The activities of compounds **326-330** range from 46% to 78%. The glucose derivatives **322**, **323** and **325** had IRI activities of 79%, 73% and 85% respectively while their galactose counterparts had increased activities of 47%, 51% and 53% for **327**, **328** and **330** respectively. The only difference between these molecules is the configuration of the stereocentre at C4. However, it was not surprising that the galactose derivatives are more active than glucose forms because it was previously shown that D-galactose is more IRI active than D-glucose as a result of its higher hydration index.^{1,15} This was the opposite of what was observed with the aryl glycosides.⁵

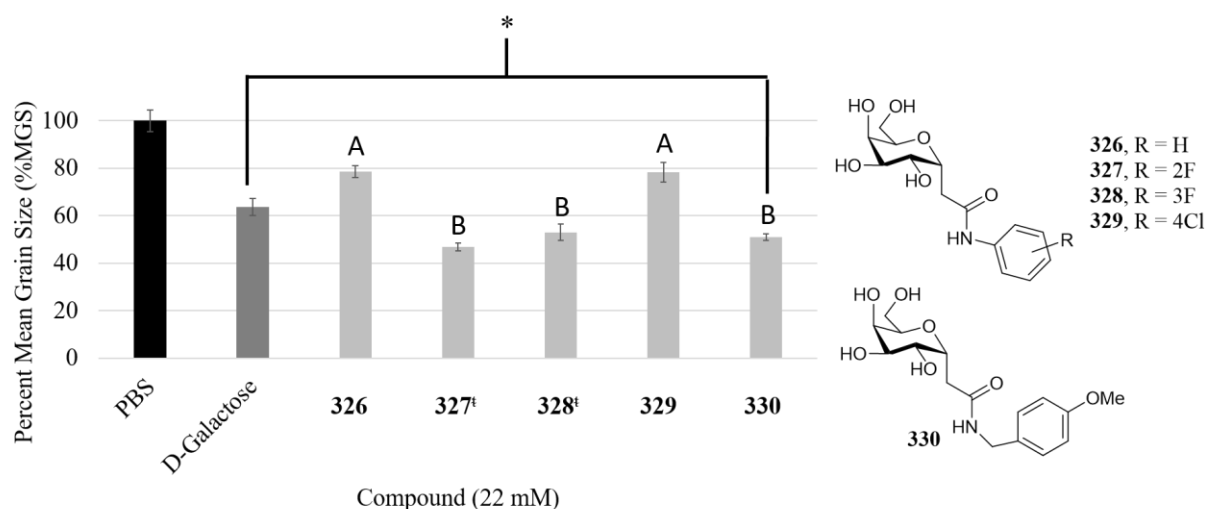


Figure 3.5. IRI activity of *N*-aryl- α -D-galactosyl-acetamide derivatives **326-330**. Compounds were assessed at 22 mM ([†]indicates assessed at 11 mM). Error bars represent standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed by the unpaired Student's T-test. Asterisks indicate significant difference compared to control. Statistical significance between compounds is indicated by different letters above bars in the graph.

Interestingly, compounds **327** and **330** were more IRI active than D-galactose and contained the same aryl substituents as the most active compounds in the aryl glycoside series. β -PMP-Glc (**166**) and β -pPPh-Glc (**170**) (Figure 3.1C) have IRI activities of 23%.⁴ While compounds **327** and **330** were the most

IRI active of the *N*-aryl- α -D-galactosyl-acetamides assessed, they were still less active than β -PMP-Glc (**166**) and β -pFPh-Glc (**170**). A major difference between the β -aryl glycoside and the *N*-aryl- α -D-pyranosyl-acetamide classes is the configuration at C1. Previous results showed that β -aryl glycosides were more IRI active than α -aryl glycoside derivatives. Therefore, the IRI activity of *N*-aryl- β -D-galactosyl-acetamides was hypothesized to be increased compared to the *N*-aryl- α -D-galactosyl-acetamides. The IRI activity of the *N*-aryl- β -D-galactosyl-acetamide derivatives is shown in Figure 3.6. There was no difference ($p > 0.05$) in IRI activity upon changing the configuration from α - to β -linkage at C1 when there were no aromatic substituents (**326** compared to **333**). However, in the fluorine substituted derivatives, the change in configuration from an α - (**327**) to a β -linkage (**334**) at C1 resulted in a decrease in IRI activity ($p < 0.001$). This was the opposite of what was observed in the aryl glycosides where a β -linkage was more favourable.

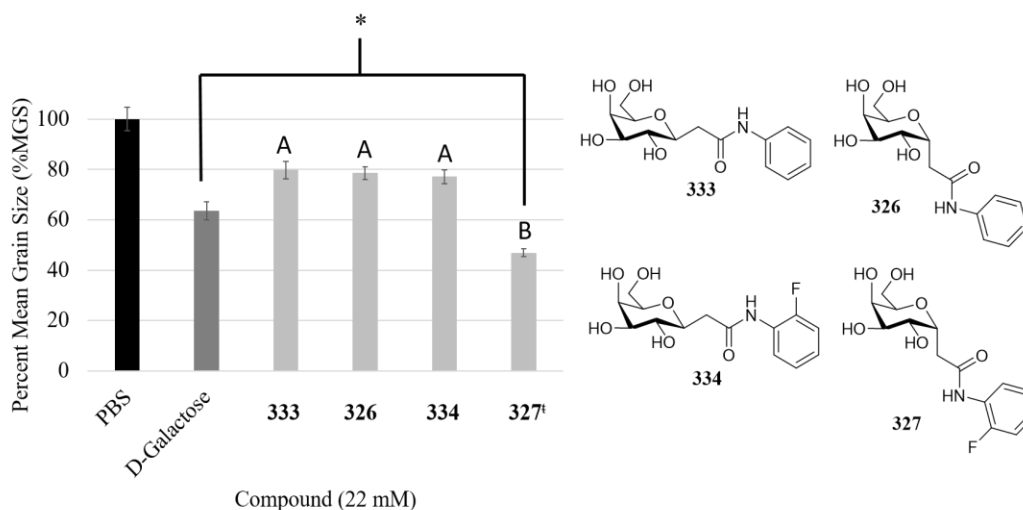


Figure 3.6. IRI activity of *N*-aryl- β -D-galactosyl-acetamide derivatives **333** and **334**. Compounds were assessed at 22 mM († indicates assessed at 11 mM). Error bars represent standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed by the unpaired Student's T-test. Asterisks indicate significant difference compared to control. Statistical significance ($p < 0.05$) between compounds is indicated by different letters above bars in the graph.

Overall, this study revealed the importance of the configuration at C1 and C4. It was found that an α -linkage at C1 was important for IRI activity. D-Galactose derivatives were more IRI active than D-glucose derivatives ($p < 0.05$). Furthermore, the presence of certain aromatic substituents had an effect on IRI activity. Derivatives with fluoro- or methoxy-substituents were more IRI active than derivatives with chloro- substituents. The compounds which possessed the highest IRI activity assessed in this chapter were galactose derivatives with an α -linkage at C1 and possessed either a fluorine substituent (**327** and **328**) or methoxy functional group (**330**) on the aromatic ring. This could be because this class more closely resembles the truncated AFGP analogues and OGG-Gal which require an α -C-linkage at C1 and galactose moiety for IRI activity.

3.5 Chapter summary

Truncated AFGP analogues with long alkyl chains were previously found to be potent inhibitors of ice recrystallization but their surfactant structure impeded their ability to act as novel cryoprotectants.³ It was found that aryl glycosides can be high IRI active and were significantly less toxic.⁵ In fact, several aryl glycosides were found to be effective cryoprotectants for RBCs (discussed in Chapter 7).⁴ It was therefore hypothesized that replacement of the alkyl chain in the truncated AFGPs with an aryl ring could result in a new class of small molecules with high IRI activity. *N*-aryl-D-pyranosyl-acetamides were rationally designed and assessed for IRI activity. The synthesis of the *N*-aryl- α -D-galactosyl-acetamide derivatives involved allyl 2,3,4,6-tetra-*O*-benzyl-D-pyranoside intermediates. The α -linked allyl derivatives were synthesized as previously described for the synthesis of glycoconjugate OGG-Gal (**102**).^{1,2} However, the β -linked allyl derivative (allyl 2,3,4,6-tetra-*O*-benzyl- β -D-galactopyranoside) required a revised synthesis using the Hosomi-Sakurai allylation to improve the overall yield.¹⁶ Subsequent oxidation and couplings furnished the desired targets.

Overall, the *N*-aryl-D-pyranosyl-acetamides possessed moderate to weak IRI activity. Compounds **327**, **328** and **330** possessed the highest activity with of 47, 51 and 53% MGS respectively. These were *N*-

aryl- α -D-galactosyl-acetamide derivatives. The α -D-galactose versions were more IRI active than the α -D-glucose derivatives ($p < 0.05$ assessed by unpaired Student's T-test). This may be attributed to the fact that D-galactose is more highly hydrated than D-glucose. In order to prove this, the calculation of hydration parameters for these molecules would be required. None of these compounds possessed a MGS $< 40\%$ and therefore were considered weakly active or inactive. The truncated AFGPs only exhibited IRI activities greater than D-galactose when the alkyl chain was very long. Therefore, perhaps the reason the *N*-aryl-D-pyranosyl-acetamides did not exhibit high IRI activity was because the hydrophobic moiety was too small.

In the truncated AFGP analogues, derivatives with longer alkyl chain lengths were more IRI active. Truncated AFGP analogues with alkyl chain lengths 7-10 carbons long were highly IRI active.³ Therefore, future work could involve the synthesis and IRI assessment of *N*-aryl- α -D-galactosyl-acetamides with an increased hydrophobic portion. This could be accomplished by using alkyl substituents on the aromatic ring or by increasing the length of the carbon spacer between the aromatic ring and the amide bond. The number of carbons in the hydrophobic portion should be between 7 and 10 carbons since truncated AFGP analogues with alkyl chain lengths 7-10 carbons were the most IRI active. Examples of future targets are shown in Figure 3.7.

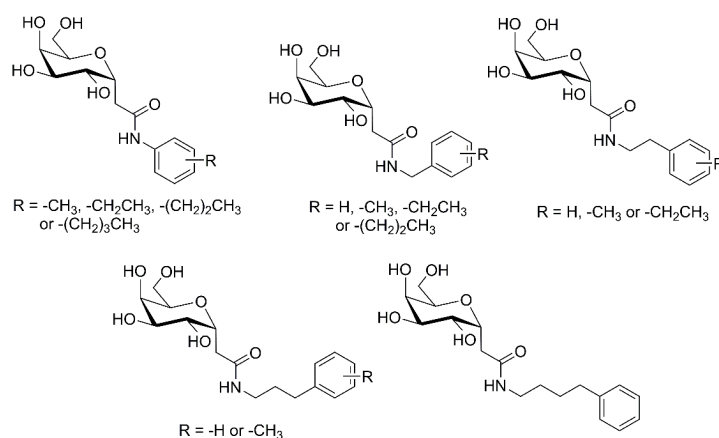


Figure 3.7. Future synthetic targets to assess the effect of increasing the hydrophobicity of the *N*-aryl- α -D-galactosyl-acetamides.

Compound **327** exhibited a reduction in IRI activity when the configuration at C1 was changed from an α - to a β -linkage (**334**) suggesting that the α -linkage at C1 was important for IRI activity. This was the opposite of what was observed in the aryl glycosides where a β -linkage was more favourable. However, the influence of the linkage at C1 in the truncated AFGP analogues has not been investigated. The *N*-aryl-D-pyranosyl-acetamides possess the same C-linkage and amide bond as the truncated AFGP analogues and were therefore more structurally similar to the truncated AFGP analogues than the aryl glycosides. In this chapter, it was found that the *N*-aryl-D-pyranosyl-acetamides with an α -linkage possessed higher IRI activity compared to those with a β -linkage. Therefore, it is hypothesized that modifying the configuration from an α - to a β -linkage at C1 in the truncated AFGP analogues would result in a decrease in IRI activity. Since the *N*-aryl-D-galactosyl-acetamides were more IRI active than the glucose derivatives it is hypothesized that glucose derivatives of the truncated AFGP analogues will also be less active. Future targets to assess the influence of the configuration at C1 and C4 on IRI activity are shown in Figure 3.8.

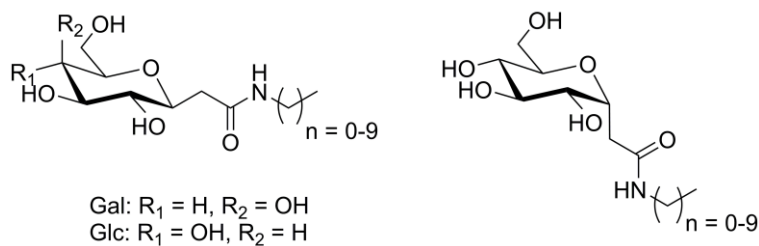


Figure 3.8. Future synthetic targets to assess the effect of configuration at C1 and C4 in the truncated AFGP analogues.

3.6 Experimental

3.6.1 Ice recrystallization inhibition (IRI) assay

IRI activity was performed using the “splat cooling” method. The analyte was dissolved in phosphate buffered saline (PBS) solution at a concentration of 22 mM. If the analyte was insoluble, serial dilutions to 11, 5.5 or 0.5 mM were performed. A 10 μ L droplet of this solution was dropped from a

height of two metres onto a block of polished aluminum precooled on dry ice to approximately -80 °C. The droplet froze instantly forming a wafer on the polished aluminum block that was approximately 1 cm in diameter and 20 µm thick. This wafer was then carefully removed from the surface of the block and transferred onto a circular glass coverslip and to a cryostage held at -6.4 °C for annealing. After a period of 30 minutes, the wafer was photographed between crossed polarizing filters using a digital camera (Nikon CoolPix 5000) fitted to the microscope. A total of three images were taken from each wafer and each solution was tested three times.

The initial ice crystals formed during flash freezing are relatively homogeneous in size and quite small. Recrystallization occurs during the 30 minute annealing cycle, resulting in an increase in ice crystal size. After the annealing process, images captured the frozen wafer and ice crystal sizes were obtained using a novel domain recognition software (DRS) program. This processing employed the Microsoft Windows Graphical User Interface to allow a user to visually demarcate and store the vertices of ice domains in a digital micrograph. The data was then used to calculate the domain areas. A total of 12 domain areas were obtained for nine images ($n = 108$). All data was plotted and analyzed using Microsoft Excel. The mean grain (or ice crystal) size (MGS) of the sample was compared to the MGS of the control PBS solution for that same day of testing and IRI activity is reported as the percentage of the MGS (%MGS) relative to the PBS control. Therefore, small percentages represent small ice crystals and this is indicative of high IRI activity. The %MGS for each sample was plotted along with its standard error of the mean (SEM). %MGS was calculated as the mean of three samples of the mean of three fields of view of the mean of 12 ice crystal areas. Statistical significance for all data was determined by unpaired Student's t-test with a 95% confidence level ($p < 0.05$ indicated with an asterisk).

3.6.2 General experimental

Anhydrous reactions were performed under a positive pressure of argon in flame-dried glassware. Air- or moisture-sensitive reagents and anhydrous solvents were transferred with oven-dried syringes.

Reactions were monitored using analytical thin layer chromatography (TLC) with 0.2 mm pre-coated silica gel aluminum plates 60 F254 (E. Merck). Components were visualized by illumination with a short-wavelength (254 nm) ultra-violet light and/or staining (ceric ammonium molybdate or orcinol/sulphuric acid stain solution). Flash column chromatography was performed with E. Merck silica gel 60 (230-400 mesh). ^1H (300, 400 or 500 MHz) and ^{13}C NMR (100 or 125 MHz) spectra were recorded on a Bruker Avance 400 or Bruker Avance 500. Deuterated chloroform (CDCl_3), DMSO ($\text{DMSO-}d_6$), methanol (MeOD), benzene (C_6D_6) or water (D_2O) were used as NMR solvents. Chemical shifts are reported in ppm using the solvent residual peak as an internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet and br, broad. Low resolution mass spectrometry (LRMS) was performed on a Micromass Quatro-LC Electrospray spectrometer with a pump rate of 20 $\mu\text{L}/\text{min}$ using electrospray ionization (ESI).

Compounds **102**, **129-132**, **166**, **170** and **171** were synthesized and assessed for IRI activity by previous members of the Ben laboratory.^{1,3-5} NMR spectra for novel compounds and final compounds assessed for IRI activity are provided.

3.6.3 Compound characterization and NMR spectra

Allyl 2,3,4,6-tetra-O-acetyl- α -D-glucopyranoside (302)

Acetobromo- α -D-glucose (1.03 g, 2.5 mmol) was dissolved in dry DCM (5 mL). Allyltributyltin (1.96 mL, 6.27 mmol) and 1 M triethyl borane (0.25 mL, x 0.1 mmol of acetobromo- α -D-glucose) was added to the dry solution. After the dry additions, oxygen from the atmosphere (20 mL) was added at a rate of 5 mL/hour via syringe pump. The reaction was monitored for completion by TLC (6:2:2 toluene, petroleum ether, ethyl acetate). After completion, the DCM was evaporated under reduced pressure. Acetonitrile (40 mL) was added and washed with pentane (3 x 40 mL). The acetonitrile layer was evaporated under reduced pressure and the product purified by flash chromatography (6:2:2 toluene, petroleum ether, ethyl acetate) to yield **302** a white solid (0.58 g, 62%). Characterization is consistent with previously published

results.¹ ¹H NMR (400 MHz, CDCl₃): δ 5.68-5.68 (m, 1H), 5.32 (t, J = 9.2 Hz, 1H), 5.05-5.16 (m, 3H), 4.96 (dd, J = 8.8, 9.5 Hz, 1H), 4.24-4.29 (m, 1H), 4.19 (dd, J = 5.4, 12.1 Hz, 1H), 4.06 (dd, J = 2.7, 12.0 Hz, 1H), 3.82-3.86 (m, 1H), 2.49-2.58 (m, 1H), 2.29-2.35 (m, 1H), 2.01-2.06 (m, 12H). ¹³C NMR (100 MHz, CDCl₃): δ 170.8, 170.4, 169.9, 169.8, 133.2, 118.1, 72.1, 70.6, 70.5, 69.0, 62.5, 30.8, 20.9, 20.9, 20.9, 20.9. LRMS (ESI): m/z calcd. for C₁₇H₂₄NaO₉ [M + Na]⁺ 395.4, found 394.9.

(2,3,4,6-tetra-O-acetyl- α -D-glucopyranosyl)acetic acid (303)

Compound **302** (0.58 g, 1.56 mmol) was dissolved in DCM (4 mL), acetonitrile (4 mL) and distilled water (6 mL). Sodium periodate (2.0 g, 9.36 mmol) and ruthenium trichloride (10 mg, 0.05 mmol, 3 mol % of **302**) were added and the mixture was allowed to stir for 16 hours. Reaction completion was monitored by TLC (8:2 DCM with ethyl acetate). Once complete by TLC, the product was extracted in DCM (40 mL) and washed with 1% HCl (40 mL). The aqueous phase was re-extracted with DCM (20 mL). The organic layers were combined and washed twice more with 1% HCl (40 mL). Saturated sodium chloride was added as needed to separate layers. The organic phase was dried over magnesium sulfate and concentrated under reduced pressure. The product was purified by flash chromatography using a gradient solvent system from 8:2 to 6:4 DCM with ethyl acetate. The product **303** was obtained as a colourless sticky solid (0.43 g, 71%). Characterization is consistent with previously published results.¹ ¹H NMR (400 MHz, CDCl₃): δ 5.24 (t, J = 8.7 Hz, 1H), 5.14 (dd, J = 5.6, 9.0 Hz, 1H), 4.98 (t, J = 8.7 Hz, 1H), 4.64-4.69 (m, 1H), 4.22 (dd, J = 5.3, 12.2 Hz, 1H), 4.09 (dd, J = 2.8, 12.3 Hz, 1H), 4.90-4.94 (m, 1H), 2.78 (dd, J = 9.3, 15.5 Hz, 1H), 2.67 (dd, J = 5.4, 15.6 Hz, 1H), 2.02-2.06 (m, 12H). ¹³C NMR (100 MHz, CDCl₃): δ 175.3, 170.9, 170.3, 169.7, 169.6, 70.3, 70.2, 69.7, 69.4, 68.4, 62.1, 33.0, 20.9, 20.8, 20.8. LRMS (ESI): m/z calcd. for C₁₆H₂₂NaO₁₁ [M + Na]⁺ 413.3, found 412.9.

Allyl 2,3,4,6-tetra-O-acetyl- α -D-galactopyranoside (305)

Acetobromo- α -D-galactose (2.0 g, 4.86 mmol) was dissolved in dry DCM (5 mL). Allyltributyltin (3.80 mL, 12.16 mmol) and 1 M triethyl borane (0.49 mL, x 0.1 mmol of acetobromo- α -D-galactose) was added

to the dry solution. After the dry additions, oxygen from the atmosphere (20 mL) was added at a rate of 5 mL/hour via syringe pump. The reaction was monitored for completion by TLC (6:4:1 toluene, petroleum ether, ethyl acetate). Once complete, the DCM was evaporated under reduced pressure. Acetonitrile (50 mL) was added and washed with pentane (3 x 50 mL). The acetonitrile layer was evaporated under reduced pressure and the product purified by flash chromatography (6:4:1 toluene, petroleum ether, ethyl acetate) to yield **305** as a white solid (0.58 g, 56%). Characterization is consistent with previously published results.¹ ¹H NMR (400 MHz, CDCl₃): δ 5.71 (m, 1H), 5.38 (dd, J = 3.1, 2.4 Hz, 1H), 5.24 (dd, J = 9.3, 5.1 Hz, 1H), 5.18 (dd, J = 9.3, 3.3 Hz, 1H), 5.08 (m, 2H), 4.26 (ddd, J = 9.9, 5.1, 4.7 Hz, 1H), 4.17 (dd, J = 12.2, 8.7 Hz, 1H), 4.05 (m, 2H), 2.44 (m, 1H), 2.24 (m, 1H), 2.08 (s, 3H), 2.03 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H). ¹³C NMR (100 MHz, CDCl₃): δ 170.6, 170.1, 169.9, 169.8, 133.3, 117.7, 71.4, 68.3, 67.9, 67.6, 61.5, 30.9, 20.8, 20.7, 20.7. LRMS (ESI): m/z calcd. for C₁₇H₂₅O₉ [M+H]⁺ 373.4, found 373.1.

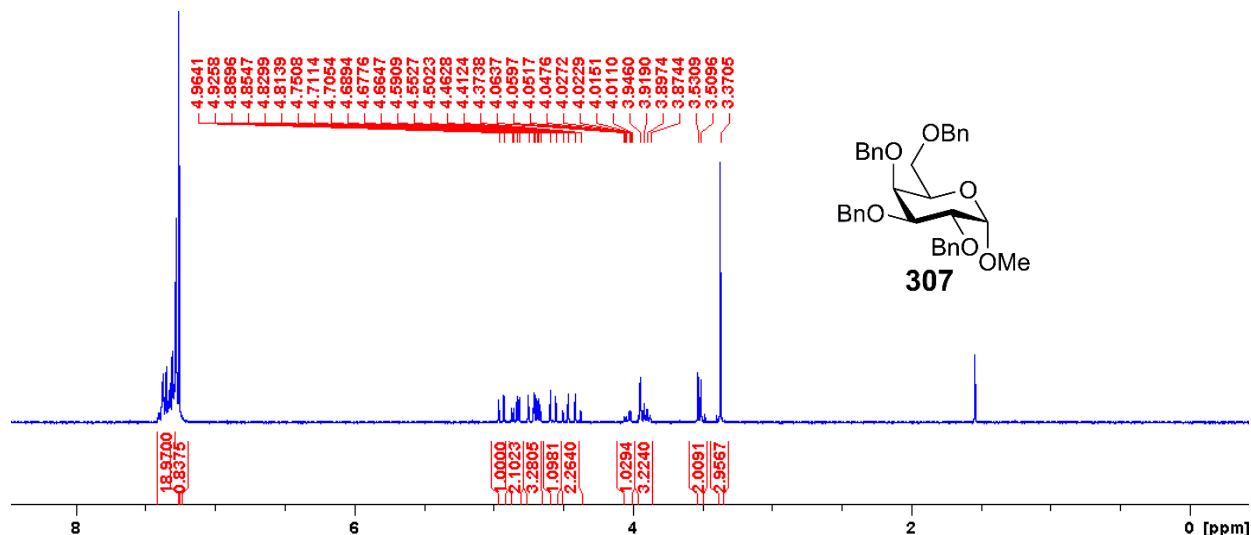
(2,3,4,6-tetra-O-acetyl- α -D-galactopyranosyl)acetic acid (306)

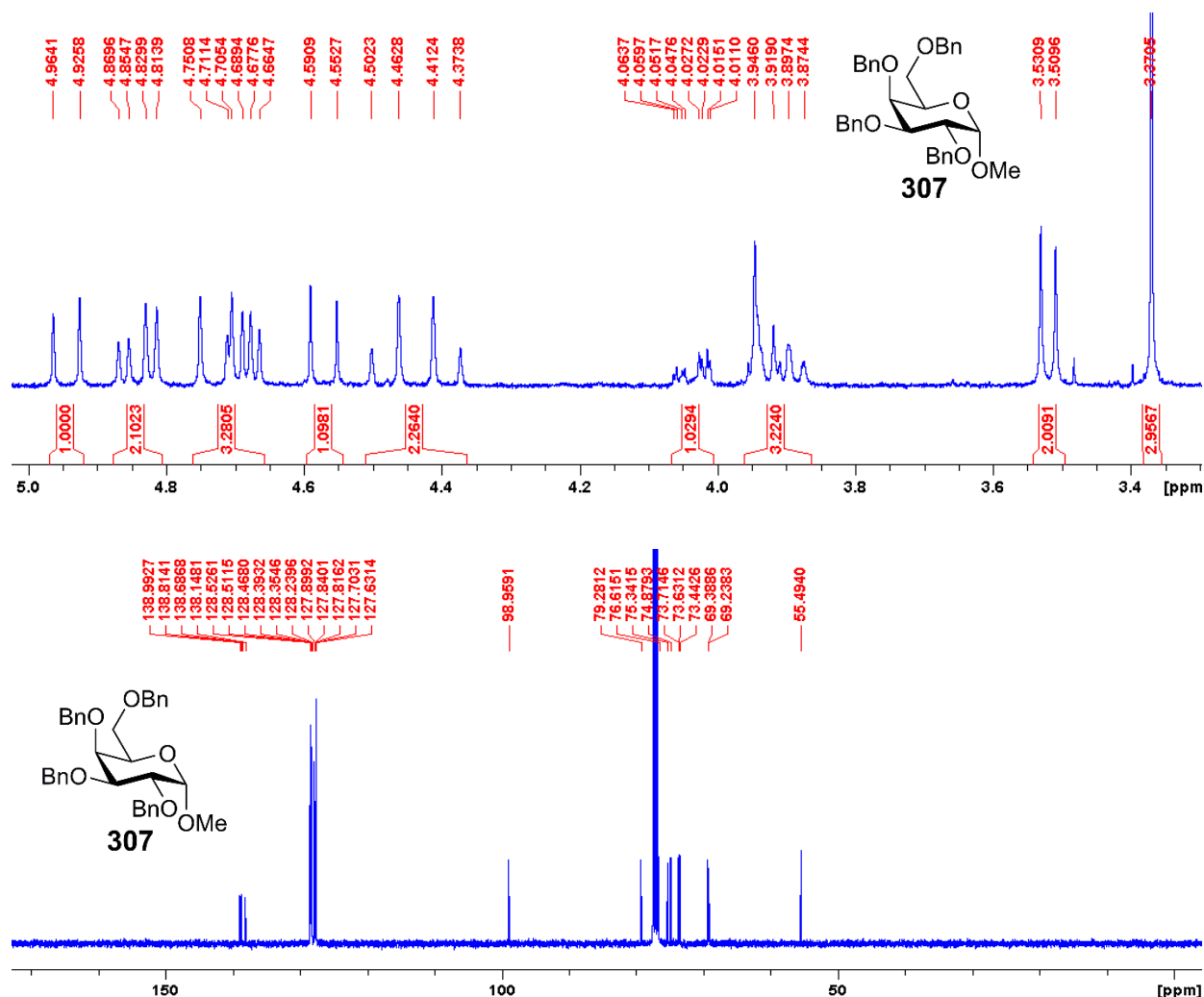
Compound **305** (1.0 g, 2.7 mmol) was dissolved in DCM (4 mL), acetonitrile (4 mL) and distilled water (6 mL). Sodium periodate (3.44 g, 16.1 mmol) and ruthenium trichloride (17 mg, 0.08 mmol, 3 mol % of **305**) were added and the mixture was allowed to stir for 16 hours. Reaction completion was monitored by TLC (8:2 DCM with ethyl acetate). Once complete by TLC, the product was extracted in DCM (40 mL) and washed with 1% HCl (40 mL). The aqueous phase was re-extracted with DCM (20 mL). The organic layers were combined and washed twice more with 1% HCl (40 mL). Saturated sodium chloride was added as needed to separate layers. The organic phase was dried over magnesium sulfate and concentrated under reduced pressure. The product was purified by flash chromatography using a gradient solvent system from 8:2 to 6:4 DCM with ethyl acetate. Product **306** was obtained as a colourless sticky solid (0.43 g, 71%). Characterization is consistent with previously published results.¹ ¹H NMR (400 MHz, CDCl₃): δ 5.43 (t, J = 2.8 Hz, 1H), 5.33 (dd, J = 8.9, 5.0 Hz, 1H), 5.17 (dd, J = 8.9, 3.3 Hz, 1H), 4.70 (ddd, J = 9.3, 8.3, 5.3 Hz, 1H), 4.30-4.08 (m, 3H), 2.73 (dd, J = 15.6, 8.7 Hz, 1H), 2.63 (dd, J = 15.6, 5.7 Hz, 1H), 2.13 (s, 3H), 2.07 (s, 3H), 2.04 (s, 6H). ¹³C NMR (100 MHz, CDCl₃): δ 175.5, 170.8, 170.1,

170.0, 169.7, 69.4, 68.9, 67.8, 67.6, 67.1, 61.2, 33.15, 20.7. LRMS (ESI): m/z calcd. for $C_{16}H_{22}NaO_{11}$ [$M + Na$] $^{+}$ 413.3, found 413.1.

2,3,4,6-Tetra-*O*-benzyl- α -methyl-galacto-*D*-pyranoside (**307**)

Dry DMF (100 mL) was added to a flame-dried flask and cooled to 0°C. 60% NaH in mineral oil (7.13 g, 179 mmol) was added slowly with stirring. Benzyl bromide (23 g, 134 mmol) was added dropwise followed by the slow addition of α -D-methoxygalactopyranose (5 g, 22.4 mmol) (100 mg per 10 minutes). The solution was brought to room temperature and stirred for 48 h while monitoring by TLC (2% EtOAc in DCM). The reaction was quenched with H_2O , then extracted in DCM with brine, and dried over $MgSO_4$. The product was purified by silica gel column chromatography (1:9 to 3:7 EtOAc:hexanes) and **307** was obtained as a clear oil (10.5 g, 85%). Characterization is consistent with previously published results.¹⁶ 1H NMR (300 MHz, $CDCl_3$): δ 7.41-7.24 (m, 20H), 4.95 (d, $J = 11.3$ Hz, 1H), 4.84 (dd, $J = 4.5$, 12.0 Hz, 2H), 4.71 (m, 3H), 4.57 (d, $J = 11.5$ Hz, 1H), 4.44 (q, $J = 11.6$, 15.1, 2H), 4.04 (m, 1H), 3.91 (m, 3H), 3.52 (d, $J = 6.4$ Hz, 2H), 3.37 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 139.0, 138.4, 138.7, 138.2, 128.5, 128.5, 128.5, 128.4, 128.4, 128.2, 127.9, 127.8, 127.8, 127.7, 127.6, 99.0, 79.3, 76.6, 75.3, 74.9, 73.7, 73.6, 73.4, 69.4, 69.2, 55.5. LRMS (ESI): m/z calcd. for $C_{35}H_{38}O_6Na$ [$M + Na$] $^{+}$ 577.3, found 577.2.

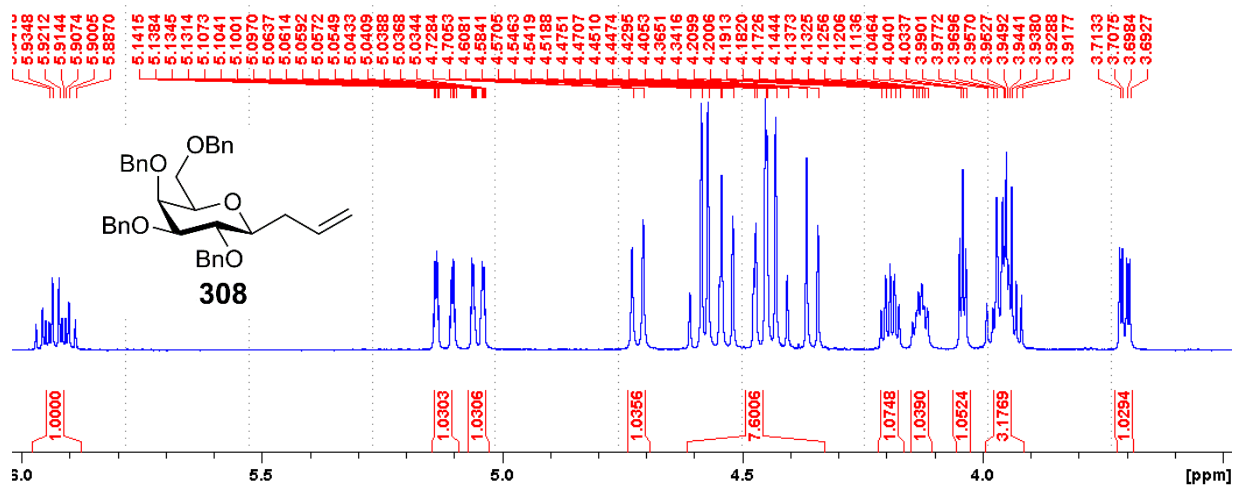
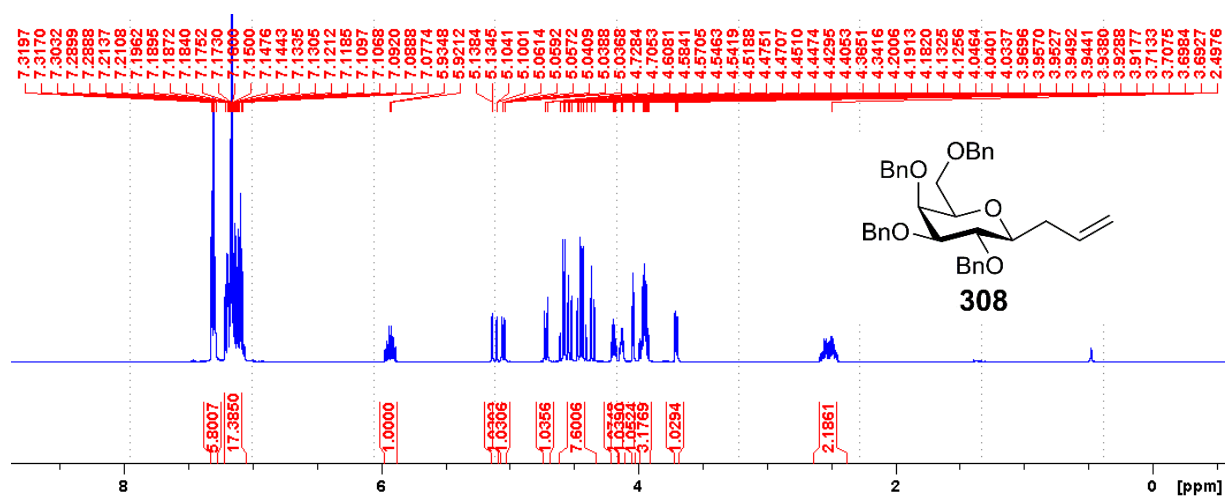


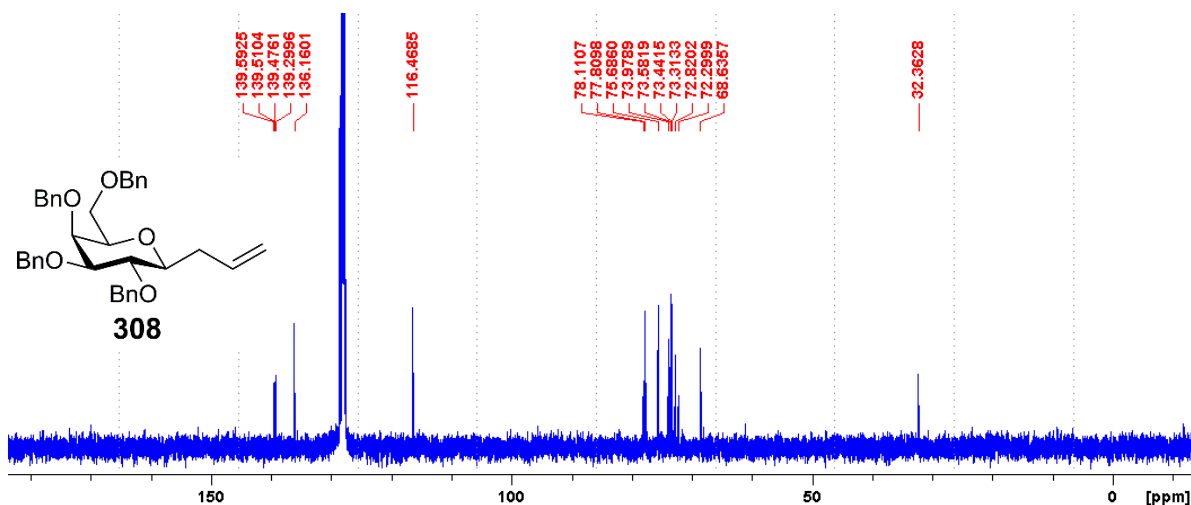


Allyl-2,3,4,6-tetra-O-benzyl-β-D-galactopyranoside (308)

Compound **307** (10.5 g, 18.9 mmol) was dissolved in dry ACN (80 mL). Allyltrimethylsilane (9 mL, 56.8 mmol) was added and the solution was stirred at ambient temperature for 30 minutes. The solution was cooled to 0°C and TMSOTf (2.5 mL, 14.3 mmol) was added dropwise. The solution was allowed to warm to room temperature and stirred under Ar for 18 hours while monitoring reaction completion by TLC (8:2 PE:Et₂O). The reaction was quenched with Et₂O and washed with NaHCO₃, H₂O, and brine, then dried over MgSO₄ and filtered. The crude product was purified by silica gel chromatography (8:2 PE:Et₂O) to obtain the pure β-anomer **308** as a pale yellow oil (3.74 g, 35%). Characterization is consistent with previously published results.¹⁶ ¹H NMR (500 MHz, C₆D₆): δ 7.33-7.28 (m, 6H), 7.22-7.06

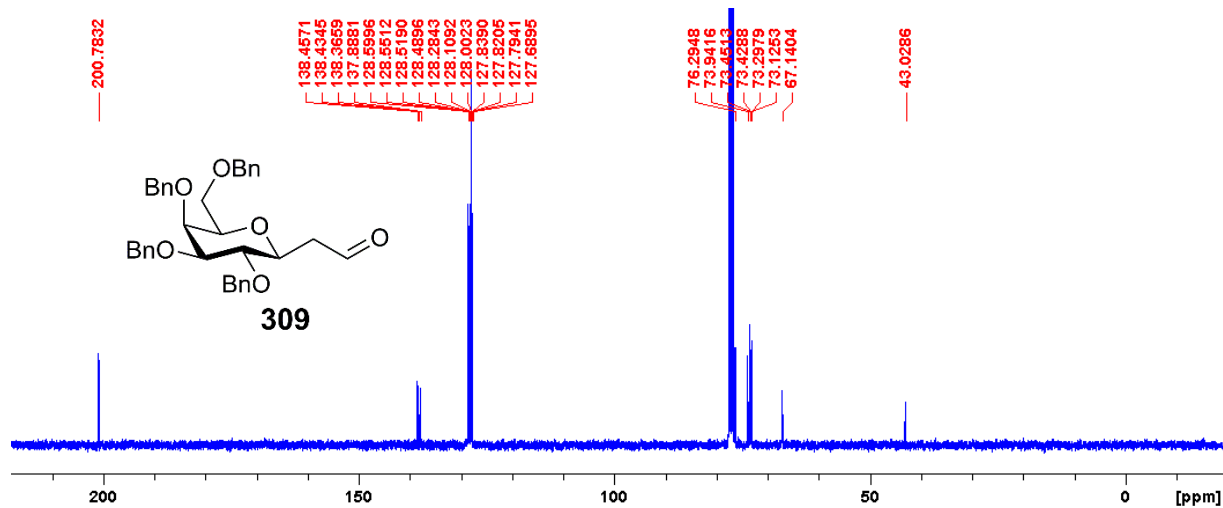
(m, 14H), 5.93 (ddt, $J = 17.2, 6.9, 10.3$ Hz, 1H), 5.12 (ddd, $J = 17.1, 3.5, 1.6$ Hz, 1H), 5.05 (ddt, $J = 10.2, 2.2, 1.1$ Hz, 1H), 4.60-4.33 (m, 8H), 4.19 (quint, $J = 4.7$ Hz, 1H), 4.13 (m, 1H), 4.04 (t, $J = 3.2$ Hz, 1H), 3.99-3.91 (m, 3H), 3.70 (dd, $J = 2.8, 7.4$ Hz, 1H), 2.59-2.45 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3 , 60°C): δ 138.85, 138.7, 138.6, 138.4, 135.3, 128.5, 128.5, 128.5, 128.4, 128.1, 128.0, 127.9, 127.9, 127.7, 127.7, 127.6, 116.9, 76.6, 74.4, 73.3, 73.2, 73.2, 72.7, 71.0, 67.4, 32.4. LRMS (ESI): m/z calcd. for $\text{C}_{37}\text{H}_{40}\text{O}_5\text{Na}$ $[\text{M} + \text{Na}]^+$ 587.3, found 587.2.





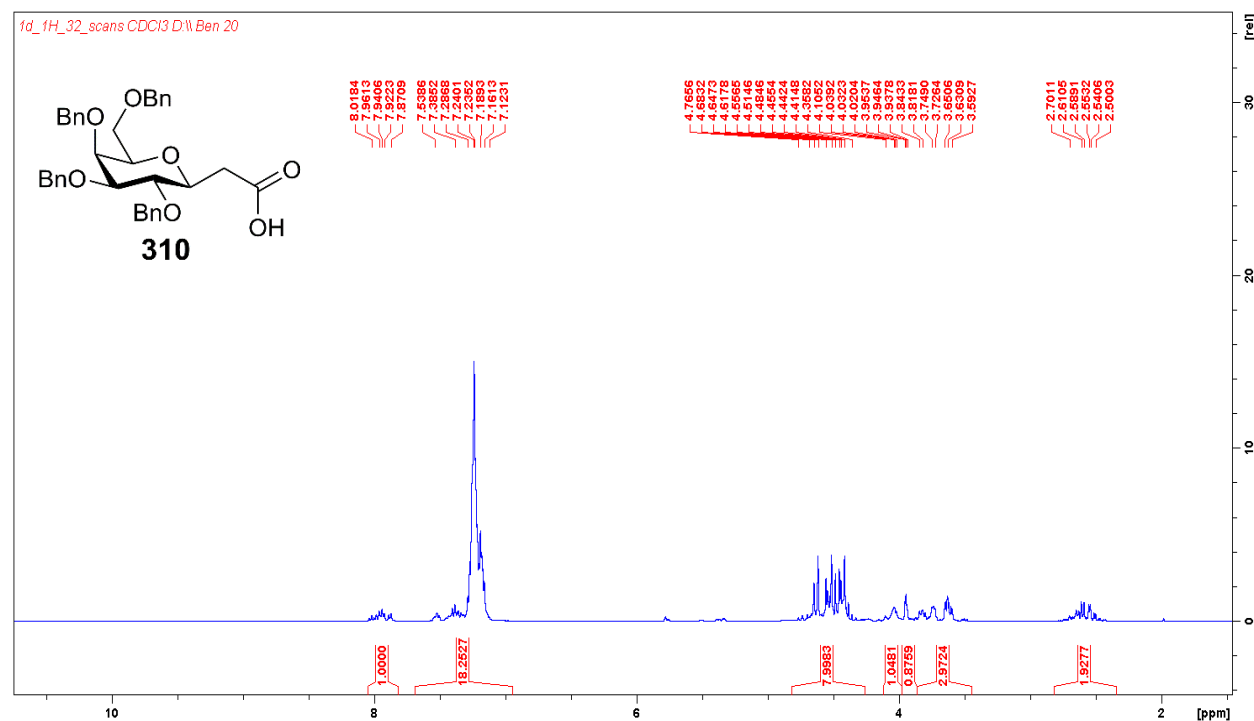
2,3,4,6-Tetra-O-benzyl- β -D-galactopyranosal (**309**)

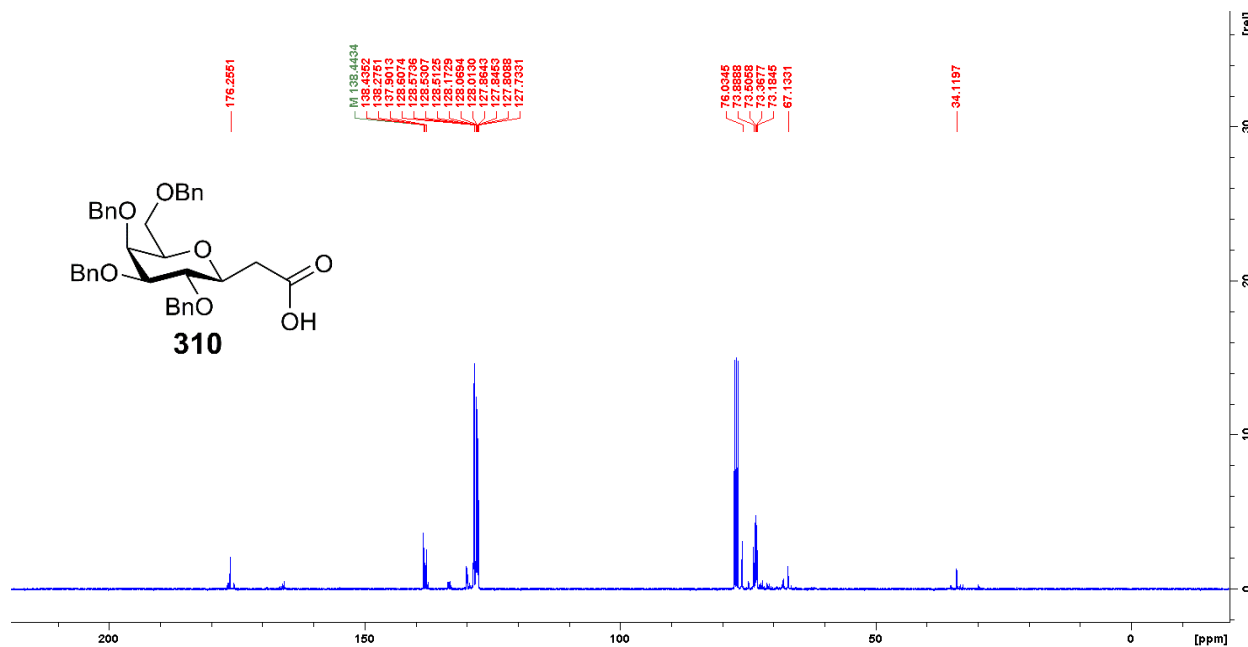
Compound **308** (3.64 g, 6.45 mmol) was dissolved in 3:1 THF:H₂O (72 mL). NaIO₄ (5.5 g, 25.8 mmol), OsO₄ (32 mg, 0.129 mmol), and 2,6-lutidine (1.38 g, 12.9 mmol) were added and the solution was stirred at RT for 1 h while monitoring by TLC (7:3 Et₂O:hexanes). Upon completion, the reaction was quenched with sodium thiosulfate (4.8 g, 30.5 mmol) and extracted with EtOAc and washed with brine, then dried over MgSO₄ and filtered. The pure product **309** was obtained as a clear oil from silica gel column chromatography (2:8 $\rightarrow$ 1:1 Et₂O:hexanes) (2.90 g, 79%). Characterization is consistent with previously published results.²⁰ ¹H NMR (400 MHz, CDCl₃): δ 9.70 (t, J = 2.2 Hz, 1H), 7.38-7.23 (m, 20H), 4.70 (d, J = 11.9 Hz, 2H), 4.53 (m, 7H), 4.04 (m, 1H), 4.00 (dd, J = 2.8, 3.9 Hz, 1H), 3.85 (dd, J = 7.5, 10.4, 1H), 3.79 (dd, J = 3.9, 6.6 Hz, 1H), 3.70 (dd, J = 2.7, 6.7 Hz, 1H), 3.65 (dd, J = 4.6, 10.6 Hz, 1H), 2.63 (dd, J = 2.1, 6.7 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃): δ 200.8, 138.5, 138.3, 138.4, 137.9, 128.6, 128.6, 128.5, 128.5, 128.3, 128.1, 128.0, 127.8, 127.8, 127.8, 127.7, 76.3, 73.9, 73.5, 73.4, 73.3, 73.3, 73.1, 67.1, 43.0. LRMS (ESI): m/z calcd. for C₃₆H₃₈O₆Na [M + Na]⁺ 589.3, found 589.2.



(2,3,4,6-Tetra-O-benzyl- β -D-galactopyranosyl)acetic acid (310)

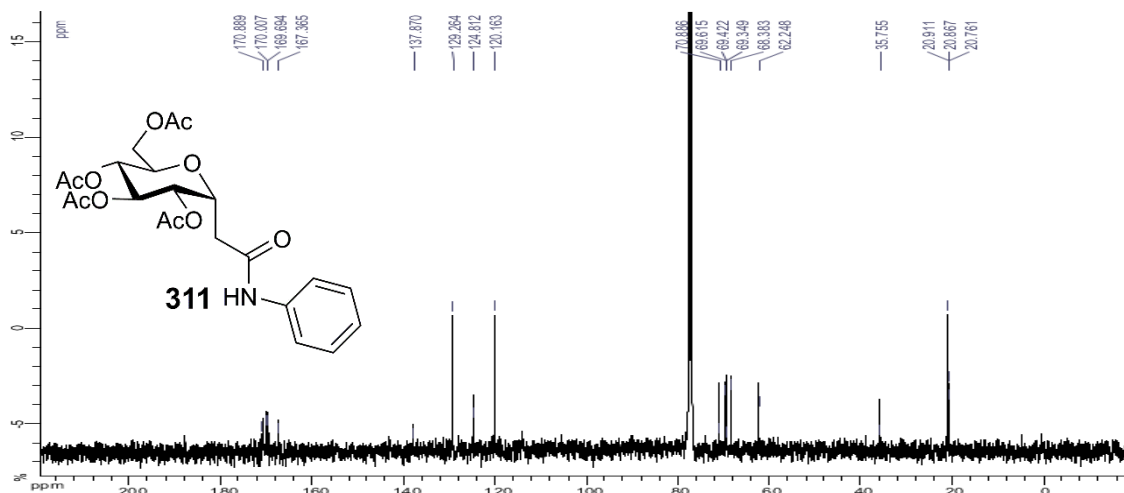
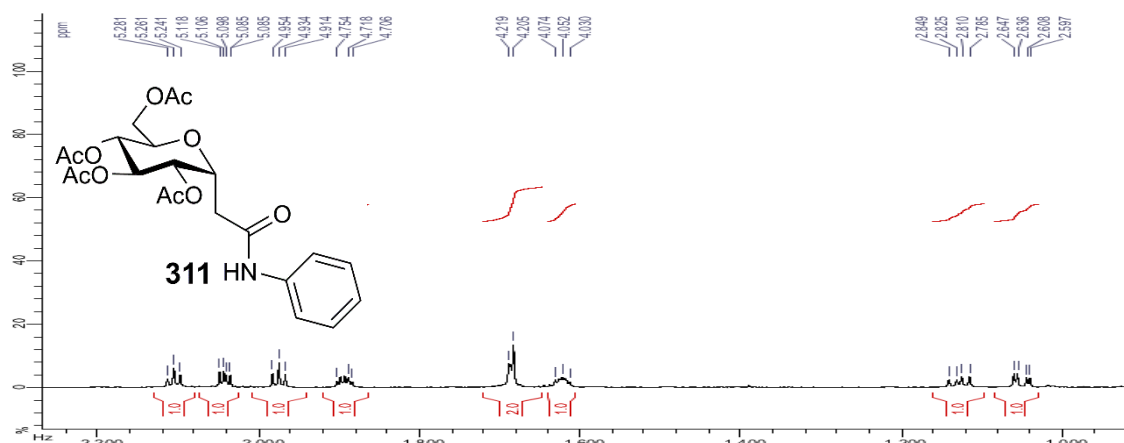
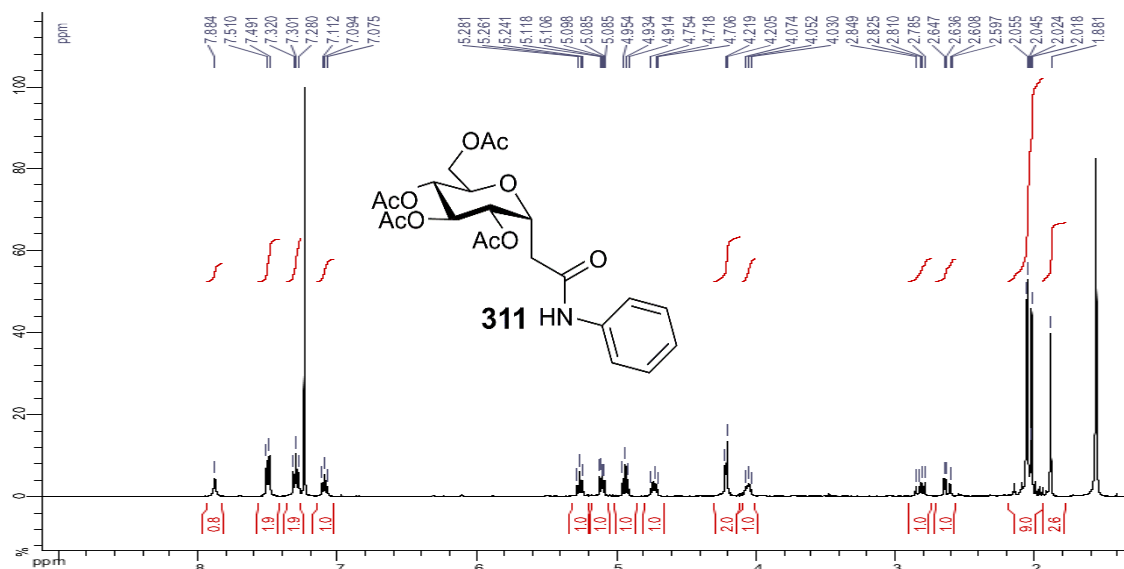
Compound **309** (2.85 g, 5.03 mmol) was dissolved in *t*-BuOH (120 mL). H₂O was added (60 mL), followed by the addition of 2-methyl-2-butene (1.93 g, 27.6 mmol), monopotassium phosphate (6.16 g, 45.3 mmol), and sodium hypochlorite (4.37 g, 48.3 mmol). The mixture was stirred at RT while monitoring by TLC (6:4 hexanes:EtOAc). After 30 min, no starting material remained and the solvent was removed under reduced pressure. The mixture was diluted with H₂O, acidified with 10% HCl to pH = 2, and extracted five times in EtOAc. The organic layers were combined, dried over MgSO₄, filtered, and the solvent was evaporated under reduced pressure. Silica gel column chromatography (2% to 5% MeOH in DCM) resulted in pure product **310** as a clear oil (1.75 g, 60%). ¹H NMR (400 MHz, CDCl₃): δ 7.87-8.02 (m, 1H), 7.12-7.54 (m, 18 H), 4.36-4.77 (m, 8H), 4.02-4.11 (m, 1H), 3.94-3.95 (m, 1H), 3.59-3.84 (m, 3H), 2.50-2.70 (m, 2H). ¹³C NMR (100 MHz, CDCl₃): δ 176.3, 138.4, 138.4, 138.3, 137.9, 128.6, 128.6, 128.5, 128.5, 128.2, 128.1, 128.0, 127.7, 127.9, 127.8, 127.7. LRMS (ESI): *m/z* calcd. for C₃₆H₃₈O₇Na [M + Na]⁺ 605.3, found 605.2.





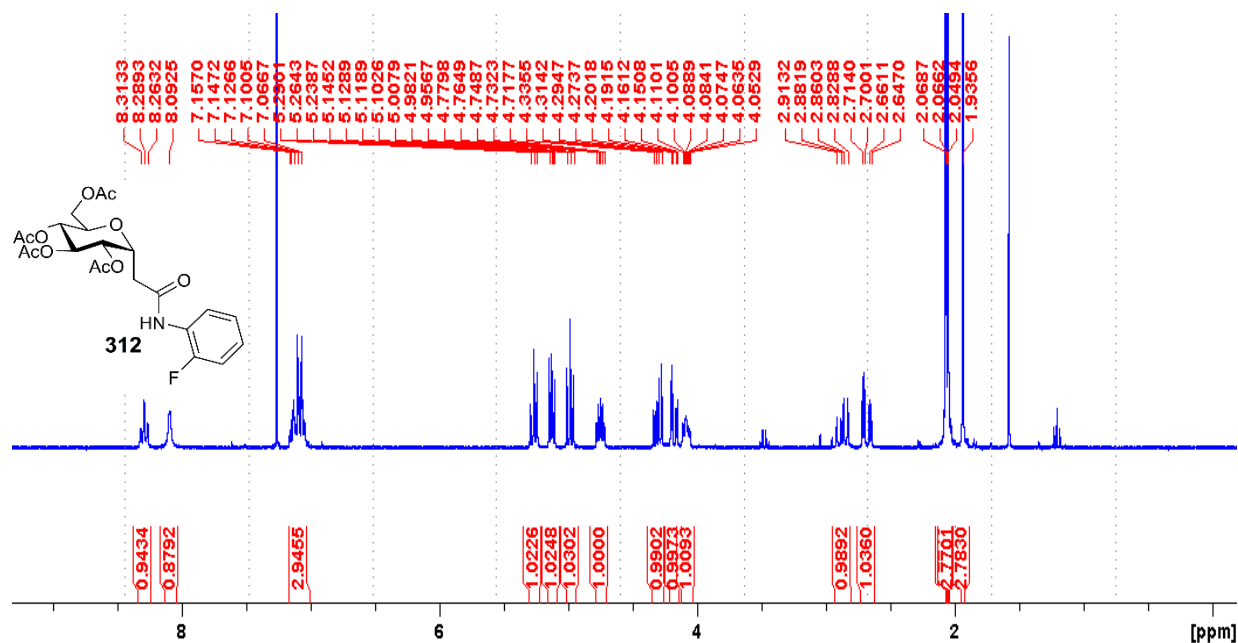
***N*-(Phenyl)-2-(2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranosyl)acetamide (311)**

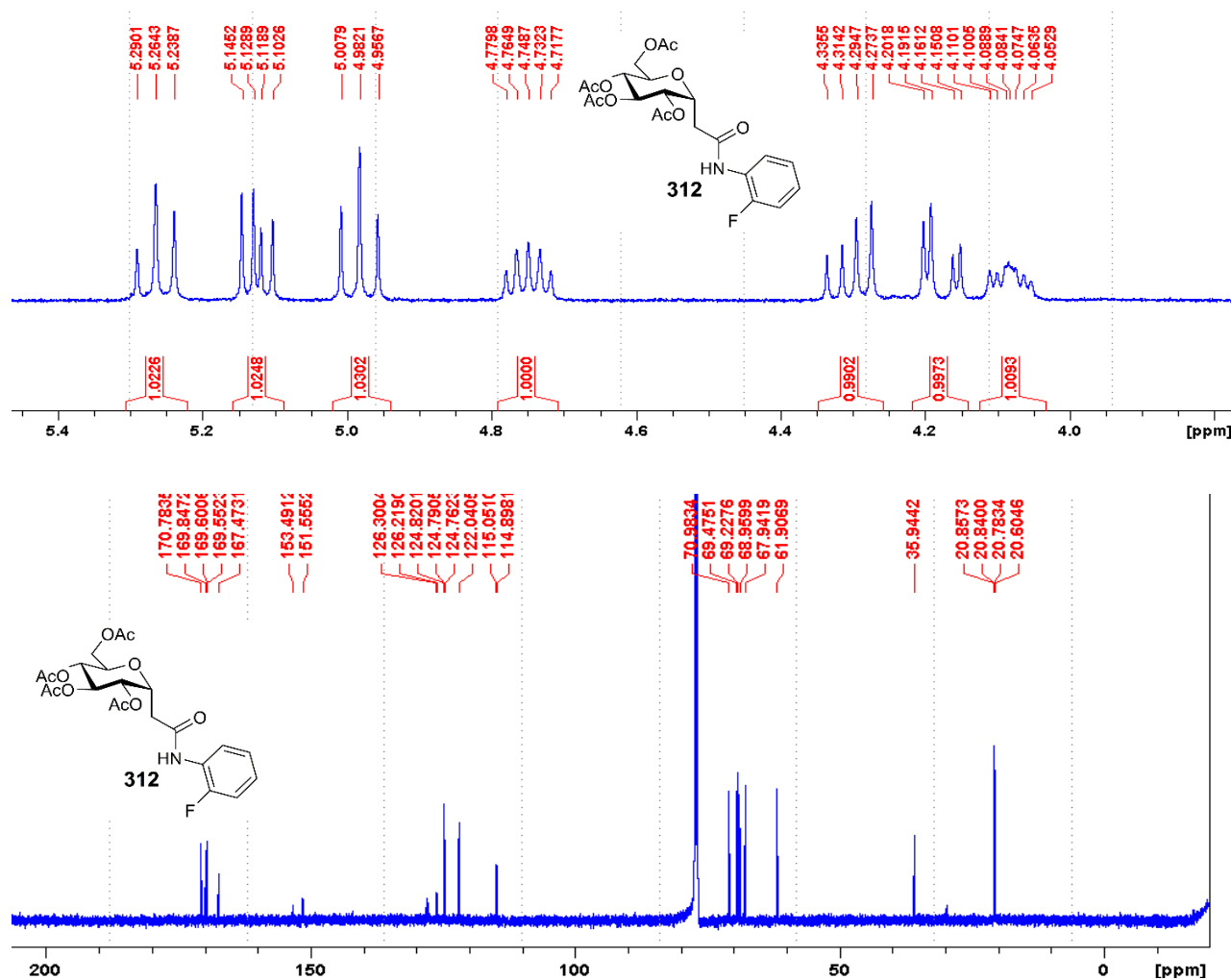
Compound **303** (170 mg, 0.44 mmol) was dissolved in dry DCM. HCTU (182 mg, 0.44 mmol) and DIPEA (100 μ L, 0.44 mmol) were added and the resulting solution was stirred for 15 min at RT. Aniline (0.12 mL, 1.32 mmol) and DIPEA (100 μ L, 0.44 mmol) were added and the resulting mixture was stirred overnight. The mixture was washed three times each with 20% HCl, NaHCO₃, brine, and extracted in DCM. The product **311** was isolated by flash chromatography (1% to 2% MeOH in DCM), and then recrystallized in Et₂O to obtain a white solid (140 mg, 68%). ¹H NMR (400 MHz, CDCl₃): δ 7.884 (br. s, 1H), 7.50 (d, J = 7.8 Hz, 2H), 7.30 (t, J = 7.7 Hz, 2H), 7.09 (t, J = 7.4 Hz, 1H), 5.26 (t, J = 7.9 Hz, 1H), 5.1 (dd, J = 5.1, 8.2 Hz, 1H), 4.93 (t, J = 7.9 Hz, 1H), 4.71-4.75 (m, 1H), 4.21-4.22 (m, 2H), 4.03-4.07 (m, 1H), 2.82 (dd, J = 9.7, 15.8 Hz, 1H), 2.63 (dd, J = 15.7, 4.3 Hz, 1H), 2.02-2.06 (m, 9H), 1.88 (s, 3H). ¹³C NMR (100 MHz, CDCl₃): δ 170.9, 170.0, 169.7, 167.4, 137.9, 129.3, 124.8, 120.2, 70.9, 69.6, 69.4, 69.4, 68.4, 62.3, 35.8, 20.9, 20.9, 20.8. LRMS (ESI): m/z calcd. for C₂₂H₂₇NNaO₁₀ [M + Na]⁺ 488.4, found 488.0.



***N*-(2-Fluorophenyl)-2-(2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranosyl)acetamide (**312**)**

Compound **303** (124 mg, 0.318 mmol) was dissolved in 15 mL dry DCM. HCTU (131 mg, 0.318 mmol) and DIPEA (55 μ L, 0.318 mmol) were added and the solution was stirred for 15 min at RT. 2-Fluoroaniline (60 μ L, 0.635 mmol) and DIPEA (55 μ L, 0.318 mmol) were added and the resulting mixture was stirred overnight. The mixture was washed with 20% HCl, NaHCO₃, brine, and extracted in DCM. The pure product was obtained after silica gel column chromatography (2% MeOH in DCM) and recrystallized in ether to form **312** as a white solid (33 mg, 21%). ¹H NMR (300 MHz, CDCl₃): δ 8.29 (t, J = 7.4 Hz, 1H), 8.09 (s, 1H), 7.08 (m, 3H), 5.26 (t, J = 7.7 Hz, 1H), 5.12 (dd, J = 4.9, 7.8 Hz, 1H), 4.98 (t, J = 7.7 Hz, 1H), 4.75 (dt, J = 4.4 Hz, 9.8 Hz, 1H), 4.30 (dd, J = 6.4, 12.3 Hz, 1H), 4.18 (dd, J = 3.1, 12.2 Hz, 1H), 4.08 (m, 1H), 2.87 (dd, J = 9.4, 15.9 Hz, 1H), 2.68 (dd, J = 4.9, 15.9 Hz, 1H), 2.07 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 1.94 (s, 3H). ¹³C NMR (125 MHz, CDCl₃): δ 170.8, 169.9, 169.6, 169.6, 167.5, 152.5 (d, $J_{C,F}$ = 242.3 Hz), 126.3 (d, $J_{C,F}$ = 10.2 Hz), 124.8 (d, $J_{C,F}$ = 3.7 Hz), 124.8 (d, $J_{C,F}$ = 7.3 Hz), 122.0, 115.0 (d, $J_{C,F}$ = 19.2 Hz), 71.0, 69.5, 69.2, 69.0, 67.9, 61.9, 35.9, 20.9, 20.8, 20.8, 20.6. LRMS (ESI): m/z calcd. for C₂₂H₂₆O₁₀Na [M + Na]⁺ 506.1, found 506.1.

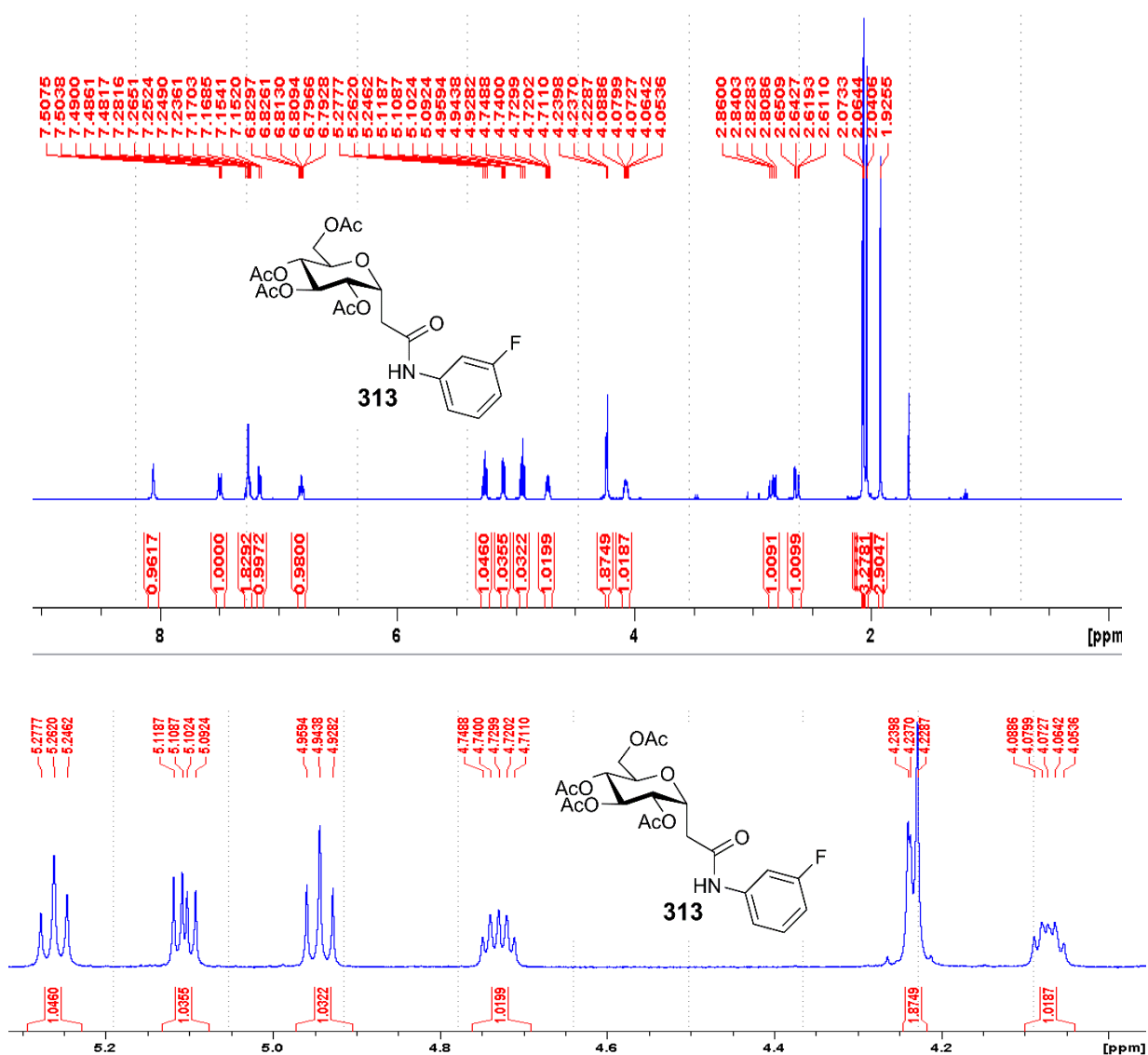


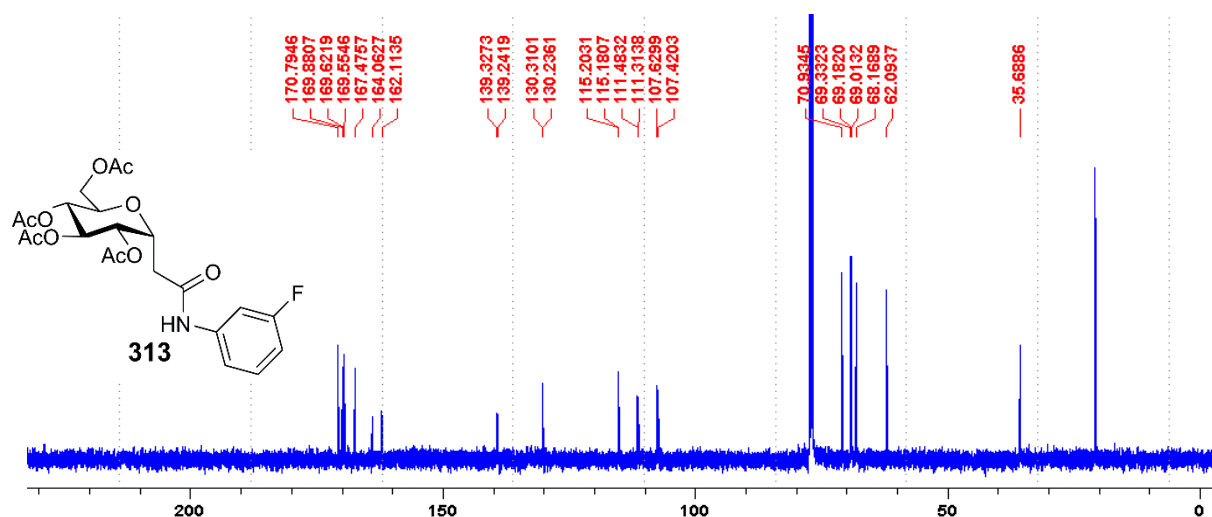


***N*-(3-Fluorophenyl)-2-(2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranosyl)acetamide (**313**)**

Compound **303** (134 mg, 0.344 mmol) was dissolved in 15 mL dry DCM. HCTU (142 mg, 0.344 mmol) and DIPEA (60 μ L, 0.344 mmol) were added and the solution was stirred for 15 min at RT. 3-Fluoroaniline (76 mg, 0.689 mmol) and DIPEA (60 μ L, 0.344 mmol) were added and the resulting mixture was stirred overnight. The mixture was washed with 20% HCl, NaHCO₃, brine, and extracted in DCM. The crude product was recrystallized in ether to obtain the pure product **313** (55 mg, 33%). ¹H NMR (500 MHz, CDCl₃): δ 8.06 (s, 1H), 7.50 (dt, *J* = 10.7, 1.9 Hz, 1H), 7.26 (td, *J* = 8.1, 6.4 Hz, 1H),

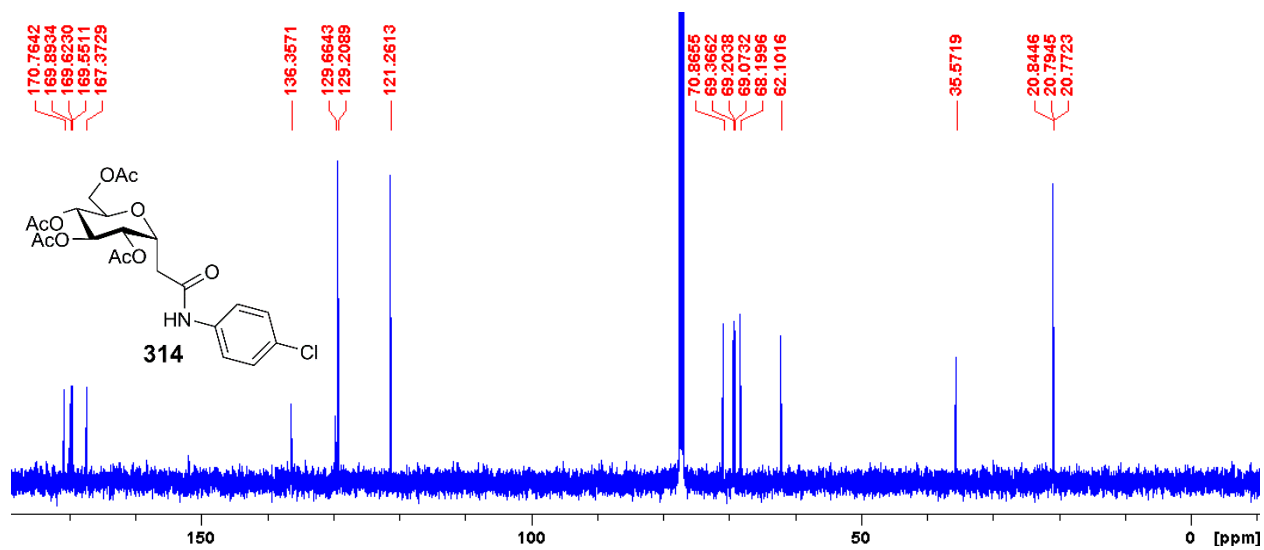
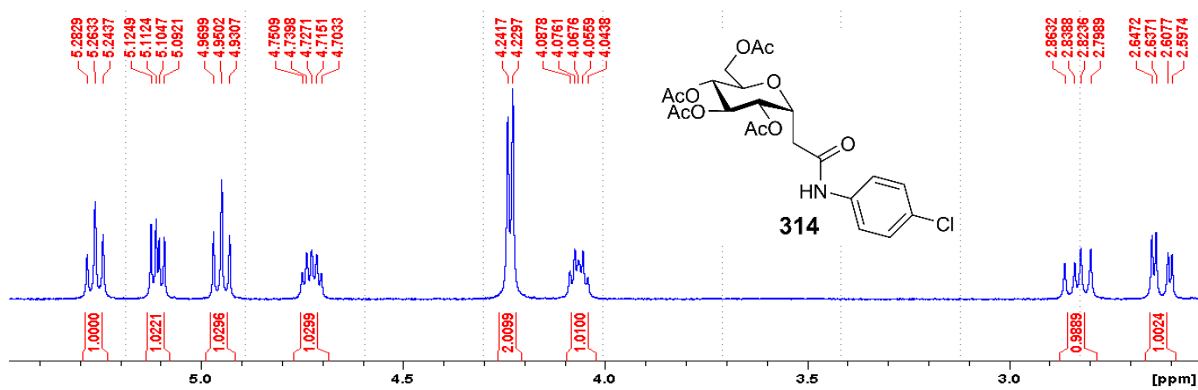
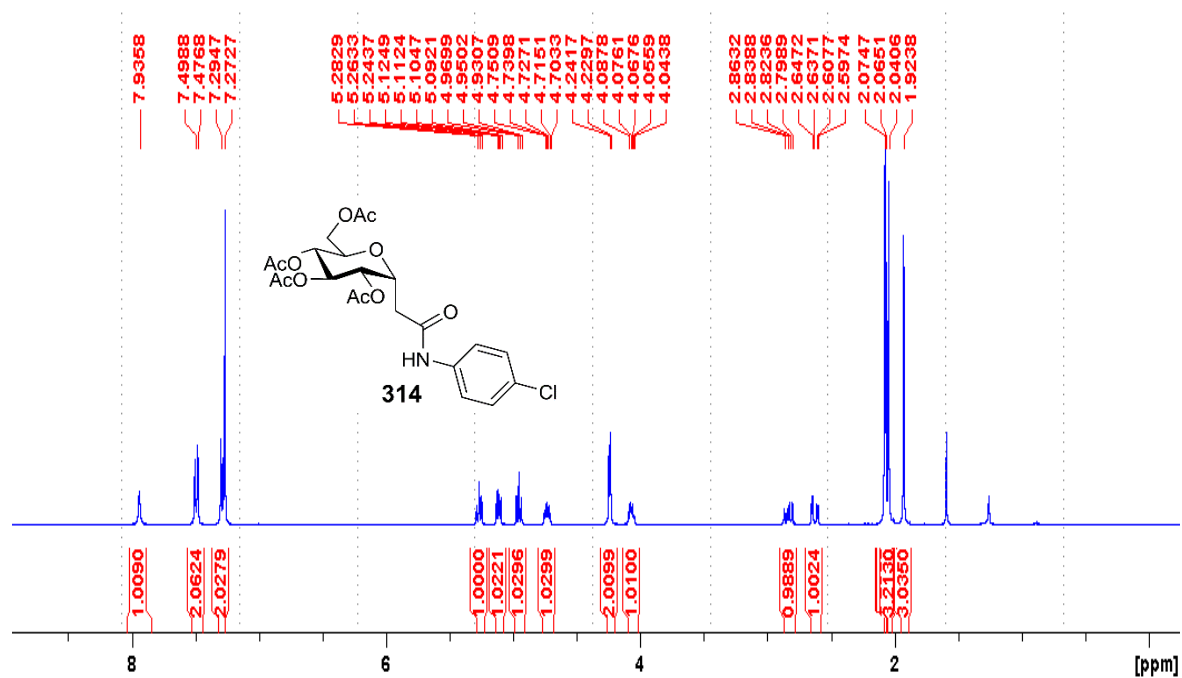
7.16 (dd, $J = 0.9, 8.1$ Hz, 1H), 6.81 (td, $J = 1.8, 8.4$ Hz, 1H), 5.26 (t, $J = 7.7$ Hz, 1H), 5.11 (dd, $J = 5.0, 8.0$ Hz, 1H), 4.94 (t, $J = 7.7$ Hz, 1H), 4.73 (quint, $J = 4.9$ Hz, 1H), 4.23 (m, 2H), 4.07 (m, 1H), 2.83 (dd, $J = 7.9, 16.0$ Hz, 1H), 2.63 (dd, $J = 4.0, 16.0$ Hz, 1H), 2.07 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 1.92 (s, 3H). ^{13}C NMR (125 MHz, CDCl_3): δ 170.8, 169.9, 169.6, 169.5, 167.5, 163.1 (d, $J_{\text{C,F}} = 245.3$ Hz), 139.3 (d, $J_{\text{C,F}} = 10.7$ Hz), 130.3 (d, $J_{\text{C,F}} = 9.2$ Hz), 115.2 (d, $J_{\text{C,F}} = 2.9$ Hz), 111.4 (d, $J_{\text{C,F}} = 21.3$ Hz), 107.5 (d, $J_{\text{C,F}} = 26.5$ Hz), 70.9, 69.3, 69.2, 69.0, 68.2, 62.1, 35.7. LRMS (ESI): m/z calcd. for $\text{C}_{22}\text{H}_{26}\text{O}_{10}\text{FNNa}$ $[\text{M} + \text{Na}]^+$ 506.1, found 506.1.





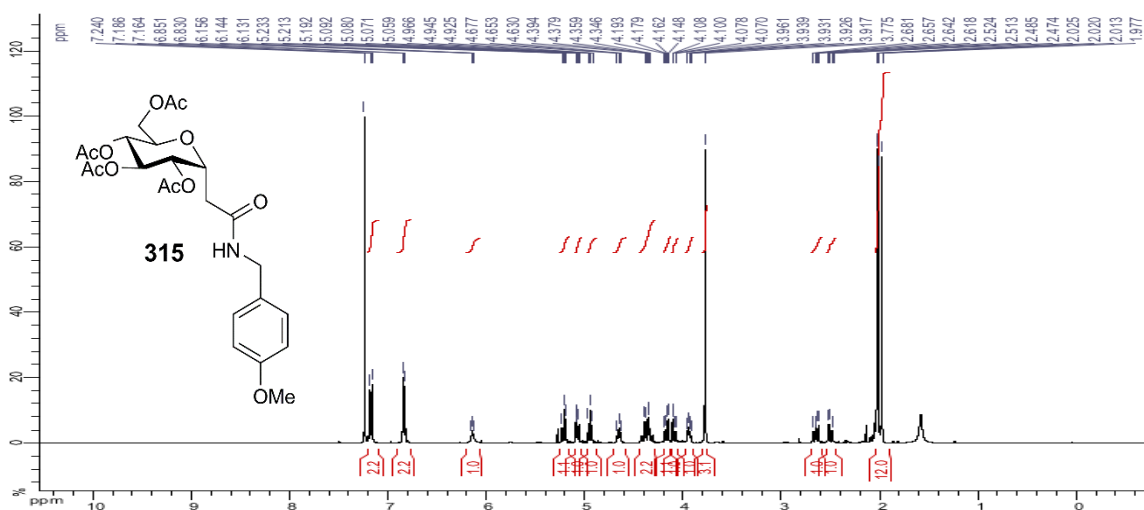
***N*-(4-Chlorophenyl)-2-(2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranosyl)acetamide (**314**)**

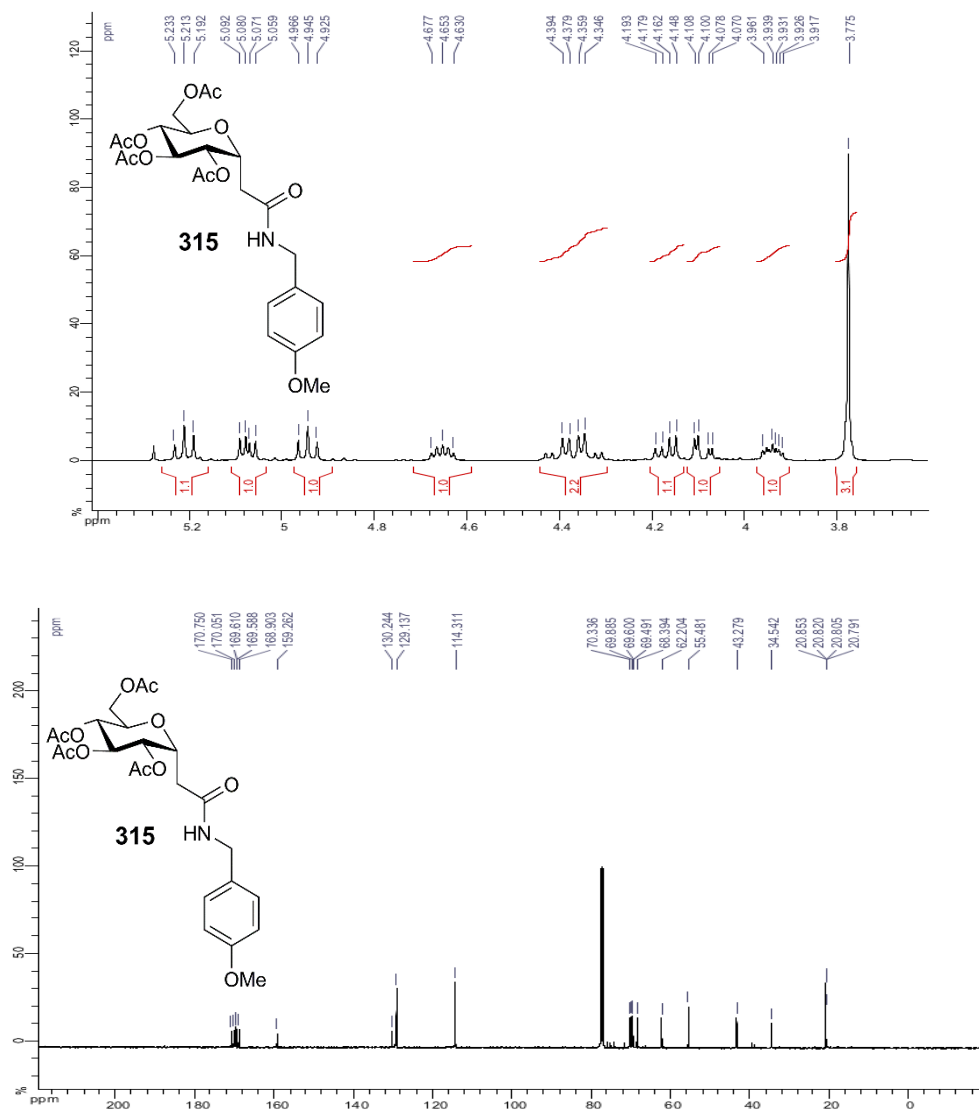
Compound **303** (100 mg, 0.256 mmol) was dissolved in dry DCM. HCTU (106 mg, 0.256 mmol) and DIPEA (90 μ L, 0.256 mmol) were added and the resulting solution was stirred for 15 min at RT. 4-Chloroaniline (65 mg, 0.512 mmol) and DIPEA (90 μ L, 0.256 mmol) were added and the resulting mixture was stirred overnight. The mixture was washed three times each with 20% HCl, NaHCO₃, brine, and extracted in DCM. The product was isolated by flash chromatography (1% to 2% MeOH in DCM), and then recrystallized in Et₂O to obtain **314** as a white solid (35 mg, 27%). ¹H NMR (500 MHz, CDCl₃): δ 7.94 (s, 1H), 7.49 (d, *J* = 8.9 Hz, 2H), 7.28 (d, *J* = 8.9 Hz, 2H), 5.26 (t, *J* = 7.9 Hz, 1H), 5.11 (dd, *J* = 5.1, 7.9 Hz, 1H), 4.95 (t, *J* = 7.9 Hz, 1H), 4.73 (m, *J* = 4.8 Hz, 1H), 4.23 (d, *J* = 4.7 Hz, 2H), 4.06 (m, *J* = 3.5 Hz, 1H), 2.83 (dd, *J* = 9.8, 15.8 Hz, 1H), 2.62 (dd, *J* = 3.7, 15.8 Hz, 1H), 2.07 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 1.92 (s, 3H). ¹³C NMR (125 MHz, CDCl₃): δ 170.8, 169.9, 169.6, 169.6, 167.4, 136.4, 129.7, 129.2, 121.3, 70.9, 69.4, 69.2, 69.1, 68.2, 62.1, 35.6, 20.8, 20.8, 20.8. LRMS (ESI): *m/z* calcd. for C₂₂H₂₆ClNO₁₀Na [M + Na]⁺ 522.1, found 522.2.



***N*-(4-Methoxybenzyl)-2-(2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranosyl)acetamide (315)**

Compound **303** (0.43 g, 1.11 mmol) was dissolved in dry DCM (10 mL). HCTU (0.46 g, 1.11 mmol) and dry DIPEA (0.19 mL, 1.11 mmol) were added and the mixture was allowed to stir for 20 minutes. 4-Methoxybenzylamine (0.36 mL, 2.78 mmol) and dry DIPEA (0.19 mL, 1.11 mmol) were added and an off-white precipitate formed. The reaction was allowed to stir for 16 hours. The reaction completion was monitored by TLC (2% MeOH in DCM). Once complete by TLC, the mixture was diluted to 40 mL with DCM. The organic phase was washed with 10% HCl (2 x 40 mL), then H₂O (2 x 40 mL), then sodium bicarbonate (2 x 40 mL), then brine (2 x 40 mL). The organic phase was dried over magnesium sulfate and concentrated. The product **315** was purified by flash chromatography using 2% MeOH in DCM (0.29 g, 51%). ¹H NMR (400 MHz CDCl₃): δ 7.18 (d, J = 8.7 Hz, 2H), 6.84 (d, J = 8.7 Hz, 2H), 6.14 (t, J = 5.1 Hz, 1H), 5.21 (t, J = 8.2 Hz, 1H), 5.08 (dd, J = 5.2, 8.4 Hz, 1H), 4.95 (t, J = 8.2 Hz, 1H), 4.63-4.68 (m, 1H), 4.37 (dd, J = 5.5, 13.6 Hz, 2H), 4.17 (dd, J = 5.6, 12.1 Hz, 1H), 4.09 (dd, J = 3.2, 12.1 Hz, 1H), 3.92-3.96 (m, 1H), 3.78 (s, 3H), 2.65 (dd, J = 9.9, 15.6 Hz, 1H), 2.5 (dd, J = 4.2, 15.5 Hz, 1H), 1.98-2.03 (m, 12H). ¹³C NMR (100 MHz CDCl₃): δ 170.6, 170.1, 169.6, 168.9, 159.3, 130.2, 129.1, 114.3, 70.3, 69.9, 69.6, 69.5, 68.4, 62.2, 55.5, 43.3, 34.5, 20.9, 20.8, 20.8, 20.8. LRMS (ESI): m/z calcd. for C₂₄H₃₁NNaO₁₁ [M + Na]⁺ 532.5, found 532.0.

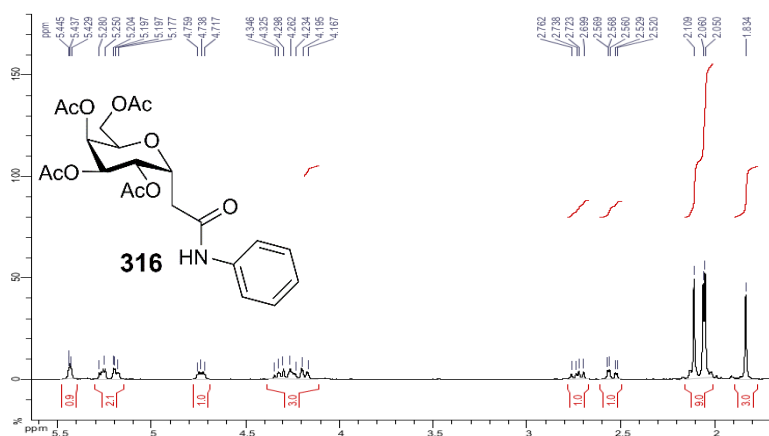
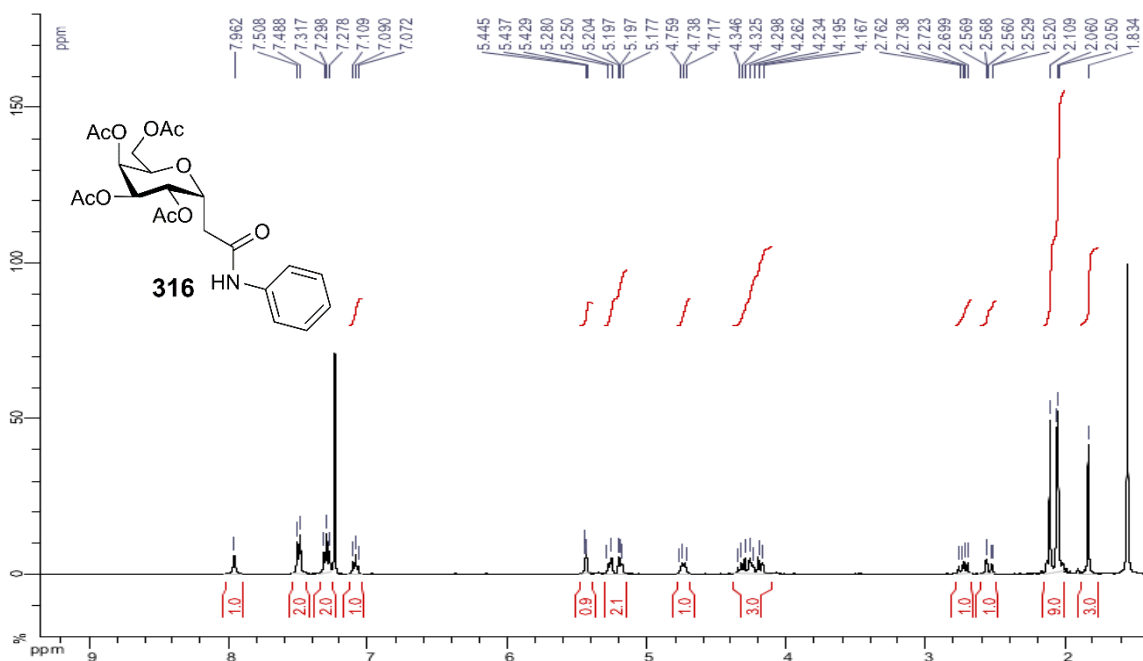


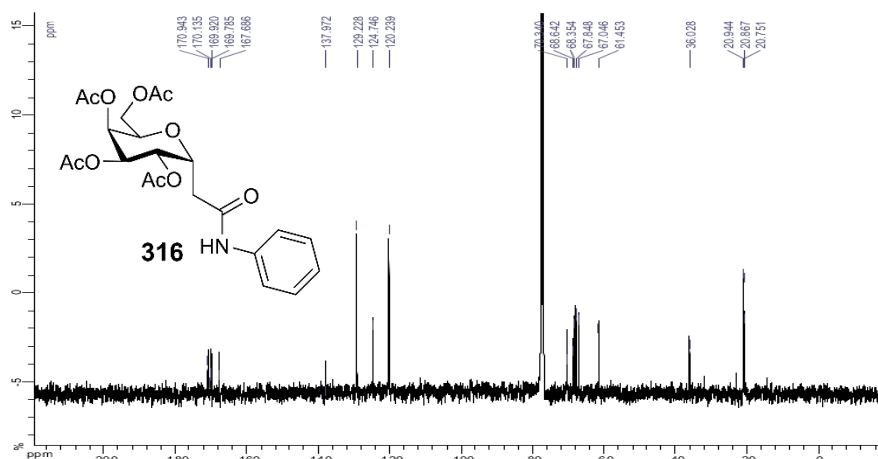


***N*-(Phenyl)-2-(2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl)acetamide (**316**)**

Compound **306** (200 mg, 0.51 mmol) was dissolved in dry DCM. HCTU (211 mg, 0.51 mmol) and DIPEA (100 μ L, 0.51 mmol) were added and the resulting solution was stirred for 15 min at RT. Aniline (0.14 mL, 1.53 mmol) and DIPEA (100 μ L, 0.51 mmol) were added and the resulting mixture was stirred overnight. The mixture was washed three times each with 20% HCl, NaHCO₃, brine, and extracted in DCM. The product was isolated by flash chromatography (1% to 2% MeOH in DCM), and then recrystallized in Et₂O to obtain **316** as a white solid (186 mg, 72%). ¹H NMR (400 MHz, CDCl₃): δ 7.96

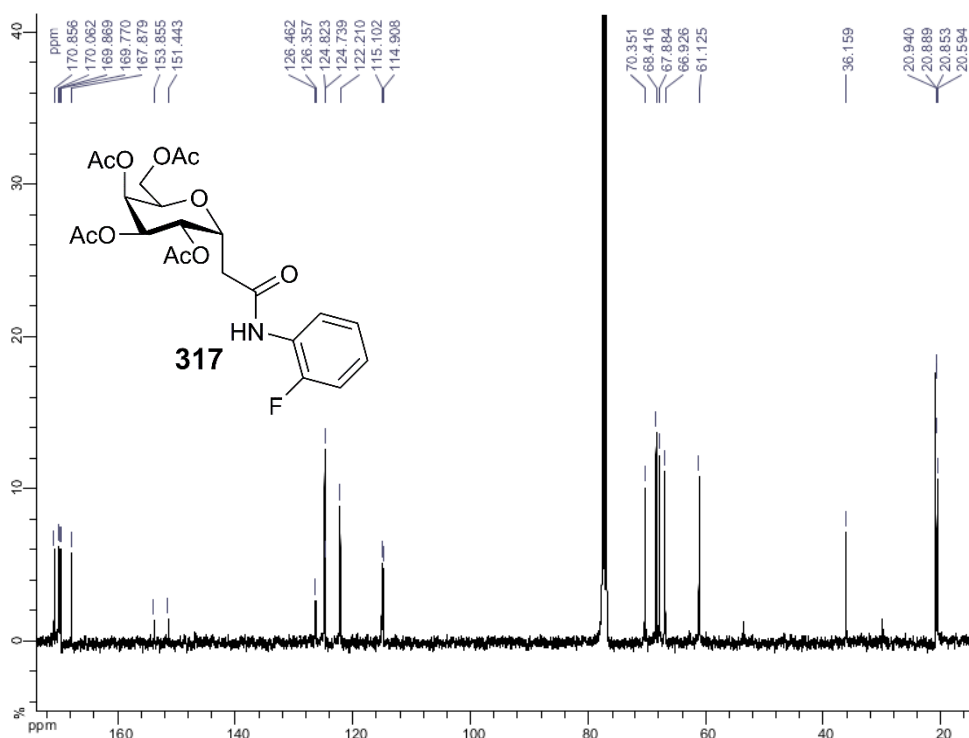
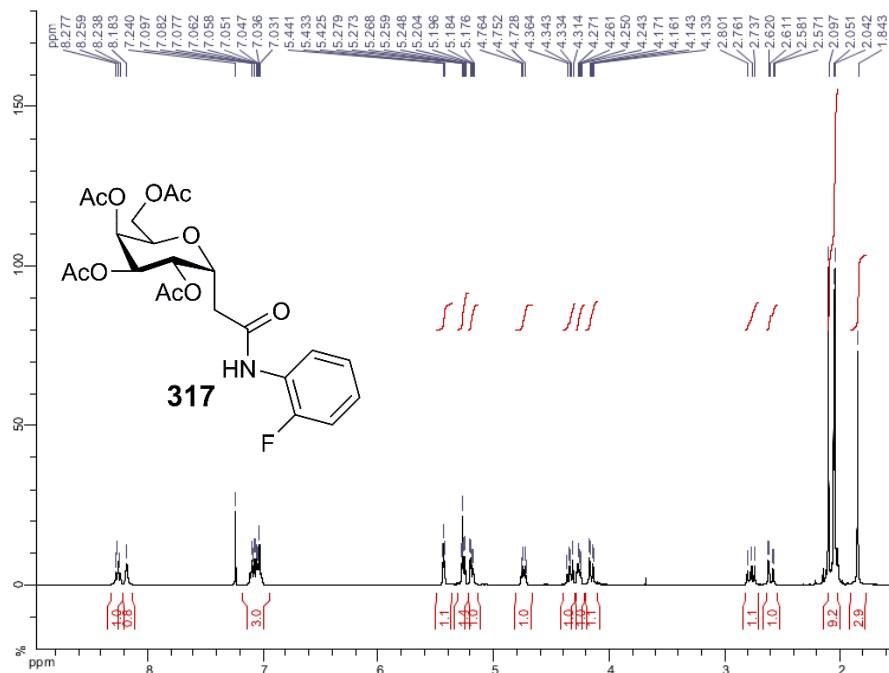
(s, 1H), 7.5 (d, $J = 7.8$ Hz, 1H), 7.3 (t, $J = 7.8$ Hz, 2H), 7.09 (t, $J = 7.2$ Hz, 1H), 5.44 (t, $J = 3.3$ Hz, 1H), 5.18-5.28 (m, 2H), 4.72-4.76 (m, 1H), 4.17-4.35 (m, 3H), 2.73 (dd, $J = 15.4, 9.7$ Hz, 1H), 2.54 (dd, $J = 4.3, 15.5$ Hz, 1H), 2.05-2.11 (m, 9H), 1.83 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ 170.9, 170.1, 169.9, 169.8, 167.7, 138.0, 129.2, 124.8, 120.2, 70.3, 68.6, 68.4, 67.9, 67.1, 61.5, 36.0, 20.9, 20.9, 20.8. LRMS (ESI): m/z calcd. for $\text{C}_{22}\text{H}_{27}\text{NNaO}_{10}$ $[\text{M} + \text{Na}]^+$ 488.4, found 488.2.





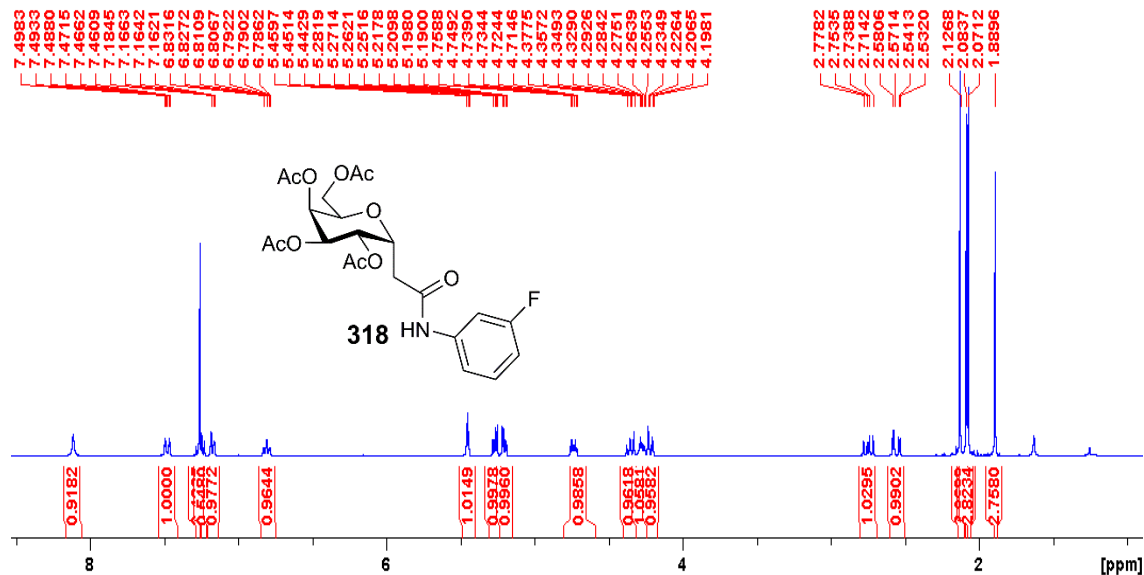
***N*-(2-Fluorophenyl)-2-(2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl)acetamide (**317**)**

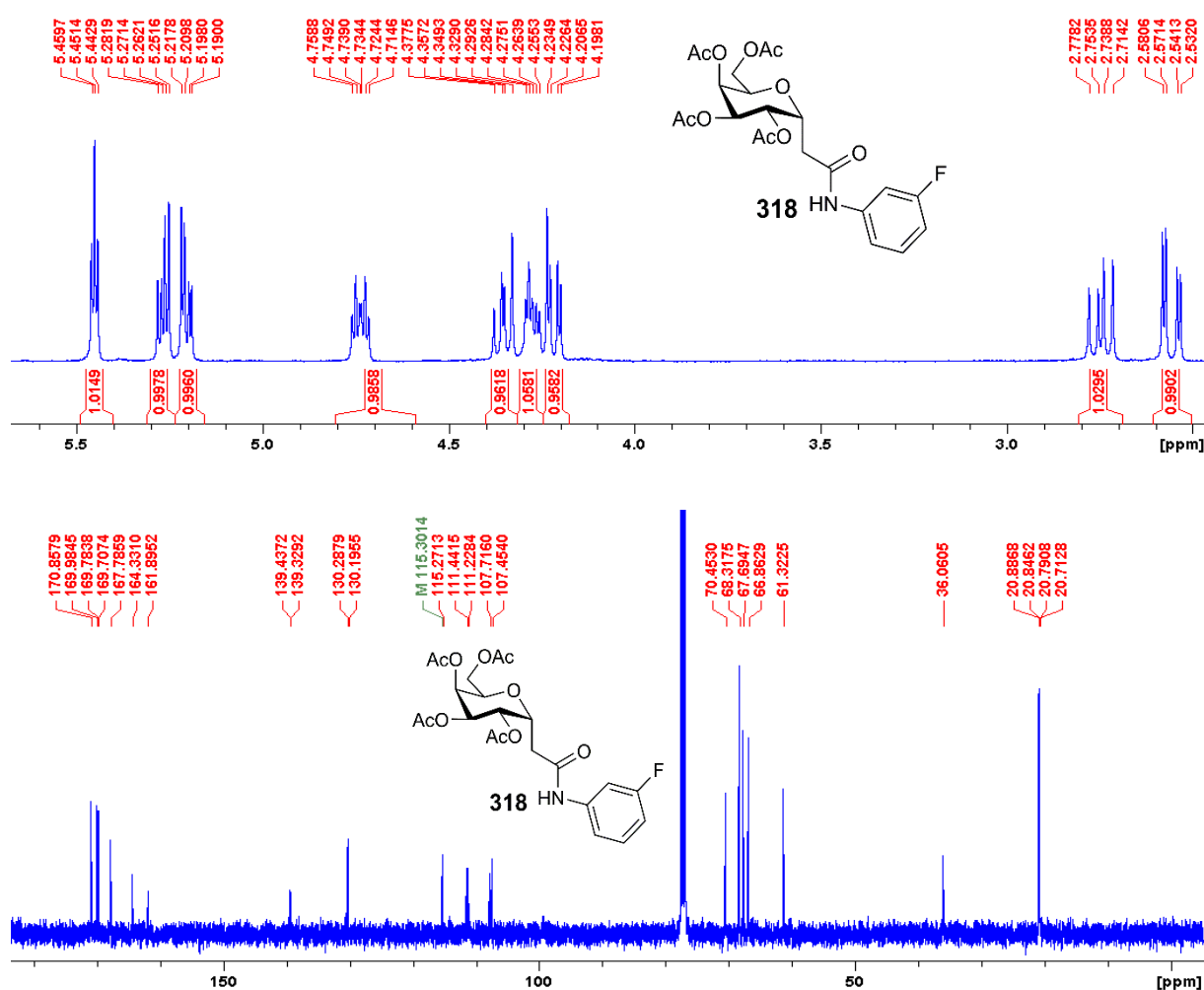
Compound **306** (100 mg, 0.26 mmol) was dissolved in 5 mL dry DCM. HCTU (110 mg, 0.26 mmol) and DIPEA (50 μ L, 0.26 mmol) were added and the solution was stirred for 15 min at RT. 2-Fluoroaniline (50 μ L, 0.52 mmol) and DIPEA (50 μ L, 0.26 mmol) were added and the resulting mixture was stirred overnight. The mixture was washed with 20% HCl, NaHCO₃, brine, and extracted in DCM. The pure product **317** was obtained after silica gel column chromatography (2% MeOH in DCM) and recrystallized in ether as a white solid (50 mg, 40%). ¹H NMR (400 MHz, CDCl₃): δ 8.26 (t, J = 7.5 Hz, 1H), 8.18 (s, 1H), 7.02-7.12 (m, 3H), 5.43 (t, J = 3.4 Hz, 1H), 5.25-5.28 (m, 1H), 5.19 (dd, J = 8.2, 3.4 Hz, 1H), 4.72-4.76 (m, 1H), 4.24-4.36 (m, 2H), 4.15 (dd, J = 11.7, 4.2 Hz, 1H), 2.74-2.80 (m, 1H), 2.60 (dd, J = 16.0, 3.9 Hz, 1H), 2.10 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 1.84 (s, 3H). ¹³C NMR (400 MHz, CDCl₃): δ 170.9, 170.1, 169.9, 169.8, 167.9, 152.7 (d, J_{C-F} = 244.0 Hz), 126.4 (d, J_{C-F} = 10.6 Hz), 124.8 (d, J_{C-F} = 8.9 Hz), 122.2, 115.0 (d, J_{C-F} = 19.6 Hz), 70.6, 68.4, 68.4, 67.9, 66.9, 61.1, 36.2, 20.9, 20.9, 20.9, 20.6. LRMS (ESI): m/z calcd. for C₂₂H₂₆NO₁₀F [M + Na]⁺ 506.1, found 506.2.



***N*-(3-Fluorophenyl)-2-(2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl)acetamide (**318**)**

Compound **306** (205 mg, 0.52 mmol) was dissolved in dry DCM. HCTU (217 mg, 0.52 mmol) and DIPEA (0.09 mL, 0.52 mmol) were added and the solution was stirred for 15 min at room temperature. 3-Fluoroaniline (0.10 mL, 1.05 mmol) and DIPEA (0.09 mL, 0.52 mmol) were added and the mixture was stirred overnight. The mixture was washed with 10% HCl, NaHCO₃, brine, and extracted in DCM. The product was purified using flash chromatography (2% MeOH/DCM), then recrystallized in ether to obtain the **318** as a white powder (65 mg, 25%). ¹H NMR (400 MHz, CDCl₃): δ 8.11 (s, 1H), 7.48 (dt, J = 1.9, 10.9 Hz, 1H), 7.65 (m, 1H), 7.17 (m, 1H), 6.81 (td, J = 1.6, 8.3 Hz, 1H), 5.45 (t, J = 3.4 Hz, 1H), 5.27 (dd, J = 4.1, 8.0 Hz, 1H), 5.20 (dd, J = 3.1, 8.0 Hz, 1H), 4.74 (m, 1H), 4.35 (dd, J = 8.0, 11.1 Hz, 1H), 4.28 (m, 1H), 4.21 (dd, J = 3.4, 11.1 Hz, 1H), 2.75 (dd, 9.8, 11.8 Hz, 1H), 2.56 (dd, 3.6, 15.8, 1H), 2.13 (s, 3H), 2.08 (s, 3H), 2.07 (s, 3H), 1.89 (s, 3H). ¹³C NMR (100 MHz, CDCl₃): δ 170.9, 170.0, 169.8, 169.7, 167.8, 163.1 (d, $J_{C,F}$ = 244.1 Hz), 139.4 (d, $J_{C,F}$ = 10.2 Hz), 130.2 (d, $J_{C,F}$ = 9.5 Hz), 115.3 (d, $J_{C,F}$ = 2.9 Hz), 111.3 (d, $J_{C,F}$ = 22.2 Hz), 107.6 (d, $J_{C,F}$ = 26.1 Hz), 70.5, 68.3, 67.7, 66.9, 61.3, 36.1, 20.9, 20.9, 20.8, 20.7. LRMS (ESI): m/z calcd. for C₂₂H₂₆FNO₁₀Na [M + Na]⁺ 506.1, found 506.4.

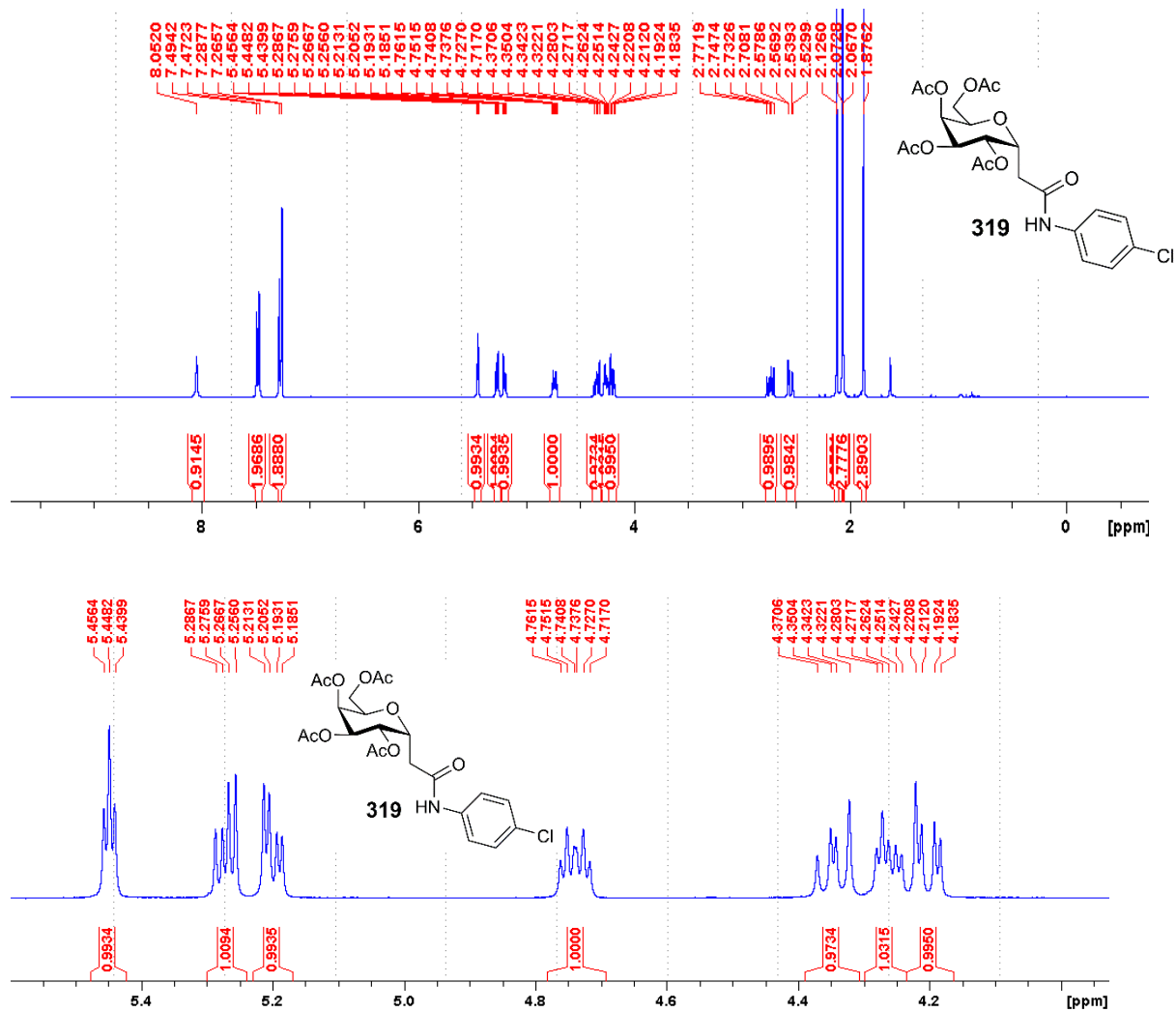


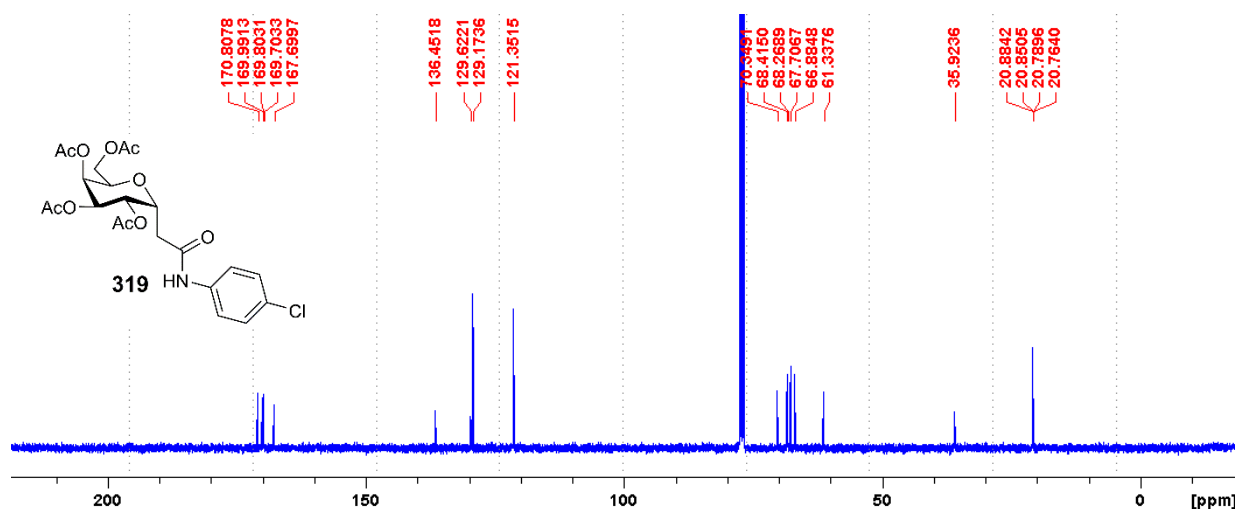


***N*-(4-Chlorophenyl)-2-(2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl)acetamide (**319**)**

Compound **306** (240 mg, 0.61 mmol) was dissolved in dry DCM. HCTU (255 mg, 0.61 mmol) and DIPEA (0.1 mL, 0.61 mmol) were added and the solution was stirred for 15 min at RT. 4-chloroaniline (156 mg, 1.22 mmol) and DIPEA (0.1 mL, 0.61 mmol) were added and the resulting mixture was stirred overnight. The mixture was washed with 10% HCl, NaHCO₃, brine, and extracted in DCM. The product was purified using flash chromatography (2% MeOH in DCM), then recrystallized in ether to obtain the pure product **319** as a white solid (60 mg, 20%). ¹H NMR (400 MHz, CDCl₃): δ 8.05 (s, 1H), 7.48 (m, 2H), 7.28 (m, 2H), 5.45 (t, *J* = 3.3 Hz, 1H), 5.27 (dd, *J* = 4.4, 8.1 Hz, 1H), 5.20 (dd, *J* = 3.2, 8.1 Hz, 1H), 4.74 (dt, *J* = 4.1, 9.7 Hz, 1H), 4.35 (dd, *J* = 8.1, 11.4 Hz, 1H), 4.27 (m, 1H), 4.20 (dd, *J* = 3.5, 11.3 Hz,

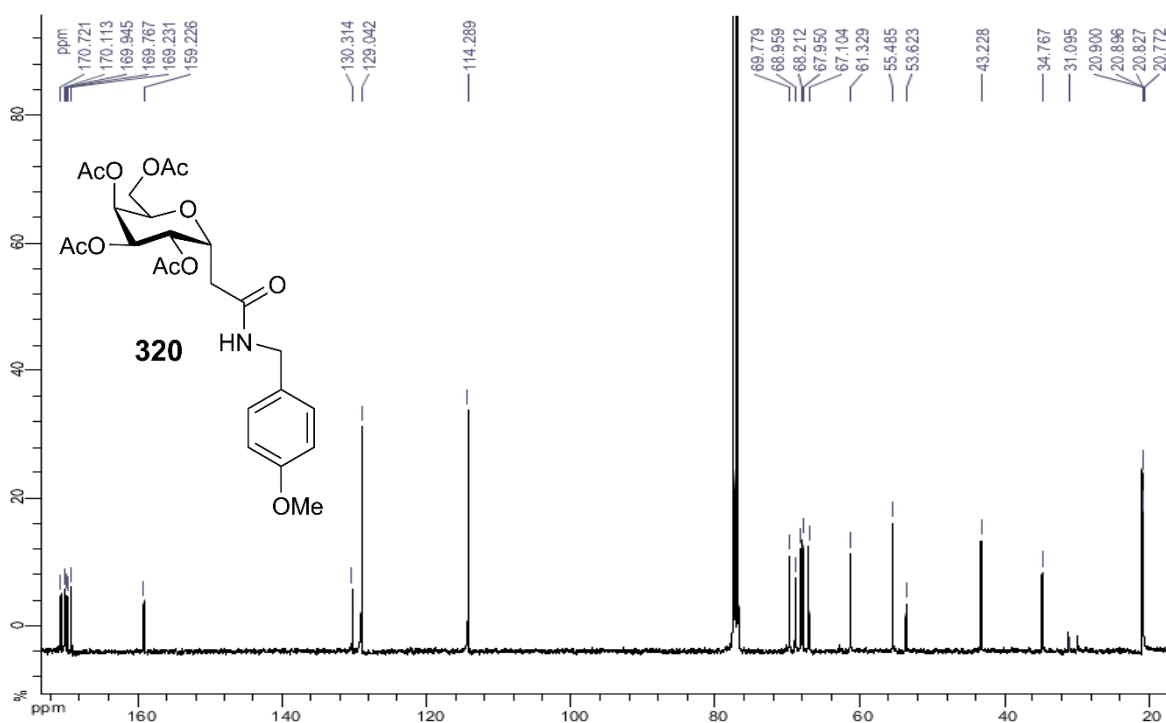
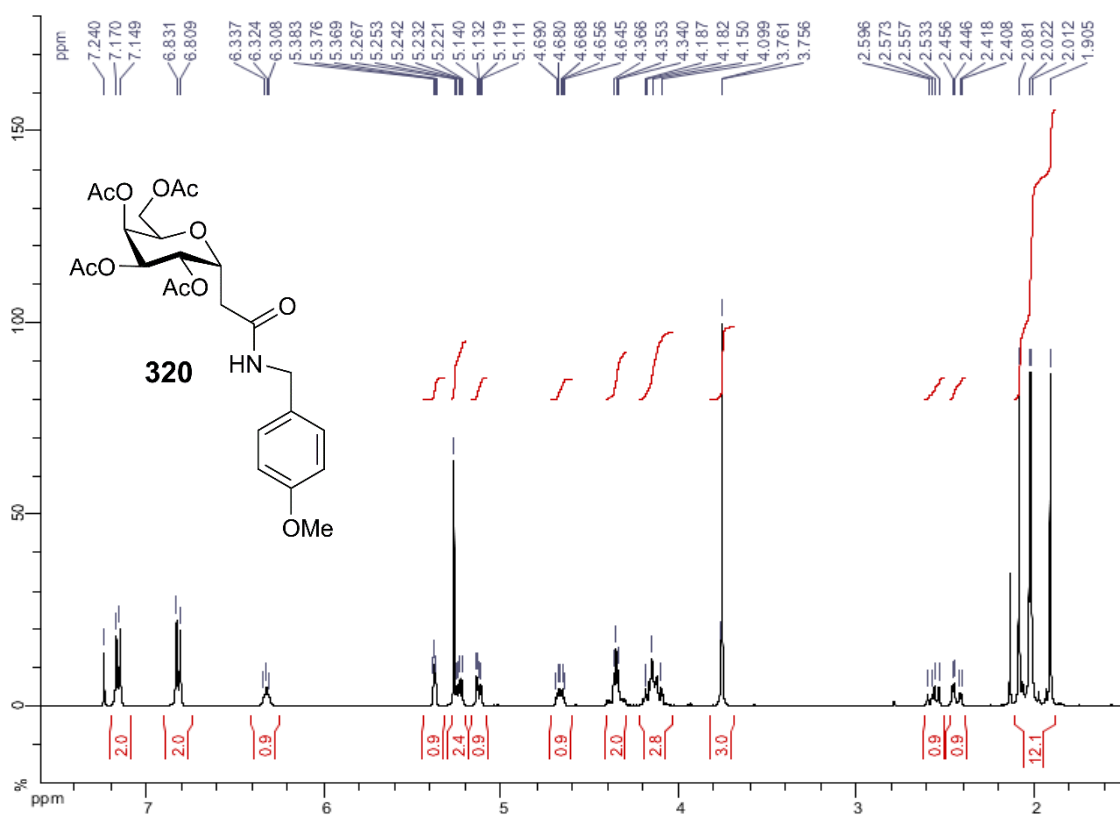
1H), 2.74 (dd, $J = 9.8, 15.7$ Hz, 1H), 2.55 (dd, $J = 3.7, 15.7$, 1H), 2.13 (s, 3H), 2.07 (s, 3H), 2.07 (s, 3H), 1.88 (s, 3H). ^{13}C (100 MHz, CDCl_3): δ 170.8, 170.0, 169.8, 169.7, 167.7, 136.5, 129.6, 129.2, 121.4, 70.4, 68.4, 68.3, 67.7, 66.9, 61.3, 35.9, 20.9, 20.9, 20.8, 20.8. LRMS (ESI): m/z calcd. for $\text{C}_{22}\text{H}_{26}\text{ClNO}_{10}\text{Na}$ $[\text{M} + \text{Na}]^+$ 522.1, found 522.1.





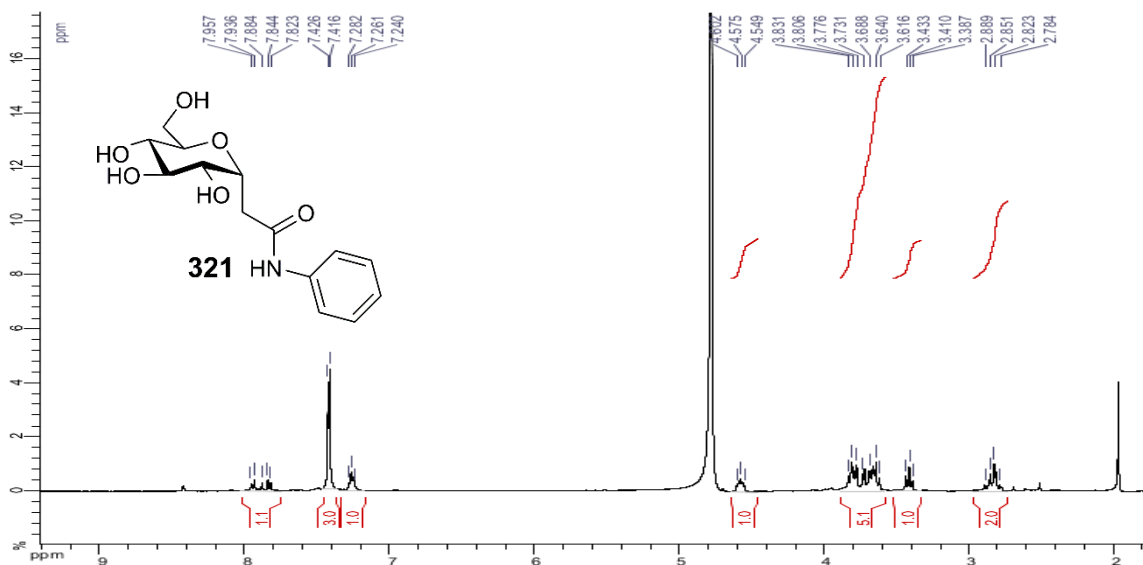
***N*-(4-Methoxybenzyl)-2-(2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl)acetamide (**320**)**

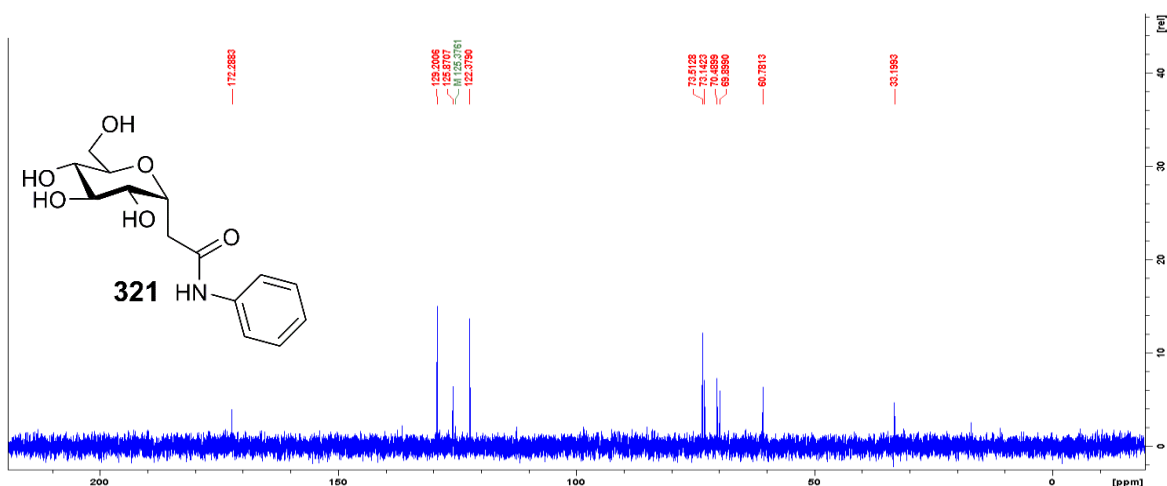
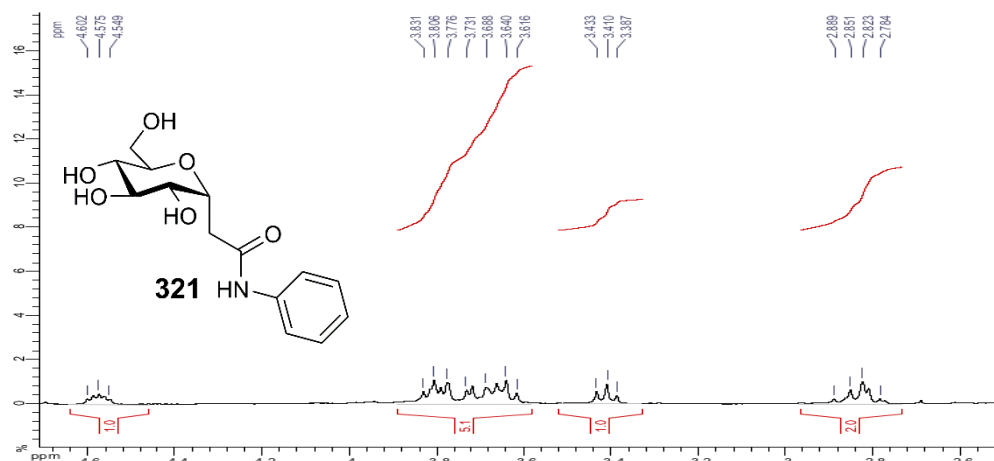
Compound **306** (0.10 g, 0.26 mmol) was dissolved in dry DCM (5 mL). HCTU (0.11 g, 0.26 mmol) and dry DIPEA (0.05 mL, 0.26 mmol) were added and the mixture was allowed to stir for 20 minutes. 4-Methoxybenzylamine (0.04 mL, 0.52 mmol) and dry DIPEA (0.05 mL, 0.26 mmol) were added and an off-white precipitate formed. The reaction was allowed to stir for 16 hours. The reaction completion was monitored by TLC (2% MeOH in DCM). Once complete by TLC, the mixture was diluted to 40 mL with DCM. The organic phase was washed with 10% HCl (2 x 40 mL), then H₂O (2 x 40 mL), then sodium bicarbonate (2 x 40 mL), then brine (2 x 40 mL). The organic phase was dried over magnesium sulfate and concentrated. The product was purified by flash chromatography (2% MeOH in DCM) to obtain **320** (0.06 g, 45%). ¹H NMR (400 MHz, CDCl₃): δ 7.16 (d, *J* = 8.6 Hz, 2H), 6.82 (d, *J* = 9.0 Hz, 2H), 6.33 (t, *J* = 5.6 Hz, 1H), 5.38 (t, *J* = 2.8 Hz, 1H), 5.27 (s, 2H), 5.24 (dd, *J* = 8.4, 4.6 Hz, 1H), 5.13 (dd, *J* = 8.4, 3.2 Hz, 1H), 4.65-4.70 (m, 1H), 4.35 (t, *J* = 5.2 Hz, 2H), 4.09-4.20 (m, 3H), 3.76 (s, 3H), 2.56 (dd, *J* = 15.3, 8.7 Hz, 1H), 2.43 (dd, *J* = 15.5, 4.2 Hz, 1H), 2.08 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 1.91 (s, 3H). ¹³C NMR (400 MHz, CDCl₃): δ 170.7, 170.1, 170.0, 169.8, 169.2, 159.2, 130.3, 129.0, 114.3, 69.8, 69.0, 68.2, 68.0, 67.1, 61.3, 55.5, 43.2, 34.8, 31.1, 20.9, 20.9, 20.8, 20.8. LRMS (ESI): *m/z* calcd. For C₂₄H₃₁NO₁₁K [M + K]⁺ 548.2, found 548.2.



***N*-(Phenyl)-2-(α -D-glucopyranosyl)acetamide (321)**

Compound **311** (140 mg, 0.30 mmol) was dissolved in MeOH (ca. 10 mL). 1 M NaOMe in MeOH was added dropwise until the solution had a pH of 12. The solution was stirred at RT for 2 h, neutralized with Amberlite (H⁺) acidic resin, and filtered. The filtrate was evaporated and recrystallized in H₂O to obtain the pure product **321** (59.5 mg, 66%). ¹H NMR (400 MHz, D₂O): δ 7.86 (m, 1H), 7.43 (d, J = 4.2 Hz, 3H), 7.24-7.28 (m, 1H), 4.55-4.60 (m, 1H), 3.62-3.83 (m, 5H), 3.41 (t, J = 9.4 Hz, 1H), 2.78-2.89 (m, 2H). ¹³C NMR (100 MHz, D₂O): δ 172.3, 129.2, 125.9, 125.4, 122.4, 73.5, 73.1, 70.5, 69.9, 60.8, 33.2. LRMS (ESI): m/z calcd. for C₁₄H₁₉NNaO₆ [M + Na]⁺ 320.3, found 320.1.



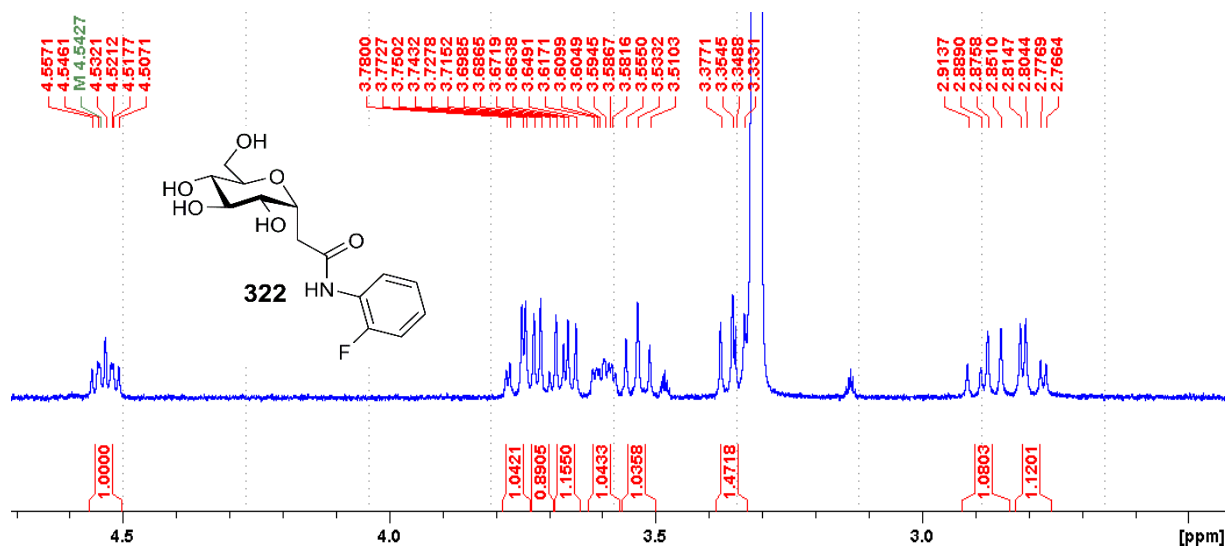
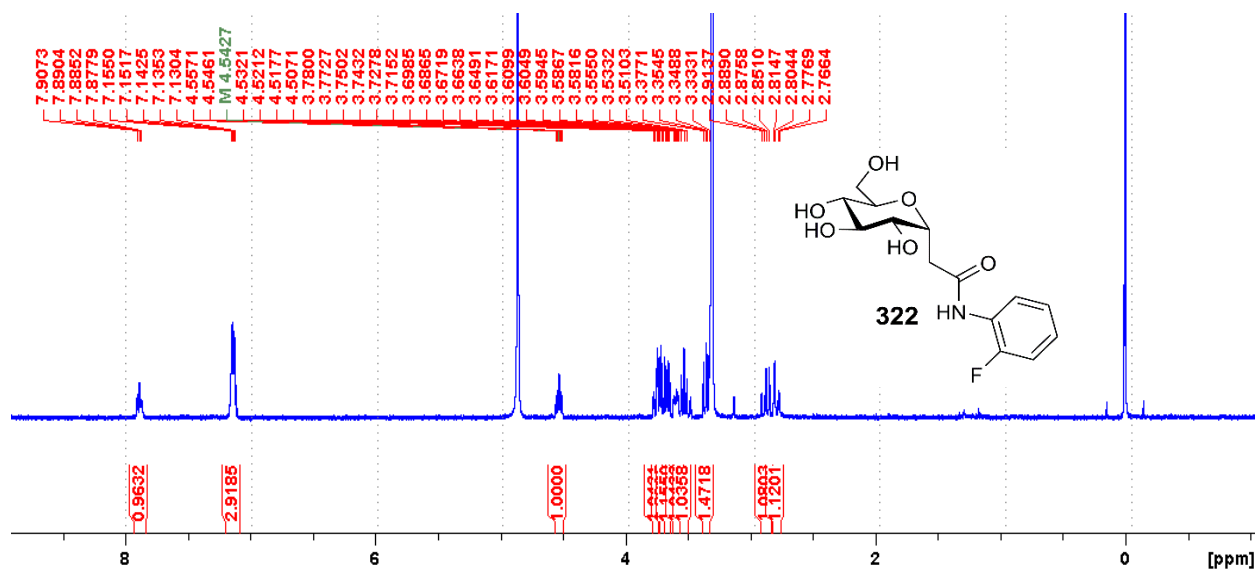


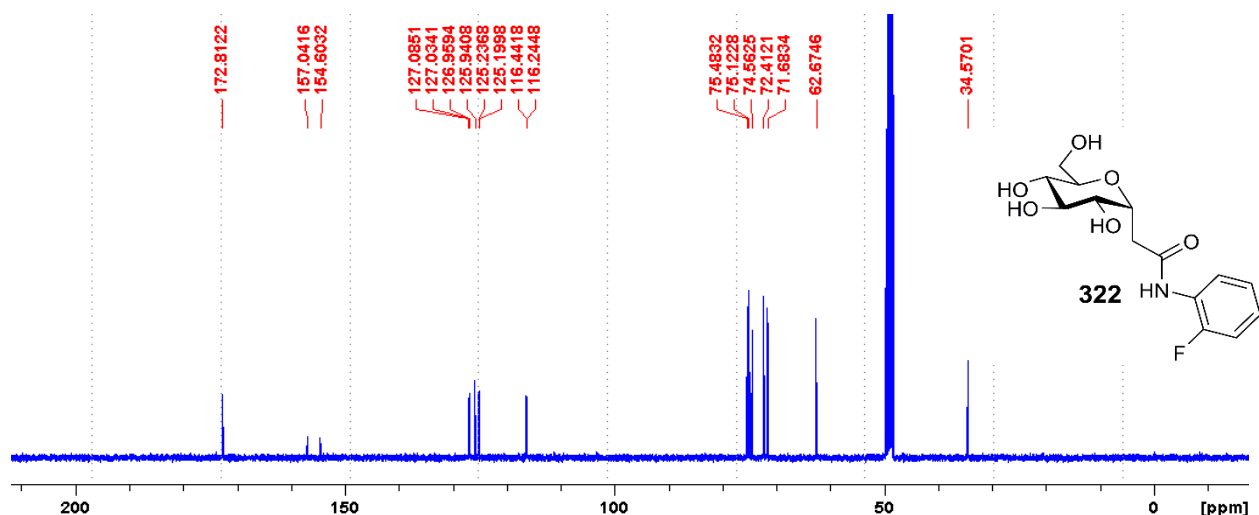
N-(2-Fluorophenyl)-2-(α -D-glucopyranosyl)acetamide (**322**)

Compound **312** (28 mg, 0.058 mmol) was dissolved in MeOH (ca. 10 mL). 1 M NaOMe in MeOH was added dropwise until the solution was strongly basic. The solution was stirred at RT for 2 h, neutralized with Amberlite (H⁺) acidic resin, and filtered. The pure product **322** was obtained as a white solid after removing the solvent and recrystallizing in EtOH (5 mg, 27%). ¹H NMR (400 MHz, MeOD): δ 7.91-7.86 (m, 1H), 7.17-7.10 (m, 3H), 4.53 (ddd, J = 4.3, 5.7, 10.0 Hz, 1H), 3.76 (dd, J = 3.0, 12.0 Hz, 1H), 3.71 (dd, J = 5.0, 11.7 Hz, 1H), 3.67 (dd, J = 5.9, 9.1 Hz, 1H), 3.62-3.57 (m, 1H), 3.53 (t, J = 9.1 Hz, 1H), 3.38-3.33 (m, 1H), 2.88 (dd, J = 9.9, 15.1 Hz, 1H), 2.79 (dd, J = 4.1, 15.1 Hz, 1H). ¹³C NMR (100 MHz, MeOD): δ 172.8, 155.8 (d, $J_{C,F}$ = 246.9 Hz), 127.0 (d, $J_{C,F}$ = 12.7 Hz), 127.0 (d, $J_{C,F}$ = 7.6 Hz), 125.9,

125.2 (d, $J_{C,F} = 3.6$ Hz), 116.4 (d, $J_{C,F} = 19.8$ Hz), 75.5, 75.1, 74.6, 72.4, 71.7, 62.8, 34.6. LRMS (ESI):

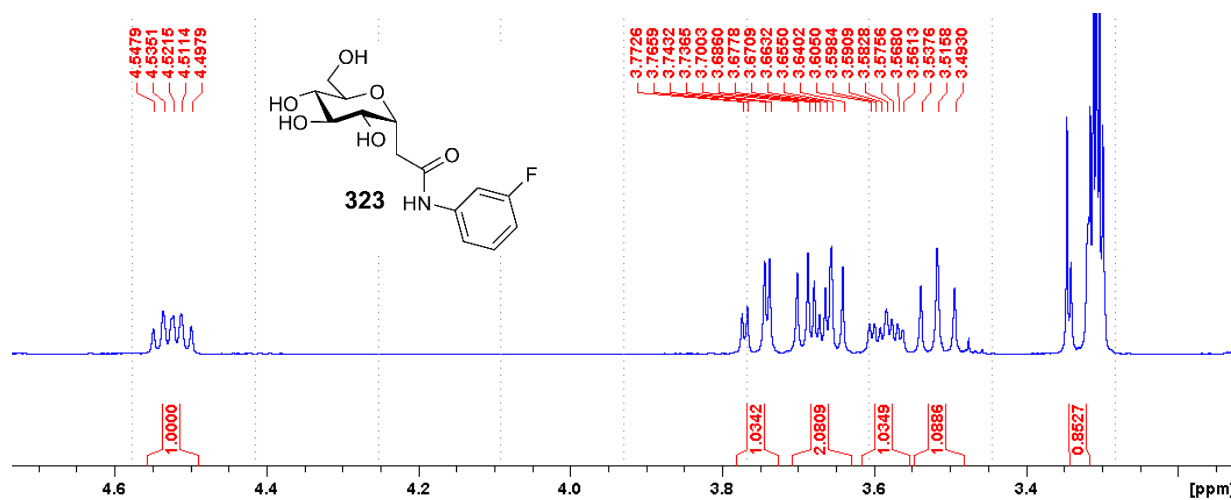
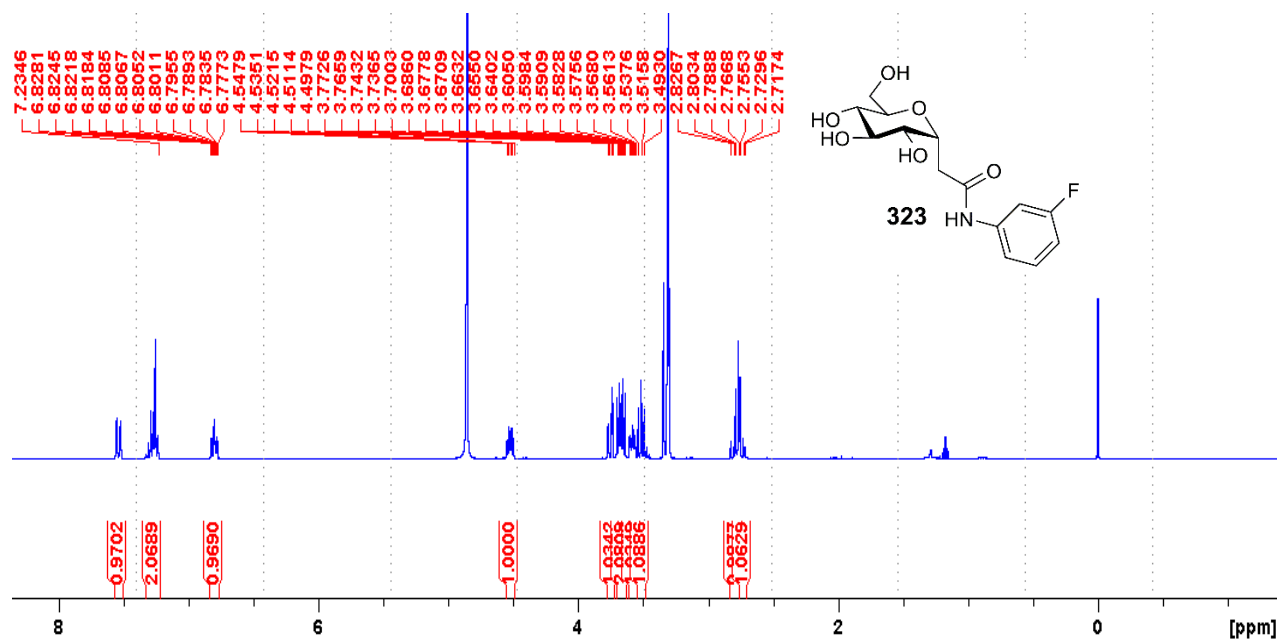
m/z calcd. for $C_{14}H_{18}O_6FNa$ $[M + Na]^+$ 338.1, found 338.0.

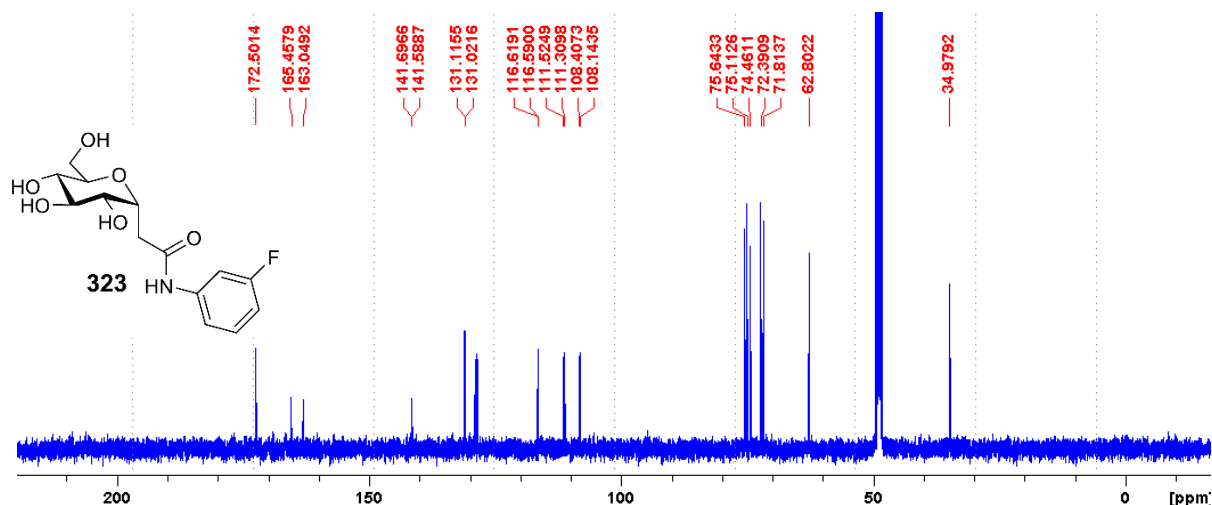




***N*-(3-Fluorophenyl)-2-(α -D-glucopyranosyl)acetamide (**323**)**

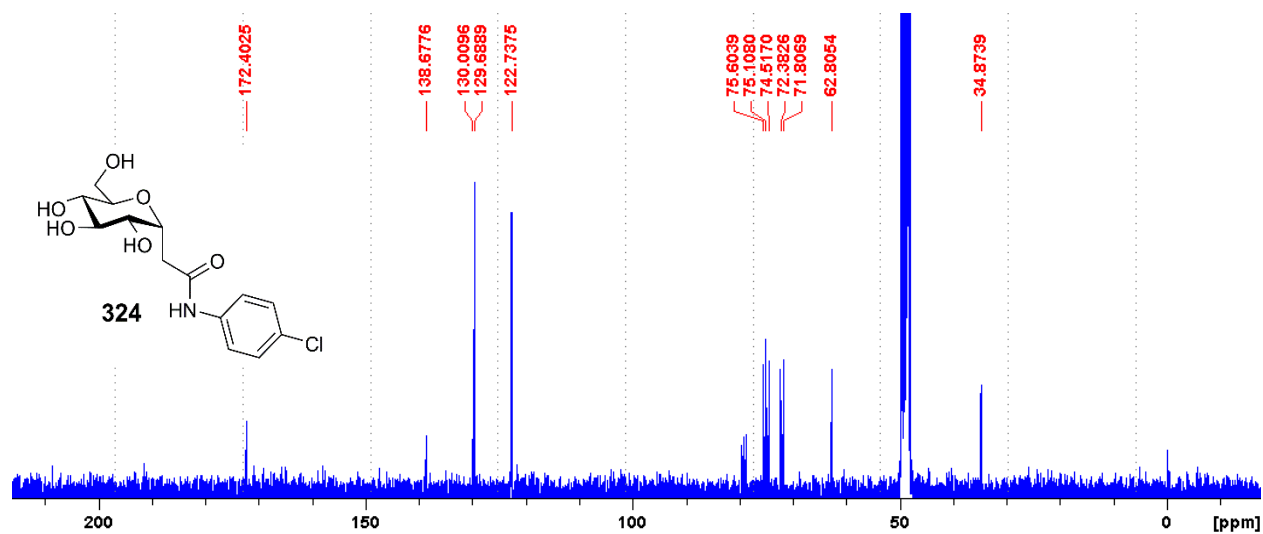
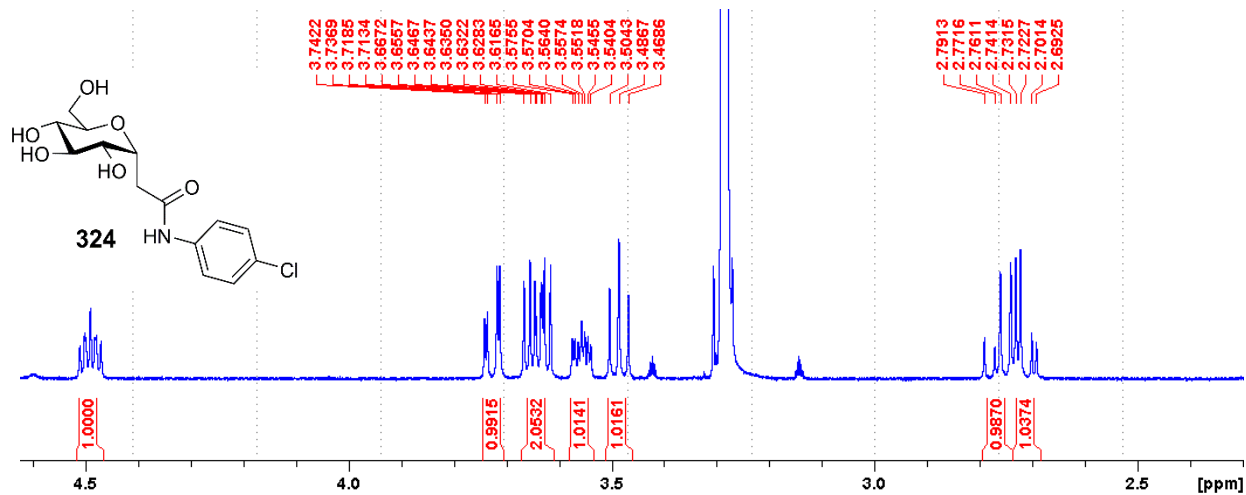
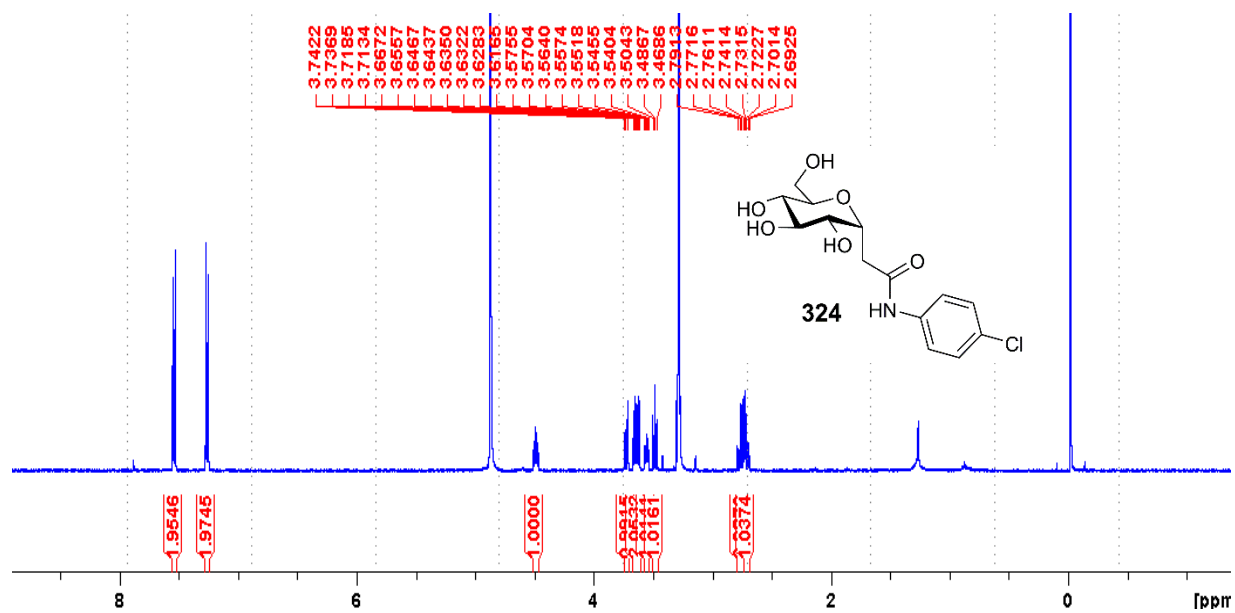
Compound **313** (35 mg, 0.072 mmol) was dissolved in MeOH (10 mL). 1 M NaOMe in MeOH was added dropwise until the solution was strongly basic. The solution was stirred at RT for two hours, neutralized with Amberlite (H⁺) acidic resin, filtered, and the solvent was evaporated to obtain the crude product. The pure product **323** was obtained from silica gel flash column chromatography (20% MeOH in DCM) as a white solid (10 mg, 44%). ¹H NMR (400 MHz, MeOD): δ 7.54 (dt, J = 2.1, 11.3 Hz, 1H), 7.28 (m, 2H), 6.80 (m, 1H), 4.52 (dt, J = 5.4, 9.5 Hz, 1H), 3.75 (dd, J = 2.7, 11.8 Hz, 1H), 3.67 (m, 2H), 3.58 (ddd, J = 2.9, 5.9, 9.1 Hz, 1H), 3.52 (t, J = 9.2 Hz, 1H), 3.32 (m, 1H), 2.80 (dd, J = 9.2, 15.0 Hz, 1H), 2.74 (dd, J = 4.9, 14.7, 1H). ¹³C NMR (100 MHz, MeOD): δ 172.5, 164.3 (d, $J_{C,F}$ = 242.8 Hz), 141.6 (d, $J_{C,F}$ = 10.8 Hz), 131.1 (d, $J_{C,F}$ = 9.2 Hz), 116.6 (d, $J_{C,F}$ = 2.9 Hz), 111.4 (d, $J_{C,F}$ = 21.6 Hz), 108.3 (d, $J_{C,F}$ = 26.5 Hz), 75.6, 75.1, 74.5, 72.4, 71.8, 62.8, 35.0. LRMS (ESI): m/z calcd. for C₁₄H₁₈O₆FNNa [M + Na]⁺ 338.1, found 338.0.





***N*-(4-Chlorophenyl)-2-(α -D-glucopyranosyl)acetamide (**324**)**

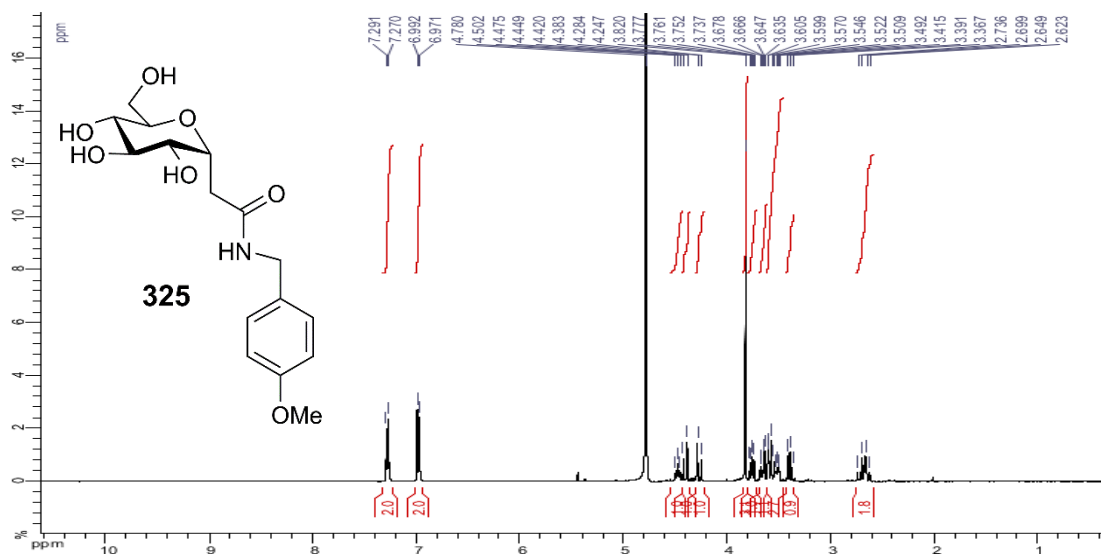
Compound **314** (35 mg, 0.070 mmol) was dissolved in MeOH (10 mL). 1 M NaOMe in MeOH was added dropwise until the solution had a pH of 12. The solution was stirred at RT for two hours, neutralized with Amberlite (H⁺) acidic resin, and filtered. The filtrate was evaporated and recrystallized in H₂O to obtain the pure product **324** (2 mg, 9%). ¹H NMR (500 MHz, MeOD): δ 7.54 (m, 2H), 7.27 (m, 2H), 4.49 (m, 1H), 3.73 (dd, J = 2.6, 11.8 Hz, 1H), 3.64 (m, 2H), 3.56 (m, 1H), 3.49 (t, J = 9.0 Hz, 1H), 2.78 (dd, J = 9.8, 15.0 Hz, 1H), 2.71 (dd, J = 4.4, 15.0 Hz, 1H). ¹³C NMR (75 MHz, MeOD): δ 172.4, 138.7, 130.0, 129.7, 122.7, 75.6, 75.1, 74.5, 72.4, 71.8, 62.8, 34.9. LRMS (ESI): m/z calcd. for C₁₄H₁₈ClNO₆Na [M + Na]⁺ 354.1, found 354.0.

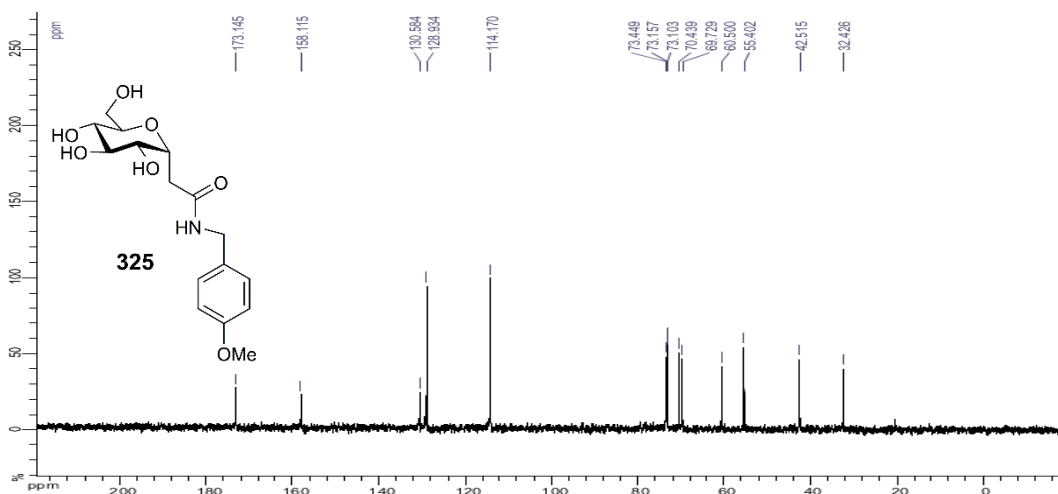
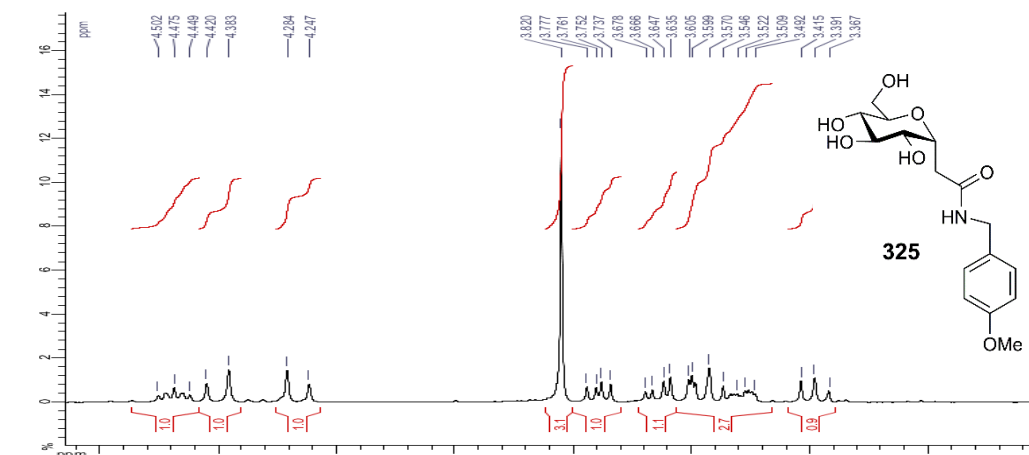


***N*-(4-Methoxybenzyl)-2-(α -D-glucopyranosyl)acetamide (325)**

Compound **315** (0.29 g, 0.56 mmol) was dissolved in 1M NaOMe in methanol (16 mL) and distilled water (4 mL) and allowed to stir for one hour. Reaction completion was monitored by TLC (10% MeOH in DCM). Once complete, the mixture was neutralized with acidic Amberlite (H⁺) resin (monitored using pH paper). The mixture was filtered and lyophilized to yield product **325** as a white powder (0.3 g, 60%).

¹H NMR (400 MHz, D₂O): δ 7.28 (d, J = 8.5 Hz, 2H), 6.98 (d, J = 8.6 Hz, 2H), 4.45-4.50 (m, 1H), 4.4 (d, J = 15.1 Hz, 1H), 4.27 (d, J = 14.9 Hz, 1H), 3.82 (s, 3H), 3.76 (dd, J = 6.3, 9.8 Hz, 1H), 3.66 (dd, J = 4.4, 12.3 Hz, 1H), 3.6 (t, J = 2.7 Hz, 1H), 3.49-3.57 (m, 2H), 3.39 (dd, J = 9.0, 9.8 Hz, 1H), 2.62-2.74 (m, 2H). ¹³C NMR (100 MHz, D₂O): δ 173.2, 158.1, 130.6, 128.9, 114.2, 73.5, 73.2, 73.1, 70.4, 69.7, 60.5, 55.4, 42.5, 32.4. LRMS (ESI): m/z calcd. for C₁₆H₂₃NNaO₇ [M + Na]⁺ 364.4, found 364.0.

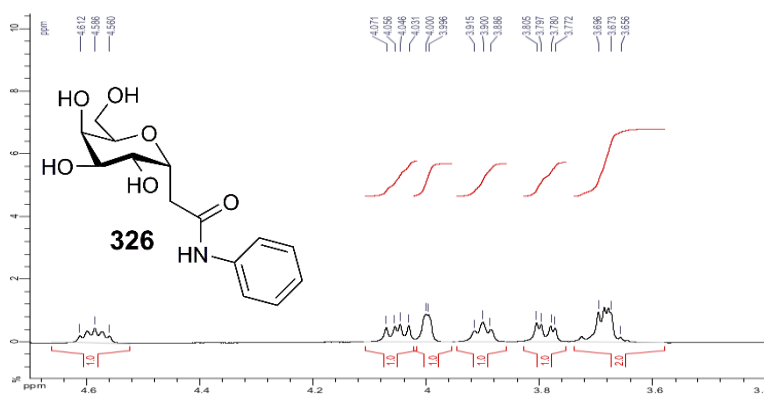
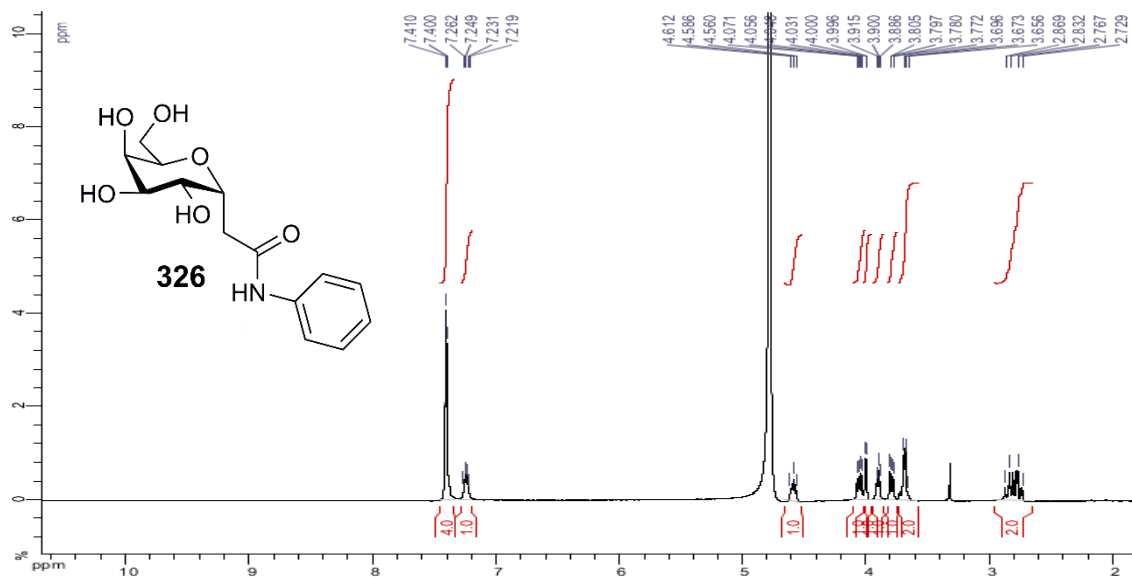


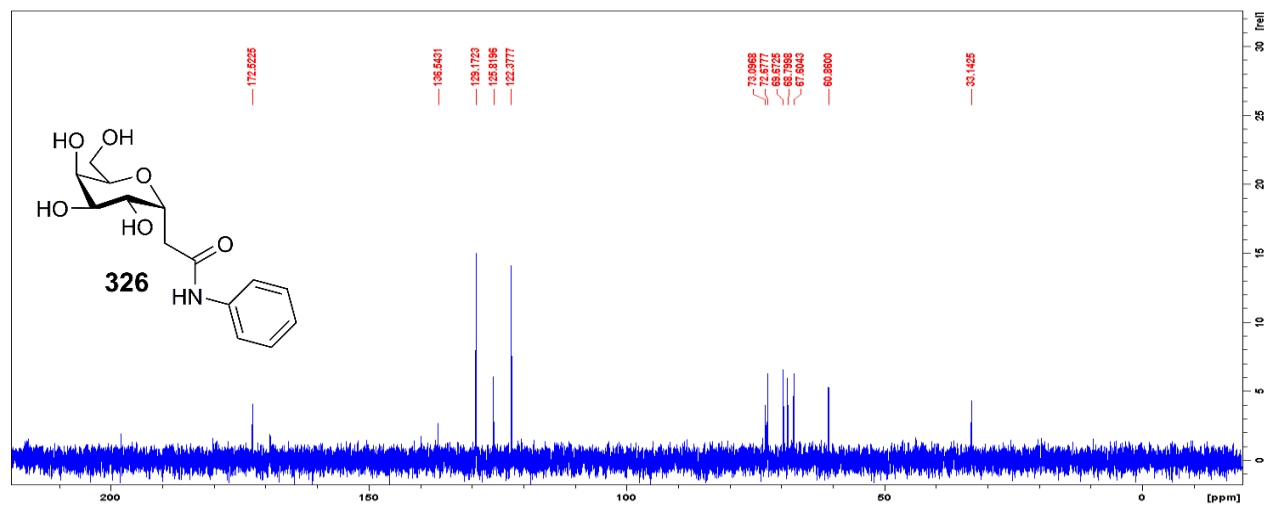


N-(Phenyl)-2-(α -D-galactopyranosyl)acetamide (**326**)

Compound **316** (186 mg, 0.40 mmol) was dissolved in MeOH (10 mL). 1 M NaOMe in MeOH was added dropwise until the solution had a pH of 12. The solution was stirred at RT for two hours, neutralized with Amberlite (H⁺) acidic resin, and filtered. The filtrate was evaporated and recrystallized in H₂O to obtain **326** (74.3 mg, 63%). ¹H NMR (400 MHz, D₂O): δ 7.40-7.41 (m, 4H), 7.22-7.26 (m, 1H), 4.56-4.61 (m, 1H), 4.05 (dd, J = 6.1, 9.9 Hz, 1H), 3.99-4.00 (m, 1H), 3.90 (t, J = 5.9 Hz, 1H), 3.79 (dd, J = 9.9, 3.2 Hz, 1H), 3.66-3.70 (m, 2H), 2.73-2.87 (m, 2H). ¹³C NMR (100 MHz, D₂O): δ 172.5, 136.5,

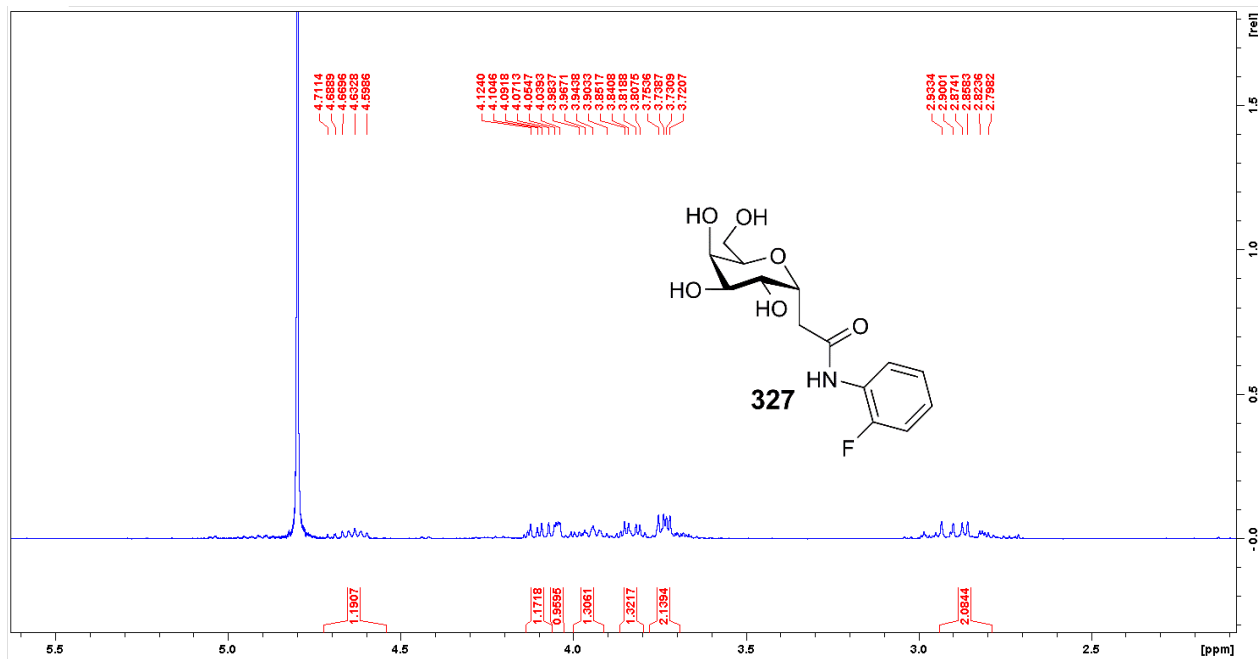
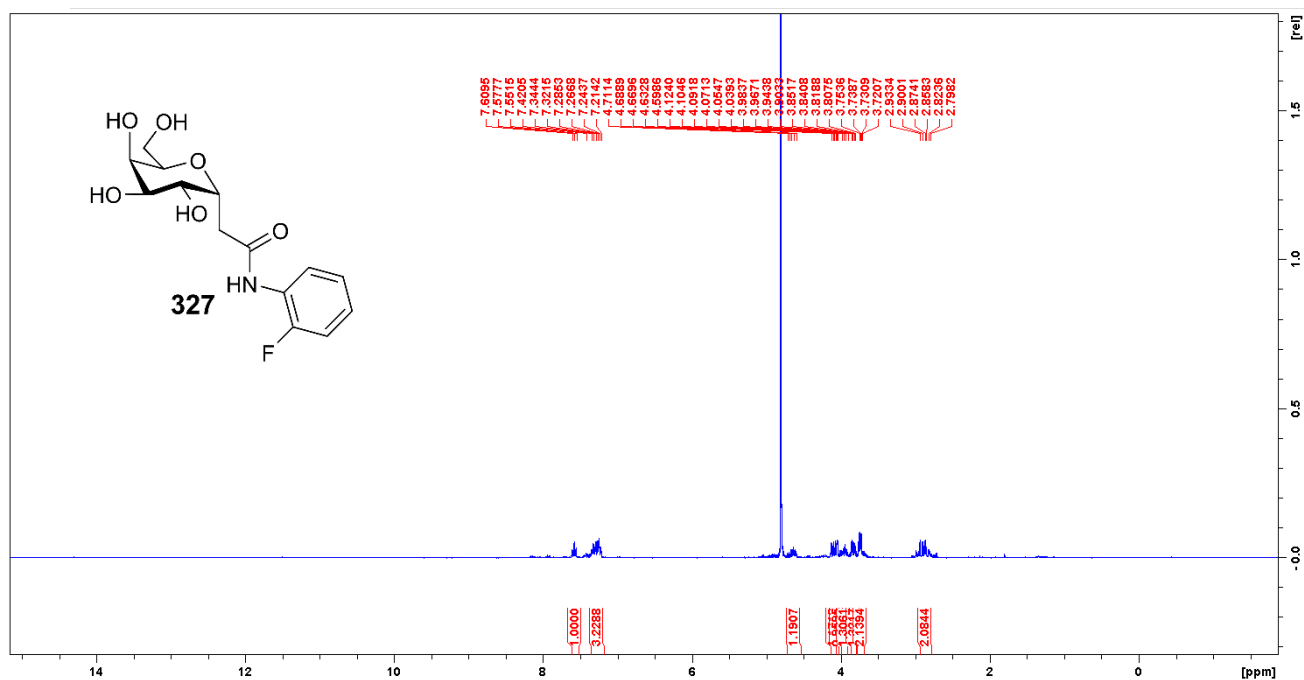
129.2, 125.8, 122.4, 73.1, 72.7, 69.8, 68.8, 67.6, 60.9, 33.1. LRMS (ESI): m/z calcd. for $C_{14}H_{19}NNaO_6$ [M + Na]⁺ 320.3, found 320.1.

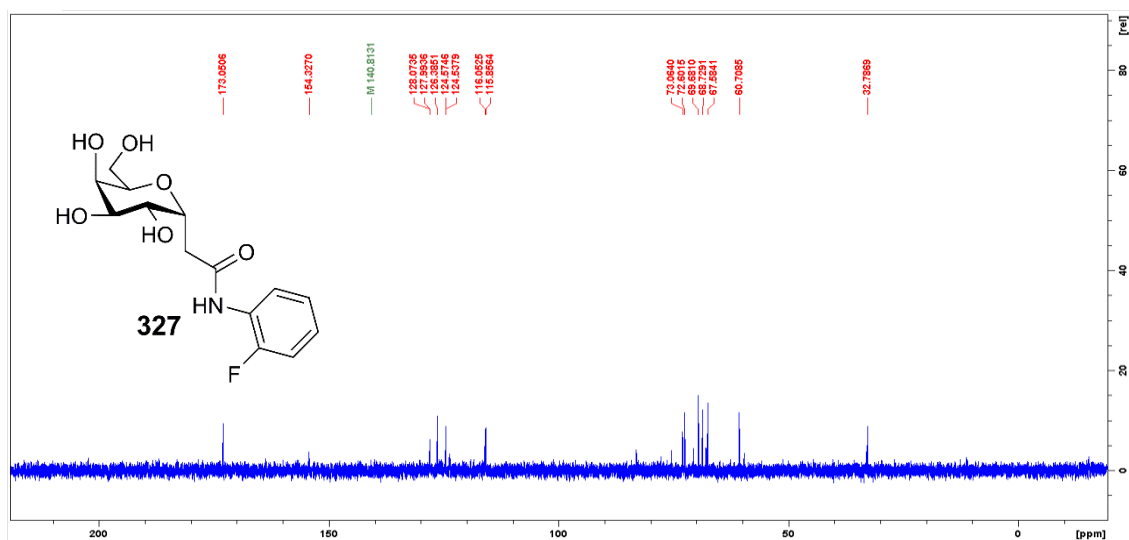




***N*-(2-Fluorophenyl)-2-(α -D-galactopyranosyl)acetamide (**327**)**

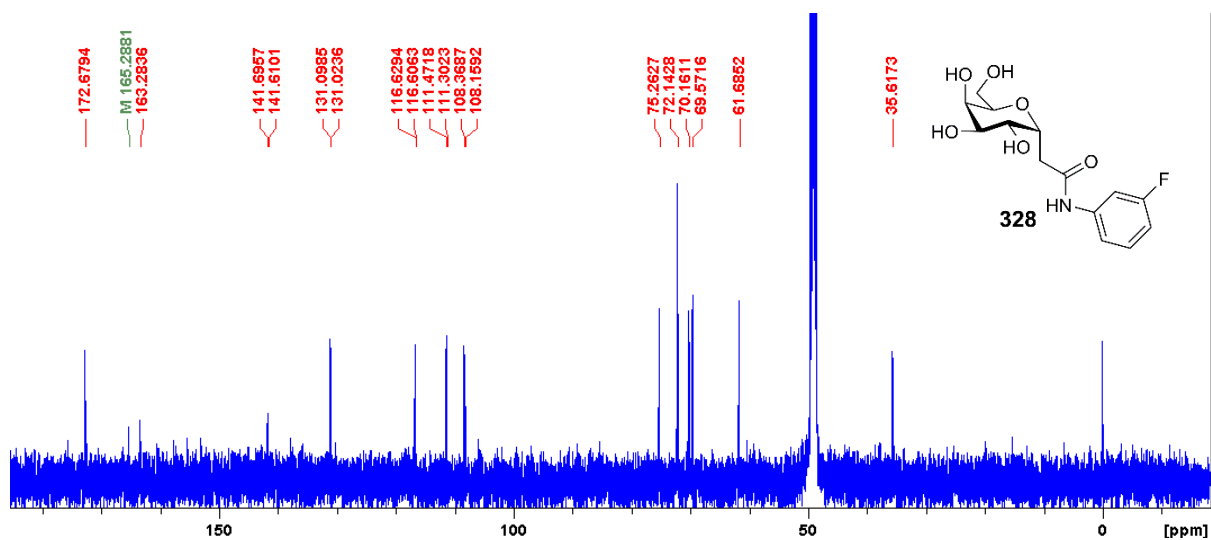
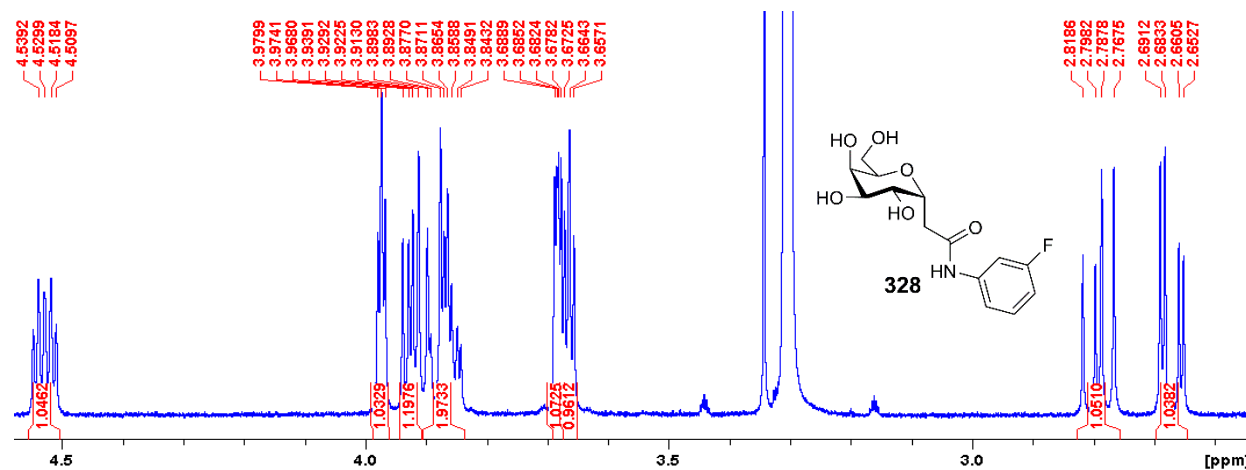
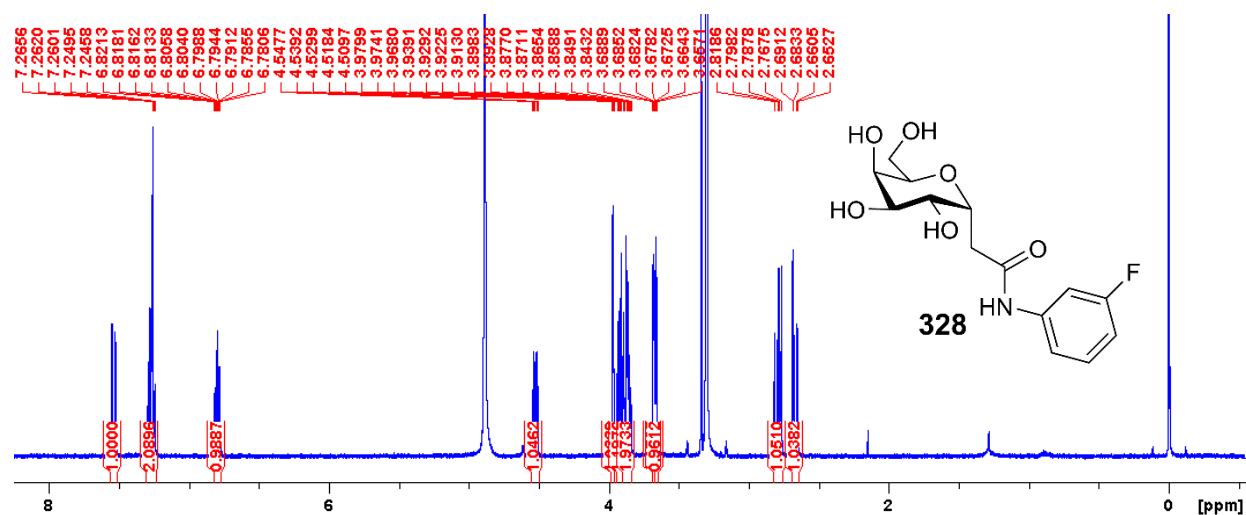
Compound **317** (50 mg, 0.10 mmol) was dissolved in 1 M NaOMe in MeOH (4 mL). The solution was stirred at RT for two hours, neutralized with Amberlite (H⁺) acidic resin, and filtered. The pure product **327** was obtained as a white solid after removing the solvent (15 mg, 47%). ¹H NMR (400 MHz, D₂O): δ 7.55-7.61 (m, 1H), 7.21-7.42 (m, 3H), 4.60-4.72 (m, 1H), 4.10 (dd, J = 9.7, 5.7 Hz, 1H), 4.04-4.05 (m, 1H), 3.90-3.98 (m, 1H), 3.83 (dd, J = 9.8, 3.2 Hz, 1H), 3.74 (dd, J = 6.8, 4.4 Hz, 1H), 2.80-2.93 (m, 2H). ¹³C NMR (100 MHz, D₂O): δ 173.1, 155.6 (d, J_{C-F} = 247.9 Hz), 128.0 (d, J_{C-F} = 8.0 Hz), 126.4, 124.6 (d, J_{C-F} = 3.6 Hz), 116.0 (d, J_{C-F} = 18.3 Hz), 73.1, 72.6, 69.7, 68.7, 67.6, 60.7, 32.8. LRMS (ES⁺): m/z calcd. for C₁₄H₁₈FNNaO₆ [M + Na]⁺ 338.1, found 338.1.





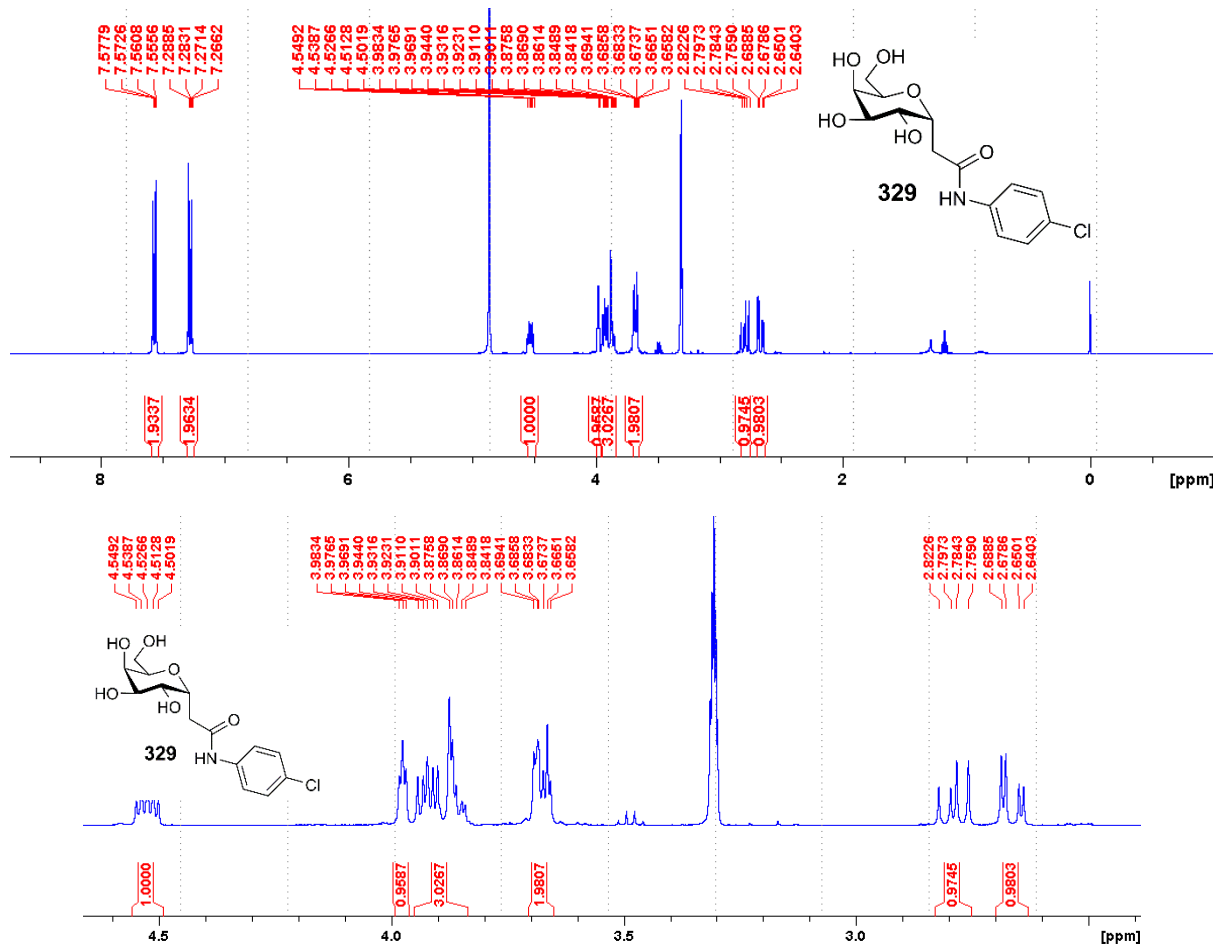
***N*-(3-Fluorophenyl)-2-(α -D-galactopyranosyl)acetamide (**328**)**

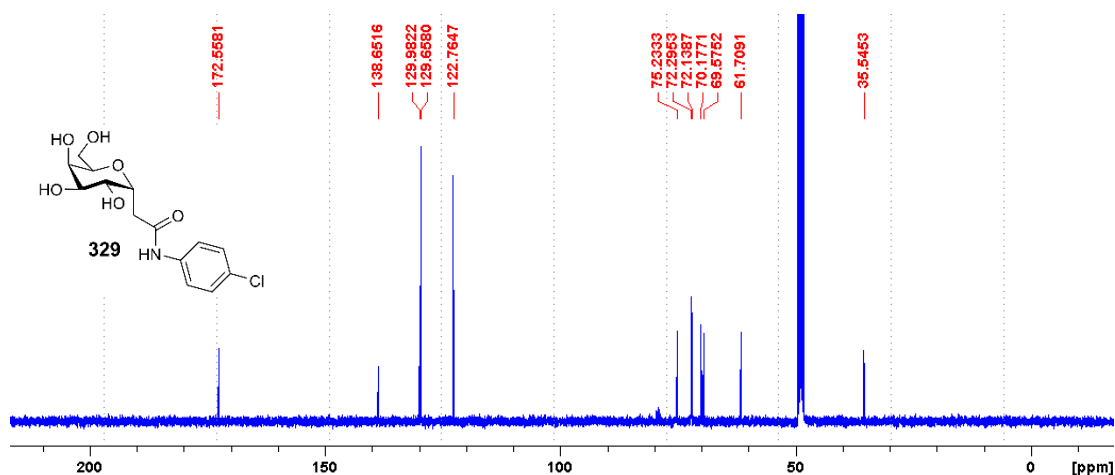
Compound **318** (55 mg, 0.11 mmol) was dissolved in MeOH (5 mL). 1 M NaOMe in MeOH was added dropwise until the solution was strongly basic. The solution was stirred at RT for 24 h, neutralized with Amberlite (H⁺) acidic resin, and filtered. The filtrate was evaporated and recrystallized in ethanol to obtain **328** as a white powder (20 mg, 58%). ¹H NMR (500 MHz, CD₃OD): δ 7.54 (dt, J = 2.3, 11.4 Hz, 1H), 7.27 (m, 2H), 6.80 (m, 1H), 4.53 (m, J = 4.8, 4.2 Hz, 1H), 3.97 (t, J = 3.0 Hz, 1H), 3.92 (dd, J = 8.3, 4.7 Hz, 1H), 3.87 (m, 2H), 3.68 (dd, J = 3.3, 1.9 Hz, 1H), 3.66 (t, J = 3.9 Hz, 1H), 2.79 (dd, J = 10.3, 15.4 Hz, 1H), 2.67 (dd, J = 3.9, 15.4 Hz, 1H). ¹³C NMR (125 MHz, CD₃OD): δ 172.7, 164.3 (d, $J_{C,F}$ = 254.1 Hz), 141.7 (d, $J_{C,F}$ = 11.3 Hz), 131.1 (d, $J_{C,F}$ = 9.4 Hz), 116.6, (d, $J_{C,F}$ = 2.9 Hz), 111.4 (d, $J_{C,F}$ = 21.2 Hz), 108.3 (d, $J_{C,F}$ = 25.8 Hz), 75.3, 72.1, 70.2, 69.6, 61.7, 35.6. LRMS (ESI): m/z calcd. for C₁₄H₁₈FO₆Na [M + Na]⁺ 338.1, found 338.0.



***N*-(4-Chlorophenyl)-2-(α -D-galactopyranosyl)acetamide (329)**

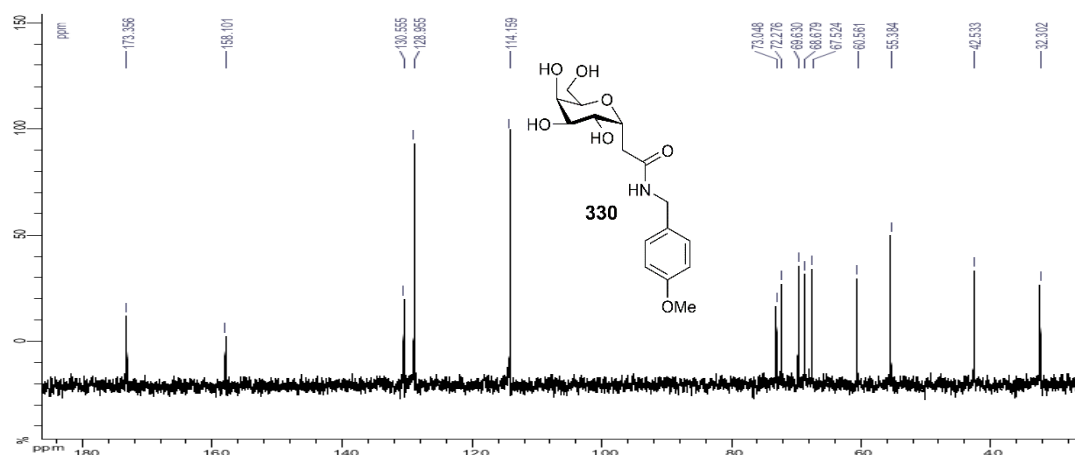
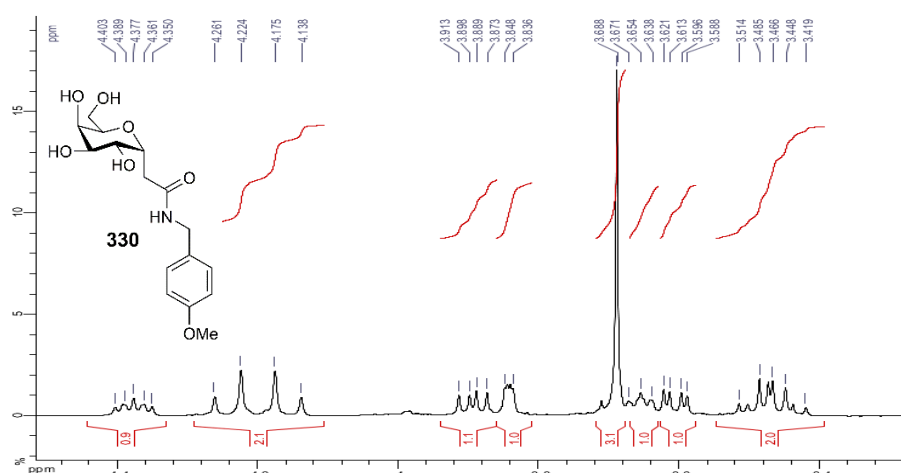
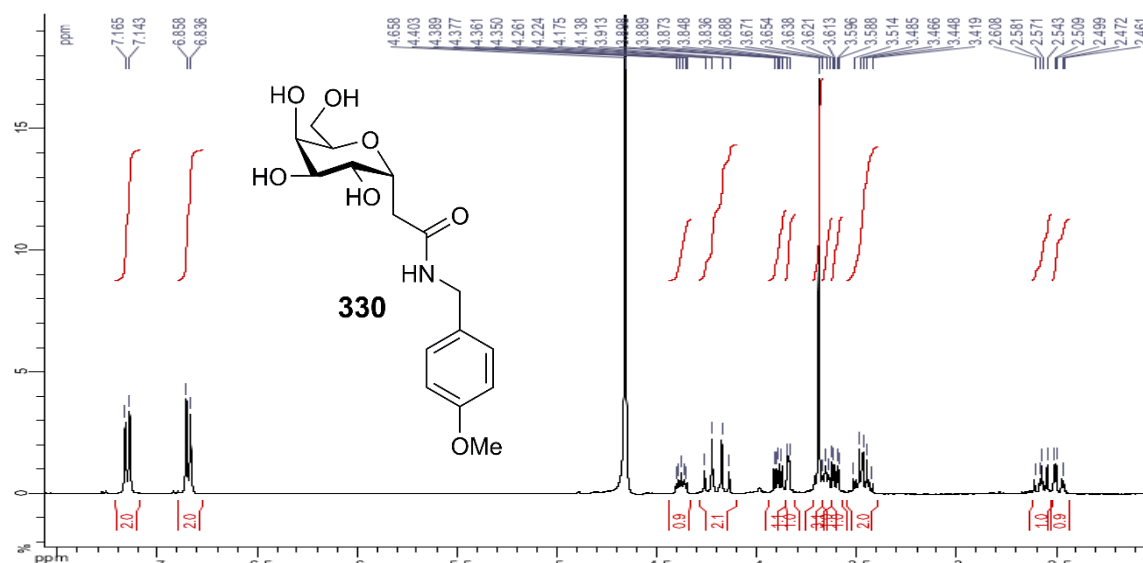
Compound **319** (44 mg, 0.088 mmol) was dissolved in MeOH (10 mL). 1 M NaOMe in MeOH was added dropwise until the solution became strongly basic, and the solution was stirred for two hours. The reaction mixture was neutralized with Amberlite (H⁺) acidic resin, filtered, and the solvent was removed to obtain **329** as a white solid (20 mg, 69%). ¹H NMR (400 MHz, MeOD): δ 7.57 (m, 2H), 7.28 (m, 2H), 4.52 (dt, J = 4.4, 10.4 Hz, 1H), 3.98 (t, J = 2.9 Hz, 1H), 3.89 (m, 3H), 3.68 (m, 2H), 2.79 (dd, J = 10.2, 15.4 Hz, 1H), 2.67 (dd, J = 4.0, 15.4 Hz, 1H). ¹³C (100 MHz, MeOD): δ 172.6, 138.7, 130.0, 129.7, 122.8, 75.2, 72.3, 72.1, 70.2, 69.6, 61.7, 35.5. LRMS (ESI): m/z calcd. for C₁₄H₁₈ClNO₆Na [M + Na]⁺ 354.1, found 354.0.





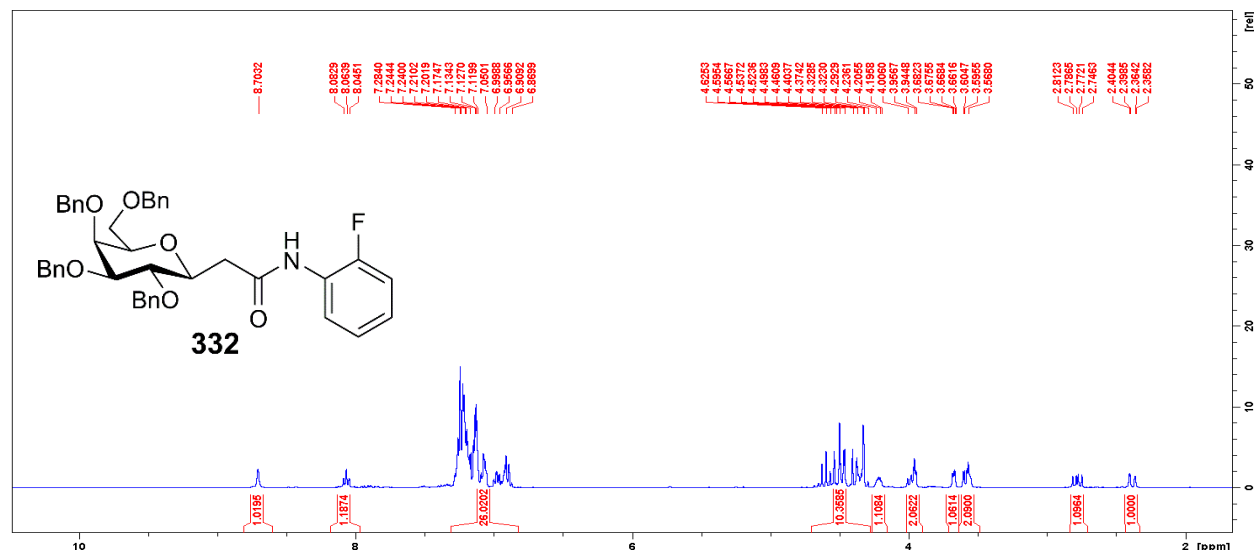
***N*-(4-Methoxybenzyl)-2-(α -D-galactopyranosyl)acetamide (**330**)**

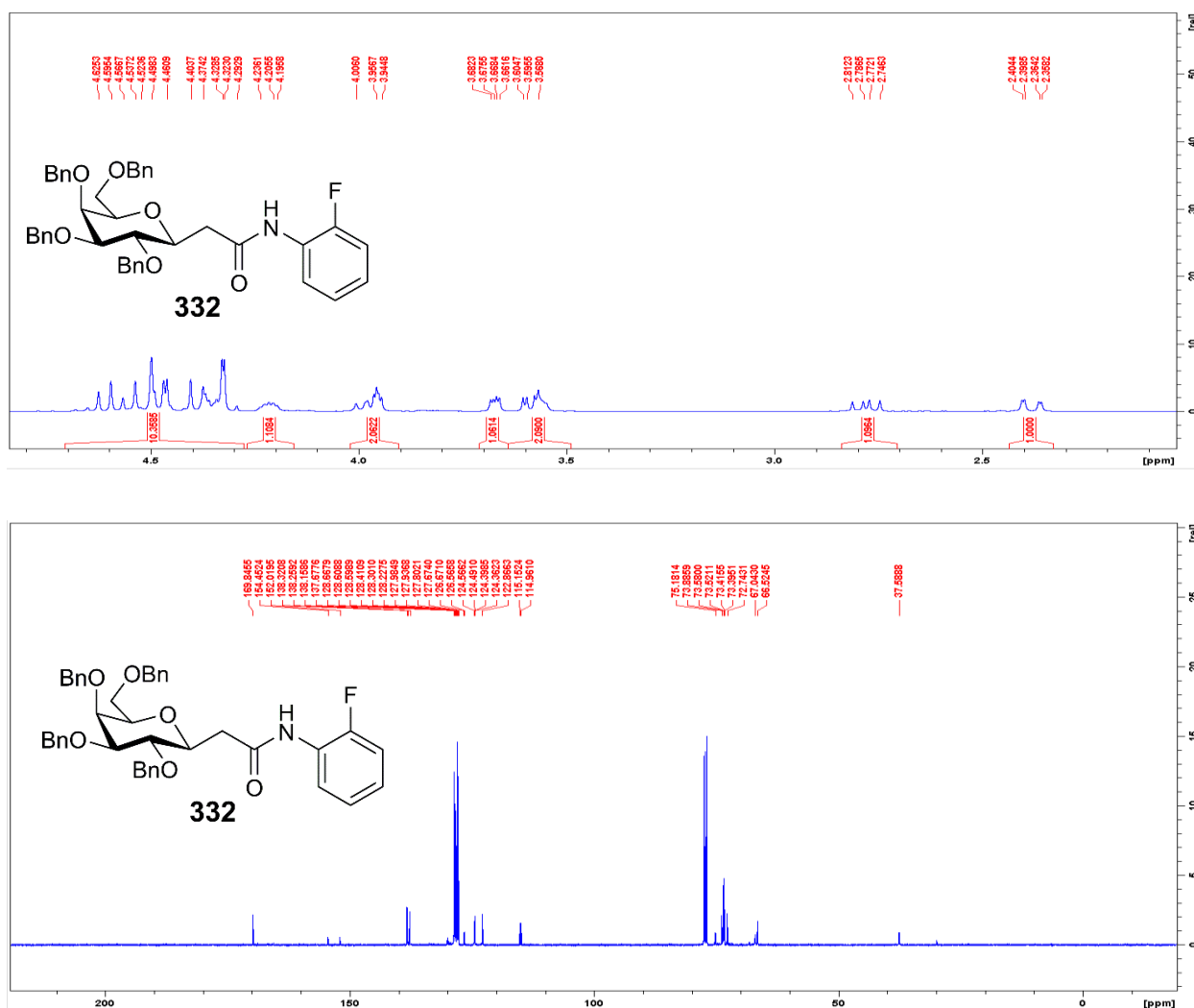
Compound **320** (0.6 g, 0.12 mmol) is dissolved in 1M NaOMe in methanol (4 mL) and distilled water (1 mL) and allowed to stir for one hour. Reaction completion was monitored by TLC (10% MeOH in DCM). Once complete, the mixture was neutralized with acidic Amberlite (H⁺) resin (monitored using pH paper). The mixture was filtered and lyophilized to yield **330** as a white powder (0.03 g, 65%). ¹H NMR (500 MHz, D₂O): δ 7.28 (d, J = 8.8 Hz, 2H), 6.97 (d, J = 8.6 Hz, 2H), 4.47-4.52 (m, 1H), 4.36 (d, J = 14.9 Hz, 1H), 4.28 (d, J = 14.8, 1H), 4.02 (dd, J = 9.8, 6.3 Hz, 1H), 3.96-3.97 (m, 1H), 3.81 (s, 3H), 3.76-3.79 (m, 1H), 3.73 (dd, J = 9.9, 3.4 Hz, 1H), 3.54-3.64 (m, 2H), 2.70 (dd, J = 15.0, 10.5 Hz, 1H), 2.61 (dd, J = 15.0, 10.5 Hz, 1H). ¹³C NMR (100 MHz, D₂O): δ 173.4, 158.1, 130.6, 129.0, 114.2, 73.1, 72.3, 69.6, 68.7, 67.5, 60.6, 55.4, 42.5, 32.3. LRMS (ESI): m/z calcd. for C₁₆H₂₃NNaO₇ [M + Na]⁺ 364.1, found 364.1.



***N*-(2-Fluorophenyl)-2-(2,3,4,6-tetra-*O*-benzyl- β -D-galactopyranosyl)acetamide (332)**

Compound **310** ((2,3,4,6-tetra-*O*-benzyl- β -D-galactopyranosyl)acetic acid) (275 mg, 0.47 mmol) was dissolved in 15 mL dry DCM. HCTU (195 mg, 0.47 mmol) and DIPEA (80 μ L, 0.47 mmol) were added and the solution was stirred for 15 min at RT. 2-Fluoroaniline (100 μ L, 1.41 mmol) and DIPEA (80 μ L, 0.47 mmol) were added and the resulting mixture was stirred overnight. The mixture was washed with 20% HCl, NaHCO₃, brine, and extracted in DCM. The pure product was obtained after silica gel column chromatography (2% MeOH in DCM) to yield **332** as a colourless oil (100 mg, 31%). ¹H NMR (400 MHz, CDCl₃): δ 8.70 (s, 1H), 8.06 (t, *J* = 7.6 Hz, 1H), 6.87-7.28 (m, 23H), 4.29-4.63 (m, 10H), 4.20-4.24 (m, 1H), 3.94-4.01 (m, 1H), 3.68 (m, dd, *J* = 5.6, 2.7 Hz, 1H), 3.57-3.60 (m, 2H), 2.78 (dd, *J* = 15.9, 10.2 Hz, 1H), 2.38 (dd, *J* = 16.1, 2.4 Hz, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 169.9, 153.3 (d, *J*_{C-F} = 244.9), 138.3, 138.3, 138.2, 137.7, 128.7, 128.6, 128.6, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 126.6 (d, *J*_{C-F} = 10.9 Hz), 124.5 (d, *J*_{C-F} = 7.5 Hz), 124.4 (d, *J*_{C-F} = 3.7 Hz), 122.9, 115.1 (d, *J*_{C-F} = 19.3 Hz), 75.2, 73.9, 73.6, 73.5, 73.4, 73.4, 72.7, 67.0, 66.5, 37.6. LRMS (ESI): *m/z* calcd. for C₄₂H₄₂FNNaO₆ [M + Na]⁺ 698.1, found 698.4.

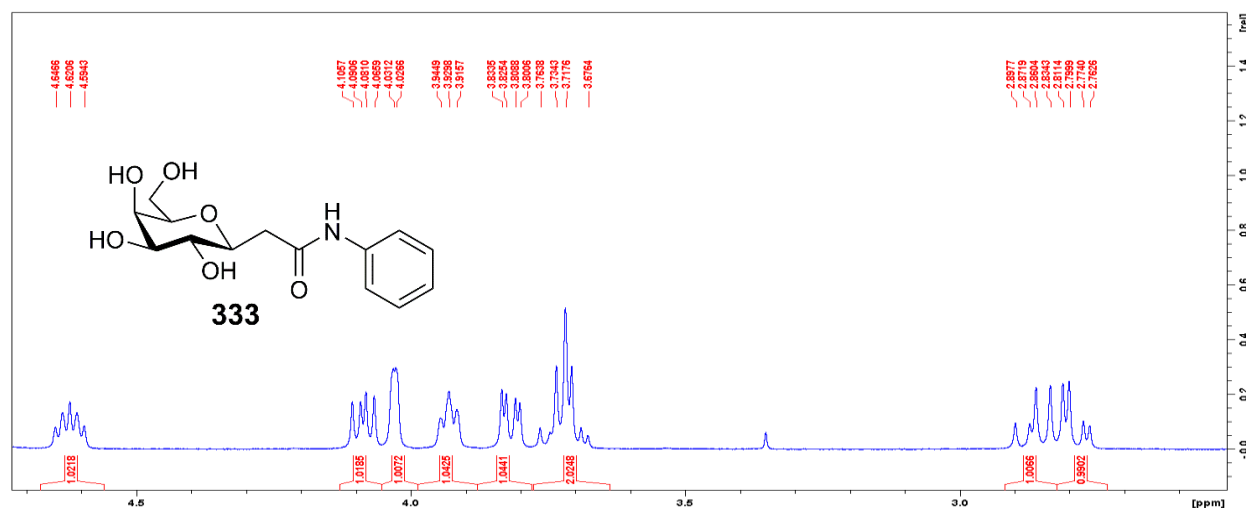
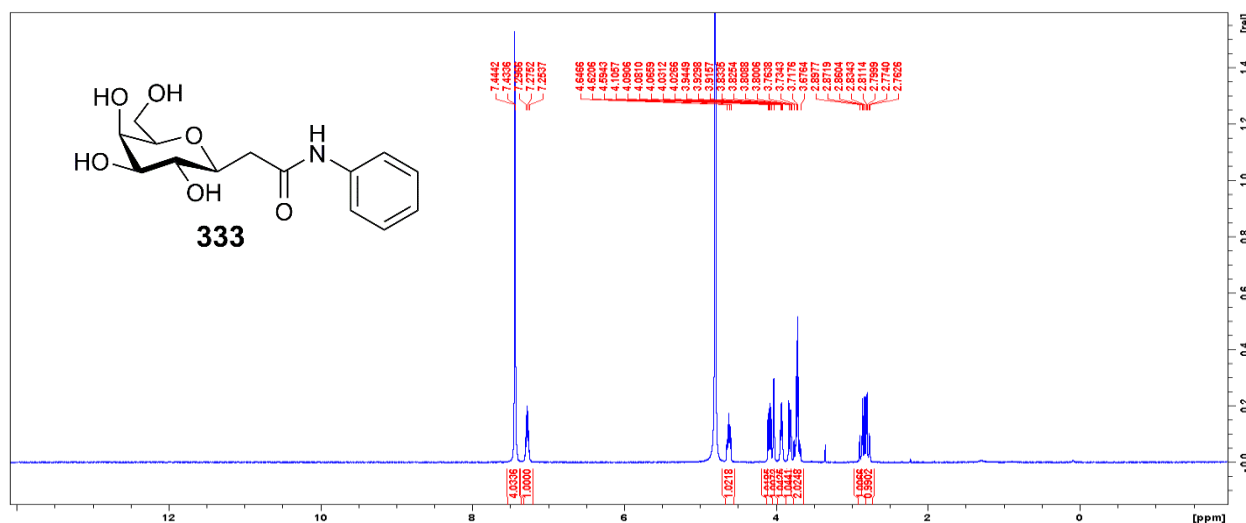


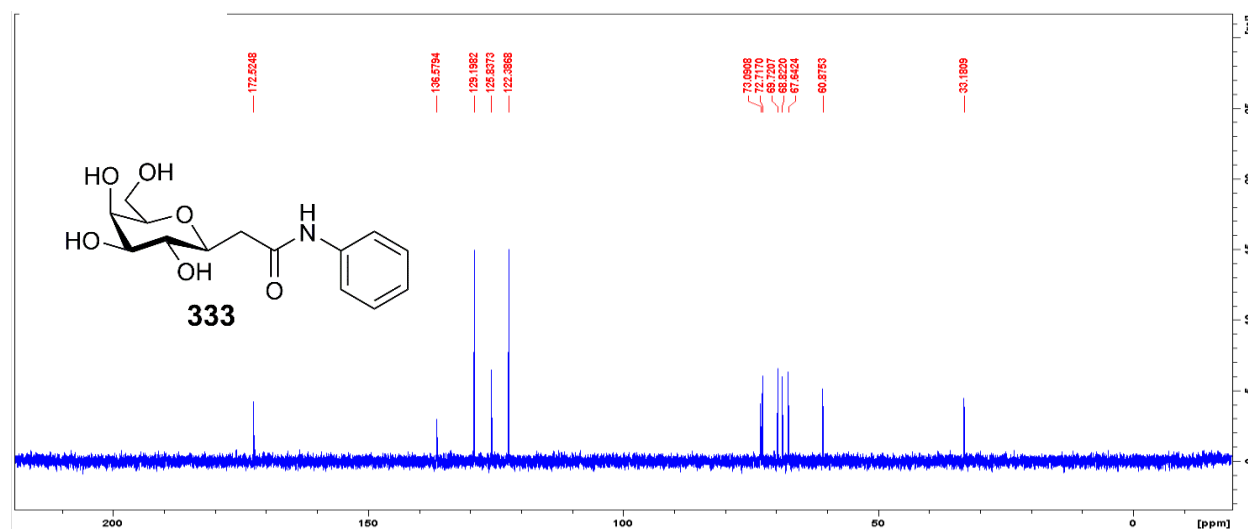


N-(Phenyl)-2-(β-D-galactopyranosyl)acetamide (**333**)

Compound **310** ((2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)acetic acid) (250 mg, 0.43 mmol) was dissolved in dry DCM. HCTU (182 mg, 0.43 mmol) and DIPEA (100 μL, 0.44 mmol) were added and the resulting solution was stirred for 15 min at RT. Aniline (0.12 mL, 1.32 mmol) and DIPEA (100 μL, 0.44 mmol) were added and the resulting mixture was stirred overnight. The mixture was washed 3x with 20% HCl, NaHCO₃, brine, and extracted in DCM. The crude product **331** was dissolved in EtOH (3 mL), MeOH (3 mL) and EtOAc (3 mL). Pd/C (5 mol %) was added, atmospheric air was removed by aspiration and hydrogen gas was introduced. The reaction mixture was stirred for 24 hours before being filtered over celite and concentrated. The pure product **333** was obtained after silica gel column chromatography (10%

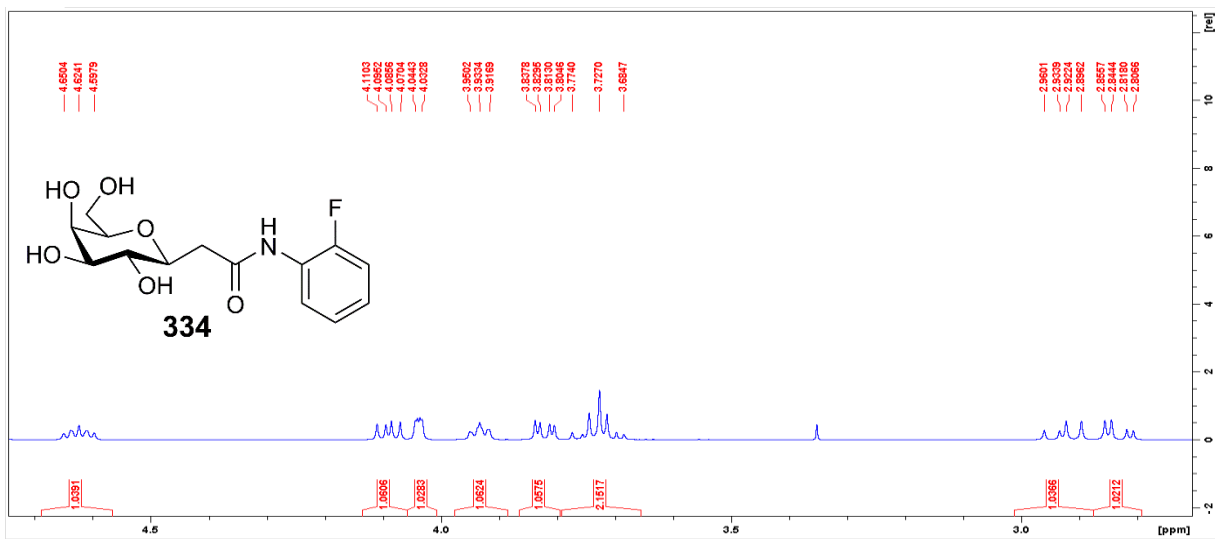
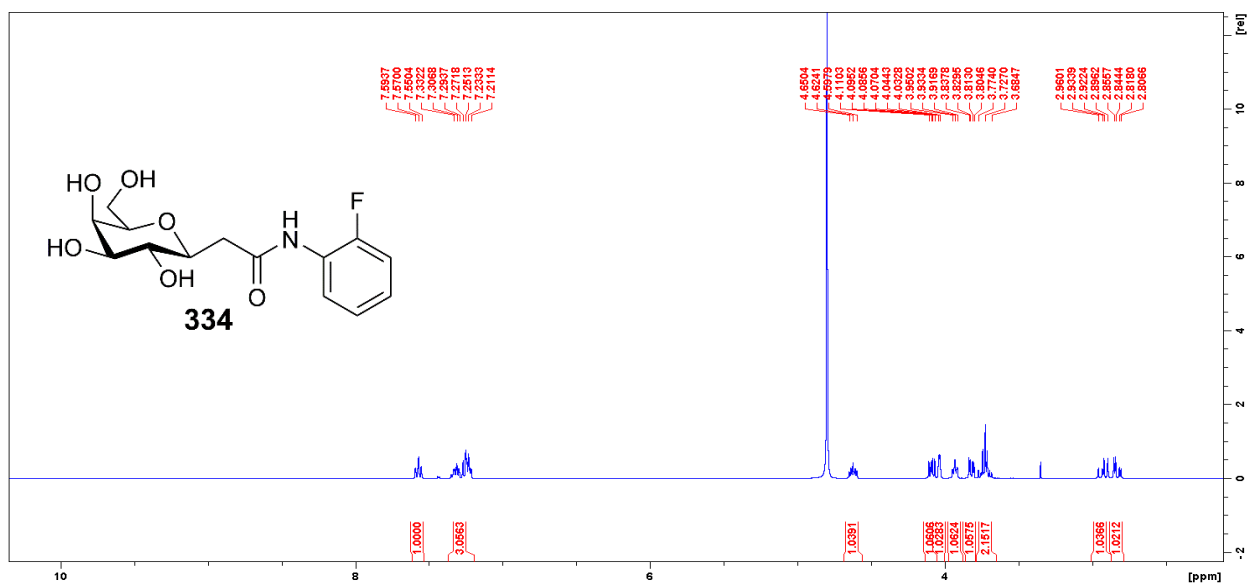
→ 20% MeOH in DCM) and recrystallization in EtOH (45 mg, 35%). ^1H NMR (400 MHz, D_2O): δ 7.44 (d, $J = 4.2$, 4H), 7.25-7.30 (m, 1H), 4.59-4.65 (m, 1H), 4.09 (dd, $J = 9.9$, 6.1 Hz, 1H), 4.03 (d, $J = 1.6$ Hz, 1H), 3.93 (t, $J = 6.3$ Hz, 1H), 3.82 (dd, $J = 9.9$, 3.3 Hz, 1H), 3.68-3.76 (m, 2H), 2.87 (dd, $J = 14.9$, 10.2 Hz, 1H), 2.79 (dd, $J = 14.9$, 4.6 Hz, 1H). ^{13}C (100 MHz, D_2O): δ 172.5, 136.6, 129.2, 125.8, 122.4, 73.1, 72.7, 69.7, 68.8, 67.6, 60.9, 33.2. LRMS (ESI): m/z calcd. for $\text{C}_{14}\text{H}_{19}\text{NNaO}_6$ $[\text{M} + \text{Na}]^+$ 320.3, found 320.1.

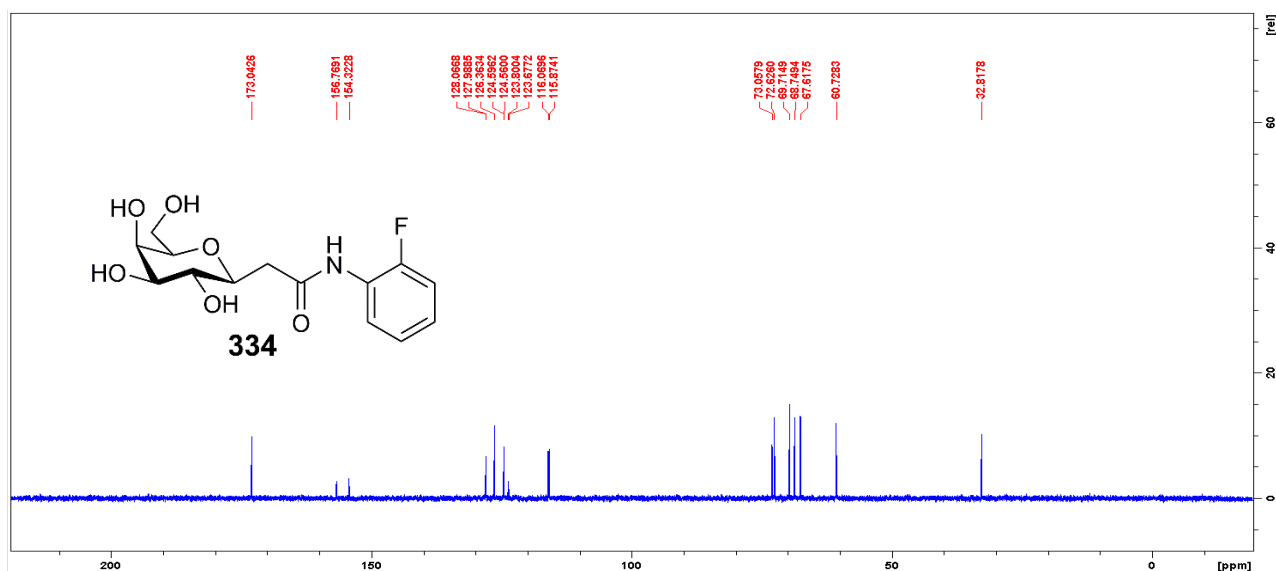




N-(2-Fluorophenyl)-2-(β -D-galactopyranosyl)acetamide (**334**)

Compound **332** (100 mg, 0.15 mmol) was dissolved in EtOH (3 mL), MeOH (3 mL) and EtOAc (3 mL). Pd/C (5 mol %) was added, atmospheric air was removed by aspiration and hydrogen gas was introduced. The reaction mixture was stirred for 24 hours before being filtered over celite and concentrated. The pure product **334** was obtained after silica gel column chromatography (10% to 20% MeOH in DCM) and recrystallization in EtOH (38 mg, 81%). ^1H NMR (400 MHz, D_2O): δ 7.55-7.59 (m, 1H), 7.21-7.33 (m, 3H), 4.60-4.65 (m, 1H), 4.10 (dd, J = 9.8, 6.0 Hz, 1H), 4.03-4.04 (m, 1H), 3.92-3.95 (m, 1H), 3.83 (dd, J = 10.0, 3.2 Hz, 1H), 3.68-3.77 (m, 2H), 2.93 (dd, J = 15.1, 10.6 Hz, 1H), 2.83 (dd, J = 15.1, 4.6 Hz, 1H). ^{13}C NMR (100 MHz, D_2O): δ 173.0, 155.6 (d, $J_{\text{C-F}}$ = 246.4 Hz), 128.0 (d, $J_{\text{C-F}}$ = 7.8 Hz), 126.4, 124.6 (d, $J_{\text{C-F}}$ = 3.7 Hz), 123.7 (d $J_{\text{C-F}}$ = 12.6 Hz), 116.0 (d $J_{\text{C-F}}$ = 19.6 Hz), 73.1, 72.6, 69.7, 68.8, 67.6, 60.7, 32.8. LRMS (ESI): m/z calcd. for $\text{C}_{14}\text{H}_{18}\text{FNNaO}_6$ $[\text{M} + \text{Na}]^+$ 338.3, found 338.1.





3.7 References

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Chapter 4: Quantifying the importance of amphiphilicity in small-molecule IRIs

4.1 Introduction

Recently, two classes of small molecules were discovered: the truncated AFGP analogues and the *N*-alkyl- and *N*-cycloalkyl-aldonamides (Figure 4.1).¹⁻⁴ The truncated AFGP analogues possess varying degrees of IRI activity depending on the length of the alkyl chain (Figure 4.1A).¹ Truncated AFGP analogues with longer alkyl chain lengths are more IRI active than analogues with shorter chains. For example, the truncated AFGP analogue with an alkyl chain length of 3 carbons (**125**) possesses moderate IRI activity (66% MGS) whereas the derivative with 7 carbons (**129**) is potently active (10% MGS). The second class, *N*-(cyclo)alkyl-aldonamides (Figure 4.1B & C), also possess varying degrees of IRI activity depending on the length of the alkyl chain. Analogue **151** (*N*-propyl-gluconamide) is inactive while analogue **153** (*N*-hexyl-gluconamide) is a very potent inhibitor of ice recrystallization.

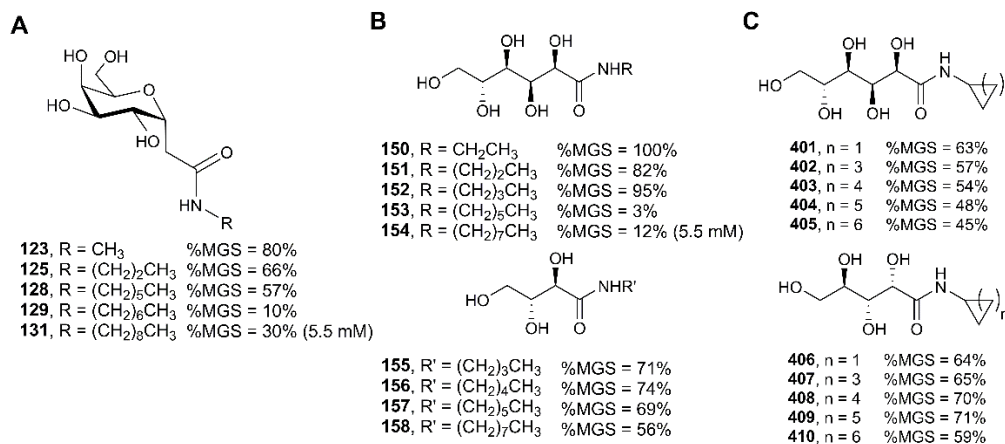


Figure 4.1. Structures of (A) truncated AFGP analogues¹, (B) *N*-alkyl-aldonamides³, and (C) *N*-cycloalkyl-aldonamides previously assessed for IRI activity (at 22 mM unless otherwise indicated).

N-cycloalkyl-aldonamides (Figure 4.1C) are weakly to moderately active.⁴ Interestingly, analysis of these derivatives reveals that the molecules with the largest cycloalkyl structure, *N*-cyclooctyl-gluconamide (**405**) and *N*-cyclooctyl-arabonamide (**410**), are the most IRI active of their class. This is consistent with the observation that *N*-alkyl-aldonamides with longer alkyl chains are more IRI active

than those with shorter chains. However, while *N*-cyclooctyl-gluconamide (**405**) is the most active of the *N*-cycloalkyl-aldonamides, it is significantly less active compared to the acyclic form (*N*-octyl-gluconamide, **154**).

The chain length of the hydrophilic moiety is also important for the IRI activity of the *N*-(cyclo)alkyl-aldonamides. For example, *N*-hexyl-gluconamide (**153**) is a very potent inhibitor of ice recrystallization whereas *N*-hexyl-erythronamide (**157**) is inactive and only differs from **153** by two hydroxyl group units. Furthermore, *N*-cycloheptyl-arabonamide (**409**) is significantly less active than *N*-cycloheptyl-gluconamide (**404**) which differs by only one fewer hydroxyl group.

It was previously determined that IRI activity is not correlated to micelle formation.² The important structural features required for IRI activity have not been elucidated and this has impeded the rational design of new IRI active small molecules. Data from previous studies suggest that a certain balance between hydrophobicity and hydrophilicity is required for IRI activity. However, this balance has yet to be quantified in a meaningful way.

In this chapter, the relationship between amphiphilicity and IRI activity is investigated and a metric to quantify the amphiphilic nature of these IRIs is presented. The results from this study have been recently submitted to *RSC Advances* entitled “Structure activity relationships of small-molecule amphiphilic ice recrystallization inhibitors”.⁵

4.2 Establishing a metric for measuring amphiphilicity

Previous structure function analyses indicate that a certain balance between hydrophobicity and hydrophilicity is required for IRI activity. Hydrophobicity is a commonly used parameter in quantitative structure activity relationships.⁶⁻⁸ The ability to quantify the amphiphilicity required for IRI activity would facilitate the rational design of potent IRIs.

One of the most classic ways to measure hydrophobicity is by obtaining octanol-water partition coefficients ($\log P$).⁶⁻⁸ The traditional stir-flask method for determining the $\log P$ of non-ionizing, non-

surfactant type chemicals is well documented.⁸ However, the ability to measure log *P* for surfactants is considered impractical due to their amphiphilic nature, which gives them a tendency to form micelles and reside at the octanol–water interface.⁶ Thus an alternative method for measuring hydrophobicity was required for the surfactant-like small molecules used in this study.

In order to effectively quantify the hydrophobic/hydrophilic balance required for IRI activity, an appropriate metric for this parameter must first be described. Using MarvinSketch™, the polar surface area (PSA) and molecular surface area (MSA) of a molecule can be predicted.^{9,10} The PSA represents the surface area of the polar atoms in the molecule while the MSA represents the surface area of the entire molecule. Using these parameters as a ratio (PSA/MSA), the fraction of the molecule which is polar can be calculated. The PSA, MSA and PSA/MSA values calculated by MarvinSketch™ for compounds **150-153** and **155-158** are shown in Table 4.1. The conformation of a given molecule in MarvinSketch™ is optimized based on one molecule in the gas phase.¹⁰ Therefore, the surface area of a molecule with a long alkyl chain might not accurately describe the conformation of that molecule in the presence of other molecules and in solution. Furthermore, the PSA/MSA ratio does not consider the absolute size of the hydrophobic portion. Therefore, a second parameter was established to measure amphiphilicity in a different way. The CH_n/OH ratio was used as an alternative parameter for hydrophobic/hydrophilic balance. This is a simple ratio between the number of carbons in the hydrophobic alkyl chain and the

Compound	PSA	MSA	PSA/MSA	Number of carbon units in alkyl chain tail (CH _n)	Number of hydroxyl groups in polyol head (OH)	CH _n /OH
150	130.25	333.49	0.39	2	5	0.40
151	130.25	364.93	0.36	3	5	0.60
152	130.25	395.08	0.33	4	5	0.80
153	130.25	456.29	0.29	6	5	1.20
155	89.79	317.48	0.28	4	3	1.33
156	89.79	348.79	0.26	5	3	1.67
157	89.79	378.86	0.24	6	3	2.00
158	89.79	440.29	0.20	8	3	2.67

Table 4.1. PSA, MSA and PSA/MSA values calculated by MarvinSketch™ and CH_n/OH ratio was calculated for compounds **150-153** and **155-158**. Compound **154** is excluded because it was insoluble at 22 mM and was tested at a lower concentration (5.5 mM).²

number of hydroxyl groups in the polyol portion. This ratio considers the absolute size of both the hydrophilic and hydrophobic portion and is not complicated by the conformation of the molecules. The CH_n/OH ratio for compounds **150-153** and **155-158** are shown in Table 4.1.

For this study, only the *N*-alkyl-aldonamides (**150-158**) were included and the truncated AFGP analogues (**123-131**) were omitted. This is because the *N*-alkyl-aldonamides belong to the same class of small molecules and therefore possess the same generic structure (Figure 4.2). These molecules all possess a hydrophilic polyol head and a hydrophobic alkyl chain tail and are classified by the length of the polyol head. *N*-Alkyl-gluconamides, -arabonamides, or -erythronamides possess five, four or three hydroxyl groups in the polyol head respectively.^{3,4} *N*-alkyl-arabonamides are truncated by one carbon unit from *N*-alkyl-gluconamides. *N*-alkyl-erythronamides are truncated by two carbon units from *N*-alkyl-gluconamides. The truncated carbon units are adjacent the amide bond and stereochemistry is conserved on the remaining carbon units. Furthermore, only compounds that were soluble at 22 mM were included in the analysis.

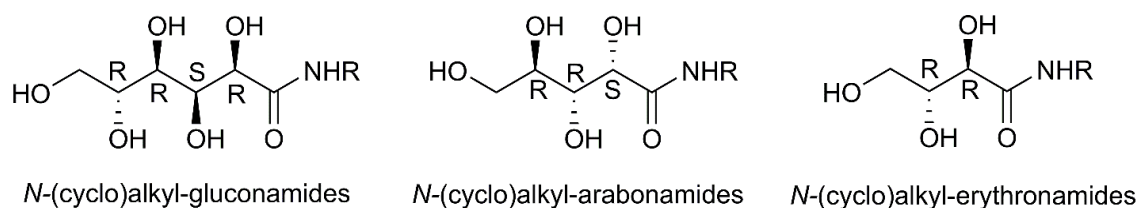


Figure 4.2. Structures of investigated aldonamides.

In order to determine if a correlation exists between IRI activity and amphiphilicity, PSA/MSA and CH_n/OH ratios were plotted with respect to the IRI activities (%MGS) of the *N*-alkyl-aldonamides and the results are shown in Figure 4.3. There was one outlier, *N*-hexyl-gluconamide (**153**, 3% MGS). Excluding this outlier, a strong linear correlation between %MGS and both PSA/MSA and CH_n/OH was observed ($R^2 > 0.84$). IRI activity decreased when the PSA fraction increased or when the hydrophobic fraction (number of CH_n groups) decreased. Overall, both graphs show that IRI activity decreases as the molecules become more polar. For simplicity, the CH_n/OH ratio will be used for future analysis.

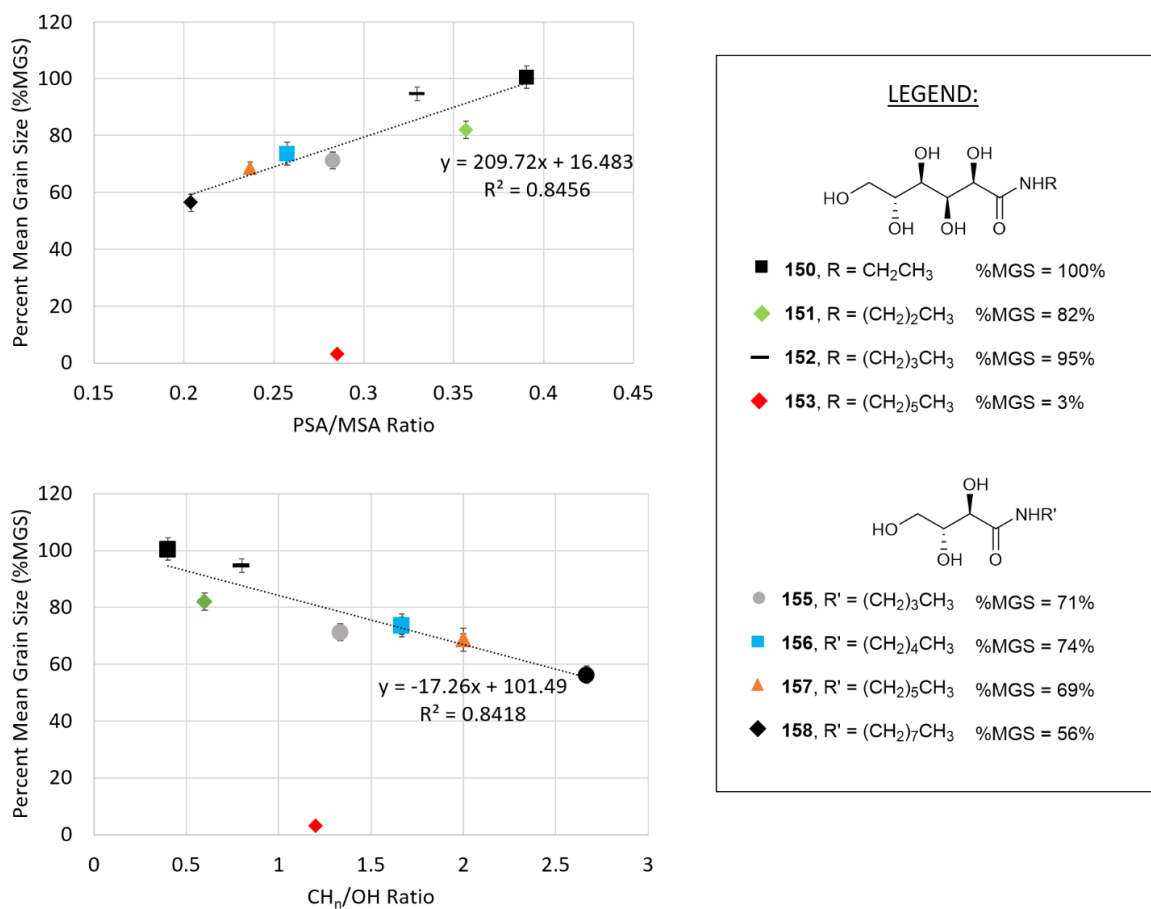


Figure 4.3. Correlation of %MGS vs. PSA/MSA ratio and %MGS vs. CH_n/OH ratio of *N*-alkyl-aldonamides.

The CH_n/OH ratio was then plotted with respect to the IRI activity of the *N*-cycloalkyl-gluconamides and –arabonamides (Figure 4.4) and it was found that IRI activity was not correlated to amphiphilicity ($R^2 = 0.0074$).

However, when graphed separately, the IRI activities of the *N*-cycloalkyl-gluconamides possessed a strong linear correlation to CH_n/OH ratios ($R^2 = 0.97$). Meanwhile, there was no correlation for the *N*-cycloalkyl-arabonamides ($R^2 = 0.01$) (Figure 4.5). The only difference between these two classes is the presence of one CH_n/OH group. Therefore, a potential reason why the *N*-cycloalkyl-gluconamides possessed a correlation to CH_n/OH ratios while the *N*-cycloalkyl-arabonamides did not could result from differences in conformations in solution as result of the extra CH_n/OH group on the gluconamides. Conformational analysis for these molecules would be required to investigate this.

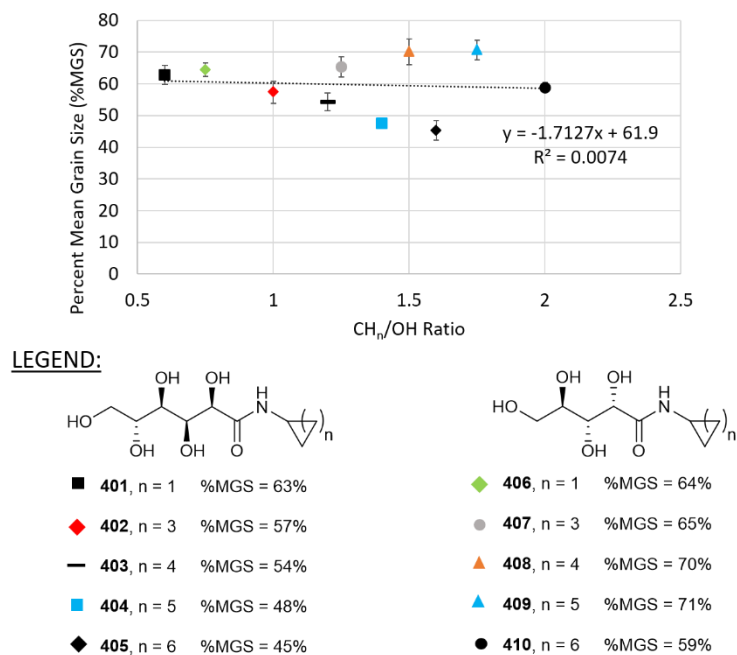


Figure 4.4. Correlation of %MGS vs. CH_n/OH ratio for all *N*-cycloalkyl-gluconamides and -arabonamides.

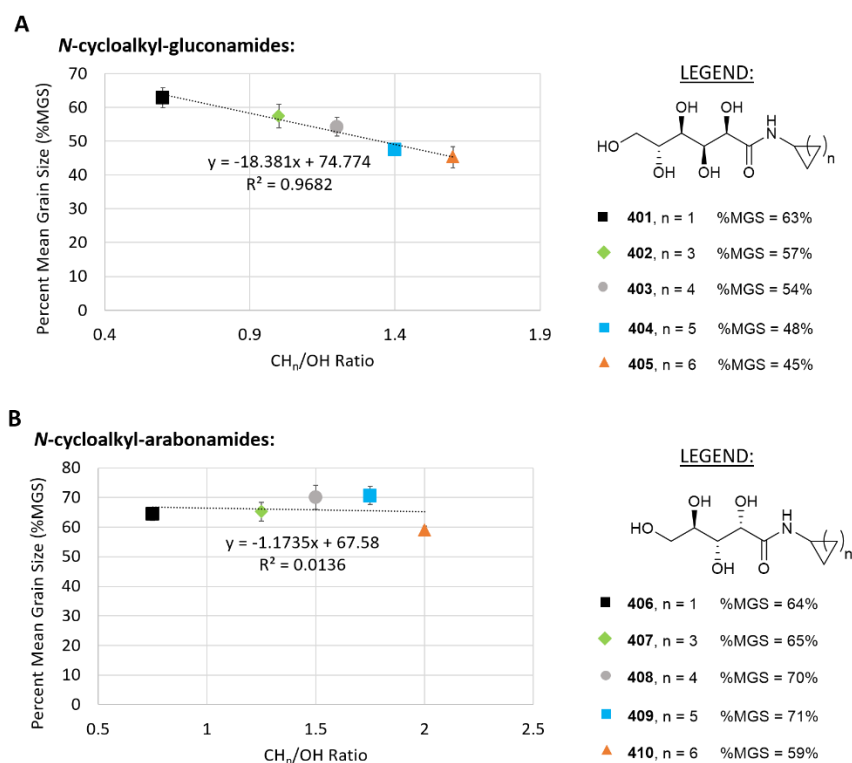


Figure 4.5. Correlation of %MGS vs. CH_n/OH ratio for (A) *N*-cycloalkyl-gluconamides and (B) %MGS vs. CH_n/OH ratio for *N*-cycloalkyl-arabonamides.

To summarize, the IRI activity of the *N*-alkyl-aldonamides is correlated to their amphiphilicity. The same correlation does not exist for the entire set of *N*-cycloalkyl-aldonamides; however, a correlation is observed with the *N*-cycloalkyl-gluconamides. It is interesting that a correlation exists for the *N*-alkyl-aldonamides but not for the *N*-cycloalkyl-aldonamides. The only difference between these two sets of compounds is the hydrophobic moiety is linear in the *N*-alkyl-aldonamide set and is cyclic in the *N*-cycloalkyl-aldonamide set. Therefore, the hydrophobic alkyl chain in the *N*-alkyl-aldonamide class may possess more degrees of rotation and therefore could adopt more conformations in solution compared to the *N*-cycloalkyl-aldonamide class. The constrained hydrophobic cycloalkyl chain in the *N*-cycloalkyl-aldonamide class could restrict the number of possible conformations therefore making it impossible to simply correlate IRI activity to amphiphilicity for these molecules. More complex conformational analysis would be required to investigate this.

In order to assess the validity of the correlations that were discovered in this section, several targets were designed, synthesized and assessed for IRI activity.

4.3 Targets and synthetic routes

Several molecules were designed to test the correlations observed in Section 4.2. The structures of these targets are shown in Figure 4.6. To test the validity of the correlation between IRI activity and amphiphilicity of the *N*-alkyl-aldonamides, *N*-hexyl-arabonamide (**411**) was synthesized and assessed for IRI activity. Possessing a six carbon alkyl chain and four hydroxyl groups, *N*-hexyl-arabonamide (**411**) contains a CH_n/OH ratio of 1.5. Using the linear correlation, the IRI activity can be predicted algebraically from the equation of the line ($y = -17.26x + 101.49$). If the correlation is valid, **411** should have an IRI activity of 76%.

To test the validity of the correlation between IRI activity and amphiphilicity of the *N*-cycloalkyl-gluconamides, *N*-cyclobutyl-gluconamide (**412**) was synthesized and assessed for IRI activity. The IRI activity for **412** is predicted algebraically to be 60% (CH_n/OH ratio is 0.8 and $y = -18.38x + 74.77$).

No correlation between IRI activity and amphiphilicity of the entire set of the *N*-cycloalkyl-aldonamides was observed. Therefore, the *N*-cycloalkyl-erythronamides **413-417** were synthesized to determine if the inclusion of these molecules in the analysis will result in a correlation between IRI activity and amphiphilicity of the *N*-cycloalkyl-aldonamides.

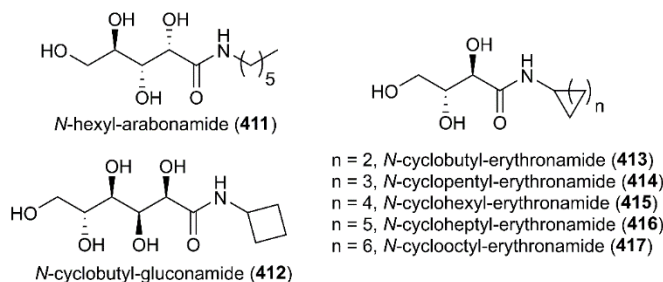
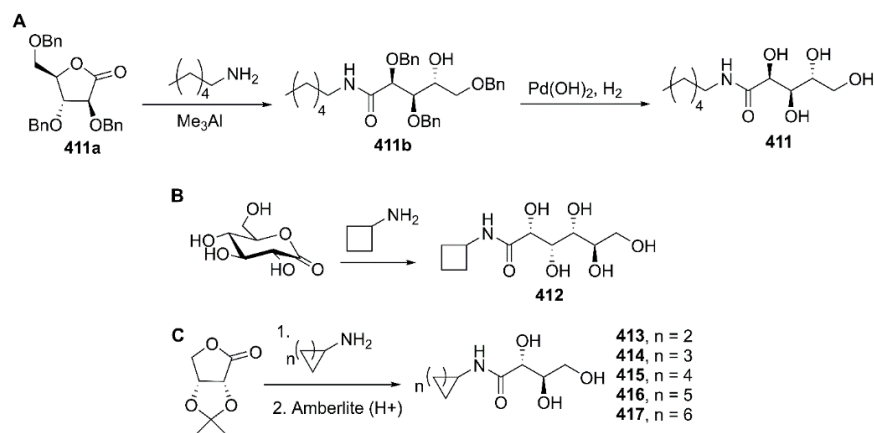


Figure 4.6. Structures of targets **411-417** to be synthesized and assessed for IRI activity.

The synthetic route to the targets **411-417** is shown in Scheme 4.1. The syntheses of all targets involves the aminolysis of lactones with various amines. Target **411** involves the lactone opening of 2,3,5-*O*-benzyl-D-arabinolactone (**411a**) with *N*-hexylamine followed by debenzylation. 2,3,5-*O*-Benzyl-D-arabinolactone (**411a**) was provided by Dr. Malay Doshi, synthesized as previously described.^{4,11} Target **412** was synthesized *via* aminolysis of δ -gluconolactone with *N*-cyclobutylamine. Targets **413-417** were synthesized *via* aminolysis of 2,3-*O*-isopropylidene-D-erythronolactone with *N*-cycloalkylamines followed by acidic isopropylidene deprotection.



Scheme 4.1. Synthetic routes for targets **411-417**.

4.4 Ice recrystallization inhibition activity and amphiphilicity of other small molecules

The IRI activities and structures of targets **411-417** are shown in Figure 4.7. *N*-Hexyl-arabonamide (**411**) was highly IRI active with a %MGS of 8%. It possesses similar activity to *N*-hexyl-gluconamide (**153**, 3% MGS).³ All other compounds assessed (**412-417**) were moderate to weak inhibitors. Interestingly, *N*-cyclooctyl-erythronamide (**417**) is the least active of the *N*-cycloalkyl-erythronamides assessed. This is the opposite of what was observed with the *N*-cycloalkyl-arabonamides and -gluconamides where the molecules possessing a cyclooctyl group (**405** and **410**), were the most IRI active of their class.

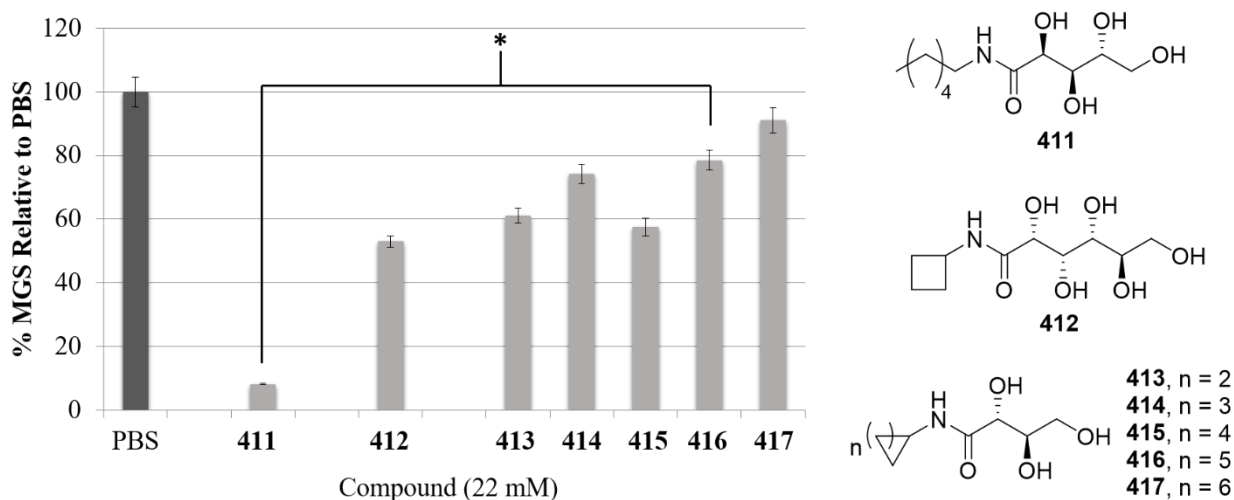


Figure 4.7. IRI activities of targets **411-417**. Statistical significance ($p < 0.05$) is indicated by an asterisks as assessed by unpaired Student's T-test.

The IRI activities of targets **411-417** were next assessed in the amphiphilicity correlation study. Compound **411** was designed to test the correlation between IRI activity and the CH_n/OH ratio for the *N*-alkyl-aldonamides. Possessing a ratio of 1.5, compound **411** was predicted to have an IRI activity of 76%. However, the experimental IRI activity was 8%. The new data point was added to the graph and is shown in Figure 4.8. Compound **411** (*N*-hexyl-arabonamide), along with **153** (*N*-hexyl-gluconamide), do not fit the linear correlation. Interestingly, both outliers are very IRI active and possess alkyl chain lengths of six

carbons ($\text{CH}_n = 6$) with a hydrophilic region of four or five hydroxyl groups ($\text{CH}_n/\text{OH} = 4$ or 5).

Shortening the hydrophilic region to three carbons ($\text{CH}_n/\text{OH} = 3$) resulted in a decrease in IRI activity (*N*-hexyl-erythronamide, **157**, 69% MGS). It is also interesting to note that compounds with alkyl chain lengths longer than six carbons ($\text{CH}_n > 6$) were not included in the graph because they were insoluble at 22 mM. For example, *N*-octyl-gluconamide (**154**) was tested at 5.5 mM and was potently active with an MGS of 12%. Therefore, while these experiments did not establish a robust trend between the CH_n/OH ratio and IRI activity, some key properties required for IRI activity were observed: the hydrophobic region must contain at least six carbons ($\text{CH}_n \geq 6$), and the hydrophilic moiety must contain at least four hydroxyl groups ($\text{CH}_n/\text{OH} \geq 4$, *N*-alkyl-gluconamides or -arabonamides). Shortening the hydrophilic moiety to three hydroxyl groups (*N*-alkyl-erythronamides) resulted in an inactive or weakly active compound, regardless of the alkyl chain length.

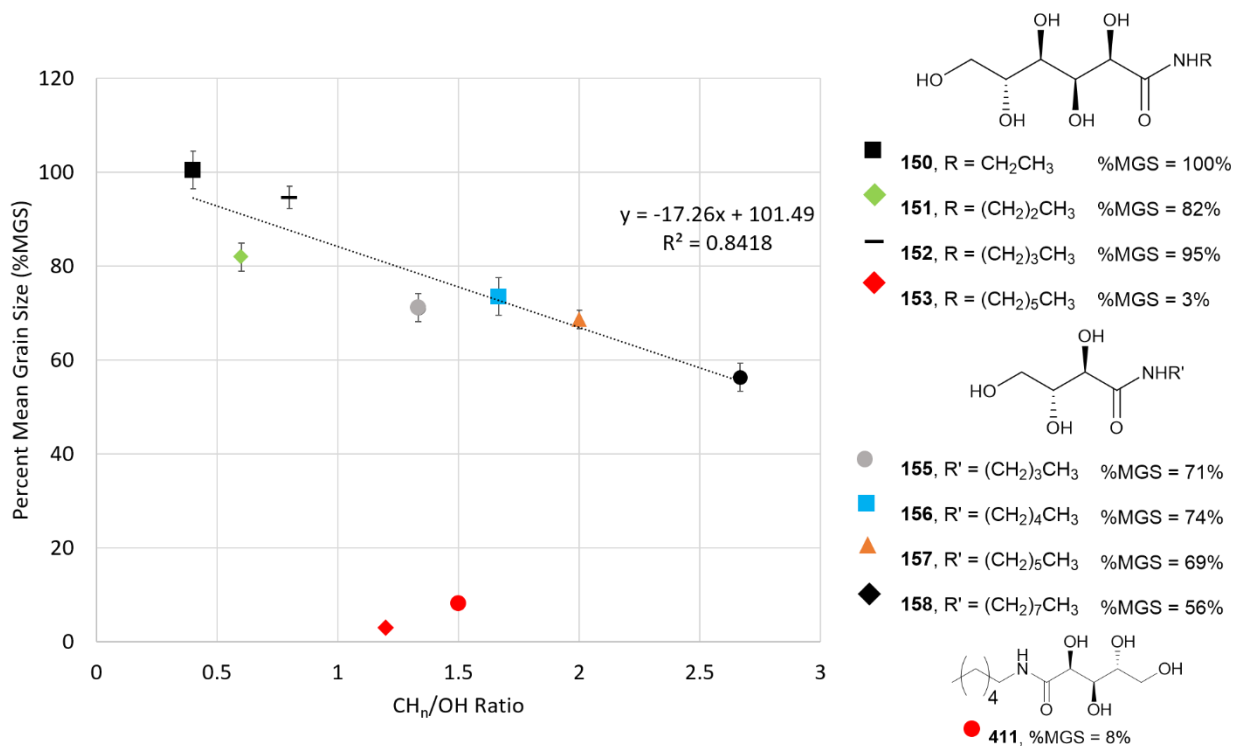


Figure 4.8. Correlation of %MGS vs. CH_n/OH ratio for all *N*-alkyl-aldonamides. Outliers, compound **153** and newly-synthesized derivative **411** are represented by red points.

Compounds **413-417** were designed to assess if these additional compounds would result in a correlation between IRI activity and the CH_n/OH ratio for the *N*-cycloalkyl-aldonamides. It was found that the amphiphilicity of the newly tested *N*-cycloalkyl-erythronamides **413-417** did not correlate well with IRI activity (Figure 4.9A & B).

Compound **412** was designed to test the correlation between IRI activities and the CH_n/OH ratios for the *N*-cycloalkyl-gluconamides. Possessing a CH_n/OH ratio of 0.8, compound **412** was predicted to have an IRI activity of 60%. The experimental IRI activity was 52% which supported the correlation between IRI activity and the amphiphilicity of *N*-cycloalkyl-gluconamides.

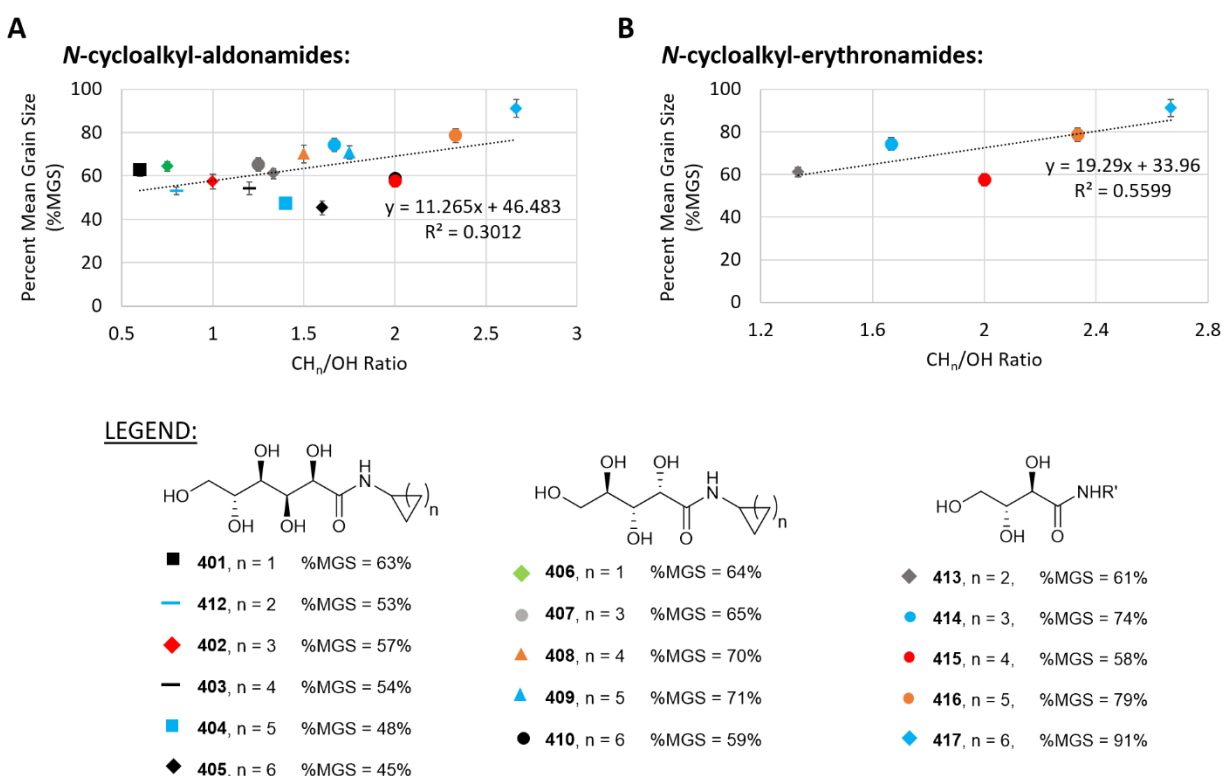


Figure 4.9. Correlation of %MGS vs. CH_n/OH ratio for (A) all *N*-cycloalkyl-gluconamides, erythronamides and arabanamides and (B) *N*-cycloalkyl-erythronamides.

To summarize, no linear correlation was observed for the entire set of *N*-cycloalkyl-aldonamides. A linear correlation was observed for the *N*-cycloalkyl-gluconamides **401-405** and this correlation was

supported by synthesizing and assessing the IRI activity of *N*-cyclobutyl-gluconamide (**412**). However, no linear correlation was observed with the *N*-cycloalkyl-arabonamides or –erythronamides. Furthermore, the linear correlation observed for *N*-alkyl-aldonamides was discredited when *N*-hexyl-arabonamide (**411**) was synthesized and assessed for IRI activity. It was found that the hydrophilic moiety must contain four or five hydroxyl groups (-arabonamides or -gluconamides) for potent IRI activity and shortening to three hydroxyl groups (-erythronamides) is not tolerated. Additionally, the hydrophobic moiety must be an alkyl chain of at least six carbons and cyclization of the alkyl chain results in a decrease in IRI activity.

4.5 Chapter summary

The results from this study reaffirm the understanding that molecules with longer alkyl chains possess increased IRI activity compared to those with shorter alkyl chains. However, IRI activity cannot be simply related to amphiphilicity. Furthermore, these surfactant-like molecules are not useful for cellular applications due to their propensity to interact with and solubilize cell membranes. Therefore, a new class of carbohydrate-based small molecules were designed to minimize the likelihood of cytotoxicity. This new class of compounds, referred to as *N*-aryl-gluconamides, feature an aromatic ring substituted with various functional groups at different positions on the ring in place of a hydrophobic alkyl chain tail. The structure-function analysis of these molecules necessitated the need for more sophisticated computational analysis, described in Chapter 5.

4.6 Experimental

4.6.1 Ice Recrystallization Inhibition (IRI) Assay

IRI activity was performed using the “splat cooling” method. The analyte was dissolved in phosphate buffered saline (PBS) solution at a concentration of 22 mM. If the analyte was insoluble, serial dilutions to 11, 5.5 or 0.5 mM were performed. A 10 μ L droplet of this solution was dropped onto a block of polished aluminum precooled on dry ice to approximately -80 °C through a two meter high plastic tube. The droplet froze instantly forming a wafer on the polished aluminum block and was approximately 1 cm

in diameter and 20 μm thick. This wafer was then carefully removed from the surface of the block and transferred onto a circular glass coverslip and to a cryostage held at $-6.4\text{ }^{\circ}\text{C}$ for annealing. After a period of 30 min, the wafer was photographed between crossed polarizing filters using a digital camera (Nikon CoolPix 5000) fitted to the microscope. A total of three images were taken from each wafer and each solution was tested three times.

The initial ice crystals formed during flash freezing are relatively homogeneous in size and quite small. Recrystallization occurs during the 30 minute annealing cycle, resulting in an increase in ice crystal size. After the annealing process, images captured the frozen wafer and ice crystal sizes were obtained using a novel domain recognition software (DRS) program. This program allows a user to visually demarcate and store the vertices of ice domains in a digital micrograph. The data was then used to calculate the domain areas. A total of 12 domain areas were obtained for nine images ($n = 108$). All data were plotted and analyzed using Microsoft Excel. The mean grain (or ice crystal) size (MGS) of the sample was compared to the MGS of the control PBS solution for that same day of testing and IRI activity is reported as the percentage of the MGS (%MGS) relative to the PBS control. Therefore, small percentages represent small ice crystals and this is indicative of high IRI activity. The %MGS for each sample was plotted along with its standard error of the mean (SEM).

4.6.2 General Experimental

Anhydrous reactions were performed under a positive pressure of dry argon in flame-dried glassware. Air or moisture-sensitive reagents and anhydrous solvents were transferred with oven dried syringes. Reactions were monitored using analytical thin layer chromatography (TLC) with 0.2 mm pre-coated silica gel aluminum plates 60 F254 (E. Merck). Components were visualized by illumination with a short-wavelength (254 nm) ultra-violet light and/or staining (ceric ammonium molybdate or orcinol/sulphuric acid stain solution). Flash column chromatography was performed with E. Merck silica gel 60 (230-400 mesh). ^1H (300, 400 or 500 MHz) and ^{13}C NMR (100 or 125 MHz) spectra were recorded on a Bruker Avance 400 or Bruker Avance 500. Deuterated chloroform (CDCl_3), DMSO ($\text{DMSO}-d_6$) or

methanol (MeOD) were used as NMR solvents. Chemical shifts are reported in ppm using the solvent residual peak as an internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet and br, broad. Low resolution mass spectrometry (LRMS) was performed on a Micromass Quatro-LC Electrospray spectrometer with a pump rate of 20 μ L/min using electrospray ionization (ESI).

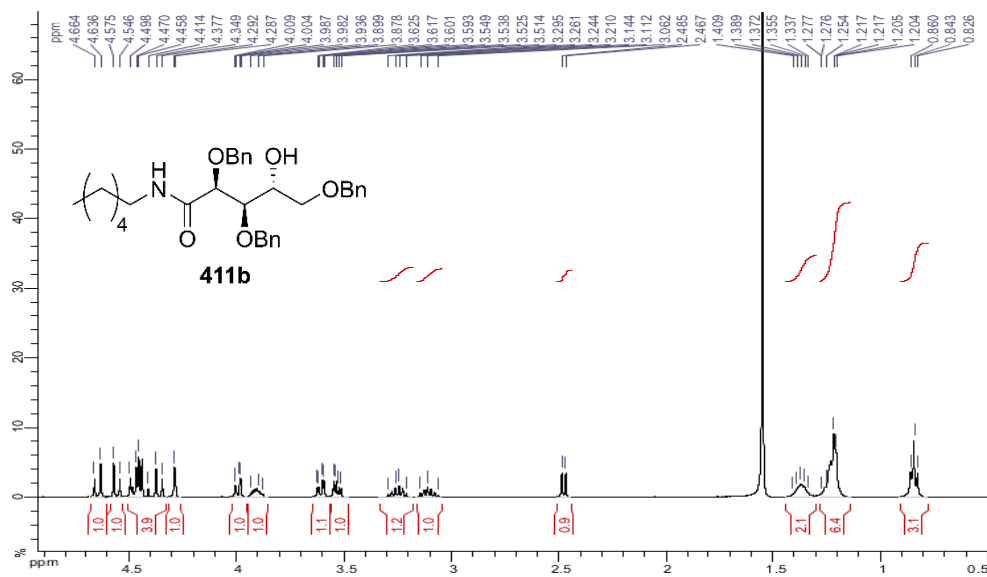
2,3,5-*O*-Benzyl-D-arabinolactone (**411a**), provided by Dr. Malay Doshi, was synthesized as previously described^{4,11} and used for the synthesis of **411**. Compounds **401-410** were synthesized and assessed for IRI activity by Dr. Malay Doshi.⁴ Compounds **150-158** were synthesized and assessed for IRI activity by previous members of the Ben laboratory; Dr. Chantelle Capicciotti^{2,3}, Kathryn Davis and Lyanne Betit. NMR spectra for novel compounds and final compounds assessed for IRI activity are provided.

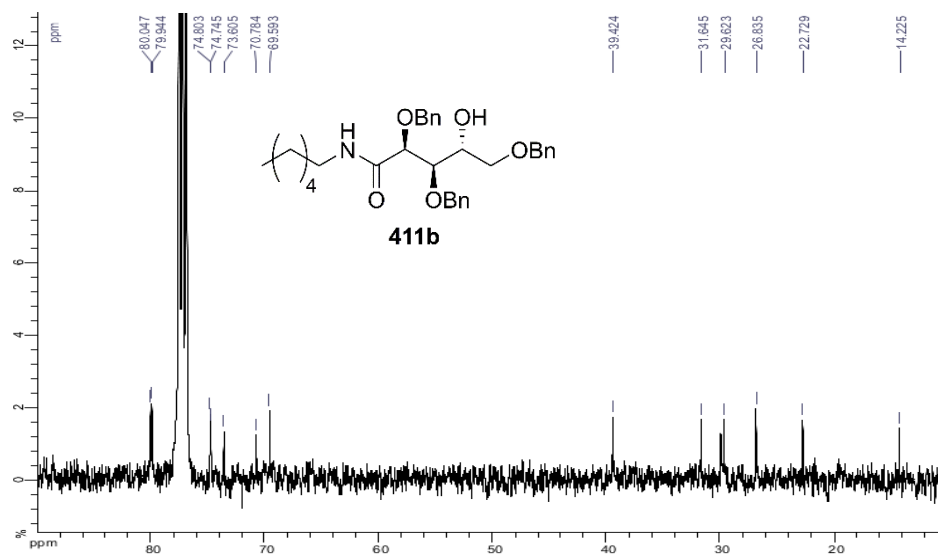
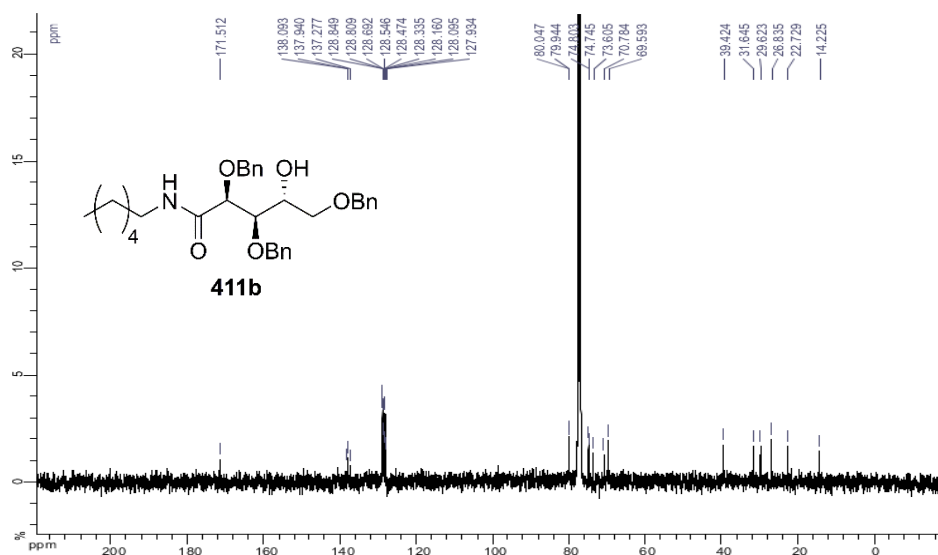
4.6.3 Compound characterization and NMR spectra

(2,3,5-*O*-Benzyl)-*N*-hexyl-arabonamide (411b)

Hexyl amine (0.1 mL, 0.24 mmol) and Me₃Al (2 M) (0.24 mL, 0.48 mmol) were dissolved in dry toluene and allowed to stir for 1 hour. 2,3,5-*O*-Benzyl-D-arabinolactone (**411a**) (100 mg, 0.24 mmol) was added and the mixture was allowed to stir for 24 hours. TLC (7:3 hexanes:EtOAc) revealed the reaction was complete. The mixture was quenched with 10% HCl until bubbling subsided. This mixture was allowed to stir for 1 hour, then filtered over celite and extracted with EtOAc and H₂O. The organic layer was dried over MgSO₄, filtered and concentrated. Purification by flash chromatography (7:3 hexanes-EtOAc) yielded pure **411b** (60 mg, 48%). ¹H NMR (400 MHz, CDCl₃): δ 7.34-7.14 (m, 15H), 6.76 (t, J = 5.4 Hz, 1H), 4.65 (d, J = 11.4 Hz, 1H), 4.57 (d, J = 11.5 Hz, 1H), 4.50-4.35 (m, 4H), 4.29 (d, J = 2.3 Hz, 1H), 4.0 (dd, J = 2.1, 8.8 Hz, 1H), 3.94-3.88 (m, 1H), 3.61 (dd, J = 3.2, 9.5 Hz, 1H), 3.53 (dd, J = 4.3, 9.5 Hz, 1H), 3.30-3.21 (m, 1H), 3.14-3.06 (m, 1H), 2.48 (d, J = 7.4 Hz, 1H), 1.41-1.34 (m, 1H), 1.28-1.20 (m, 6H), 0.84 (t, J = 6.7 Hz, 3H). ¹³C NMR (100 MHz, CDCl₃): δ 171.5, 138.1, 137.9, 137.3, 128.9, 128.8, 128.7,

22.7, 14.2. LRMS (ESI): m/z calcd. for $C_{32}H_{41}NNaO_5$ $[M+Na]^+$ 542.3; found, 542.2.

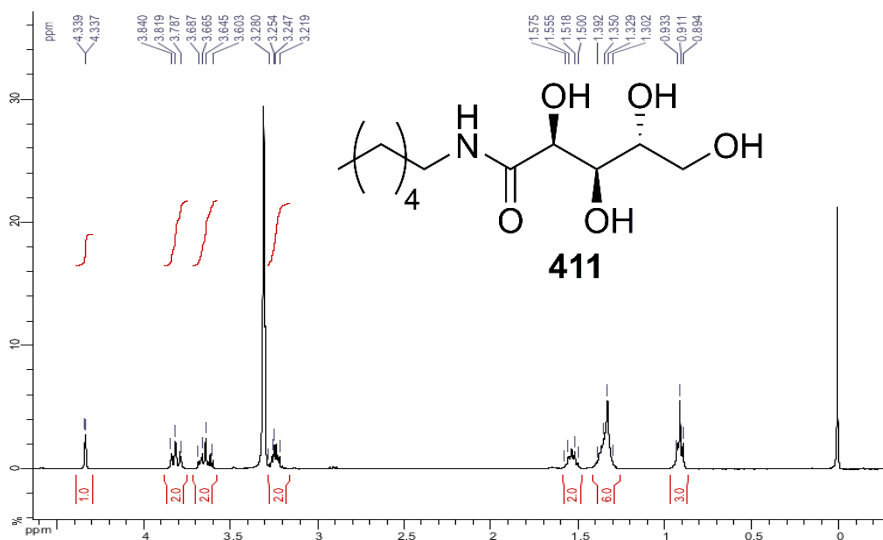
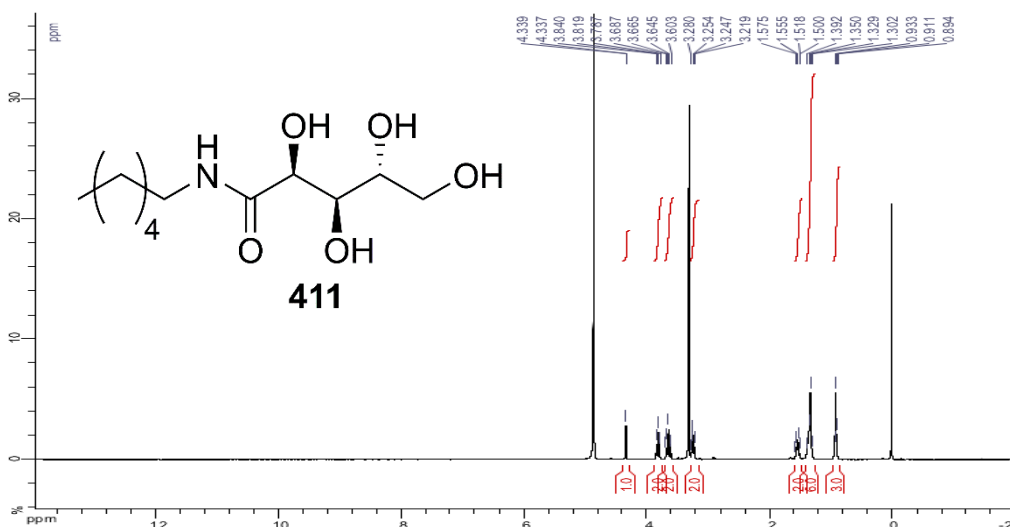


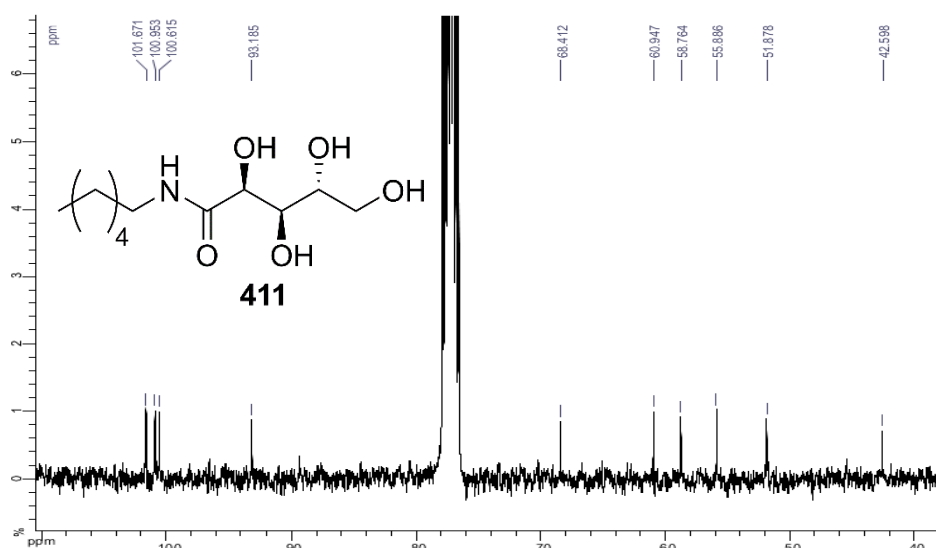
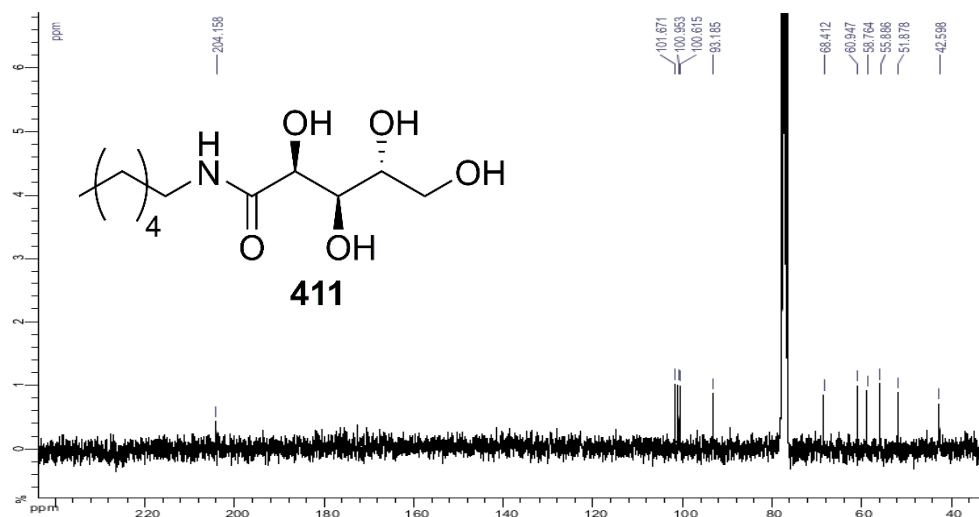


N-Hexyl-arabonamide (**411**)

Compound **411b** (58 mg, 0.11 mmol) was dissolved in ethanol (10 mL) and Pd(OH)₂ (10 mol %) was added. Atmospheric air was removed by aspiration and H₂ (g) was introduced. The mixture was allowed to stir for 3 hours before TLC (1:1 Hexanes/EtOAc) revealed reaction completion. The reaction mixture was filtered over a bed of celite and concentrated to obtain crude white powder. The crude product was dissolved in a minimum amount of methanol and precipitated with hexanes to yield **411** as pure crystals

(20 mg, 73%). ^1H NMR (400 MHz, MeOD): δ 4.34 (d, J = 1.6 Hz, 1H), 3.84-3.79 (m, 2H), 3.69-3.60 (m, 2H), 3.28-3.22 (m, 2H), 1.58-1.50 (m, 2H), 1.39-1.30 (m, 6H), 0.93-0.89 (m, 3H). ^{13}C NMR (100 MHz, MeOD): δ 204.2, 101.7, 101.0, 100.6, 93.2, 68.4, 61.0, 58.8, 55.9, 51.9, 42.7. LRMS (ESI): m/z calcd. for $\text{C}_{11}\text{H}_{23}\text{NNaO}_5$ $[\text{M}+\text{Na}]^+$ 272.2; found, 272.1.

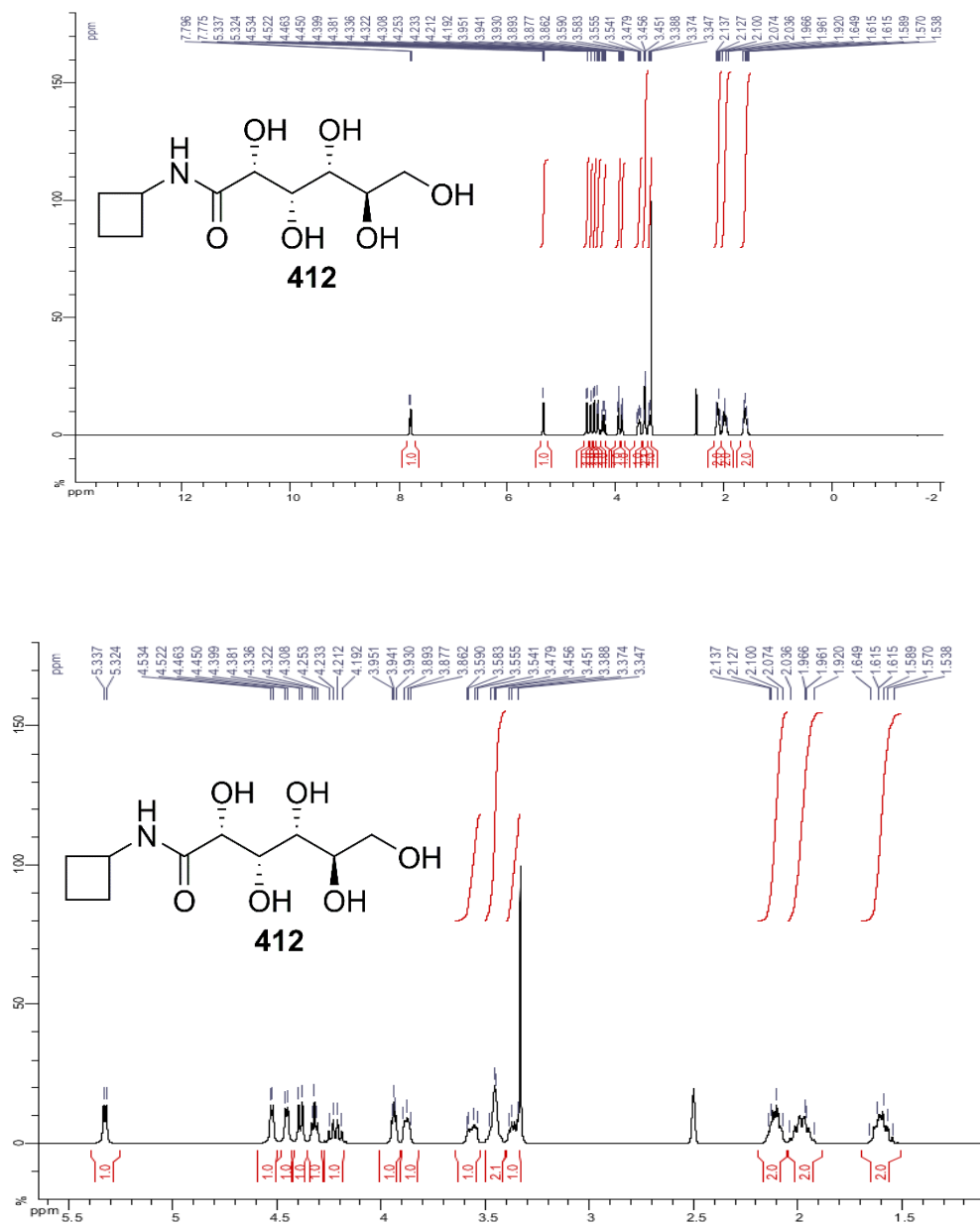


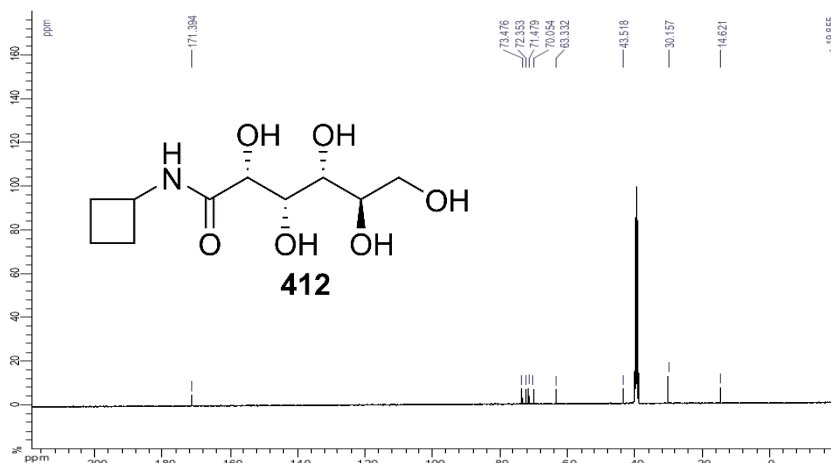


N-Cyclobutyl-gluconamide (**412**)

δ -Gluconolactone (0.1 g, 0.56 mmol) and cyclobutylamine (0.1 mL, 1.12 mmol) were dissolved in MeOH and refluxed for 24 hours. The solvent was evaporated and product recrystallized in hot EtOH to yield **412** as white crystals (137 mg, 98%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 7.79 (d, J = 8.2 Hz, 1H), 5.33 (d, J = 5.2 Hz, 1H), 4.53 (d, J = 4.8 Hz, 1H), 4.46 (d, J = 4.6 Hz, 1H), 4.39 (d, J = 7.5 Hz, 1H), 4.32 (t, J = 5.9 Hz, 1H), 4.25-4.19 (m, 1H), 3.95-3.93 (m, 1H), 3.89-3.86 (m, 1H), 3.59-3.54 (m, 1H), 3.48-3.45 (m, 2H), 3.39-3.35 (m, 1H), 2.14-2.07 (m, 2H), 2.04-1.92 (m, 2H), 1.65-1.54 (m, 2H). ^{13}C NMR (100 MHz,

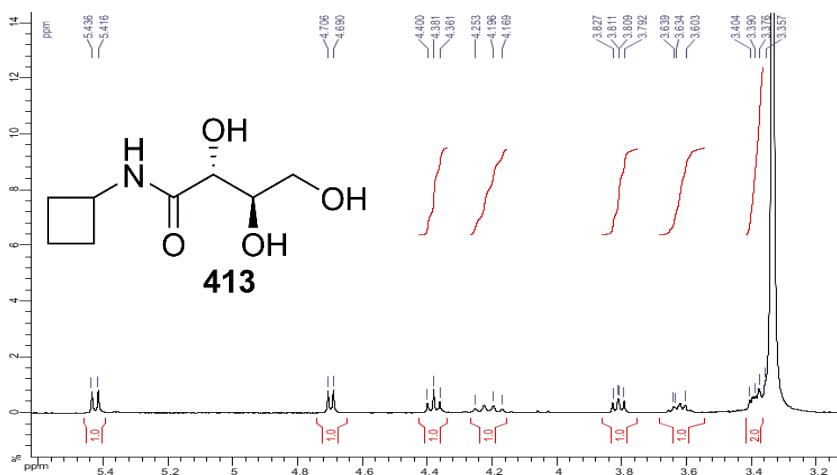
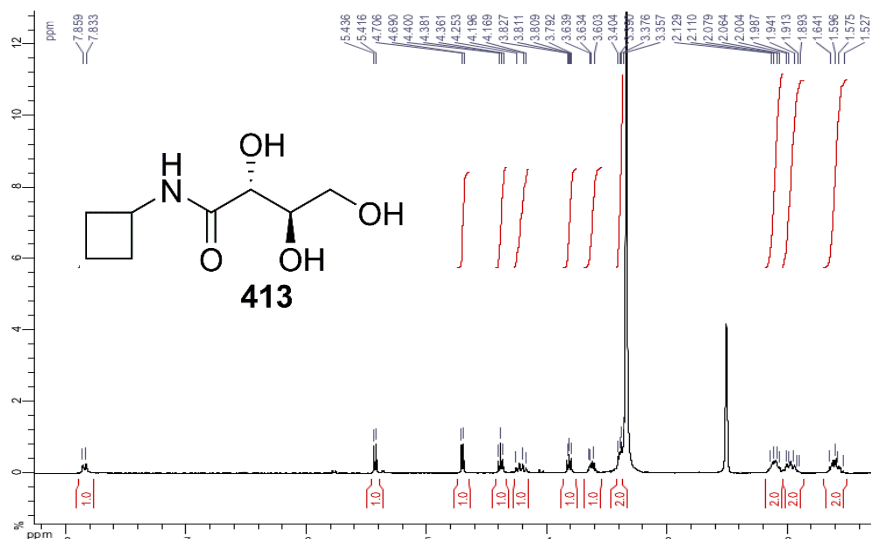
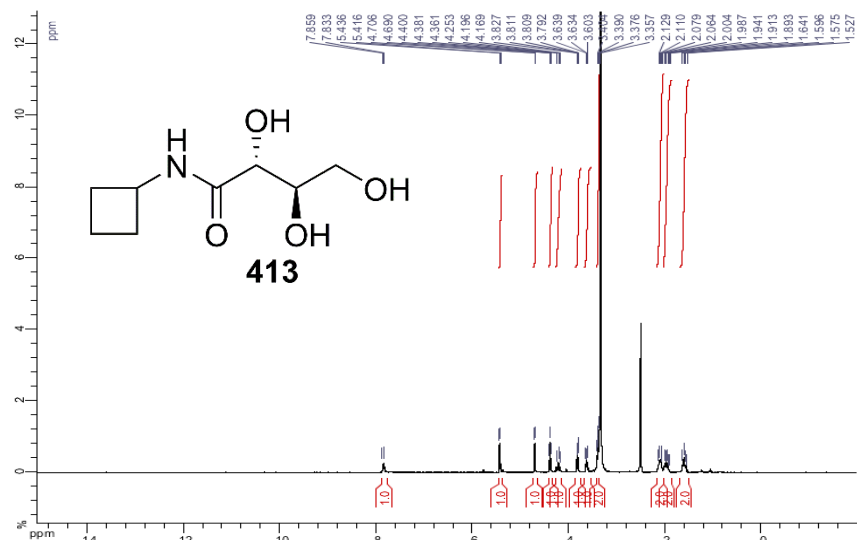
DMSO- d_6): δ 171.9, 74.0, 72.9, 72.0, 70.6, 63.8, 44.0, 30.7, 15.1. LRMS (ESI): m/z calcd. for $C_{10}H_{19}NO_6Na$ $[M+Na]^+$ 272.1; found 271.9.

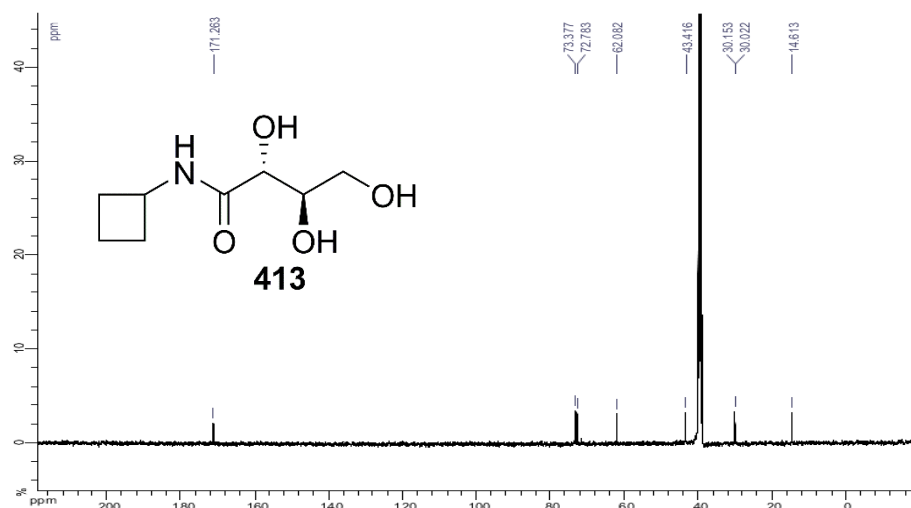




N-Cyclobutyl-erythronamide (**413**)

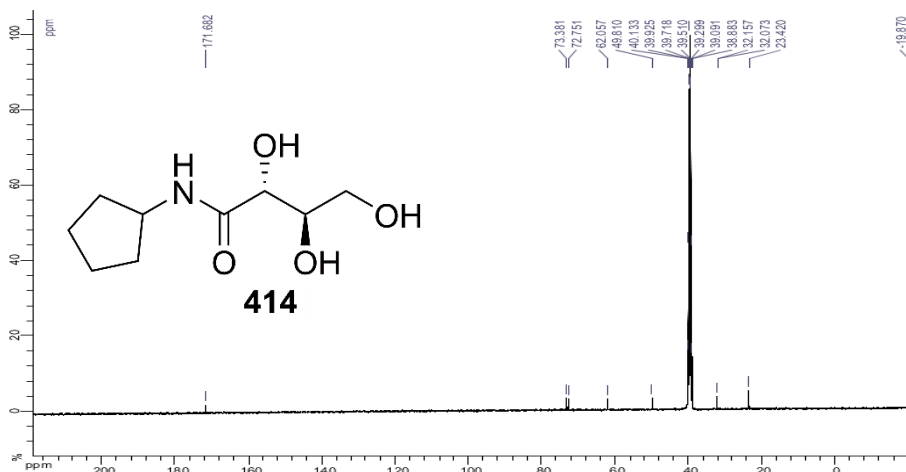
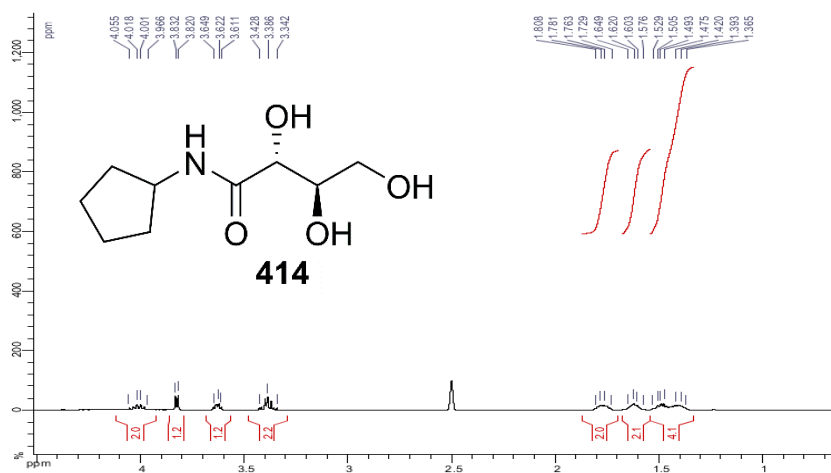
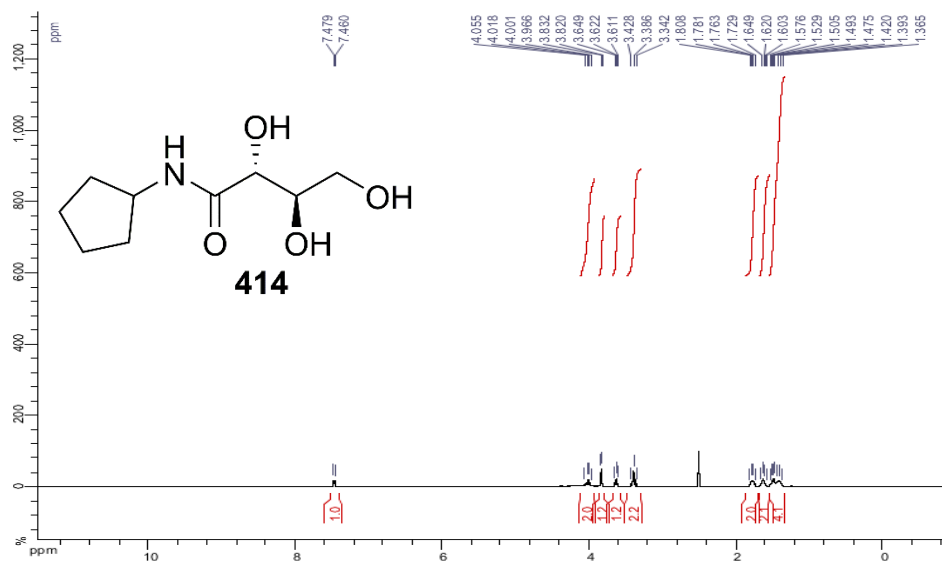
(-)-2,3-*O*-Isopropylidene-D-erythronolactone (0.1 g, 0.64 mmol) and cyclobutylamine (54 μ L, 0.64 mmol) were dissolved in dry DCM (4 mL) and allowed to stir for 24 hours until the reaction was complete by TLC (2% MeOH in DCM). The mixture was then diluted with saturated sodium bicarbonate. The crude product was extracted in DCM and washed three times with saturated sodium bicarbonate. The organic layer was dried over MgSO_4 and concentrated. The crude product was dissolved in MeOH and Amberlite® (IR-120) ion exchange resin (H^+) was added and stirred for 24 hours. The mixture was filtered over celite and concentrated to yield **413** as a white powder (20 mg, 16%). ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 7.85 (d, J = 7.9 Hz, 1H), 5.43 (d, J = 5.9 Hz, 1H), 4.7 (d, J = 5.0 Hz, 1H), 4.38 (t, J = 5.8 Hz, 1H), 4.17-4.25 (m, 1H), 3.81 (dd, J = 4.7, 5.9 Hz, 1H), 3.60-3.64 (m, 1H), 3.36-3.40 (m, 2H), 2.06-2.13 (m, 2H), 1.89-2.00 (m, 2H), 1.53-1.64 (m, 2H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 171.3, 73.4, 72.8, 62.1, 43.4, 30.2, 30.0, 14.6. LRMS (ESI): m/z calcd. for $\text{C}_8\text{H}_{15}\text{NaNO}_4$ $[\text{M}+\text{Na}]^+$ 212.2; found, 212.0.





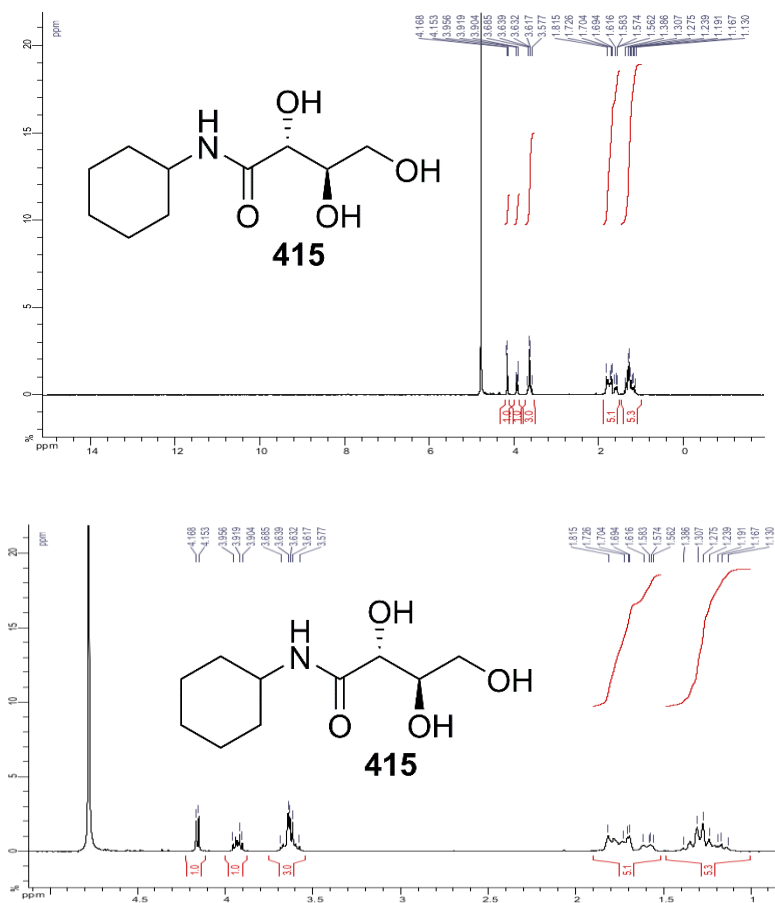
N-Cyclopentyl-erythronamide (**414**)

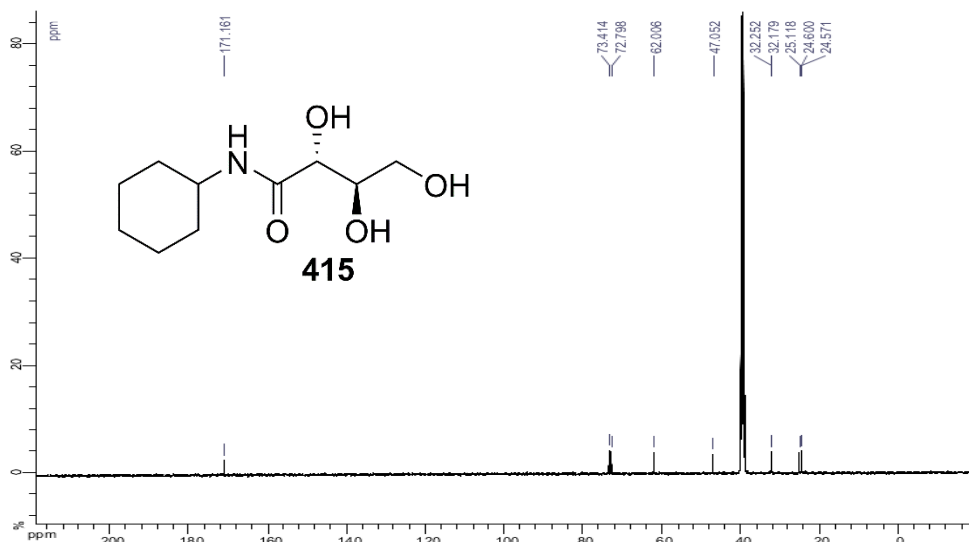
(-)-2,3-*O*-Isopropylidene-D-erythronolactone (0.1 g, 0.64 mmol) and cyclopentylamine (63 μ L, 0.64 mmol) were dissolved in dry DCM (4 mL) and allowed to stir for 24 hours. TLC (2% MeOH in DCM) showed reaction was complete and mixture was diluted with saturated sodium bicarbonate. The crude product was extracted in DCM and washed three times with saturated sodium bicarbonate. The organic layer was dried over MgSO_4 and concentrated. The crude product was dissolved in MeOH and Amberlite® (IR-120) ion exchange resin (H^+) was added and stirred for 24 hours. The mixture was filtered over celite and concentrated to yield **414** as a white powder (26 mg, 20%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 7.47 (d, $J = 7.7$ Hz, 1H), 4.06-3.97 (m, 2H), 3.83 (d, $J = 4.9$ Hz, 1H), 3.65-3.61 (m, 1H), 3.43-3.34 (m, 2H), 1.81-1.73 (m, 2H), 1.65-1.58 (m, 2H), 1.53-1.37 (m, 4H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 171.7, 73.4, 72.8, 62.1, 49.8, 32.2, 32.1, 23.4. LRMS (ESI): m/z calcd. for $\text{C}_9\text{H}_{17}\text{NaNO}_4$ $[\text{M}+\text{Na}]^+$ 226.2; found, 226.1.



***N*-Cyclohexyl-erythronamide (415)**

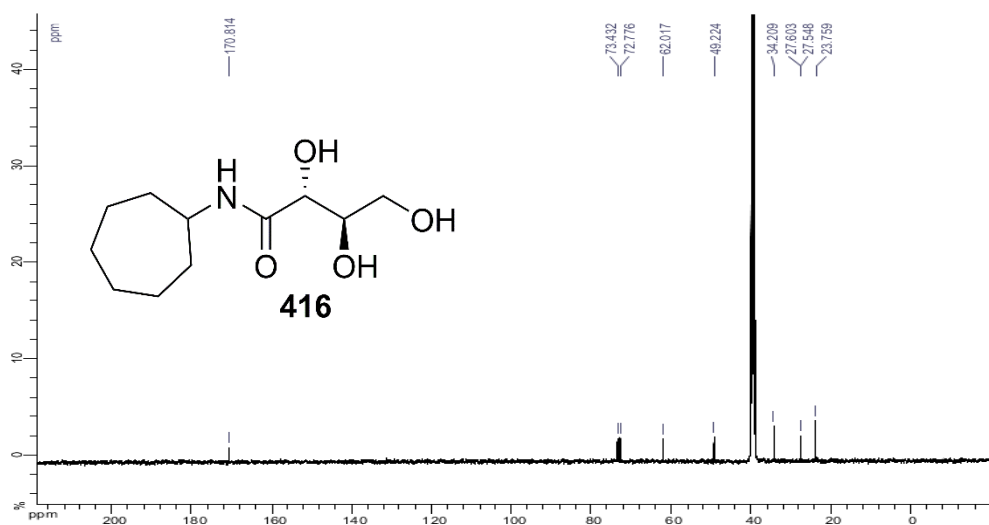
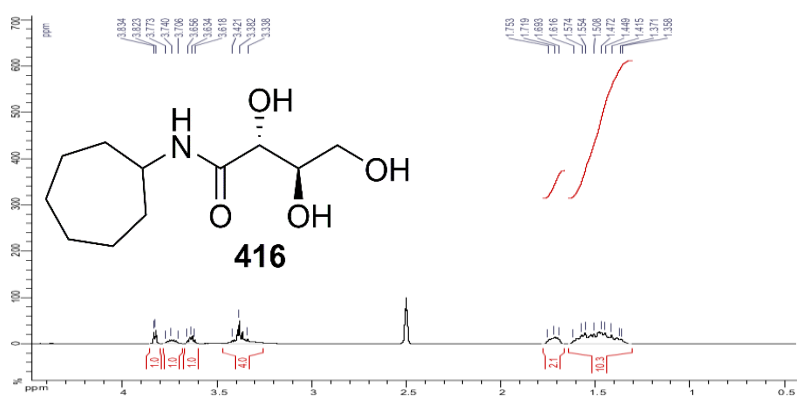
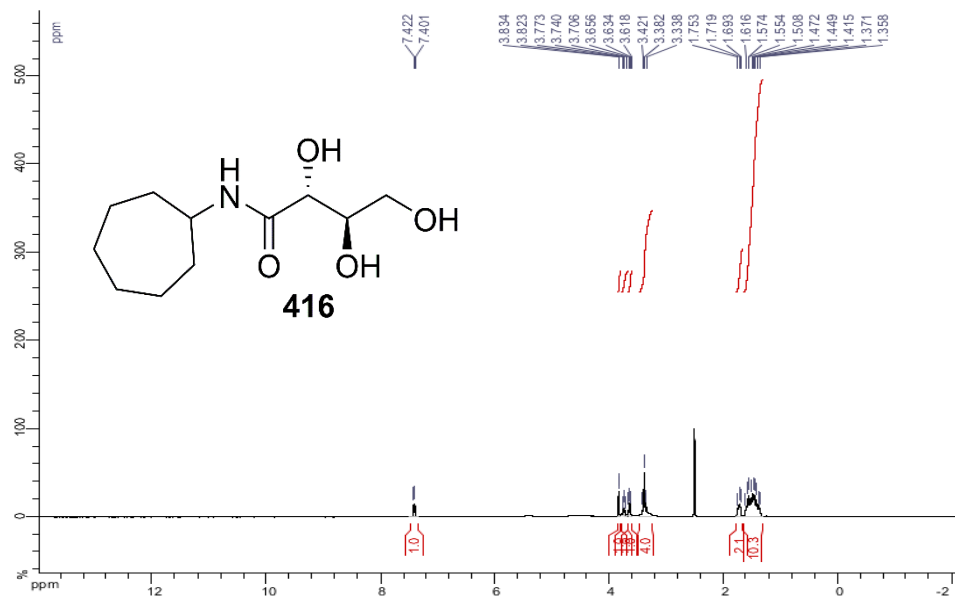
(-)-2,3-*O*-Isopropylidene-D-erythrionolactone (0.1 g, 0.64 mmol) and cyclohexylamine (73 μ L, 0.64 mmol) were dissolved in dry DCM (4 mL) and allowed to stir for 24 hours. TLC (2% MeOH in DCM) showed reaction was complete and mixture was diluted with saturated sodium bicarbonate. The crude product was extracted in DCM and washed three times with saturated sodium bicarbonate. The organic layer was dried over MgSO_4 and concentrated. The crude product was dissolved in MeOH and Amberlite® (IR-120) ion exchange resin (H^+) was added and stirred for 24 hours. The mixture was filtered over celite and concentrated to yield **415** as a white powder (31 mg, 22%). ^1H NMR (300 MHz, D_2O): δ 4.16 (d, J = 4.6 Hz, 1H), 3.96-3.90 (m, 3H), 3.69-3.58 (m, 3H), 1.82-1.56 (m, 5H), 1.39-1.13 (m, 5H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 171.2, 73.4, 72.8, 62.0, 47.1, 32.3, 32.2, 25.1, 24.1, 24.6. LRMS (ESI): m/z calcd. for $\text{C}_{10}\text{H}_{19}\text{NaNO}_4$ [$\text{M}+\text{Na}$] $^+$ 240.3; found, 240.1.





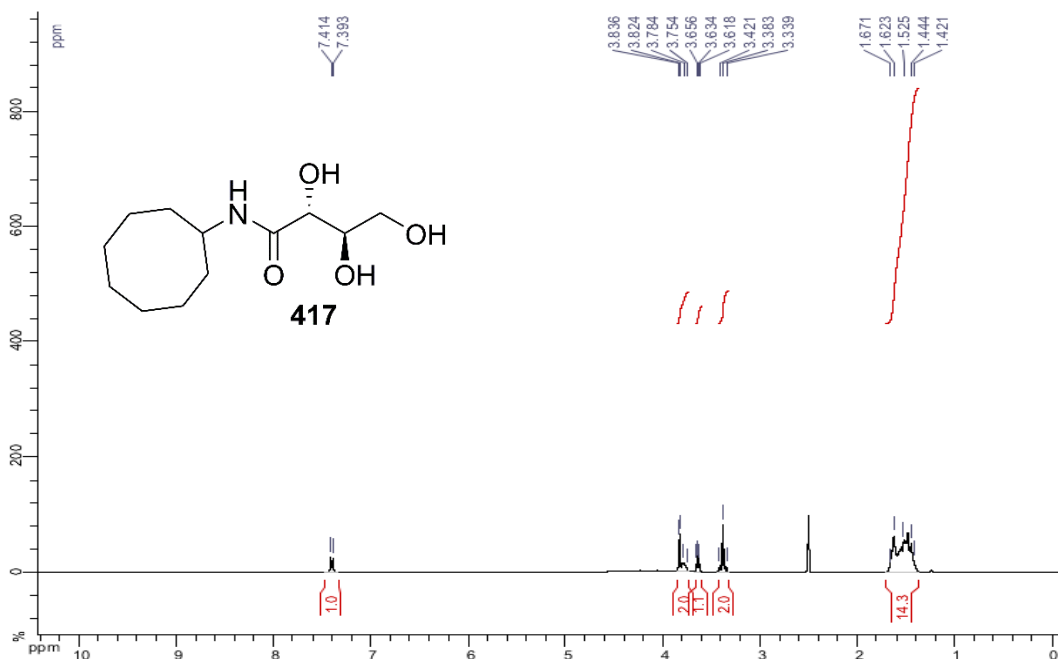
N-Cycloheptyl-erythronamide (**416**)

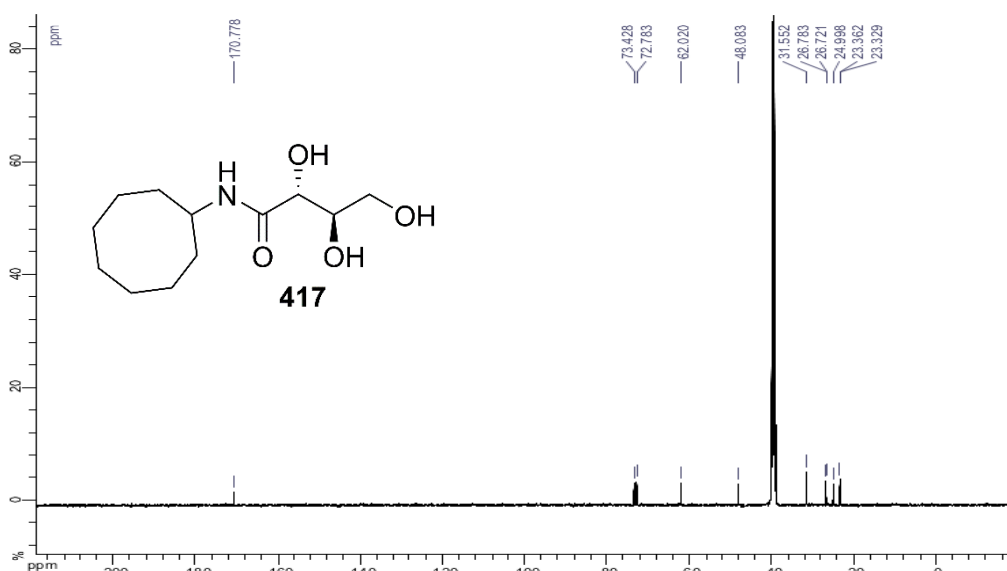
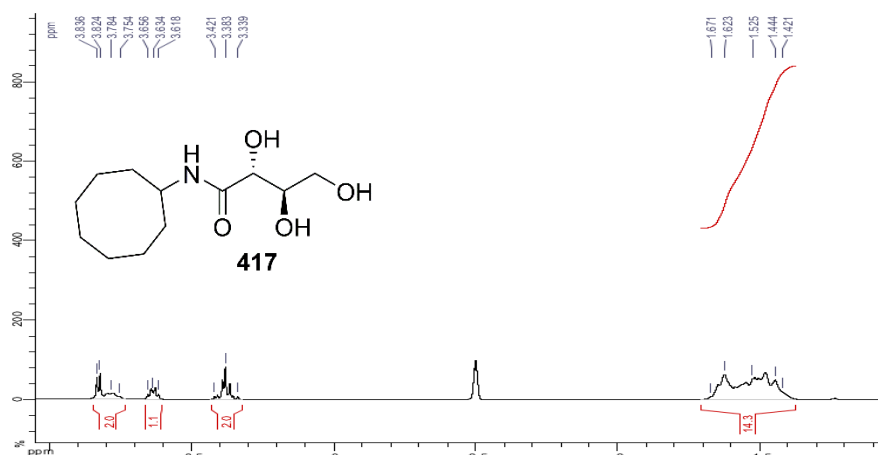
(-)-2,3-*O*-Isopropylidene-D-erythronolactone (0.1 g, 0.64 mmol) and cycloheptylamine (82 μ L, 0.64 mmol) were dissolved in dry DCM (4 mL) and allowed to stir for 24 hours. TLC (2% MeOH in DCM) showed reaction was complete and mixture was diluted with saturated sodium bicarbonate. The crude product was extracted in DCM and washed three times with saturated sodium bicarbonate. The organic layer was dried over MgSO_4 and concentrated. The crude product was dissolved in MeOH and Amberlite® (IR-120) ion exchange resin (H^+) was added and stirred for 24 hours. The mixture was filtered over celite and concentrated to yield **416** as a white powder (39 mg, 27%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 7.41 (d, J = 8.3 Hz, 1H), 3.83 (d, J = 4.6 Hz, 1H), 3.77-3.71 (m, 1H), 3.66-3.62 (m, 1H), 3.42-3.34 (m, 4H), 1.75-1.69 (m, 2H), 1.62-1.36 (m, 10H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 179.8, 73.4, 72.8, 62.0, 49.2, 34.2, 27.6, 27.6, 23.8. LRMS (ESI): m/z calcd. for $\text{C}_{11}\text{H}_{21}\text{NaNO}_4$ $[\text{M}+\text{Na}]^+$ 254.3; found, 254.1.



N-Cyclooctyl-erythronamide (**417**)

(-)-2,3-O-Isopropylidene-D-erythronolactone (0.1 g, 0.64 mmol) and cyclooctylamine (88 μ L, 0.64 mmol) were dissolved in dry DCM (4 mL) and allowed to stir for 24 hours. TLC (2% MeOH in DCM) showed reaction was complete and mixture was diluted with saturated sodium bicarbonate. The crude product was extracted in DCM and washed three times with saturated sodium bicarbonate. The organic layer was dried over MgSO_4 and concentrated. The crude product was dissolved in MeOH and Amberlite® (IR-120) ion exchange resin (H^+) was added and stirred for 24 hours. The mixture was filtered over celite and concentrated to yield **417** as a white powder (42 mg, 27%). ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 7.40 (d, J = 8.3 Hz, 1H), 3.84-3.75 (m, 2H), 3.66-3.62 (m, 1H), 3.42-3.34 (m, 2H), 1.67-1.42 (m, 14H). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ 170.8, 73.4, 72.8, 62.0, 48.1, 31.6, 26.8, 26.7, 25.0, 23.4, 23.3. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{23}\text{NaNO}_4$ $[\text{M}+\text{Na}]^+$ 268.3; found, 268.1.





4.7 References

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Chapter 5: Determining the influence of aryl moieties on the ice recrystallization inhibition of gluconamides

5.1 Introduction

It was previously shown by the Ben laboratory that simple monosaccharides and disaccharides possess moderate IRI activity, which was attributed to their ability to disrupt the structure of bulk water.^{1,2} A correlation between the carbohydrates' hydration index (HI) and IRI activities was thus established.^{1,2} Subsequently, the IRI activities of carbohydrate-based surfactants was assessed due to their ability to disrupt or alter the structure of bulk water.³ This led to the discovery of the first small molecule ice recrystallization inhibitors. The most potent IRIs discovered in this study were the pyranose-based surfactant β -octyl-Gal (**164**) and the aldonamide-based hydrogelators *N*-octyl-gluconamide (**154**) and *N*-hexyl-gluconamide (**153**) shown in Figure 5.1A.³ β -Octyl-Gal (**164**) and *N*-hexyl-gluconamide (**153**)

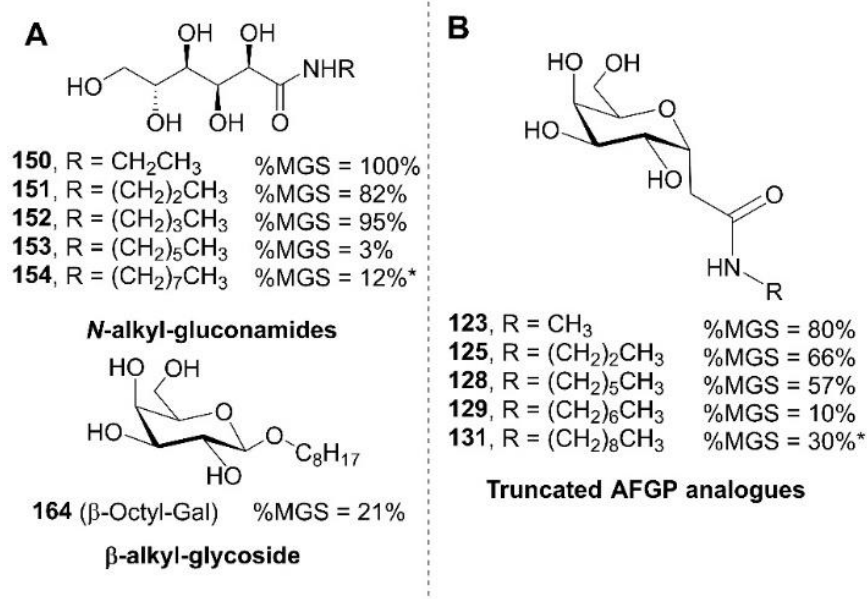


Figure 5.1. Structures of IRIs previously assessed for IRI activity at 22 mM (asterisks indicates IRI activity assessed at 5.5 mM). (A) *N*-alkyl-gluconamides and β -alkyl-glycoside **164**³, (B) truncated AFGP analogues.⁴

exhibited potent IRI activity at 22 mM (21% and 3% MGS respectively) and *N*-octyl-gluconamide was highly IRI active at a significantly lower concentration of 0.5 mM (12% MGS).³ The *N*-alkyl-

gluconamide derivatives with shorter alkyl chain lengths were shown to be inactive, suggesting that an amphiphilic balance is necessary for IRI activity. This was further demonstrated by our laboratory with the truncated AFGP analogues (Figure 5.1B).⁴ The truncated AFGP analogues which possessed long alkyl chain lengths were potent inhibitors of ice recrystallization whereas derivatives with shorter alkyl chain lengths were significantly less IRI active.⁴ These structure-function analyses revealed the importance of hydrophobic moieties for IRI activity.

Due to the amphiphilic nature of these molecules, they were not well suited to cryopreservation applications. These molecules are surfactant-like and therefore solubilize cell membranes.^{5,6} Subsequently, the aryl glycoside class of IRIs were explored and their structures are shown in Figure 5.2.^{7,8} These molecules are β -alkyl-glycoside analogues in which the long alkyl chain has been replaced with an aromatic ring. The aromatic ring provides a six-carbon hydrophobic moiety and thus the aryl glycosides maintain amphiphilicity while being less likely to interact with or solubilize cell membranes. Many derivatives were explored with different aromatic substituents at various positions on the aryl ring. This study resulted in the discovery of several potent inhibitors of ice recrystallization. These molecules are not surfactants and therefore are significantly less toxic than the pyranose-based surfactant β -octyl-Gal (**164**) and the aldonamide-based hydrogelators *N*-octyl-gluconamide (**154**) and *N*-hexyl-gluconamide (**153**). These molecules were shown to be effective cryoprotectants for RBCs.⁸

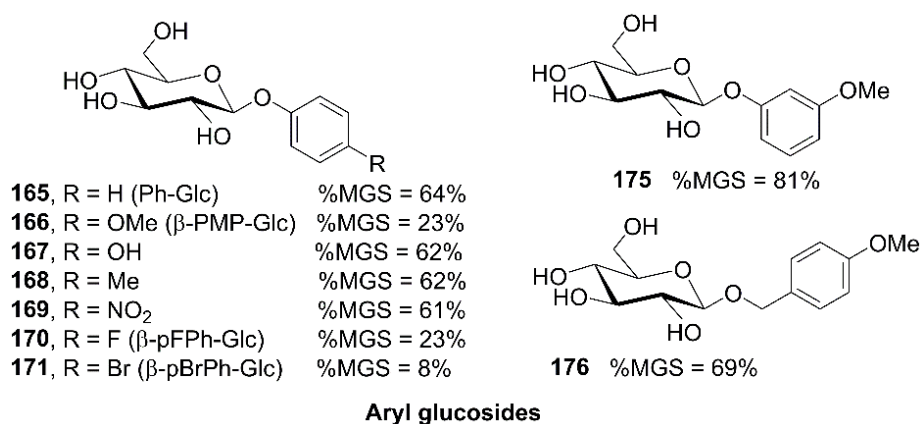


Figure 5.2. Structures of phenolic glucosides previously assessed for IRI activity.⁷

Substitution of the alkyl chain with an aromatic ring with different substituents resulted in several IRI active small-molecules. For example, β -PMP-Glc (**166**), β -pFPh-Glc (**170**) and β -pBrPh-Glc (**171**) have IRI activities of 23%, 23% and 8% MGS respectively.^{7,8} Since the substitution of the alkyl chain with an aromatic ring in the β -alkyl-glycosides resulted in the discovery of potent IRIs, it was hypothesized that the same replacement in the *N*-alkyl-gluconamides (Figure 5.3A) could also result in the discovery of potent IRIs. This new class of compounds, referred to as *N*-aryl-gluconamides, feature an aromatic ring in the place of an alkyl chain (Figure 5.3B).

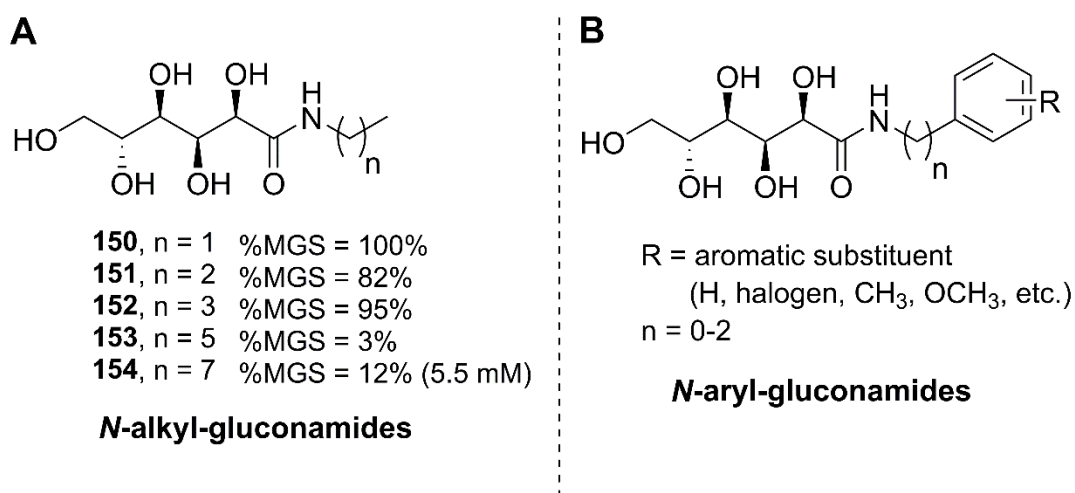


Figure 5.3. General structure of (A) *N*-alkyl-gluconamides and (B) *N*-aryl-gluconamides.

Structure-function analyses of the aryl glycosides revealed that the identity of the aromatic substituent had a dramatic effect on the IRI activity of the molecule (Figure 5.2).⁷ For example, aryl-glucosides with a *para*-fluorine (**170**) and bromine (**171**) atom resulted in very effective inhibitors of ice recrystallization. A *para*-methoxy group is also favourable (**166**, 23% MGS); however, a *para*-hydroxyl group resulted in moderate IRI activity (**167**, 62% MGS).

The location of the aromatic substituent on the ring also impacted IRI activity. When the methoxy group was moved from the *para* to the *meta* position, IRI activity was reduced (**175**, 81% MGS). Furthermore, addition of a carbon spacer between the aromatic ring and the pyranose ring in β -PMP-Glc (**166**) was also shown to reduce IRI activity (**176**, 69% MGS). Therefore, *N*-aryl-gluconamides with

various aryl substituents at different positions on the aromatic ring will be investigated in an effort to better understand this relationship. Furthermore, the effect of adding carbon or nitrogen spacers between the amide and aromatic ring will be assessed. The proposed structures to be synthesized and assessed for IRI activity are shown in Figure 5.4.

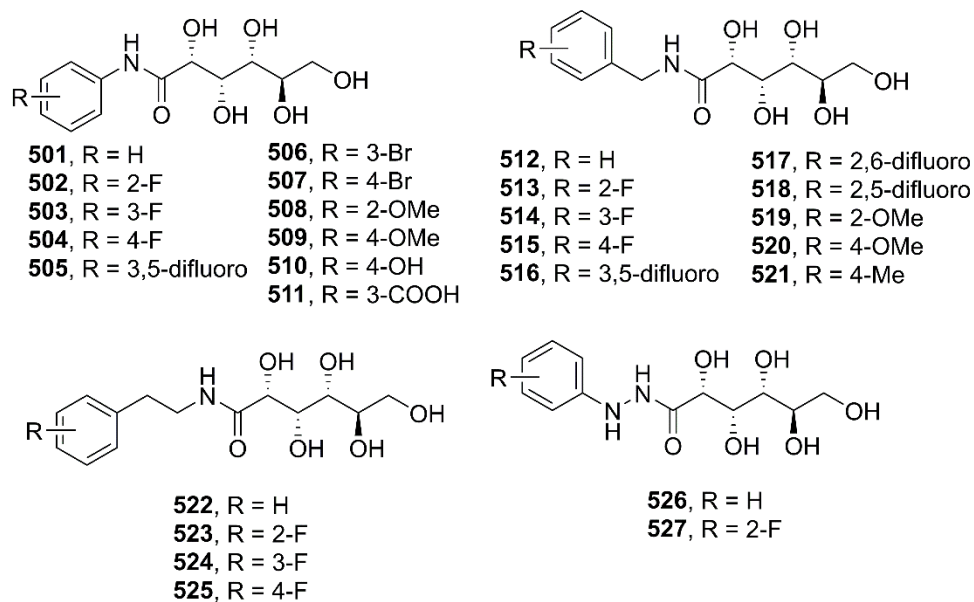
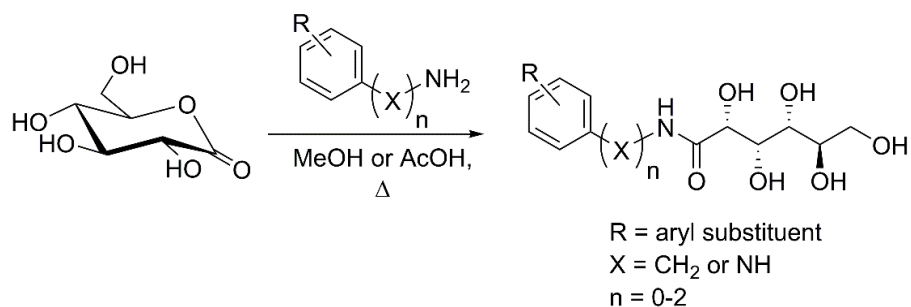


Figure 5.4. Structures of *N*-aryl-gluconamides to be synthesized and assessed for IRI activity.

In this chapter, a QSAR model was developed to facilitate the rational design of *N*-aryl-D-gluconamides possessing potent IRI activity. This work was done in collaboration with Prof. Tom Woo and his graduate students, Phil De Luna and Michael Fernandez and is in a manuscript entitled “QSAR accelerated discovery of potent ice recrystallization inhibitors” recently accepted to *Scientific Reports* (May 2016).⁹

5.2 Synthesis of *N*-aryl-gluconamides and their IRI activity

The *N*-aryl-gluconamides are synthesized by aminolysis of δ -gluconolactone with various aryl amines as described in Scheme 5.1.



Scheme 5.1. Synthesis of *N*-aryl-gluconamides.

The IRI activities of *N*-aryl-gluconamides **501-511** are shown in Figure 5.5. The *N*-aryl-gluconamide that does not possess any aromatic substituents, **501**, is a moderate inhibitor with a %MGS of 66%. This is similar to the aryl glucoside possessing no aromatic substituents (Ph-Glc, **165**), which has a %MGS of 64%. Aryl-glucosides containing *para*-substituted halogens were shown to be very IRI active (8-23% MGS), therefore *N*-aryl-gluconamides possessing halogen substituents were also expected to possess significant IRI activity. β -pFPh-Glc (**170**) is a potent inhibitor of ice recrystallization whereas the *N*-aryl-gluconamide with a fluorine in the *para* position (**504**) is inactive. However, when a fluorine substituent is placed in the *ortho* or *meta* position (**502** and **503**), there is a dramatic increase in IRI activity to 3% and 9% MGS respectively. 2,5-Difluoro substitution (**505**) also results in a very potent inhibitor (4% MGS). Therefore, fluorine substitution in the *para* position is not favourable compared to *ortho* or *meta* substitution for the *N*-aryl-gluconamides. Bromine substitution followed the same trend as fluorine; *para* substitution (**510**) was less favourable than *meta*-substitution (**509**). This is interesting because β -pBrPh-Glc (**171**) and β -pFPh-Glc (**170**) which possess a halogen in the *para*-position are highly IRI active. These initial results suggest that, similar to the aryl-glucosides, IRI activity is dependent on the location and identity of the functional group.

Next, a methoxy functional group was assessed because the aryl-glucoside, β -PMP-Glc (**166**) was highly IRI active (23% MGS). Compound **509** with a *para*-methoxy functional group is very IRI active with a MGS of 4%. Moving the methoxy group from the *para* position in β -PMP-Glc (**166**) resulted in a decrease in IRI activity. This was also seen with the *N*-aryl-gluconamides where compound **508** with an

ortho-methoxy functional group is inactive (100% MGS). The aryl-glucoside β -PMP-Glc (**166**) did not tolerate removal of the methyl group (*para*-hydroxyl substitution, **167**). Therefore, *N*-aryl-gluconamide with *para*-hydroxyl substituent (**510**) was assessed and resulted in a dramatic decrease in IRI activity from 4% to 76% MGS. The presence of an ionizable group at the *para*-position resulted in a moderately active molecule (**511**).

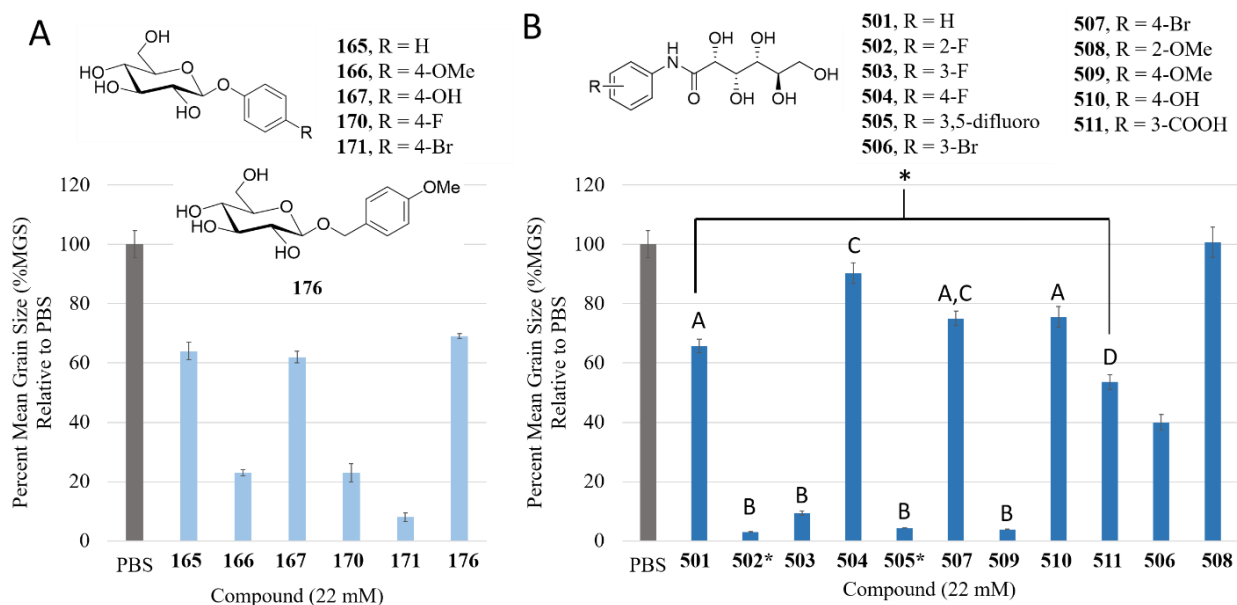


Figure 5.5. Structures and IRI activities of (A) compounds **165-167**, **170**, **171** and **176**; and (B) compounds **501-511**. Compounds were assessed for IRI activity at 22 mM unless otherwise noted. *Tested at 11 mM. Error bars represent standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons. Asterisks indicate significant difference compared to control. Statistical significance ($p < 0.05$) between compounds is indicated by different letters above bars in the graph.

Addition of a carbon spacer between the aromatic ring and the pyranose ring in β -PMP-Glc (**166**) was shown to reduce IRI activity (**176**, 69% MGS). Therefore, the effect of adding a carbon spacer between the amide and the aromatic ring in the *N*-aryl-gluconamides was assessed in compounds **512-521**. These compounds are collectively referred to as *N*-benzyl-gluconamides. The IRI activities of *N*-

benzyl-gluconamides **512-521** are shown in Figure 5.6. Addition of a carbon spacer to the *N*-aryl-gluconamide without aromatic substituents (**501**) resulted in an increase in IRI activity from 66% to 38% (**512**). This may be attributed to the increase in hydrophobicity. The polar surface area (PSA) to molecular surface area (MSA) ratio (calculated by MarvinSketch) for **501** and **512** were 0.35 and 0.32 respectively indicating that **512** is in fact less polar (more hydrophobic). However, addition of a carbon spacer to the mono-fluorinated *N*-aryl-gluconamides **502-504** resulted in a decrease in IRI activity except with *para*-fluorinated derivative **515**. Interestingly, in the *N*-aryl-gluconamide series, *ortho*- and *meta*-fluoro substitution resulted in very potent analogues **502** and **503** and *para*-fluoro substitution resulted in an inactive molecule (**504**). This is the opposite of what is observed in the *N*-benzyl-gluconamide series, where the *para*-substituted derivative **515** is more active than the *ortho*- and *meta*-fluoro substituted analogues **513** and **514**. 3,5-Difluoro substituted *N*-benzyl-gluconamide derivative **516** was weakly active (72% MGS) compared to the aryl analogue **505** (4% MGS). However, 2,6-difluoro and 2,5-difluoro substituted *N*-benzyl-gluconamide derivatives **517** and **518** have potent IRI activity (13% and 10% MGS respectively).

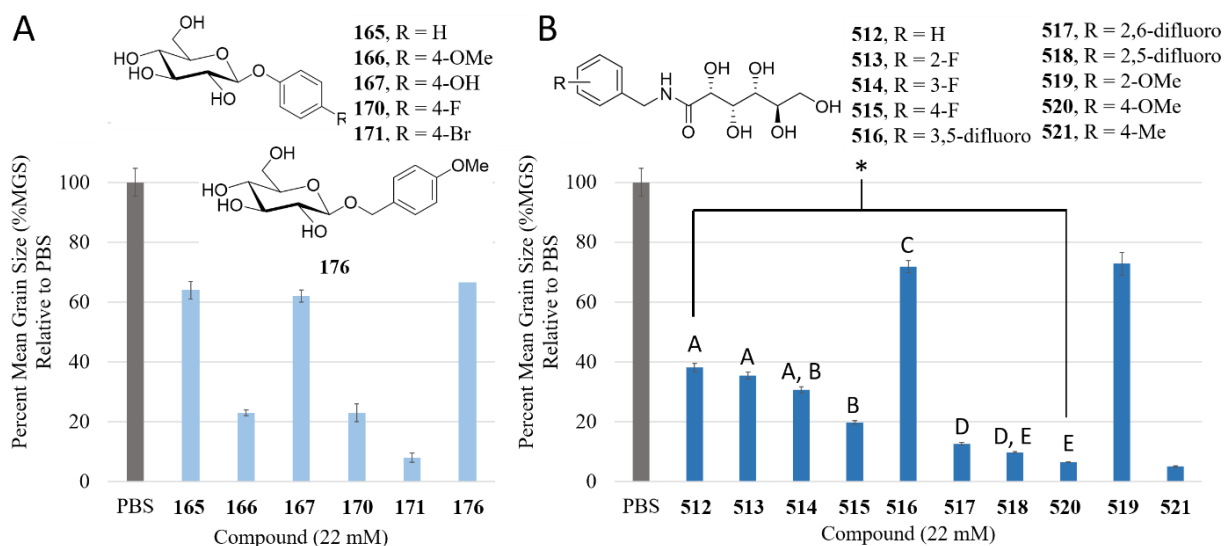


Figure 5.6. Structures and IRI activities of (A) compounds **165-167**, **170**, **171** and **176**; and (B) compounds **512-521**. Compounds were assessed for IRI activity at 22 mM unless otherwise noted. *Tested at 11 mM. Error bars

represent standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons. Asterisks indicate significant difference compared to control. Statistical significance ($p < 0.05$) between compounds is indicated by different letters above bars in the graph.

Addition of a carbon spacer between the aromatic ring and the pyranose ring in β -PMP-Glc (**166**) was shown to reduce IRI activity (**176**, 69% MGS). Since **509** was found to be highly IRI active, we investigated the effect of addition of a carbon spacer (**520**). Addition of a carbon spacer to *para*-methoxy substituted *N*-aryl-gluconamide **509** to give *N*-benzyl-gluconamide **520** did not affect IRI activity and these molecules possess a MGS $< 10\%$. Removal of the oxygen in **520** to give the *para*-methyl *N*-benzyl-gluconamide **521** did not affect IRI activity. The *ortho*-methoxy substituted analogue **519** is weakly active with MGS of 73%, similar to the *N*-aryl-gluconamide derivative **508**.

The *N*-aryl-gluconamides were further elongated by addition of a two carbon spacer to give *N*-(2-phenethyl)-gluconamides **522-525**. The IRI activities of **522-525** are shown in Figure 5.7. Compared to the *N*-aryl-gluconamide and *N*-benzyl-gluconamide (**501** and **512**), *N*-2-phenethyl-gluconamide with no aromatic substituent is more IRI active (**522**, 29% MGS). Elongating the hydrophobic portion by addition of one or two carbon spacers increases the IRI activity from 66% (**501**) to 38% (**512**) and 29% (**522**) MGS, respectively. This supports the previous studies that found that compounds with longer hydrophobic moieties were more IRI active (Chapter 4). Compounds **501**, **512** and **522** were found to be progressively more hydrophobic possessing PSA/MSA ratios of 0.35, 0.32 and 0.30, respectively. IRI activity was also influenced by the presence of aromatic substituents. *Ortho*-fluoro substitution (**523**) resulted in an increase in IRI activity compared to **522** containing no aromatic substituents, while *meta*-fluoro substitution (**524**) did not affect activity, and *para*-substitution (**525**) decreased activity.

It was hypothesized that the increase in IRI activity with the addition of one and two carbon spacers in **501** to give **512** and **522** was a result of the increased hydrophobicity. Therefore, it was hypothesized that replacement of the carbon spacer in **512** with a nitrogen spacer would result in a

reduction in IRI activity. *N*-phenylhydrazine-gluconamide derivatives **526** and **527** were also assessed in which the carbon spacer in the *N*-benzyl-gluconamides is replaced with nitrogen. These molecules were both weakly active (81 and 69% MGS, respectively) and less active than the *N*-benzyl-gluconamides **512** and **513**, attributed to the increased hydrophilicity (Figure 5.7). Compounds **512** and **513** are less polar (PSA/MSA ratios of 0.32 and 0.32 respectively) compared with **526** and **527** (PSA/MSA ratios of 0.36 and 0.35 respectively).

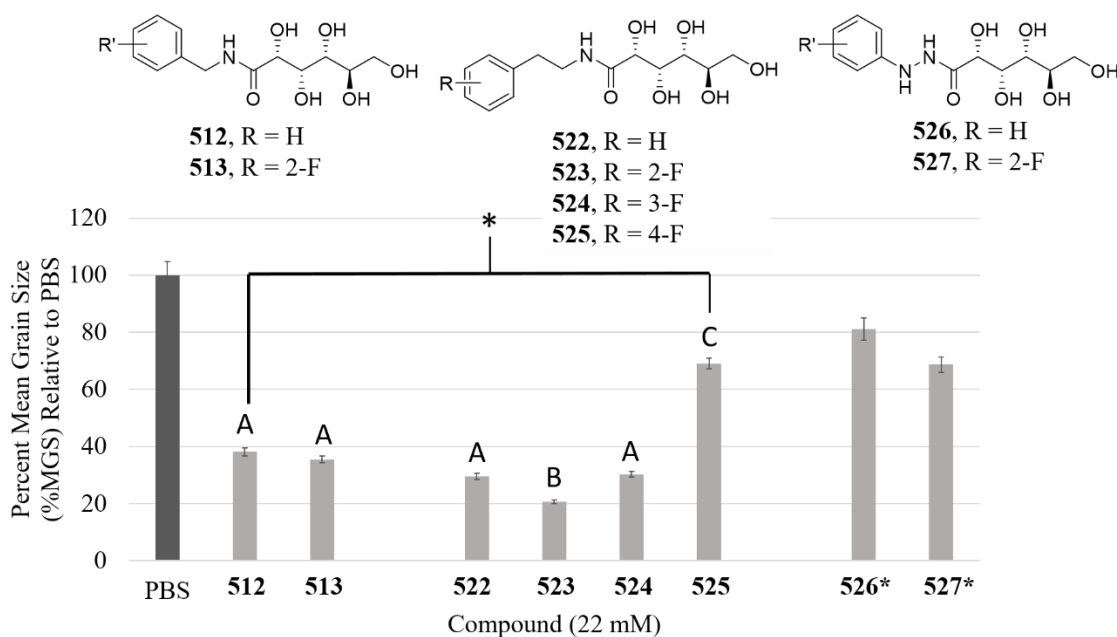
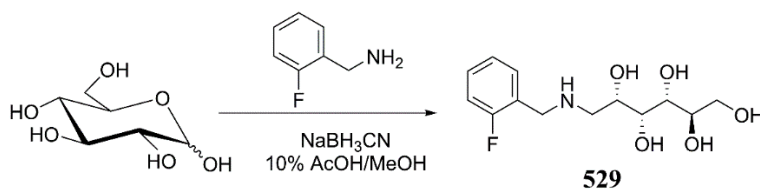


Figure 5.7. Structures and IRI activities of compounds **522-527**. Compounds were assessed for IRI activity at 22 mM unless otherwise noted. *Tested at 11 mM. Error bars represent standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons. Asterisks indicate significant difference compared to control. Statistical significance ($p < 0.05$) between compounds is indicated by different letters above bars in the graph.

Since elongating the hydrophobic portion by the addition of one or two carbon spacers resulted in an increase in IRI activity from 66% (**501**) to 38% (**512**) and 29% (**522**) MGS respectively, it was hypothesized that an increase in IRI activity could be obtained by increasing the size of the hydrophobic

moiety. To avoid using a long alkyl chain spacer, this was accomplished by using a naphthalene moiety (**528**). Derivative **528** is inactive.

N-octyl-gluconamide (**154**) was one of the first carbohydrate-based small molecules discovered to be highly IRI active. Structure-function analysis determined that replacing the amide with an ether linkage resulted in loss of activity.³ Therefore, the effect of altering the amide bond in *N*-benzyl-gluconamide **513** (possessing an *ortho*-fluoro substituent) was assessed by removal of the carbonyl (**529**). Compound **529** was obtained through the reductive amination of D-glucose with 2-fluorobenzylamine (Scheme 5.2) and its IRI activity is shown in Figure 5.8. Removal of the carbonyl did not affect IRI activity (30% MGS compared with 35% MGS for **513**), suggesting that the amide bond may not be essential for IRI activity.



Scheme 5.2. Synthesis of **529** by reductive amination.

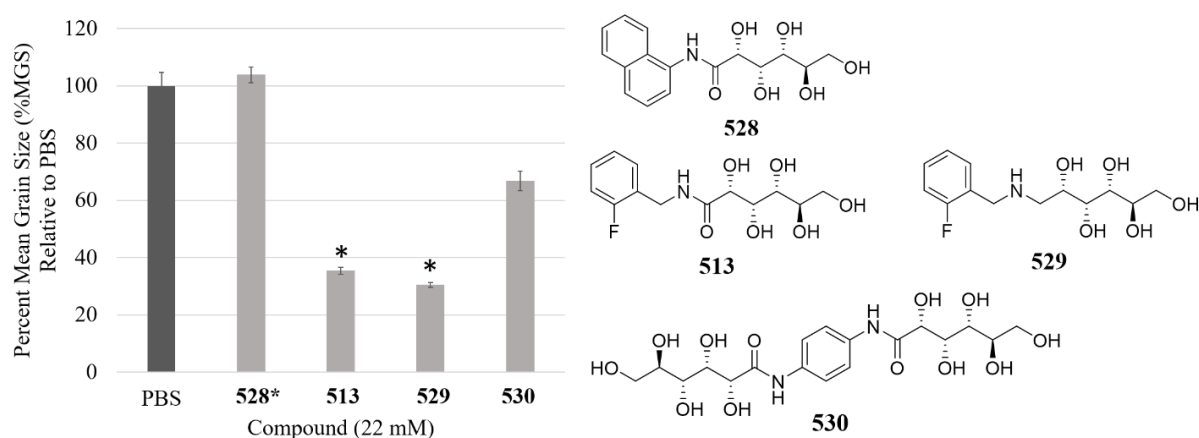


Figure 5.8. Structures and IRI activities of compounds **528-530**. Compounds were assessed for IRI activity at 22 mM unless otherwise noted. *Tested at 11 mM. Error bars represent standard error of the mean (SEM). Statistical

significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons. Asterisks indicate significant difference compared to control.

Finally, compound **530** was synthesized and assessed for IRI activity (Figure 5.8) to determine the influence of two gluconamide moieties. Compound **530** was found to have similar moderate activity to the *N*-aryl-gluconamide with no aromatic substituents (**501**) possessing a %MGS of 67%.

To summarize these results, lengthening the hydrophobic moiety by addition of a carbon spacer between the amide bond and aromatic ring resulted in an increase in IRI activity. Activity was further increased when the hydrophobic moiety was further increased by addition of a two carbon unit spacer. This is consistent with the previous structure-function analyses indicating that long hydrophobic tails are important for IRI activity. However, addition of a nitrogen spacer in lieu of a carbon spacer resulted in less active molecules probably due to the resulting decrease in hydrophobicity. An attempt to increase hydrophobicity by using a naphthyl ring instead of an aryl ring resulted in an inactive compound.

Addition of aromatic substituents complicated the analysis and there does not appear to be a clear trend with respect to the nature or location of the aromatic substituent. For example, fluorine and bromine are favourable in the *ortho*- and *meta*-positions of the *N*-aryl-gluconamides compared to the *para*-substituted derivatives which are inactive. A methoxy functional group is more favourable in the *para*-position than *ortho*-substitution. Together, these results suggest that electron-withdrawing groups are favourable in the *ortho*- or *meta*-position and electron-donating groups will be favourable in the *para*-position. However, a hydroxyl group in the *para*-position resulted in an inactive molecule. Fluorine substitution on the aryl ring in the *N*-benzyl-gluconamides is more favourable in the *para*-position than in the *ortho*- and *meta*-position which is the opposite of what is seen for the *N*-aryl-gluconamide derivatives. It is structure-function correlations of this nature that make it very difficult to rationally design new and improved small molecule ice recrystallization inhibitors. Consequently, quantitative structure activity relationship (QSAR) models were sought to accelerate the development of new IRI active compounds. This work was done in collaboration with Prof. Tom Woo and his graduate students, Phil De Luna and

Michael Fernandez and is in a manuscript entitled “QSAR accelerated discovery of potent ice recrystallization inhibitors” recently accepted to *Scientific Reports* (May 2016).⁹

5.3 QSAR models

Quantitative structure activity relationship (QSAR) models relate the chemical structures of molecules to a specific activity or property using regression models of one or more structural descriptor variables.¹⁰ QSAR modelling is an established tool in the pharmaceutical industry where it has been well documented to reduce drug discovery timelines.¹⁰ For example, a biological activity, such as a therapeutic activity of a drug, can be expressed quantitatively in the form of a concentration. Therefore, when physicochemical properties or structural features are also expressed quantitatively, one can find a mathematical relationship between the two, referred to as a ‘quantitative’ SAR, or QSAR. This mathematical expression can then be used to predict the response or biological activity of other chemical structures. In this example, the biological activity is the ‘response’ or the dependent variable which is affected by the physicochemical properties of the molecule which are the ‘descriptors’, or independent variables. We hypothesized that a QSAR model could be used to accelerate the discovery of IRI active molecules. In order to understand how the QSAR model was built, a few concepts in quantum mechanics must first be discussed.

5.3.1 Quantum mechanical concepts

The physical laws of motion that govern microscopic particles, such as atoms and molecules, are described by the theories of quantum mechanics.^{11,12} One of the theories of quantum mechanics states that a wavefunction ($\Psi(x,t)$) describes the state of a quantum mechanical system which depends on the particle’s position (x) and time (t).^{11,12} The wavefunction is a mathematical function which describes all of the physical properties of the particle.¹² Associated with each measurable property in the wavefunction is an operator. The wave function allows for the calculation of energy using the Hamiltonian operator ($\hat{H}$) in the time-independent Schrödinger equation (Eq. 5.1).^{12,13} The Hamiltonian operator, associated with the

system energy, is a sum of the kinetic energy ($\frac{-\hbar^2}{2\mu} \frac{\partial^2}{\partial x^2}$, where $\hbar$ is Planck's constant, μ is the reduced mass, and $\partial^2/\partial x^2$ is the second partial derivative with respect to position (x) and potential energy ($V(x)$) (Eq. 5.2).¹² The Schrödinger equation can only be solved for one-electron systems such as hydrogen; however, approximations to the Schrödinger equation allow for more complex systems to be solved.^{12,13} For example, the Hartree-Fock method¹⁴ is used to solve for the energies of multi-electron atoms or molecules using several approximations including the Born-Oppenheimer approximation: the assumption that the motion of atomic nuclei and electrons in a molecule can be separated.^{14,15} Computer programs that apply methods with different versions of approximations, called force fields, are used to solve the energies of complex systems. Since the energy of a molecule depends on its conformation, the lowest energy conformer is determined first using conformational searching.

$$\hat{H}\psi(x) = E\psi(x) \quad \text{Equation 5.1}$$

$$\hat{H} = \frac{-\hbar^2}{2\mu} \frac{\partial^2}{\partial x^2} + V(x) \quad \text{Equation 5.2}$$

5.3.1.1 Conformational searching

Conformational searching is a method to determine a molecule's preferred (or lowest energy) conformation. As a simple example, *n*-butane can exist in four main conformations: *syn*-eclipsed, *gauche*-staggered, eclipsed and *anti*-staggered depending on the dihedral angle. The lowest energy conformer is the *anti*-staggered in which there are no eclipsing interactions and the methyl groups are as far away from each other as possible. For larger molecules with many dihedral bonds, the energy calculations from iterative dihedral bond angle rotations becomes time consuming. The Monte-Carlo algorithm^{16,17} employs a random conformational search to more quickly identify lower energy conformations (local energy minima). First, the energy of a random conformation is calculated. Next, the dihedral angles are randomly perturbed and the energy is calculated. If the energy of the new conformation is lower than the first, this conformation is accepted. If the energy of the new conformation is higher than the first, it is either accepted or rejected based on established acceptance criteria. This is done until no new conformations can

be obtained. It is important to note that multiple low energy conformations are kept for the QSAR model since the lowest energy conformer is not always the active form. The search is performed to eliminate conformations that are higher in energy and likely do not exist. After the conformational search is complete, physicochemical properties, or descriptors, of the molecules are determined.

5.3.1.2 Descriptors

A QSAR model correlates physicochemical properties, called descriptors, to the biological activity of a molecule. Descriptors can encompass a wide variety of properties including constitutional (molecular weight, number of specific atoms, etc.), topological (connectivity), geometrical (molecular volume, surface area, etc.), electrostatic (polarizability, dipole moment, etc.) or quantum chemical (atomic charges, molecular orbital energies, total energy, etc.) properties.^{18,19} Partial least squares regression (PLSR) is a statistical technique used to relate the descriptors (independent variables) to the biological activity (dependent variable).²⁰ In order for the PLSR to accurately predict the biological activity of a molecule, the number of descriptors needs to be high enough to describe the differences in structure and activity but not so high that over-fitting occurs.²¹ Over-fitting occurs when a model is too complex, using too many descriptors and therefore results in poor predictive performance. Optimization algorithms have been developed to prevent over-fitting by selecting the most relevant descriptors for QSAR modelling.

The Genetic Algorithm (GA) is an optimization algorithm used to select the most relevant descriptors for construction of the QSAR model so that over-fitting does not occur.²² First, the descriptors from the QSAR model are randomly selected to build a trial set of QSAR equations. Each QSAR equation is then measured for accuracy, by assessing the ability to predict the activity of a molecule. Descriptors from the two most accurate equations, referred to as ‘parent’ equations, are randomly taken from each ‘parent’ and combined, or ‘mated’, to produce ‘offspring’ QSAR equations. These equations are then subsequently ranked, ‘parent’ equations are ‘mated’ and this process is repeated until an optimal set of descriptors is found.

5.3.2 Development of QSAR model to accelerate the discovery of IRI active small molecules

We hypothesized that a QSAR model could be used to accelerate the discovery of IRI active molecules. To construct a QSAR model, the molecular structures and IRI activities of a set of molecules are required. A set of 124 molecules was chosen for this study and their structures are shown in Figures 5.13-14. This set of 124 molecules includes the newly-synthesized *N*-aryl-alidonamides **501-530** from section 5.2. A conformational search using the Monte-Carlo algorithm^{16,17} was performed to identify the lowest energy conformation of each molecule. While traditional QSAR models include several low energy conformations for each molecule, only the lowest energy conformer was used in this study. This is because the majority of IRI activity is believed to be performed by the lowest energy conformation since this conformation is the most likely to be present at low temperatures. The energies were calculated using the Spartan¹⁶ program which employs a Merck Molecular Force Field (MMFF).²³ The MMFF is designed for pharmaceutical compounds and accurately describes conformational energetics and non-bonding interactions.

Next, descriptors were generated for the set of molecules. In computational drug design, comparative molecular field analysis (CoMFA) methods are used.^{10,24} In CoMFA methods, the energies of steric and electrostatic interactions are calculated between the molecule of interest and a “probe atom” placed at nodes of a consistently spaced three dimensional lattice. These interactions are calculated for all the molecules in the set and used as descriptors. However, this method is highly alignment-dependent and therefore the comparison between molecules is only valid if the interactions are calculated from the same reference orientation. If the molecules do not have the same parent structure then the comparisons between molecules will be skewed. Therefore, alignment-independent methods are required for the diverse library used in this study.

Grid-independent descriptors (GRIND) were used to describe the set of molecules.²⁵ Briefly, a regular rectangular grid with 0.176-Å spacing on the surface of each molecule was constructed. By only looking at the distances between nodes and not their orientation within space, GRIND eliminates the need

to have all molecules oriented the same way. At each grid point the surface curvature and the electrostatic potential (ESP) of the molecule was evaluated.^{26,27} Grid points were paired to obtain all combinations of pairs of grid points. These were then sorted based on their distance and placed in bins ranging from 0.33 to 25.83 Å in 0.5-Å increments. Then for each pair of grid points, the product of the two ESP values and two curvature values (auto-correlation), and the cross-correlation of the ESP-curvature were calculated. The maximum product value for the ESP-ESP, curvature-curvature, and ESP-curvature at each distance bin was used to create a unique fingerprint for each molecule. Each maximum product represents the unique interaction between nodes at the molecular surface and thus is unique to each specific molecular structure.

Once these descriptors were calculated for all molecules in the set, the molecules were separated into a *training* set or a *test* set. The *training* set was comprised of 84 compounds and the *test* set was comprised of 40 compounds. Next, PLSR models²⁰ were calibrated using the descriptors for the *training* set. The number of descriptors was too high, with approximately 150 descriptors. Therefore, a genetic algorithm (GA)²² was utilized to optimize the descriptors for the QSAR model. The resulting QSAR model contained 26 descriptors and is a linear equation of the form $y = m_i x_i + c$, where m is the optimized coefficient from the PLSR model for the descriptor i and x is the type of descriptor (curvature-curvature, ESP-ESP or ESP-curvature) at a certain distance. The optimal QSAR formula is shown in Figure 5.9.

For any new molecular structure, a score can be calculated based the descriptors in the QSAR equation using the computational methods described previously. The QSAR model was designed to produce a score between 0 and 1, where 0 is inactive (MGS > 70%) and 1 is active (MGS < 70%) and the cut-off for classification is 0.5. Therefore, any molecule with a score between 0 and 0.5 is predicted to be inactive whereas a score between 0.5 and 1 is predicted to be active. The cut-off for activity was chosen to be a MGS of 70%. This is because compounds with IRI activity > 70% have been previously described as weakly active to inactive.^{1,2} Furthermore, this cut-off was found to yield the best correlation between chemical similarity (using the GRIND descriptors) and activity (%MGS).

Descriptor coefficient
Interaction Distance (Å)

$$\begin{aligned}
 \text{Scores} = & -12.88209813 + \textcolor{gray}{-1.55349518}*\textcolor{red}{RR08} + 1.26493429*\textcolor{red}{RR09} + -0.71879784*\textcolor{red}{RR13} + \\
 & 0.37435381*\textcolor{red}{RR20} + 0.63260225*\textcolor{red}{RR25} + 1.09873932*\textcolor{red}{RR27} + -1.02370836*\textcolor{red}{RR39} + \\
 & 22.27632064*\textcolor{blue}{PP06} + -51.55378289*\textcolor{blue}{PP08} + 67.70262385*\textcolor{blue}{PP11} + -22.17870625*\textcolor{blue}{PP14} + \\
 & 32.91374802*\textcolor{blue}{PP15} + -45.62170944*\textcolor{blue}{PP18} + -9.44374889*\textcolor{blue}{PP29} + 34.25290347*\textcolor{blue}{PP34} + - \\
 & 94.74108231*\textcolor{blue}{PP37} + 147.33610196*\textcolor{blue}{PP38} + -52.49714414*\textcolor{blue}{PP39} + -26.91971935*\textcolor{blue}{PP41} + \\
 & 0.62665292*\textcolor{blue}{PP48} + -6.52771421*\textcolor{green}{RP08} + 15.66194217*\textcolor{green}{RP10} + -63.15678236*\textcolor{green}{RP14} + \\
 & 46.98504881*\textcolor{green}{RP15} + -7.46979402*\textcolor{green}{RP17} + 0.57636719*\textcolor{green}{RP50}
 \end{aligned}$$

RR = curvature-curvature auto-correlation
PP = ESP-ESP auto-correlation
RP = curvature-ESP cross-correlation

Figure 5.9. Optimal QSAR linear equation as found after 100 GA runs. RR_X, PP_X, and RP_X are the maximum steric-steric autocorrelation, electrostatic-electrostatic autocorrelation, and steric-electrostatic cross correlation at a certain distance in angstroms (X) respectively.

Finally, the precision of the QSAR model was assessed. The optimum 3D-QSAR model successfully identified IRI active compounds in the *training* set with accuracy of 95%. We consider these results good considering the diversity of molecules in the *training* set and the fact that only 37% of the molecules in the *test* set are IRI active. The model was further tested by performing a cross-validation.²⁸ A leave-one-out (LOO) cross-validation^{29,30} was performed where a single molecule is left out from the *training* set, then the model is refitted, and the predictive activity for the molecule that was left out is compared to its actual activity. This is done for each individual molecule being omitted. The LOO cross validation^{29,30} resulted in an accuracy of 85%, sensitivity of 82% and precision of 72%. Sensitivity is the number of truly active molecules identified divided by the total number of active molecules in the set and it is a measure of the probability that an active molecule will be identified by the model. On the other hand, precision is the number of truly active molecules out of the total number of molecules identified as such by the model, which relates to the probability that a molecule identified by the model as active is truly active. Therefore, there is always a trade-off between sensitivity and precision.

Although the QSAR model performed well on the *training* set, these were molecules for which the model parameters and descriptors were optimized to. The important measure of the predictive capability of the QSAR model is the performance on molecules not used in calibration. When applied to the *test* set of 40 molecules that were not part of the *training* set, the 3D-QSAR model successfully recovered 67% of the active molecules with similar precision and higher sensitivity of ~80%. The statistics for the different structural groups in the *test* set are shown in Table 5.1, where 100% of the active aryl-aldonamides and alkyl-aldonamides were successfully identified in comparison to only 60% of the aryl-glycosides.

Structures	Sensitivity	Specificity	Number of Molecules	Precision
Entire test set	0.67	0.80	40	0.67
Aryl aldonamides	1.00	0.75	6	0.67
Alkyl aldonamides	1.00	0.67	6	0.75
Aryl glycosides	0.60	0.79	24	0.67

Table 5.1. Prediction statistics for different classes of IRIs.

5.3.2.1 Most relevant interactions

With the GRIND approach, it is possible to trace back the most relevant interactions from the QSAR equation to chemical structure. The most relevant features and their specific correlations for aryl-glycosides, β -pClPh-Glc (**550**) (left), possessing potent IRI activity, and β -pCH₃Ph-Gal (**551**) (right), which does not possess IRI activity, are shown in Figure 5.10. The structures of **550** and **551** are shown in Figure 5.13.

A prominent ESP-ESP interaction occurs at ~ 10 Å between the aryl substituent and the farthest hydroxyl group for both compounds as shown by the green line. Because this interaction is prominent for both active and inactive compounds, this feature is not important for IRI activity. However, the most prominent ESP-ESP interactions (~ 6 Å) and curvature-ESP interactions (~ 3 Å) occur between atoms in the aromatic ring in IRI active **550**, whereas these prominent interactions occur within the carbohydrate region in inactive compound **551**. Therefore, it is unfavourable for interactions to occur between the aromatic ring and carbohydrate moiety.

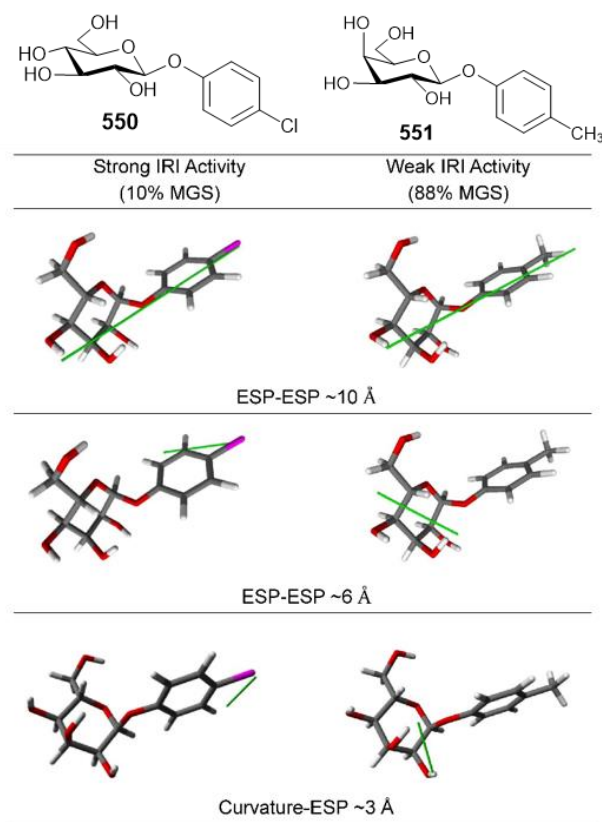


Figure 5.10. Most relevant features and their specific correlations for compound **550** (left), possessing potent IRI activity and compound **551** (right) which does not possess IRI activity.

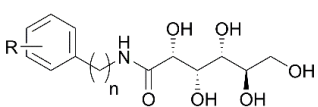
5.3.2.2 Experimental application

Although the QSAR model performed well on the *training* and cross-validation sets, these sets were made up of molecules that were used to build the model. The important measure of the predictive capability of the QSAR model is the performance on molecules not used in calibration. As discussed earlier, when applied to the *test* set of 40 molecules that were not part of the *training* set, the 3D-QSAR model successfully recovered 67% of the active molecules with a sensitivity of 80%.

Next, the QSAR model was used to screen for IRI active molecules that had not yet been synthesized. For this purpose, a small library of 29 aryl-alditol structures as shown in Figure 5.11 was created and the QSAR model applied to them. The library was limited to aryl-alditols as they were deemed to be most easily synthesized. Seventeen of these molecules (compounds **531-547**) were

synthesized and tested for IRI activity. Eleven of these compounds were predicted to be active (with a score between 0.5 and 1) and 6 were predicted to be inactive (with a score between 0 and 0.5). The experimental activities of **531-547** is shown in Figure 5.12.

N-aryl-gluconamides **531-541** and *N*-benzyl-gluconamides **542-547** were synthesized (described in Experimental Section 5.5.8 & 5.5.9) and assessed for IRI activity (Figure 5.12). *N*-aryl-gluconamide with chlorine in the *para*-position (**538**) resulted in a more IRI active molecule than *ortho*-substitution (**537**) with activities of 35% and 100% MGS respectively. 2,5-Dichloro substitution (**531**) resulted in a



Entry	A/I	n	R	Entry	A/I	n	R
531	A	0	2,5-dichloro	546	A	1	2-OCF ₃
532	A	0	4-CH ₃	547	A	1	4-OCF ₃
533	A	0	2-OH	548	A	0	2-CF ₃
534	A	0	3-CF ₃	549	I	1	2-Cl
535	A	0	4-CF ₃	550	I	0	2,6-dichloro
536	A	0	4-OCF ₃	551	A	0	3-OCF ₃
537	I	0	2-Cl	552	A	0	2-OCF ₃
538	I	0	4-Cl	553	I	1	3-OCF ₃
539	I	0	2-CH ₃	554	A	1	3,5-dimethyl
540	I	0	2,6-dimethyl	555	I	1	2,5-dimethyl
541	I	0	2,5-dimethyl	556	A	1	4-OH
542	I	1	4-Cl	557	A	1	2-CF ₃
543	A	1	2-CH ₃	558	I	1	2,4-dichloro
544	A	1	3-CF ₃	559	A	0	3-OH
545	A	1	4-CF ₃				

Figure 5.11. Structures of *N*-aryl-gluconamides and *N*-benzyl-gluconamides predicted by the QSAR model to be active indicated as “A” (%MGS < 70%) or inactive indicated as “I” (%MGS > 70%).

molecule with moderate activity. A *para*-methyl substituent resulted in a potent inhibitor (**532**, 6% MGS) and possessed similar activity to the *para*-methoxy analogue **509**. On the other hand, an *ortho*-methyl substituent resulted in an inactive molecule (**539**, 90% MGS). Di-*ortho*-methyl substitution however was favourable (**540**, 32% MGS) while 2,5-dimethyl substitution was not (**541**, 84% MGS). A *para*-trifluoromethoxy functional group was active (**536**, 15% MGS), but less active than the non-fluorinated analogue **509**. Replacement of the *para*-methyl substituent with a *para*-trifluoromethyl functional group

(535) resulted in a decrease in IRI activity from 6% to 28%. However, 535 was more IRI active than the *meta*-substituted analogue 534 (87% MGS).

The *para*-chloro-substituted *N*-benzyl-gluconamide is highly active (542, 7% MGS) and more active than the aryl derivative 538 (35% MGS). Trifluoromethyl-substituted *N*-benzyl-gluconamides 544 and 545 possess similar activity to the *N*-aryl-gluconamides 534 and 535 where *para*-substitution is more favourable. Addition of a carbon spacer to *para*-trifluoromethoxy *N*-aryl-gluconamide 536 resulted in a slight decrease in IRI activity (547, 24% MGS) but was more active than the *ortho*-trifluoromethoxy *N*-benzyl-gluconamide 546 which possessed an activity of 61%.

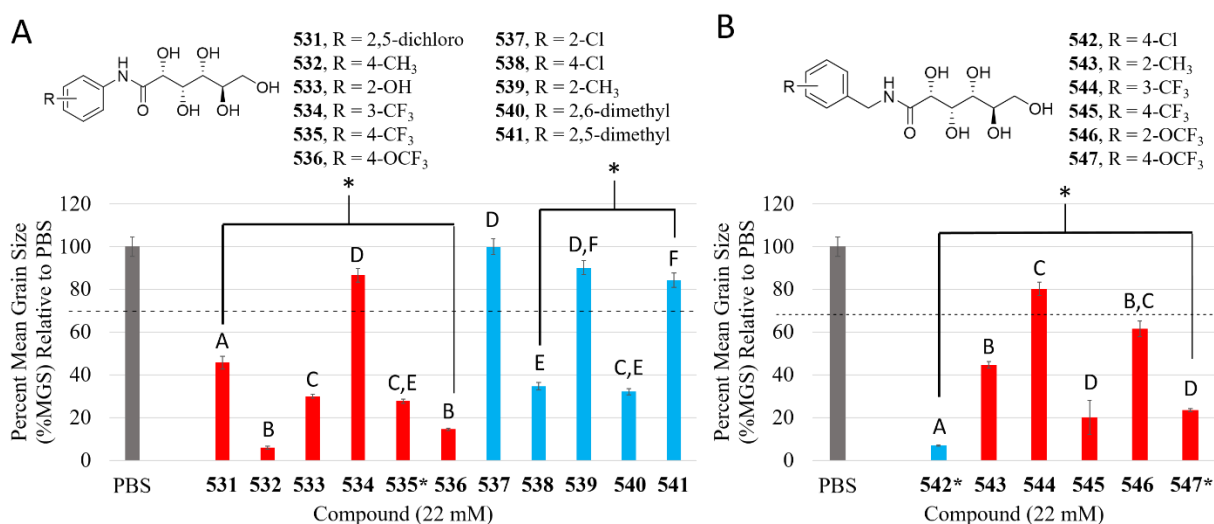


Figure 5.12. Structures and IRI activities of compounds 531-547. Compounds were assessed for IRI activity at 22 mM (except compounds indicated with an asterisks which were tested at 11 mM). Red bars represent compounds which were predicted to be active (MGS < 70%) and blue bars represent compounds which were predicted to be inactive (MGS > 70%). The cut-off for activity is indicated by the dotted line. Error bars represent standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons. Asterisks indicate significant difference compared to control. Statistical significance ($p < 0.05$) between compounds is indicated by different letters above bars in the graph.

This small library of 29 aryl-alditol structures (Figure 5.11) was created for the QSAR model to predict whether the molecules were likely to be active or inactive. Eighteen of the molecules were predicted to be IRI active and eleven were predicted to be inactive. Seventeen of these molecules were synthesized and tested for IRI activity – eleven of which were predicted to be active and six that were predicted to be inactive. The results are summarized in Figure 5.12 which shows the experimental IRI activity of the seventeen compounds synthesized. Compounds shown in red were predicted to be IRI active by the QSAR model with a MGS < 70%. Compounds in blue were predicted to be inactive with a MGS > 70%. Figure 5.12 shows that nine out of eleven (82%) of the compounds predicted to be IRI active were determined experimentally to be active. This sensitivity (true positive rate) is consistent with the *training* and *test* set true positive rates of 80%. Of the six compounds that were predicted to be IRI inactive, three were experimentally found to be active with a MGS < 70%. This is not surprising because the QSAR model was optimized to enhance the true positive rate rather than the true negative rate (specificity). A cut-off value was used that increased the precision of predicting active compounds at the cost of lower sensitivity. While the model is not effective at predicting compounds which are inactive, the ability of the model to predict molecules that will be experimentally active is a great achievement and has accelerated the discovery of IRI active small molecules that were previously discovered through a laborious trial-and-error process.

5.4 Chapter summary

Since the replacement of the alkyl chain with an aromatic ring in the β -alkyl-glycosides resulted in the discovery of potent IRIs, it was hypothesized that the same replacement in the *N*-alkyl-gluconamides could also result in the discovery of potent IRIs. Thirty derivatives (**501-530**) of this new class of compounds, referred to as *N*-aryl-gluconamides, were synthesized and assessed for IRI activity. This resulted in several very potent IRIs being discovered. Unfortunately, structure-function analyses of these derivatives did not result in the identification of key structural attributes required for inhibition of ice

recrystallization. This is unfortunate because it hampers the rational design of new inhibitors and has required an arduous trial-and-error discovery process.

Since the mechanism by which these molecules inhibit ice recrystallization is not known and this has impaired the rational design of new IRI active compounds, a 3D-QSAR model was designed and used to pre-screen compounds for IRI potency to minimize labour-intensive chemical synthesis. This ultimately accelerated the discovery process. Using a diverse set of 124 molecules with known IRI activities, a 3D-QSAR model was calibrated that was able to successfully identify 80% of the IRI active molecules in both a *training* set of 84 molecules and a *test* set of 40 molecules.

The GRIND descriptors highlighted structural features that had the greatest impact on IRI activity. It was found that a prominent curvature-ESP interaction between the aryl ring atoms at 3 Å occurred in IRI active molecules, while this interaction occurred between carbohydrate atoms in molecules which were inactive. These interactions may provide a basis in which to further understand the mechanism of recrystallization inhibition and this is something that will be explored in future work. Furthermore, it was previously shown that IRI activity is correlated to the hydration of a molecule.^{1,2} In future studies, renewed building, testing and optimization of the current QSAR model to include hydration parameters are required in order to better understand the role of hydration in the mechanism of ice recrystallization inhibition.

The QSAR model was used to prescreen a small library of novel IRI active compounds that were then synthesized and tested with a sensitivity (success rate) of 82%. This success rate is comparable to that of other QSAR aided drug discoveries. For example, QSAR models were shown to have a 70% success rate for their prediction of blood-brain barrier permeation and central nervous system (CNS) activity.³¹ However, of the molecules assessed which were predicted to be inactive, only 50% were experimentally found to be inactive. This is problematic as it results in missing compounds which could potentially be highly IRI active. It was previously shown that IRI activity is correlated to the hydration of a molecule.^{1,2} Therefore, future work will attempt to improve the model by incorporating hydration parameters.

While the model is not effective at predicting compounds that are inactive, the ability of the model to predict molecules that will be experimentally active has greatly accelerated the discovery of IRI active small molecules that were previously discovered through a laborious trial-and-error process. Thus, QSAR modeling can be used to enrich the sampling of active IRI compounds even though rational design principles for IRI activity do not yet exist. This is a strategy which has drastically improved upon the current trial-and-error process and has allowed for the more rapid discovery of molecules possessing IRI activity.

5.5 Experimental

5.5.1 *Ice Recrystallization Inhibition (IRI) Assay*

IRI activity was performed using the “splat cooling” method.³² The analyte was dissolved in phosphate buffered saline (PBS) solution at a concentration of 22 mM. If the analyte was insoluble, serial dilutions to 11, 5.5 or 0.5 mM were performed. A 10 μ L droplet of this solution was dropped from a height of two metres onto a block of polished aluminum precooled on dry ice to approximately -80 °C. The droplet froze instantly forming a wafer on the polished aluminum block and was approximately 1 cm in diameter and 20 μ m thick. This wafer was then carefully removed from the surface of the block and transferred onto a circular glass coverslip and to a cryostage held at -6.4 °C for annealing. After a period of 30 min, the wafer was photographed between crossed polarizing filters using a digital camera (Nikon CoolPix 5000) fitted to the microscope. A total of three images were taken from each wafer and each solution was tested three times.

The initial ice crystals formed during flash freezing are relatively small and homogeneous.

Recrystallization occurs during the 30 minute annealing cycle, resulting in an increase in ice crystal size. After the annealing process, images captured the frozen wafer and ice crystal sizes were obtained using a novel domain recognition software (DRS) program. This processing employed the Microsoft Windows Graphical User Interface to allow a user to visually demarcate and store the vertices of ice domains in a digital micrograph.³³ The data was then used to calculate the domain areas. A total of 12 domain areas

were obtained for nine images taken from three separate drops ($n = 108$). All data were plotted and analyzed using Microsoft Excel. The mean grain (or ice crystal) size (MGS) of the sample was compared to the MGS of the control PBS solution for that same day of testing and IRI activity is reported as the percentage of the MGS (%MGS) relative to the PBS control. Therefore, small percentages represent small ice crystals and this is indicative of high IRI activity. The %MGS for each sample was plotted along with its standard error of the mean (SEM).

5.5.2 Training and test set structures for QSAR model

The experimental synthesis and characterization of newly synthesized compounds **501-530** is described below. The remaining 94 molecules used to build the QSAR model were previously synthesized and tested for IRI activity by other members of the Ben laboratory. The chemical structures of the molecules used to build the QSAR model are shown in Figures 5.13-14.

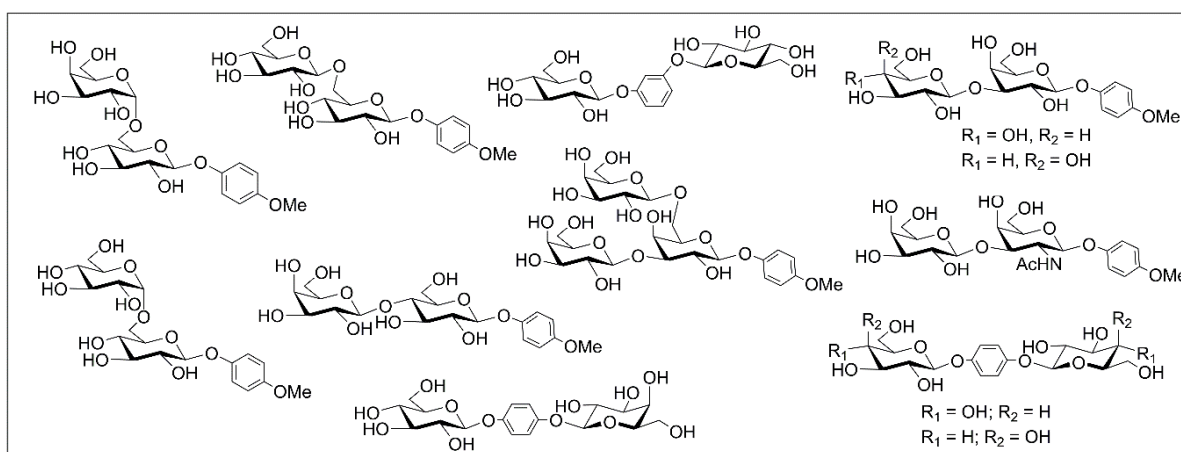
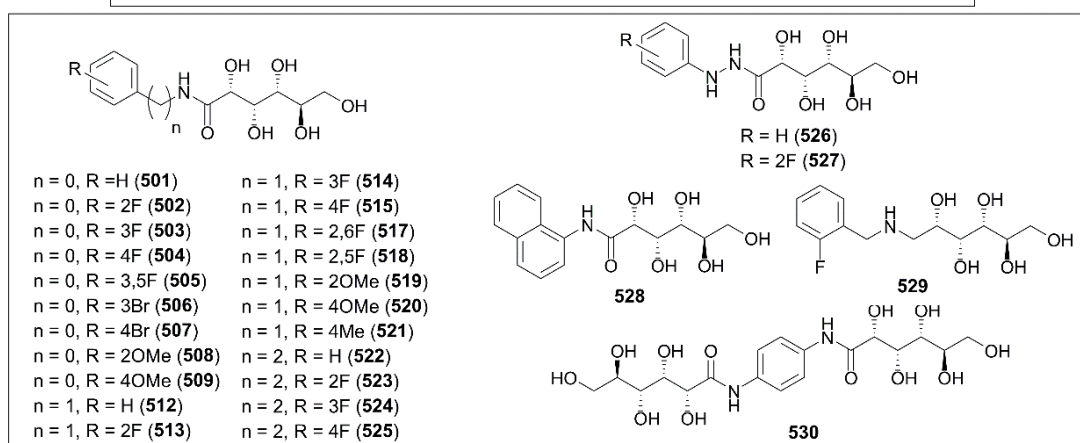
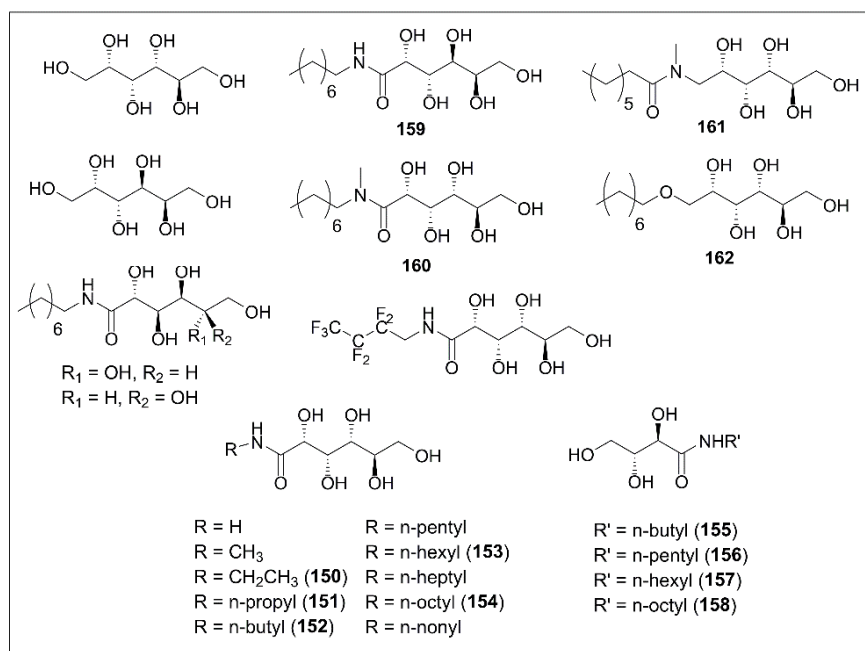


Figure 5.14 Structure of alky- and aryl-aldonamides and di- and trisaccharide derivatives used for the *training* and *test* sets for building the QSAR model.

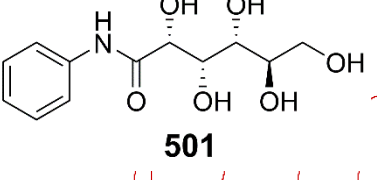
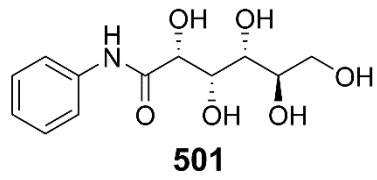
5.5.3 General Experimental

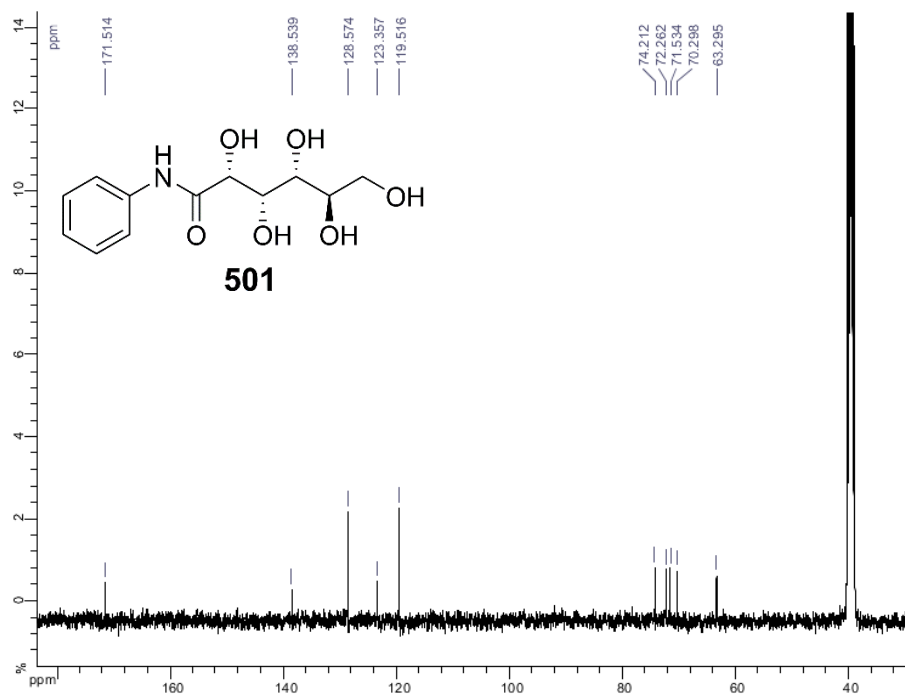
Anhydrous reactions were performed under a positive pressure of dry argon in flame-dried glassware. Air- or moisture-sensitive reagents and anhydrous solvents were transferred with oven-dried syringes. Reactions were monitored using analytical thin layer chromatography (TLC) with 0.2 mm pre-coated silica gel aluminum plates 60 F254 (E. Merck). Components were visualized by illumination with a short-wavelength (254 nm) ultra-violet light and/or staining (ceric ammonium molybdate or orcinol/sulphuric acid stain solution). ^1H (300, 400 or 500 MHz) and ^{13}C NMR (100 or 125 MHz) spectra were recorded on a Bruker Avance 300, 400, or 500 spectrometer. Deuterated DMSO ($\text{DMSO-}d_6$) was used as the NMR solvent. Chemical shifts are reported in ppm using the solvent residual peak as an internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet and br, broad. Low resolution mass spectrometry (LRMS) was performed on a Micromass Quatro-LC Electrospray spectrometer with a pump rate of 20 $\mu\text{L}/\text{min}$ using electrospray ionization (ESI).

5.5.4 Characterization data and spectra of *N*-aryl-gluconamides (501-511)

N-Phenyl-D-gluconamide (501)

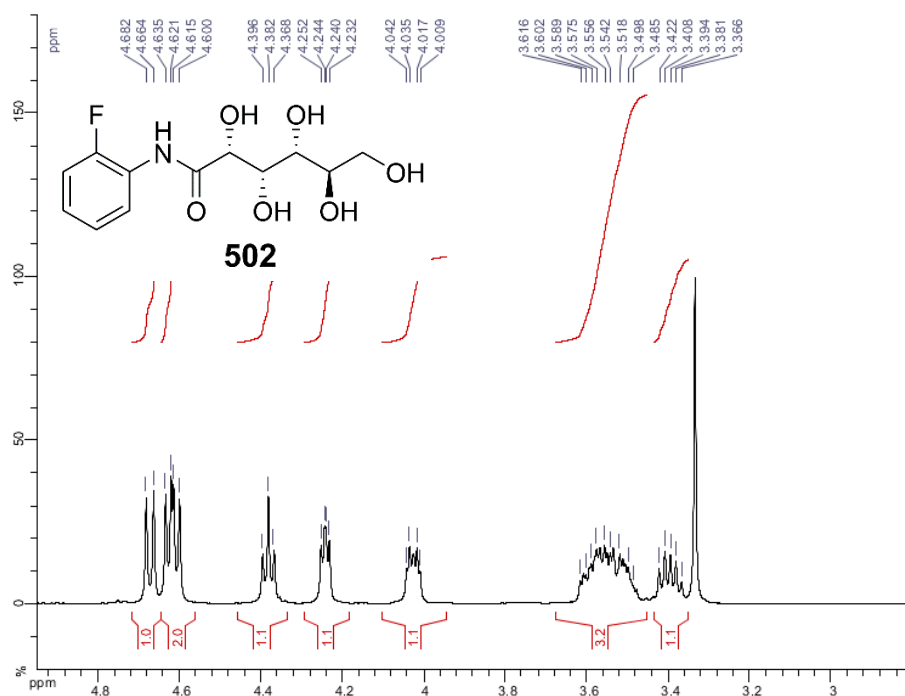
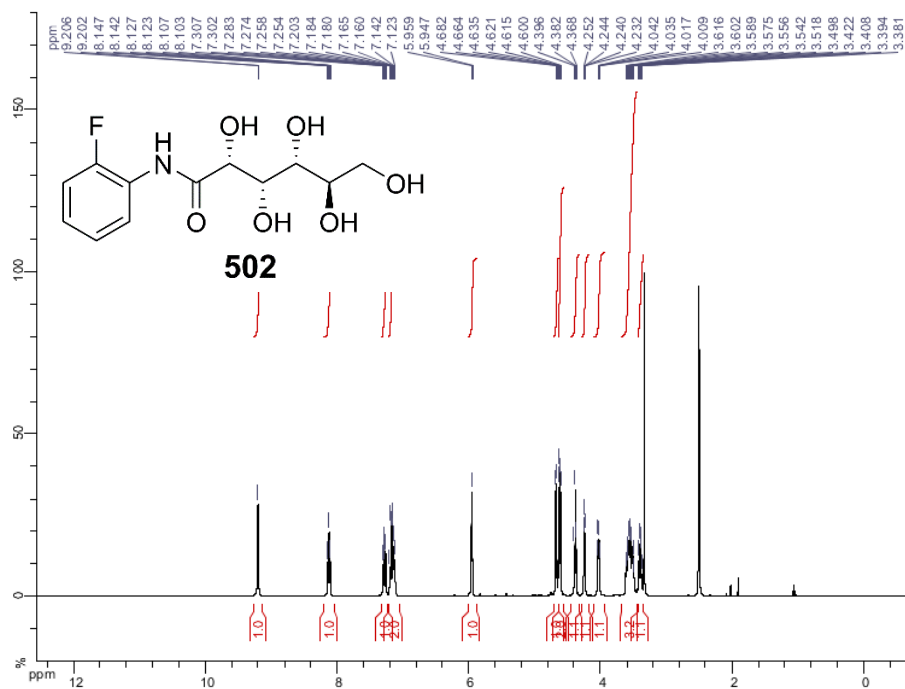
To a solution of D-gluconic acid- δ -lactone (0.50 g, 2.81 mmol) in MeOH (10 mL) was added aniline (0.26 mL, 2.81 mmol). The mixture was stirred under reflux for 48 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **501** as a pale brown solid (87%). Characterization data is consistent with that of previously reported data.³⁴ ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 8.68 (s, 1H), 6.86 (d, $J = 8.1$ Hz, 2H), 6.56 (t, $J = 8.1$ Hz, 2H), 6.21 (t, $J = 8.1$ Hz, 1H), 4.86 (d, $J = 5.3$ Hz, 1H), 3.76 (d, $J = 5.2$ Hz, 1H), 3.70-3.72 (m, 2H), 3.53 (t, $J = 5.9$ Hz, 1H), 5.33 (t, $J = 4.8$ Hz, 1H), 3.16-3.19 (m, 1H), 2.73-2.78 (m, 1H), 2.68 (t, $J = 2.7$ Hz, 2H), 2.52-2.58 (m, 1H). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ 171.5, 138.5, 128.6, 123.4, 119.5, 74.2, 72.3, 71.5, 70.3, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{18}\text{NO}_6$ $[\text{M} + \text{H}]^+$ 272.3, found 272.2.

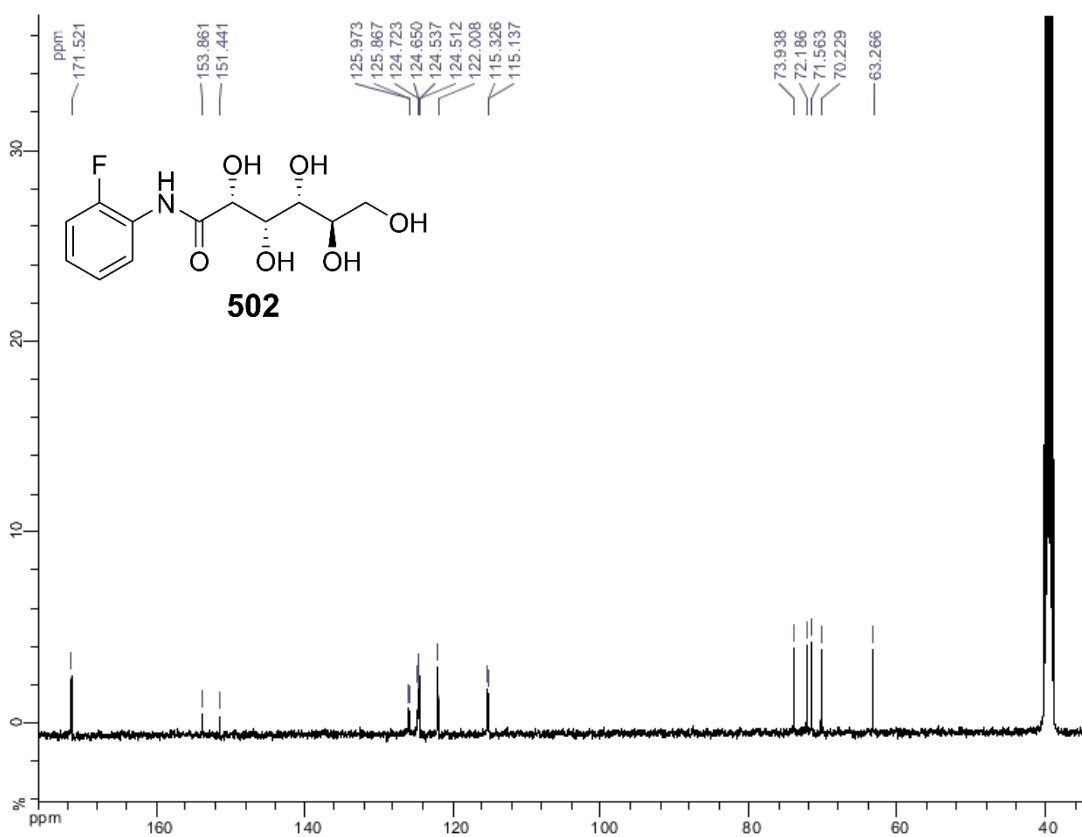




N-2-Fluorophenyl-D-gluconamide (**502**)

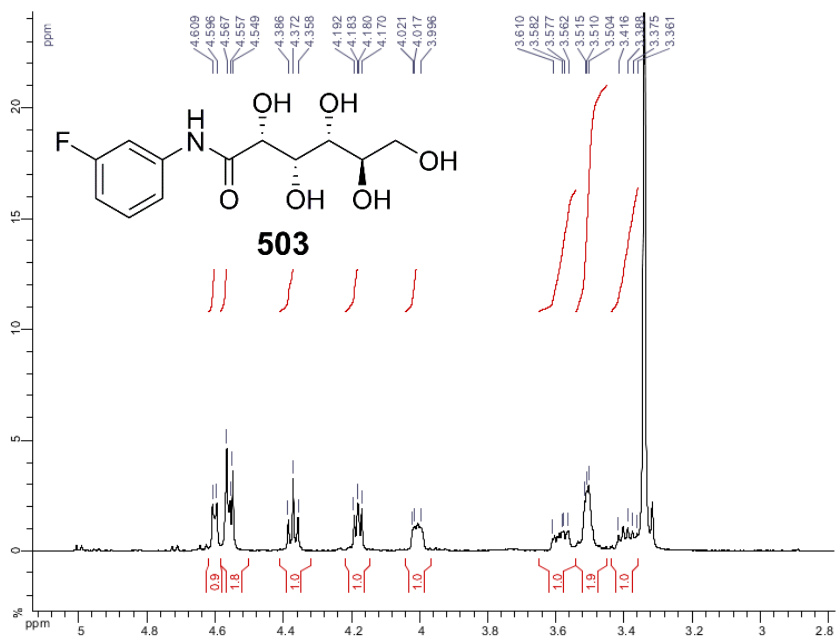
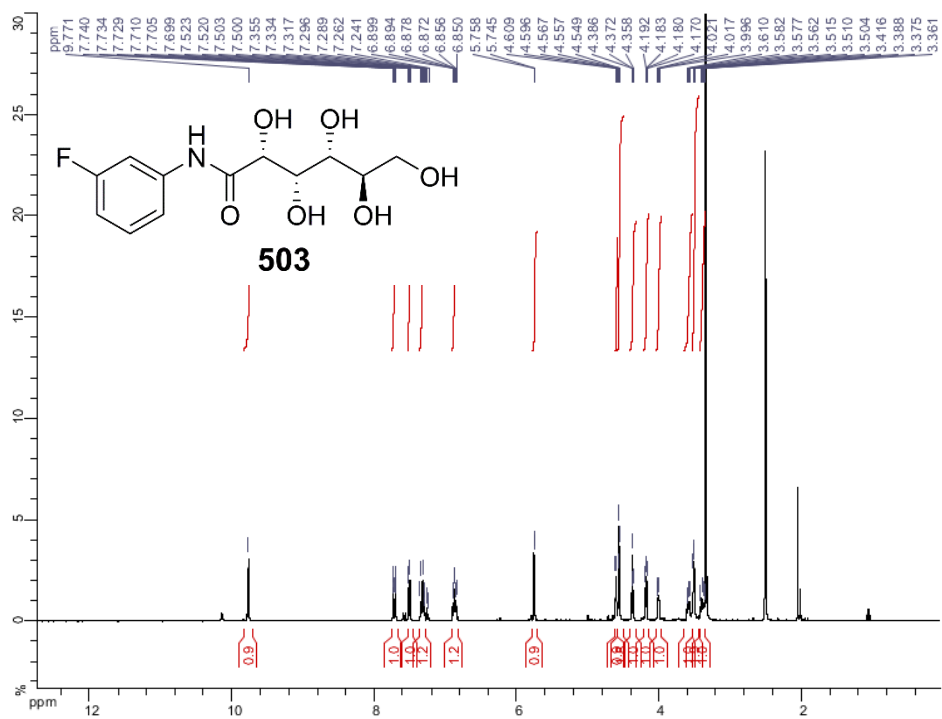
To a solution of D-gluconic acid- δ -lactone (0.50 g, 2.81 mmol) in acetic acid (5 mL) was added 2-fluoroaniline (0.81 mL, 8.42 mmol). The mixture was stirred under reflux for 1 hour. The crude product was precipitated with hexanes and filtered to obtain a dark brown sludge. The solid was recrystallized in EtOH to afford **502** as a white powder (33%). ^1H NMR (400 MHz, DMSO- d_6): δ 9.21 (br. s, 1H), 8.13 (td, J = 7.9 Hz, 2.1 Hz, 1H), 7.31-7.26 (m, 1H), 7.20-7.12 (m, 2H), 5.95 (d, J = 6.0 Hz, 1H), 4.67 (d, J = 6.9 Hz, 1H), 4.62 (dd, J = 8.4 Hz, 5.4 Hz, 2H), 4.38 (t, J = 5.7 Hz, 1H), 4.24 (dd, J = 4.9 Hz, 3.4 Hz, 1H), 4.04-4.01 (m, 1H), 3.62-3.49 (m, 3H), 3.42-3.33 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 171.5, 152.5 (d, $J_{\text{C,F}}$ = 241.6 Hz), 125.9 (d, $J_{\text{C,F}}$ = 10.7 Hz), 124.7 (d, $J_{\text{C,F}}$ = 7.3 Hz), 124.5 (d, $J_{\text{C,F}}$ = 3.4 Hz), 122.0, 115.2 (d, $J_{\text{C,F}}$ = 18.8 Hz), 73.9, 72.2, 71.6, 70.2, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{15}\text{FNO}_6$ $[\text{M-H}]^-$ 288.1, found 288.1.

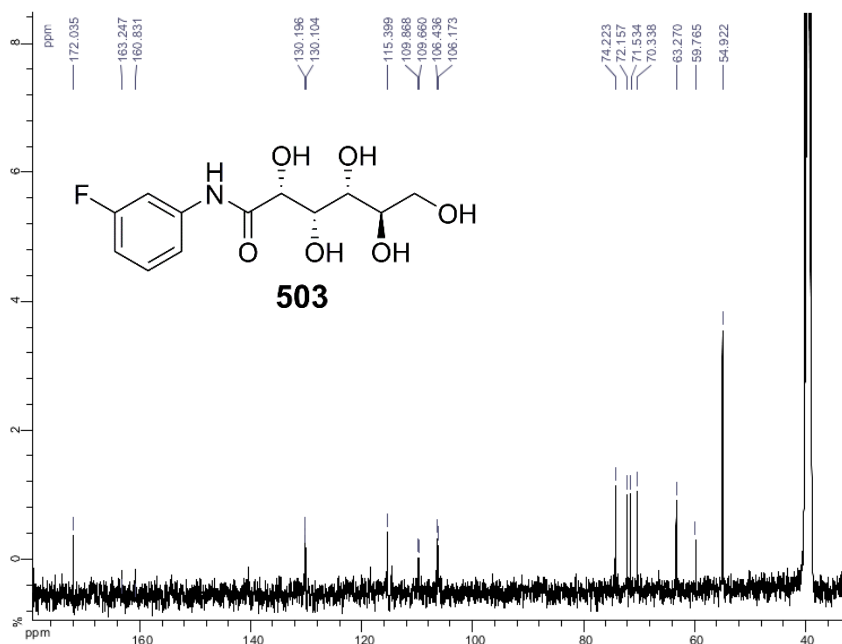




N-3-Fluorophenyl-D-gluconamide (**503**)

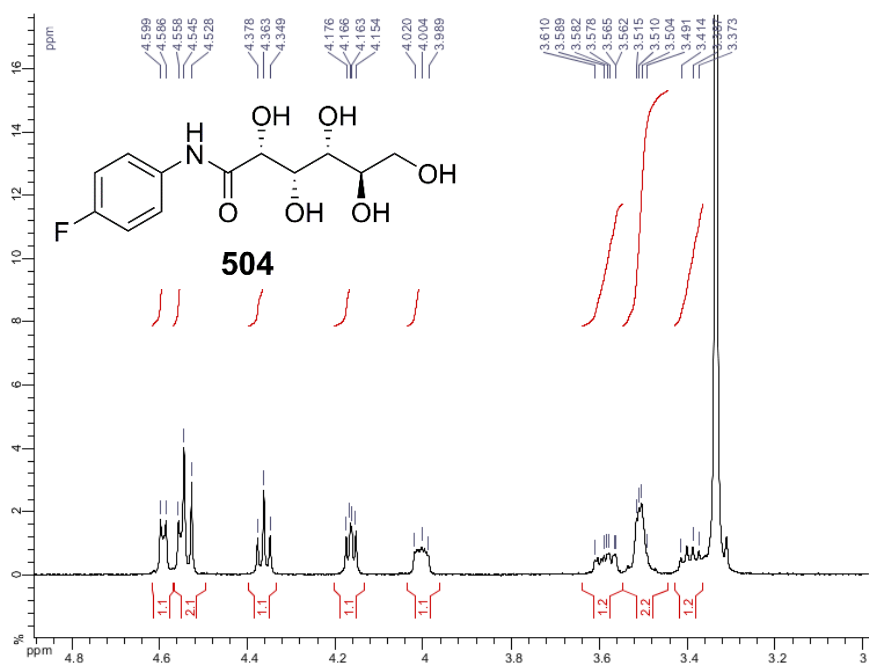
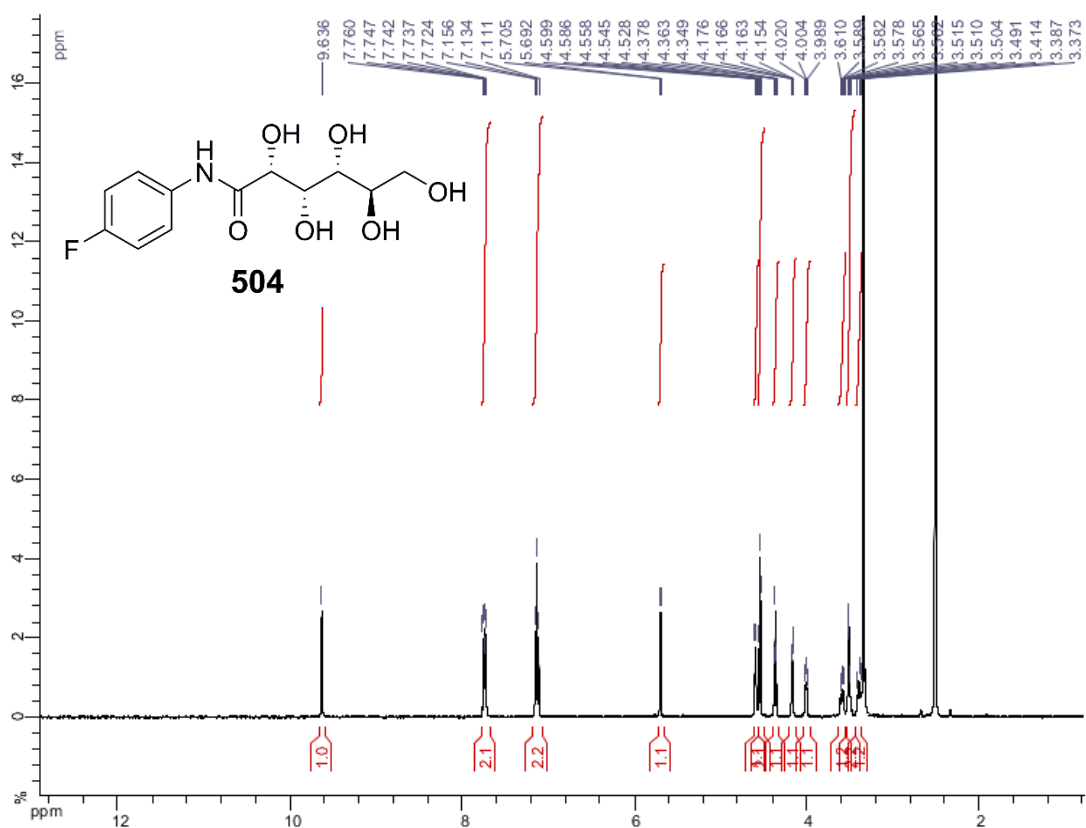
To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in acetic acid (10 mL) was added 3-fluoroaniline (0.36 mL, 3.36 mmol). The mixture was stirred under reflux for 3 hours after which crude product was precipitated with hexanes and filtered to collect a dark brown solid. Recrystallization in EtOH afforded **503** as an off-white powder (62%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 9.77 (s, 1H), 7.72 (dt, $J = 11.9, 2.3$ Hz, 1H), 7.51 (d, $J = 8.4$ Hz, 1H), 7.30-7.36 (m, 1H), 6.85-6.90 (m, 1H), 5.75 (d, $J = 5.3$ Hz, 1H), 4.60 (d, $J = 5.0$ Hz, 1H), 4.55-4.57 (m, 2H), 4.37 (t, $J = 5.6$ Hz, 1H), 4.18 (t, $J = 4.6$ Hz, 1H), 4.00-4.02 (m, 1H), 3.56-3.61 (m, 1H), 3.50-3.52 (m, 2H), 3.36-3.42 (m, 1H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 172.0, 162.0 (d, $J_{\text{C,F}} = 242.4$ Hz), 140.4 (d, $J_{\text{C,F}} = 2.9$ Hz), 130.2 (d, $J_{\text{C,F}} = 9.5$ Hz), 115.4 (d, $J_{\text{C,F}} = 8.2$ Hz), 109.8 (d, $J_{\text{C,F}} = 20.6$ Hz), 106.3 (d, $J_{\text{C,F}} = 26.1$ Hz), 74.2, 72.2, 71.5, 70.3, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{15}\text{FNO}_6$ $[\text{M}-\text{H}]^-$ 288.1, found 288.1.

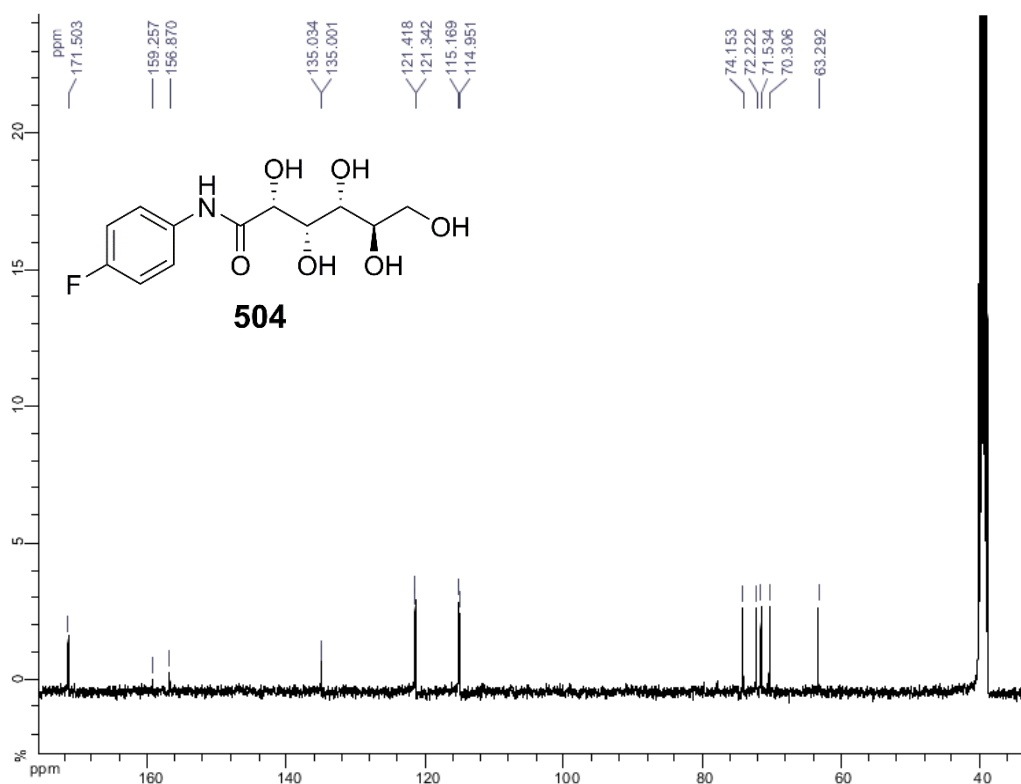




***N*-4-Fluorophenyl-D-gluconamide (504)**

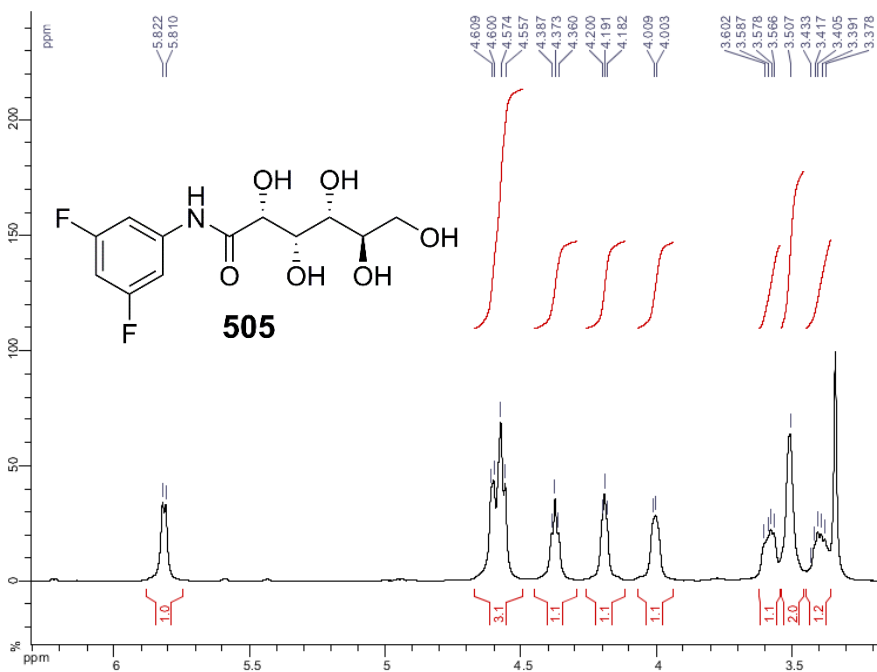
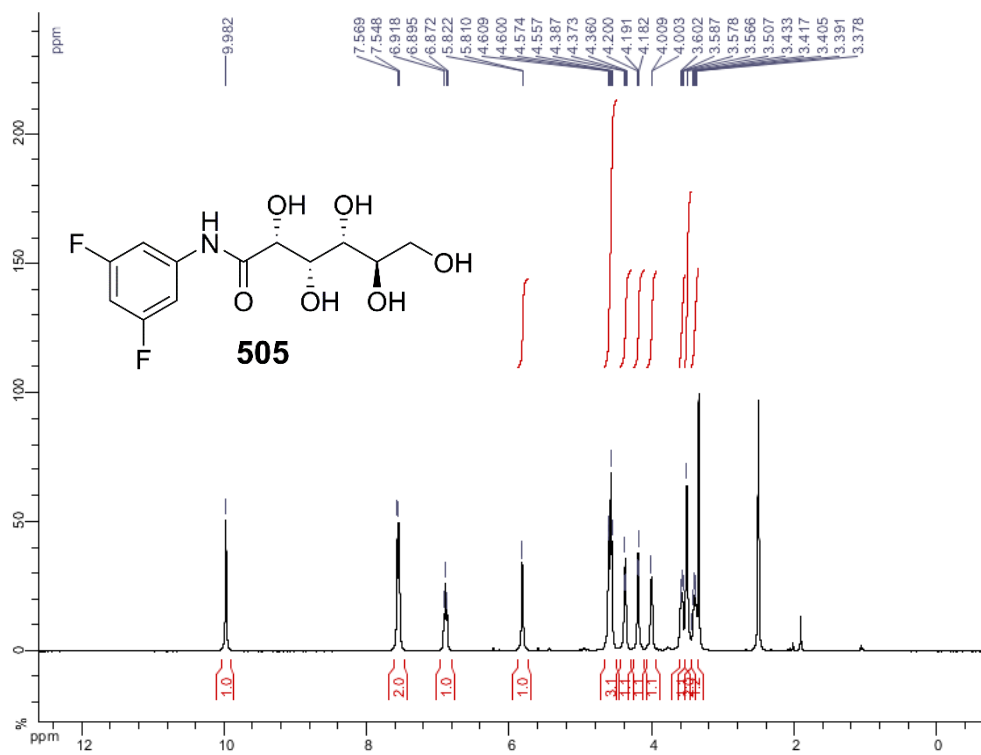
To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added 4-fluoroaniline (0.11 mL, 1.12 mmol). The mixture was stirred under reflux for 48 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **504** as a white powder (30%). ^1H NMR (400 MHz, DMSO- d_6): δ 8.80 (s, 1H), 6.90 (q, J = 5.5 Hz, 2H), 6.30 (t, J = 9.2 Hz, 2H), 4.86 (d, J = 5.0 Hz, 1H), 3.76 (d, J = 5.4, 1H), 3.69-3.72 (m, 2H), 3.53 (t, J = 6.0 Hz, 1H), 3.33 (t, J = 5.0 Hz, 1H), 3.16-3.19 (m, 1H), 2.73-2.77 (m, 1H), 2.67-2.69 (m, 2H), 2.53-2.58 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 171.5, 158.1 (d, $J_{\text{C,F}}$ = 239.9 Hz), 135.0 (d, $J_{\text{C,F}}$ = 2.2 Hz), 121.4 (d, $J_{\text{C,F}}$ = 7.7 Hz), 115.1 (d, $J_{\text{C,F}}$ = 22.0 Hz), 74.2, 72.2, 71.5, 70.3, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{16}\text{FNNaO}_6$ $[\text{M}+\text{Na}]^+$ 312.3; found 312.2.

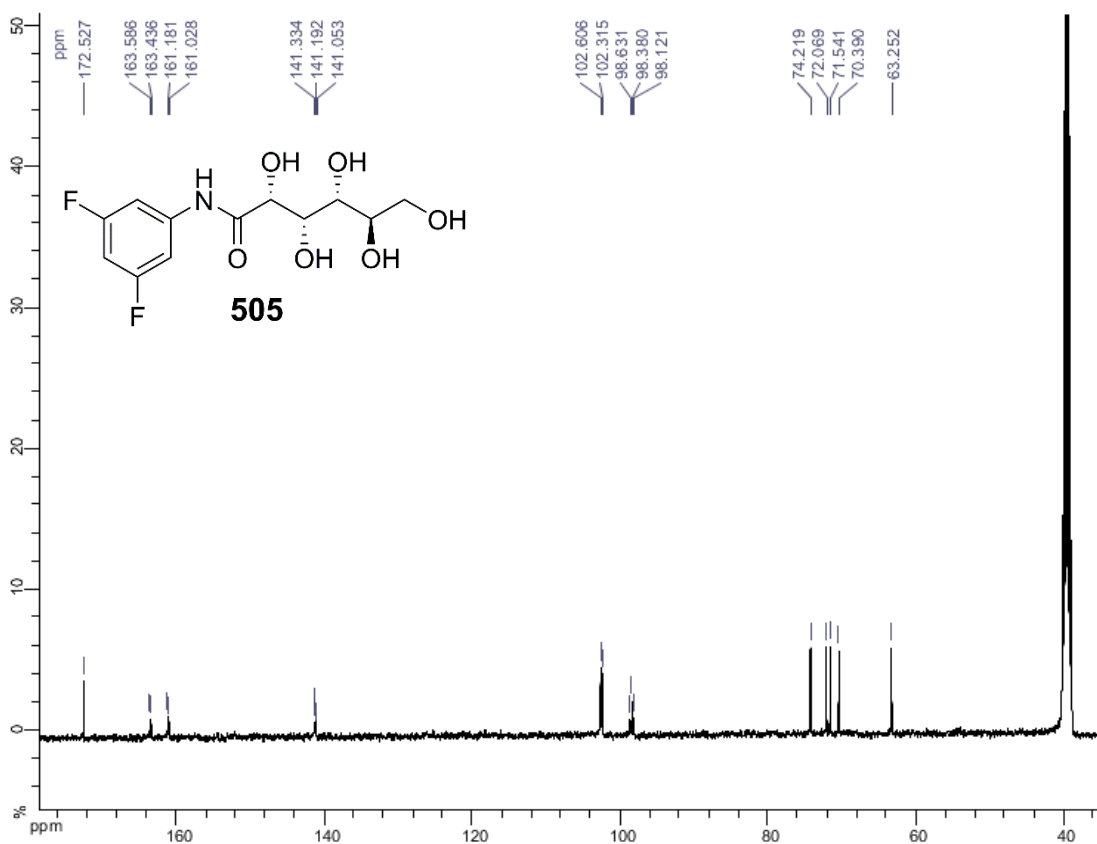




N-3,5-Difluorophenyl-D-gluconamide (**505**)

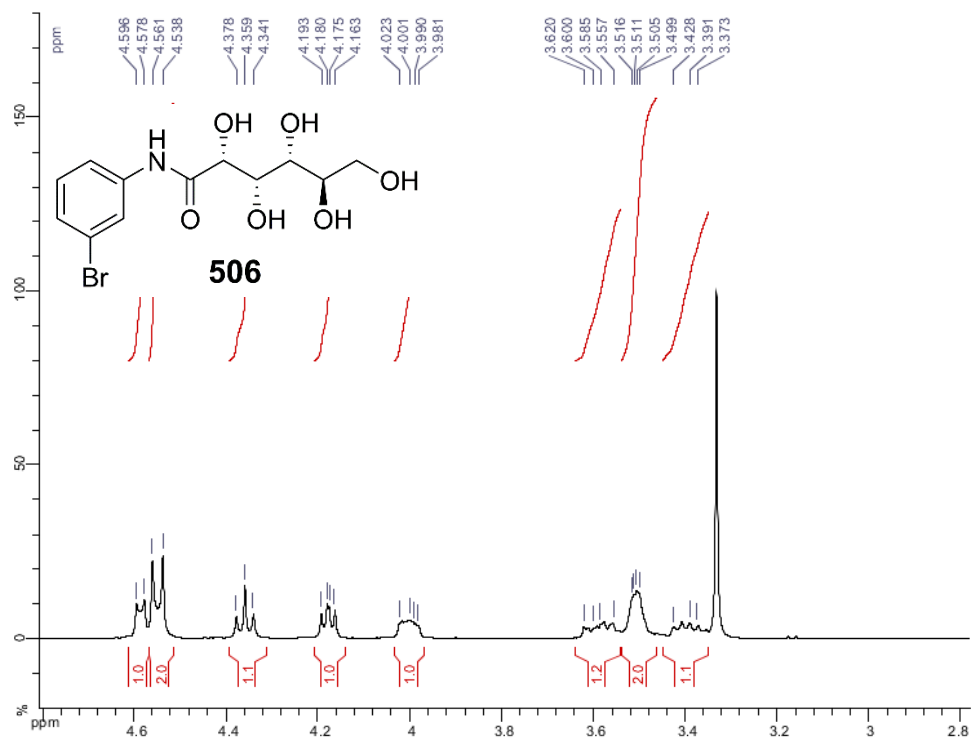
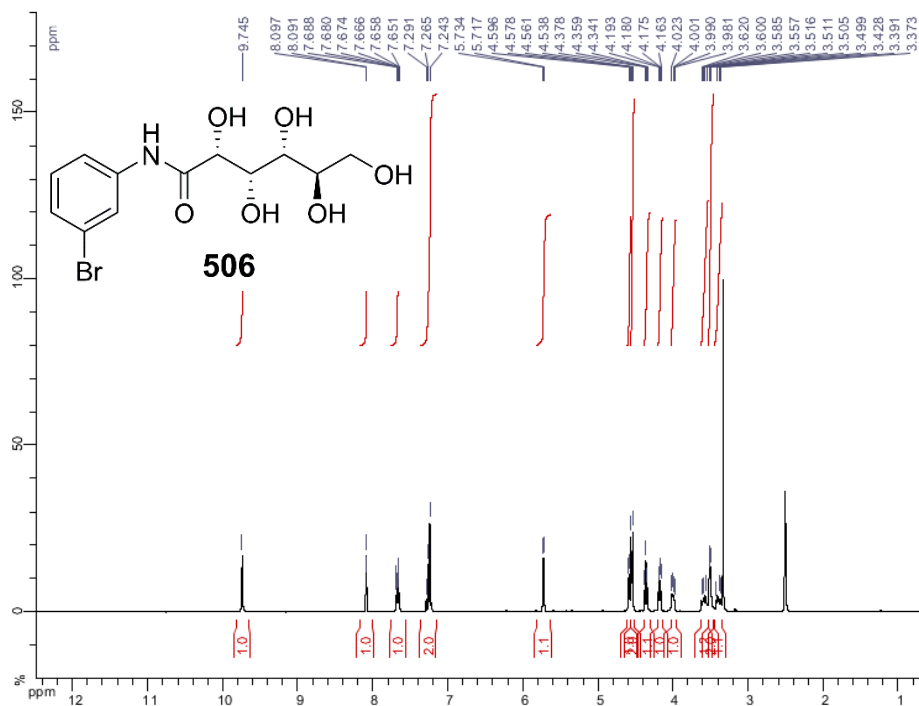
To a solution of D-gluconic acid- δ -lactone (0.23 g, 1.29 mmol) in acetic acid (5 mL) was added 3,5-difluoroaniline (0.5 g, 3.87 mmol). The mixture was stirred under reflux for 1 hour. The crude product was precipitated with hexanes and filtered to obtain a dark brown sludge. The solid was recrystallized in EtOH to afford **505** as a white powder (22%). ^1H NMR (400 MHz, DMSO- d_6): δ 9.98 (s, 1H), 7.55 (d, J = 9.5 Hz, 2H), 6.90 (t, J = 9.5 Hz, 1H), 5.82 (d, J = 4.6 Hz, 1H), 4.61–4.56 (m, 3H), 4.37 (t, J = 5.6 Hz, 1H), 4.19 (t, J = 3.8 Hz, 1H), 4.02–4.00 (m, 1H), 3.60–3.57 (m, 1H), 3.51 (br. s, 2H), 3.43–3.38 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 172.5, 162.5 (d, $J_{\text{C,F}}$ = 245.0 Hz), 162.0 (d, $J_{\text{C,F}}$ = 242.8 Hz), 141.2 (t, $J_{\text{C,F}}$ = 14.2 Hz), 102.51 (dd, $J_{\text{C,F}}$ = 21.6 Hz, 8.3 Hz), 98.4 (t, $J_{\text{C,F}}$ = 26.4), 74.2, 72.1, 71.5, 70.4, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{14}\text{F}_2\text{NO}_6$ [$\text{M}-\text{H}$] $^-$ 306.1, found 306.1.

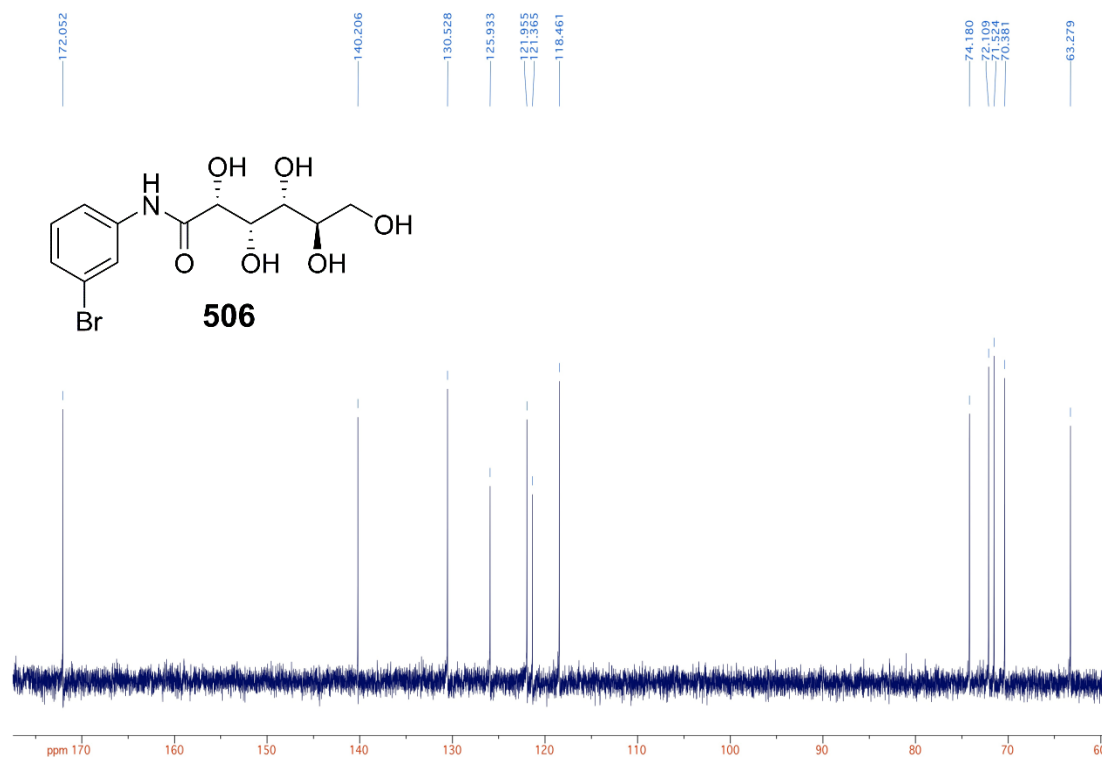




N-3-Bromophenyl-D-gluconamide (**506**)

To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (15 mL) was added 3-bromoaniline (0.15 mL, 1.12 mmol). The mixture was stirred under reflux for 48 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **506** as white crystals (60%). ^1H NMR (300 MHz, DMSO- d_6): δ 9.75 (s, 1H), 8.09 (d, J = 1.9 Hz, 1H), 7.65-7.69 (m, 1H), 7.24-7.29 (m, 2H), 5.73 (d, J = 5.1 Hz, 1H), 4.59 (d, J = 5.2 Hz, 1H), 4.55 (d, J = 6.8 Hz, 2H), 4.36 (t, J = 5.5 Hz, 1H), 4.18 (dd, J = 5.0 Hz, 3.7 Hz, 1H), 3.98-4.02 (m, 1H), 3.56-3.62 (m, 1H), 3.50-3.52 (m, 2H), 3.37-3.42 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 172.1, 140.2, 130.5, 125.9, 122.0, 121.4, 118.5, 74.2, 72.1, 71.5, 70.4, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{16}\text{BrKNO}_6$ $[\text{M}+\text{K}]^+$ 390.0, found 390.0.

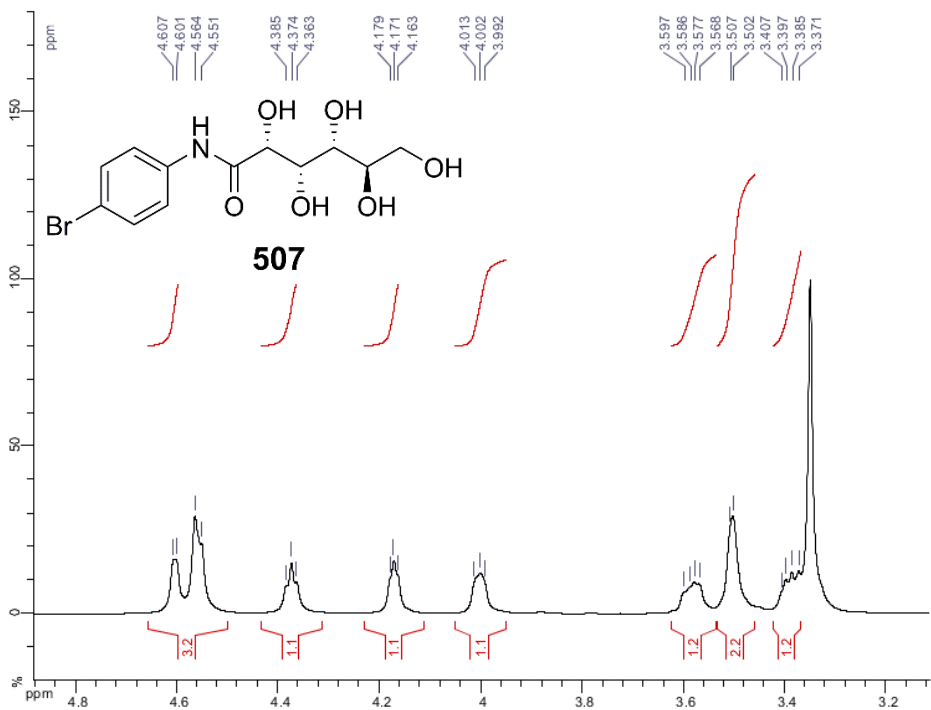
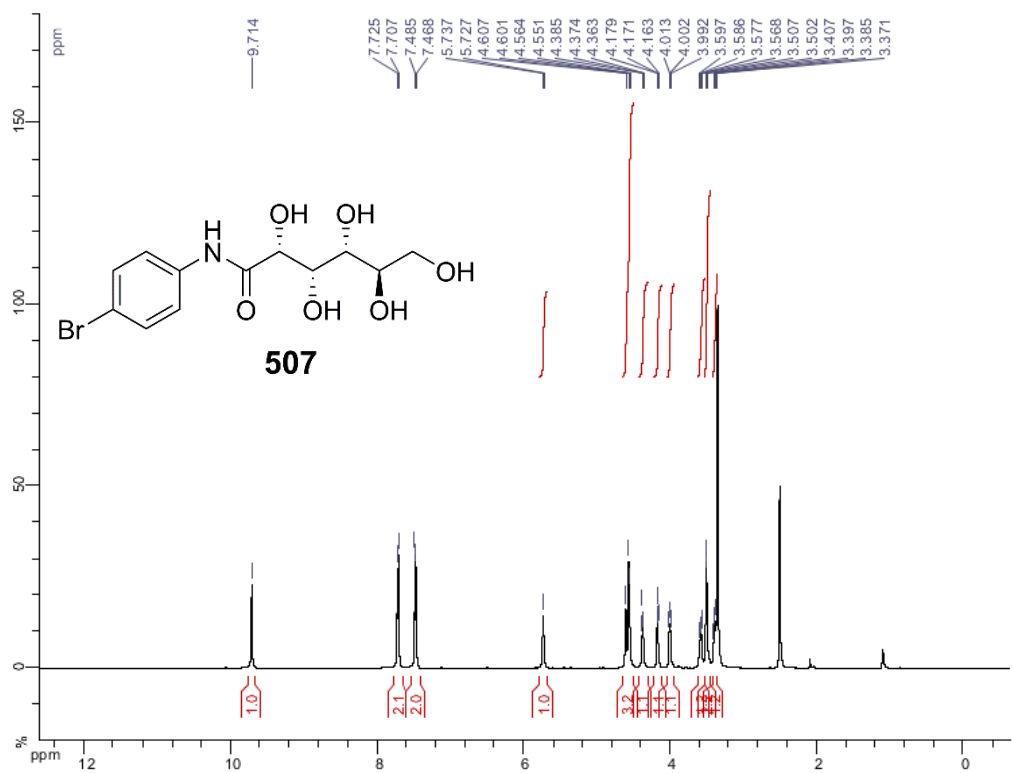


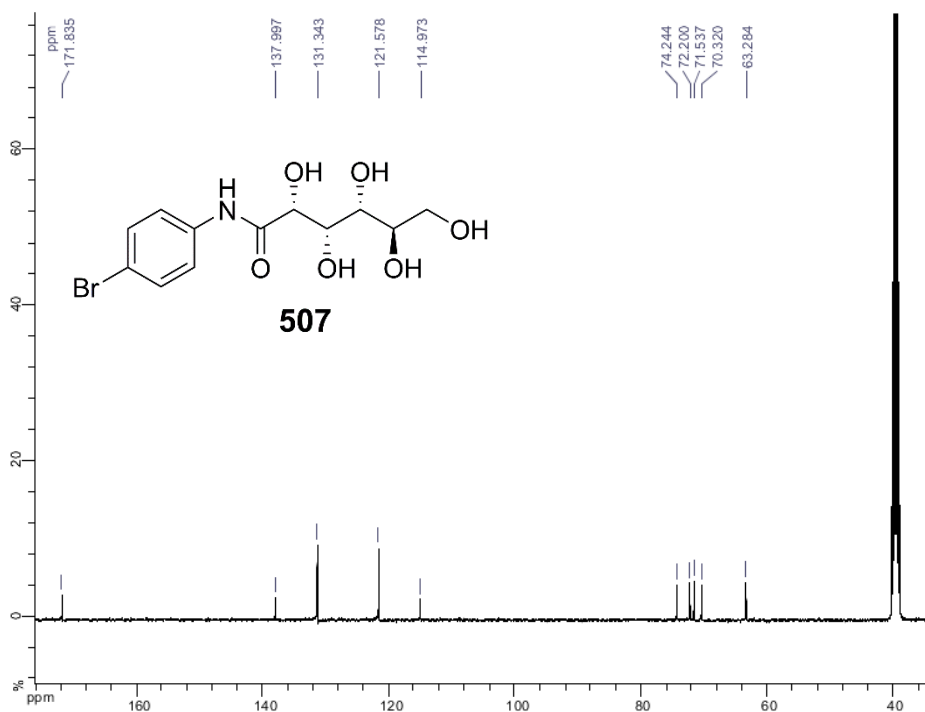


N-4-Bromophenyl-D-gluconamide (**507**)

To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added 4-bromoaniline (0.15 mL, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **507** as a white powder (22%).

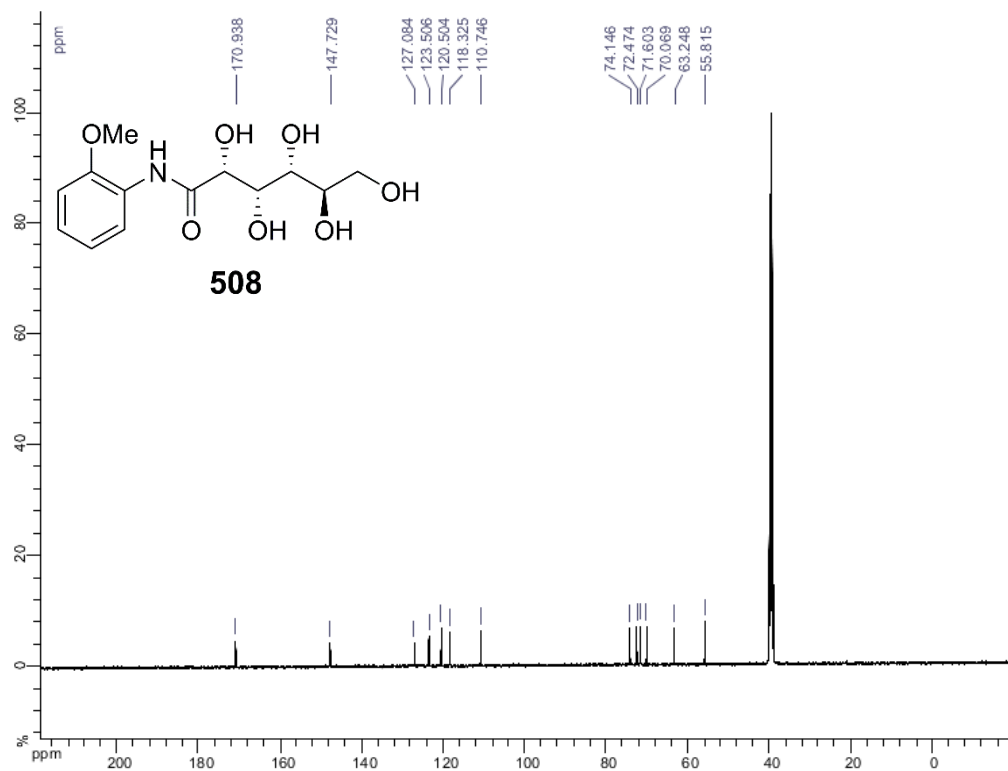
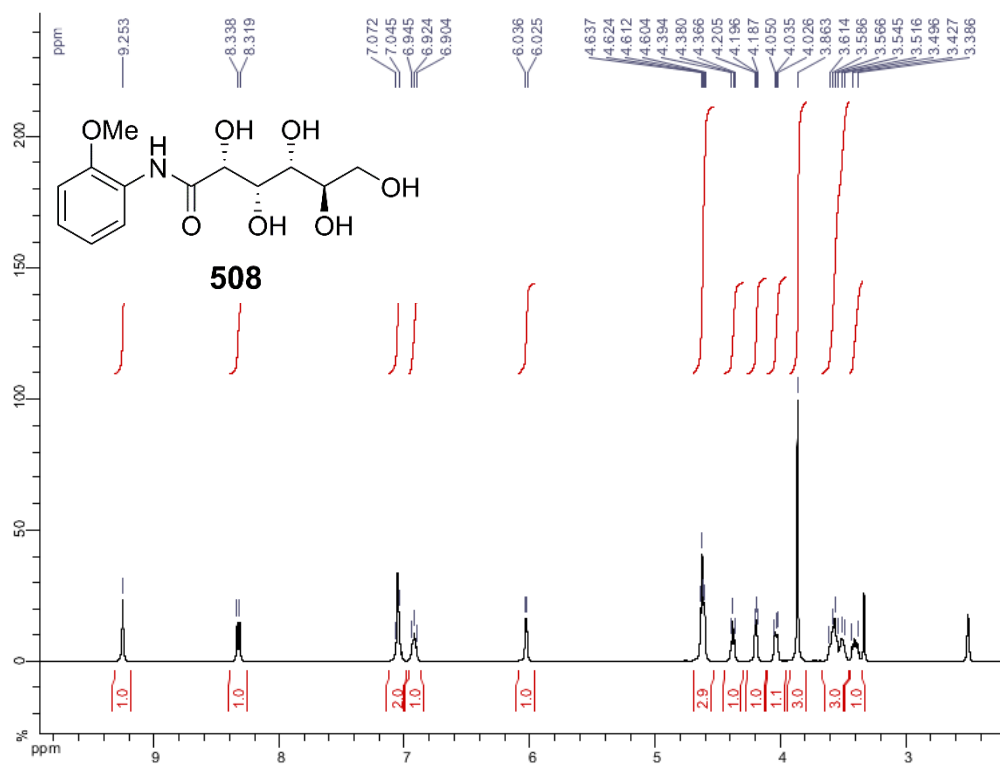
Characterization data is consistent with that previously published.³⁵ ¹H NMR (500 MHz, DMSO-*d*₆): δ 9.71 (s, 1H), 7.72 (d, *J* = 8.7 Hz, 2H), 7.48 (d, *J* = 8.7 Hz, 2H), 5.74 (d, *J* = 5.0 Hz, 1H), 4.61 (br. s, 1H), 4.56 (d, *J* = 7.1 Hz, 2H), 4.37 (t, *J* = 5.6 Hz, 1H), 4.17 (t, *J* = 4.2 Hz, 1H), 4.00 (br. s, 1H), 3.58 (m, 1H), 3.50 (br. s, 1H), 3.39 (m, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 171.8, 138.0, 131.3, 121.6, 115.0, 74.2, 72.2, 71.5, 70.3, 63.3. LRMS (ESI): *m/z* calcd. for C₂₇H₃₉N₂O₁₃Br₂ [2M + C₃H₇O]⁺ 759.4, found 759.1.





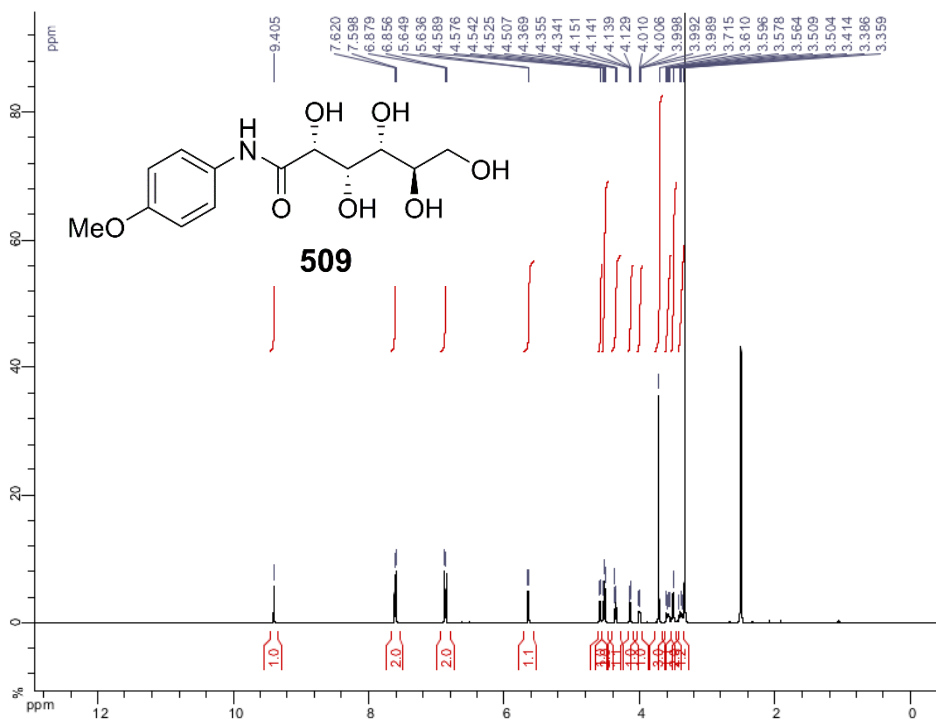
N-2-Methoxyphenyl-D-gluconamide (**508**)

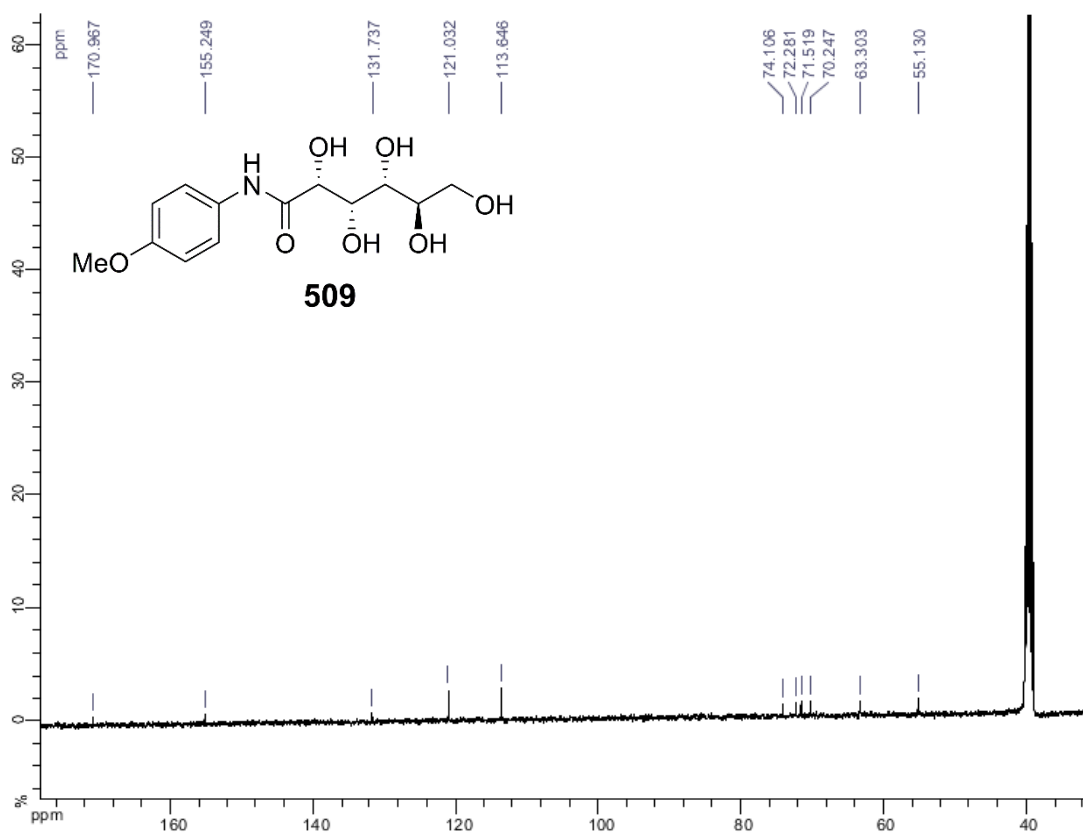
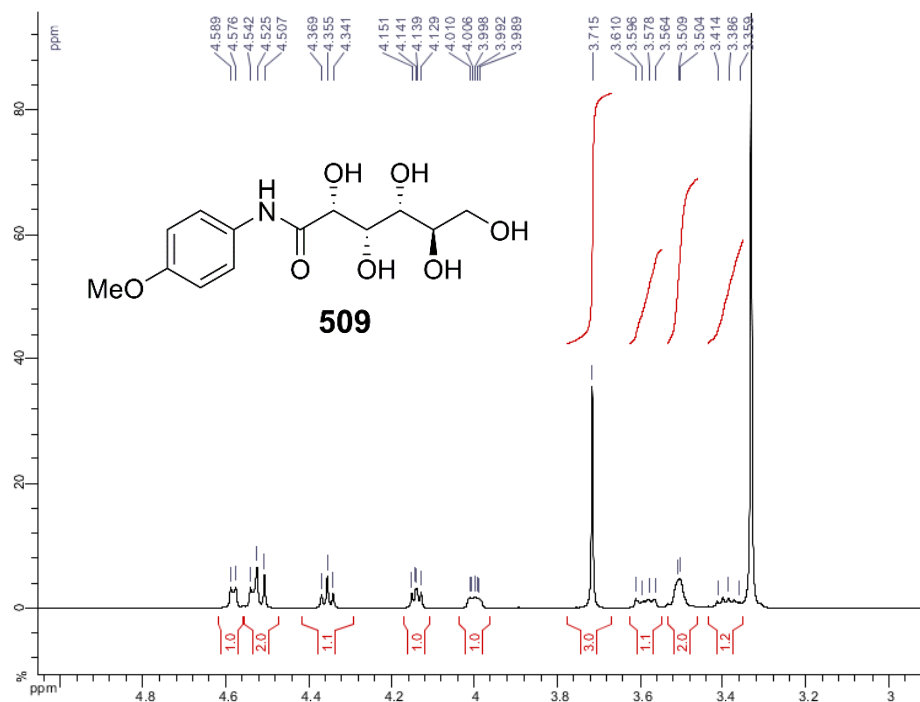
To a solution of D-gluconic acid- δ -lactone (0.10 g, 0.56 mmol) in MeOH (10 mL) was added *o*-anisidine (0.07 g, 0.56 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **508** as white crystals (65%). ^1H NMR (400 MHz; DMSO- d_6): δ 9.25 (s, 1H), 8.33 (d, J = 8.0 Hz, 1H), 7.07-7.05 (m, 2H), 6.95-6.90 (m, 1H), 6.03 (d, J = 4.6 Hz, 1H), 4.64-4.60 (m, 3H), 4.38 (t, J = 5.6 Hz, 1H), 4.20 (t, J = 3.4 Hz, 1H), 4.05-4.03 (m, 1H), 3.86 (s, 3H), 3.61-3.50 (m, 3H), 3.43-3.39 (m, 1H). ^{13}C NMR (100 MHz; DMSO- d_6): δ 170.9, 147.7, 127.1, 123.5, 120.5, 118.1, 110.8, 74.2, 72.5, 71.6, 70.1, 63.3, 55.8. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{19}\text{NNaO}_7$ $[\text{M}+\text{Na}]^+$ 324.1, found 324.0.



***N*-4-Methoxyphenyl-D-gluconamide (509)**

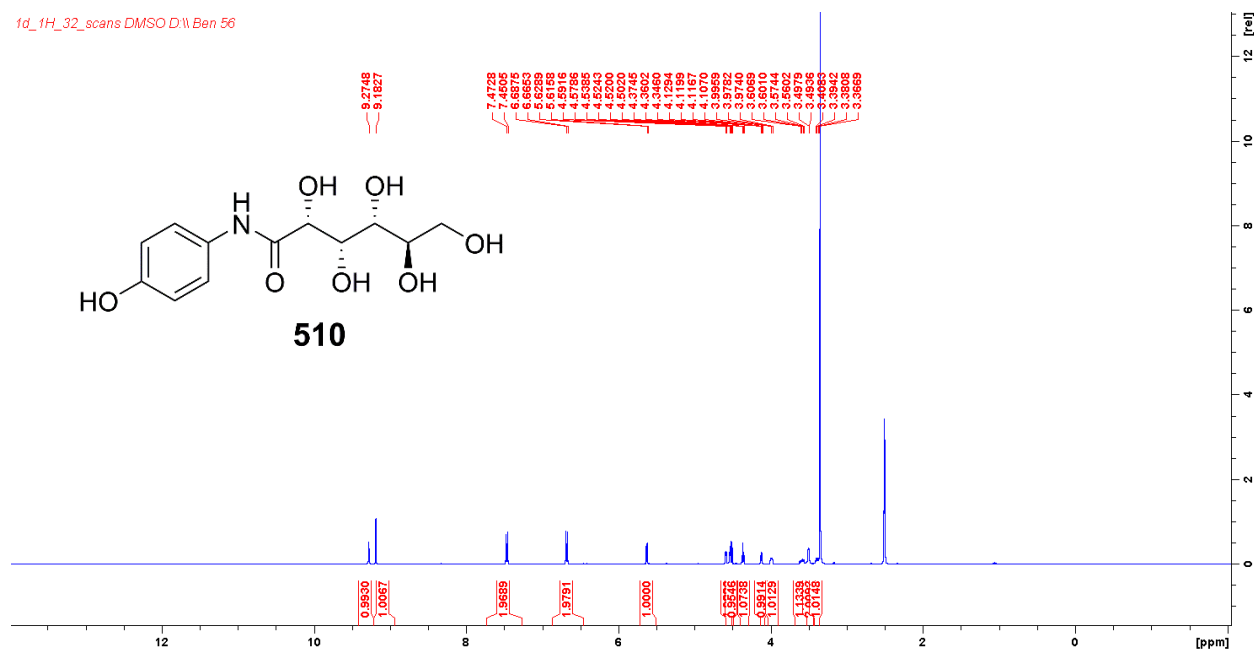
To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (15 mL) was added *p*-anisidine (0.14 g, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **509** as white crystals (21%). Characterization data is consistent with that previously published.³⁵ ¹H NMR (400 MHz, DMSO-*d*₆): δ 9.41 (s, 1H), 7.61 (d, *J* = 9.3 Hz, 2H), 6.86 (d, *J* = 9.0 Hz, 2H), 5.65 (d, *J* = 5.3 Hz, 1H), 4.58 (d, *J* = 5.1 Hz, 1H), 4.53-4.51 (m, 2H), 4.36 (t, *J* = 5.9 Hz, 1H), 4.14 (dd, *J* = 4.1 Hz, 5.2 Hz, 1H), 4.01-3.99 (m, 1H), 3.72 (s, 3H), 3.61-3.56 (m, 1H), 3.51-3.49 (m, 2H), 3.41-3.36 (m, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 171.0, 155.3, 131.7, 121.0, 113.7, 74.1, 72.3, 71.5, 70.3, 63.3, 55.1. LRMS (ESI): *m/z* calcd. for C₁₃H₁₈NO₇ [M-H]⁻ 300.1, found 300.1.

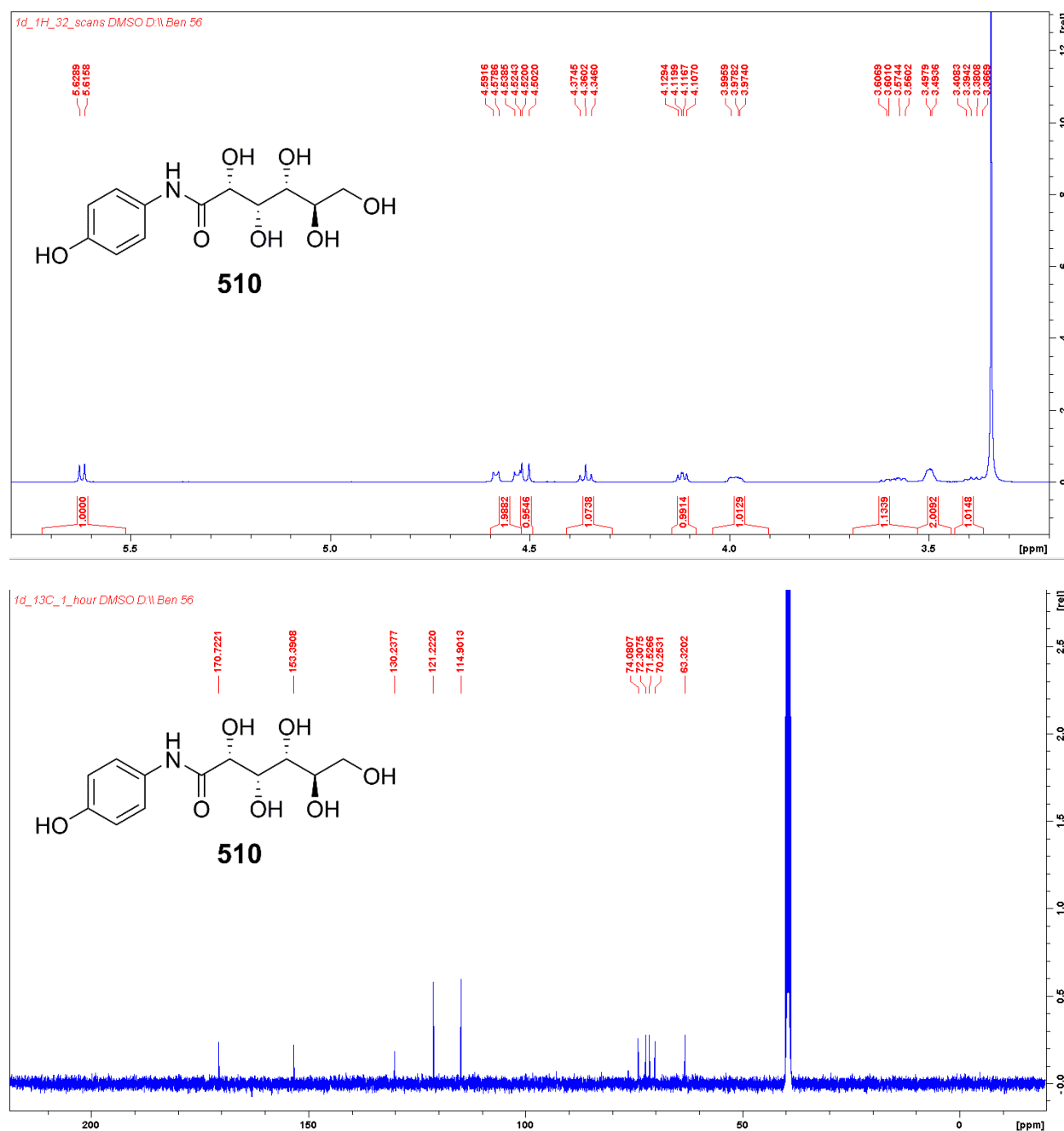




N-4-Hydroxyphenyl-D-gluconamide (**510**)

To a solution of D-gluconic acid- δ -lactone (0.1 g, 0.56 mmol) in methanol (10 mL) was added 4-aminophenol (60 mg, 0.56 mmol). The mixture was stirred under reflux for 24 hours. The crude product was precipitated with hexanes, filtered and crude solid was recrystallized in EtOH to afford **510** as white crystals (83 mg, 52%). ^1H NMR (400 MHz, DMSO- d_6): δ 9.27 (s, 1H), 9.18 (s, 1H), 7.46 (d, J = 9.0, 2H), 6.68 (d, J = 9.1 Hz, 2H), 5.63 (d, J = 5.3 Hz, 1H), 4.56 (dd, J = 21.3, 5.0 Hz, 2H), 4.51 (d, J = 7.1 Hz, 1H), 4.36 (t, J = 5.7 Hz, 1H), 4.1 (dd, J = 5.1, 3.8 Hz, 1H), 3.97-4.00 (m, 1H), 3.56-3.61 (m, 1H), 3.49-3.50 (m, 2H), 3.37-3.41 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 170.7, 153.4, 130.2, 121.2, 114.9, 74.1, 72.3, 71.5, 70.3, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{17}\text{NNaO}_7$ $[\text{M}+\text{Na}]^+$ 310.1, found 309.9.





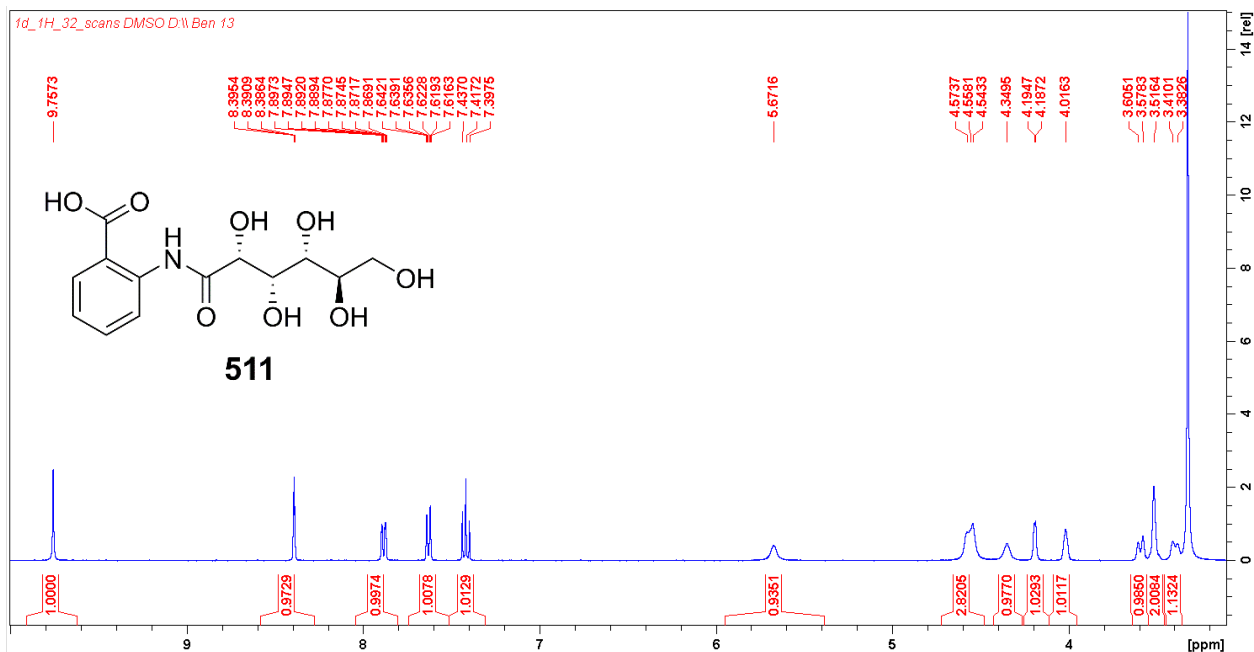
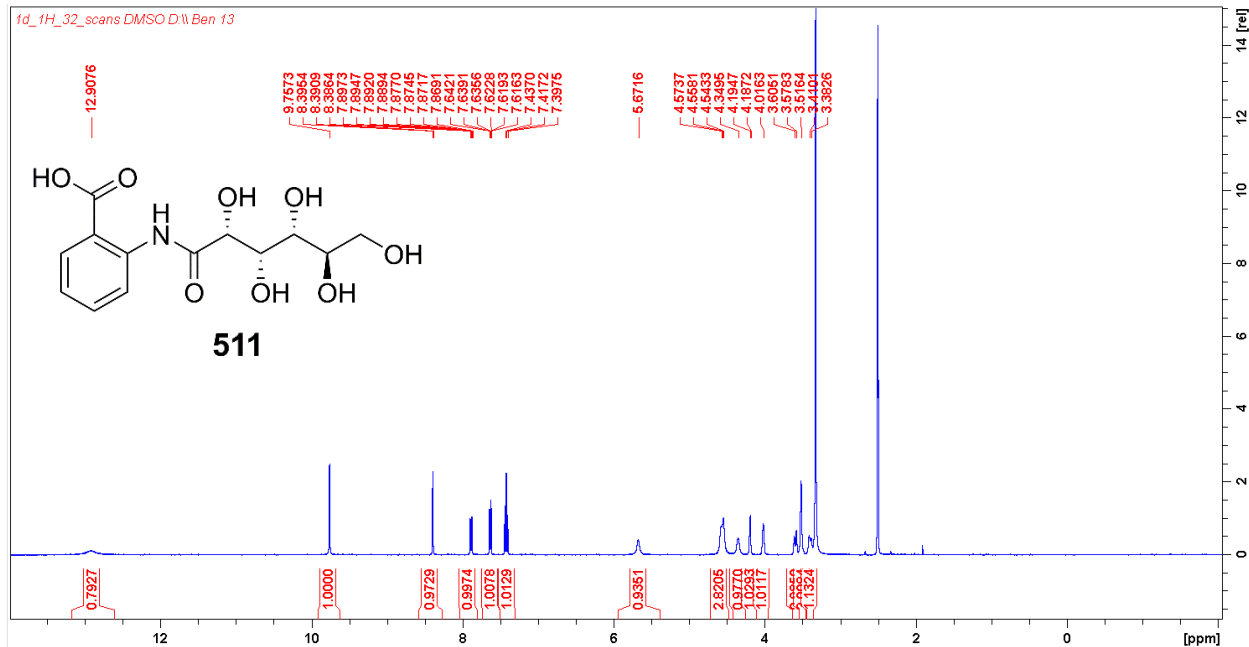
N-3-(Carboxy)phenyl-D-gluconamide (**511**)

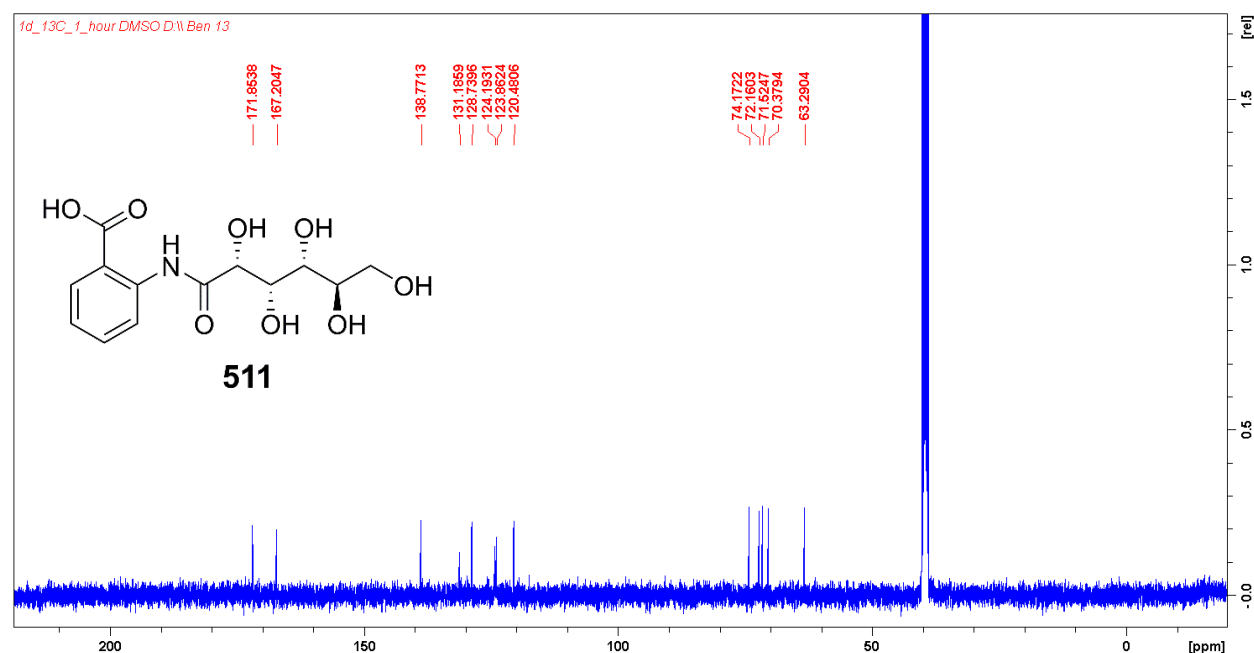
To a solution of D-gluconic acid- δ -lactone (0.50 g, 2.80 mmol) in acetic acid (10 mL) was added 3-aminobenzoic acid (0.77 g, 5.60 mmol). The mixture was stirred under reflux for 1 hour. The crude product was precipitated with hexanes, filtered and crude solid was recrystallized in EtOH to afford **511** as white crystals (640 mg, 73%). ¹H NMR (400 MHz, DMSO-*d*₆): δ 12.91 (br. s, 1H), 9.76 (s, 1H), 8.39 (d, *J* = 1.8 Hz, 1H), 7.87-7.90 (m, 1H), 7.62-7.64 (m, 1H), 7.42 (t, *J* = 8.1 Hz, 1H), 5.67 (s, 1H), 4.54-4.57 (m, 3H),

4.35 (m, 1H), 4.18-4.19 (m, 1H), 4.02 (m, 1H), 4.59 (d, $J = 10.9$ Hz, 1H), 3.52 (m, 2H), 3.38-3.41 (m, 1H).

^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 171.9, 167.2, 138.8, 131.2, 128.7, 124.2, 123.9, 120.5, 74.2, 72.2, 71.5,

70.4, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{17}\text{NNaO}_8$ $[\text{M}+\text{Na}]^+$ 338.1, found 337.9.



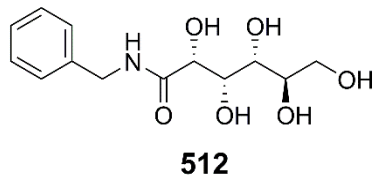
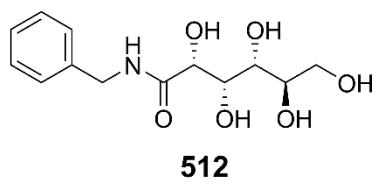


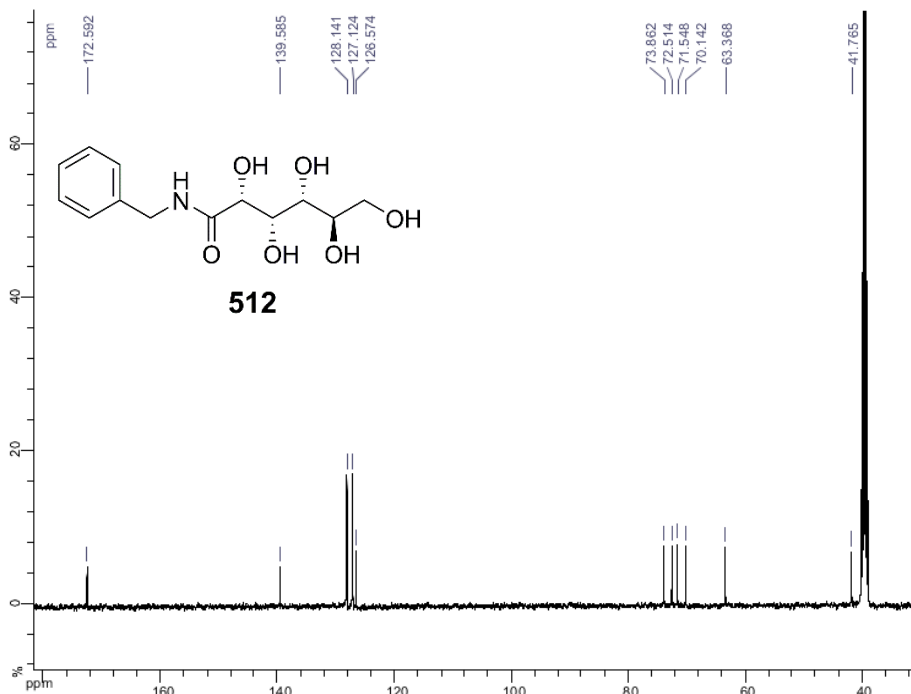
5.5.5 Characterization data and spectra of *N*-benzyl-gluconamides (512-521)

N-Benzyl-*D*-gluconamide (512)

To a solution of *D*-gluconic acid- δ -lactone (0.89 g, 5.02 mmol) in MeOH (30 mL) was added benzylamine (0.69 mL, 5.02 mmol). The mixture was stirred under reflux for 48 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **512** as white crystals (97%).

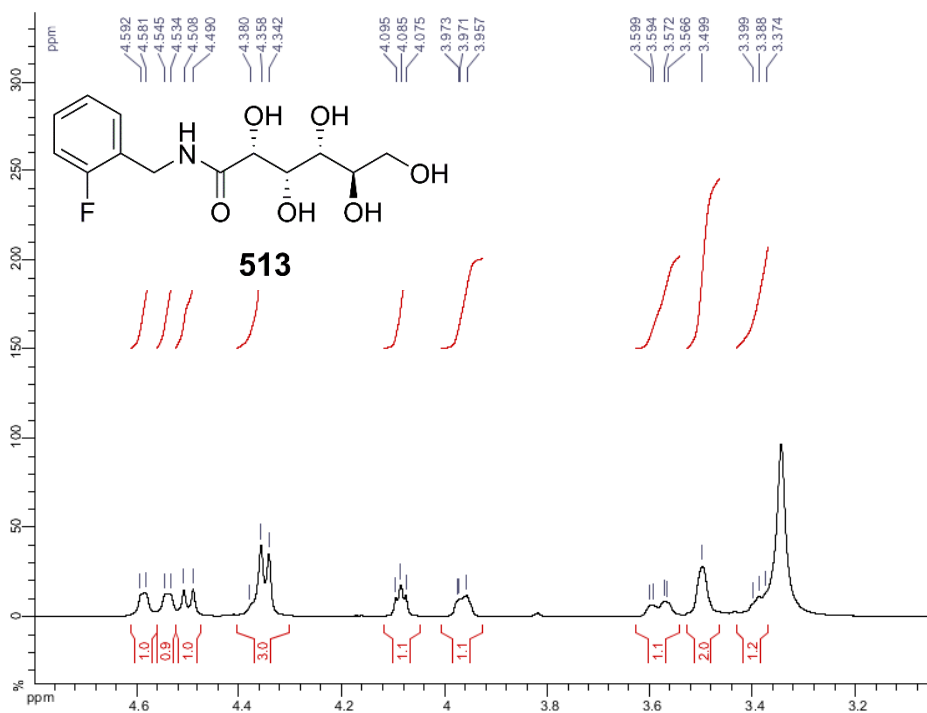
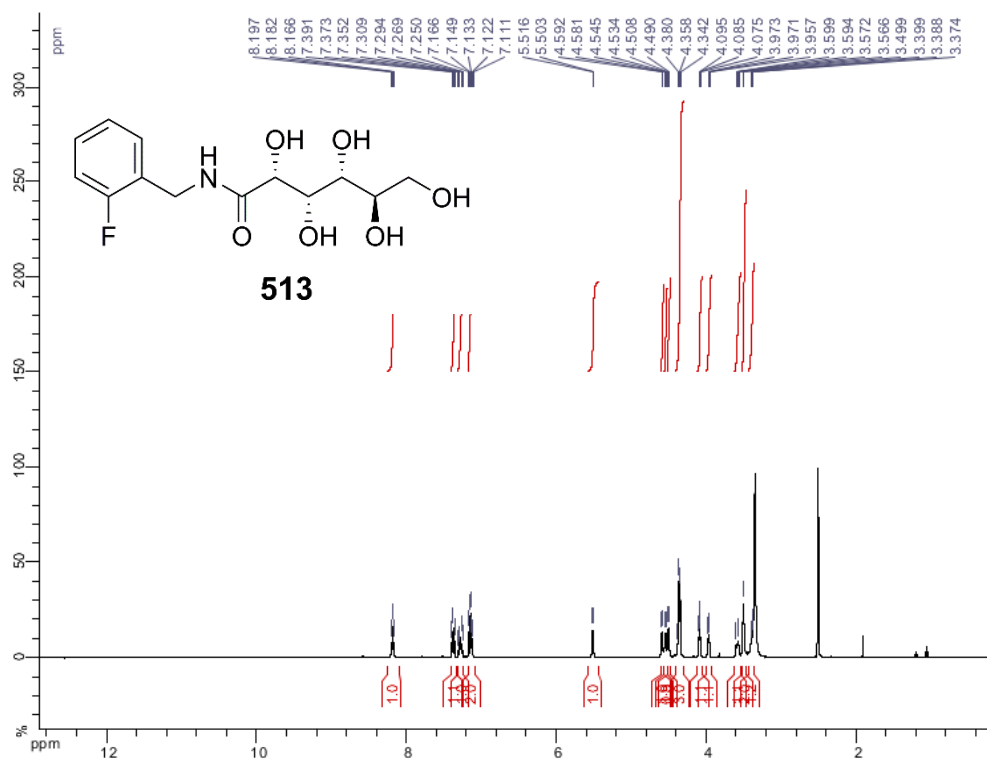
Characterization data is consistent with that of previously reported data.³⁴ ^1H NMR (400 MHz, DMSO- d_6): δ 8.17 (t, J = 6.4 Hz, 1H), 7.21-7.3 (m, 5H), 5.46 (d, J = 5.1 Hz, 1H), 4.58 (d, J = 4.8 Hz, 1H), 4.52 (d, J = 5.4, 1H), 4.47, (d, J = 7.7 Hz, 1H), 4.36 (t, J = 5.8 Hz, 1H), 4.31 (d, J = 6.5 Hz, 2H), 4.07 (t, J = 4.5 Hz, 1H), 3.96-3.98 (m, 1H), 3.56-3.58 (m, 1H), 3.51 (s, 2H), 3.36-3.39 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 172.6, 139.6, 128.1, 127.1, 126.6, 73.7, 72.5, 71.6, 70.1, 63.4, 41.8. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{20}\text{NO}_6$ $[\text{M}+\text{H}]^+$ 286.1, found 286.1.

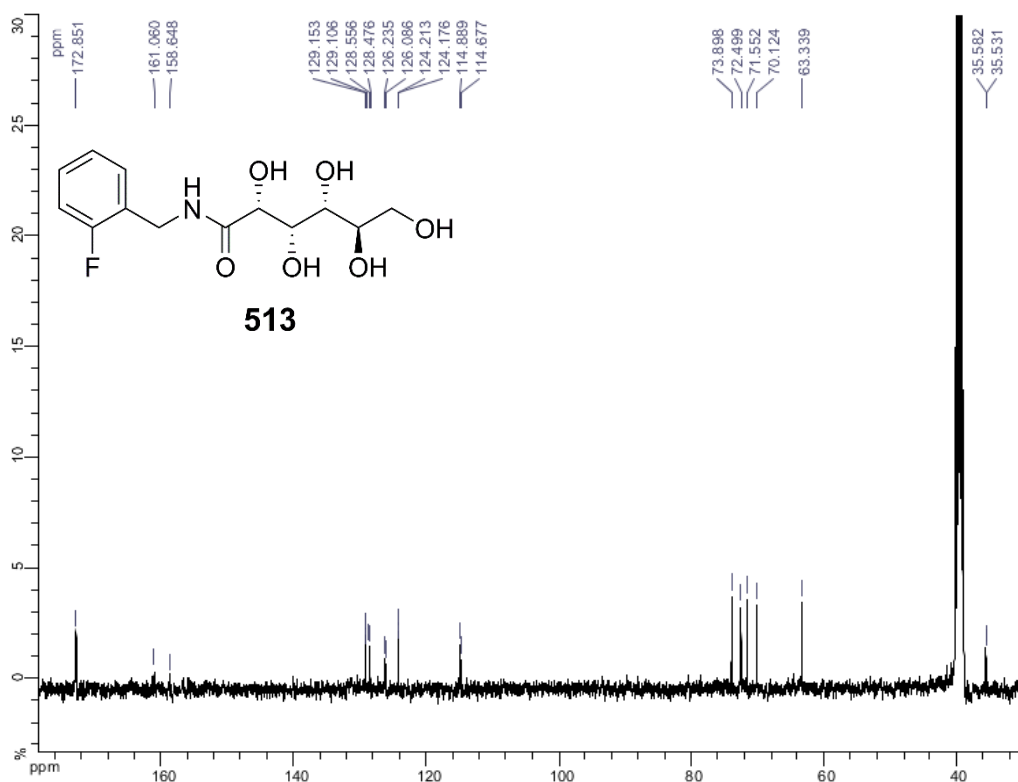




***N*-2-Fluorobenzyl-*D*-gluconamide (513)**

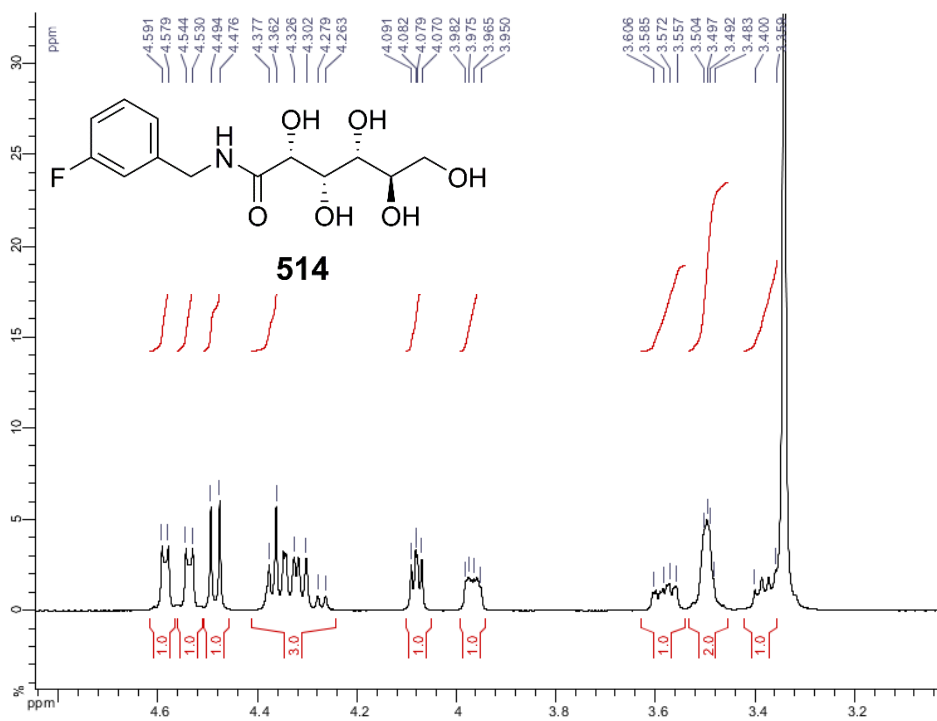
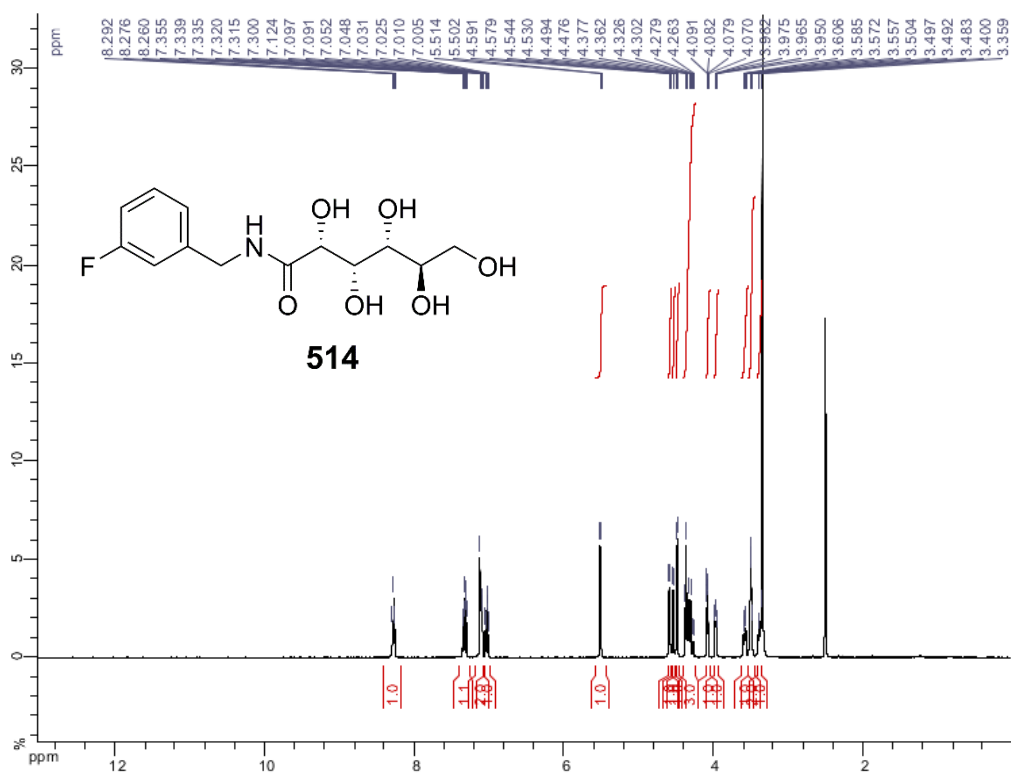
To a solution of *D*-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added 2-fluorobenzylamine (0.13 mL, 1.12 mmol). The mixture was stirred under reflux for 48 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **513** as white crystals (7%). ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 7.34 (t, J = 6.1 Hz, 1H), 6.54 (t, J = 7.0 Hz, 1H), 7.29-7.25 (m, 1H), 7.17-7.11 (m, 2H), 5.51 (d, J = 5.3 Hz, 1H), 4.59 (d, J = 4.3 Hz, 1H), 4.54 (d, J = 4.6 Hz, 1H), 4.50 (d, J = 7.3 Hz, 1H), 4.38-4.34 (m, 3H), 4.09 (t, J = 4.3 Hz, 1H), 3.97-3.96 (m, 1H), 3.60-3.57 (m, 1H), 3.45 (s, 2H), 3.40-3.37 (m, 1H). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ 172.9, 159.9 (d, $J_{\text{C,F}}$ = 244.0 Hz), 129.1 (d, $J_{\text{C,F}}$ = 4.8 Hz), 128.5 (d, $J_{\text{C,F}}$ = 8.2 Hz), 126.2 (d, $J_{\text{C,F}}$ = 15.1 Hz), 124.2 (d, $J_{\text{C,F}}$ = 3.3 Hz), 114.8 (d, $J_{\text{C,F}}$ = 21.4 Hz), 73.9, 72.5, 71.6, 70.1, 63.3, 35.6 (d, $J_{\text{C,F}}$ = 5.1 Hz). LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{19}\text{FNO}_6$ $[\text{M}+\text{H}]^+$ 304.3; found 304.2.

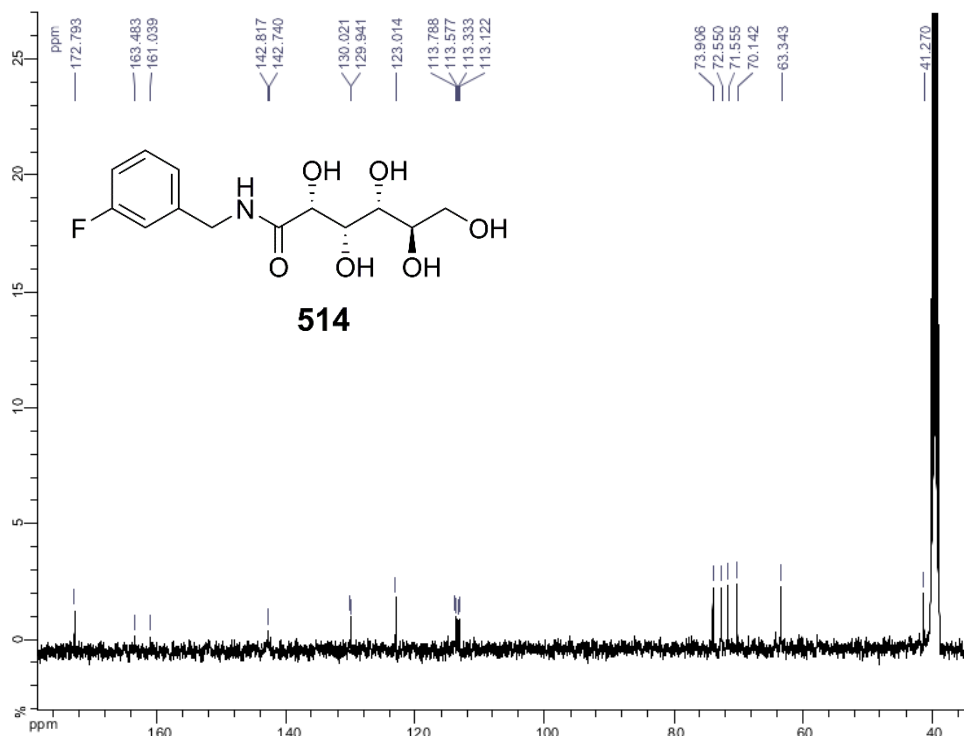




N-3-Fluorobenzyl-*D*-gluconamide (**514**)

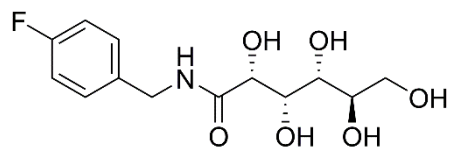
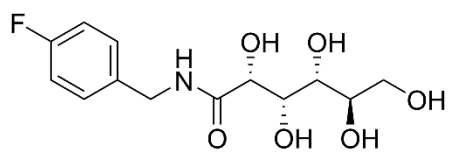
To a solution of *D*-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added 3-fluorobenzylamine (0.13 mL, 1.12 mmol). The mixture was stirred under reflux for 48 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **514** as white crystals (62%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 7.35 (t, J = 6.68 Hz, 1H), 6.48 (q, J = 8.0 Hz, 1H), 6.27 (m, 2H), 6.19 (m, 1H), 4.72 (d, J = 5.2 Hz, 1H), 3.74, (d, J = 5.2 Hz, 1H), 3.69 (d, J = 5.6 Hz, 1H), 3.64 (d, J = 7.3 Hz, 1H), 3.42-3.54 (m, 3H), 3.24 (t, J = 3.8 Hz, 1H), 3.12-3.14 (m, 1H), 2.72-2.76 (m, 1H), 2.66 (t, J = 2.7 Hz, 2H), 2.53-2.56 (m, 1H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 172.8, 162.3 (d, $J_{\text{C,F}}$ = 246.2 Hz), 142.8 (d, $J_{\text{C,F}}$ = 7.4 Hz), 130.0 (d, $J_{\text{C,F}}$ = 8.1 Hz), 123.0 (d, $J_{\text{C,F}}$ = 2.3 Hz), 113.7 (d, $J_{\text{C,F}}$ = 21.3 Hz), 113.2 (d, $J_{\text{C,F}}$ = 21.4 Hz), 73.9, 72.6, 71.6, 70.1, 63.3, 41.3. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{18}\text{FNNaO}_6$ $[\text{M}+\text{Na}]^+$ 326.3; found 326.2.

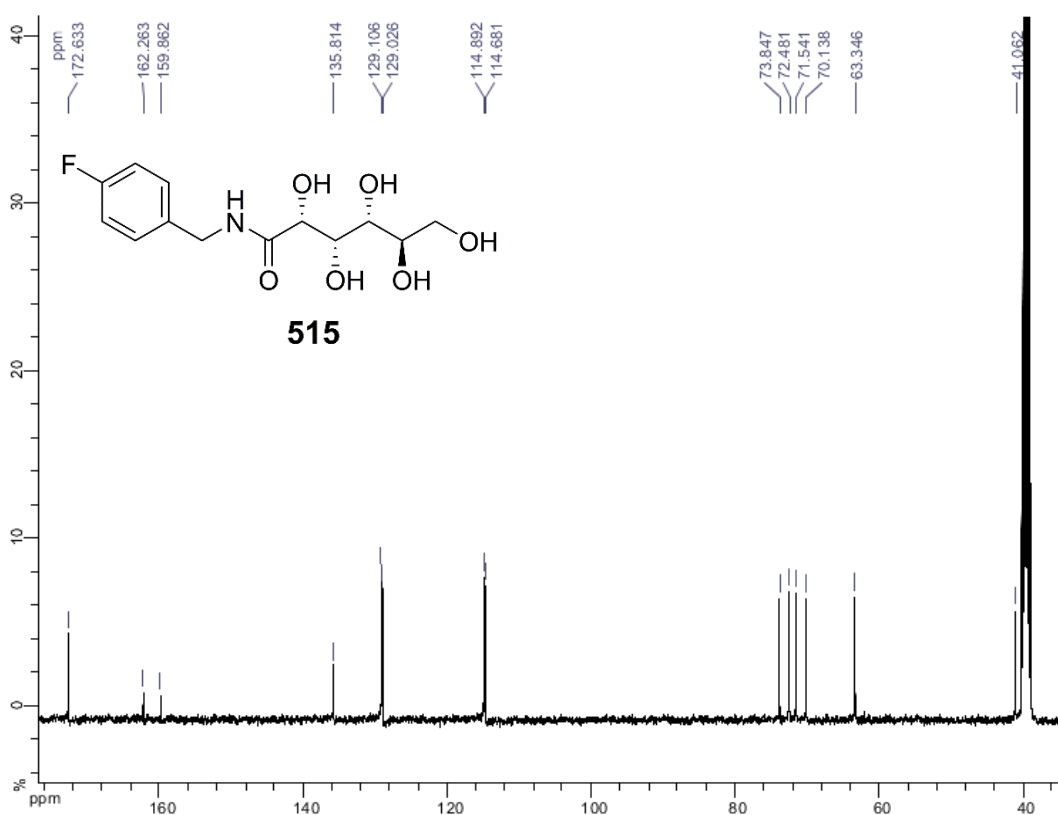




N-4-Fluorobenzyl-D-glucosamine (515)

To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added 4-fluorobenzylamine (0.13 mL, 1.12 mmol). The mixture was stirred under reflux for 48 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **515** as white crystals (62%). ^1H NMR (400 MHz, DMSO- d_6): δ 8.22 (t, J = 6.5 Hz, 1H), 7.31 (t, J = 7.1 Hz, 2H), 7.11, (t, J = 9.6 Hz, 2H), 5.46 (d, J = 5.0 Hz, 1H), 4.57 (d, J = 5.2 Hz, 1H), 4.52 (d, J = 5.6, 1H), 4.46 (d, J = 7.3, 1H), 4.36 (t, J = 5.6, 1H), 4.28 (d, J = 6.0, 2H), 4.06 (t, J = 4.7, 1H), 3.97-3.95 (m, 1H), 3.60-3.56 (m, 1H), 3.49 (t, J = 2.5, 2H), 3.40-3.36 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 172.6, 161.1 (d, $J_{\text{C,F}}$ = 241.7 Hz), 135.8 (d, $J_{\text{C,F}}$ = 2.5 Hz), 129.1 (d, $J_{\text{C,F}}$ = 8.1 Hz), 114.8 (d, $J_{\text{C,F}}$ = 21.4 Hz), 73.9, 72.5, 71.5, 70.1, 63.4, 41.1. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{19}\text{FNO}_6$ $[\text{M}+\text{H}]^+$ 304.3; found 304.2.





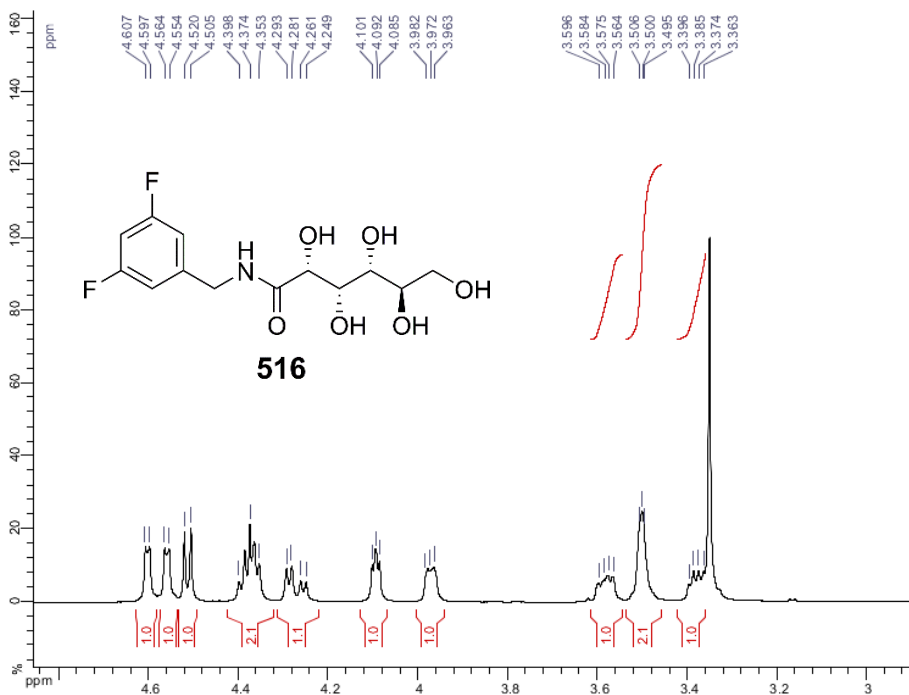
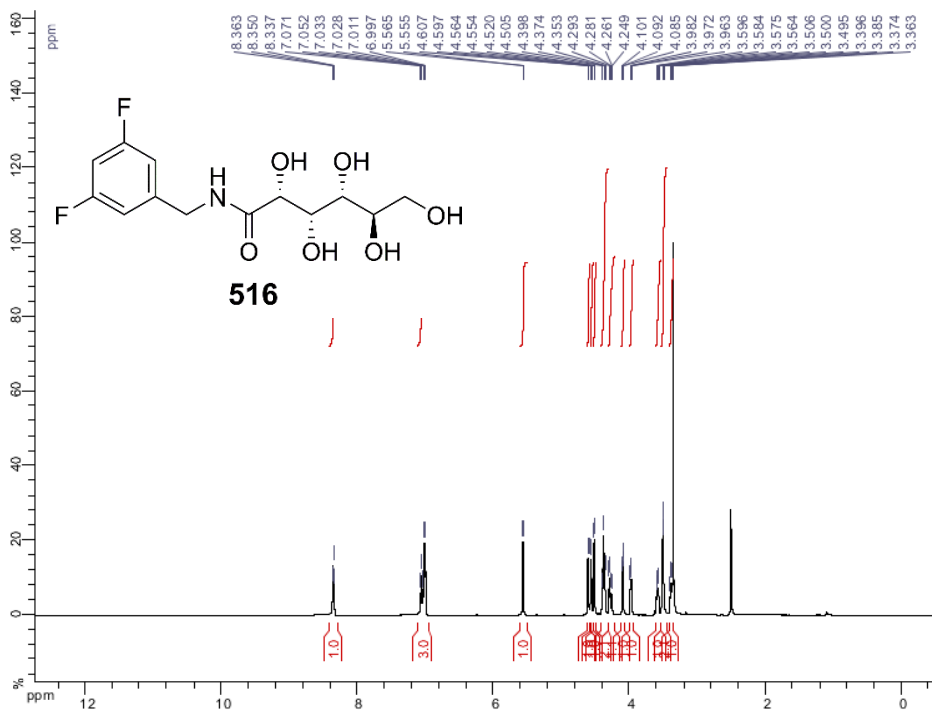
N-3,5-Difluorobenzyl-*D*-gluconamide (**516**)

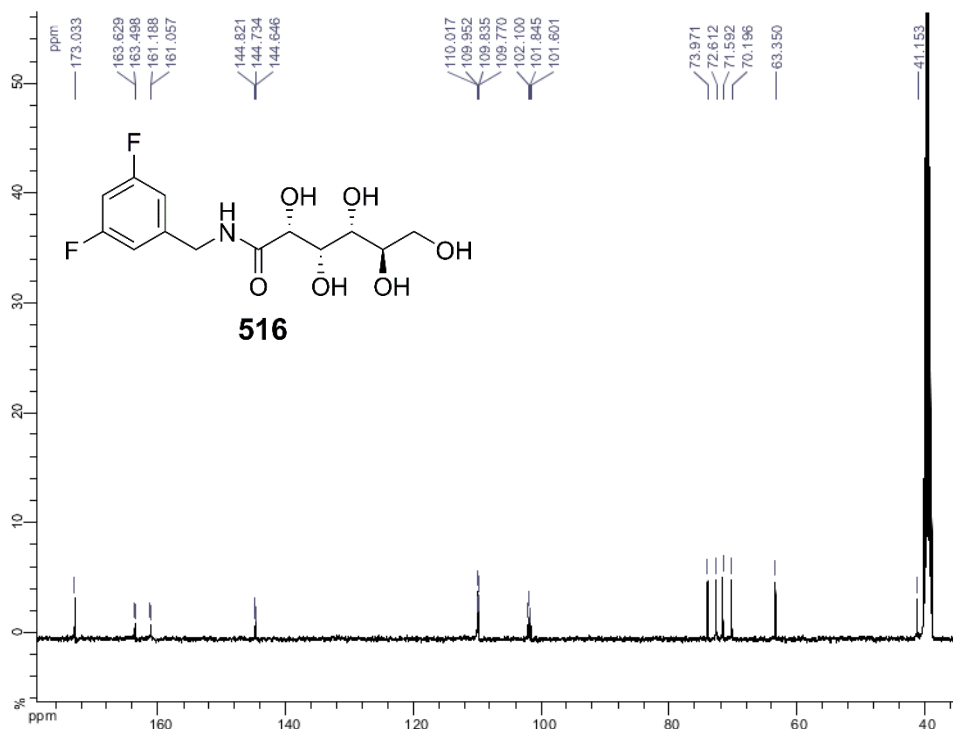
To a solution of *D*-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added 3,5-difluorobenzylamine (0.15 mL, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **516** as white crystals (0.31 g, 87%).

^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ 8.35 (t, $J = 6.3$ Hz, 1H), 6.70-7.07 (m, 3H), 5.56 (d, $J = 4.9$ Hz, 1H), 4.60 (d, $J = 4.5$ Hz, 1H), 4.56 (d, $J = 5.3$ Hz, 1H), 4.51 (d, $J = 7.3$ Hz, 1H), 4.35-4.40 (m, 2H), 4.25-4.30 (m, 1H), 4.09 (t, $J = 4.3$ Hz, 1H), 3.96-3.98 (m, 1H), 3.56-3.60 (m, 1H), 3.50 (s, 2H), 3.36-3.39 (m, 1H).

^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ 173.0, 162.4 (d, $J_{\text{C,F}} = 246.1$ Hz), 162.3 (d, $J_{\text{C,F}} = 245.7$ Hz), 144.7 (t, $J_{\text{C,F}} = 8.8$ Hz), 109.9 (dd, $J_{\text{C,F}} = 24.8, 6.4$ Hz), 101.9 (t, $J_{\text{C,F}} = 26.0$ Hz), 74.0, 72.6, 71.6, 70.2, 63.4, 41.2.

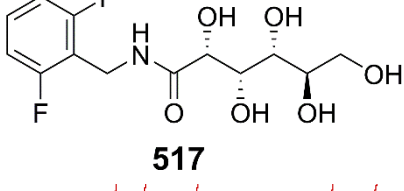
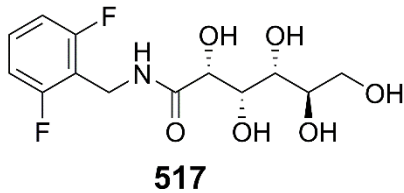
LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{17}\text{NO}_6\text{F}_2$ $[\text{M} + \text{H}]^+$ 322.1, found 322.2.

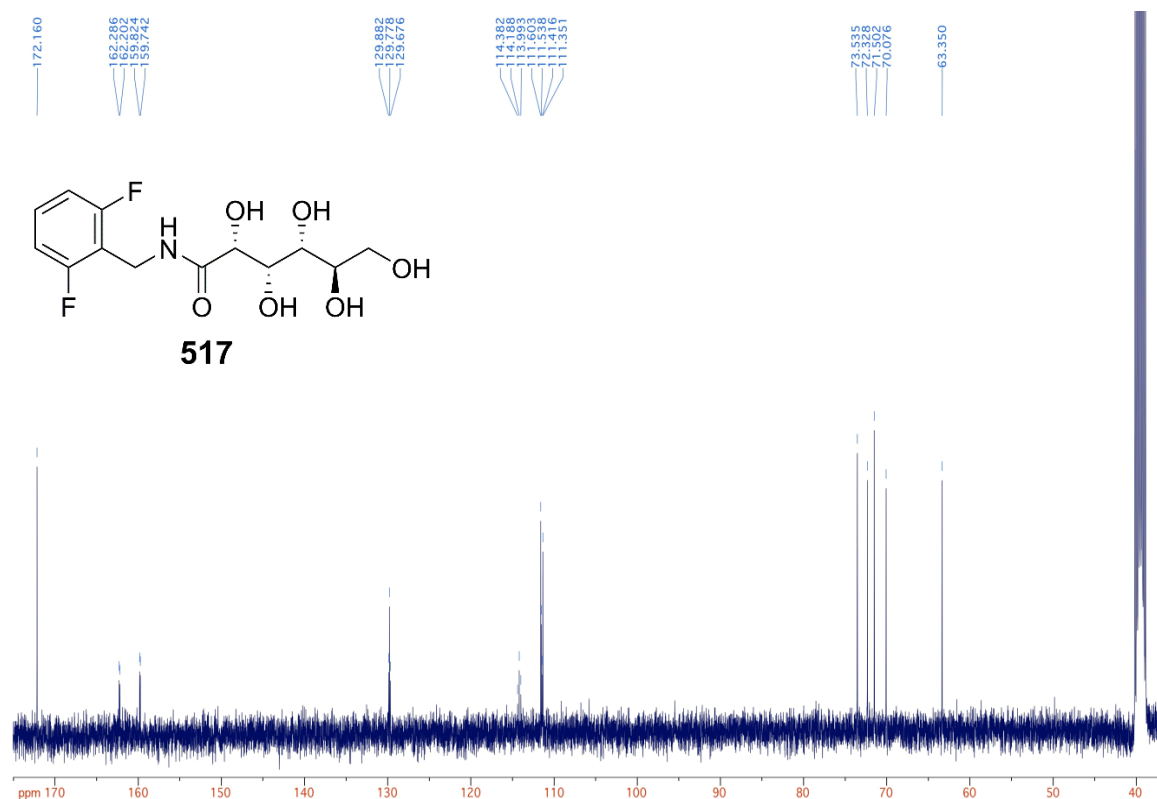




N-2,6-Difluorobenzyl-*D*-gluconamide (**517**)

To a solution of *D*-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (15 mL) was added 2,6-difluoroaniline (0.13 mL, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **517** as white crystals (89%). ¹H NMR (300 MHz, DMSO-*d*₆): δ 7.89 (t, *J* = 5.7 Hz, 1H), 7.33-7.43 (m, 1H), 7.07 (t, *J* = 8.1 Hz, 2H), 5.35 (d, *J* = 5.3 Hz, 1H), 4.53 (d, *J* = 5.0 Hz, 1H), 4.38-4.48 (m, 3H), 4.27-4.34 (m, 1H), 4.00 (dd, *J* = 5.2 Hz, 3.8 Hz, 1H), 3.87-3.91 (m, 1H), 3.53-3.59 (m, 1H), 3.44-3.45 (m, 2H), 3.35-3.39 (m, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 172.2, 162.2 (d, *J*_{C,F} = 8.4 Hz), 159.8 (d, *J*_{C,F} = 8.2 Hz), 129.8 (t, *J*_{C,F} = 10.4 Hz), 114.2 (t, *J*_{C,F} = 19.6 Hz), 111.5 (dd, *J*_{C,F} = 18.9 Hz, 6.5 Hz), 73.5, 72.3, 71.5, 70.1, 63.4. LRMS (ESI): *m/z* calcd. for C₁₃H₁₇F₂KNO₆ [M+K]⁺ 360.1, found 360.1.

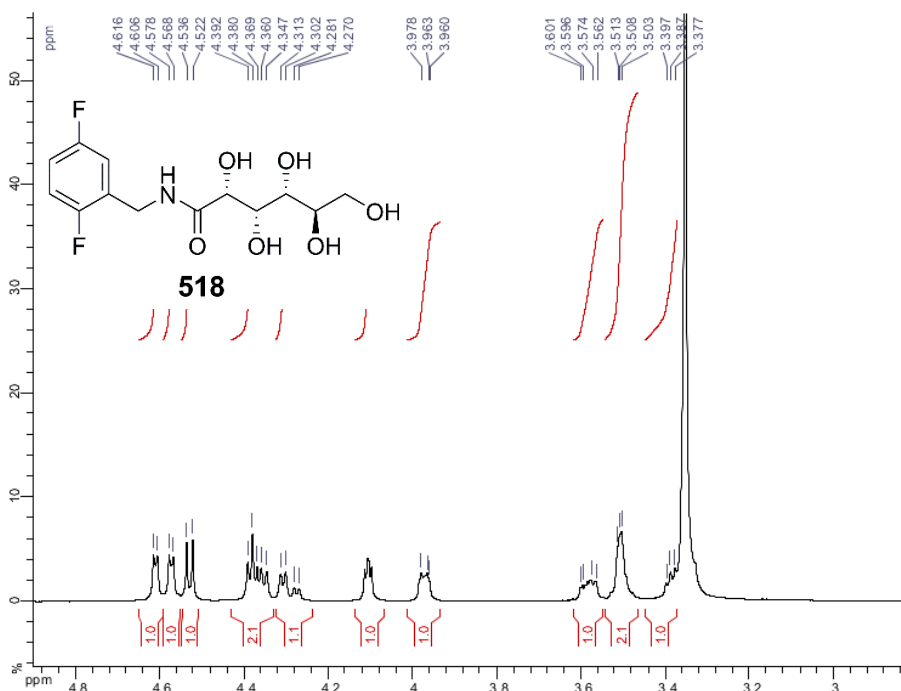


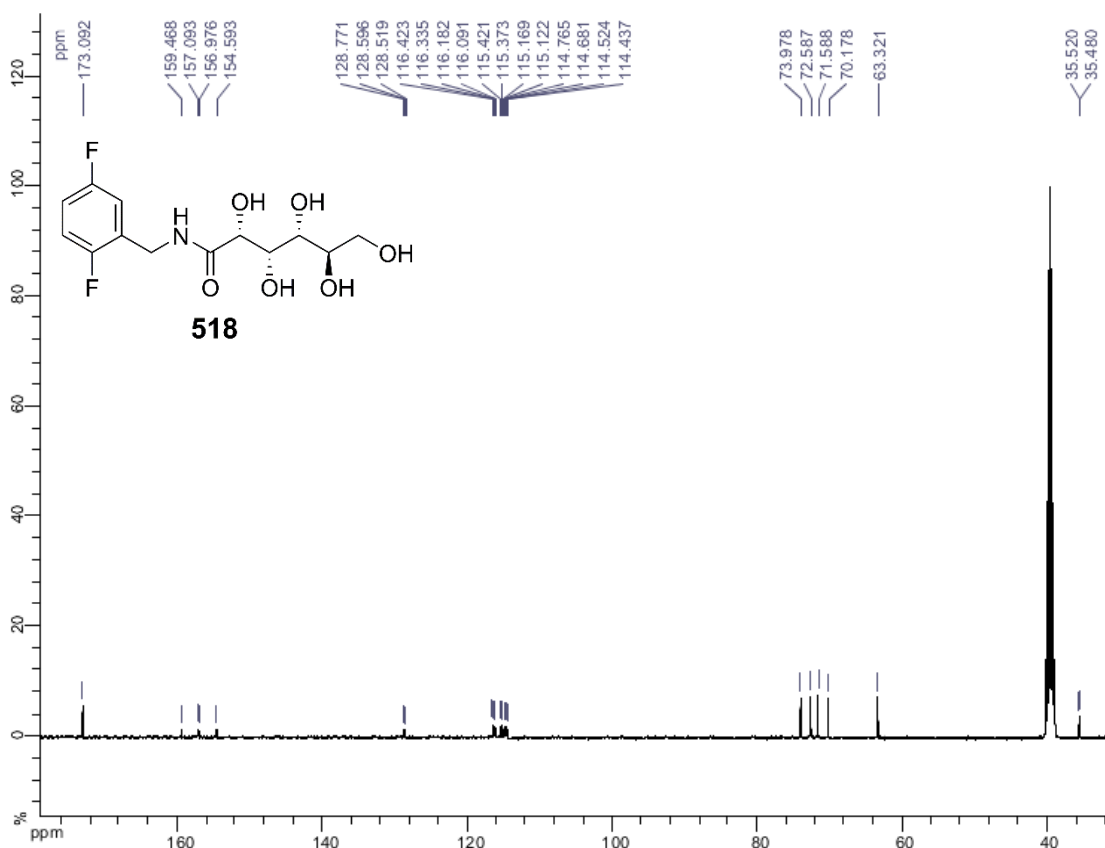


N-2,5-Difluorobenzyl-*D*-gluconamide (**518**)

To a solution of *D*-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added 2,5-difluorobenzylamine (0.15 mL, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **518** as white crystals (79%).

^1H NMR (500 MHz, $\text{DMSO}-d_6$): δ 8.30 (t, J = 6.1 Hz, 1H), 7.18-7.23 (m, 2H), 7.08-7.12 (m, 1H), 5.59 (d, J = 5.0 Hz, 1H), 4.61 (d, J = 5.0 Hz, 1H), 4.57 (d, J = 5.4 Hz, 1H), 4.53 (d, J = 7.2 Hz, 1H), 4.35-4.39 (m, 2H), 4.27-4.31 (m, 1H), 4.11 (t, J = 4.0 Hz, 1H), 3.97-3.98 (m, 1H), 3.57-3.60 (m, 1H), 3.51 (s, 2H), 3.38-3.40 (m, 1H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 173.1, 158.3 (d, $J_{\text{C,F}}$ = 240.4 Hz), 155.8 (d, $J_{\text{C,F}}$ = 240.4 Hz), 128.7 (dd, $J_{\text{C,F}}$ = 17.5 Hz, 7.8 Hz), 116.3 (dd, $J_{\text{C,F}}$ = 24.3 Hz, 8.9 Hz), 115.3 (dd, $J_{\text{C,F}}$ = 25.3 Hz, 4.8 Hz), 114.6 (dd, $J_{\text{C,F}}$ = 24.2 Hz, 8.4 Hz), 74.0, 72.6, 71.6, 70.2, 63.3, 35.5 (d, $J_{\text{C,F}}$ = 4.0 Hz). LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{18}\text{F}_2\text{NO}_6$ $[\text{M}+\text{H}]^+$ 322.1, found 322.2.

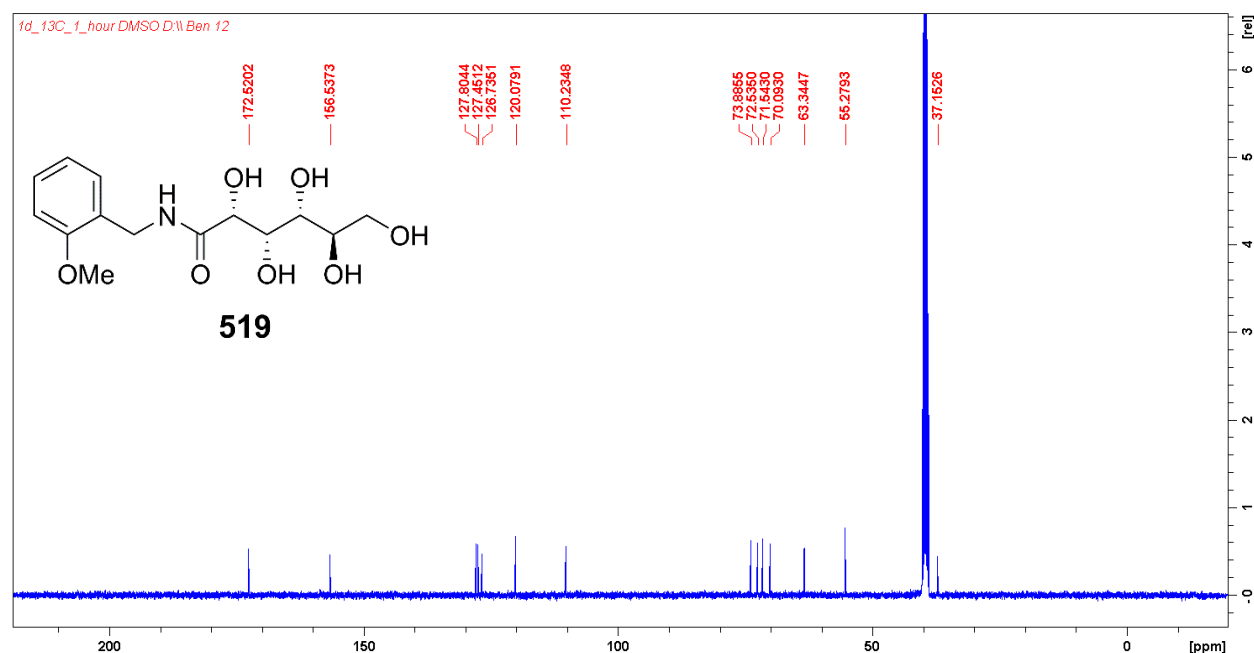




N-2-Methoxybenzyl-D-gluconamide (**519**)

To a solution of D-gluconic acid- δ -lactone (0.10 g, 0.56 mmol) in MeOH (10 mL) was added 2-methoxybenzylamine (0.08 mL, 0.58 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **519** as white crystals (51%).

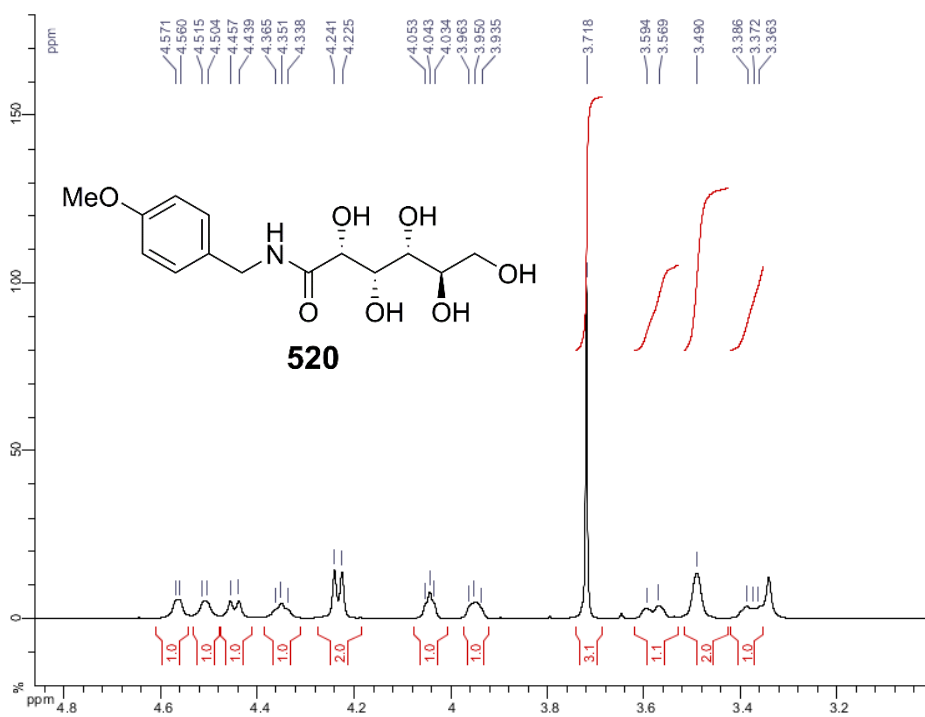
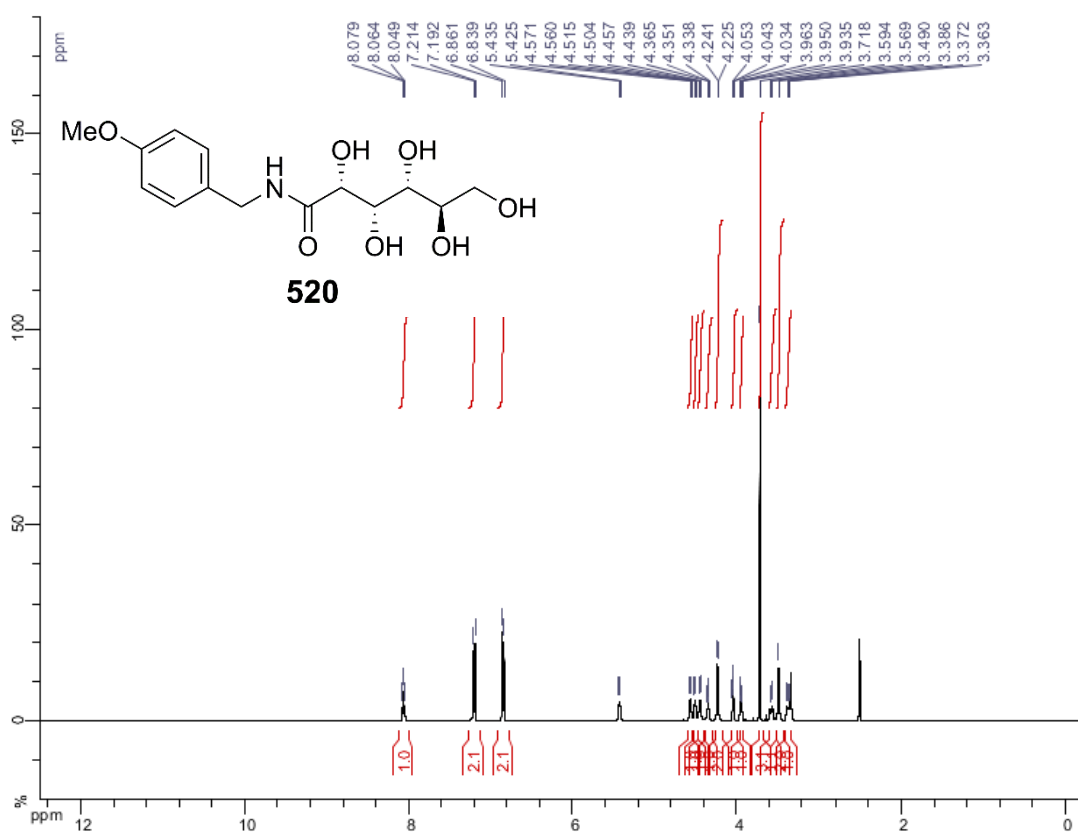
^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 7.95 (t, J = 6.3 Hz, 1H), 7.20-7.24 (m, 2H), 6.95-6.97 (m, 1H), 6.86-6.89 (m, 1H), 5.50 (d, J = 4.7 Hz, 1H), 4.55 (dd, J = 18.5, 5.0 Hz, 2H), 4.48 (d, J = 7.2 Hz, 1H), 4.35 (t, J = 5.5 Hz, 1H), 4.27 (d, J = 6.2 Hz, 2H), 4.06-4.08 (m, 1H), 3.95-3.97 (m, 1H), 3.80 (s, 3H), 3.56-3.60 (m, 1H), 3.48-3.50 (m, 2H), 3.37-3.40 (m, 1H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 172.5, 156.5, 127.8, 127.5, 126.7, 120.1, 110.2, 73.9, 72.5, 71.5, 70.1, 63.3, 55.3, 37.2. LRMS (ESI): m/z calcd. for $\text{C}_{14}\text{H}_{20}\text{NO}_7$ $[\text{M}-\text{H}]^-$ 314.1, found 314.1.

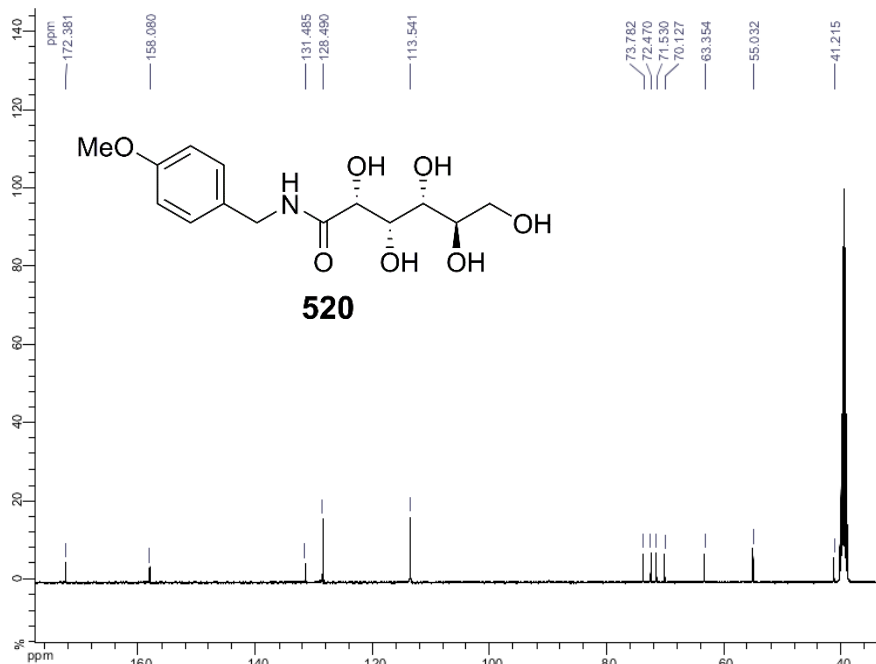


N-4-Methoxybenzyl-D-gluconamide (**520**)

To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (15 mL) was added 4-methoxybenzylamine (0.15 mL, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **520** as white crystals (62%).

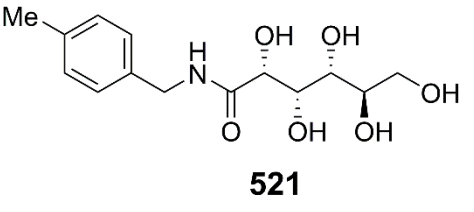
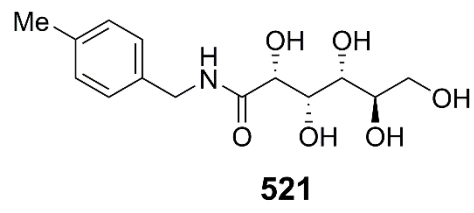
¹H NMR (400 MHz, DMSO-*d*₆): δ 8.06 (t, *J* = 6.2 Hz, 1H), 7.20 (d, *J* = 8.7 Hz, 2H), 6.85 (d, *J* = 8.8 Hz, 2H), 5.43 (d, *J* = 4.4 Hz, 1H), 4.57 (br.s, 1H), 4.51 (br. s, 1H), 4.44 (d, *J* = 7.4 Hz, 1H), 4.35 (t, *J* = 5.4 Hz, 1H), 4.23 (d, *J* = 6.8 Hz, 2H), 4.04 (t, *J* = 3.4 Hz, 1H), 3.96-3.94 (m, 1H), 3.71 (s, 3H), 3.60-3.56 (m, 1H), 3.49 (br. s, 2H), 3.40-3.36 (m, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 172.4, 158.1, 131.5, 128.5, 113.5, 73.8, 72.5, 71.5, 70.1, 63.4, 55.0, 41.2. LRMS (ESI): *m/z* calcd. for C₁₄H₂₀NO₇ [M-H]⁻ 314.1, found 314.1.

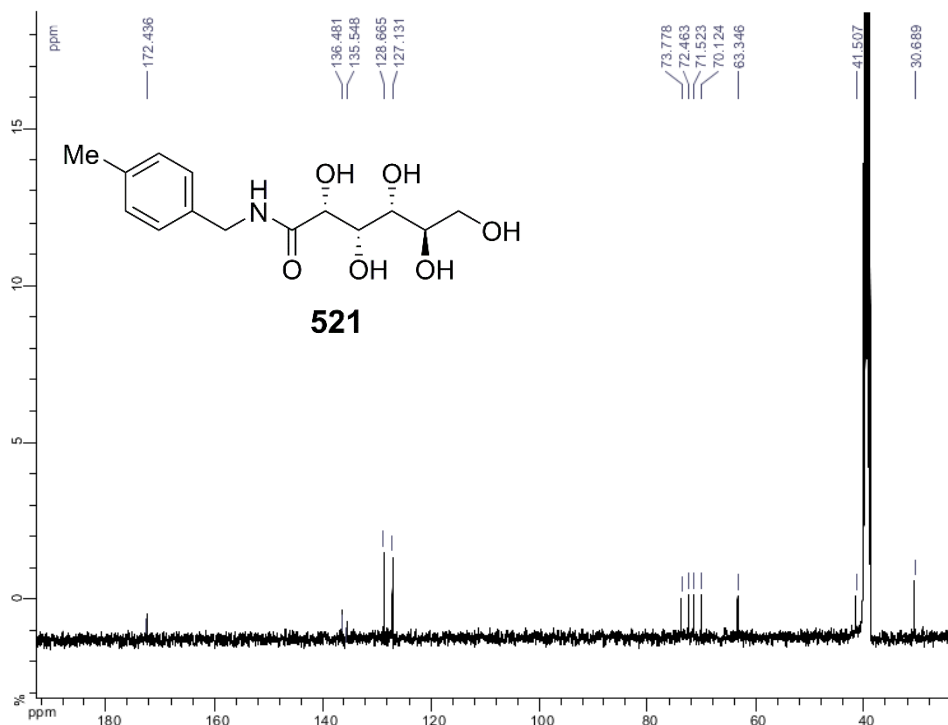




***N*-4-Methylbenzyl-D-gluconamide (521)**

To a solution of D-gluconic acid- δ -lactone (0.10 g, 0.56 mmol) in MeOH (10 mL) was added 4-methylbenzylamine (0.07 mL, 0.56 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **521** as white crystals (88%). ^1H NMR (400 MHz; $\text{DMSO-}d_6$): δ 8.08 (t, J = 6.1 Hz, 1H), 7.17-7.08 (dd, 4H), 5.43 (d, J = 5.1 Hz, 1H), 4.53 (dd, J = 22.4, 5.4 Hz, 2H), 4.44 (d, J = 7.2 Hz, 1H), 4.34 (t, J = 5.7 Hz, 1H), 4.26 (d, J = 6.2 Hz, 2H), 4.05 (dd, J = 5.1, 3.7 Hz, 1H), 3.95 (m, 1H), 3.60-3.57 (m, 1H), 3.49 (m, 2H), 3.37 (dd, J = 11.0, 5.7 Hz, 1H), 2.26 (s, 3H). ^{13}C NMR (100 MHz; $\text{DMSO-}d_6$): δ 172.4, 136.5, 135.6, 128.7, 127.1, 73.8, 72.5, 71.5, 70.1, 63.4, 41.5, 30.7. LRMS (ESI): m/z calcd. for $\text{C}_{14}\text{H}_{20}\text{NO}_6$ $[\text{M-H}]^-$ 298.1, found 298.1.

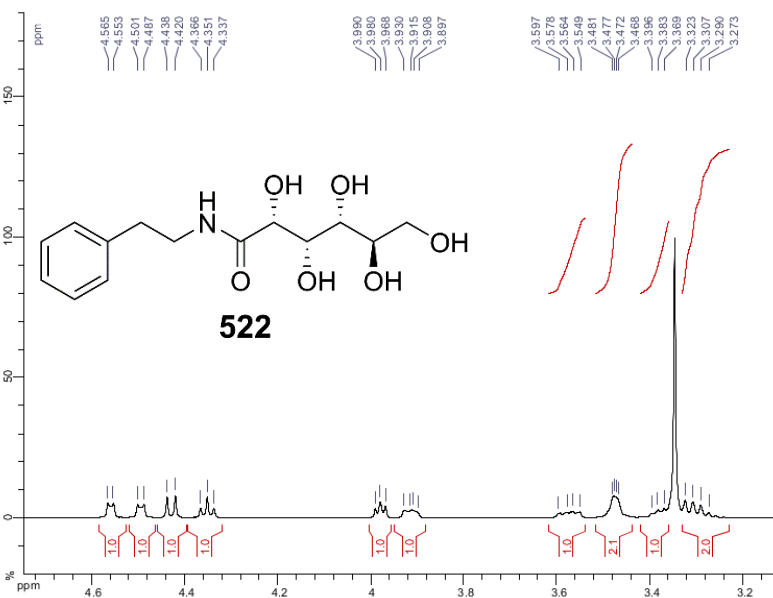


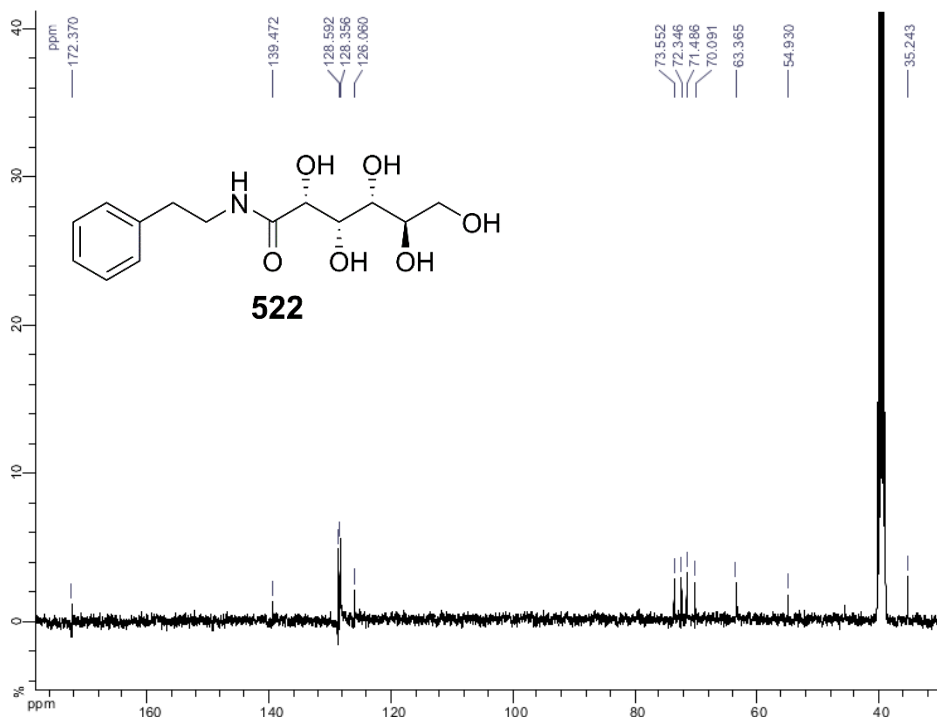


5.5.6 Characterization data and spectra of *N*-phenethyl-gluconamides and *N*-phenylhydrazine-gluconamides (522-527)

N-2-Phenylethyl-*D*-gluconamide (522)

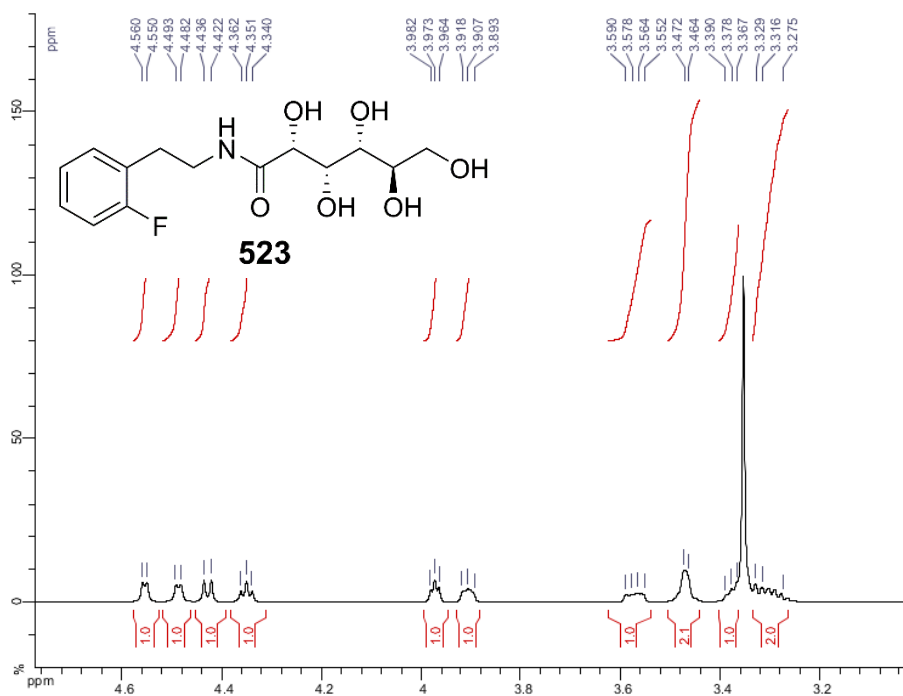
To a solution of *D*-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added phenethylamine hydrochloride (0.18 g, 1.12 mmol). Triethylamine (0.16 mL, 1.12 mmol) was added and the mixture was stirred under reflux for 48 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **522** as white crystals (50%). Characterization data are consistent with those of previously reported data.³⁴ ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 7.70 (t, J = 6.2 Hz, 1H), 7.18-7.31 (m, 5H), 5.40 (d, J = 5.2 Hz, 1H), 4.56 (d, J = 5.1 Hz, 1H), 4.50 (d, J = 5.9 Hz, 1H), 4.30 (d, J = 7.3 Hz, 1H), 4.35 (t, J = 6.1 Hz, 1H), 3.98 (t, J = 4.0 Hz, 1H), 3.90-3.93 (m, 1H), 3.55-3.59 (m, 1H), 3.47-3.48 (m, 2H), 3.38 (t, J = 6.0 Hz, 1H), 3.27-3.32 (m, 2H), 2.73 (t, J = 7.7 Hz, 2H). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ 172.4, 139.5, 128.6, 128.4, 126.1, 73.6, 72.4, 71.5, 70.1, 63.4, 54.9, 35.2. LRMS (ESI): m/z calcd. for $\text{C}_{14}\text{H}_{20}\text{NO}_6$ $[\text{M-H}]^-$ 298.1, found 298.1.

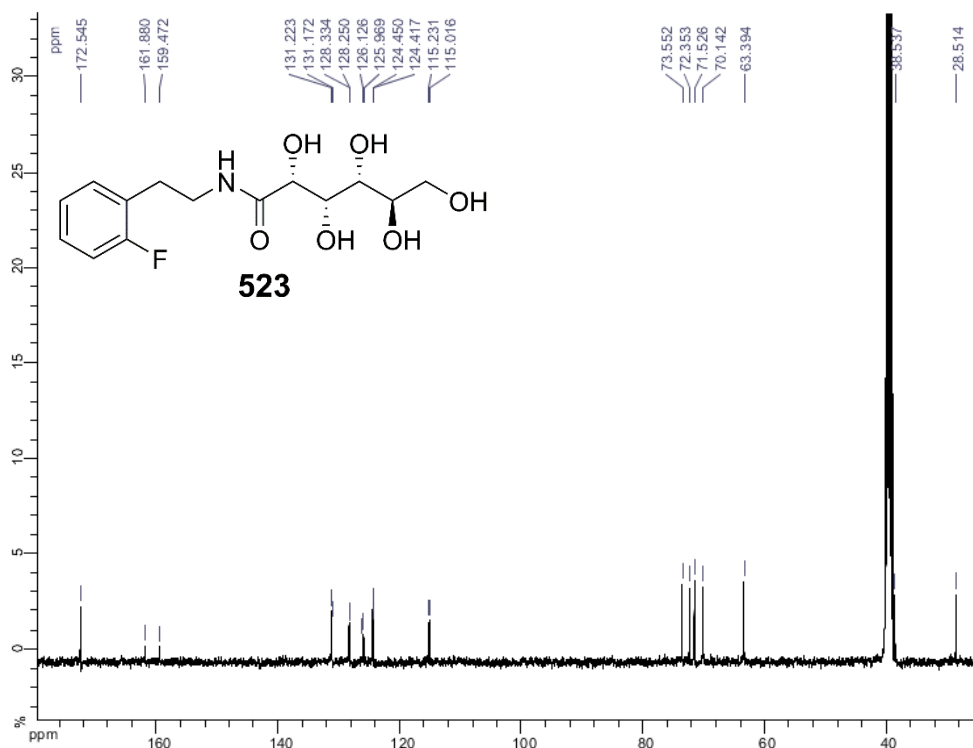




N-2-(2-Fluoro)phenylethyl-*D*-gluconamide (**523**)

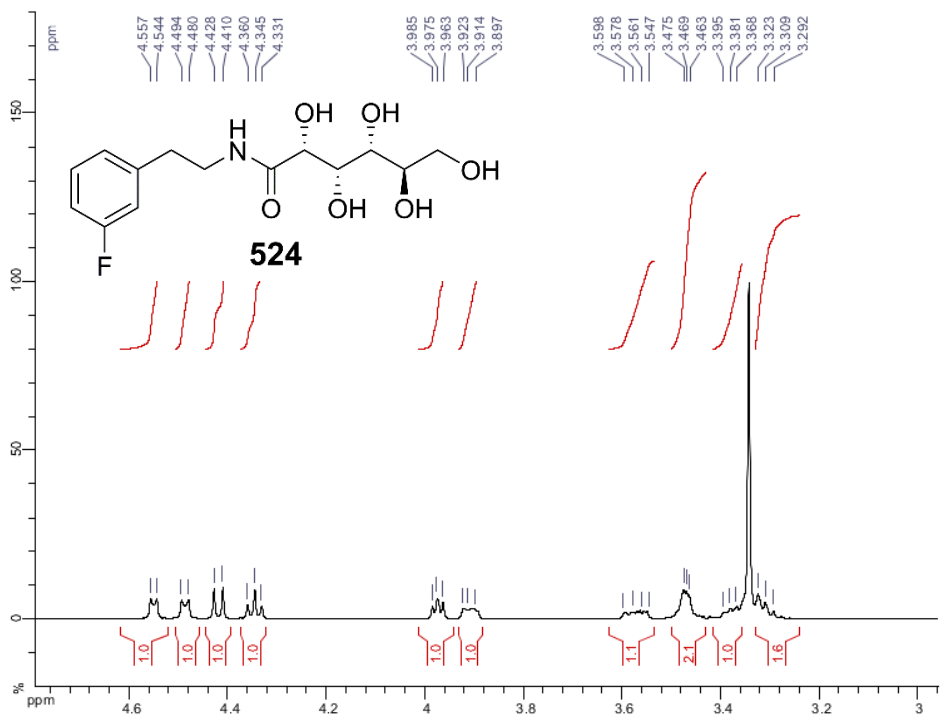
To a solution of *D*-gluconic acid-*d*-lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added 2-fluorophenethylamine (0.15 mL, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **523** as a white powder (51%). ¹H NMR (500 MHz, DMSO-*d*₆): δ 7.80 (t, *J* = 6.0 Hz, 1H), 7.28 (m, 2H), 7.14 (m, 2H), 5.39 (d, *J* = 6.0 Hz, 1H), 4.56 (d, *J* = 4.8 Hz, 1H), 4.49 (d, *J* = 5.1 Hz, 1H), 4.43 (d, *J* = 7.2 Hz, 1H), 4.35 (t, *J* = 5.2 Hz, 1H), 3.97 (t, *J* = 4.2 Hz, 1H), 3.90 (m, 1H), 3.57 (m, 1H), 3.47 (m, 2H), 3.37 (m, 1H), 3.30 (m, 2H), 2.76 (m, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 172.6, 160.7 (d, *J*_{C,F} = 244.9 Hz), 131.2 (d, *J*_{C,F} = 5.1 Hz), 128.3 (d, *J*_{C,F} = 8.4 Hz), 126.1 (d, *J*_{C,F} = 15.5 Hz), 124.4 (d, *J*_{C,F} = 2.9 Hz), 115.1 (d, *J*_{C,F} = 21.7), 73.6, 72.4, 71.5, 70.1, 63.4, 38.5, 28.5. LRMS (ESI): *m/z* calcd. for C₁₄H₂₁FNO₆ [M+H]⁺ 318.1, found 318.2.

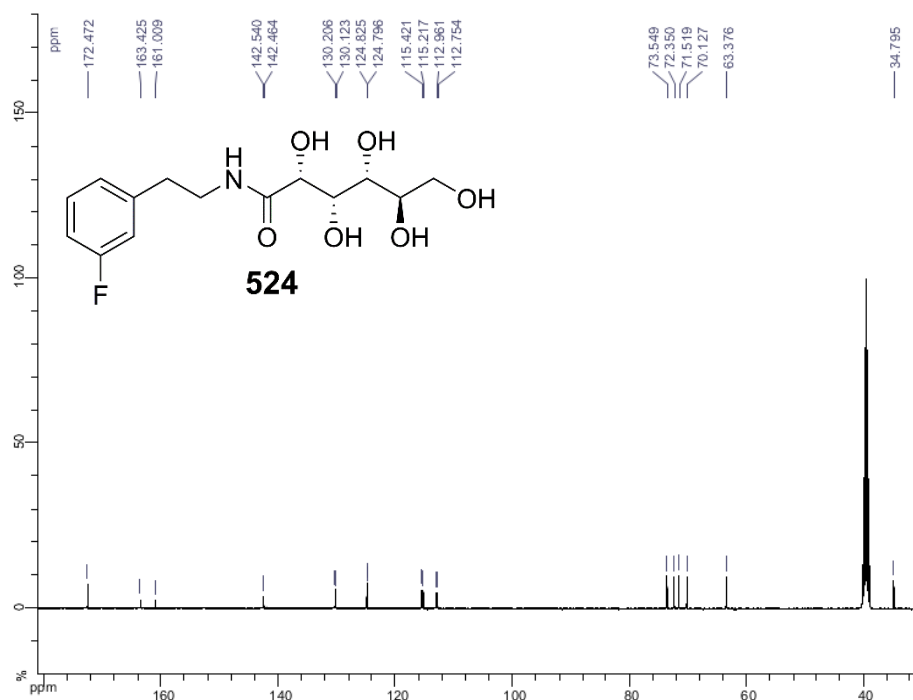




N-2-(3-Fluoro)phenylethyl-*D*-gluconamide (**524**)

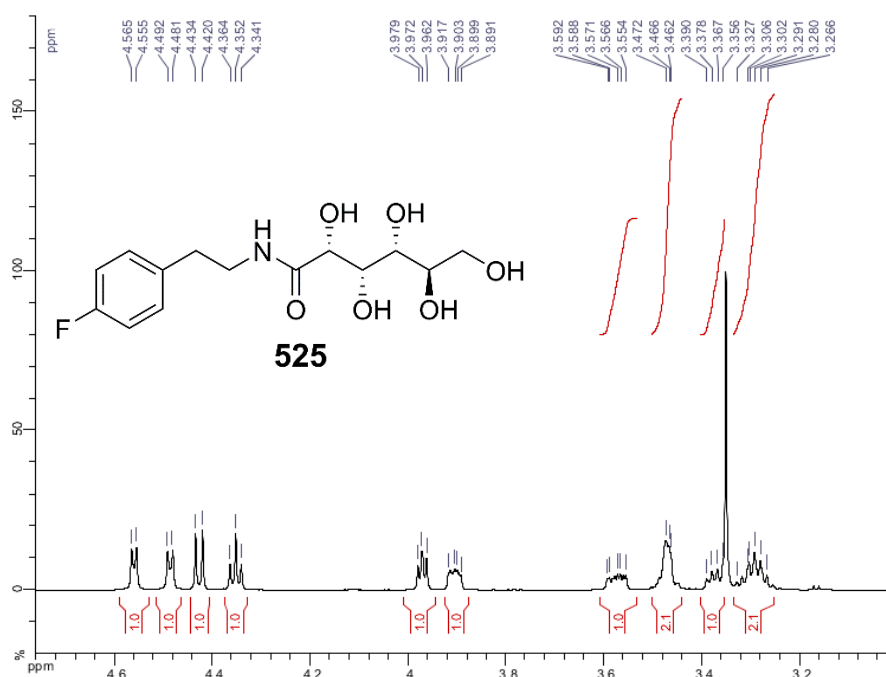
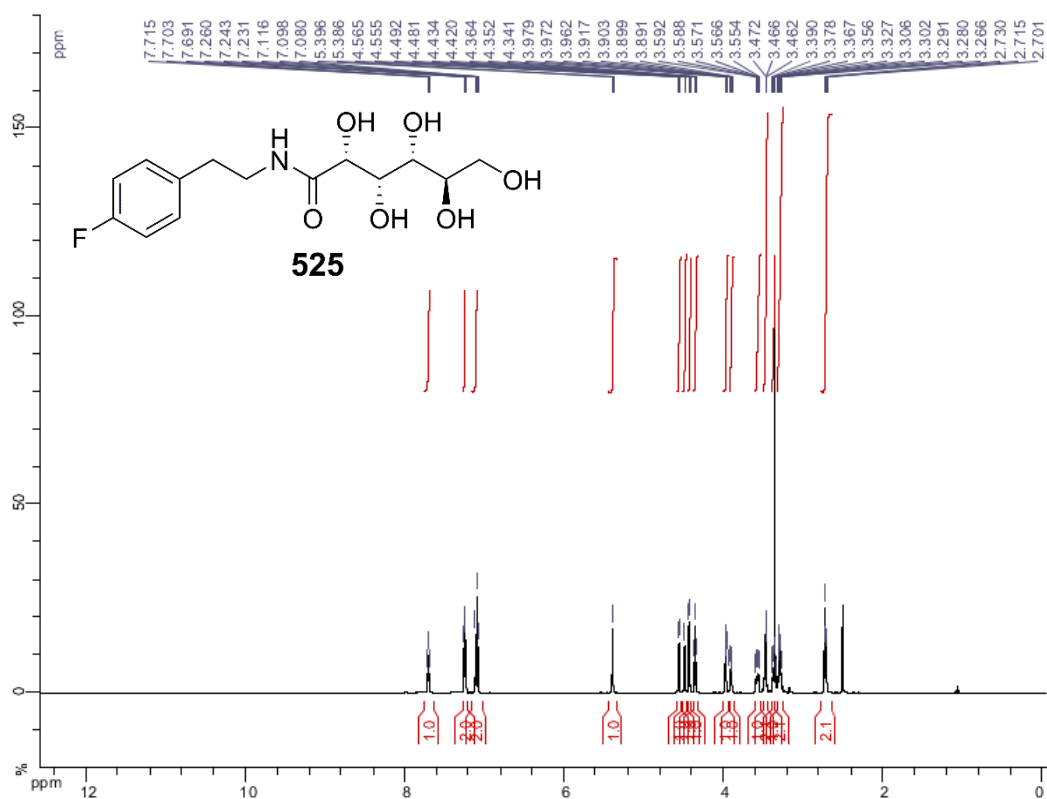
To a solution of *D*-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added 3-fluorophenethylamine (0.15 mL, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **524** as a white powder (52%). ^1H NMR (400 MHz, DMSO- d_6): δ 7.72 (t, J = 5.9 Hz, 1H), 7.32 (m, 1H), 7.05 (m, 3H), 5.39 (d, J = 5.2 Hz, 1H), 4.55 (d, J = 5.2 Hz, 1H), 4.49 (d, J = 5.6 Hz, 1H), 4.42 (d, J = 7.4 Hz, 1H), 4.35 (t, J = 6.0 Hz, 1H), 3.98 (t, J = 4.0 Hz, 1H), 3.91 (m, 1H), 3.57 (m, 1H), 3.45 (m, 2H), 3.38 (m, 1H), 3.31 (m, 2H), 2.75 (t, J = 7.2 Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 172.5, 162.2 (d, $J_{\text{C,F}}$ = 245.7 Hz), 142.5 (d, $J_{\text{C,F}}$ = 7.5 Hz), 130.17 (d, $J_{\text{C,F}}$ = 8.4 Hz), 124.8 (d, $J_{\text{C,F}}$ = 2.2 Hz), 115.3 (d, $J_{\text{C,F}}$ = 21.1 Hz), 112.9 (d, $J_{\text{C,F}}$ = 20.8 Hz), 73.6, 72.4, 71.5, 70.1, 63.4, 34.8. LRMS (ESI): m/z calcd. for $\text{C}_{14}\text{H}_{21}\text{FNO}_6$ $[\text{M}+\text{H}]^+$ 318.1, found 318.2.

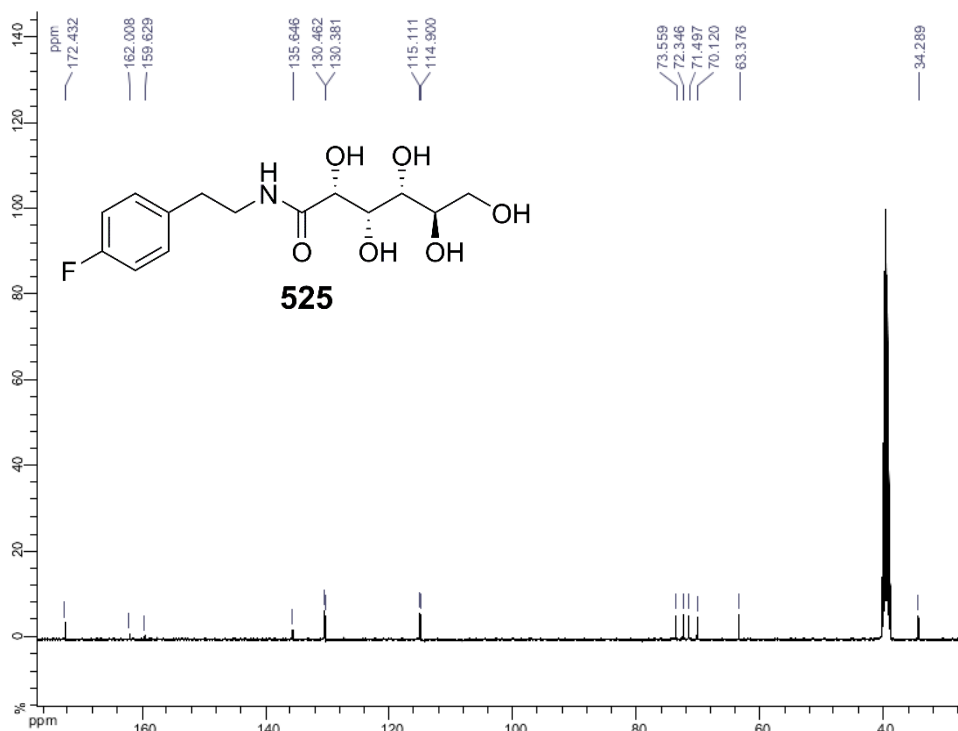




***N*-2-(4-Fluoro)phenylethyl-*D*-gluconamide (**525**)**

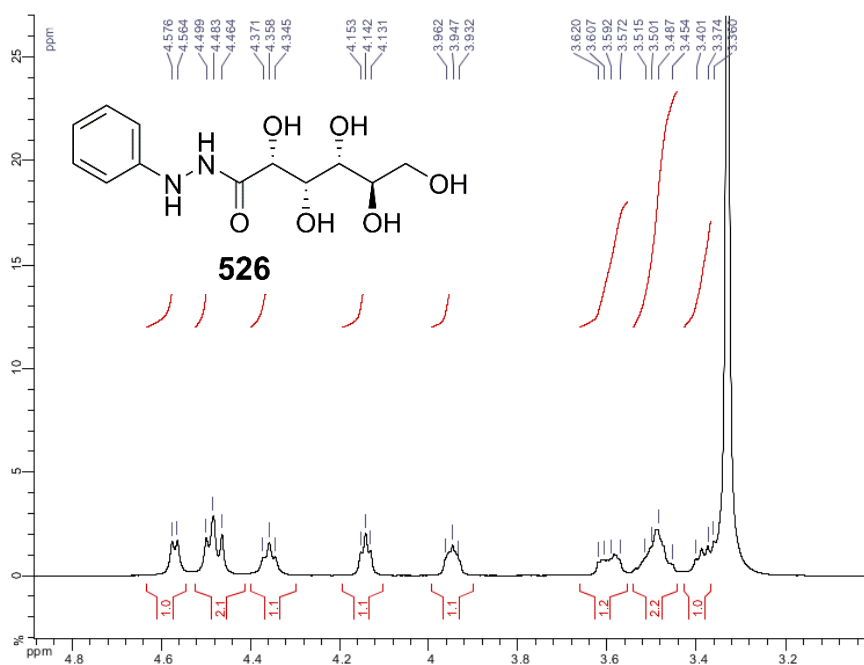
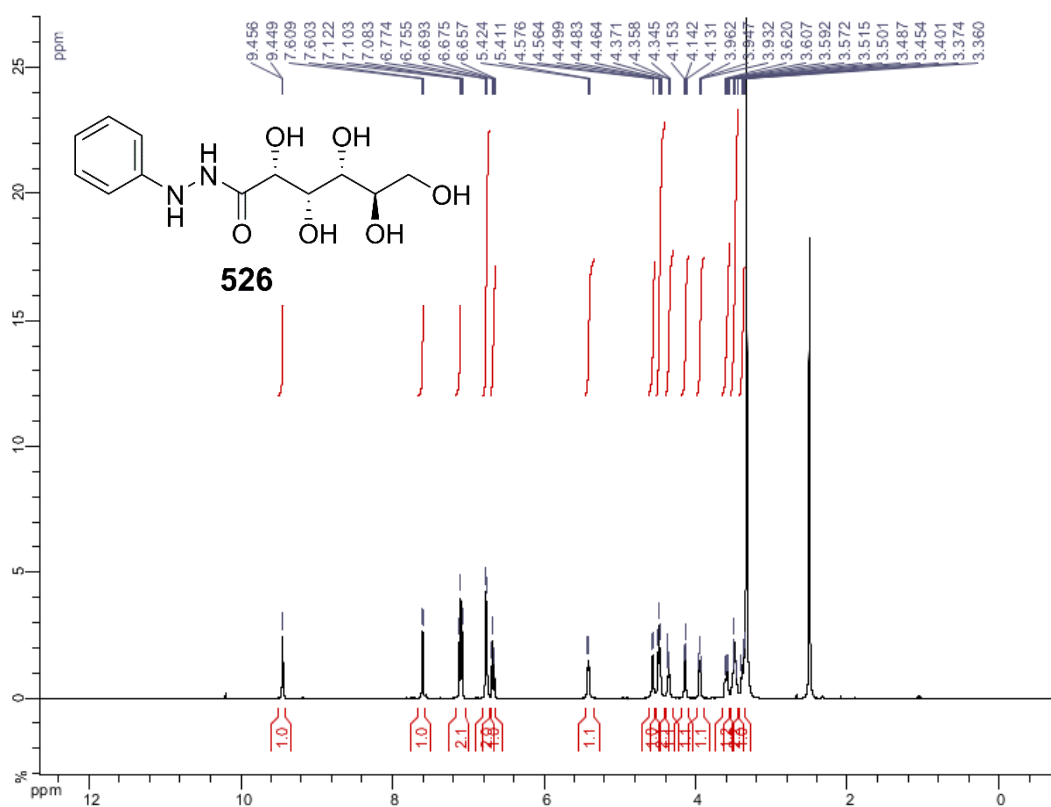
To a solution of *D*-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (10 mL) was added 4-fluorophenethylamine (0.15 mL, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **525** as a white powder (97%). ^1H NMR (500 MHz, DMSO- d_6): δ 7.70 (t, J = 6.0 Hz, 1H), 7.25 (m, 2H), 7.10 (t, J = 9.0 Hz, 2H), 5.39 (d, J = 5.1 Hz, 1H), 4.56 (d, J = 5.2 Hz, 1H), 4.48 (d, J = 5.7 Hz, 1H), 4.42 (d, J = 7.2 Hz, 1H), 4.35 (t, J = 5.7 Hz, 1H), 3.97 (t, J = 3.9 Hz, 1H), 3.90 (m, 1H), 3.57 (m, 1H), 3.45 (m, 2H), 3.38 (m, 1H), 3.30 (m, 2H), 2.72 (t, J = 7.4 Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 172.4, 160.8 (d, $J_{\text{C,F}}$ = 242.0 Hz), 135.6 (d, $J_{\text{C,F}}$ = 2.7 Hz), 130.4 (d, $J_{\text{C,F}}$ = 7.8 Hz), 115.0 (d, $J_{\text{C,F}}$ = 21.0 Hz), 73.6, 72.3, 71.5, 70.1, 63.4, 34.3. LRMS (ESI): m/z calcd. for $\text{C}_{14}\text{H}_{21}\text{FNO}_6$ $[\text{M}+\text{H}]^+$ 318.1, found 318.2.

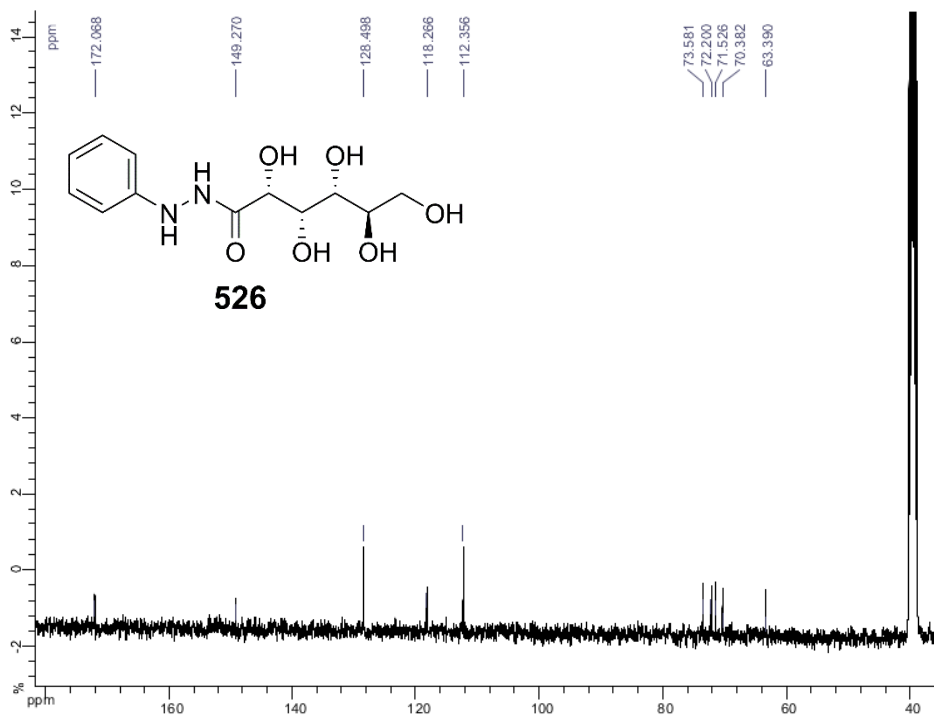




2-Phenylhydrazide-D-gluconic acid (**526**)

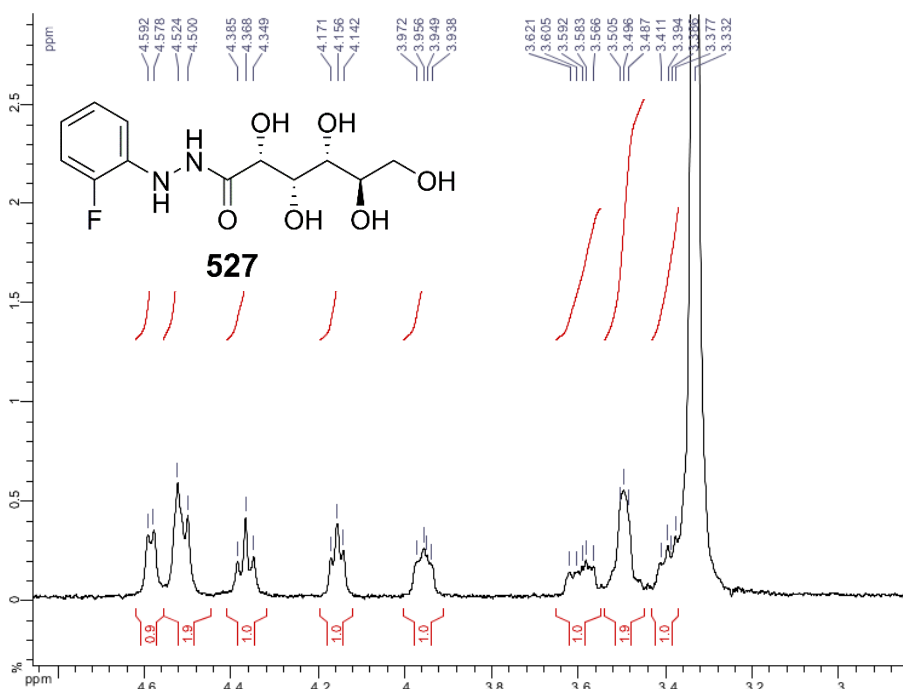
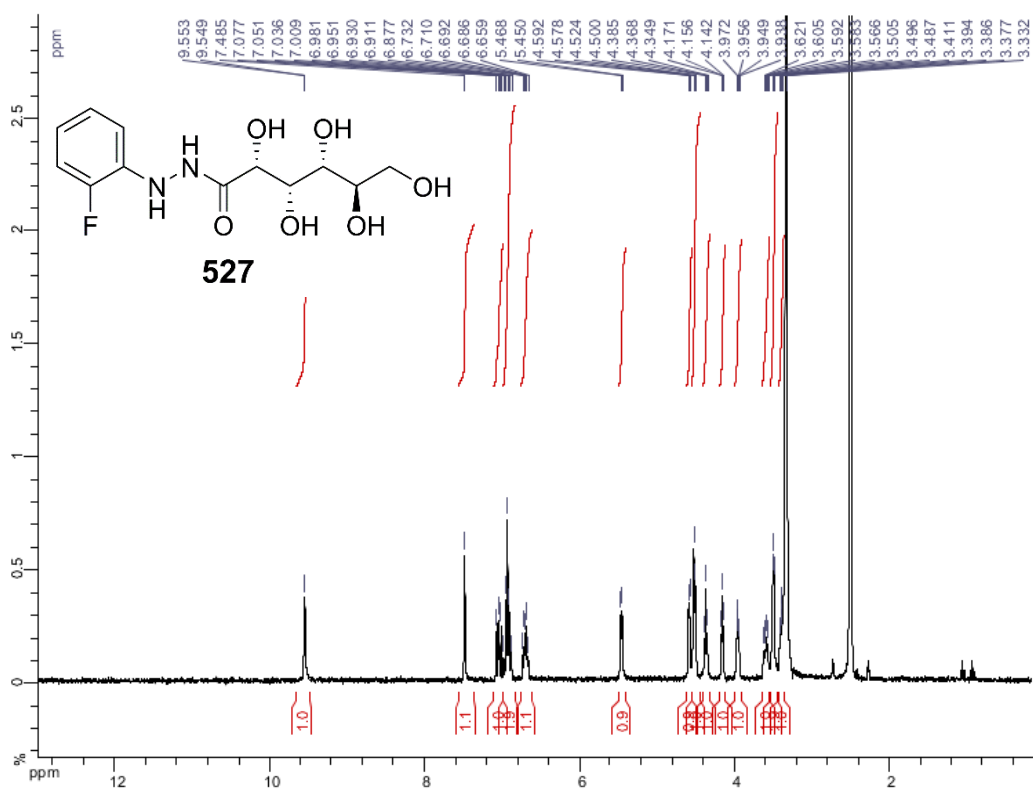
To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (15 mL) was added phenylhydrazine (0.11 mL, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **526** as white crystals (83%). ^1H NMR (400 MHz, DMSO- d_6): δ 9.45 (d, J = 2.9 Hz, 1H), 7.61 (d, J = 2.5 Hz, 1H), 7.10 (dd, J = 8.3, 7.5 Hz, 2H), 6.76 (d, J = 8.2 Hz, 2H), 6.68 (t, J = 7.0 Hz, 1H), 5.42 (d, J = 5.1 Hz, 1H), 4.57 (d, J = 4.5 Hz, 1H), 4.48 (t, J = 6.3 Hz, 2H), 4.36 (t, J = 5.6 Hz, 1H), 4.14 (t, J = 4.6 Hz, 1H), 3.94-3.95 (m, 1H), 3.57-3.62 (m, 1H), 3.45-3.52 (m, 2H), 3.36-3.40 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 172.1, 149.3, 129.0, 118.7, 112.8, 73.9, 72.5, 71.9, 70.8, 63.8. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{18}\text{KN}_2\text{O}_6$ $[\text{M}+\text{K}]^+$ 325.1, found 325.1.

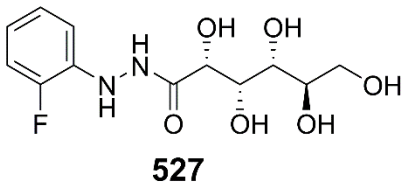


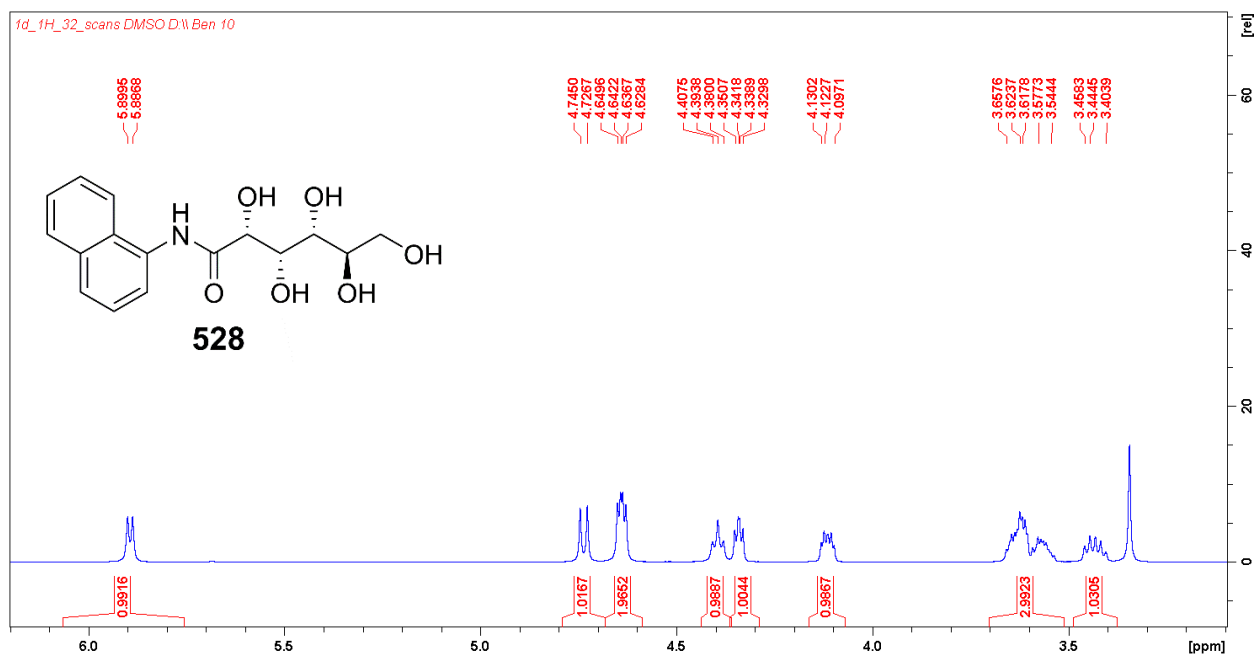
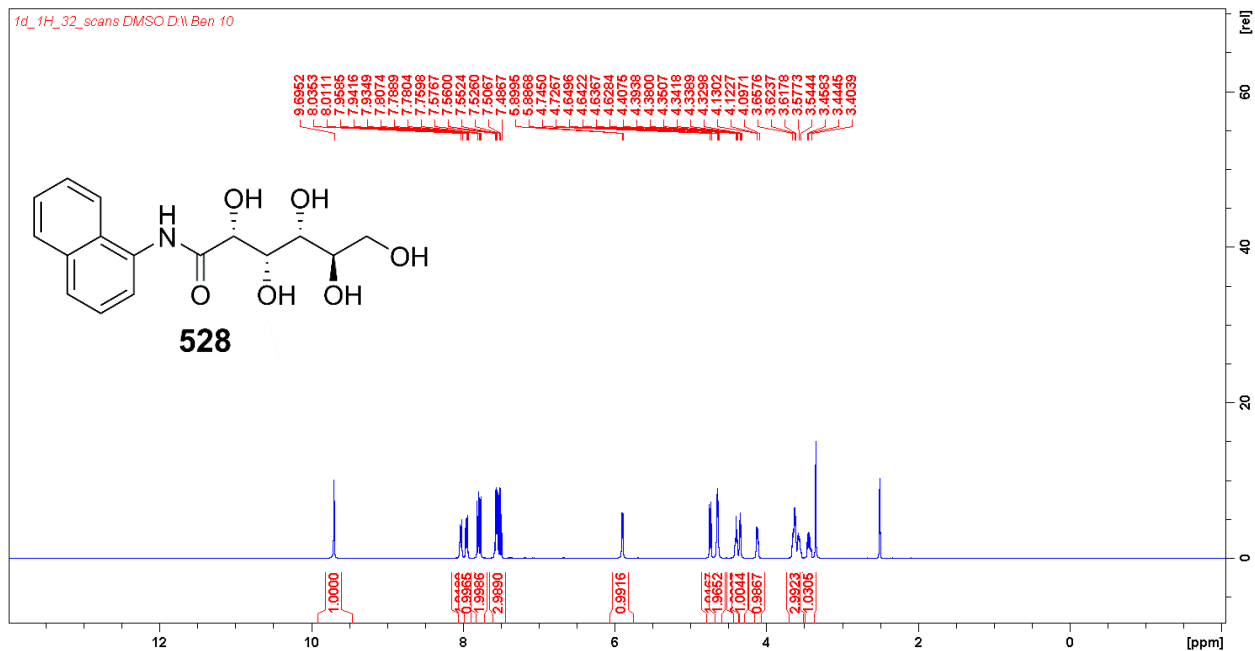


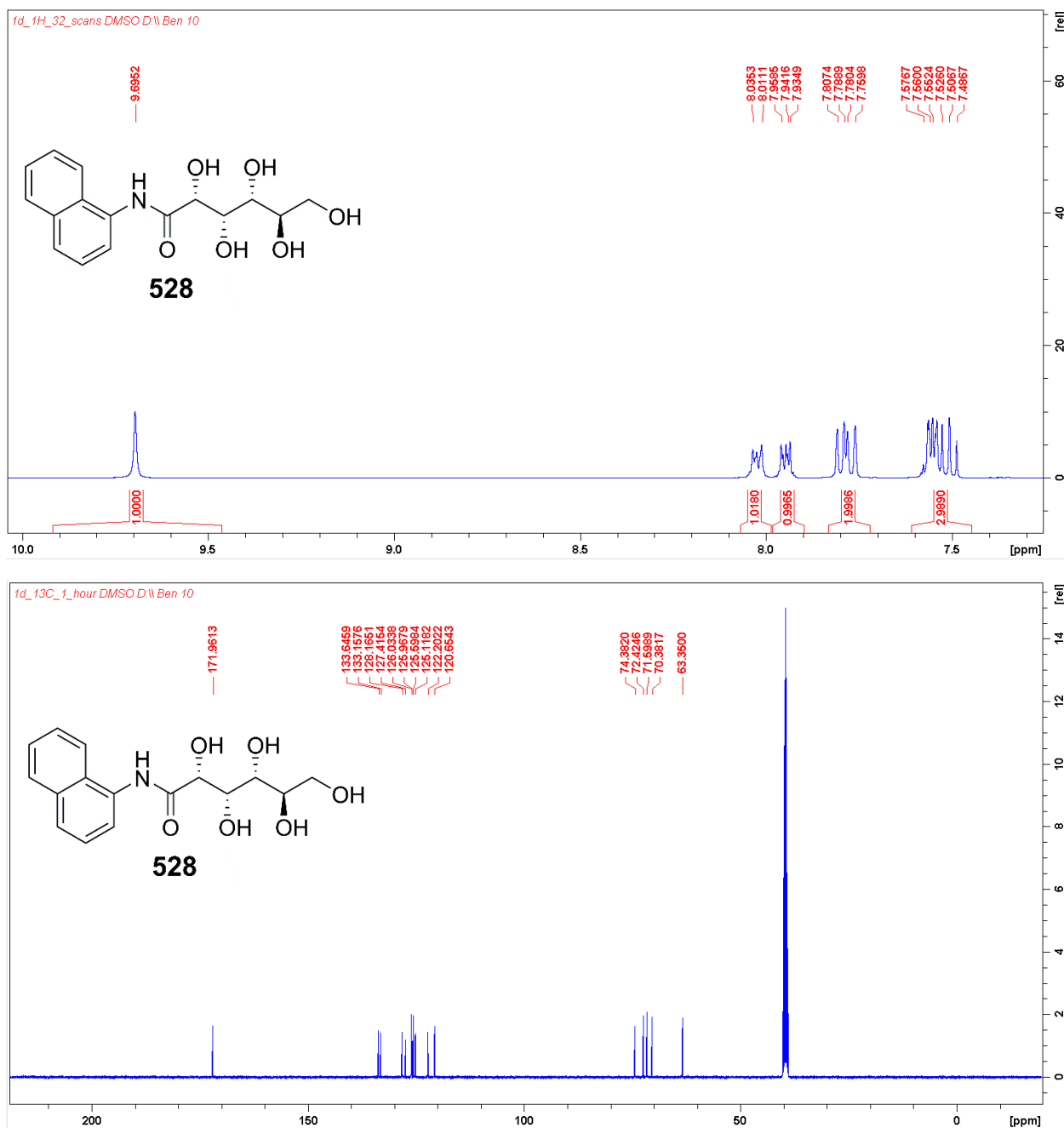
2-(2-Fluorophenyl)hydrazide-D-gluconic acid (**527**)

To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in MeOH (15 mL) was added 2-fluorophenylhydrazine hydrochloride (0.18 g, 1.12 mmol). Triethylamine (0.51 mL, 1.12 mmol) was added and the mixture was stirred under reflux for 24 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **527** as white crystals (39%). ^1H NMR (400 MHz, DMSO- d_6): δ 9.55 (d, J = 2.3 Hz, 1H), 7.49 (br.s, 1H), 7.02-7.07 (m, 1H), 6.89-6.97 (m, 2H), 6.67-6.72 (m, 1H), 5.46 (d, J = 5.4 Hz, 1H), 4.59 (d, J = 4.8 Hz, 1H), 4.50-4.53 (m, 2H), 4.34-4.38 (m, 1H), 4.16 (dd, J = 5.1, 4.2 Hz, 1H), 3.94-3.97 (m, 1H), 3.57-3.62 (m, 1H), 3.49-3.52 (m, 2H), 3.37-3.41 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 172.2, 150.2 (d, $J_{\text{C,F}}$ = 232.6 Hz), 136.8 (d, $J_{\text{C,F}}$ = 10.5 Hz), 124.4 (d, $J_{\text{C,F}}$ = 2.9 Hz), 118.5 (d, $J_{\text{C,F}}$ = 6.6 Hz), 114.5 (d, $J_{\text{C,F}}$ = 17.3 Hz), 114.1 (d, $J_{\text{C,F}}$ = 3.1 Hz), 73.6, 72.2, 71.5, 70.4, 63.4. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{17}\text{FKN}_2\text{O}_6$ $[\text{M}+\text{K}]^+$ 343.1, found 343.1.





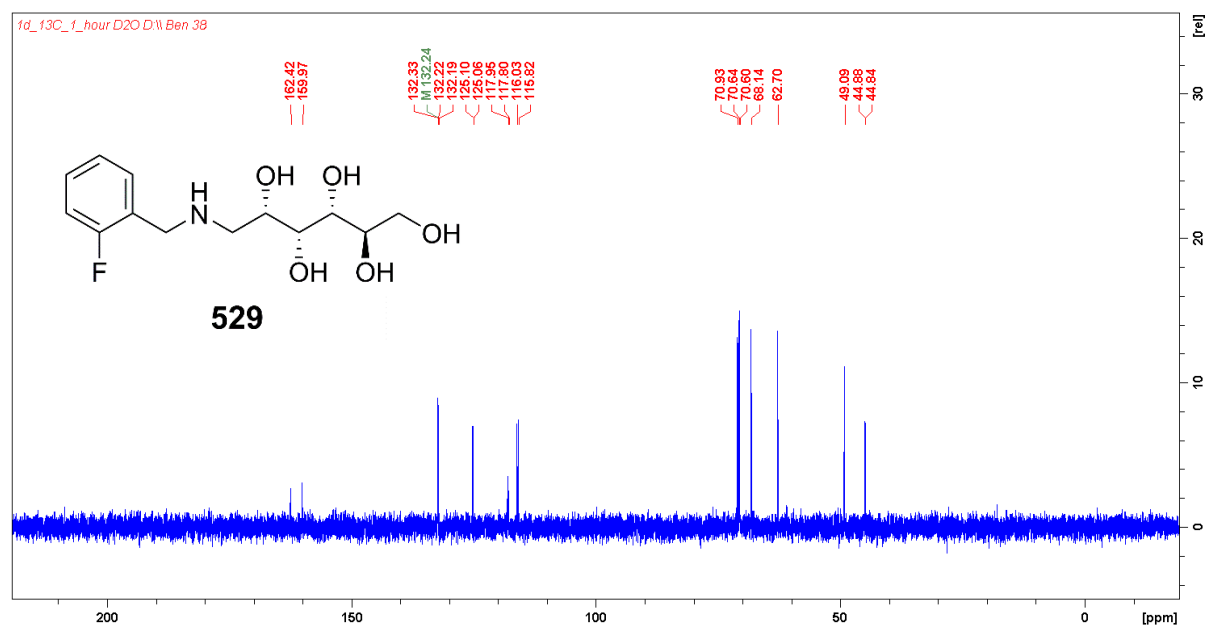
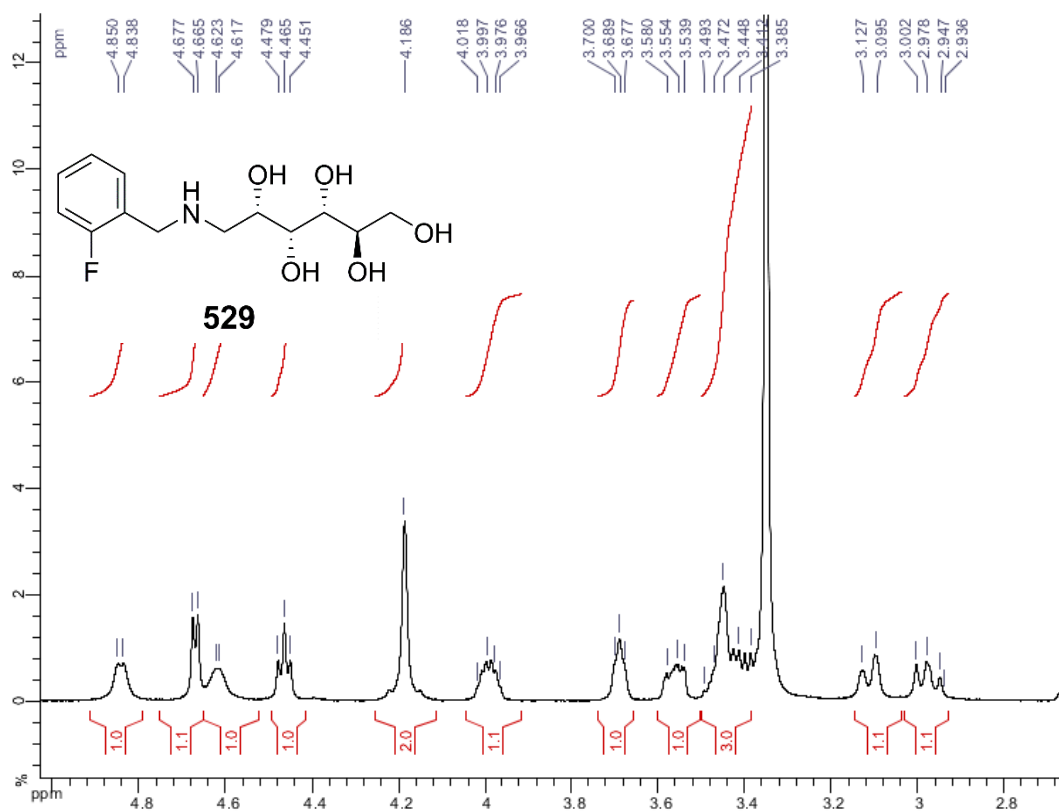




1-Deoxy-1-[(2-fluorophenylmethyl)amino]-D-glucitol (529)

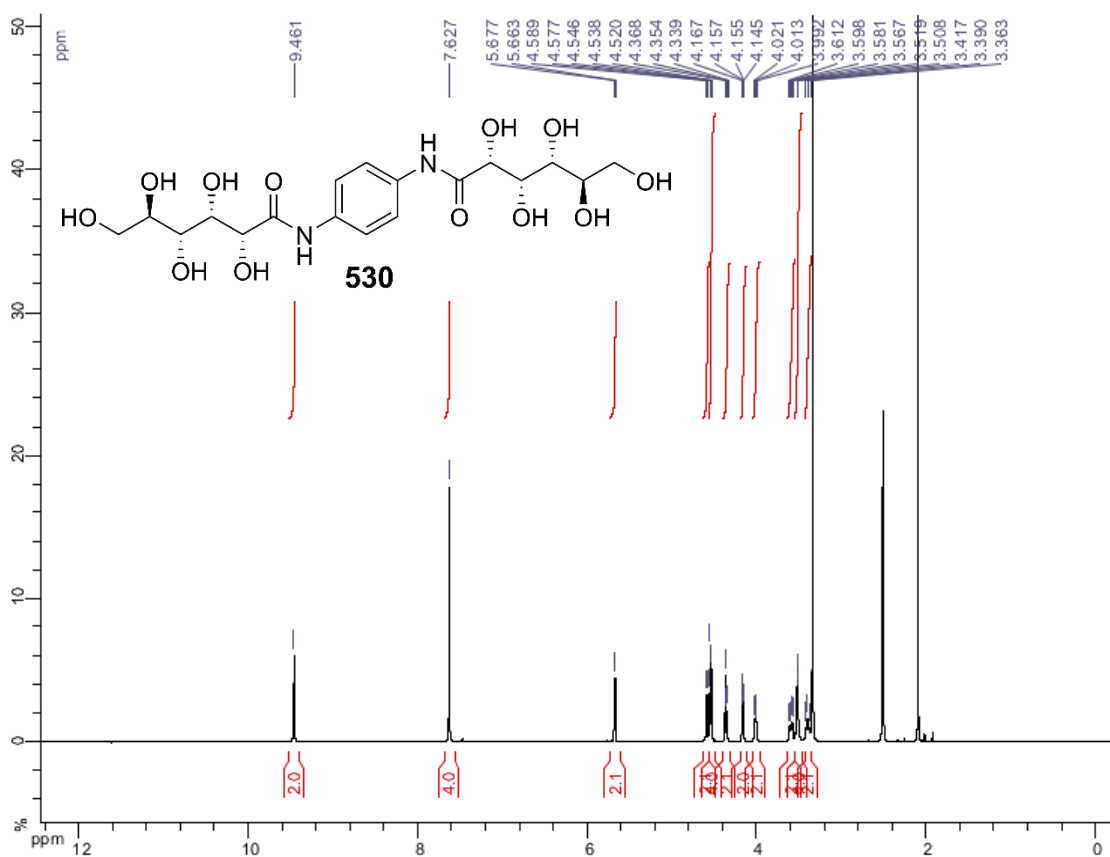
To a solution of D-glucose (0.1 g, 0.56 mmol) and 2-fluorobenzylamine (0.45 mL, 3.9 mmol) in 10% acetic acid in methanol (15 mL), was added sodium cyanoborohydride (0.1 g, 1.67 mmol). The mixture was stirred under reflux for 3 hours. The solution was allowed to cool to room temperature and concentrated hydrochloric acid was added dropwise until the solution became slightly acidic. The solvent

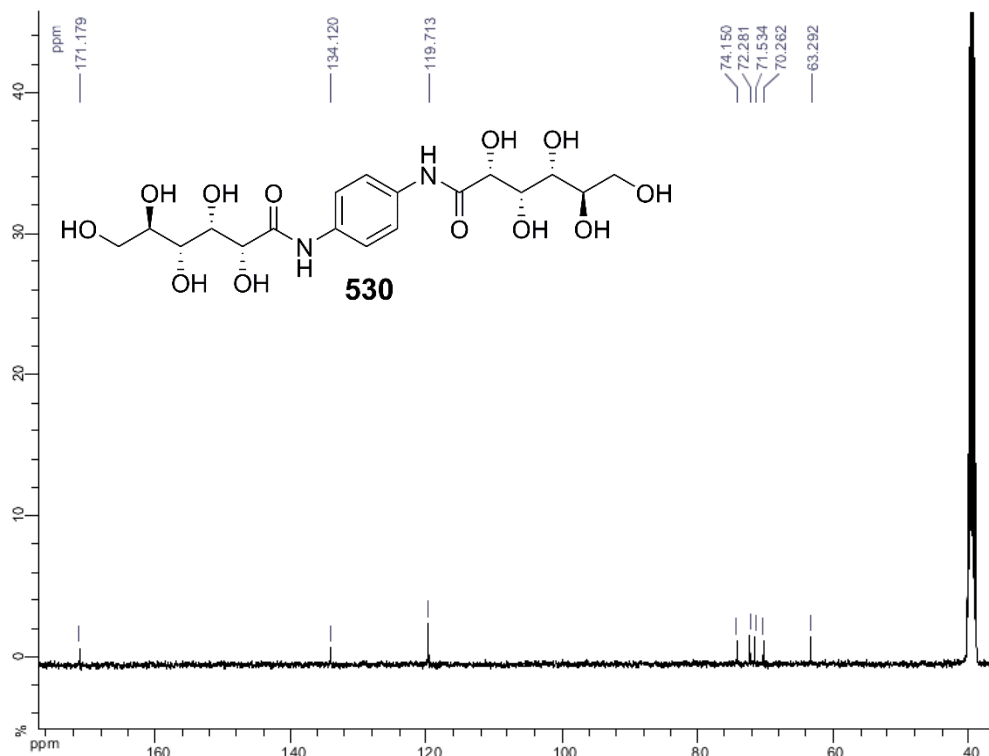
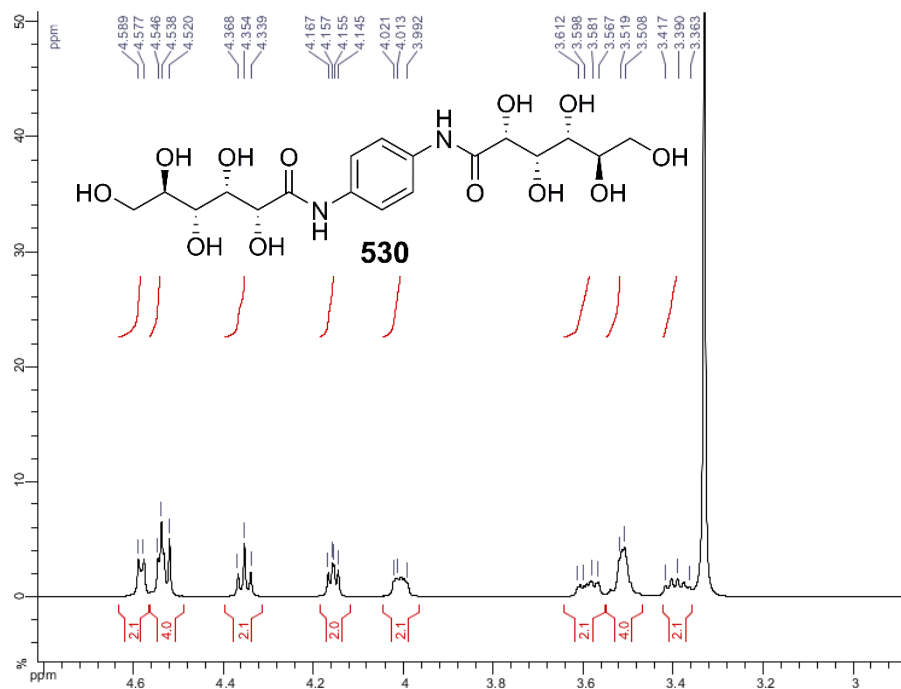
[illegible]



1,4-Bis-(D-gluconamide)benzene (530)

To a solution of D-gluconic acid- δ -lactone (0.40 g, 2.25 mmol) in acetic acid (10 mL) was added *p*-phenylenediamine (0.12 g, 1.12 mmol). The mixture was stirred under reflux for 4 hours. The crude product was precipitated with hexanes and collected by suction filtration, then recrystallized in EtOH to afford **530** as a light brown powder (45%). ^1H NMR (400 MHz; DMSO- d_6): δ 9.47 (s, 2H), 7.63 (s, 4H), 5.67 (d, J = 5.3 Hz, 2H), 4.58 (d, J = 5.0 Hz, 2H), 4.52-4.55 (m, 4H), 4.35 (t, J = 5.7 Hz, 2H), 4.16 (dd, J = 5.0, 3.8 Hz, 2H), 3.99-4.02 (m, 2H), 3.57-3.61 (m, 2H), 3.49-3.52 (m, 4H), 3.36-3.42 (m, 2H). ^{13}C NMR (100 MHz; DMSO- d_6): δ 171.2, 134.1, 119.7, 74.2, 72.3, 71.5, 70.3, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{18}\text{H}_{27}\text{N}_2\text{O}_{12} [\text{M}-\text{H}]^-$ 463.2, found 463.2.

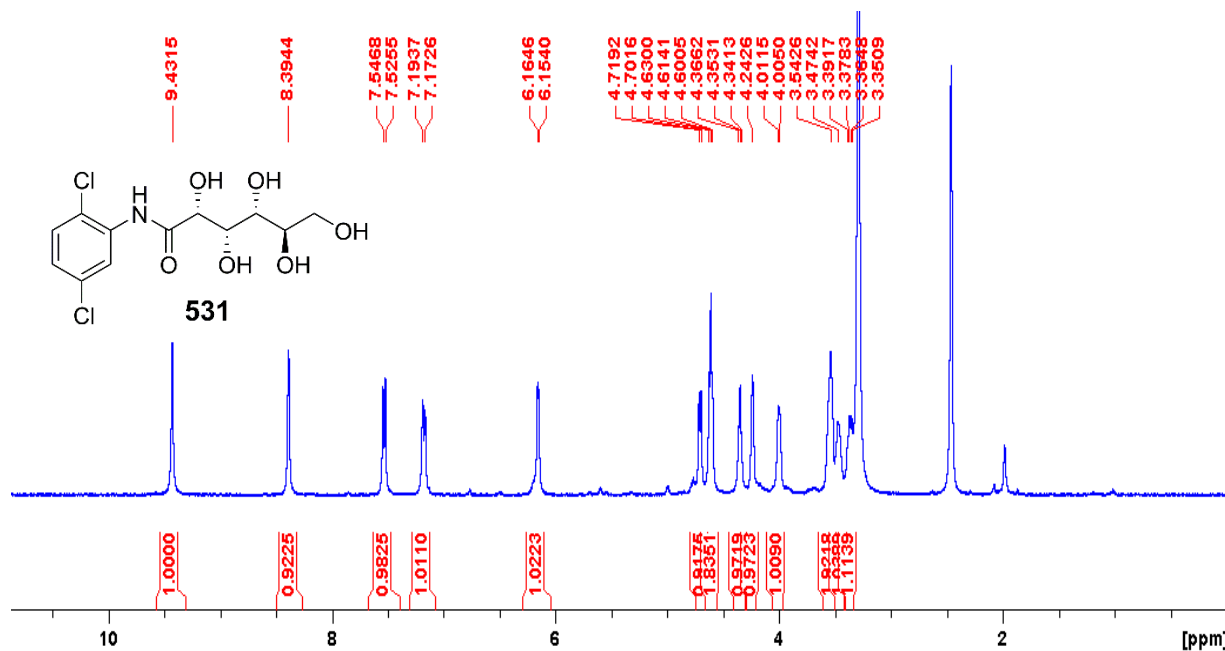


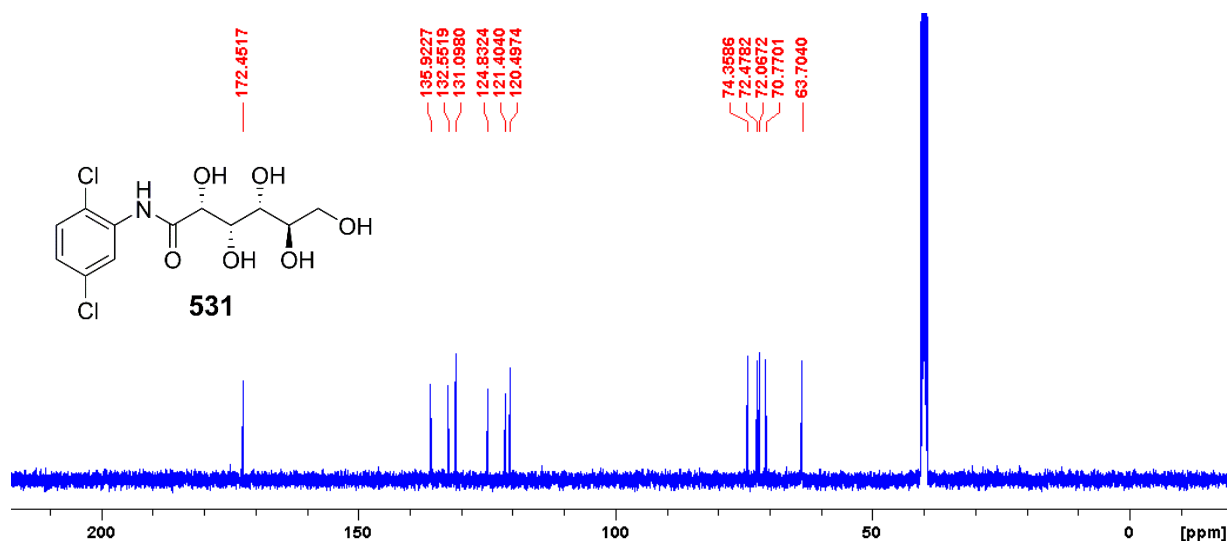
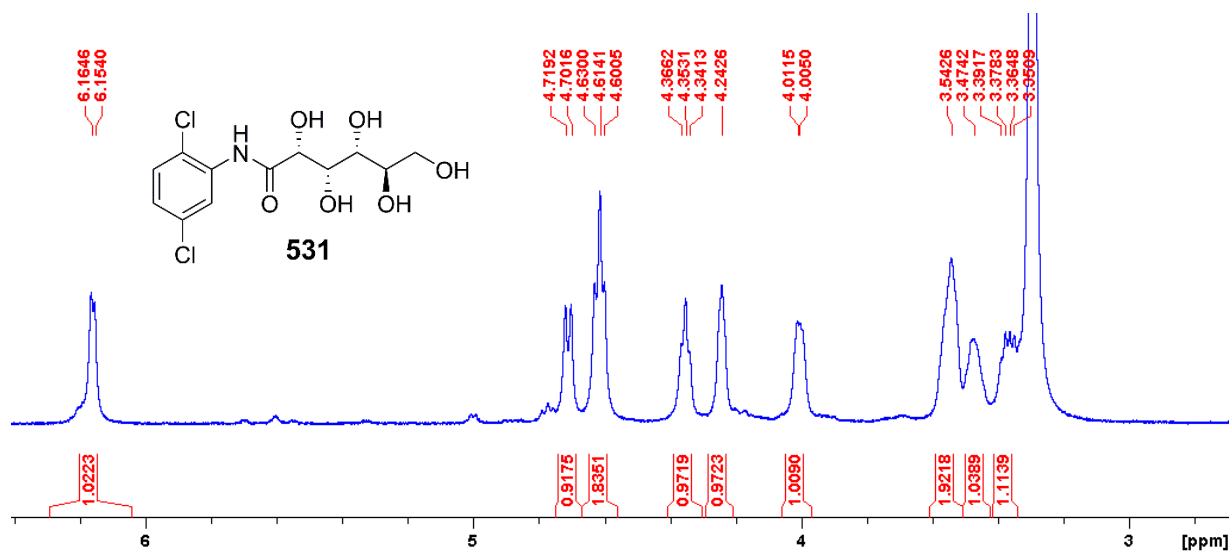


5.5.8 Characterization data and spectra of *N*-aryl-gluconamdes (531-541)

N-2,5-(Dichloro)phenyl-D-gluconamide (531)

To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in acetic acid (10 mL) was added 2,5-(dichloro)aniline (0.22 g, 1.34 mmol). The mixture was stirred under reflux for 3 hours. The crude product was precipitated with hexanes, filtered and crude solid was recrystallized in EtOH to afford **531** as white crystals (17 mg, 5%). ^1H NMR (400 MHz, DMSO- d_6): δ 9.43 (s, 1H), 8.39 (s, 1H), 7.53 (d, J = 8.7 Hz, 1H), 7.18 (d, J = 8.2 Hz, 1H), 6.16 (d, J = 4.3 Hz, 1H), 4.71 (d, J = 6.9 Hz, 1H), 4.61 (t, J = 6.1 Hz, 2H), 4.35 (t, J = 6.1 Hz, 1H), 4.24 (s, 1H), 4.00 (s, 1H), 3.54 (s, 2H), 3.47 (s, 1H), 3.36 (quint, J = 5.2, 5.6 Hz, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 172.5, 135.9, 132.6, 131.1, 124.8, 121.4, 120.5, 74.4, 72.5, 72.1, 70.8, 63.7. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{15}\text{Cl}_2\text{NNaO}_6$ $[\text{M}+\text{Na}]^+$ 363.1, found 363.9.





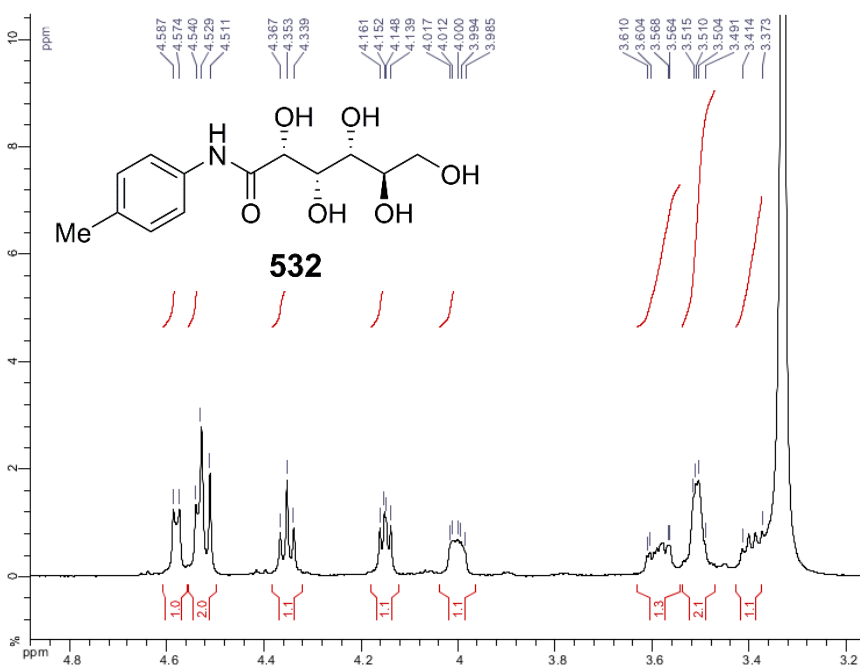
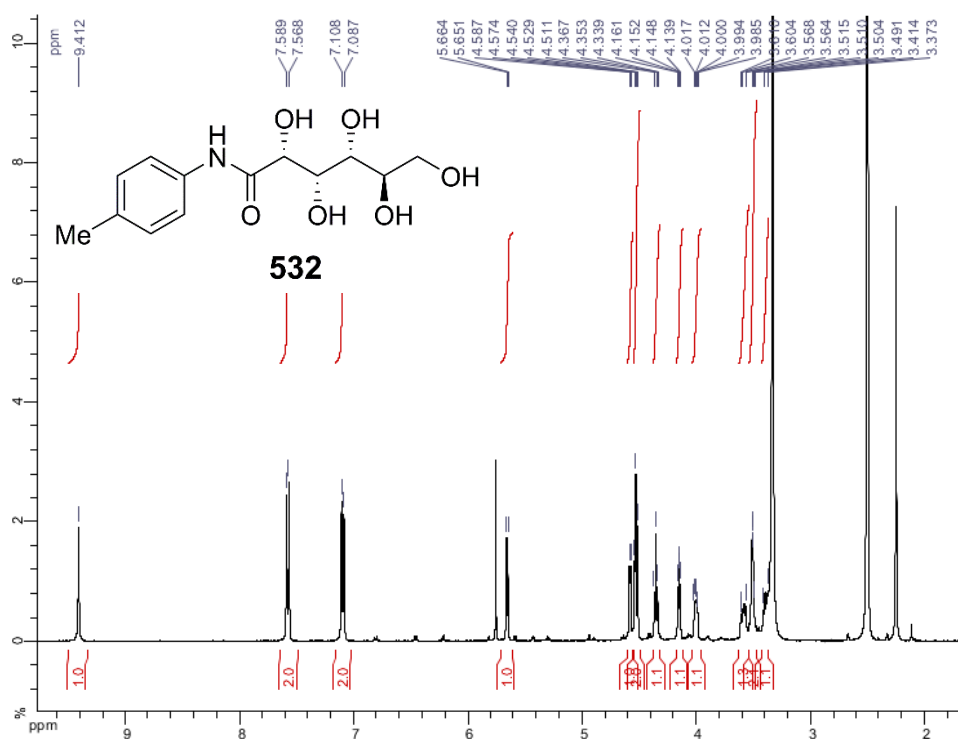
N-4-Methylphenyl-*D*-gluconamide (**532**)

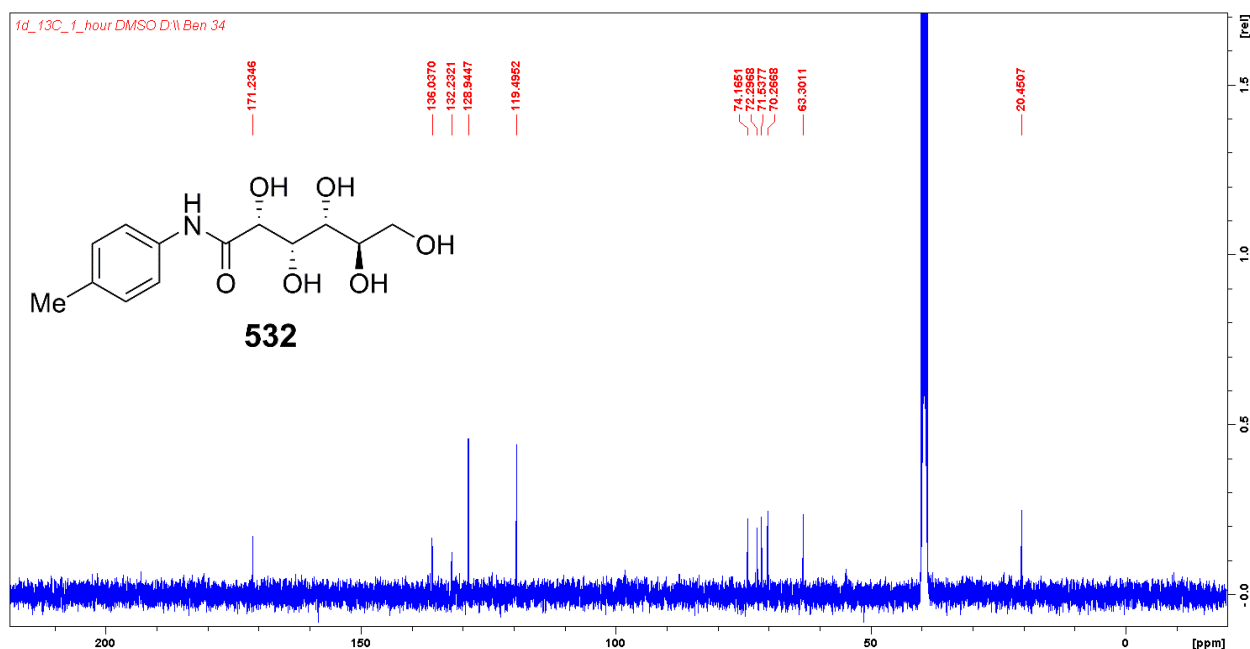
To a solution of *D*-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in methanol (10 mL) was added 4-methylaniline (0.12 g, 1.12 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the crude product was recrystallized in EtOH to afford **532** as white crystals (12 mg, 5%).

¹H NMR (400 MHz, DMSO-*d*₆): δ 9.41 (s, 1H), 7.58 (d, *J* = 8.4 Hz, 2H), 7.10 (d, *J* = 8.6 Hz, 2H), 5.66 (d, *J* = 5.3 Hz, 1H), 4.58 (d, *J* = 5.1 Hz, 1H), 4.51-4.54 (m, 2H), 4.35 (t, *J* = 5.7 Hz, 1H), 4.15 (dd, *J* = 4.8, 3.8 Hz, 1H), 3.99-4.02 (m, 1H), 3.56-3.61 (m, 1H), 3.49-3.52 (m, 2H), 3.37-3.41 (m, 1H), ¹³C NMR

(100 MHz, DMSO- d_6): δ 172.2, 136.0, 132.2, 128.9, 119.5, 74.2, 72.3, 71.5, 70.3, 63.3, 20.5. LRMS

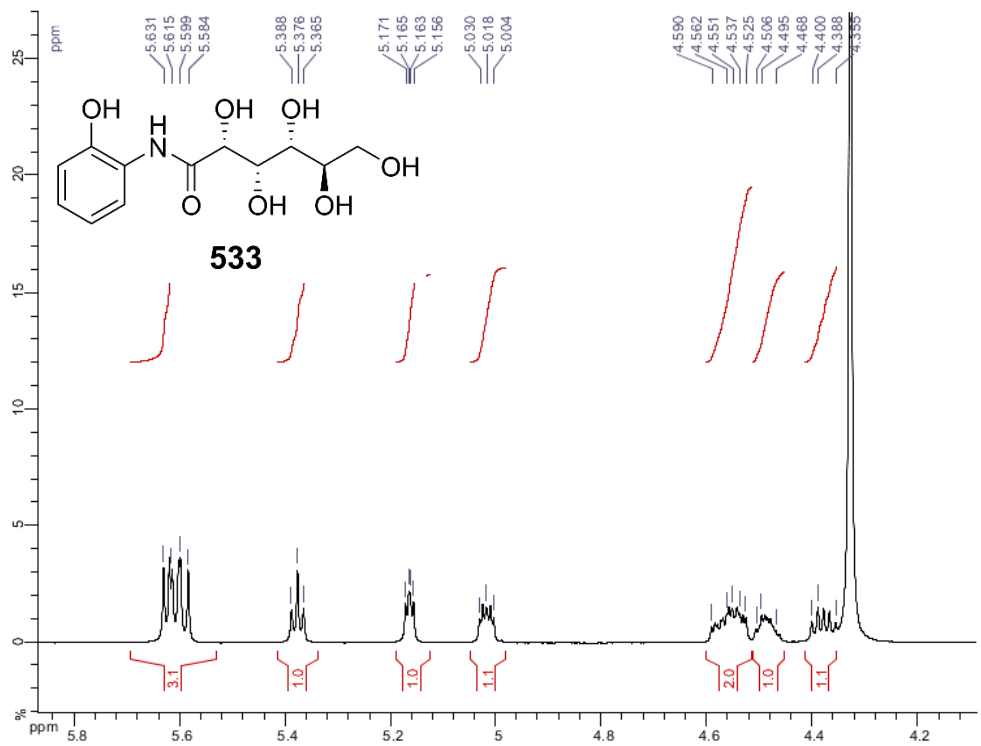
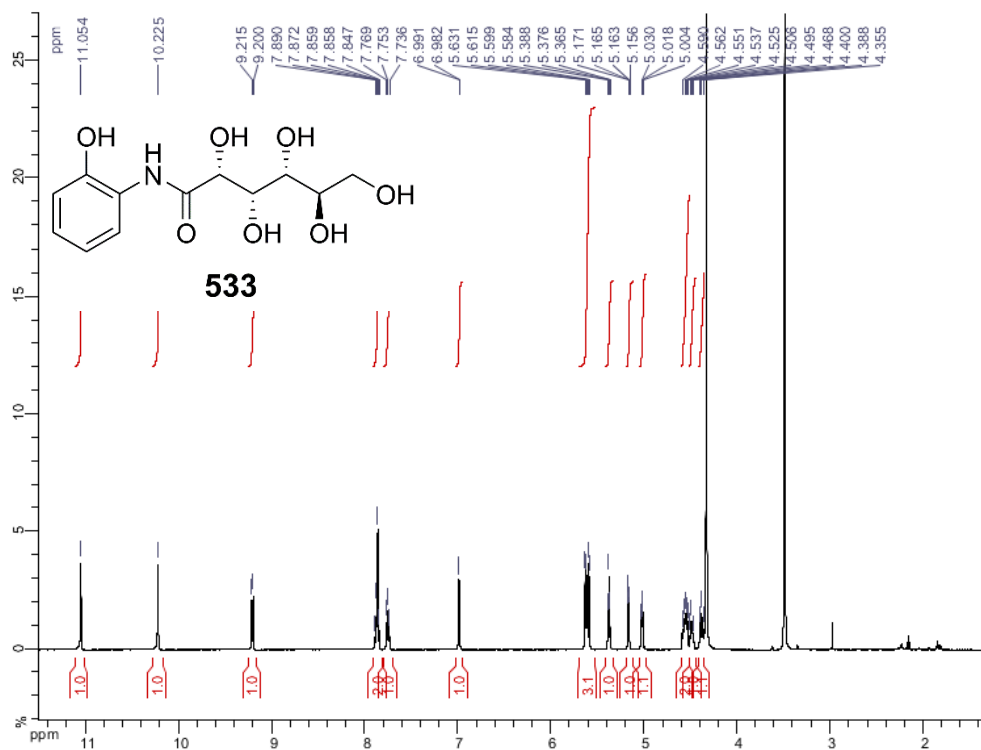
(ESI): m/z calcd. for $C_{13}H_{19}NNaO_6$ $[M+Na]^+$ 308.1, found 308.1.

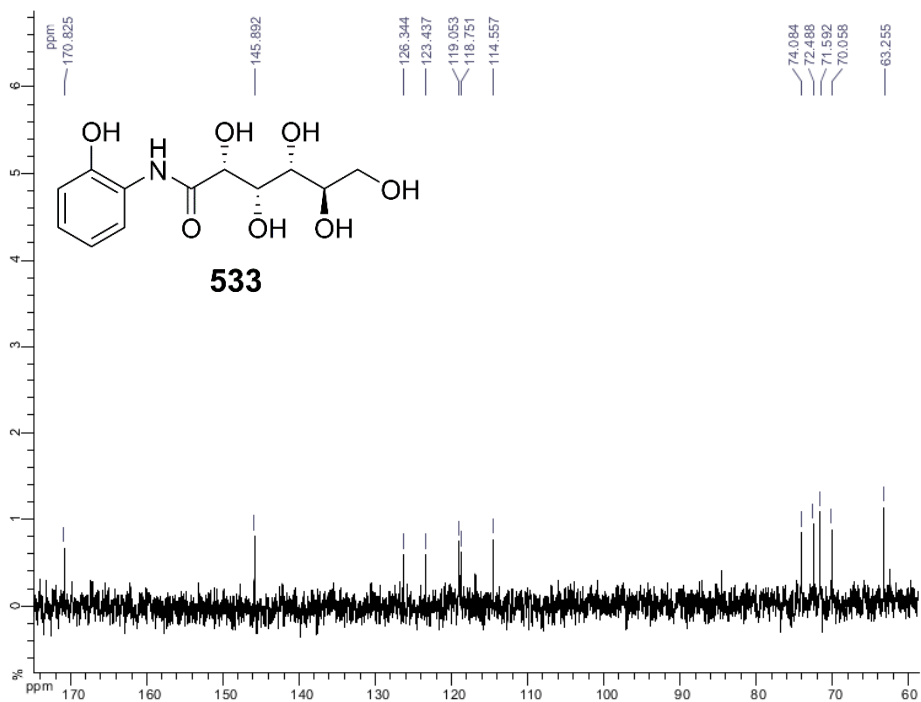
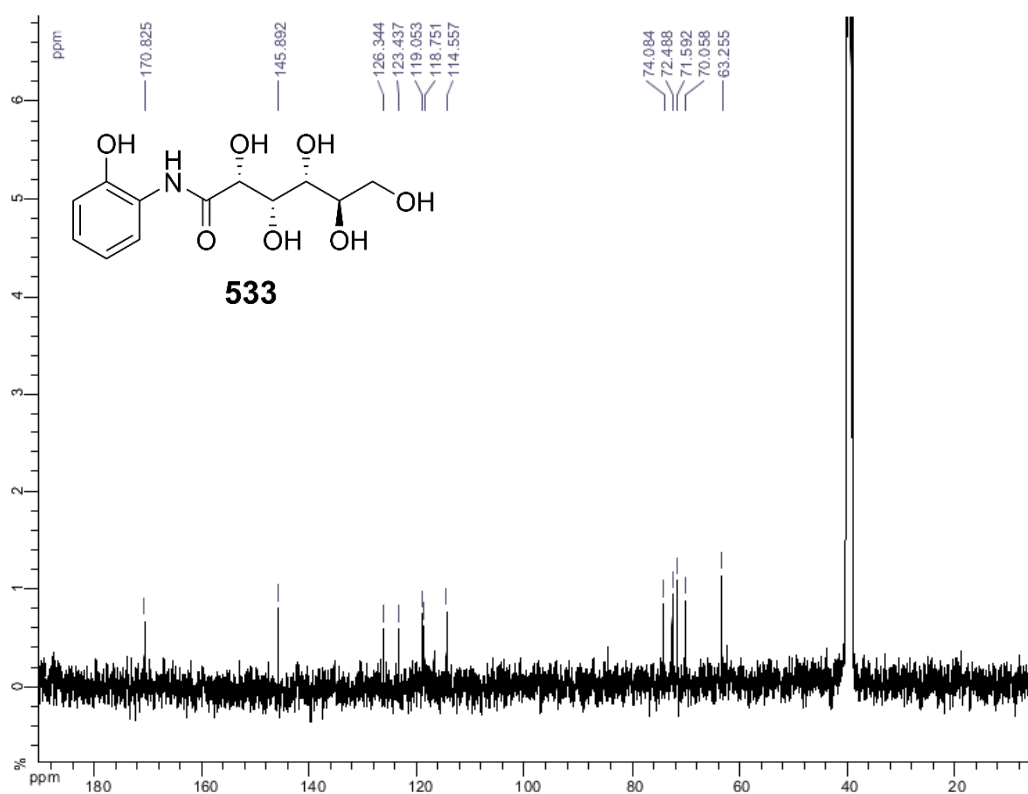




N-2-Hydroxyphenyl-*D*-gluconamide (**533**)

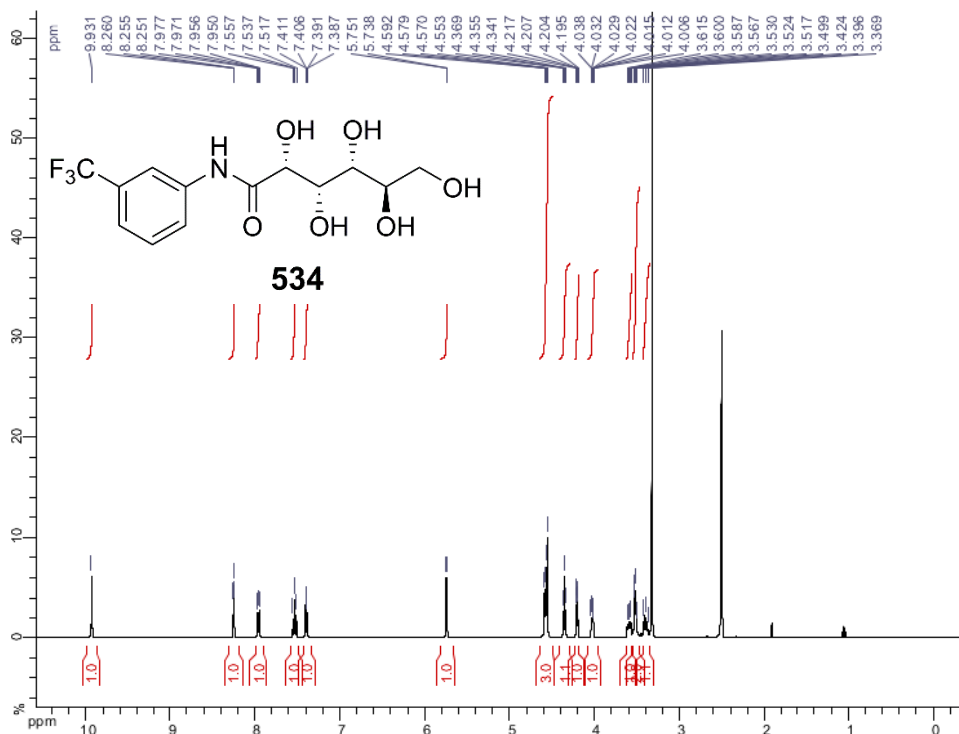
To a solution of *D*-gluconic acid- δ -lactone (0.10 g, 0.56 mmol) in acetic acid (5 mL) was added 2-aminophenol (0.12 g, 1.12 mmol). The mixture was stirred under reflux for 2 hours. The crude product was precipitated with hexanes, filtered and crude solid was recrystallized in EtOH to afford **533** as white crystals (100 mg, 62%). ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ 11.05 (s, 1H), 10.23 (s, 1H), 9.21 (dd, J = 1.4, 7.7 Hz, 1H), 7.85-7.89 (m, 2H), 7.74-7.77 (m, 1H), 6.99 (d, J = 4.6 Hz, 1H), 5.59-5.63 (m, 3H), 5.38 (t, J = 5.7 Hz, 1H), 5.16 (dd, J = 3.1, 4.6 Hz, 1H), 5.00-5.03 (m, 1H), 4.53-4.59 (m, 2H), 4.47-4.51 (m, 1H), 4.36-4.40 (m, 1H). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ 170.8, 145.9, 126.3, 123.4, 119.1, 118.8, 114.6, 74.1, 72.5, 71.6, 70.1, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{17}\text{NNaO}_7$ $[\text{M}+\text{Na}]^+$ 310.1, found 310.1.

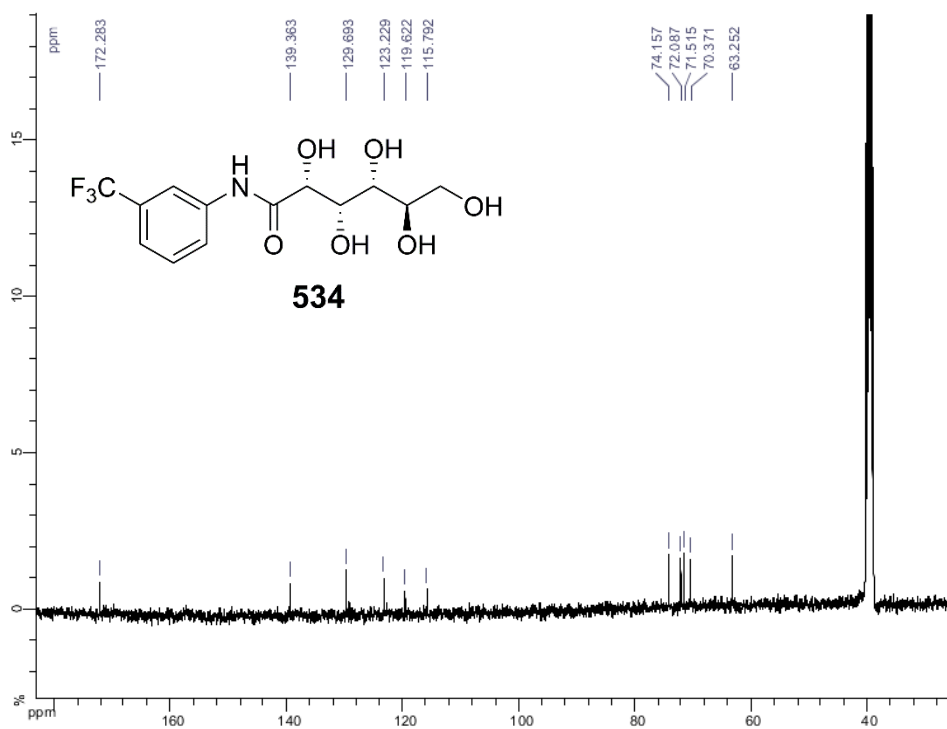
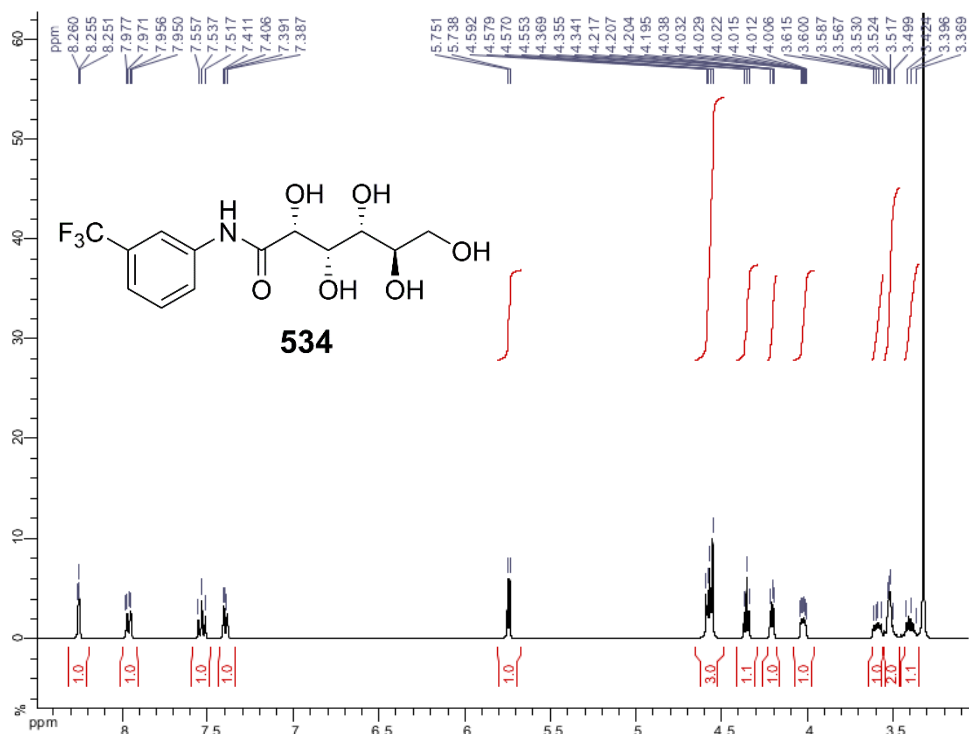




***N*-3-(Trifluoromethyl)phenyl-D-gluconamide (534)**

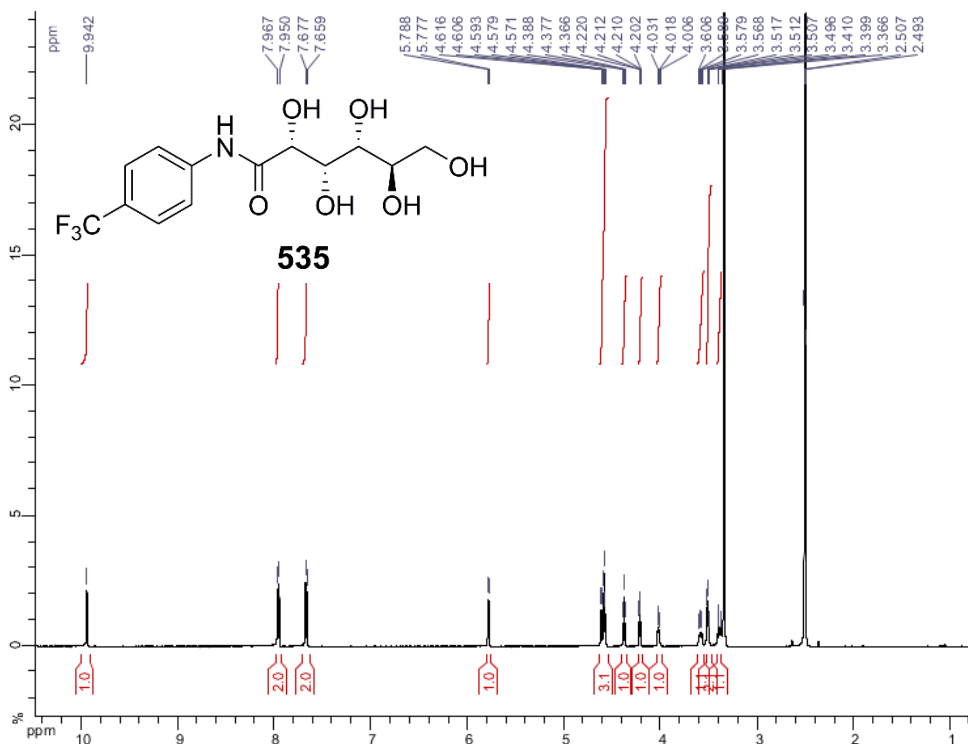
To a solution of D-gluconic acid- δ -lactone (0.60 g, 3.37 mmol) in acetic acid (10 mL) was added 3-(trifluoromethyl)aniline (0.84 mL, 6.74 mmol). The mixture was stirred under reflux for 1 hour. The crude product was precipitated with hexanes, filtered and crude solid was recrystallized in EtOH to afford **534** as white crystals (685 mg, 59%). ^1H NMR (400 MHz, DMSO- d_6): δ 9.93 (s, 1H), 8.26 (t, J = 2.0 Hz, 1H), 7.96 (dd, J = 2.3, 8.4 Hz, 1H), 7.54 (t, J = 8.1 Hz, 1H), 7.4 (dd, J = 1.8, 7.7 Hz, 1H), 5.74 (d, J = 5.2 Hz, 1H), 4.55-4.59 (m, 3H), 4.36 (t, J = 5.6 Hz, 1H), 4.21 (dd, J = 3.7, 5.2 Hz, 1H), 4.01-4.04 (m, 1H), 3.57-3.62 (m, 1H), 3.50-3.53 (m, 2H), 3.37-3.42 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 172.3, 139.4, 129.7, 123.2, 119.6, 115.8, 74.2, 72.1, 71.5, 70.4, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{16}\text{F}_3\text{NNaO}_6$ $[\text{M}+\text{Na}]^+$ 362.1, found 361.9.

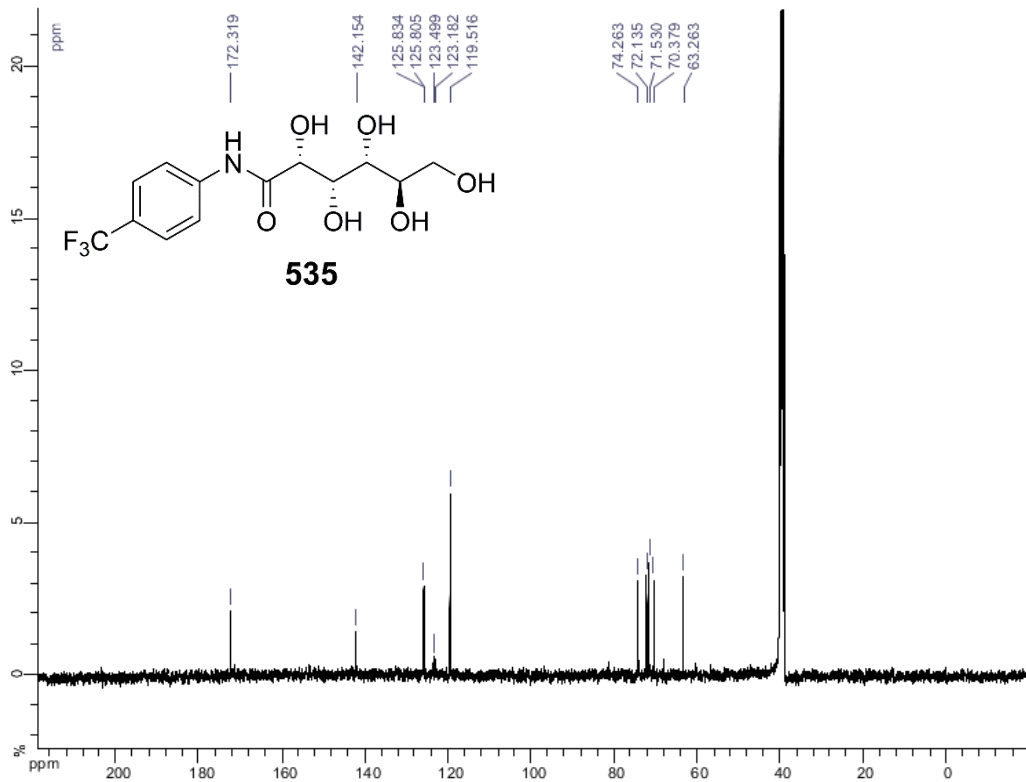
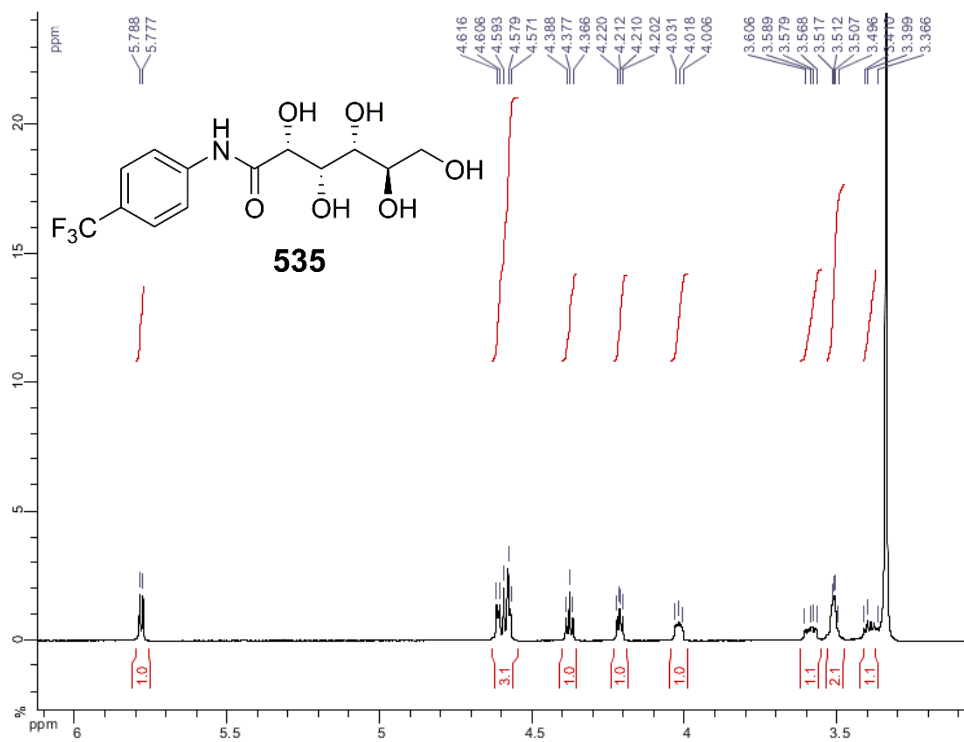




***N*-4-(Trifluoromethyl)phenyl-D-gluconamide (535)**

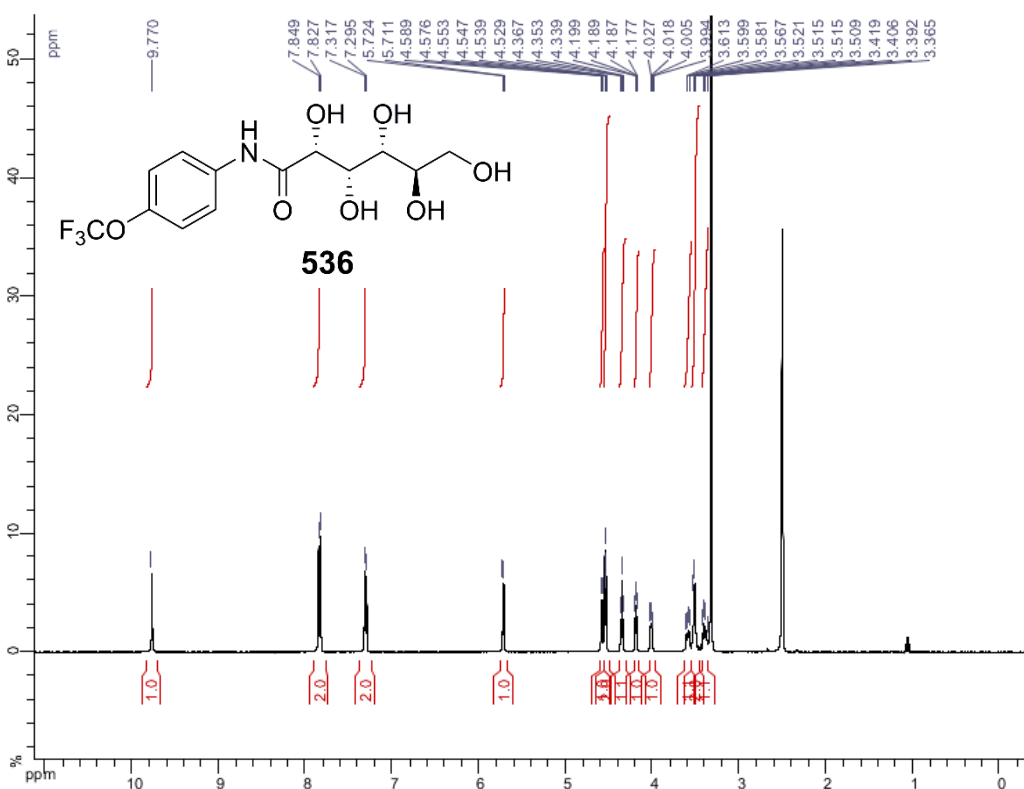
To a solution of D-gluconic acid- δ -lactone (0.10 g, 0.56 mmol) in acetic acid (5 mL) was added 4-(trifluoromethyl)aniline (0.10 mL, 0.56 mmol). The mixture was stirred under reflux for 2 hours. The crude product was precipitated with hexanes, filtered and recrystallized in EtOH to afford **535** as white crystals (89 mg, 47%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$): δ 9.94 (s, 1H), 7.96 (d, $J = 8.9$ Hz, 2H), 7.67 (d, $J = 8.9$ Hz, 2H), 5.78 (d, $J = 5.2$ Hz, 1H), 4.57-4.62 (m, 3H), 4.38 (t, $J = 5.7$ Hz, 1H), 4.21 (dd, $J = 3.9$, 5.3 Hz, 1H), 4.01-4.03 (m, 1H), 3.57-3.61 (m, 1H), 3.50-3.52 (m, 2H), 3.37-3.41 (m, 1H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 172.3, 142.2, 125.8 (d, $J_{\text{C,F}} = 3.8$ Hz), 123.3 (d, $J_{\text{C,F}} = 33.0$ Hz), 119.5, 74.3, 72.1, 71.5, 70.4, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{16}\text{F}_3\text{NNaO}_6$ $[\text{M}+\text{Na}]^+$ 362.1, found 362.0.

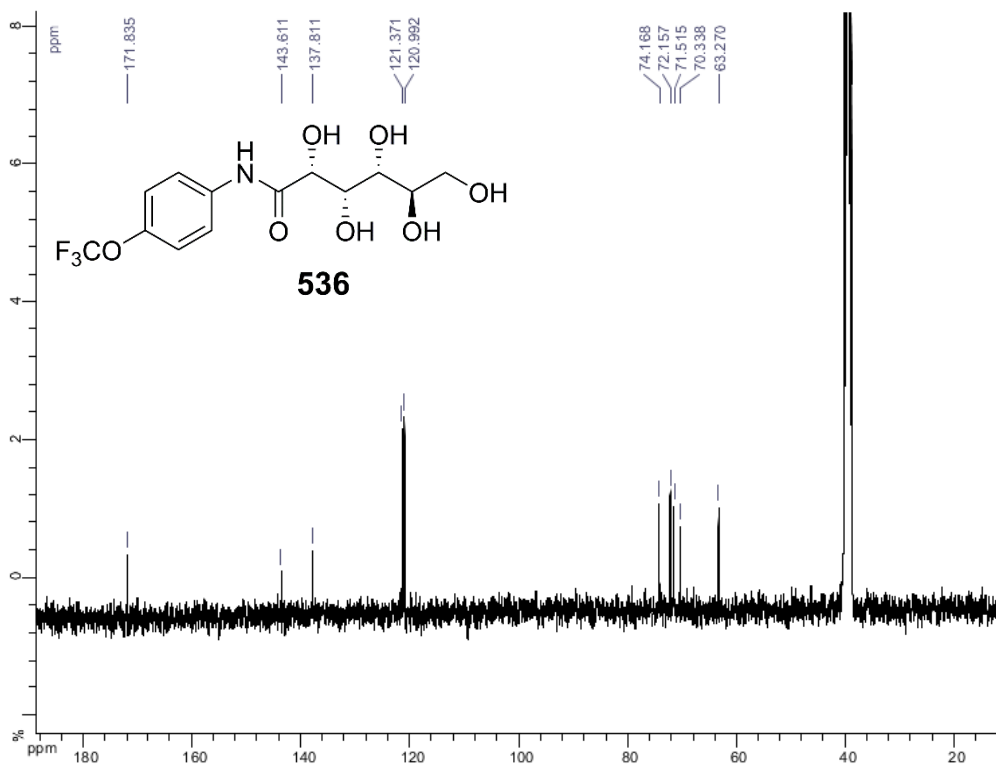
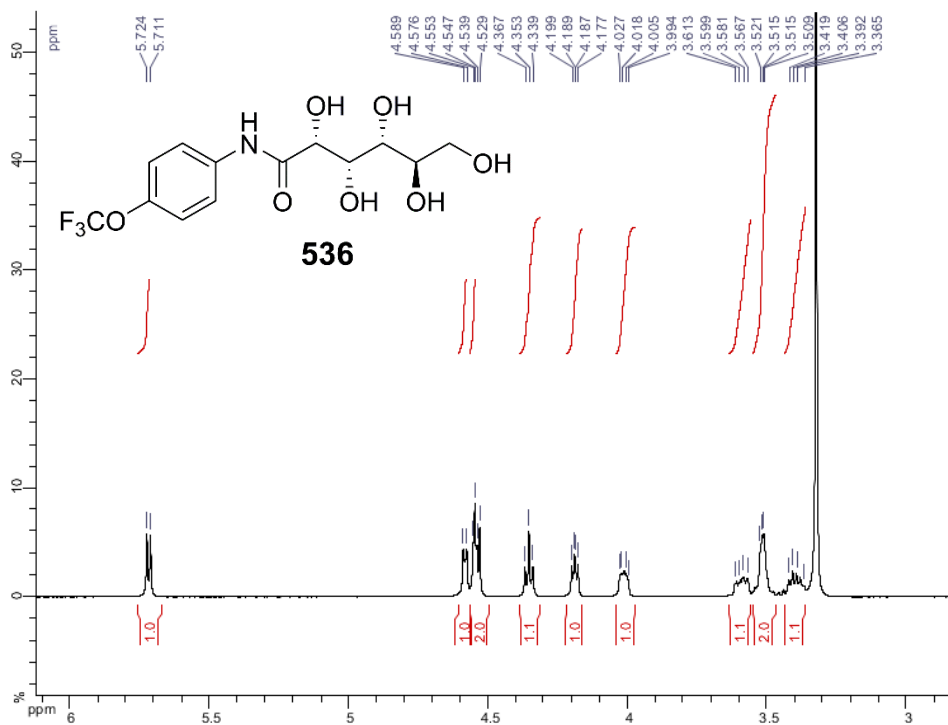




***N*-4-(Trifluoromethoxy)phenyl-D-gluconamide (536)**

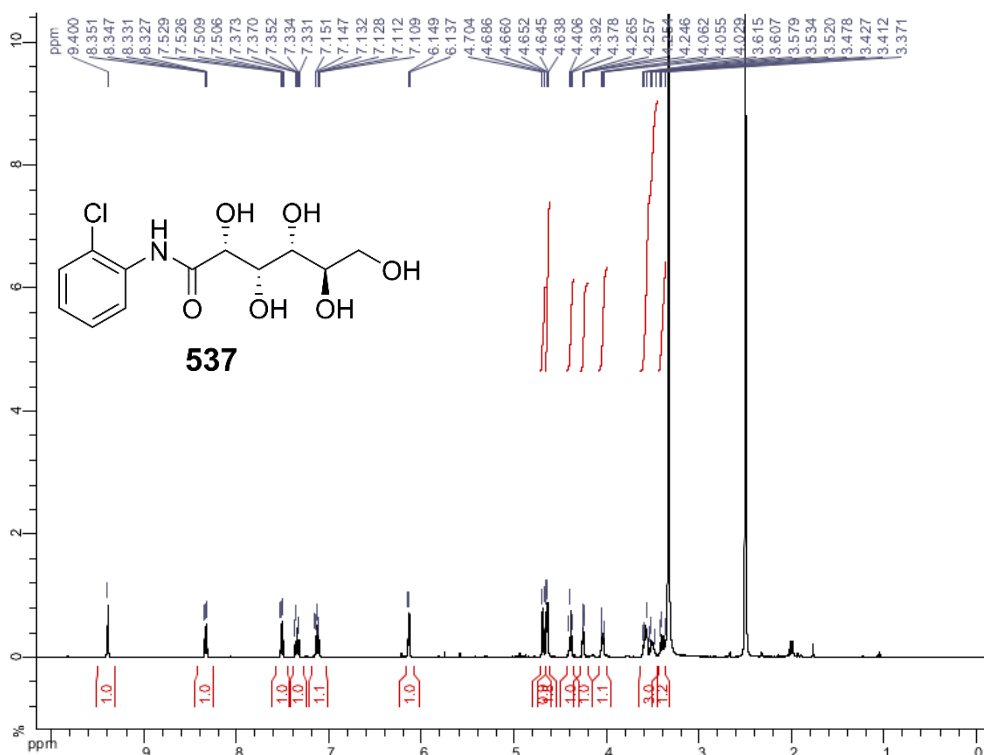
To a solution of D-gluconic acid- δ -lactone (0.50 g, 2.80 mmol) in acetic acid (10 mL) was added 4-(trifluoromethoxy)aniline (0.75 mL, 5.60 mmol). The mixture was stirred under reflux for 1 hour. The crude product was precipitated with hexanes, filtered and the crude solid was recrystallized in EtOH to afford **536** as white crystals (580 mg, 58%). ^1H NMR (400 MHz, DMSO- d_6): δ 9.77 (s, 1H), 7.84 (d, J = 9.1 Hz, 2H), 7.31 (d, J = 9.0 Hz, 2H), 5.72 (d, J = 5.2 Hz, 1H), 4.58 (d, J = 4.0 Hz, 1H), 4.53-4.55 (m, 2H), 4.35 (t, J = 5.7 Hz, 1H), 4.19 (dd, J = 3.9, 5.3 Hz, 1H), 3.99-4.03 (m, 1H), 3.57-3.60 (m, 1H), 3.51-3.52 (m, 2H), 3.37-3.42 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 171.8, 143.6, 137.8, 121.4, 121.0, 74.2, 72.2, 71.5, 70.3, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{16}\text{F}_3\text{NNaO}_7$ $[\text{M}+\text{Na}]^+$ 378.1, found 377.9.

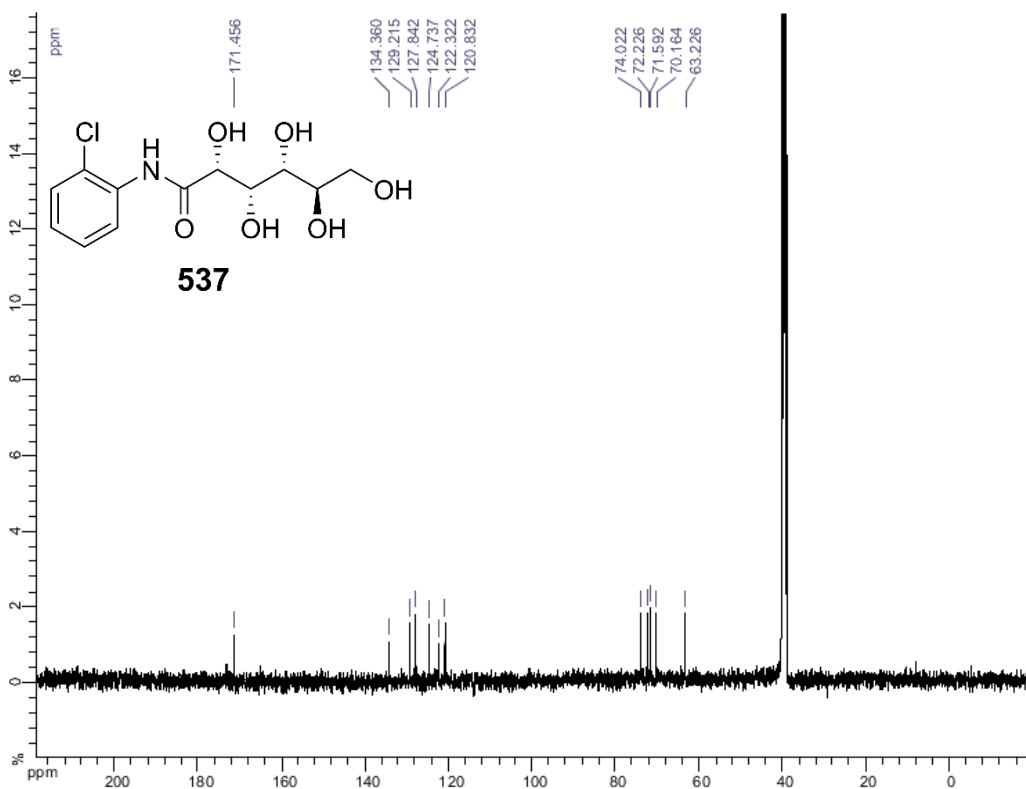
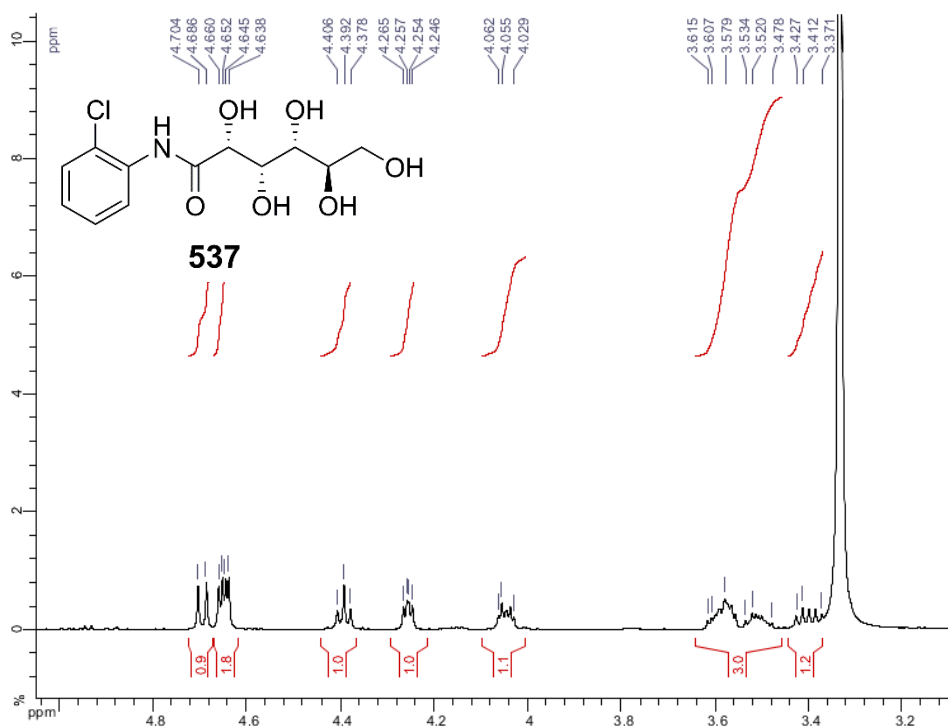




N-2-Chlorophenyl-*D*-gluconamide (**537**)

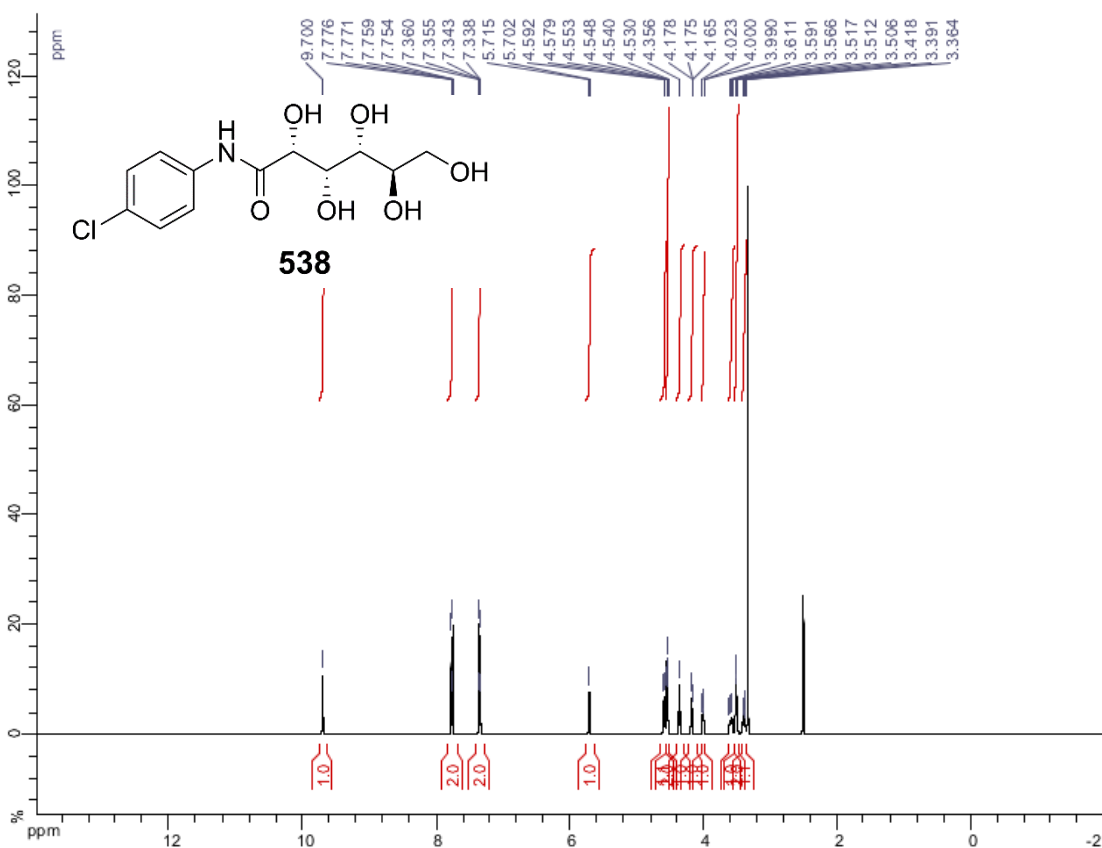
To a solution of *D*-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in acetic acid (5 mL) was added 2-chloroaniline (0.12 mL, 1.12 mmol). The mixture was stirred under reflux for 2 hours. The crude product was precipitated with hexanes, filtered and recrystallized in EtOH to afford **537** as white crystals (10 mg, 3%). ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 9.40 (s, 1H), 8.34 (dd, $J = 1.6, 8.3$ Hz, 1H), 7.52 (dd, $J = 1.5, 8.0$ Hz, 1H), 7.35 (dt, $J = 1.6, 7.8$ Hz, 1H), 7.13 (dt, $J = 1.6, 7.7$ Hz, 1H), 6.14 (d, $J = 4.7$ Hz, 1H), 4.7 (d, $J = 7.4$ Hz, 1H), 4.65 (dd, $J = 2.8, 6.0$ Hz, 2H), 4.39 (t, $J = 5.7$ Hz, 1H), 4.26 (dd, $J = 3.1, 4.8$ Hz, 1H), 4.05 (td, $J = 3.0, 7.5$ Hz, 1H), 3.48-3.61 (m, 3H), 3.37-3.43 (m, 1H). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ 171.5, 134.4, 129.2, 127.8, 124.7, 122.3, 120.8, 74.0, 72.2, 71.6, 70.2, 63.2. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{16}\text{ClNNaO}_6$ $[\text{M}+\text{Na}]^+$ 328.1, found 328.0.

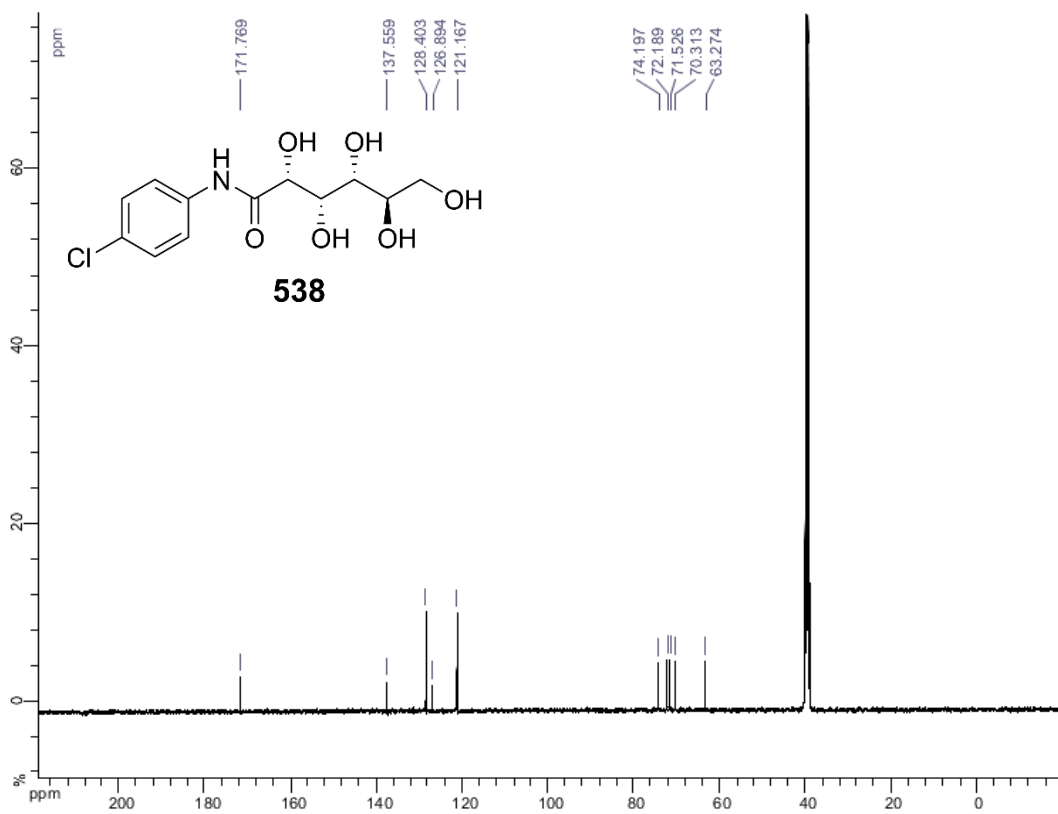
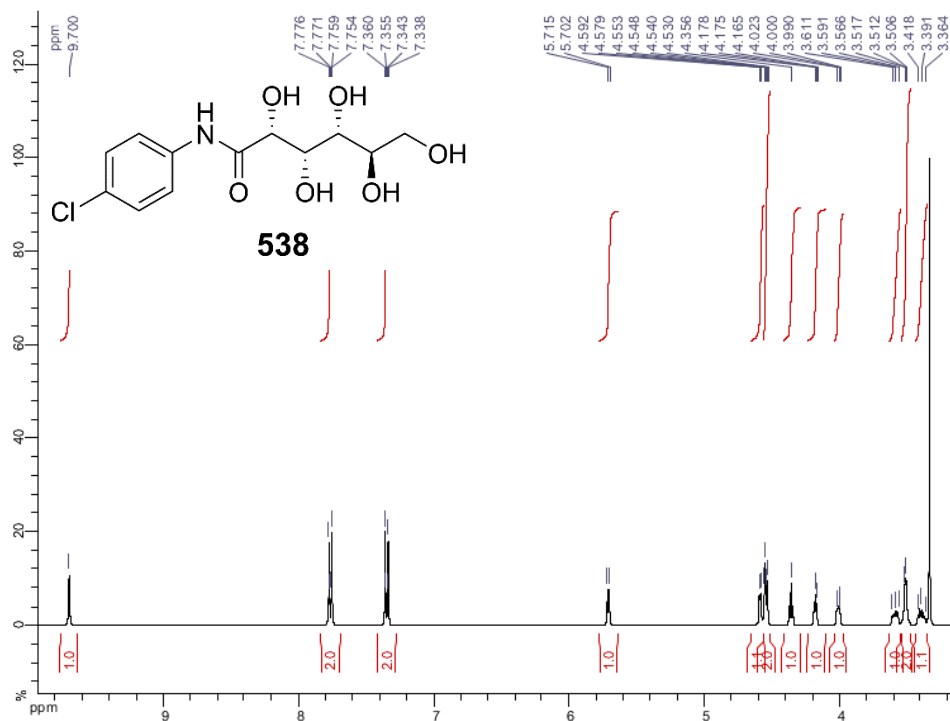




***N*-4-Chlorophenyl-D-gluconamide (538)**

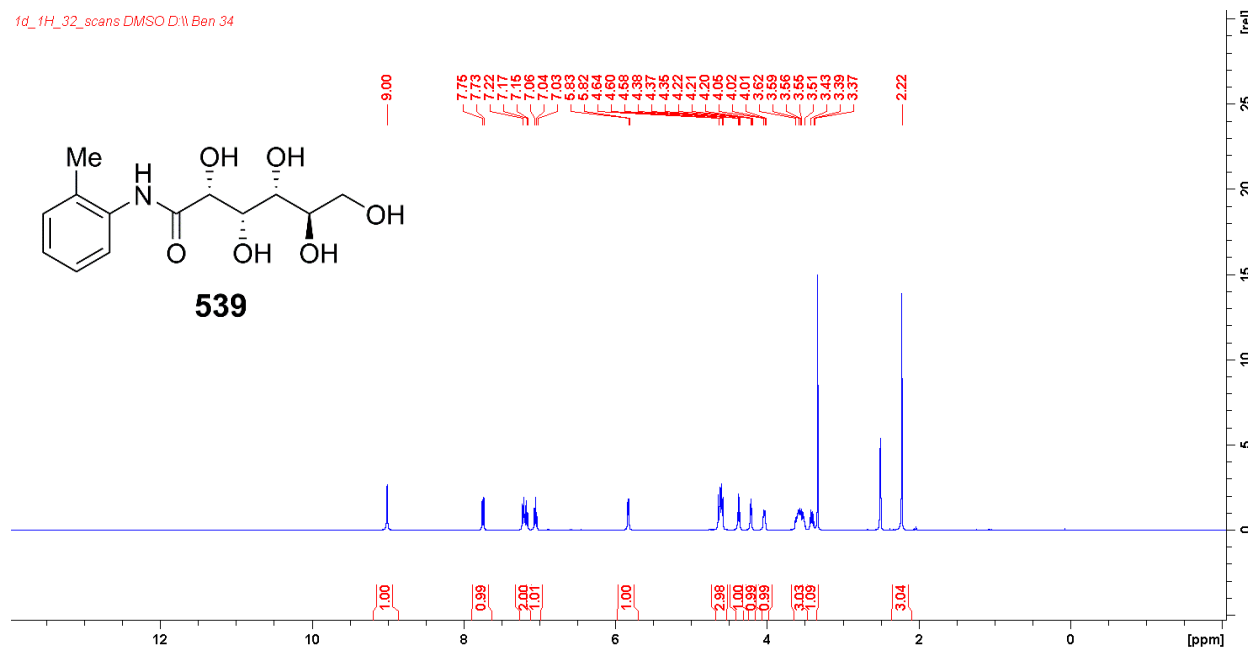
To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in acetic acid (5 mL) was added 4-chloroaniline (0.12 mL, 1.12 mmol). The mixture was stirred under reflux for 2 hours. The crude product was precipitated with hexanes, filtered and recrystallized in EtOH to afford **538** as white crystals (180mg, 52%). ^1H NMR (400 MHz, DMSO- d_6): δ 9.70 (s, 1H), 7.80 (d, J = 9.1 Hz, 2H), 7.35 (d, J = 8.8 Hz, 2H), 5.71 (d, J = 5.3 Hz, 1H), 4.59 (d, J = 4.9 Hz, 1H), 4.55-4.53 (m, 2H), 4.36 (t, J = 5.7 Hz, 1H), 4.18 (dd, J = 5.1, 3.7 Hz, 1H), 4.02-3.99 (m, 1H), 3.61-3.57 (m, 1H), 3.52-3.51 (m, 2H), 3.42-3.36 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 171.8, 137.6, 128.4, 126.9, 121.2, 74.2, 72.2, 71.5, 70.3, 63.3. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{16}\text{ClNNaO}_6$ $[\text{M}+\text{Na}]^+$ 328.1, found 328.0.

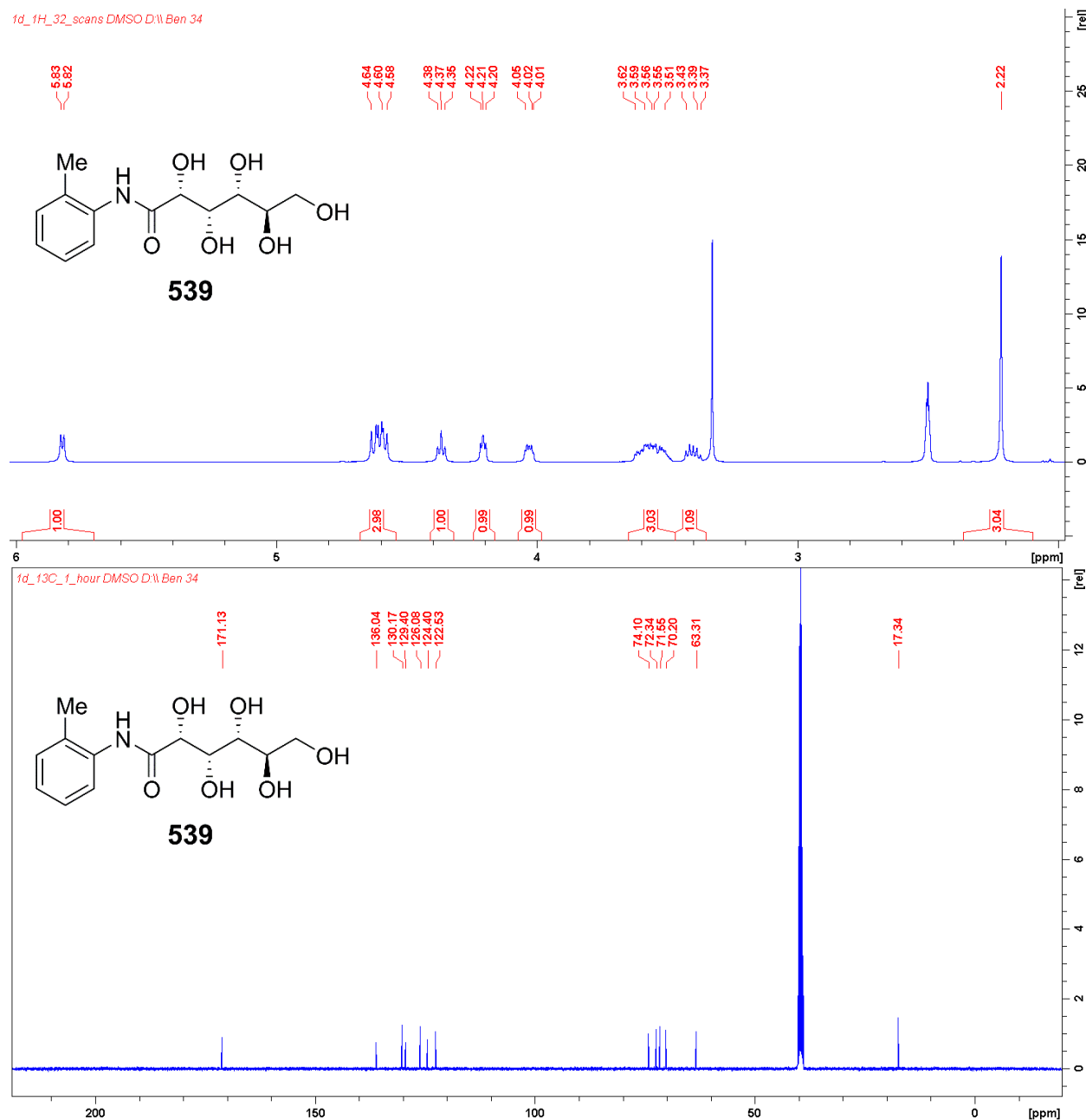




***N*-2-Methylphenyl-*D*-gluconamide (539)**

To a solution of *D*-gluconic acid-*d*-lactone (0.50 g, 2.81 mmol) in acetic acid (5 mL) was added 2-methylaniline (0.6 mL, 5.62 mmol). The mixture was stirred under reflux for two hours. The crude product was precipitated with hexanes, filtered and crude solid was recrystallized in EtOH to afford **539** as white crystals (705 mg, 88%). ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 9.00 (s, 1H), 7.74 (d, $J = 7.6$ Hz, 1H), 7.15-7.22 (m, 2H), 7.03-7.06 (m, 1H), 5.83 (d, $J = 4.8$ Hz, 1H), 4.58-4.64 (m, 3H), 4.37 (t, $J = 5.9$ Hz, 1H), 4.20-4.22 (m, 1H), 4.01-4.05 (m, 1H), 3.51-3.62 (m, 3H), 3.37-3.43 (m, 1H), 2.22 (s, 3H). ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ 171.1, 136.0, 130.2, 129.4, 126.1, 124.4, 122.5, 74.1, 72.3, 71.6, 70.2, 63.3, 17.3. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{19}\text{NaNO}_6$ $[\text{M}+\text{Na}]^+$ 308.1, found 308.2.

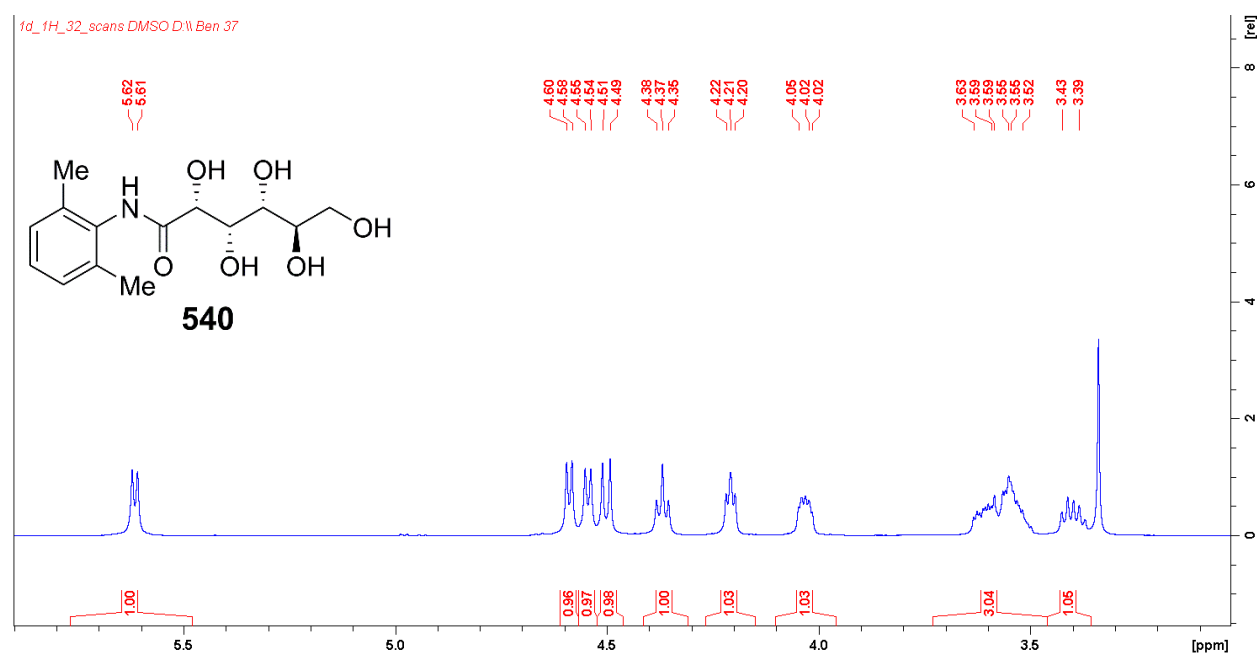
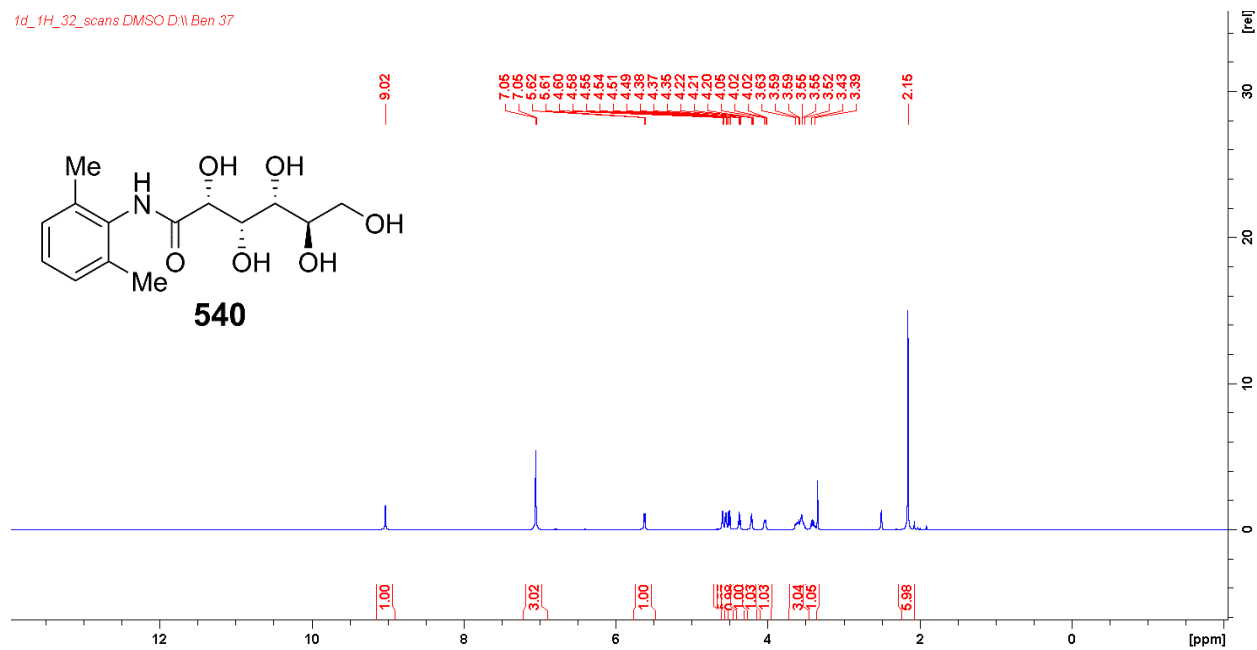


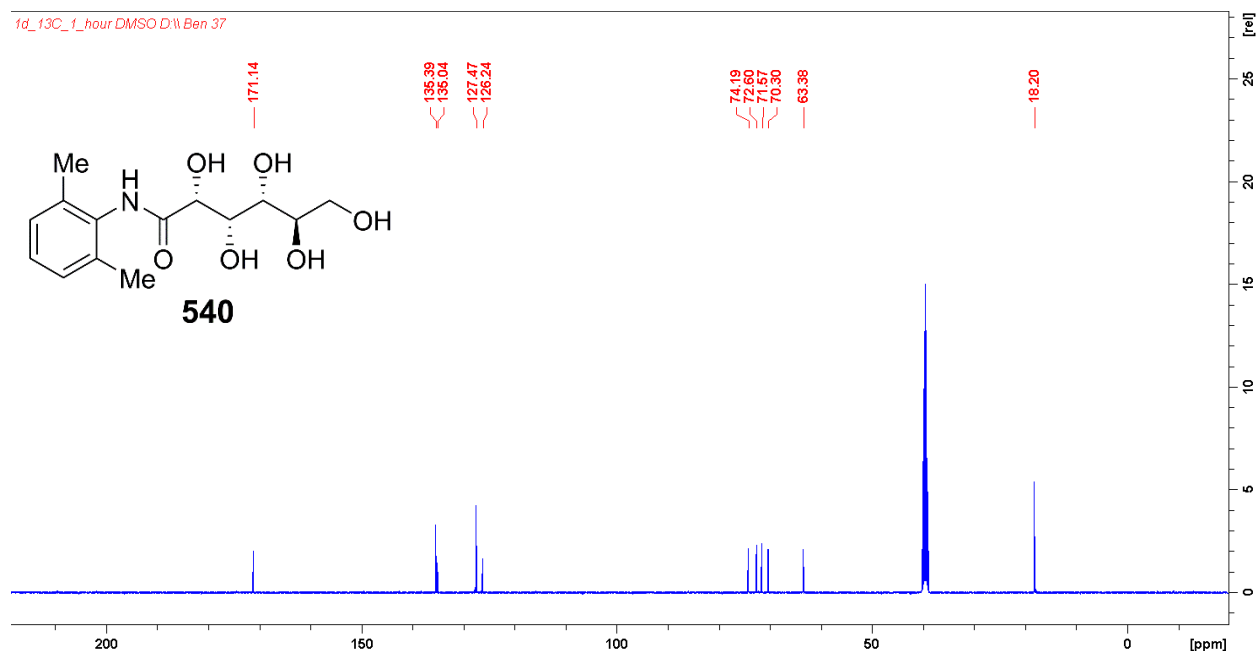


N-2,6-Dimethylphenyl-D-gluconamide (**540**)

To a solution of D-gluconic acid- δ -lactone (0.50 g, 2.81 mmol) in acetic acid (5 mL) was added 2,6-dimethylaniline (0.7 mL, 5.62 mmol). The mixture was stirred under reflux for two hours. The crude product was precipitated with hexanes, filtered and recrystallized in EtOH to afford **540** as white crystals (690 mg, 82%). ^1H NMR (400 MHz, DMSO- d_6): δ 9.02 (s, 1H), 7.05-7.07 (m, 3H), 5.62 (d, J = 5.0, 1H), 4.59 (d, J = 5.0 Hz, 1H), 4.55 (d, J = 5.4 Hz, 1H), 4.50 (d, J = 7.3 Hz, 1H), 4.37 (t, J = 5.5 Hz, 1H), 4.21

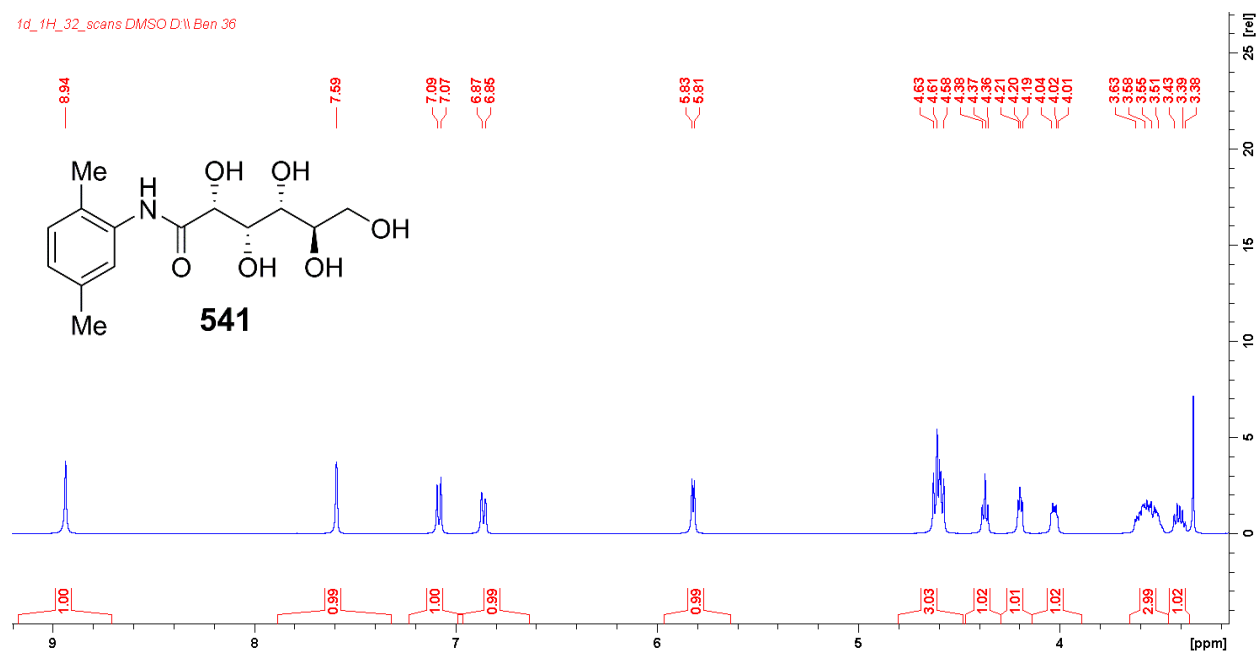
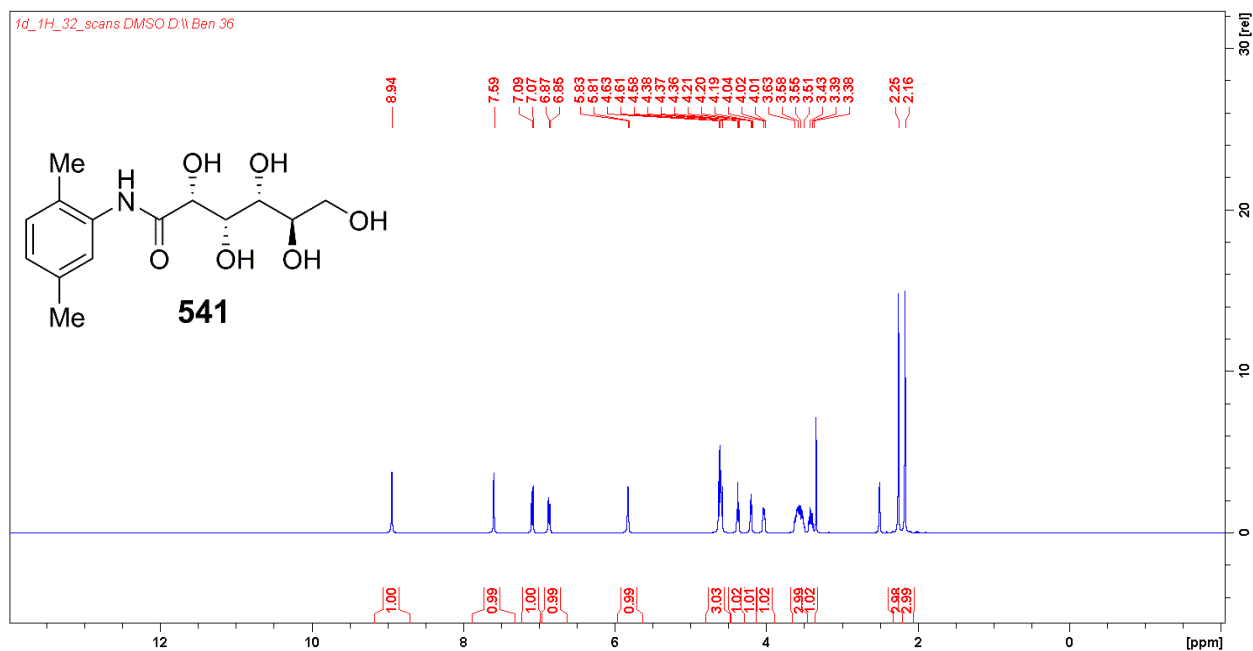
(t, $J = 4.1$ Hz, 1H), 4.02-4.05 (m, 1H), 3.52-3.63 (m, 3H), 3.39-3.43 (m, 1H), 2.15 (s, 6H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 171.1, 135.4, 135.0, 127.5, 126.2, 74.2, 72.6, 71.6, 70.3, 63.4, 18.2. LRMS (ESI): m/z calcd. for $\text{C}_{14}\text{H}_{21}\text{NNaO}_6$ $[\text{M}+\text{Na}]^+$ 322.1, found 322.2.

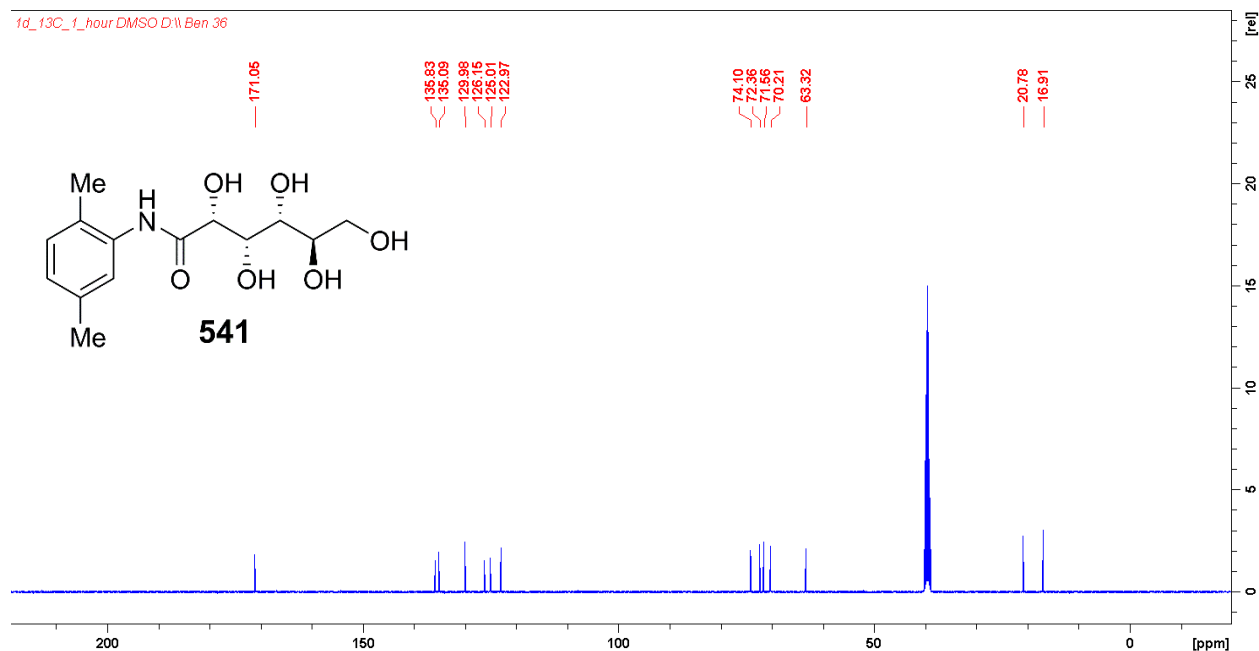




N-2,5-Dimethylphenyl-D-gluconamide (**541**)

To a solution of D-gluconic acid- δ -lactone (0.50 g, 2.81 mmol) in acetic acid (5 mL) was added 2,5-dimethylaniline (0.7 mL, 5.62 mmol). The mixture was stirred under reflux for two hours. The crude product was precipitated with hexanes, filtered and recrystallized in EtOH to afford **541** as white crystals (650 mg, 77%). ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.94 (s, 1H), 7.59 (s, 1H), 7.08 (d, *J* = 7.7 Hz, 1H), 6.86 (d, *J* = 7.4 Hz, 1H), 5.82 (d, *J* = 4.4 Hz, 1H), 4.58 (m, 3H), 4.37 (t, *J* = 5.6 Hz, 1H), 4.19-4.21 (m, 1H), 4.01-4.04 (m, 1H), 3.51-3.63 (m, 3H), 3.38-3.49 (m, 1H), 2.25 (s, 3H), 2.16 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 171.1, 135.8, 135.1, 130.0, 126.2, 125.0, 123.0, 74.1, 72.4, 71.6, 70.2, 63.3, 20.8, 16.9. LRMS (ESI): *m/z* calcd. for C₁₄H₂₁NNaO₆ [M+Na]⁺ 322.1, found 322.2.

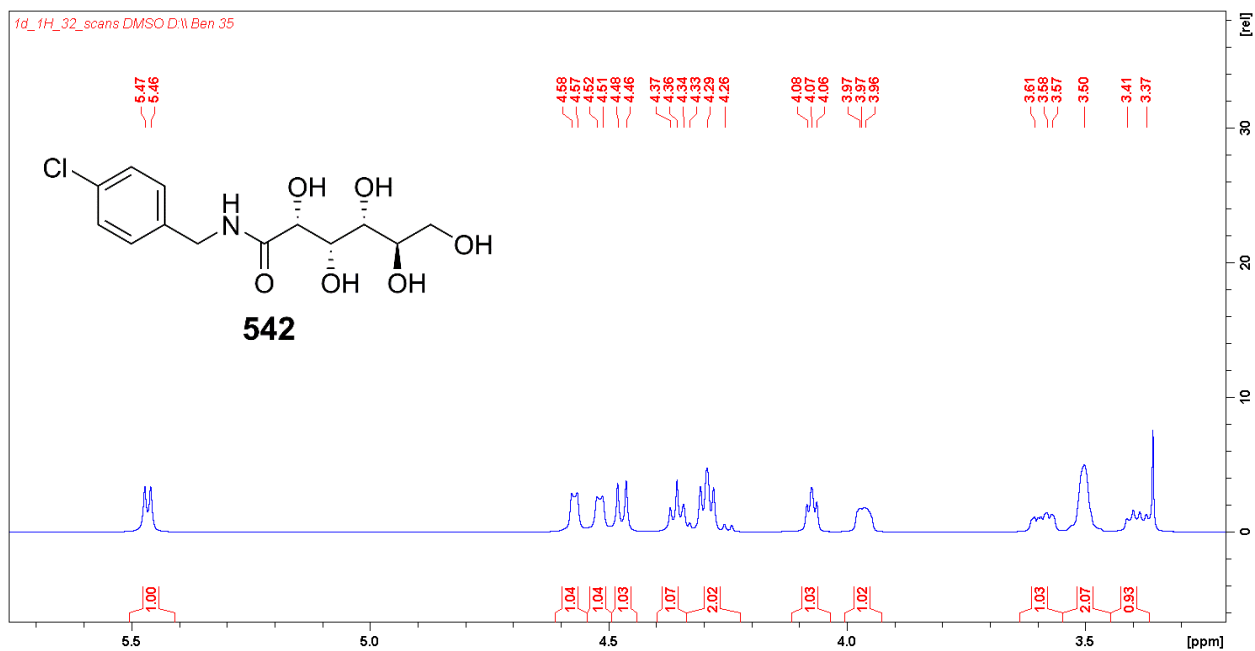
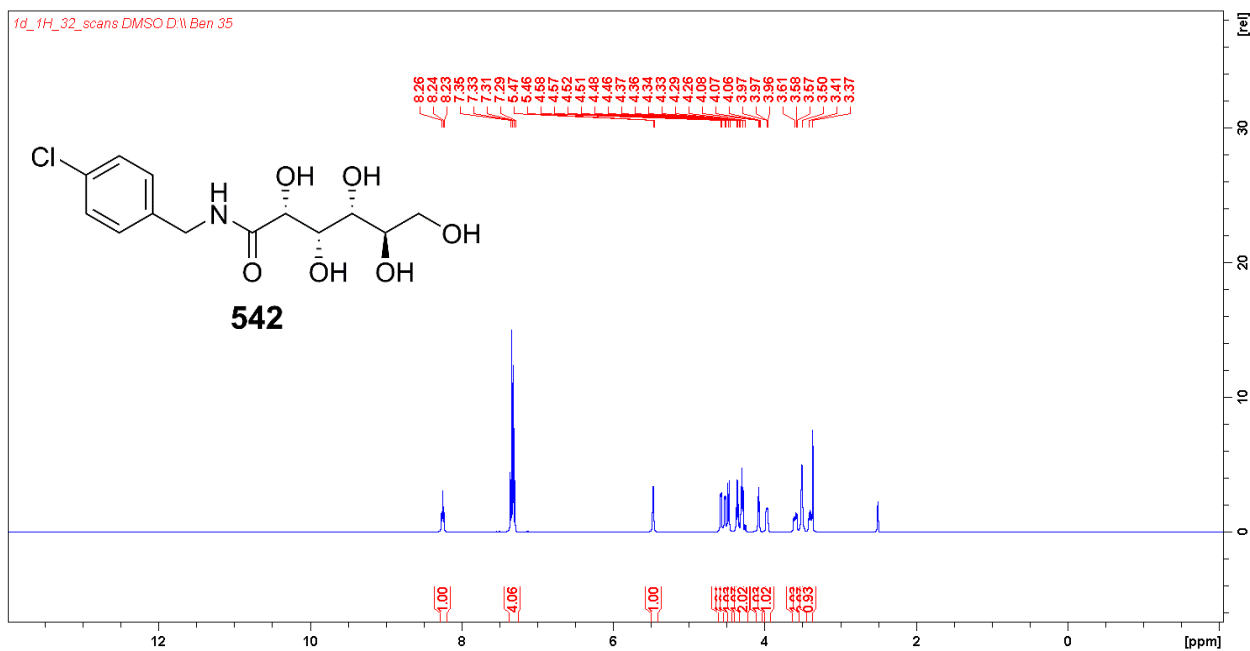


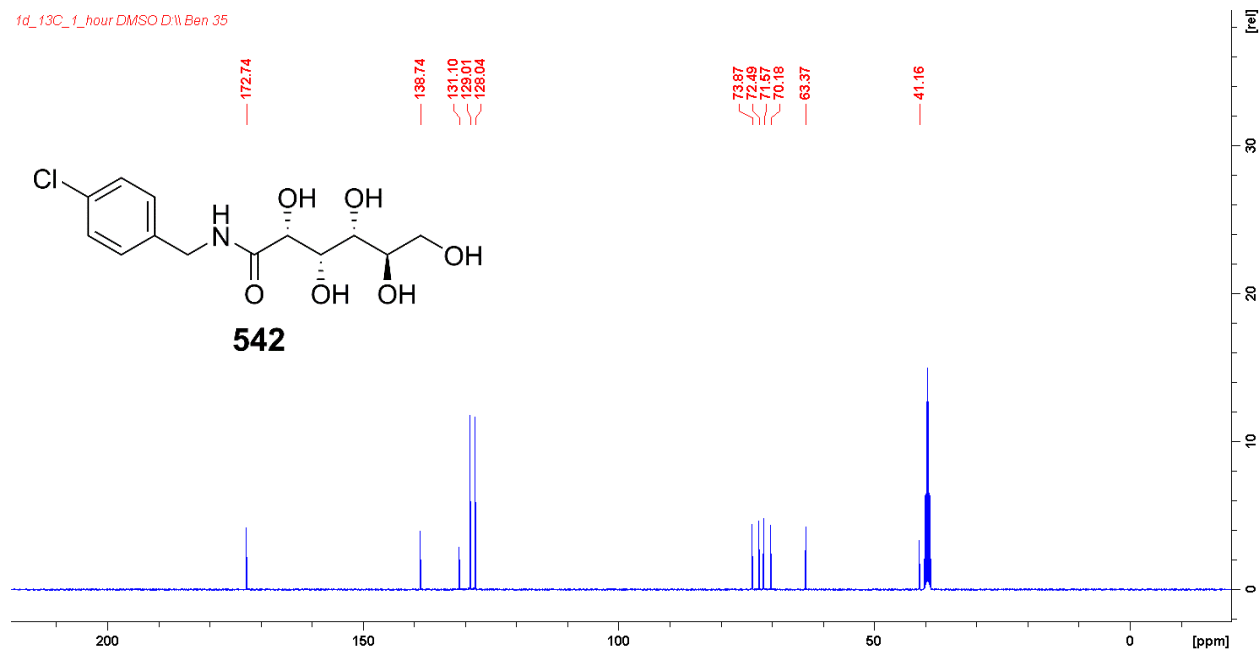


5.5.9 Characterization data and spectra of *N*-benzyl-gluconamides (542-547)

N-4-Chlorobenzyl-D-gluconamide (542)

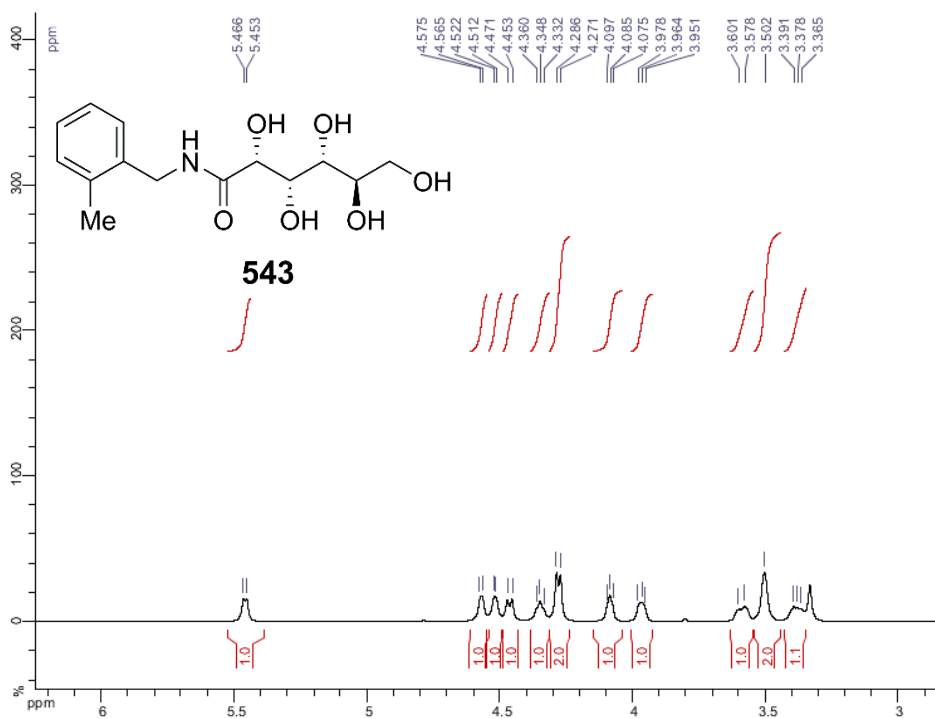
To a solution of D-gluconic acid- δ -lactone (0.89 g, 5.02 mmol) in methanol (30 mL) was added 4-chlorobenzylamine (0.8 mL, 5.02 mmol). The mixture was stirred under reflux for 48 hours. The solvent was evaporated and the residue was recrystallized in EtOH to afford **542** as white crystals (760 mg, 85%). Characterization data are consistent with those of previously reported data.³⁴ ^1H NMR (400 MHz, DMSO- d_6): δ 8.24 (t, J = 6.3 Hz, 1H), 7.32 (dd, J = 16.1, 8.8 Hz, 4H), 5.47 (d, J = 5.0 Hz, 1H), 4.58 (d, J = 4.7 Hz, 1H), 4.52 (d, J = 4.6 Hz, 1H), 4.47 (d, J = 7.2 Hz, 1H), 4.36 (t, J = 5.6 Hz, 1H), 4.26-4.33 (m, 2H), 4.07 (t, J = 4.0 Hz, 1H), 3.96-3.97 (m, 1H), 3.57-3.61 (m, 1H), 3.50 (m, 2H), 3.37-3.41 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 172.7, 138.7, 131.1, 129.0, 128.0, 73.9, 72.5, 71.6, 70.2, 63.4, 41.2. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{18}\text{ClNNaO}_6$ $[\text{M}+\text{Na}]^+$ 342.1, found 342.2.

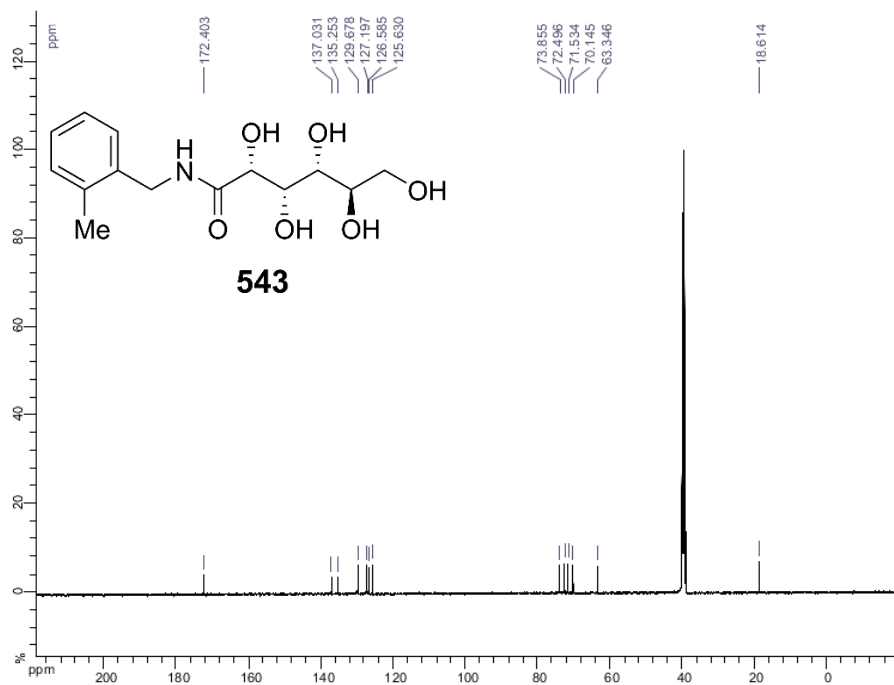




N-2-Methylbenzyl-*D*-gluconamide (**543**)

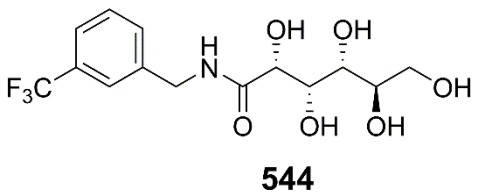
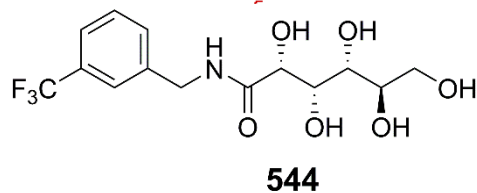
To a solution of *D*-gluconic acid- δ -lactone (0.10 g, 0.56 mmol) in methanol (10 mL) was added 2-methylbenzylamine (0.10 mL, 0.56 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the crude product was recrystallized in EtOH to afford **543** as white crystals (123 mg, 73%). ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.97 (t, *J* = 6.1 Hz, 1H), 7.27-7.11 (m, 4H), 5.46 (d, *J* = 4.8 Hz, 1H), 4.58 (d, *J* = 3.9 Hz, 1H), 4.52 (d, *J* = 4.2 Hz, 1H), 4.46 (d, *J* = 6.3 Hz, 1H), 4.35 (t, *J* = 4.8 Hz, 1H), 4.28 (d, *J* = 5.9 Hz, 2H), 4.10 (t, *J* = 4.6 Hz, 1H), 3.98-3.95 (m, 1H), 3.60-3.58 (m, 1H), 3.50 (s, 2H), 3.39-3.37 (m, 1H), 2.26 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 172.4, 137.0, 135.3, 129.7, 127.2, 126.6, 125.6, 73.9, 72.5, 71.5, 70.2, 63.4, 18.6. LRMS (ESI): *m/z* calcd. for C₁₄H₂₂NO₆ [M + H]⁺ 300.1, found 300.1.

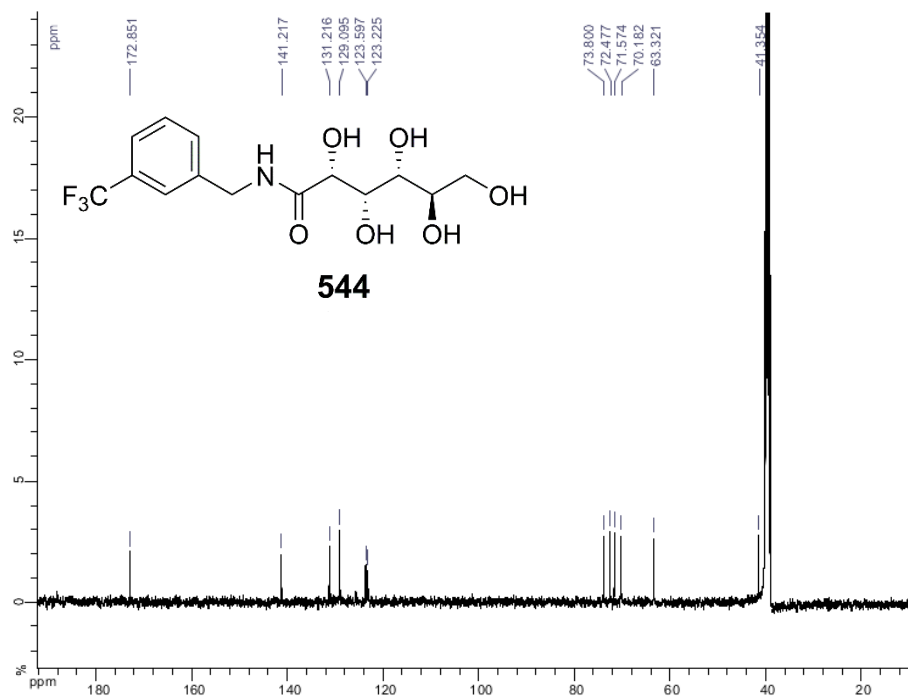




***N*-3-(Trifluoromethyl)benzyl-D-gluconamide (544)**

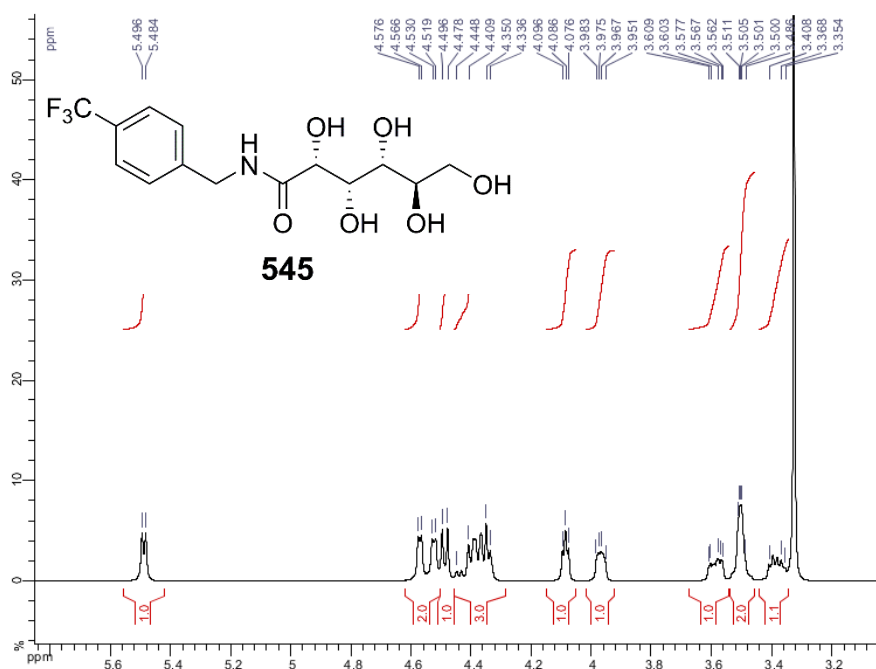
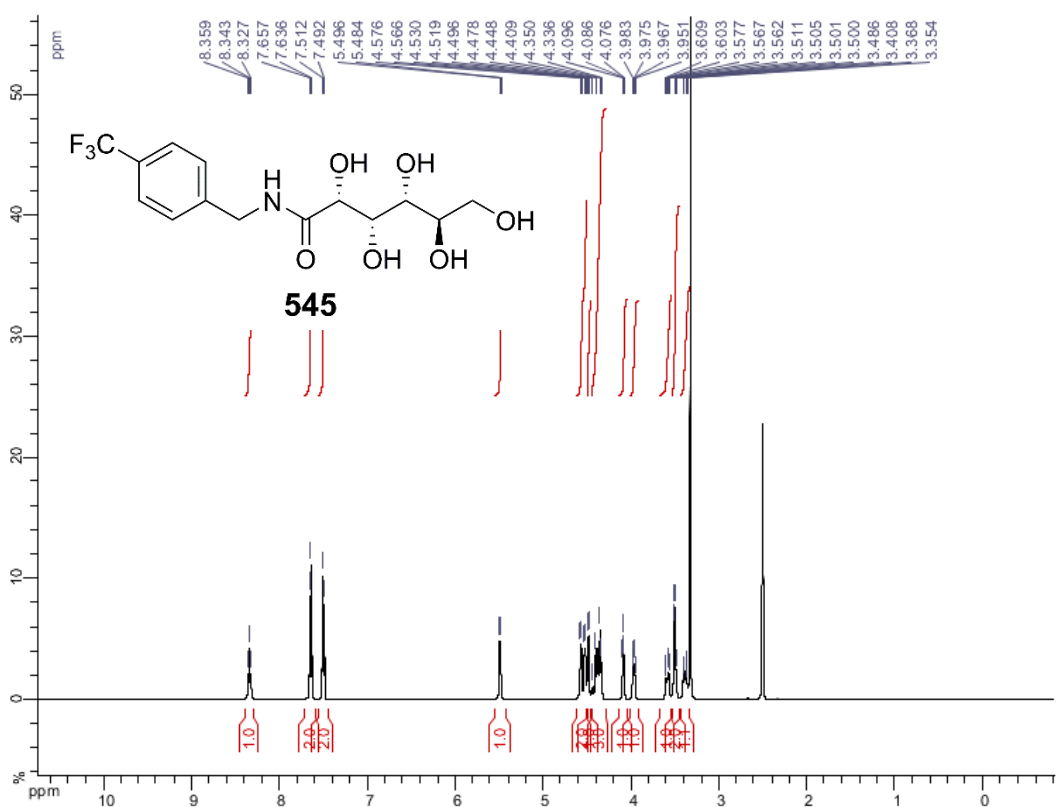
To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in methanol (10 mL) was added 3-(trifluoromethyl)benzylamine (0.32 mL, 2.24 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the crude product was recrystallized in EtOH to afford **544** as white crystals (245 mg, 62%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 8.36 (t, J = 6.4 Hz, 1H), 7.51-7.65 (m, 4H), 5.5 (d, J = 5.1 Hz, 1H), 4.55 (dd, J = 17.8, 5.1 Hz, 2H), 4.34-4.45 (m, 4H), 4.09 (dd, J = 3.5, 5.1 Hz, 1H), 3.95-3.98 (m, 1H), 3.56-3.61 (m, 1H), 3.49-3.51 (m, 2H), 3.35-3.40 (m, 1H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 172.9, 141.2, 131.2, 129.1, 123.6, 123.2, 73.8, 72.5, 71.6, 70.2, 63.3, 41.4. LRMS (ESI): m/z calcd. for $\text{C}_{14}\text{H}_{18}\text{F}_3\text{NNaO}_6$ $[\text{M}+\text{Na}]^+$ 376.1, found 375.9.

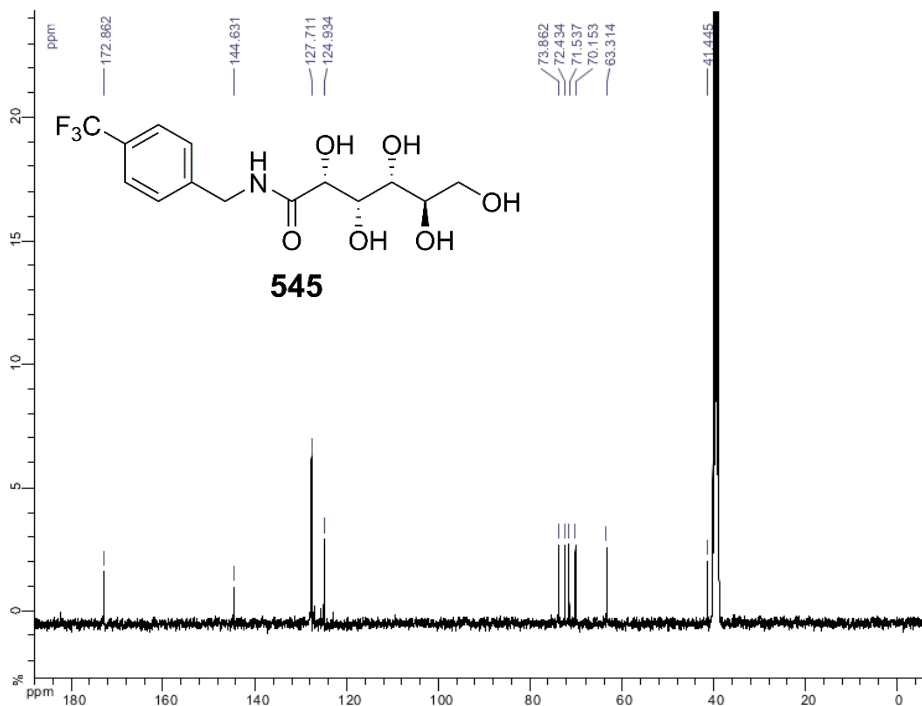




N-4-(Trifluoromethyl)benzyl-D-gluconamide (**545**)

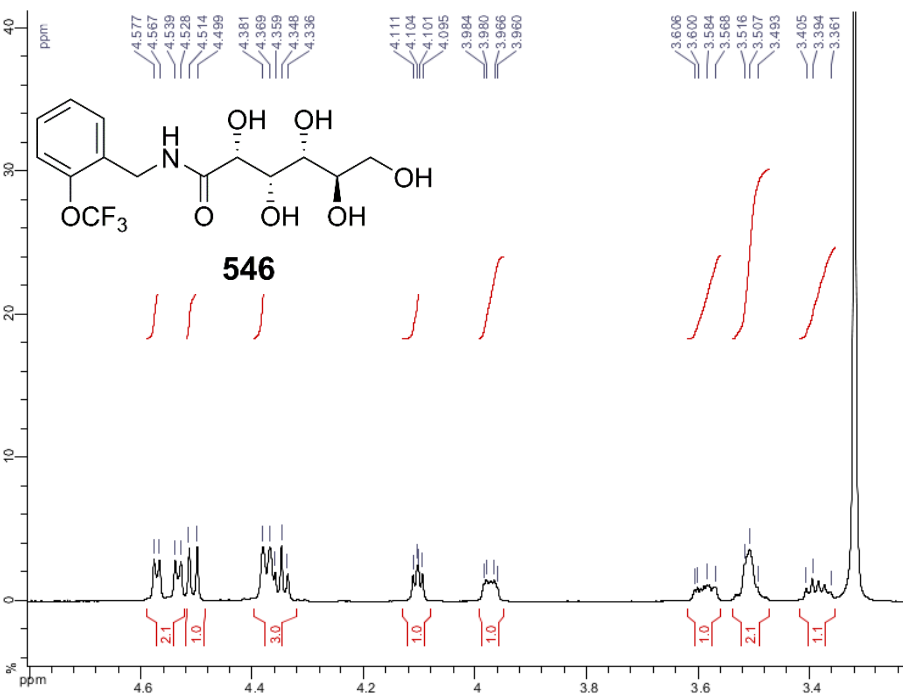
To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in methanol (10 mL) was added 4-(trifluoromethyl)benzylamine (0.32 mL, 2.24 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the crude product was recrystallized in EtOH to afford **545** as white crystals (367 mg, 93%). ^1H NMR (400 MHz, DMSO- d_6): δ 8.34 (t, J = 6.4 Hz, 1H), 7.65 (d, J = 8.5 Hz, 2H), 7.50 (d, J = 8.2 Hz, 2H), 5.49 (d, J = 4.9 Hz, 1H), 4.55 (dd, J = 18.6, 4.1 Hz, 2H), 4.49 (d, J = 7.3 Hz, 1H), 4.34-4.45 (m, 3H), 4.09 (t, J = 3.7 Hz, 1H), 3.95-3.98 (m, 1H), 3.56-3.61 (m, 1H), 3.49-3.51 (m, 2H), 3.35-3.41 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 172.9, 144.6, 127.7, 124.9, 73.9, 72.4, 71.5, 70.2, 63.3, 41.5. LRMS (ESI): m/z calcd. for $\text{C}_{14}\text{H}_{18}\text{F}_3\text{NNaO}_6$ $[\text{M}+\text{Na}]^+$ 376.1, found 375.9.

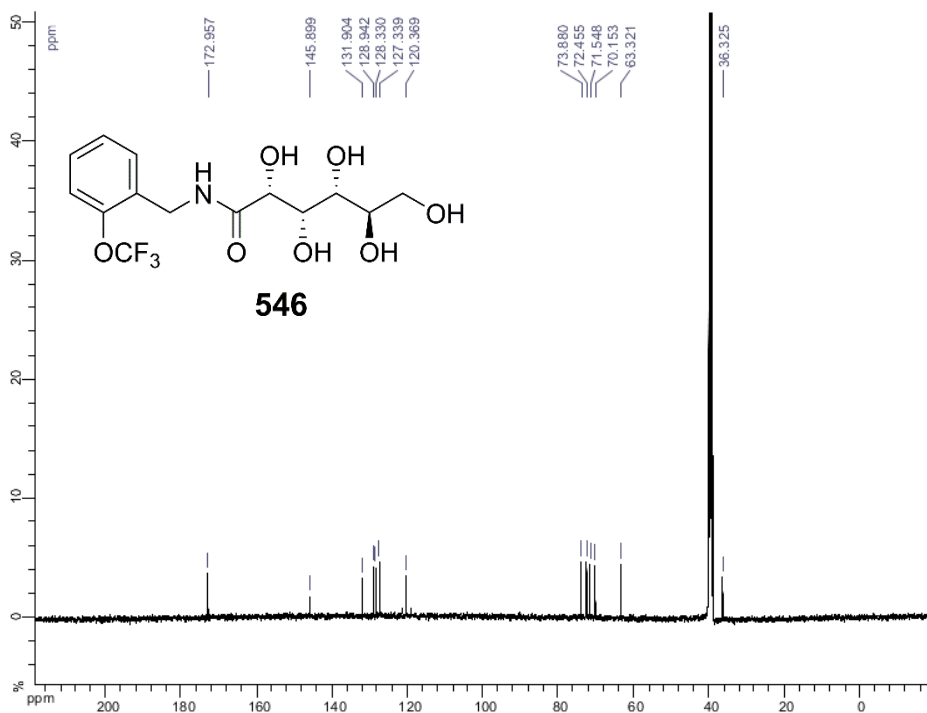




***N*-2-(Trifluoromethoxy)benzyl-D-gluconamide (546)**

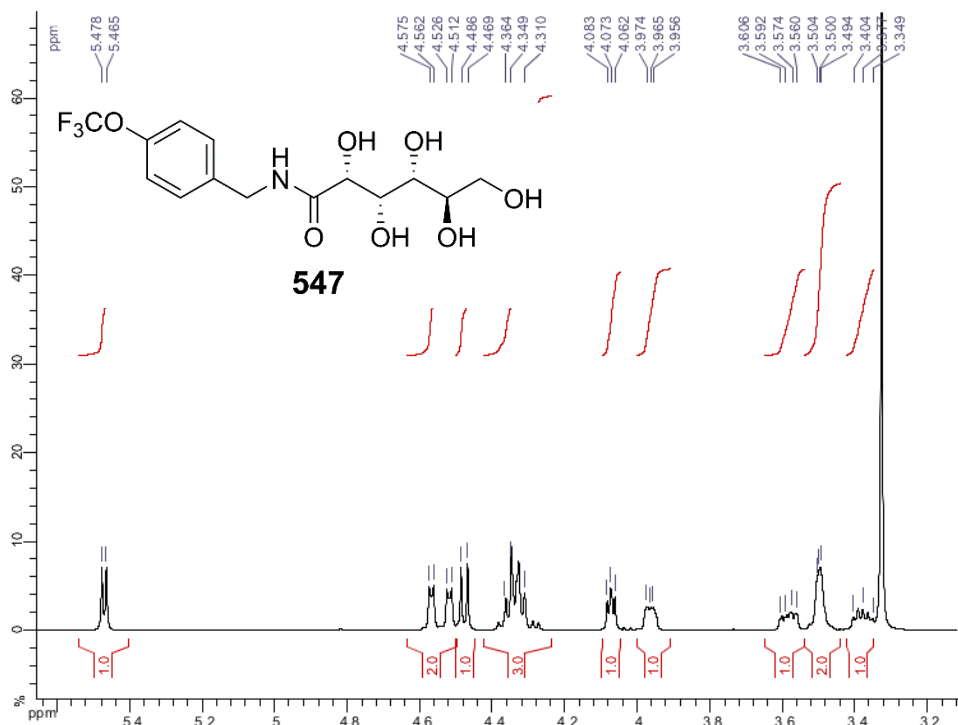
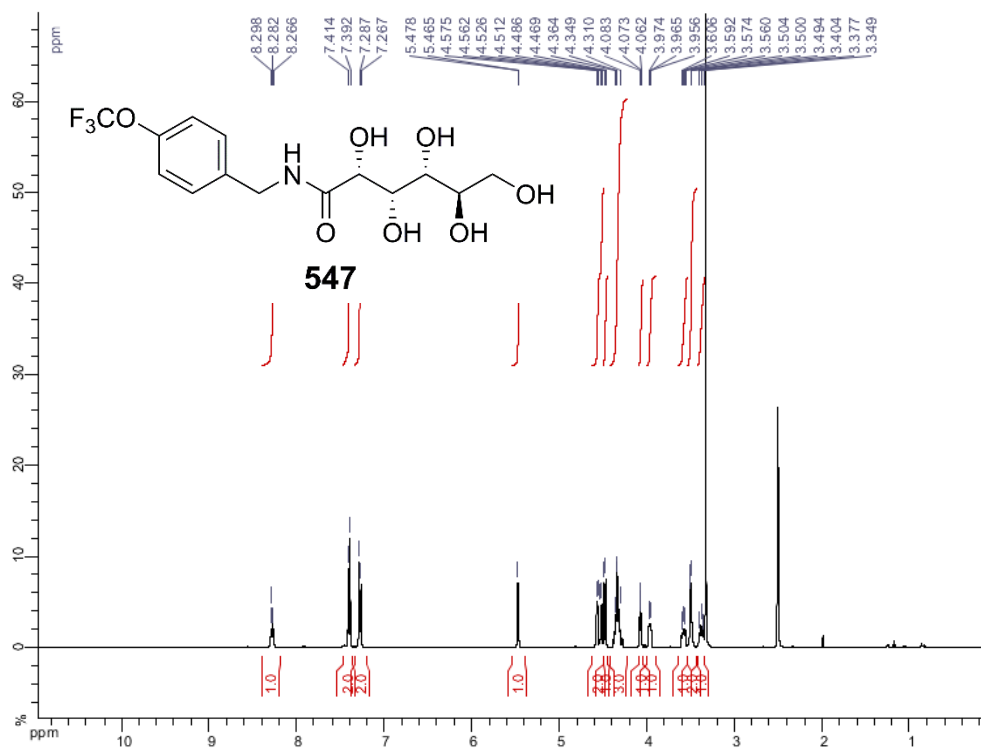
To a solution of D-gluconic acid- δ -lactone (0.20 g, 1.12 mmol) in methanol (10 mL) was added 2-(trifluoromethoxy)benzylamine (0.43 g, 2.24 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the crude product was recrystallized in EtOH to afford **546** as white crystals (267 mg, 64%). ^1H NMR (500 MHz, DMSO- d_6): δ 8.23 (t, J = 6.2 Hz, 1H), 7.47 (dd, J = 2.3, 7.2 Hz, 1H), 7.31-7.38 (m, 3H), 5.52 (d, J = 5.0 Hz, 1H), 4.56 (dd, J = 18.7, 4.7 Hz, 2H), 4.51 (d, J = 7.0 Hz, 1H), 4.34-4.38 (m, 3H), 4.10 (dd, J = 3.6, 5.1 Hz, 1H), 3.96-3.98 (m, 1H), 3.57-3.61 (m, 1H), 3.49-3.52 (m, 2H), 3.36-3.41 (m, 1H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 173.0, 145.9, 131.9, 128.9, 128.3, 127.3, 120.4, 73.9, 72.5, 71.6, 70.2, 63.3, 36.3. LRMS (ESI): m/z calcd. for $\text{C}_{14}\text{H}_{18}\text{F}_3\text{NNaO}_7$ $[\text{M}+\text{Na}]^+$ 392.1, found 392.0.

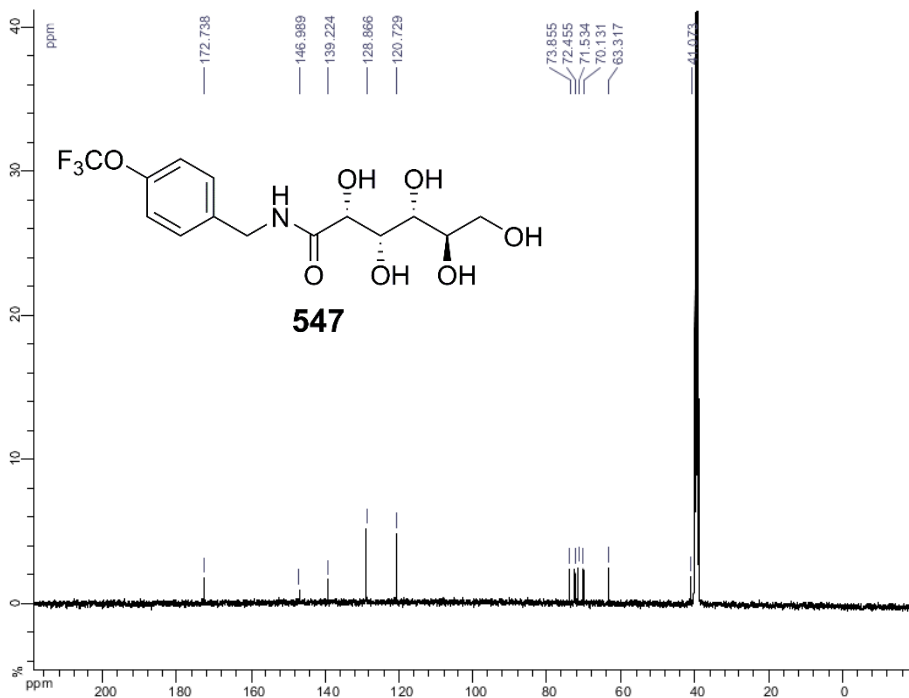




N-4-(Trifluoromethoxy)benzyl-*D*-gluconamide (**547**)

To a solution of *D*-gluconic acid- δ -lactone (0.10 g, 0.56 mmol) in methanol (10 mL) was added 4-(trifluoromethoxy)benzylamine (0.17 mL, 0.56 mmol). The mixture was stirred under reflux for 24 hours. The solvent was evaporated and the crude product was recrystallized in EtOH to afford **547** as white crystals (180 mg, 87%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$): δ 8.28 (t, J = 6.4 Hz, 1H), 7.4 (d, J = 8.6 Hz, 2H), 7.28 (d, J = 8.6 Hz, 2H), 5.47 (d, J = 5.0 Hz, 1H), 4.55 (dd, J = 19.2, 5.1 Hz, 2H), 4.48 (d, J = 7.4 Hz, 1H), 4.27-4.36 (m, 3H), 4.07 (dd, J = 3.6, 5.0 Hz, 1H), 3.96-3.87 (m, 1H), 3.56-3.61 (m, 1H), 3.49-3.50 (m, 2H), 3.35-3.40 (m, 1H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 172.7, 147.0, 139.2, 128.9, 120.7, 73.9, 72.5, 71.5, 70.1, 63.2, 41.1. LRMS (ESI): m/z calcd. for $\text{C}_{14}\text{H}_{18}\text{F}_3\text{NNaO}_7$ $[\text{M}+\text{Na}]^+$ 392.1, found 392.0.





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Chapter 6: Assessing the potential of IRIs to improve the cryopreservation of HSCs from UCB

6.1 Introduction

Hematopoietic stem cells (HSCs) are routinely used for regenerative medicine therapies. HSC transplantation offers life-saving treatment for patients suffering from hematological cancers (leukemias and lymphomas), hematological diseases, anemias, and immunodeficiencies.^{1,2} HSCs are identified as CD34-positive (CD34⁺) stem cells. CD34 is a stage-specific glycoprotein and identifies cells that are in the early stages of hematopoietic differentiation.³ There are several sources of CD34⁺ cells including bone marrow (BM), peripheral blood (PB) and umbilical cord blood (UCB). HSC transplantation from UCB offers advantages over treatments using BM and PB including reduced risk of graft-versus-host disease (GVHD), reduced risk of blood-borne infections, absence of risk to donor, and less stringent human leukocyte antigen (HLA) matching requirements.⁴ In select situations, fresh stem cells can be used for allogenic transplants if the cells are transplanted within 72 hours of harvest.⁵ However, virtually all autologous and allogenic transplants require that the cells are cryopreserved.⁵ Since the success of HSC transplantation is directly correlated to both the number and quality of cells transplanted, optimization of cryopreservation protocols for HSCs is an important issue for UCB banks.^{6–13}

Cryopreservation is particularly important for UCB HSCs because these cells are harvested at birth for use at a later, undetermined date. Clinical protocols for the cryopreservation of UCB HSCs employ the use of 10% dimethyl sulfoxide (DMSO) as the cryoprotectant solution.¹⁴ While a 10% DMSO solution is very effective and maintains high levels of cell viability, there are significant drawbacks associated with the use of DMSO as a cryoprotectant.^{5,15} These drawbacks are explained in detail in Chapter 1 (Section 1.2.4). Firstly, DMSO is toxic resulting in several side effects after transfusion of DMSO into patients including headache, nausea, vomiting, hypertension, anaphylactic shock, and many others.^{15–20} Efforts to reduce toxicity include the removal of DMSO prior to transfusion which is expensive, time consuming, and results in cell loss.^{21–23} Secondly, DMSO as a cryoprotectant fails to protect the functionality of HSCs

post-thaw.^{24–28} Approximately 20% of patients receiving a UCB transplant will fail to engraft, in part due to the inadequate potency of the unit as a result of cryopreservation.²⁷ Despite all of the problems associated with DMSO as a cryoprotectant, it remains the “gold standard” cryoprotectant for many cell types, including HSCs.

Due to the inadequacies of DMSO as a cryoprotectant, efforts have been made to improve the cryopreservation of HSCs. These efforts have centered on alternate freezing and storage conditions, or improved cryoprotectant solution formulations. Woods *et al.* have demonstrated that lower concentrations of DMSO (5%) can be used if alternate freezing conditions are used (freezing rate of 4 °C/min to -44 °C before plunging in liquid nitrogen).²⁹ Other studies suggest that DMSO can be eliminated with the storage of cells by alginate encapsulation.^{30–32} However, the majority of efforts to improve the cryopreservation of HSCs involves the incorporation of additives to the conventional 10% DMSO cryoprotectant solution, or the elimination or reduction of DMSO content using alternative cryoprotectants.

The main mechanisms of cryoinjury, which are described in detail in Chapter 1 (Section 1.2.2) can be divided into three categories: (1) osmotic imbalances arising from disruption in solute concentration during ice formation and melting, (2) cell membrane damage caused by thermotropic phase transitions, and (3) mechanical cell damage caused by ice recrystallization injury.^{33–35} DMSO functions as a permeating cryoprotectant by replacing water within the cell, diluting high intracellular solutes and maintaining cell volume.³⁶ However, DMSO is not capable of stabilizing cell membranes or controlling ice growth or recrystallization. Furthermore, cells experience hypoxia during cryopreservation which results in multiple cellular metabolic changes, including the catabolism of adenine nucleotides.³⁷ Hypoxanthine accumulates as a result of adenine nucleotide catabolism, which is subsequently converted into reactive oxygen species (ROS) with the reperfusion of oxygen that occurs upon transplantation.³⁷ The free radicals generated in this process cause oxidative damage such as lipid peroxidation, protein oxidation, and DNA damage.^{37,38} Therefore, efforts to design new and improved cryoprotective solutions

have focused on: (1) stabilizing cell membranes; (2) neutralizing reactive oxygen species, and; (3) controlling ice nucleation and recrystallization.

6.1.1 Membrane stabilizers as cryoprotectants

During cryopreservation, cell membranes can leak and allow intracellular components to seep out.³⁹ When cells are cooled, their membranes undergo a liquid crystalline-to-gel phase transition, known as a thermotropic phase transition, during which the permeability of the membrane is increased.^{40–42} Therefore the addition of membrane stabilizers, such as trehalose, to cryosolutions has been a strategy investigated thoroughly in the last 20 years.^{43–48}

Disaccharides such as trehalose and sucrose (Figure 6.1) have been widely used as natural cryoprotectants.⁴⁹ In nature, organisms such as yeasts and nematodes accumulate trehalose and survive for extended periods of time in a desiccated state with up to 99% water loss.^{50–53} Trehalose has been shown to stabilize and preserve cell structures during freezing and drying due to its protective interaction with lipid membranes.^{54–59} As such, it has been used in the preservation of various cells such as red blood cells (RBCs), platelets, oocytes and pancreatic islets.^{60–68}

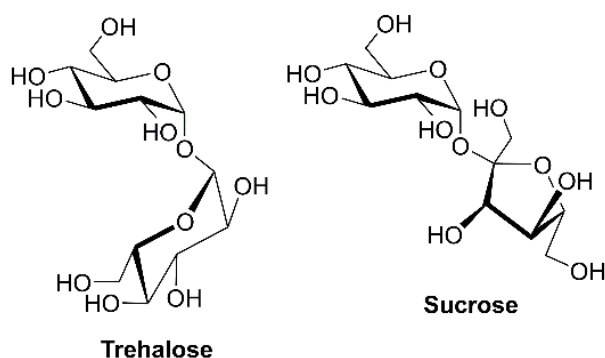


Figure 6.1. Chemical structures of disaccharides trehalose and sucrose.

Many research groups have investigated the effectiveness of trehalose and/or sucrose for the cryopreservation of HSCs from UCB. Rodrigues and coworkers screened many different combinations of trehalose and sucrose and identified the optimal concentrations with reduced DMSO content.⁶⁹ The best concentrations of DMSO, sucrose and trehalose (43 different combinations of cryoprotectants) were determined using hematopoietic cell lines, KG1 and K562, and bone marrow stromal cell line, S17.⁶⁹

These cell lines are used as a model for HSCs. Post-thaw viability assessment revealed 10 promising combinations for use with UCB cells. These new cryosolutions were then assessed for their ability to improve the cryopreservation of UCB HSCs. Cryopreservation outcomes were measured by post-thaw CD34⁺ recovery, viability and colony forming unit (CFU) counts.⁶⁹ CFU dose has been recognized as the best indicator of engraftment success and is a measure of the ability of the sample to proliferate and differentiate.²⁷ This study concluded that the DMSO content could be reduced from 10% to 2.5% with the addition of 30 mM trehalose or to 5% with the addition of 60 mM sucrose without sacrificing total CFU counts or CD34⁺ viability in cryopreserved UCB compared to the conventional 10% DMSO cryosolution.⁶⁹ Motta *et al.* also saw an increase in CD34⁺ cell recovery in HSCs cryopreserved with 5% DMSO and 60 mM sucrose compared DMSO alone.³⁸

Trehalose and sucrose are promising additives^{38,69–71} to the conventional 10% DMSO cryosolution, and studies like those described above indicate that the DMSO content required to cryopreserve HSCs can be reduced to as little as 2.5% with the addition of these disaccharides.⁶⁹ However, the use of these disaccharides alone without any DMSO is not effective. Rodrigues and coworkers found that cryopreservation of K562, KG1 and S17 cells with sucrose and trehalose alone would not permit more than 50% cell survival.⁶⁹ Furthermore, cryopreservation of TF-1 α cells, used as a model cell line for HSCs from UCB (CD34⁺ cells which possess the ability to differentiate), with 0.2 M trehalose without DMSO resulted in a significant decrease in CFU counts compared to 10% DMSO.^{70,71}

As discussed earlier, the major drawbacks to the use of DMSO as a cryoprotectant for HSCs is the toxicity of DMSO and the inability of DMSO to preserve the functionality of HSCs. Cryosolution formulations containing sucrose and/or trehalose have allowed for a reduction in the amount of DMSO required for CD34⁺ viability and recovery. The degree of toxicity is directly related to the amount of DMSO transfused into the patient and therefore these new cryosolutions would reduce the toxic effects associated with the transfusion of DMSO. However, these solutions do not improve the functionality of HSCs post-thaw. This is important because CFU dose is the best indicator of engraftment success.

6.1.2 Antioxidants as cryoprotectants

Generation of free radicals upon hypoxia during freezing is one of the factors responsible for cell damage during cryopreservation.^{37,72–74} Cells mediate this damage by employing antioxidants such as ascorbic acid, α -tocopheryl acetate, and enzymes such as peroxidases including catalase.⁷⁵ As such, antioxidants and catalase have been incorporated as additives to cryosolutions to help mitigate damage associated with the free radical formation during cryopreservation.

Limaye *et al.* evaluated the antioxidants ascorbic acid, catalase and α -tocopheryl acetate (Figure 6.2) for their potential to improve the cryopreservation of UCB HSCs by using them as additives to the conventional 10% DMSO cryosolution.⁷⁶ The addition of ascorbic acid or α -tocopheryl acetate to 10% DMSO did not show any beneficial effect.⁷⁶ However, the addition of catalase improved total CFU count compared to 10% DMSO alone.⁷⁶ Addition of both catalase and trehalose to the 10% DMSO cryosolution further improved CFU recovery and was better than the individual additives used alone.⁷⁶

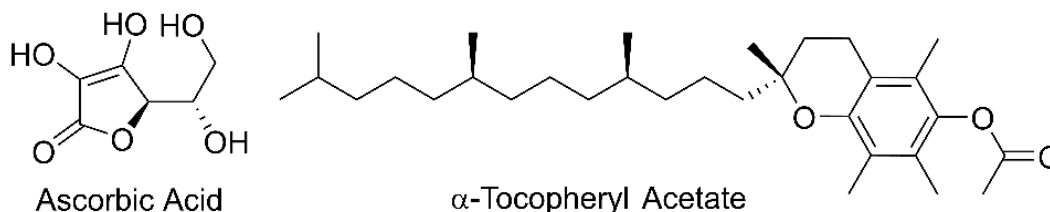


Figure 6.2. Chemical structures of ascorbic acid and α -tocopheryl acetate.

In 2003, Sasnoor *et al.* extended this study to examine the response of HSCs to growth factors after cryopreservation with catalase and trehalose.⁷² Successful transplantation relies on the rapid response of transplanted HSCs to growth factors and subsequent differentiation.^{72,77} This is especially important when cells are infused in an irradiated and severely immunocompromised host where the transplant could be exposed to limiting concentrations of growth factors.^{78,79} In addition, successful transplantation also relies on the interaction between the HSC transplant and bone marrow stroma, required for the homing and engraftment of the cells.⁸⁰ Hattori *et al.* have shown that L-selectin, an adhesion molecule responsible for homing and engraftment, is decreased post-cryopreservation.⁸¹ Sasnoor and coworkers found that a combination of catalase (100 μ g/mL) and trehalose (25 μ g/mL) with 10% DMSO resulted in the improved

growth factor responsiveness as well as expression of growth factor receptors and adhesion molecules and adhesive properties.⁷² Cells cryopreserved with catalase and trehalose additives responded better to suboptimal growth factor concentrations and had increased expression of cell-surface receptors responsible for successful homing and engraftment, self-renewal, proliferation and differentiation.⁷² Furthermore, HSCs frozen with catalase and trehalose additives had increased binding to the bone marrow stromal cell line, M210B4, compared to cells frozen without additives.⁷²

Overall, these studies found that the addition of the antioxidant, catalase, and membrane stabilizer, trehalose, to the conventional 10% DMSO cryosolution improved the functionality of HSCs post-thaw. Motta *et al.* assessed the ability of catalase and ascorbic acid with trehalose and sucrose to enhance the cryopreservation of HSCs from UCB with reduced concentrations of DMSO.³⁸ An increase in the percentage of CD34⁺ cells cryopreserved with 5% DMSO with 60 mM trehalose or sucrose and 100 µg/mL ascorbic acid was observed post-thaw compared to 5% DMSO alone.³⁸ However, there was no significant difference in CFU counts.³⁸

The successful engraftment of cryopreserved HSCs depends on not only CD34⁺ recovery, but also preservation of functionality.^{24–28} Response to growth factors, expression of adhesion molecules, and the ability of HSCs to interact with the stromal microenvironment are properties that must be preserved during the cryopreservation of these cells.^{72,77–79} Addition of antioxidants and membrane stabilizers appear to protect these functionalities better than the conventional 10% DMSO cryosolution alone.

6.1.3 Ice recrystallization inhibitors as cryoprotectants

The phenomenon of ice recrystallization is a major cause of cellular injury during cryopreservation.^{35,82–85} Ice recrystallization is the thermodynamically driven growth of large ice crystals into larger ice crystals at the expense of smaller ones and this process is most active in the thawing phase of cryopreservation.^{86–89} Ice recrystallization leads to an increase in the mean ice crystal size in a sample which can cause mechanical damage to cell membranes. Slow thawing of cryopreserved cells has been demonstrated to exacerbate ice recrystallization and in turn reduces cell viability.^{90,91} As a result, cryopreserved cells are commonly thawed rapidly to minimize the occurrence of ice recrystallization.

Previous work from our laboratory assessed the ice recrystallization inhibition (IRI) activity of several mono- and disaccharides.⁹² The carbohydrates assessed in this study possessed moderate to weak activity.⁹² These sugars were assessed for their ability to function as cryoprotectants for CD34⁺ cells derived from UCB.⁹³ Interestingly, the IRI activity of the mono- and disaccharides was found to correlate with post-thaw CD34⁺ cell viability.⁹³ When HSCs were frozen with carbohydrates with moderate IRI activity and thawed using conditions to increase ice recrystallization (slow thawing), an increase in the yield of viable CD34⁺ cells was observed compared to samples cryopreserved with carbohydrates possessing weaker IRI activity.⁹³ Furthermore, early apoptosis was significantly reduced within the CD34⁺ population with samples cryopreserved with carbohydrates that were more IRI active.⁹³ A correlation between preservation of CFU count and IRI activity of the carbohydrate used in the cryosolution was also established.⁹³

This study was extended in 2012 when the Ben laboratory performed a comprehensive study of the relationship between IRI activity, cytotoxicity and the cryopreservation ability of carbohydrates with several human cell lines.⁹⁴ Two of the three cell lines selected were human liver cells because liver cells have been identified as a potential source of stem cells, and thus were used as a model for HSCs.⁹⁵ The results from this study confirmed the correlation between cryopreservation ability and the IRI activity of the carbohydrate.⁹⁴ All carbohydrates assessed were found to have equal or less cytotoxicity compared to 2.5 or 5% DMSO.⁹⁴ However, it was found that disaccharides that were superior at cryopreservation were also those that were more cytotoxic.⁹⁴ For example, lactose, sucrose, or trehalose (220 mM) resulted in high post-thaw cell viabilities; however, these were among the most cytotoxic carbohydrates examined. D-Galactose (220 mM) exhibited the best balance between cytotoxicity and cryopreservation ability.⁹⁴ Interestingly, D-galactose is the most IRI active monosaccharide of those examined in the study.

We hypothesized that compounds which are highly IRI active and which are also non-toxic, could represent a new class of cryoprotectants capable of improving the cryopreservation of HSCs from UCB. The results from this chapter are included in a manuscript submitted to *ACS Chemical Biology* entitled

“Small-molecule ice recrystallization inhibitors improve the functionality of hematopoietic stem cells after cryopreservation”.⁹⁶

6.2 Cryopreservation of TF-1 α cells

TF-1 α cells are CD34⁺ immortalized human bone marrow erythroblasts that are often used as a model for studying human primitive myeloid progenitor cells.⁹⁷ These cells are easily cultured and therefore this cell line was utilized to pre-screen compounds for their cryopreservation ability. It was hypothesized that compounds that were effective at cryopreserving TF-1 α cells would also be effective at cryopreserving HSCs from UCB. We first assessed the simple mono- and disaccharides D-galactose and D-sucrose. Previous work by the Ben laboratory found that D-galactose (220 mM) possessed an ideal balance between cytotoxicity and HSC cryopreservation ability.⁹⁴ D-Galactose was also the most IRI active of the mono- and disaccharides assessed.⁹⁴ D-Sucrose (220 mM) also resulted in high post-thaw cell viabilities but was found to be cytotoxic.⁹⁴ We were interested in assessing D-sucrose because previous studies demonstrated that the DMSO content could be reduced to 5% with the addition of 60 mM D-sucrose without sacrificing CD34⁺ viability in cryopreserved UCB.⁶⁹ In fact, Motta *et al.* saw an increase in CD34⁺ cell recovery using this cryosolution.³⁸

We first assessed the cryopreservation ability of D-galactose and D-sucrose using TF-1 α cells. TF-1 α cells were cryopreserved using 0, 2, and 10% DMSO cryosolutions supplemented with D-galactose (110 mM) or D-sucrose (110 mM). The carbohydrates were insoluble at 220 mM in cell medium (RPMI-1640) and therefore were assessed at 110 mM. TF-1 α cells were frozen (1 °C/min) to -80 °C followed by storage at -196 °C for 48 hours. The samples were thawed quickly in a 37 °C water bath. Tf-1 α cell recovery was measured as the percentage of cells recovered post-thaw compared to the number of cells frozen as assessed by hemocytometer count. No differences in post-thaw cell recoveries were observed (Figure 6.3). Post-thaw viability and apoptosis were measured by flow cytometry using 7-AAD and Annexin-V staining procedures (See Experimental) (Figure 6.4). Cryosolutions containing 0, 2, and 10% DMSO

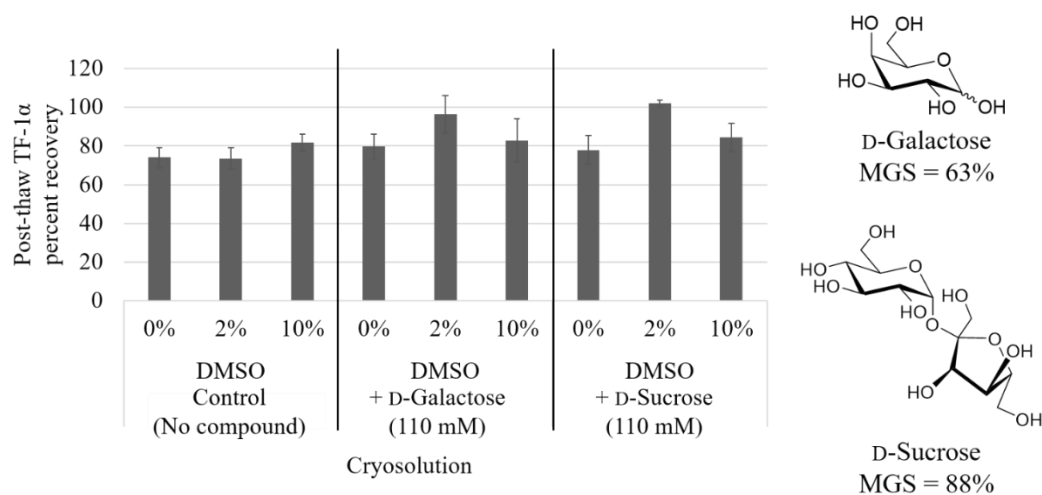


Figure 6.3. Post-thaw percent recovery of TF-1α cells cryopreserved with D-galactose or D-sucrose. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test.

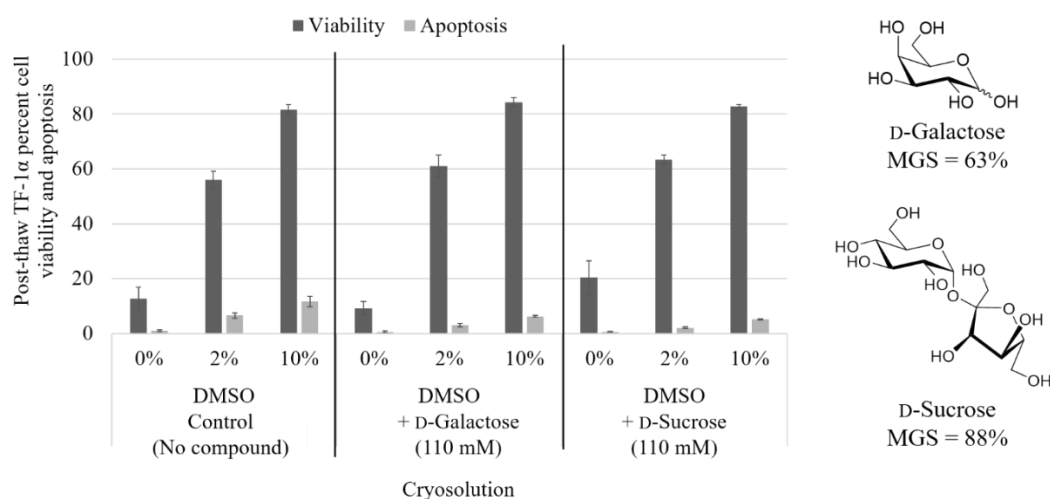


Figure 6.4. Post-thaw percent viability and apoptosis of TF-1α cells cryopreserved with D-galactose or D-sucrose. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test.

without D-galactose or D-sucrose resulted in post-thaw viabilities of 13%, 56%, and 81% respectively. Viability was significantly decreased ($p < 0.05$) when the DMSO in the cryosolution was reduced from 10% to 2% or lower. Supplementation of the DMSO solutions with D-galactose or D-sucrose did not influence post-thaw viability ($p > 0.05$). While there was no difference in viability or recovery upon supplementation with D-galactose or D-sucrose, cryosolutions containing these carbohydrates resulted in lower apoptosis levels which was consistent with previous reports.⁹⁴

Next, cryopreservation outcomes were measured on samples thawed using slow thawing conditions by allowing the samples to thaw at room temperature. This was done in order to exacerbate ice recrystallization injury during thawing. It was hypothesized that D-galactose would provide better protection against this increased ice recrystallization injury than D-sucrose because it is a more IRI active molecule. TF-1 α cells were cryopreserved using 0, 2, and 10% DMSO cryosolutions supplemented with D-galactose (110 mM) or D-sucrose (110 mM). Post-thaw recoveries were increased for 0% DMSO when D-galactose or D-sucrose were supplemented; however, there was no difference in recovery for 2 and 10% DMSO upon supplementation with D-galactose or D-sucrose (Figure 6.5). Post-thaw viability and apoptosis were also measured (Figure 6.6). Under slow thawing conditions, samples frozen with 0, 2, and 10% DMSO alone resulted in post-thaw viabilities of 1%, 44% and 65% respectively compared to 13%, 56% and 81% for fast thawing conditions. This reduction in viability was attributed to the increase in ice recrystallization during thawing. Viability was reduced ($p < 0.05$) with 2% DMSO + D-sucrose (110 mM) compared to the 2% DMSO cryosolution alone (20% compared to 44%). Ben *et al.* previously reported that D-sucrose was cytotoxic at 200 mM and 500 mM in human embryonic liver cells (WRL-68 cells).⁹⁴ The reduction in viability with 2% DMSO + D-sucrose (110 mM) could be a result of the toxic effects TF-1 α cells experienced during longer exposure times associated with the slower thawing rate. Cytotoxicity was not previously assessed at 110 mM and would need to be assessed in order to prove this. Viability was increased with 10% DMSO + D-galactose (110 mM) compared to the 10% DMSO cryosolution alone. D-Galactose was the most IRI active monosaccharide assessed.⁹² Therefore, this

increase in post-thaw viability under slow thawing conditions could be a result of the IRI activity of D-galactose.

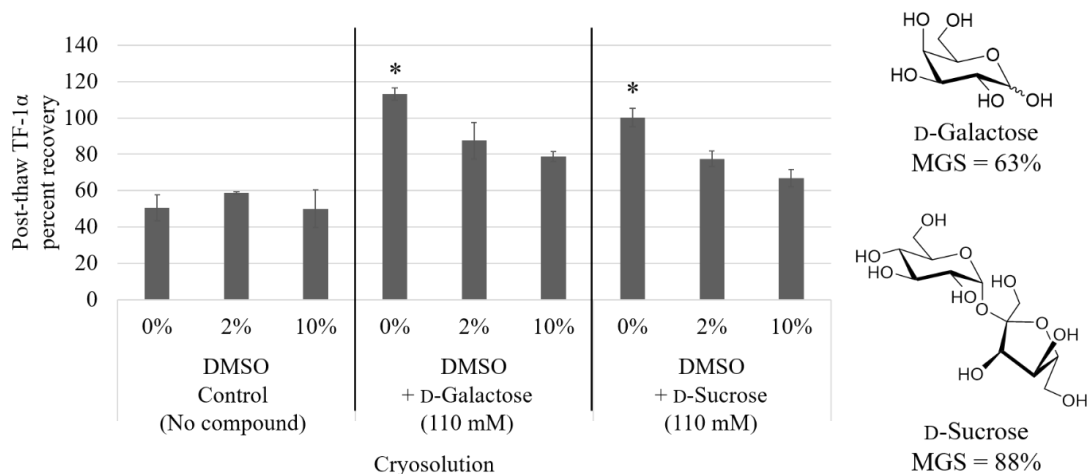


Figure 6.5. Post-thaw percent recovery of TF-1α cells cryopreserved with D-galactose or D-sucrose. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed slowly at room temperature. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control.

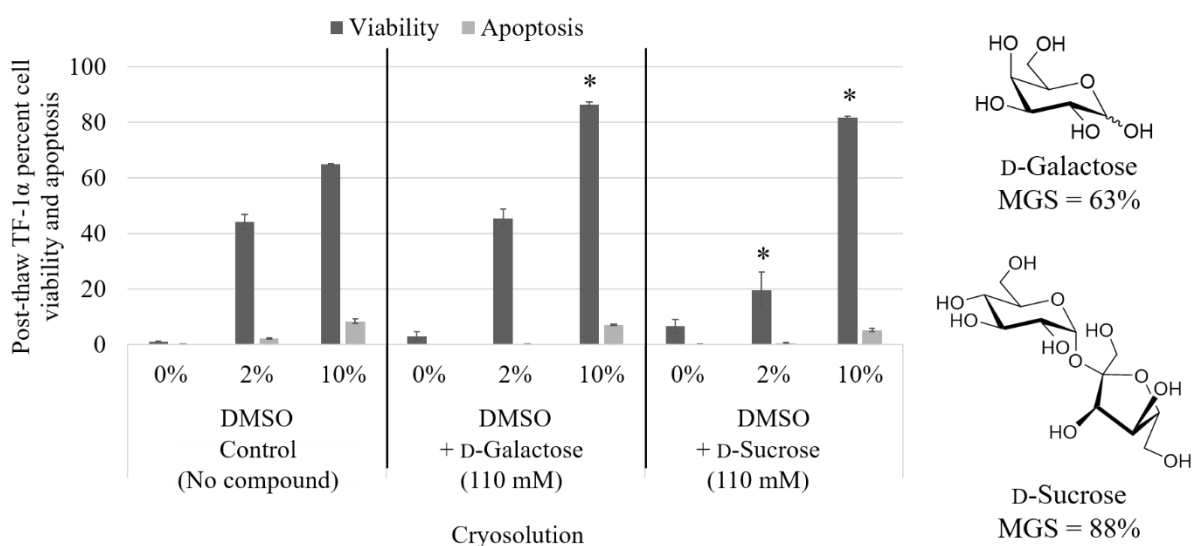


Figure 6.6. Post-thaw percent viability and apoptosis of TF-1α cells cryopreserved with D-galactose or D-sucrose. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed slowly at

room temperature. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control.

Increased post-thaw viabilities and recoveries were observed when TF-1 α cells were frozen with DMSO solutions supplemented with D-galactose. D-Galactose was the most IRI active monosaccharide assessed in our laboratory; however, our laboratory has discovered more highly IRI active small carbohydrate-based molecules. Therefore, we investigated the cryoprotective ability of other small molecules that exhibit greater IRI activity than D-galactose. Aryl glycosides, β -PMP-Glc (**166**), β -pFPh-Glc (**170**) and β -pBrPh-Glc (**171**) are small molecules that are very effective inhibitors of ice recrystallization (MGS 8-23% at 22 mM).⁹⁸ Furthermore, these molecules were previously shown to be effective cryoprotectants for RBCs with reduced glycerol concentrations (15% v/v compared to the clinically used concentration of 40% v/v).⁹⁹ Therefore, **166**, **170** and **171** were assessed for their ability to cryopreserve TF-1 α cells. TF-1 α cells were cryopreserved using 0, 2, and 10% DMSO cryosolutions supplemented with **166** (110 mM), **170** (110 mM), or **171** (110 mM). TF-1 α cells were frozen (1 °C/min) to -80 °C followed by storage at -196 °C for 48 hours. The samples were thawed quickly in a 37 °C water bath and post-thaw viability and apoptosis was measured by flow cytometry (Figure 6.7). Greatly reduced post-thaw viabilities were observed upon addition of these aryl glycosides to the cryosolutions and some yielded no viable cells post-thaw. This was hypothesized to be a result of the toxicity of these compounds at the concentrations utilized. The cytotoxicity of **166** was assessed using the MTT assay with HepG2 cells (Supplemental Figure S6.7) and was found to be significantly toxic at 110 mM. Compounds **170** and **171** were also previously found to be cytotoxic at concentrations higher than 11 mM.⁹⁸ There was no difference ($p > 0.05$) in post-thaw recovery upon supplementation with aryl glycosides **166**, **170** or **171** (Supplemental Figure S6.1)

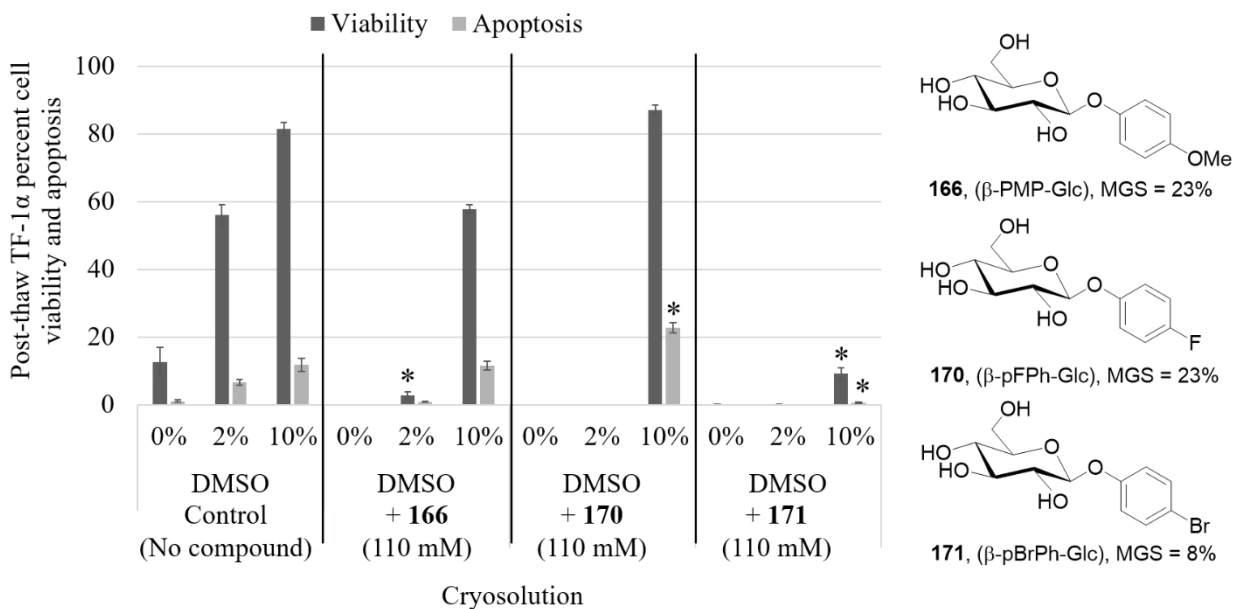


Figure 6.7. Post-thaw percent viability and apoptosis of TF-1α cells cryopreserved with aryl glycosides β-PMP-Glc (**166**), β-pFPh-Glc (**170**) and β-pBrPh-Glc (**171**). Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control.

Aryl glycosides were found to increase cell damage during cryopreservation of TF-1α cells. This is the opposite of what is observed with RBCs where a cryosolution containing 15% glycerol with **166** (110 mM) resulted in higher post-thaw intact RBCs compared to the 15% glycerol control.⁹⁹ A possible explanation for this could be that TF-1α cells are more complex than RBCs and therefore more sensitive to the addition of these molecules. The decrease in viability of TF-1α cells frozen with these aryl glycosides was attributed to their cytotoxicity at 110 mM. Whether or not these compounds would be beneficial additives at lower concentrations was not investigated and is the subject of future work.

Chapter 5 described the rational design of the novel class of small molecule IRIs, the *N*-aryl-alidonamides. Several of these molecules are highly IRI active (MGS < 10%). For example, **502** possesses an IRI activity of 3%. These molecules were designed to be less toxic than the *N*-alkyl-alidonamides by

replacing the long alkyl chains with an aromatic ring (Figure 6.8). Aromatic substituents were strategically chosen to consist of substituents that resulted in highly IRI active aryl glycosides. The cytotoxicity of several of these *N*-aryl-aldonamides was assessed using the MTT assay (Section 6.6.2 Supplementary Figures and Data). For comparison, the cytotoxicity of DMSO and *N*-octyl-gluconamide (**154**, Figure 6.8A) at various concentrations is also shown (Supplemental Figure S6.5 & S6.6). After 48 hours of incubation, only 4% of HepG2 cells were viable in the presence of only 5 mM of **154**. The *N*-aryl-aldonamides were significantly less toxic at higher concentrations. This was thought to be a result of the replacement of the alkyl chain with an aromatic ring, making the *N*-aryl-aldonamides less likely to interact with and solubilize cell membranes. It was therefore hypothesized that IRI active *N*-aryl-aldonamides could be beneficial cryoprotectants for TF-1 α cells.

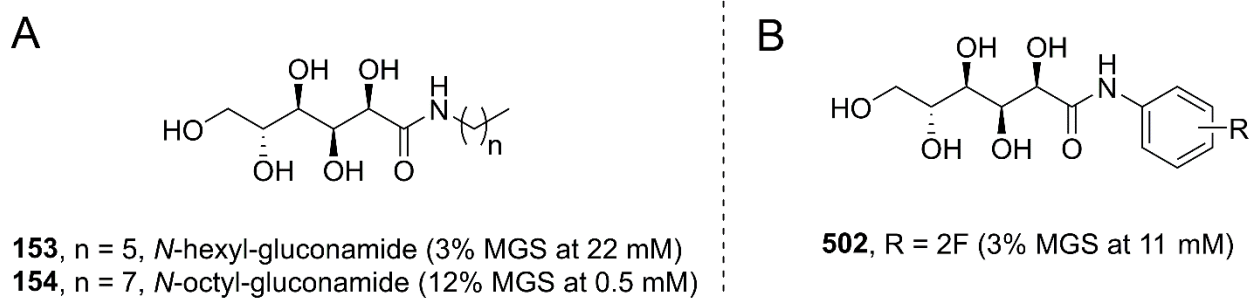


Figure 6.8. General structure of (A) *N*-alkyl-aldonamides and (B) *N*-aryl-aldonamides with example structures of IRI active molecules in each class.

TF-1 α cells were cryopreserved using 0, 2, and 10% DMSO cryosolutions supplemented with *N*-aryl-aldonamides, **501-503**, **509**, **517**, **520** or **521**. These molecules were less soluble than the aryl glycoside class and therefore were assessed at their maximum solubility of 55 mM. TF-1 α cells were frozen (1 °C/min) to -80 °C followed by storage at -196 °C for 48 hours. The samples were thawed quickly in a 37 °C water bath and post-thaw viability and apoptosis was measured by flow cytometry (Figure 6.9). Post-thaw viabilities of TF-1 α cells frozen with 0, 2, and 10% DMSO were 13%, 56%, and 81% respectively. A decrease in cell viability was observed when the 2% DMSO cryosolution was

supplemented with **501** or **521** ($p < 0.05$). The most interesting results were with the 2% DMSO cryosolution supplemented with **502**, **509** and **520**. TF-1 α cells frozen with 2% DMSO resulted in 56% cell viability. Remarkably, supplementation of the 2% DMSO solution with **502**, **509** or **520** (55 mM) resulted in significantly increased cell viabilities (81%, 74% and 80% respectively) ($p < 0.05$). This was a similar post-thaw viability to what was observed with the conventional 10% DMSO cryosolution (81%) ($p > 0.05$). There were no significant differences in post-thaw recoveries for any cryosolutions (Supplemental Figure S6.2).

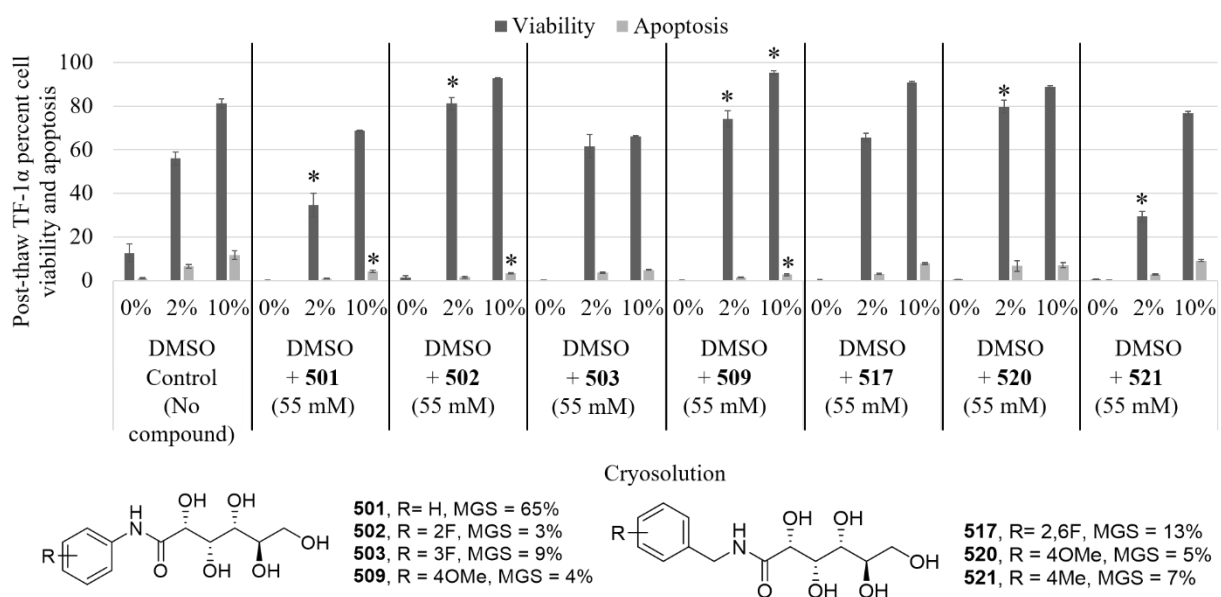


Figure 6.9. Post-thaw percent viability and apoptosis of TF-1 α cells cryopreserved with aryl aldnamides, **501-503**, **509**, **517**, **520** and **521**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control (no compound).

The DMSO content could be reduced from 10% to 2% with the addition of IRIs **502**, **509**, or **520** (55 mM) while maintaining cell viabilities comparable to the conventional 10% DMSO cryosolution. Next, we assessed whether the concentration of IRI in the cryosolution could be reduced without affecting post-thaw viability. TF-1 α cells were frozen with 0, 2, and 10% DMSO solutions supplemented with **502**,

509 or **520** at concentrations of 55 mM or 30 mM. Reducing the concentration from 55 mM to 30 mM was not tolerated with compounds **502** or **520** (Figure 6.10). Post-thaw viability and apoptosis of TF-1 α cells frozen with **502** (30 mM) was statistically similar to the controls ($p > 0.05$). Reducing the concentration of **520** from 55 mM to 30 mM resulted in a significantly lower post-thaw viability for the 2% DMSO solution ($p < 0.05$). However, a reduction in the concentration of **509** to 30 mM was tolerated: 2% DMSO + **509** (30 mM) resulted in a similar post-thaw TF-1 α cell viability as when 55 mM of **509** was used (approx. 75% viable) ($p > 0.05$). Furthermore, there were no differences in post-thaw recovery when the concentration of **509** was reduced to 30 mM ($p > 0.05$) (Supplemental Figure S6.3).

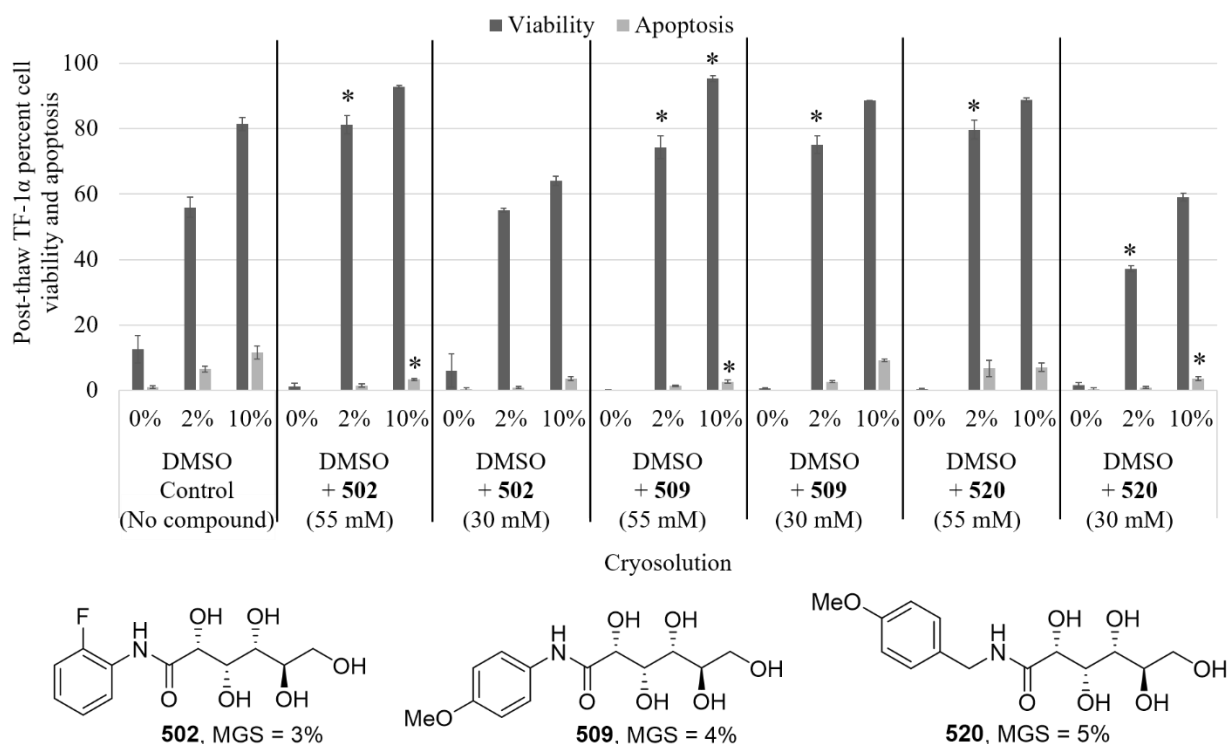


Figure 6.10. Post-thaw percent viability and apoptosis of TF-1 α cells cryopreserved with different concentrations of aryl aldnamides, **502**, **509** and **520**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C.

Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control (no compound).

Compounds **502**, **509** and **520** were effective at maintaining high cell viability post-thaw with reduced amounts of DMSO. This was attributed to their high IRI activity. In order to confirm this, slow thawing conditions were employed to deliberately increase ice recrystallization injury. It was hypothesized that the addition of IRIs **502** or **509** would result in increased viability compared to the DMSO control cryosolutions when using slow thawing conditions. TF-1 α cells were cryopreserved using 0, 2, and 10% DMSO cryosolutions supplemented with **502** or **509** (55 mM). TF-1 α cells were frozen (1 °C/min) to -80 °C followed by storage at -196 °C for 48 hours. The samples were thawed slowly by allowing the samples to thaw at room temperature. Post-thaw viability and recovery was measured by flow cytometry (Figure 6.11). There were no significant differences in post-thaw recoveries for all cryosolutions (Supplemental Figure S6.4). When the 2% DMSO cryosolution was supplemented with **502** or **509** a reduction in TF-1 α cell viability was observed. This could suggest that these molecules are not functioning by inhibiting ice recrystallization and are exerting a cryoprotective effect through an alternate mechanism. A second explanation could suggest that the increased exposure time of the cells with these IRIs during slow thawing resulted in reduced cell viabilities caused by the cytotoxicity of the compounds.

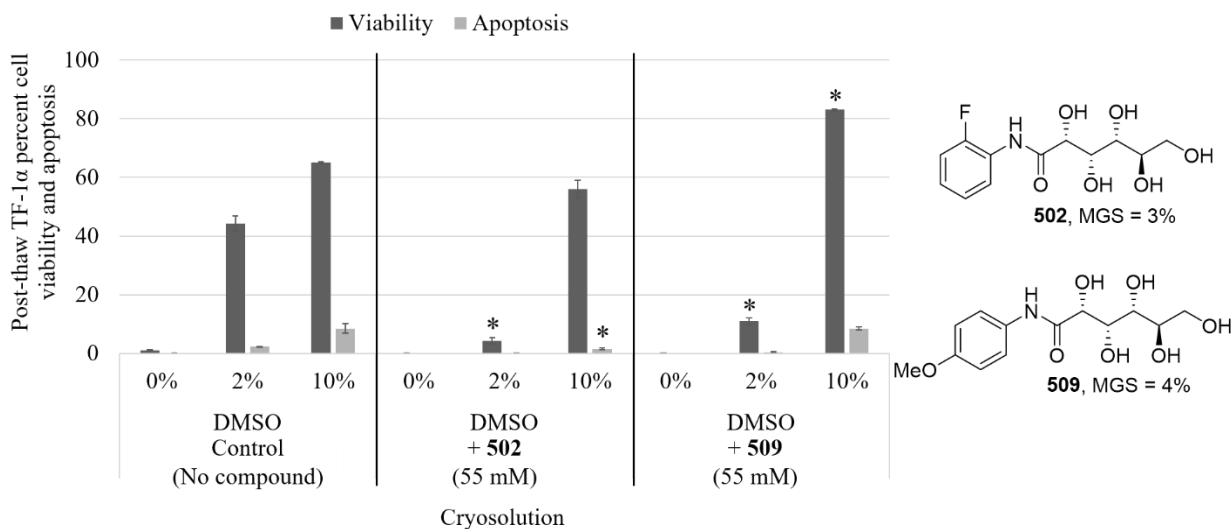


Figure 6.11. Post-thaw percent viability and apoptosis of TF-1 α cells cryopreserved with aryl aldonamides, **502** and **509**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were slowly at room temperature. Error bars are reported as the standard error of the mean (SEM). Statistical significance (p < 0.05) was

assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control (no compound).

To summarize this section, post-thaw viabilities of TF-1 α cells frozen with D-galactose and D-sucrose were consistent with previous results. D-Galactose provided enhanced protection against slow thawing which was attributed to the moderate IRI activity of D-galactose. Aryl glycosides **166**, **170**, and **171** resulted in decreased post-thaw viabilities compared to the control DMSO cryosolutions. This was attributed to the cytotoxicity of these compounds at the concentrations used. Remarkably, *N*-aryl-aldoamides **502**, **509** and **520** were found to be effective cryoprotectants for TF-1 α cells allowing for the reduction of DMSO from 10% to 2% without sacrificing post-thaw viability. It was therefore hypothesized that *N*-aryl-aldoamides, which are highly IRI active, would be beneficial additives for the cryopreservation of HSCs from UCB.

6.3 Cryopreservation of HSCs from UCB

6.3.1 *N*-Aryl-aldoamides as cryoprotective agents

It was hypothesized that compounds which were effective at cryopreserving TF-1 α cells would also be beneficial for the cryopreservation of HSCs from UCB. This is because TF-1 α cells are CD34⁺ and can be used as a model for progenitor cells. In the previous section, *N*-aryl-aldoamides, **502**, **509**, and **520** were found to be effective cryoprotectants for TF-1 α cells allowing for the reduction of DMSO from 10% to 2% without sacrificing post-thaw viability. Therefore, HSCs from UCB were cryopreserved using cryosolutions containing 0, 2, 5, and 10% DMSO with *N*-aryl-aldoamides **502** and **509**. Compound **520** was found to be extremely cytotoxic using the MTT assay (Supplemental Figure S6.14) and was therefore not initially assessed in HSCs.

Most currently used protocols for HSC cryopreservation employ 10% DMSO as a cryoprotectant solution. In the clinic, leukocyte concentrates (LCs) consisting of total nucleated cells (TNCs) are isolated from UCB units by RBC depletion using hydroxyethyl starch. UCB LCs are extracted from UCB and the

total number of viable CD34⁺ cells is quantified by flow cytometry. Small scale cryopreservation experiments were designed herein to mimic what is done clinically in order to effectively compare the cryopreservation success of HSCs cryopreserved with IRI active molecules. LCs were cryopreserved using protocols previously described by Rubinstein *et al.* using a 1:5 dilution of 50% DMSO (in a 5% dextran/0.9% saline solution) with the LC for a final DMSO concentration of 10%.¹⁴

The processing of UCB was performed as follows: UCB was separated into Falcon tubes and depleted of RBCs by addition of Hespan (6% hydroxyethyl starch in 0.9% sodium chloride). After a 10 minute incubation of RBCs with Hespan on ice, the blood was centrifuged at 50 x g for 15-20 minutes depending on total volume. This resulted in sedimentation of the RBCs. The top layer was carefully collected, containing plasma and leukocytes. The top layer was further centrifuged at 400 x g for 10 minutes at 10 °C to pellet the leukocytes which were then resuspended in 20 mL plasma to obtain the LC. Extra plasma was kept aside on ice. The concentration and viability of CD34⁺ and CD45⁺ cells was obtained by flow cytometry using the ISHAGE gating strategy.¹⁰⁰ An aliquot of 50,000 CD34⁺ cells was added to cryovials, centrifuged at 400 x g for 5 minutes, supernatant removed and the pellet was resuspended in 80 µL of plasma. Next, 20 µL of cryosolution was added for final DMSO concentrations of 0, 2, 5 and 10% DMSO using a 1:5 dilution with CD34⁺ cells in plasma. These cryosolutions were also supplemented with *N*-aryl-aldonamides **502** and **509**. Due to solubility limits, **502** and **509** were assessed at concentrations of 5 mM and 11 mM respectively. Samples were frozen (1 °C/min) to -80 °C, then stored in a liquid nitrogen dewar for 48 hours prior to thawing quickly in a 37 °C water bath.

Most transplant centres base donor selection on total nuclear cell (TNC) count.^{101,102} However, using this approach, approximately 20% of patients fail to engraft.²⁷ Recent reports have indicated that engraftment success was more closely correlated to CD34⁺ cell dose (compared to TNC dose).^{27,103,104} Therefore, cryopreservation success was measured by post-thaw CD34⁺ recovery and viability. Typical post-thaw recovery in a cryopreserved unit was > 80% for CD34⁺ cells.²⁷ Post-thaw cell recovery was assessed by comparing the total number of cells cryopreserved with the absolute number of cells obtained

post-thaw (Figure 6.12). Post-thaw recovery for CD45⁺ cells was also included for comparison. CD45 (also known as the leukocyte common antigen (LCA)) is found on all leukocytes. Interestingly, post-thaw CD45⁺ and CD34⁺ recoveries were significantly better with the addition of **509** to the 0, 2 and 5% DMSO cryosolutions ($p < 0.05$). Post-thaw CD34⁺ recoveries were similar to what is observed clinically ($>80\%$)²⁷ with the addition of **509** to the 0 and 2% DMSO cryosolutions. It is impressive that CD34⁺ recovery is this high with only 0 or 2% DMSO in the cryosolution when **509** is supplemented. Post-thaw CD34⁺ cell viability was assessed by flow cytometry and represents the percentage of viable CD34⁺ recovered post-thaw. As seen in Figure 6.13, there was no difference in cell viability when **502** or **509** were supplemented compared to the control ($p > 0.05$).

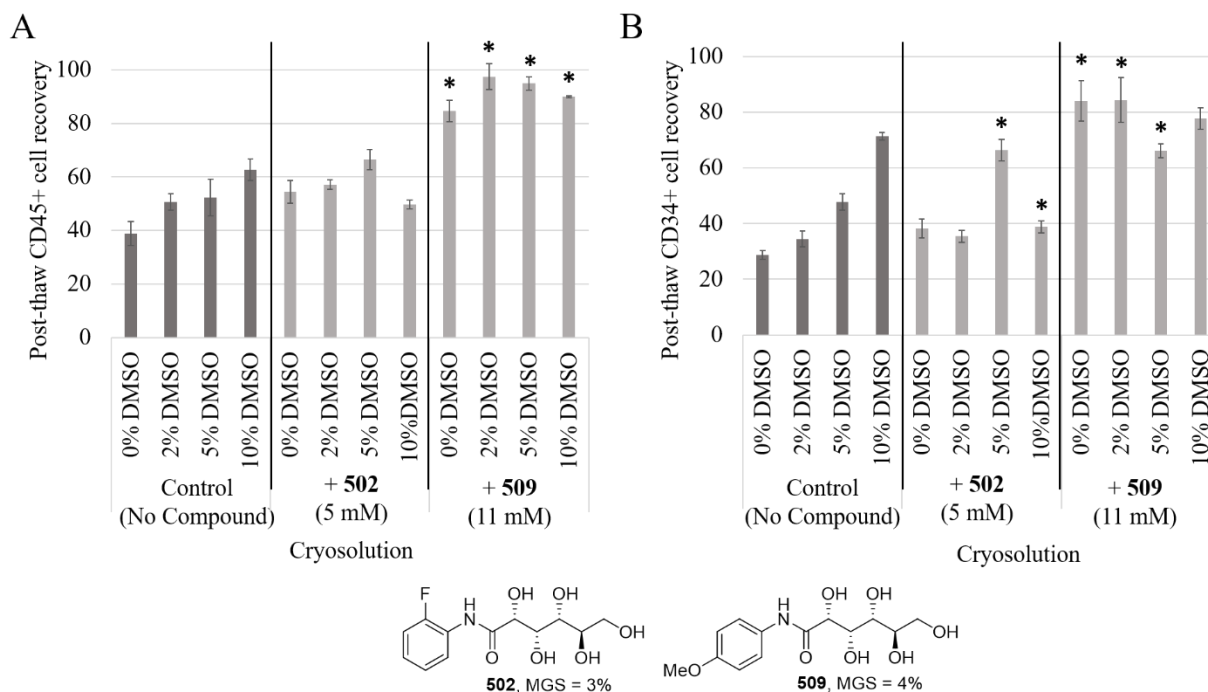


Figure 6.12. Post-thaw percent recovery of (A) CD45⁺ and (B) CD34⁺ cells from UCB cryopreserved with *N*-aryl-aldonamides **502** and **509**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control (no compound).

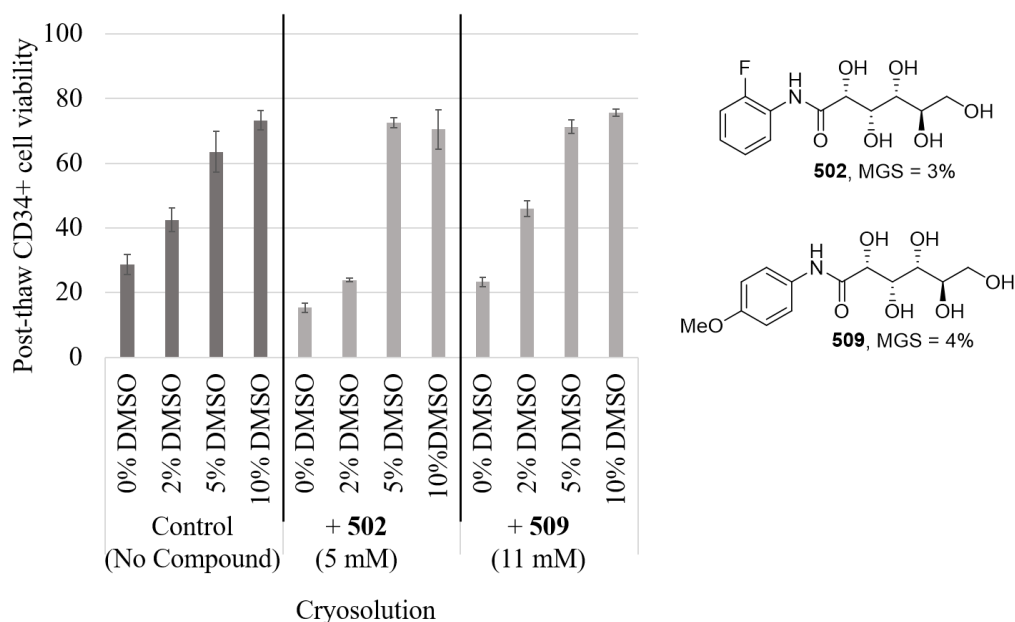


Figure 6.13. Post-thaw percent viability of CD34⁺ cells from UCB cryopreserved with *N*-aryl-aldonamides **502** and **509**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to the control (no compound).

The post-thaw CD34⁺ cell viability was not consistent with what was observed for TF-1 α cells frozen with **502** or **509**, in which an increase in cell viability was observed when the 2% DMSO cryosolution was supplemented with these compounds. Therefore, the TF-1 α cell line was deemed an inappropriate model for predicting the cryopreservation outcomes for HSCs. As such, other *N*-aryl-aldonamides were assessed for their ability to effectively cryopreserve CD34⁺ cells from UCB.

CD34⁺ cells were frozen with 0, 2, 5, and 10% DMSO supplemented with *N*-aryl-aldonamides, **501**, **503**, **533**, and **538** and post-thaw viability is shown in Figure 6.14. There was no significant difference in post-thaw viability when the DMSO solutions were supplemented with **501**, **503**, **533**, and **538** compared to the controls ($p > 0.05$) (with the exception of 10% DMSO + **533** which resulted in a significant decrease in viability compared to 10% DMSO alone). While there were no increases in post-

thaw viability upon supplementation of the DMSO cryosolutions with **501**, **503**, **533**, or **538**, increased post-thaw recoveries were observed (Figure 6.15). Similar to or higher CD34⁺ recoveries ($p < 0.05$) were observed when the 0%, 2%, and 5% DMSO cryosolutions were supplemented with **501**, **503**, **533**, or **538** (Figure 6.15). In fact, 0% DMSO and 2% DMSO supplemented with **501**, **503** or **538** resulted in statistically similar post-thaw CD34⁺ recoveries as the conventional 10% DMSO cryosolution ($p > 0.05$). This is significant because post-thaw CD34⁺ recovery is correlated with engraftment success.^{6,7,11} Significant increases in CD45⁺ cell recovery were also observed when 0, 2 and 10% DMSO was supplemented with **501** and when 10% DMSO was supplemented with **533** ($p < 0.05$) (Supplemental Figure S6.17).

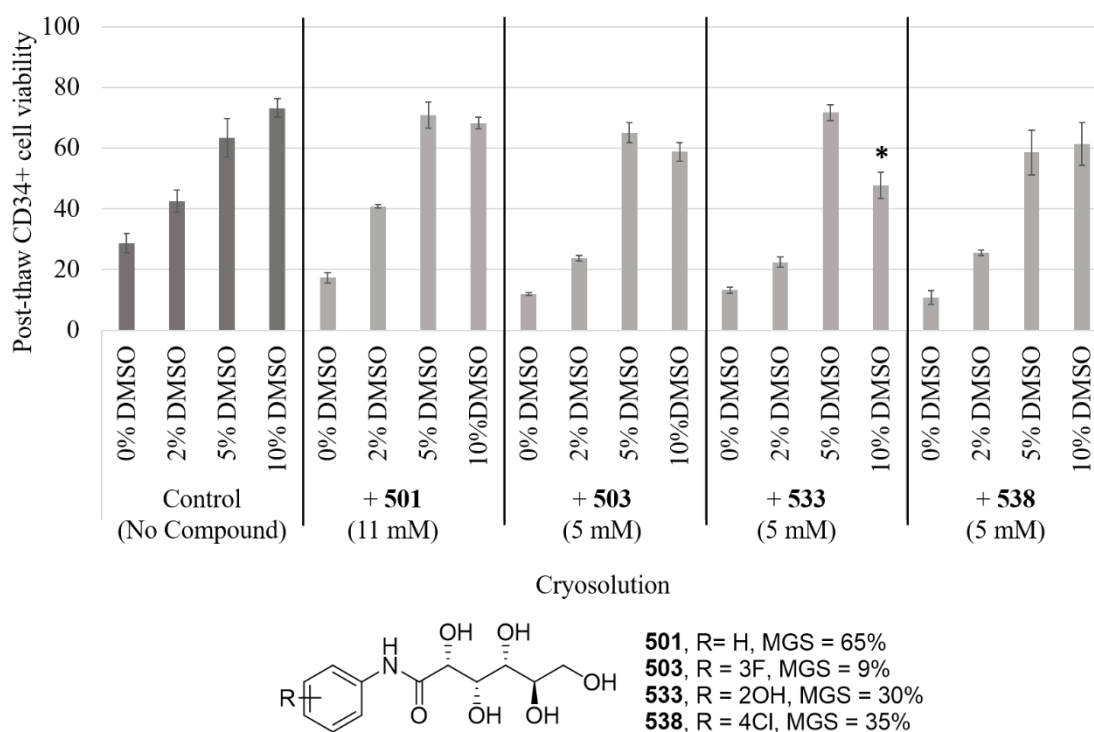


Figure 6.14. Post-thaw percent viability of CD34⁺ cells from UCB cryopreserved with *N*-aryl-aldonamides **501**, **503**, **533** and **538**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test.

Asterisks indicate significant difference compared to control (no compound).

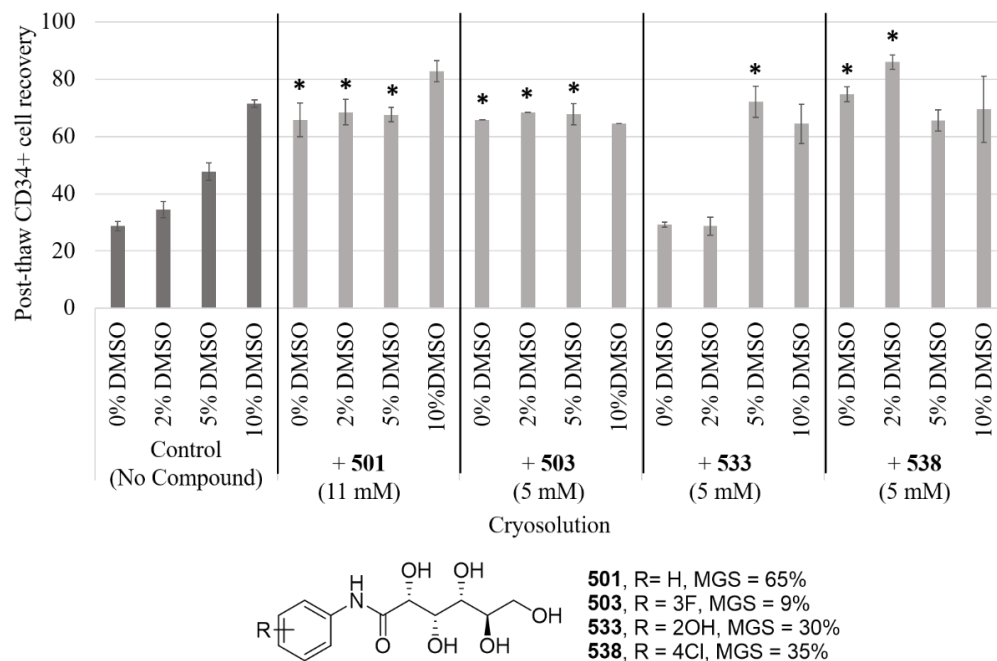


Figure 6.15. Post-thaw percent recovery of CD34⁺ cells from UCB cryopreserved with *N*-aryl-aldoxamides **501**, **503**, **533** and **538**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test.

Asterisks indicate significant difference compared to control (no compound).

N-aryl-aldoxamides possessing a carbon linker between the amide nitrogen and aromatic ring, **512**, **515**, **517**, **520**, or **521** were also assessed for their ability to cryopreserve CD34⁺ cells (Figures 6.16 & 6.17). Similar to the other *N*-aryl-aldoxamides assessed, there was no significant difference in post-thaw viability upon supplementation of the DMSO solutions with **512**, **515**, **517**, **520** or **521** ($p > 0.05$) (Figure 6.16). However, significantly higher CD34⁺ post-thaw recoveries ($p < 0.05$) were observed when the 0%, 2% and 5% DMSO cryosolutions were supplemented with **515** or **517** (Figure 6.17). When the 0 and 2% DMSO solutions were supplemented with **515** or **521** an increase in CD45⁺ cell recovery was observed ($p < 0.05$) (Supplemental Figure S6.18).

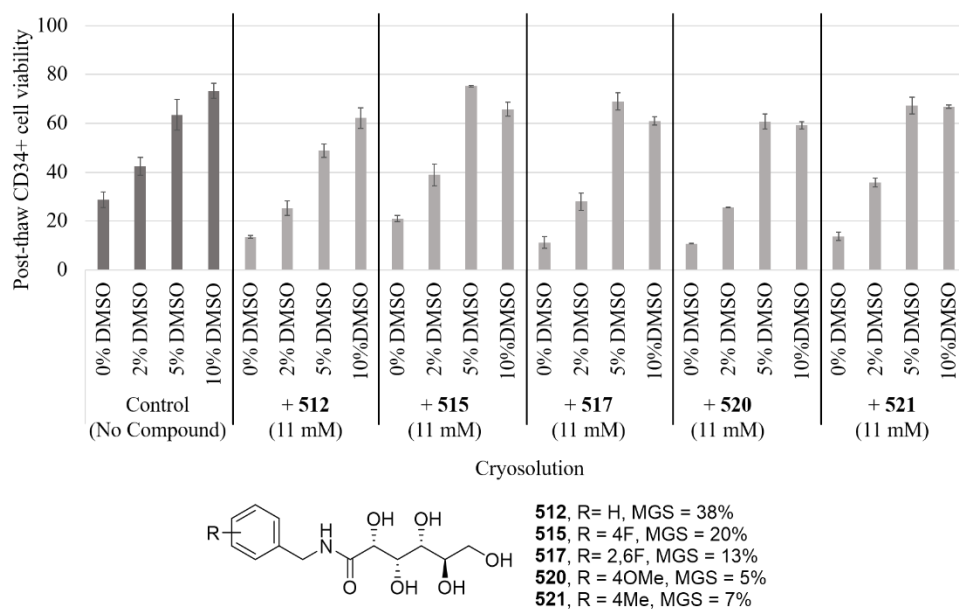


Figure 6.16. Post-thaw percent viability of CD34⁺ cells from UCB cryopreserved with *N*-phenyl-aldonamides **512**, **515**, **517**, **520**, and **521**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control (no compound).

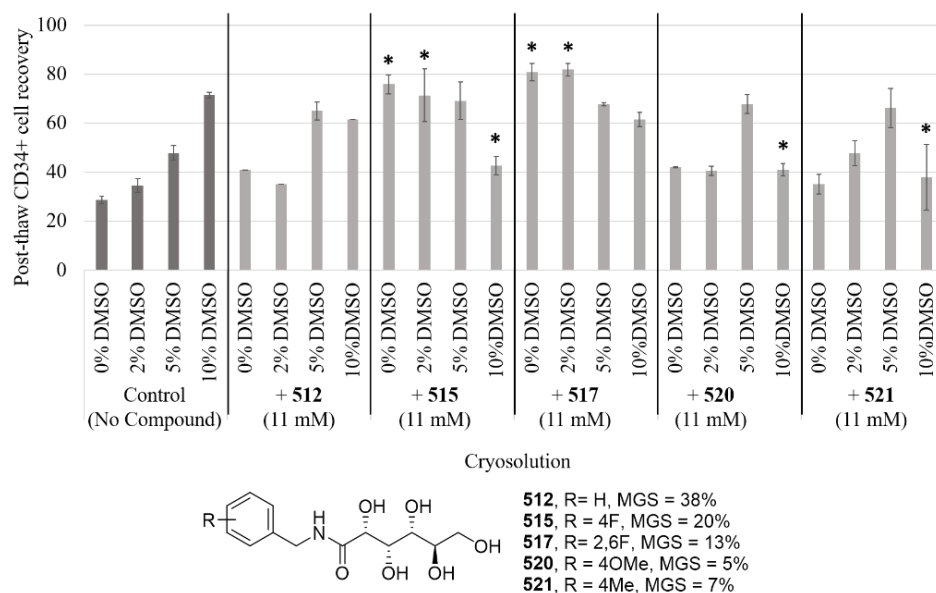


Figure 6.17. Post-thaw percent recovery of CD34⁺ cells from UCB cryopreserved with *N*-phenyl-aldonamides **512**, **515**, **517**, **520**, and **521**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples

were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control (no compound).

To summarize, supplementation of 0%, 2%, 5% or 10% DMSO cryosolutions with *N*-aryl-alidonamides did not result in an increase in post-thaw CD34⁺ viability ($p > 0.05$). However, some compounds resulted in improved post-thaw recovery with reduced DMSO concentrations. This is important because the absolute number of CD34⁺ cells transplanted is correlated with engraftment success.^{23,105} In other words, a reduction in cell viability could be tolerated if more CD34⁺ cells were recovered. This would be particularly beneficial if lower concentrations of DMSO were present in the sample.

While both post-thaw CD34⁺ cell viability and recovery are important, these cells must also be functional. Cryopreservation has been shown to impair the ability of HSCs to proliferate and differentiate.^{24–28} Up to 20% of patients receiving a UCB transplant will fail to engraft, in part due to inadequate potency of the unit.^{102,106–108} A recent study used pre-cryopreservation (pre-cryo) and post-thaw graft characteristics, including total nuclear cell (TNC), mononuclear cell (MNC), and CD34⁺ cell counts as well as colony forming unit (CFU) dose, to correlate the best indicators for successful engraftment.²⁷ The study concluded that CFU dose was the best indicator of engraftment success.²⁷ Typical post-thaw recovery in a cryopreserved unit was $> 80\%$ for TNC, MNC and CD34⁺, but the yield of colonies obtained in CFU assays after thawing was only 21.2%.²⁷ Based upon this measure of graft adequacy, only 2.8% of the inventory at a large cord blood bank (the CCBB at Duke) would provide an adequate dose to ensure timely engraftment (19.1×10^4 CFU/kg based on pre-cryo content) for a patients weighing more than 50kg.²⁷ To overcome this significant limitation, two or three separate UCB units are often transplanted in adult recipients to obtain a sufficient dose.¹⁰⁹ Therefore, optimization of cryopreservation protocols for HSCs, with emphasis on the preservation of clonogenic CFU potential, is an important issue for optimizing storage in UCB banks.

6.3.2 *Clonogenic potential of HSCs frozen with N-aryl-aldoenamides*

With the understanding that CFU dose is a better indicator of engraftment success than the viable CD34⁺ count,^{27,110} we wanted to assess the post-thaw clonogenic potential HSCs cryopreserved with IRI active *N*-aryl-aldoenamides. This work is included in a manuscript submitted to *ACS Chemical Biology* entitled “Small-molecule ice recrystallization inhibitors improve the functionality of hematopoietic stem cells after cryopreservation”.⁹⁶ Experiments were performed in which HSCs were cryopreserved in a 10% DMSO cryosolution supplemented with *N*-aryl-aldoenamides, **502**, **509**, **517** and **538**. Post-thaw colony formation was measured on samples cryopreserved with 10% DMSO cryosolutions because the majority of these cells were found to be viable post-thaw (Figures 6.14 & 6.16). Upon thawing, cryopreservation outcome was measured by performing the CFU assay. Briefly, the CFU assay involves plating cells in a semisolid medium that contains supplements and growth factors to support proliferation and differentiation.¹¹¹ Colony formation is a quantitative and reliable method to measure the capacity of cryopreserved cells to restore normal hematopoiesis and therefore the number of colonies formed is considered an indicator of increased efficiency of the cryoprotectant in terms of maintaining the quality of the graft.^{111,112} The total number of colonies formed post-thaw is shown in Figure 6.18. These results were from one UCB unit and repeated four times for each cryosolution. Remarkably, significant increases in colony formation were observed upon supplementation of the 10% DMSO cryosolution with **502** (5 mM) and **517** (11 mM).

Interestingly, increased colony formation was observed with concentrations of compounds that were much lower than those used to assess IRI activity. However, higher concentrations of IRIs were not possible using this current cryopreservation method due to the solubility limit of the IRIs. Therefore, the protocol was modified to utilize different dilutions of plasma with cryosolutions in order to obtain higher IRI concentrations in the final frozen HSC samples. A concentration scan was performed on two separate UCB units, repeated 4-8 times for each cryosolution (Figure 6.19). Remarkably, significant increases ($p < 0.001$, 0.01 , or 0.05) in the number of colonies formed were observed when the cryosolution was

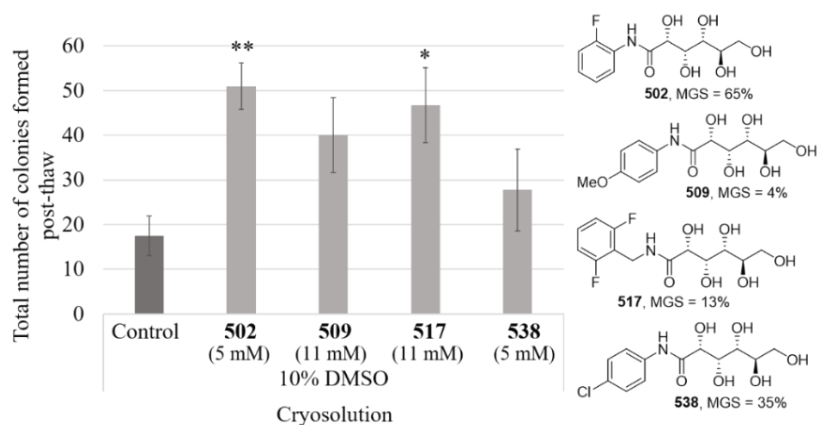


Figure 6.18. Total number of colonies formed after cryopreservation with 10% DMSO supplemented with IRI active molecules **502**, **509**, **517** and **538**. These results are from one cord blood unit and repeated four times for each cryosolution. Error bars are reported as the standard error of the mean (SEM). Statistical significance, marked by asterisks, was assessed using Student's unpaired T-test for comparison to the control (10% DMSO) with a 95% ($p < 0.05$, *) or 99% ($p < 0.01$, **) confidence level.

supplemented with IRI active molecules and this was concentration dependent. Compound **502** was most effective at 12.5 and 25 mM ($p < 0.05$ and 0.01), producing 61 and 63 colonies, respectively, compared to 31 colonies for the 10% DMSO control. Compound **502** (25 mM) also resulted in an increase in post-thaw CD34⁺ and CD45⁺ recovery compared to 10% DMSO alone ($p < 0.05$) (Figure 6.20 & Supplemental Figure S6.19). Compound **509** at 27.5 mM produced the most colonies (75) out of the compounds tested, more than 2.4 times produced than the 10% DMSO cryosolution and also resulted in a significant increase in CD45⁺ recovery ($p < 0.05$) (Supplemental Figure S6.19). Compound **517** was most effective at 18 mM, producing 57 colonies post-thaw and also resulted in a significant increase in post-thaw CD34⁺ and CD45⁺ recovery ($p < 0.05$) (Figure 6.20 & Supplemental Figure S6.19). Compound **538** did not produce significantly more colonies than the control ($p > 0.05$). What was interesting was that there was no significant difference in post-thaw viability of CD34⁺ cells when compared to the standard 10% DMSO cryosolution (Figure 6.21) ($p > 0.05$). Post-thaw CD34⁺ cell viability and colony formation were not correlated ($R^2 = 0.20$). These results further validate that post-thaw viability is not an accurate measure of cryopreservation success.

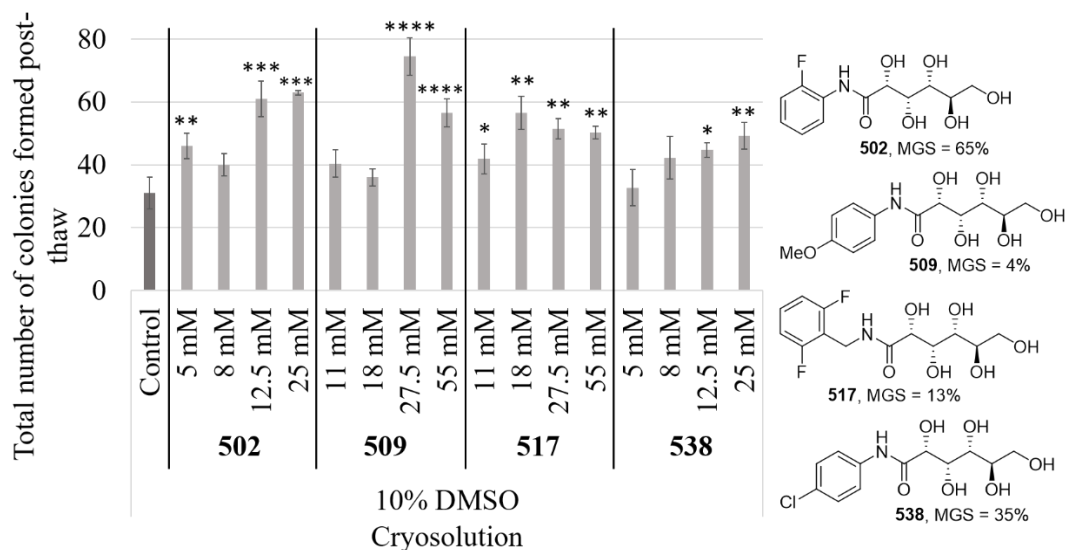


Figure 6.19. Total number of colonies formed after cryopreservation with 10% DMSO supplemented with IRI active molecules **502**, **509**, **517**, and **538** at various concentrations. These results are from two cord blood units and repeated four to eight times for each cryosolution. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to the control (10% DMSO) with a 95% ($p < 0.05$, *), 99% ($p < 0.01$, **), 99.9% ($p < 0.001$, ***) or 99.99% ($p < 0.0001$, ****) confidence level.

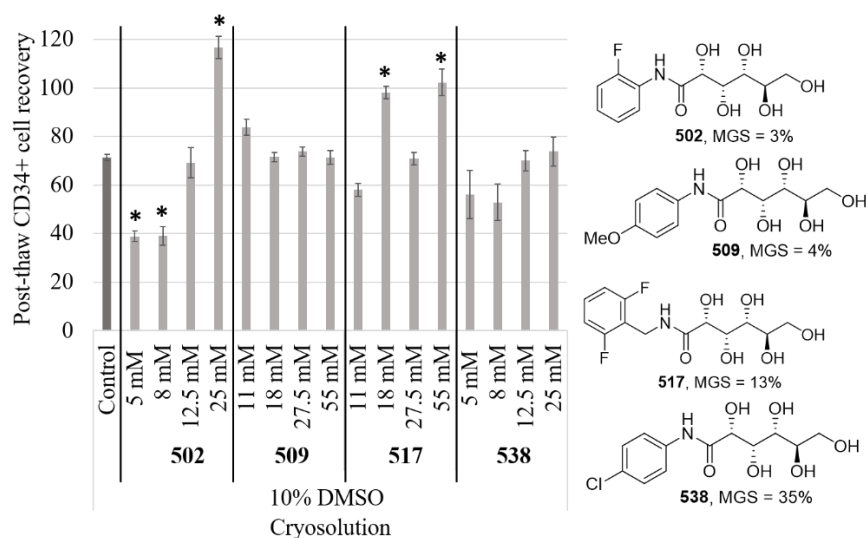


Figure 6.20. Post-thaw percent recovery of CD34⁺ cells from UCB cryopreserved with *N*-aryl-aldonamides **502**, **509**, **517** and **538** at various concentrations. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -

196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control.

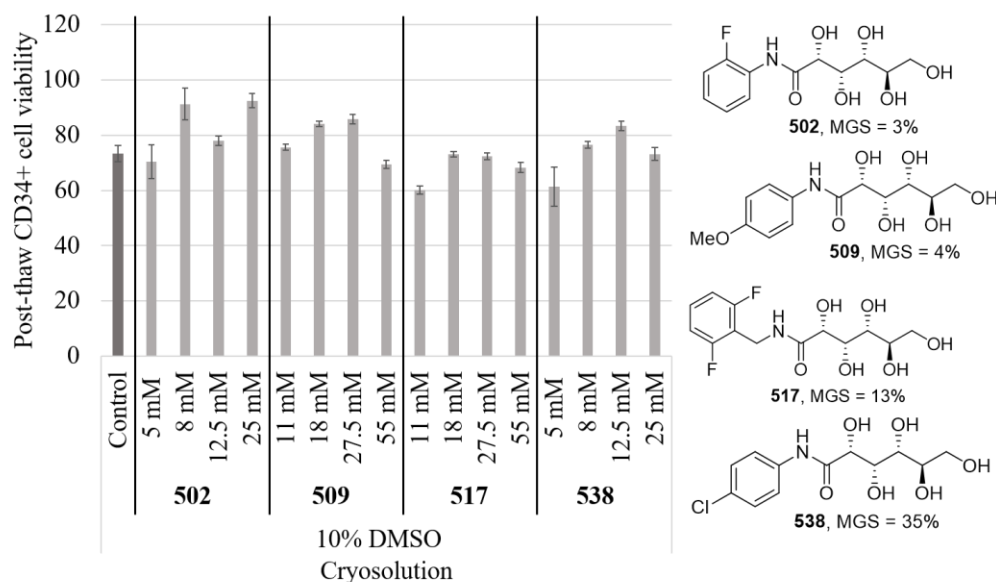


Figure 6.21. Post-thaw percent viability of CD34⁺ cells from UCB cryopreserved with *N*-aryl-aldonamides **502**, **509**, **517** and **538** at various concentrations. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test.

The increased recovery of progenitors with increased CFU suggested that these compounds can improve post-thaw recovery of immature UCB cells compared to the conventional 10% DMSO cryosolution. To address whether the cryoprotective property of these IRIs extends to more immature progenitors, we measured the frequency of multipotent progenitors in UCB HSCs using the long-term culture initiating cell (LTC-IC) assay. The frequency of LTC-IC was measured using limiting dilution analysis on two separate UCB units (Figure 6.22). This assay is frequently used to measure the frequency of progenitors with self-renewal and differentiation activities significantly superior to progenitors

detected using the CFU assay.¹¹³ The frequency of LTC-IC in UCB HSCs post-thaw with IRI **502** (25 mM) and **509** (27.5 mM) were increased compared to the 10% DMSO control (Figure 6.22).

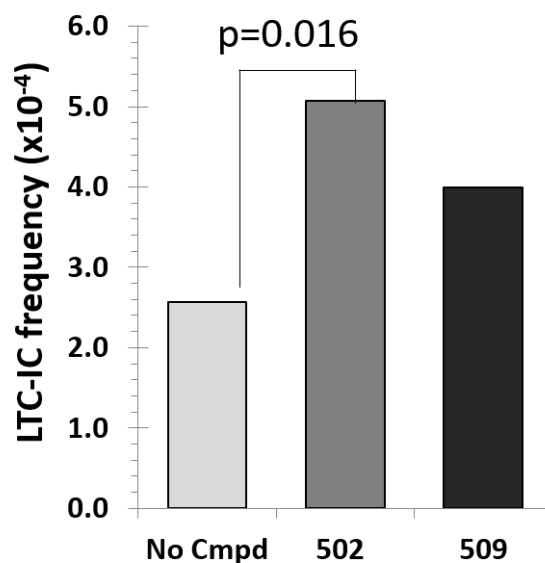


Figure 6.22. Frequency of LTC-IC in post-thaw LCs after cryopreservation with 10% DMSO supplemented or not with IRI **502** (25 mM) and **509** (27.5 mM).

To summarize, supplementation of the conventional 10% DMSO cryosolution with small molecule IRIs, **502**, **509**, and **517** resulted in a significant increase in clonogenic potential post-thaw. This could suggest one of two things. Either (1) during cryopreservation, ice recrystallization injury results in an impairment of HSC clonogenic potential post-thaw and therefore supplementation of the cryoprotectant solution with IRIs results in increased colony formation post-thaw; or (2) IRIs **502**, **509**, and **517** are stimulating colony growth. In either scenario, compounds that are capable of controlling ice recrystallization are able to preserve the functionality of HSCs post-thaw. Improved cryopreservation methods for UCB resulting in increased clonogenic potential would increase the effective dose of cells available in stored units and therefore allow greater access to UCB transplantation.¹¹⁴ Overall, the supplementation of the current cryoprotectant solution with small molecules capable of controlling ice growth and recrystallization resulted in an increase in CFU recovery and frequency of multipotent progenitors in UCB units. Ultimately, this would reduce the percentage of engraftment failure and allow

for a larger proportion of cord blood banks' inventory to provide an adequate dose for patients requiring transplants. This would also reduce the costs associated with UCB transplantation. The cost per day for patients with engraftment failure is approximately seven times that of a patient who does not fail to engraft.¹¹⁵

6.3.3 CFU analysis

In the previous section (Section 6.3.2), it was found that the total number of colonies formed post-thaw was significantly increased when the cryosolution was supplemented with **502**, **509**, or **517**. During the CFU assay, several different types of colonies are formed. Colony types are differentiated by morphology shown in Figure 6.23. Single cell type colonies include CFU-M (macrophages), CFU-G (granulocytes) and BFU-E (burst-forming unit-erythroids) while multi-cell type colonies include CFU-GM or CFU-GEMM, which are comprised of a mixture of granulocytes, macrophages, erythrocytes and megakaryocytes.

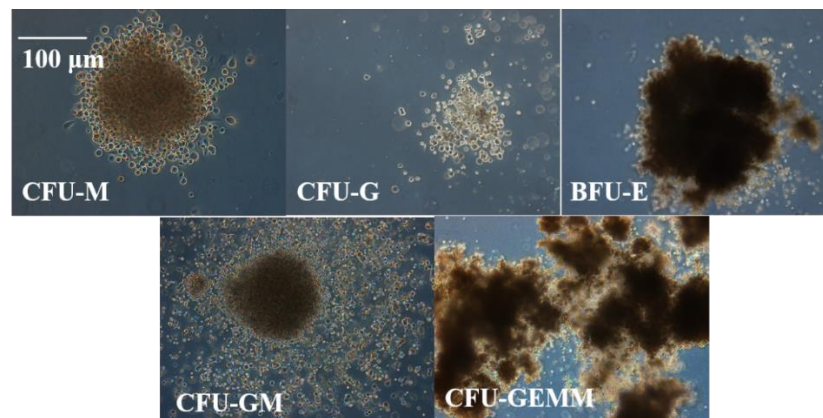


Figure 6.23. Images of different types of colonies formed in the CFU assay.

The composition of the colonies formed post-thaw with different cryosolutions was determined (Figure 6.24). With the conventional 10% DMSO cryosolution, the composition of colonies were 50% BFU-E, 22% CFU-GM, 16% CFU-G, 9% CFU-GEMM and 2% CFU-M. It is interesting to note that the composition of the types of colonies changed when HSCs were cryopreserved with 10% DMSO

supplemented with IRIs **502**, **509**, or **517**. For example, a larger portion of the colonies formed post-thaw with 10% DMSO + **502** were BFU-E. A larger portion of the colonies formed with 10% DMSO + **509** were CFU-GEMM. With the 10% DMSO + **517** (18 mM) cryosolution, more CFU-M colonies were formed compared to the control. This could suggest one of two things; either ice recrystallization during cryopreservation is impairing the ability of the HSCs to form these types of colonies or that these molecules are acting as growth factors for specific colonies. Since the type of colony that was increased post-thaw was different for each IRI, it was hypothesized that the latter was occurring. This could allow for the customization of the cryosolution to favour the formation of certain cells post-thaw depending on the desired application. This is something that will be investigated in future work.

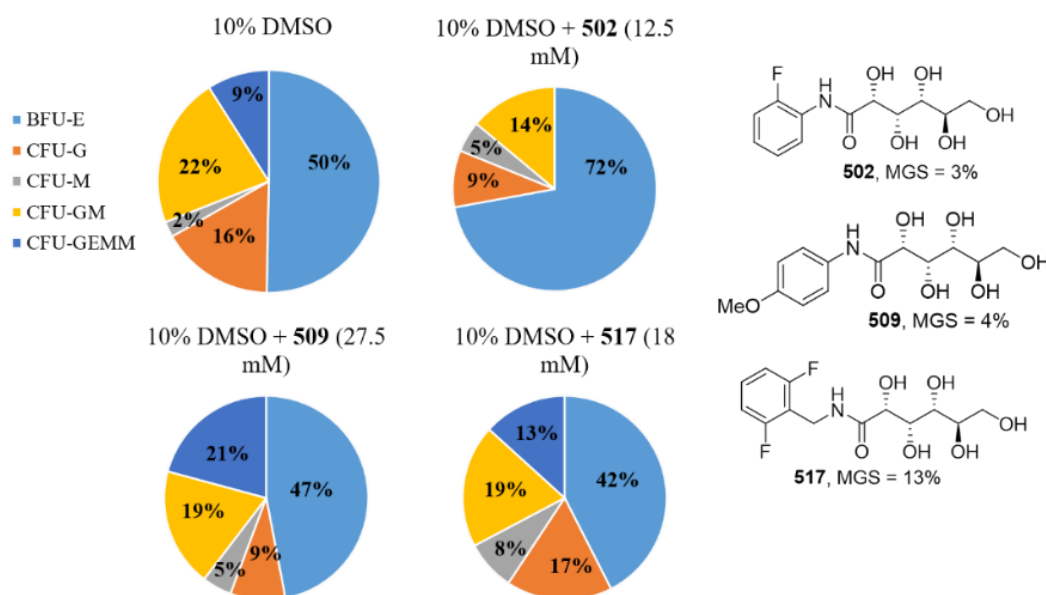


Figure 6.24. Composition of colonies formed after cryopreservation with IRIs **502**, **509**, **517** and **538**.

6.3.4 Mechanism of cryoprotection

6.3.4.1 Inhibition of ice recrystallization

In the previous section (Section 6.3.3), it was found that the total number of colonies formed post-thaw was significantly increased when the cryosolution was supplemented with IRI active molecules **502**, **509**, and **517**. In order to investigate whether this enhanced functional protection can be attributed to

the molecules' ability to inhibit ice recrystallization, HSCs frozen with molecules that were structurally similar to **502**, **509**, **517**, and **538** but which were not IRI active. HSCs were frozen with 10% DMSO supplemented with **504**, **508**, **516**, or **537** which possess IRI activities of 90%, 100%, 72% and 100% respectively. There were no significant differences in colony formation when the 10% DMSO cryosolution was supplemented with small molecules that are not IRI active (Figure 6.25). Furthermore, there was no significant difference in post-thaw cell viability (Figure 6.26). There was also no significant difference in post-thaw CD45⁺ recovery (Supplemental Figure S6.20). However, a reduction in post-thaw CD34⁺ recovery was observed for several cryosolutions (Figure 6.27).

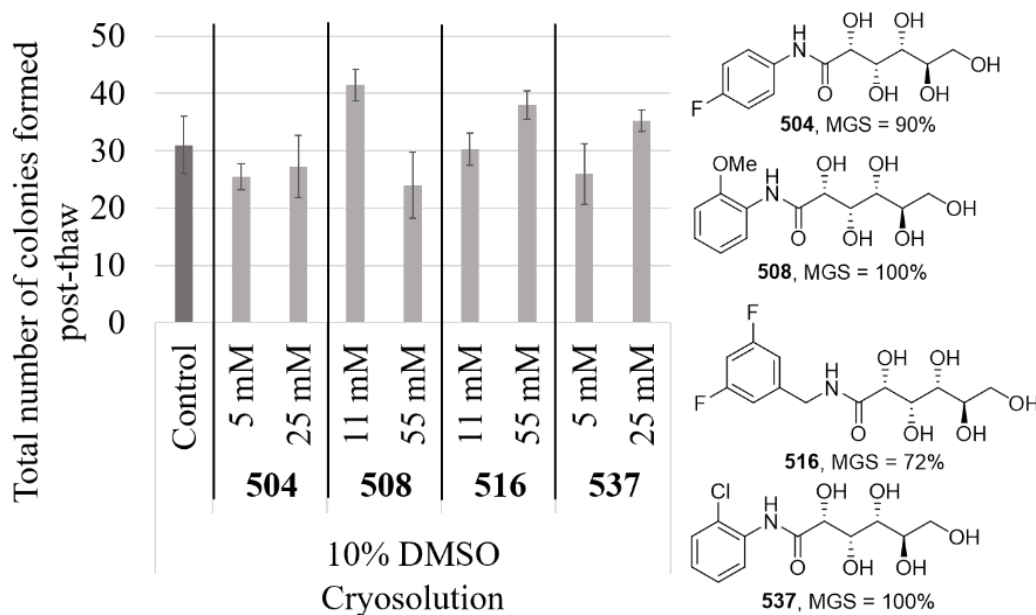


Figure 6.25. Total number of colonies formed after cryopreservation with 10% DMSO supplemented with IRI active molecules **504**, **508**, **516** and **537** at various concentrations. These results are from two cord blood units and repeated four to eight times for each cryosolution. Error bars are reported as the standard error of the mean (SEM).

Statistical significance ($p < 0.05$) was assessed by the Student's unpaired T-test for comparison to control.

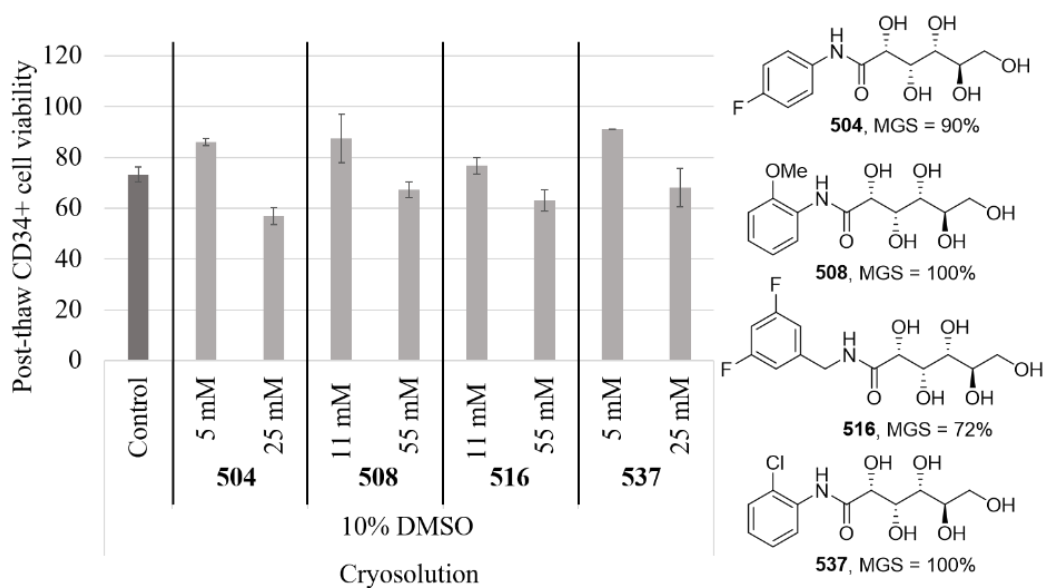


Figure 6.26. Post-thaw percent viability of CD34⁺ cells from UCB cryopreserved with *N*-aryl-aldonamides **504**, **508**, **516** and **537** at various concentrations. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed by the Student's unpaired T-test for comparison to control.

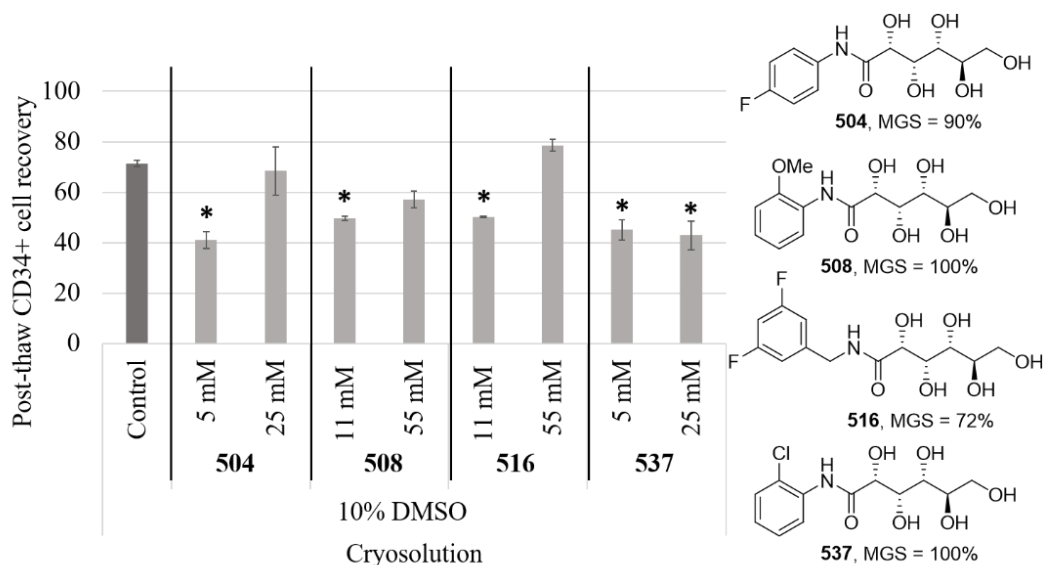


Figure 6.27. Post-thaw percent recovery of CD34⁺ cells from UCB cryopreserved with *N*-aryl-aldonamides **504**, **508**, **516** and **537** at various concentrations. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -

196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Asterisks indicate significant difference ($p < 0.05$) compared to the controls as assessed by the Student's unpaired T-test for comparison to control.

The IRI activity of the compounds used for the cryopreservation of HSCs was measured utilizing PBS solutions containing 22 mM of compound. While this is our standard method for measuring the IRI activity of newly synthesized molecules, the cryosolutions were comprised of *N*-aryl-alidonamides dissolved in water (0.9% saline/0.5% dextran) at concentrations different than 22 mM and were in the presence of plasma and HSCs. When assessed in PBS, compounds **502**, **509**, **517**, and **538** were IRI active (3%, 4%, 13% and 35% MGS respectively) and compounds **504**, **508**, **516**, and **537** were inactive (90%, 100%, 72% and 100% respectively). We predicted that ice crystals should be noticeably smaller in size in the presence of an ice recrystallization inhibitor. Thus, solutions of HSCs in cryosolution and plasma were assessed for IRI activity.

We assessed the IRI activity of plasma and compounds **502**, **509**, **517** and **538** in the presence of HSCs (Figure 6.28). The chemical structures and IRI activities of **502**, **509**, **517** and **538** in PBS are shown in Figure 6.29A for reference. Compared to PBS, blood plasma alone possessed remarkable IRI activity, with an MGS of 13%. This was not surprising as plasma contains many different lipids, carbohydrates, proteins and salts. HSCs suspended in plasma and saline/dextran (cryosolution without DMSO or IRI) were less active (24% MGS) compared to plasma ($p < 0.05$ assessed by unpaired Student's T-test) alone which was attributed to the dilution of plasma with saline/dextran. HSCs in plasma and saline/dextran supplemented with compounds **502**, **509**, **517**, and **538** show increased IRI activity compared to HSCs in plasma and saline/dextran alone ($p < 0.05$ assessed by unpaired Student's T-test). Interestingly, the solutions which were the most IRI active were those that produced the most colonies post-thaw. A solution of HSCs with **502** (12.5 mM) or **509** (27.5 mM) possessed IRI activities of 9% and 3% respectively compared to without compound (24% MGS). HSCs frozen with **502** (12.5 mM) or **509** (27.5 mM) produced the most colonies (61 and 75, respectively) compared to 31 colonies for the control.

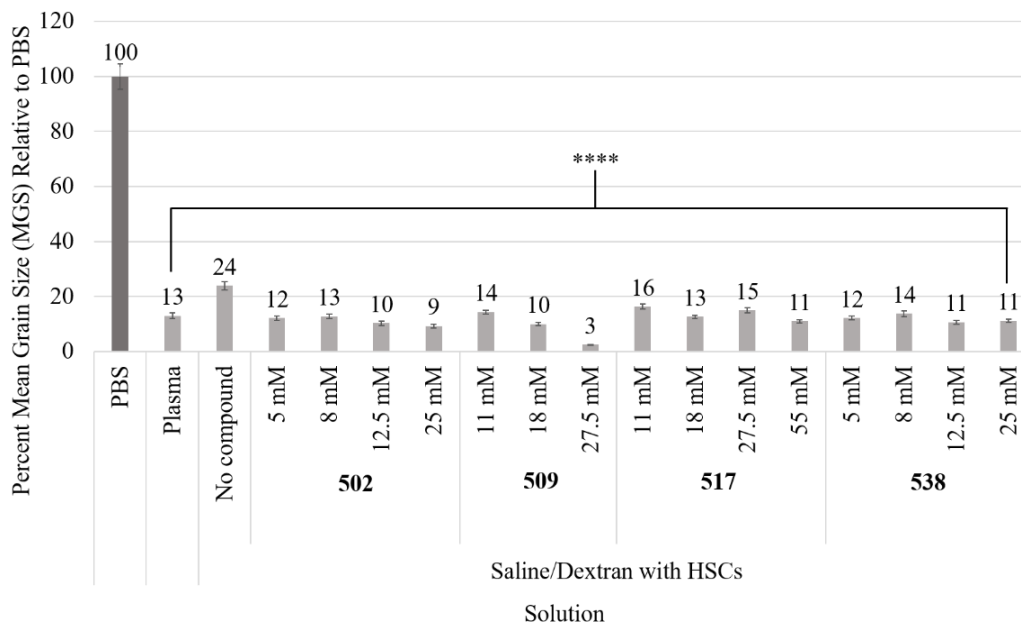


Figure 6.28. IRI activity of HSC cryosolution (saline/dextran and plasma supplemented with IRIs). Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.0001$) was assessed using a one-way analysis of variance (ANOVA) with a Dunnett post-test for comparison to control (PBS).

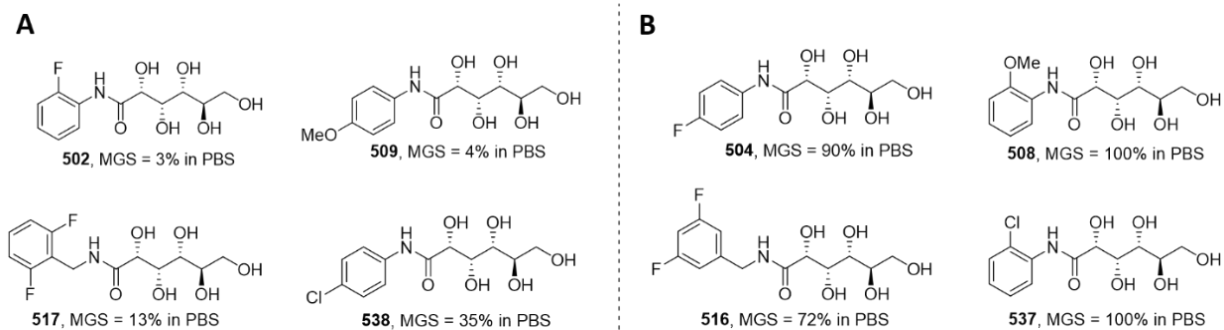


Figure 6.29. Chemical structures and IRI activities (in PBS) of *N*-aryl-aldonamides.

We next assessed the IRI activity of compounds **504**, **508**, **516**, and **537** in the presence of HSCs (Figure 6.30). The chemical structures and IRI activities of **504**, **508**, **516**, and **537** in PBS are shown in Figure 6.29B for reference.

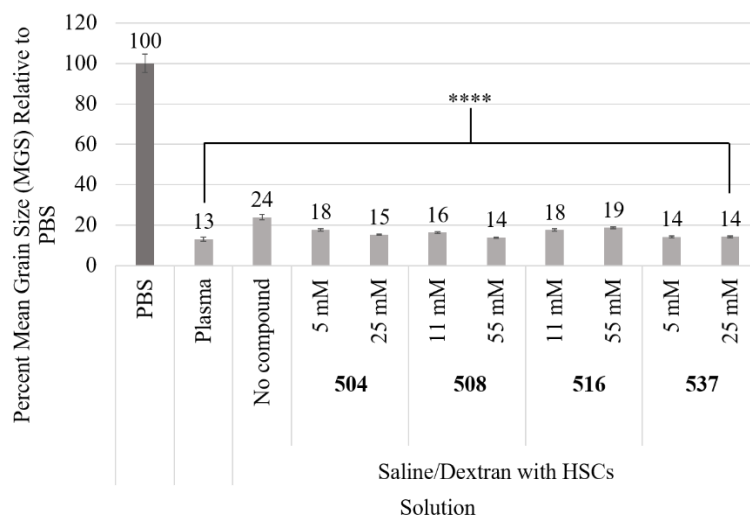


Figure 6.30. IRI activity of HSC cryosolution (saline/dextran and plasma supplemented with compounds **504**, **508**, **516** and **537**). Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.0001$) was assessed using a one-way analysis of variance (ANOVA) with a Dunnett post-test for comparison to control (PBS).

As expected, solutions of **504**, **508**, **516**, and **537** in the presence of HSCs had weaker IRI activity than solutions of **502**, **509**, **517** and **538** in the presence of HSCs. Solutions of HSCs containing compounds **502** (12.5 mM and 25 mM) or **509** (27.5 mM) were significantly more IRI active ($p < 0.05$ assessed by Student's T-test) than any HSC solution containing inactive compounds **504**, **508**, **516**, or **537**. Overall, solutions that were more IRI active in the presence of cells resulted in the most colonies formed post-thaw whereas less IRI active solutions did not result in increased colony formation compared to the control. This suggests that ice recrystallization is inhibited in the presence of cells when IRI active molecules are supplemented and that this leads to an increase in colony formation of HSCs frozen with these molecules.

6.3.4.2 Further analyses of IRI activity of important *N*-aryl-alidonamides

In Chapter 5, the IRI activities of 47 *N*-aryl-alidonamides were assessed using our traditional method for measuring IRI activity. The traditional method used by the Ben laboratory to measure IRI

activity is the “splat cooling” assay.¹¹⁶ This method measures the average ice crystal size after thirty minutes of recrystallization at -6.4 °C. The major downfall with this method is that it fails to take into account the time and concentration dependence of ice recrystallization and the heterogeneity of ice crystal sizes. Recently, the Ben lab, in collaboration with Prof. Keillor, identified a more accurate way to measure IRI activity that takes these factors into account.¹¹⁷ This work was done by a previous Ben lab member, Stephanie Abraham and undergraduate student, Kerkeslin Keillor. The new method allows IRI activity to be quantified by an IC₅₀ value.¹¹⁷ This was done by using a modified version of the “splat cooling” assay. Briefly, the ice crystal sizes in a sample are placed in different bins according to size. When the sample is first frozen, there are many ice crystals that are very small and therefore are all in the first, smallest bin size (Bin 1). After five minutes of recrystallization, the ice crystals may grow and enter the next larger bin sizes. The change in proportion of crystals in Bin 1 after five minutes allows one to calculate an initial rate of growth (v_{norm}) which can be normalized to the PBS control.¹¹⁷ Thus, a dose-response curve can be generated and IRI activity can then be quantified based on IC₅₀ values.¹¹⁷ The IC₅₀ value is the concentration of compound in which 50% of the crystals remain small and in Bin 1 after five minutes of recrystallization.¹¹⁷

The dose-response curves generated by Stephanie are shown in Figure 6.31.¹¹⁷ For a compound that was previously determined to be inactive, such as D-glucose, a sigmoidal curve was not observed and therefore IC₅₀ concentration cannot be obtained (Figure 6.31A). For previously identified IRI active molecules, β -PMP-Glc (**166**, 23% MGS) and β -pBrPh-Glc (**171**, 8% MGS), a sigmoidal curve was obtained and the IC₅₀ values were found to be 19.1 mM and 13.5 mM respectively (Figure 6.31B & C).¹¹⁷

This more thorough analysis of IRI activity was performed on the *N*-aryl-aldonamides which improved the cryopreservation of HSCs (**502**, **509**, **517** and **538**). The dose-response curves for the *N*-aryl-aldonamides, **502**, **509**, **517** and **538**, were generated (Figure 6.32). Compounds **502**, **509**, **517** and **538** were found to have IC₅₀ values of 4.1 mM, 5.1 mM, 10.8 mM and 12.0 mM respectively. It is interesting to note that **502** and **509** have low IC₅₀ values and were also the most effective compounds at

cryopreserving HSCs. This could suggest that the IC_{50} value is a better indicator of potential cryopreservation success for HSCs than the traditional MGS. Therefore, new compounds with low IC_{50} values should be investigated for their ability to improve the cryopreservation of HSCs.

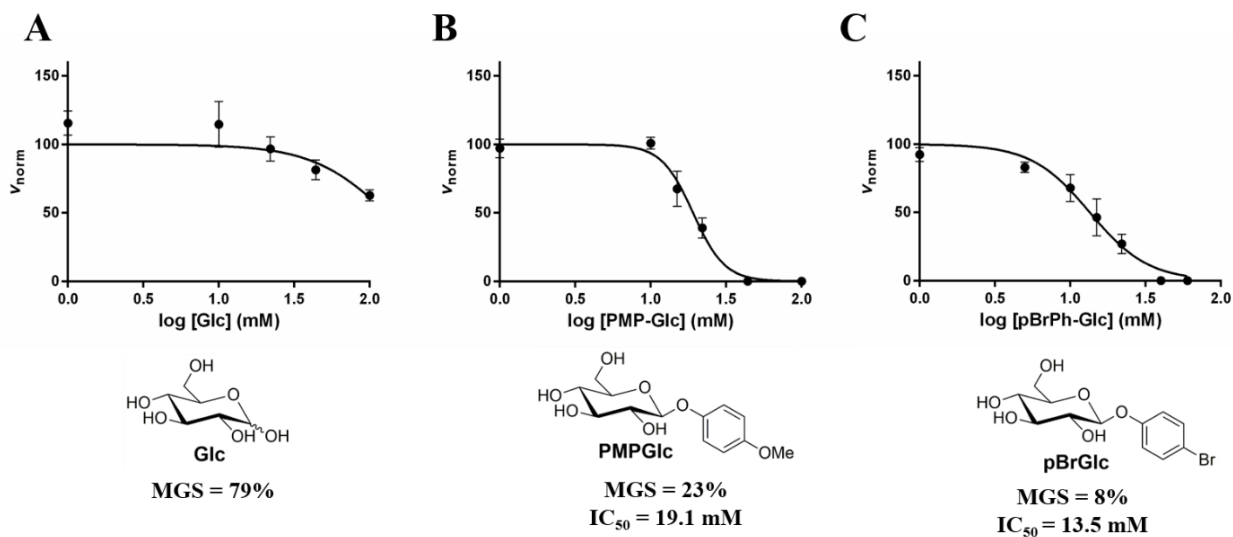


Figure 6.31. Dose response curves for D-glucose, β -pBrPh-Glc and β -PMP-Glc.¹¹⁷

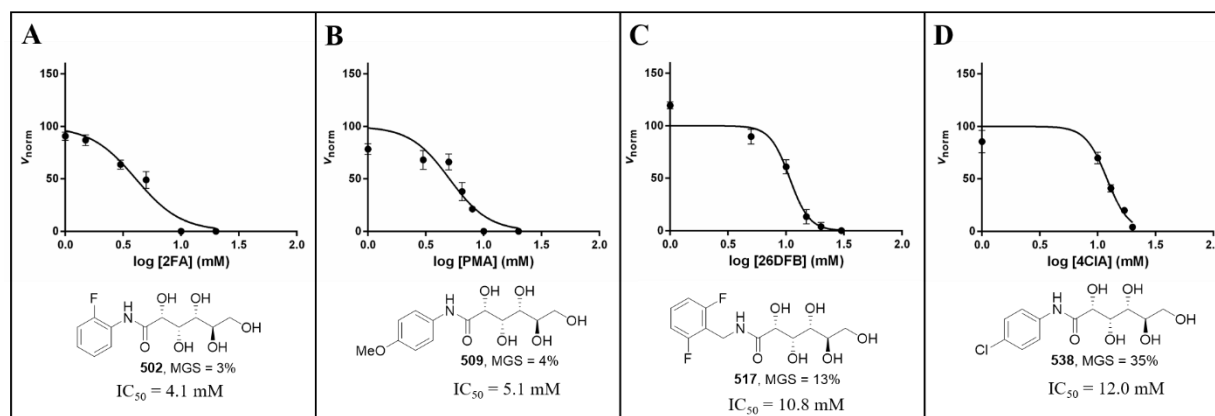


Figure 6.32. Dose response curves for *N*-aryl-aldonamides.

6.3.4.3 *The effect of transient warming on HSC recovery, viability and clonogenic potential*

IRI active compounds, **502**, **509**, **517**, and **538** were capable of improving the clonogenic potential of HSCs after cryopreservation. This was attributed to the ability of these molecules to inhibit ice recrystallization because structurally similar compounds that did not possess IRI activity did not improve the clonogenic potential post-thaw. We therefore hypothesized that these compounds would protect cells against injury resulting from ice recrystallization during transient warming events (TWEs). As discussed in the introductory section, UCB units can theoretically be stored indefinitely.^{118,119} Broxmeyer and coworkers determined that HSCs from UCB which had been stored in liquid nitrogen for 21-23.5 years readily engrafted into mice.¹¹⁸ However, temperature fluctuations (due to TWEs) experienced in stored UCB units when the tank is accessed or when a unit is being transferred to and from different blood banks can negatively impact the quality of the unit.¹²⁰ TWEs have been recognized as a significant contributor to reduced post-thaw viabilities in sperm,¹²¹ placental cord blood,^{122,123} peripheral blood mononuclear cells (PBMNCs)^{124,125} and tissue allografts.¹²⁶ Furthermore, high levels of apoptosis PBMNCs stored in liquid nitrogen were observed as a result of improper storage and temperature cycling.¹²⁰

In order to investigate the effects of TWEs on HSCs frozen with cryosolutions containing IRIs, an experiment was designed to mimic transient warming. HSCs suspended in cryosolution were frozen to -80 °C (1 °C/min) followed by storage at -196 °C for at least 48 hours. Samples were then placed in dry ice and the temperature was allowed to equilibrate. The temperature was monitored by a temperature probe inserted into a control cryovial. After equilibration, the samples were partially thawed to -20 °C by placement in a cold ethylene glycol bath. After stabilization at -20 °C, the sample was cooled again to -80 °C. This process was repeated for several cycles (one, three, or five) before the samples were fully thawed. A schematic representation of this cycling process is shown in Figure 6.33.

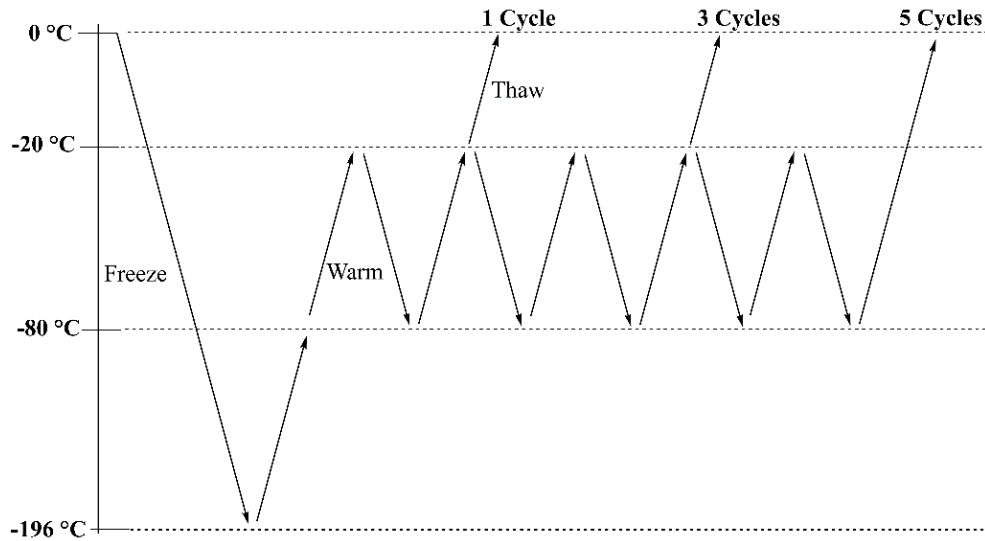


Figure 6.33. Illustration of the exacerbation of ice recrystallization related injury and transient warming effects.

Each -80 °C to -20 °C to -80 °C represents one cycle of transient warming.

The post-thaw recovery, viability, and clonogenic potential of HSCs frozen with 10% DMSO supplemented with **502**, **509**, or **517** were measured immediately post-thaw (no transient warming) and after one, three, and five cycles of transient warming (Figures 6.34, 6.35 & 6.36). As discussed in Section 6.3.2, immediately post-thaw CD34⁺ recovery was significantly increased when the 10% DMSO cryosolution was supplemented with **502** (25 mM) or **517** (18 mM) compared to the 10% DMSO control (Figure 6.34) ($p < 0.05$). This increase in CD34⁺ recovery was also observed after one cycle of transient warming ($p < 0.05$). After three cycles of transient warming, there was no improvement in post-thaw recovery with the addition of the IRIs to the 10% DMSO cryosolution ($p > 0.05$). However, CD34⁺ recovery is significantly increased after five cycles of transient warming with the addition of **517** (18 mM) compared to the control ($p < 0.05$).

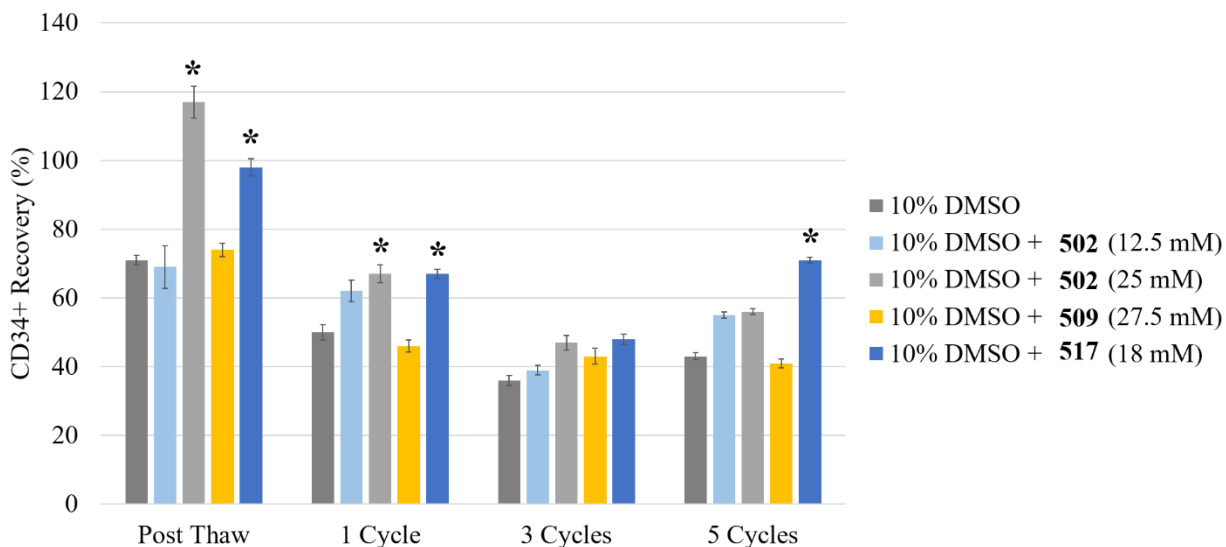


Figure 6.34. Post-thaw CD34⁺ recovery immediately post-thaw and after one, three, or five cycles of transient warming for HSCs frozen with 10% DMSO supplemented with **502**, **509**, or **517**. Error bars are reported as the standard error of the mean (SEM). Statistical significance was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference ($p < 0.05$) compared to control.

Viability immediately post-thaw and after one, three and five cycles of transient warming was the same with the addition of IRIs compared to the controls ($p > 0.05$) (Figure 6.35). This was consistent with results in previous sections that found that post-thaw viability was not altered with the addition of small-molecule IRIs (Section 6.3.2 & 6.3.3). Visual inspection of Figure 6.35 indicated that a progressive decrease in post-thaw viability was experienced after successive transient warming cycles. However, post-thaw viability did not significantly decrease ($p > 0.05$) even after five cycles of transient warming when the HSCs were frozen with 10% DMSO or 10% DMSO + **517** (18 mM) as assessed by ANOVA with a Tukey post-test. Post-thaw viability significantly decreased after five cycles of transient warming when HSCs were frozen with 10% DMSO + **502** (12.5 mM) or 10% DMSO + **509** (27.5 mM) ($p < 0.05$). With 10% DMSO + **502** (25 mM), post-thaw viability significantly decreased after only three cycles of transient warming ($p < 0.05$).

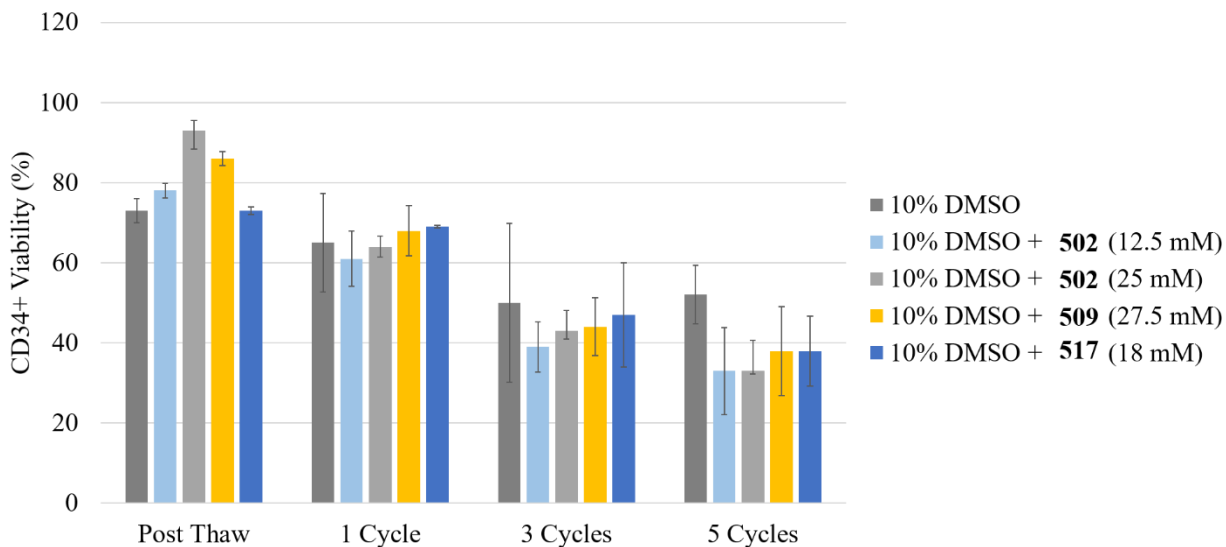


Figure 6.35. Post-thaw CD34⁺ viability immediately post-thaw and after one, three, or five cycles of transient warming for HSCs frozen with 10% DMSO supplemented with **502**, **509**, or **517**. Error bars are reported as the standard error of the mean (SEM). Statistical significance was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test.

As discussed in Section 6.3.3, the addition of **502**, **509**, or **517** to the 10% DMSO cryosolution resulted in a significant increase in the clonogenic potential of HSCs post-thaw. However, there was no significant increase in colony formation when the HSCs were frozen with these IRIs and subjected to TWEs (one, three, or five cycles) compared with the 10% DMSO control (Figure 6.36). Interestingly, colony formation was not significantly decreased even after three cycles of transient warming when the HSCs were frozen with the 10% DMSO control. However, colony formation was significantly decreased when the 10% DMSO cryosolution was supplemented with **502** (12.5 or 25 mM) after just one cycle of transient warming and after three cycles with **509** (27.5 mM) or **517** (18 mM). It was hypothesized that the addition of molecules that are capable of inhibiting ice recrystallization to the cryosolution for HSCs would improve the functionality of the cells subjected to TWEs post-thaw. However, these results suggested that the addition of IRIs **502**, **509** and **517** to the 10% DMSO cryosolution was actually detrimental to the post-thaw clonogenic potential when the HSCs were subjected to TWEs. This

suggested that these molecules were functioning by an alternate mechanism and were not increasing the clonogenic potential of HSCs post-thaw as a result of their ability to inhibit ice recrystallization.

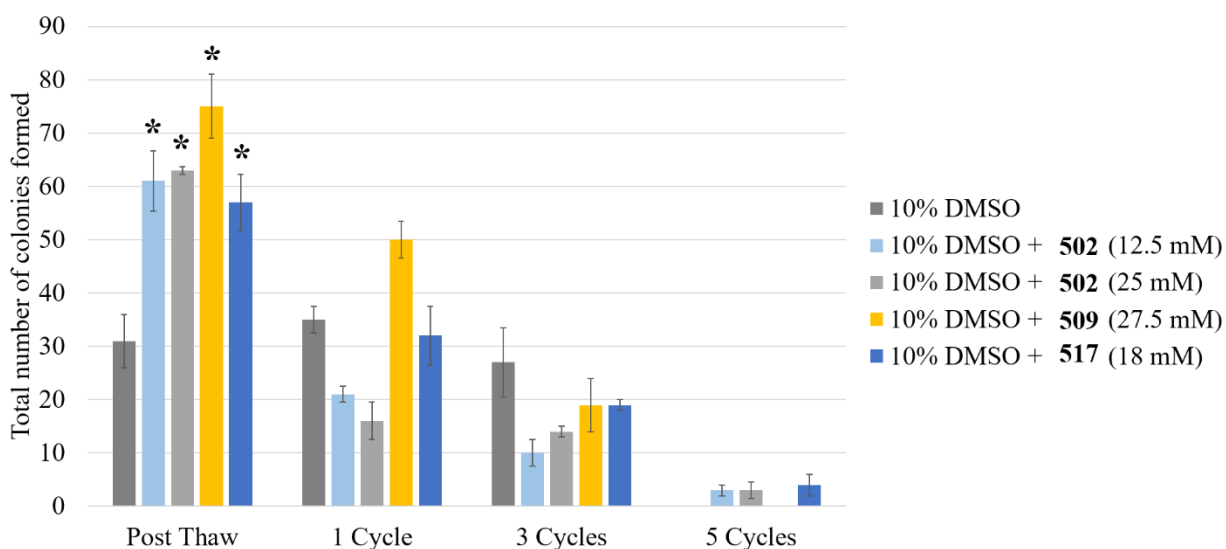


Figure 6.36. Total number of colonies formed immediately post-thaw and after one, three, or five cycles of transient warming for HSCs frozen with 10% DMSO supplemented with **502**, **509**, or **517**. Error bars are reported as the standard error of the mean (SEM). Statistical significance was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference ($p < 0.05$) compared to control.

To summarize this section, supplementation of the 10% DMSO cryosolution with IRIs **502**, **509** or **517** does not improve the cryopreservation outcome of HSCs subjected to TWEs. The clonogenic potential of HSCs was significantly decreased after one, three and five cycles of transient warming. This suggested that IRIs **502**, **509** and **517** were functioning by a mechanism different than their ability to inhibit ice recrystallization.

6.3.4.4 Expression of cell surface markers

In Section 6.3.3, it was found that the cryopreservation of HSCs with cryosolutions supplemented with small molecule IRIs **502**, **509**, **517**, and **538** resulted in improved clonogenic potential of HSCs post-thaw. Supplementation of the 10% DMSO cryosolution with structurally similar molecules which did not possess IRI activity did not significantly increase colony formation post-thaw. Therefore, it was

hypothesized that the increase in clonogenic potential was a result of the ability of these molecules to inhibit ice recrystallization. However, IRI active molecules, **502**, **509**, **517**, and **538** were not able to preserve the functionality of frozen HSCs which were subjected to TWEs suggesting that these molecules were functioning by an alternate mechanism. As such, the expression of several characteristic cell surface markers on HSCs that have been frozen with 10% DMSO supplemented with **502**, **509**, **517**, or **538** was investigated.

It has been reported that the cryopreservation of HSCs results in the shedding of surface receptors.¹²⁷ As a result, growth factor responsiveness is impaired. HSCs are often infused in an irradiated and severely immunocompromised host where the stroma may also be affected and therefore could be exposed to limiting concentrations of growth factors.^{78,79} Therefore, the ability of HSCs to respond to growth factors, expression of adhesion molecules, and their ability to interact with stromal microenvironment must be preserved during cryopreservation. It was hypothesized that small-molecule IRIs capable of improving the clonogenic potential of HSCs post-thaw could also be protecting cell surface receptors important for homing and engraftment.

Homing of HSCs to bone marrow and their successful interaction with the stromal cells and other matrix molecules present in the marrow are mediated by specific adhesion molecules expressed on the surface of these cells.¹²⁸ HSCs express CD43 and CD44, which are known to play important roles in successful homing and engraftment.⁸¹ Furthermore, expression of CD44 on CD34⁺ cells in stem cell grafts has been shown to have direct correlation with the time of neutrophil and platelet recovery after high-dose chemotherapy.¹²⁹ We therefore studied the expression of these molecules on HSCs cryopreserved with 10% DMSO supplemented with small molecule IRIs.

HSCs were frozen with 10% DMSO supplemented with **502**, **509**, **517**, or **538**. CD43 and CD44 expression on CD34⁺ cells was measured post-thaw (Figure 6.37). It was found that 98% of CD34⁺ cells expressed CD43 and CD44 from HSCs frozen with the conventional 10% DMSO cryosolution. Supplementation of the cryosolution with **502**, **509**, **517**, or **538** resulted in no differences or decreases in

CD43 and CD44 expression. Similar results were obtained when HSCs were frozen with IRI inactive compounds **504**, **508**, **516**, or **537** (Figure 6.38). These results suggest that addition of small molecule *N*-aryl-aldonamides did not preserve the cell surface receptors, CD43 or CD44, during cryopreservation. Therefore, the increased clonogenic potential of HSCs cryopreserved with IRI active small molecules was not related to the expression of cell surface markers CD43 or CD44.

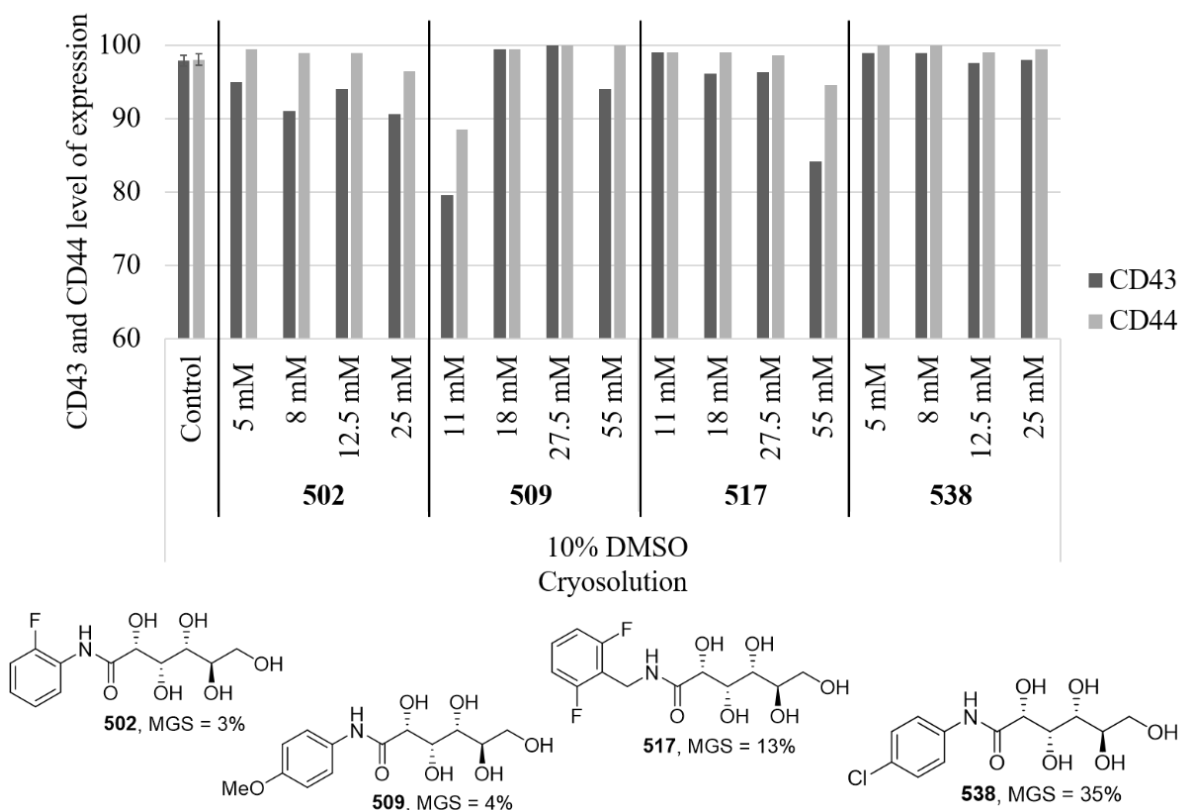


Figure 6.37. Post-thaw percent expression of CD43 and CD44 of CD34⁺ cells from UCB cryopreserved with *N*-aryl-aldonamides **502**, **509**, **517** and **538** at various concentrations. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath.

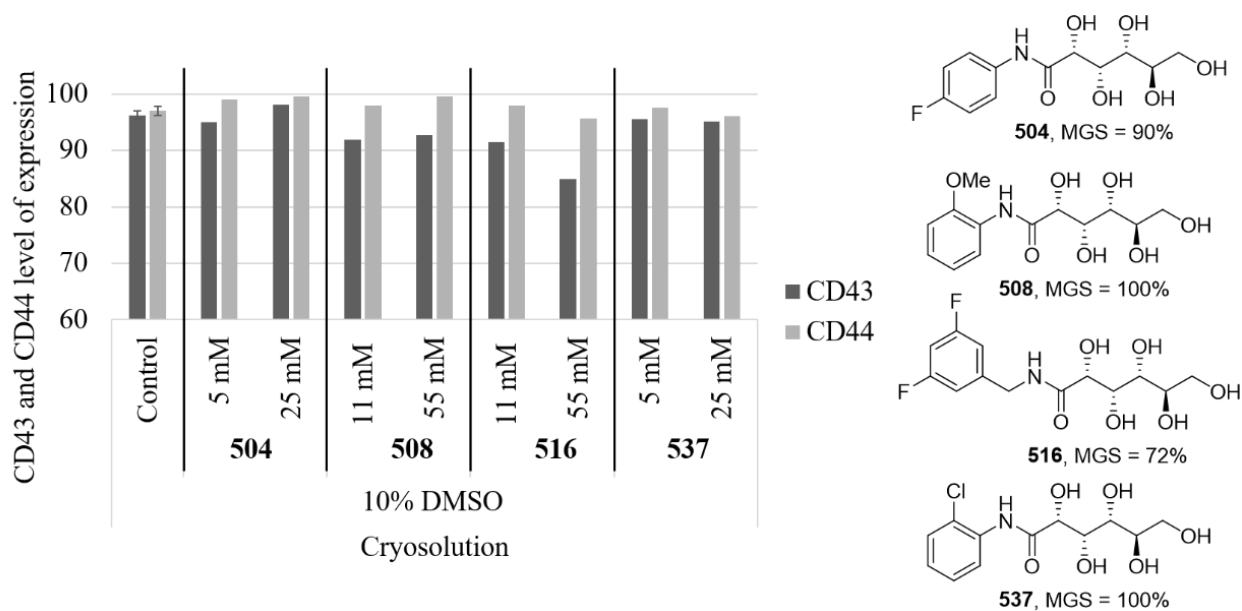


Figure 6.38. Post-thaw percent expression of CD43 and CD44 of CD34⁺ cells from UCB cryopreserved with *N*-aryl-aldonamides **504**, **508**, **516**, or **537** at various concentrations. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath.

Several studies have indicated that HSCs can exhibit high levels of post-thaw apoptosis and as a result, significant cell loss is experienced post-thaw.^{26,130–132} Cosentino *et al.* determined that improper storage of frozen PBMNCs resulted in higher levels of post-thaw apoptosis.¹²⁰ Hubel and coworkers found that the fraction of UCB cells expressing early markers of apoptosis significantly increased with time of improper storage in a -80 °C freezer.¹³³ This was significant because UCB HSC samples with signs of apoptosis (Annexin V⁺) did not engraft when transplanted into animal models.²⁶ Previous work from the Ben laboratory determined that early apoptosis was significantly reduced within the CD34⁺ population of HSCs with samples cryopreserved with carbohydrates that were more IRI active.⁹³ Therefore, the fraction of viable but apoptotic CD34⁺ cells (Sytox⁻/Annexin V⁺) post-thaw was measured in HSC samples frozen with IRIs **502**, **509** and **517**.

The degree of apoptosis was measured post-thaw and the results are shown in Table 6.1. These experiments were performed by Suria Jahan supervised by Prof. Nicolas Pineault from CBS. CD34⁺ cells

were identified by flow cytometry using the ISHAGE gating strategy.¹⁰⁰ Apoptotic CD34⁺ cells were identified as Annexin V⁺/Sytox⁻. When HSCs were cryopreserved with 10% DMSO supplemented with **502** (12.5 mM) or **509** (27.5 mM), less apoptosis was observed post-thaw.

Cryosolution	% CD34 ⁺ /Sytox ⁻ /Annexin V ⁺
10% DMSO	26%
10% DMSO + 502 (12.5 mM)	19%
10% DMSO + 502 (25 mM)	28%
10% DMSO + 509 (27.5 mM)	17%
10% DMSO + 517 (18 mM)	21%

Table 6.1. Percentage of viable CD34⁺ cells (Sytox⁻) that are apoptotic (Annexin V⁺) post-thaw.

Primitive HSCs can be identified by the expression of intracellular enzymes involved in cellular differentiation.¹³⁴ HSCs exhibit elevated activities of aldehyde dehydrogenase (ALDH) and staining for this enzyme can be used to discriminate these cells.^{135,136} Lee *et al.* recently determined that ALDH analysis allowed for the rapid quality assessment of post-thaw UCB units.¹³⁷ Together, the expression of high ALDH activity (ALDH^{br}) and the cell surface marker CD34 in UCB are used to identify a distinct subset of the progenitor cell population that is enriched for LTC-IC (long-term culture-initiating cells).¹³⁸ Lee *et al.* determined that the numbers of both CFU-GMs and CFU-GEMMs were strongly correlated with the numbers of CD34⁺ cells with high ALDH activity.¹³⁷ Interestingly, the number of CFU-GMs has been reported to be associated with the transplantation outcome of HSCs from autologous BM and UCB.^{110,139–143} Furthermore, it has been reported that successfully transplanted UCB units had a higher number of CFU-GMs than those that were not transplanted.¹⁴⁴ Thus, we measured the ALDH activity of HSCs frozen with 10% DMSO supplemented with **502**, **509**, or **517**. These experiments were performed by Suria Jahan supervised by Prof. Nicolas Pineault from CBS. Additionally, we measured the number of CFU-GM and CFU-GEMM colonies formed post-thaw.

HSCs were frozen with 10% DMSO supplemented with **502** (12.5 or 25 mM), **509** (27.5 mM), or **517** (18 mM). After thawing, the ALDH activity of CD34⁺ cells was measured (Table 6.2). Remarkably, HSCs frozen with 10% DMSO + **502** (12.5 mM) have a greater percentage of CD34⁺/ALDH^{br} cells. Since the numbers of both CFU-GMs and CFU-GEMMs were strongly correlated with the numbers of CD34⁺ cells with high ALDH activity, the numbers of CFU-GM and CFU-GEMM colonies formed post-thaw were also measured (Table 6.2). HSCs frozen with 10% DMSO supplemented with **509** or **517** resulted in a significant increase ($p < 0.0001$ and 0.05) in CFU-GEMM formation post-thaw. Furthermore, HSCs frozen with 10% DMSO supplemented with **509** resulted in a significant increase ($p < 0.05$) in CFU-GM colonies post-thaw compared to 10% DMSO alone. This is significant because the number of CFU-GM colonies is correlated to engraftment success.¹⁴⁴

Cryosolution	% CD34⁺/ALDH^{br}	Number of CFU-GMs post-thaw	Number of CFU-GEMMs post-thaw
10% DMSO	0.02%	7 (± 1.5)	3 (± 1.0)
10% DMSO + 502 (12.5 mM)	0.05%	9 (± 2.5)	0 (± 0.0)
10% DMSO + 502 (25 mM)	0.01%	11 (± 1.1)	2 (± 0.7)
10% DMSO + 509 (27.5 mM)	0.02%	14 (± 0.7)*	16 (± 2.5)*
10% DMSO + 517 (18 mM)	0.03%	11 (± 0.7)	8 (± 0.4)*

Table 6.2. Percentage of viable CD34⁺ cells (Sytox⁻) with high ALDH activity (ALDH^{br}) post-thaw. Number of CFU-GM and CFU-GEMM colonies formed post-thaw. Asterisks indicate significant difference ($p < 0.05$) compared to the control as assessed using Student's T-test.

6.3.4.5 Examining interaction with cell membranes

An active area of research involves the use of trehalose as an alternative cryoprotectant for the cryopreservation of HSCs from UCB.^{43–48} Trehalose exerts its cryoprotective effects by stabilizing the cell membrane during freezing and thawing.^{54–59} We therefore wanted to assess if small molecule IRIs, **502**, **509**, **517**, or **538** were interacting with cell membranes. This was done by measuring the retention of carboxyfluorescein (CF) dye that has been loaded into liposomes after treatment with IRIs, **502** or **538**. This work was done by Prof. Willem F. Wolkers from Leibniz Universität Hannover. Experiments were performed as previously described.¹⁴⁵ Briefly, liposomes containing CF inside were incubated with **502** or

538 (5 mM) in water at 37 °C for 7 hours and 24 hours. The amount of dye retained within the liposome was measured (Figure 6.39). Incubation of the liposomes with water alone and DMSO were used as a control. After 7 hours of incubation with **502**, there was no significant dye leakage (approximately 90% of CF is retained). After 24 hours of incubation with **502**, only 30% CF was retained. This suggested that over time, the presence of **502** resulted in CF leakage potentially caused by membrane interactions. After 7 hours of incubation with **538**, only 20% CF was retained and after 24 hours, all of the dye had leaked out of the liposome. This suggested that **538** was interacting with the cell membrane to a greater extent than **502**. This could explain why **538** did not significantly improve the cryopreservation of HSCs; the IRI activity of **538** was beneficial but perhaps the excessive interaction of **538** with the cell membranes of HSCs was negating this positive effect.

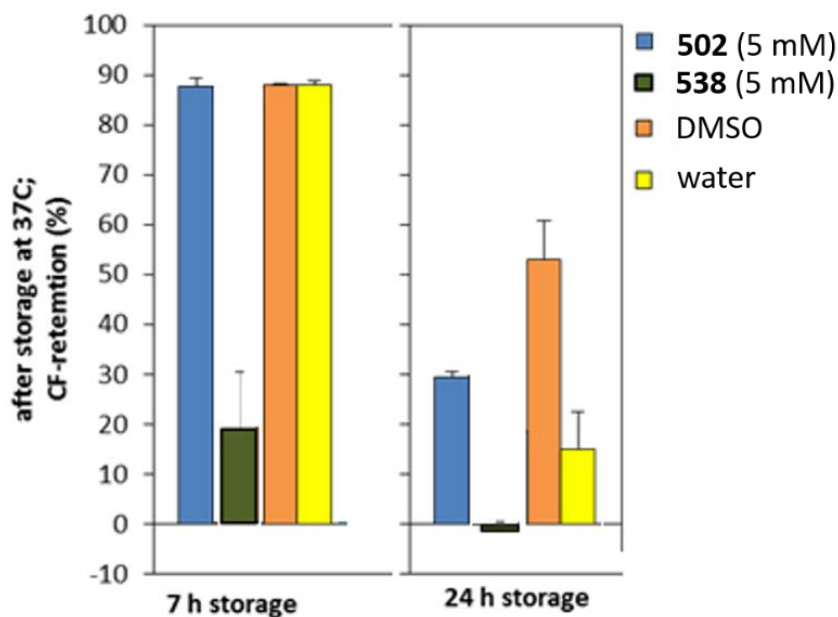


Figure 6.39. Percentage of dye retained in liposomes after 7 and 24 hours of incubation with **502** (5 mM), **538** (5 mM), DMSO, or water.

These initial results suggested that **502** and **538** were interacting with cell membranes because they caused CF dye leakage over time from liposomes loaded with CF. However, this experiment did not

accurately represent the complex composition of HSC cell membranes. Therefore, an experiment was designed to assess potential membrane interactions using TF-1 α cells.

TF-1 α cells incubated with IRIs were treated with propidium iodide (PI). PI does not permeate cell membranes and therefore does not enter cells which have intact cell membranes.¹⁴⁶ If cells have compromised membranes, PI can enter the cell where it intercalates DNA and its fluorescence increases dramatically.¹⁴⁶ Therefore, cells with compromised membranes will fluoresce and absorbance can be detected. It was hypothesized that TF-1 α cells incubated with IRIs that interact with cell membranes would fluoresce more than cells incubated with IRIs that do not interact with cell membrane. PI is often used as a cell viability dye because cells that are non-viable can have compromised cell membranes and thus PI can enter. Therefore, as a control, Calcein AM (CAM) was also utilized. The CAM assay is based on the conversion of the cell permeant non-fluorescent CAM to the fluorescent CAM by intracellular esterase activity in living cells. Therefore living cells will fluoresce and absorbance can be detected.¹⁴⁶

TF-1 α cells were aliquoted into a 96-well plated, treated with the desired compound, and incubated for 10 minutes at 37 °C. After incubation, CAM and PI were added and incubated at 37 °C for 30 minutes after which fluorescence was measured using a microplate fluorescence plate reader. Cells that were non-viable as determined by the CAM assay were subtracted. Background fluorescence was corrected for using wells which contained only cells in DPBS (no dye). Positive and negative controls included no treatment (cells in DPBS with dyes) and cells treated with the non-ionic surfactant Triton X. TF-1 α cells were >80% viable after treatment with **502**, **509**, **517**, and **538** (Figure 6.40). After incubation with **502** and **538**, an increase in PI fluorescence was observed (Figure 6.41). This suggested that these compounds were somehow disrupting TF-1 α cell membranes, allowing PI to enter the cells. This was consistent with the results obtained by Prof. Willem Wolkers that showed that incubation of liposomes with **502** and **538** caused CF dye leakage. However, compounds **509** and **517** did not increase the fluorescence of PI, suggesting that these compounds were not interacting with cell membranes and therefore PI was excluded from these cells.

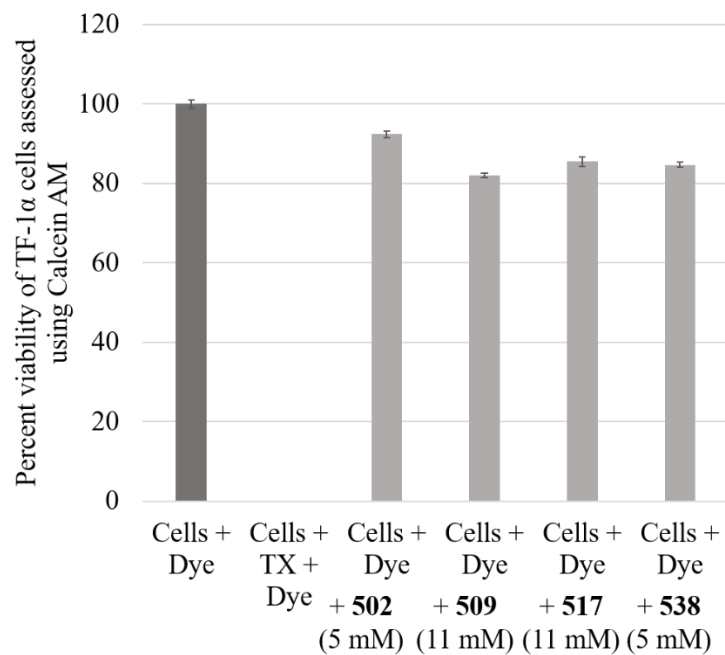


Figure 6.40. Percentage of viable TF-1 α cells after incubation with **502**, **509**, **517** and **538**. TF-1 α cells were assessed for viability using Calcein AM.

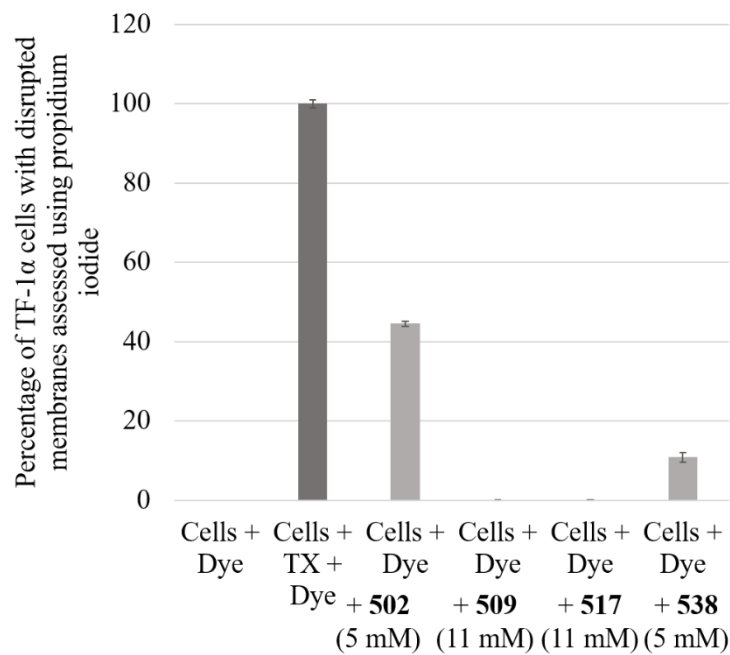


Figure 6.41. Percentage of TF-1 α cells with disrupted cell membranes after incubation with **502**, **509**, **517** and **538**. TF-1 α cells were assessed for membrane disruption using propidium iodide.

6.4 Chapter summary

To summarize this chapter, several *N*-aryl-alidonamides were found to be beneficial additives to the conventional 10% DMSO cryosolution for HSCs from UCB. TF-1 α cells were used as a model cell line to pre-screen different cryosolutions containing IRIs. Unfortunately, this cell line did not accurately predict the cryopreservation outcome for HSCs from UCB, requiring the screening of IRI active molecules in UCB HSCs. The supplementation of the conventional 10% DMSO cryosolution with *N*-aryl-alidonamides, **502**, **509**, **517**, or **538** did not increase post-thaw viability but resulted in an increase in HSC clonogenic potential post-thaw. HSCs cryopreserved with **502** (12.5 or 25 mM), **509** (27.5 mM) or **517** (18 mM) resulted in a significant increase in colony formation post-thaw and **502** (25 mM) and **509** (27.5 mM) were found to increase the frequency of LTC-IC. Furthermore, these solutions resulted in less apoptosis post-thaw and HSCs cryopreserved with **502** (12.5 mM) were found to have increased ALDH levels. Future studies would involve the investigation of successful engraftment in mice of HSCs frozen with these IRIs.

Compounds that were found to be IRI active in PBS, **502**, **509**, **517** and **538**, possessed higher IRI activity in the presence of HSCs and plasma than compounds that were found to be inactive in PBS, implying that these molecules are effective at inhibiting ice recrystallization even in the presence of HSCs and in solutions that are used to cryopreserve HSCs. Furthermore, the most IRI active solutions, HSCs in plasma and saline/dextran with **502** (25 mM) or **509** (27.5 mM), were the cryosolutions that also resulted in the most colonies formed post-thaw. In addition, compounds that did not possess IRI activity did not result in increased colony formation post-thaw compared to the 10% DMSO control. This suggested that the ability to inhibit ice recrystallization was an important feature for a cryoprotectant for HSCs allowing preservation of clonogenic potential.

Frozen HSC samples subjected to transient warming events (TWEs) resulted in decreased viability, recovery, and CFU. It was hypothesized that the addition of IRIs to the cryosolution would protect HSCs from the enhanced ice recrystallization injury during transient warming and therefore result in improved

quality of HSCs post-thaw. Unfortunately, this did not occur and the addition of IRIs to the cryosolution actually increased the damage to HSCs during transient warming and subsequent thawing. It was hypothesized that these molecules were therefore functioning by an alternate mechanism.

HSCs frozen with IRIs **502** and **509** were found to have decreased levels of apoptosis post-thaw. This is significant because HSCs expressing signs of apoptosis were found to not engraft into animal models.²⁶ Several studies have found that damage to the mitochondria during cryopreservation causes the release of caspases that subsequently results in cell losses due to apoptosis.^{130–132} Caspase expression occurs early in the apoptosis cascade and is therefore an earlier marker of damage compared to the traditionally used apoptosis marker, Annexin V.¹⁴⁷ Furthermore, caspase expression has been associated with freeze-thaw damage.¹⁴⁸ Hubel *et al.* found increased caspase expression on HSC samples improperly stored at -80 °C.¹³³ Therefore, future work would involve the measurement of caspase expression in HSC samples frozen with 10% DMSO supplemented with IRIs.

Lee *et al.* determined that the numbers of both CFU-GMs and CFU-GEMMs were strongly correlated with the numbers of CD34⁺ cells with high ALDH activity.¹³⁷ Supplementation of **502** did not significantly increase the number of CFU-GEMM colonies; however, this cryosolution was found to result in increased ALDH activity post-thaw. It was also found that supplementation of IRIs **509** and **517** resulted in increased CFU-GM and CFU-GEMM colonies post-thaw. This is significant because the number of CFU-GMs has been reported to be associated with the transplantation outcome of HSCs from autologous BM and UCB.^{110,139–143} Furthermore, it has been reported that successfully transplanted UCB units had a higher number of CFU-GMs than those that were not transplanted.¹⁴⁴

Future work should include investigation into the mechanisms of action of **502** and **509**. Preliminary studies suggest these types of molecules could be interacting with the cell membrane. Whether or not these molecules are being internalized is something that should be investigated. Furthermore, it is unclear whether these molecules are providing protection of clonogenic potential due to their ability to inhibit ice recrystallization. Compounds that were inactive but structurally similar did not improve the clonogenic

potential of HSCs post-thaw. This suggested that the improved post-thaw clonogenic potential of HSCs frozen with IRI active molecules was correlated to IRI activity. However, IRI active molecules, **502**, **509**, and **517** did not preserve functionality when frozen HSC samples were subjected to transient warming. Interestingly, the composition of colonies formed post-thaw was altered with the addition of IRIs to the cryosolution. It is therefore possible that rather than improving functionality post-thaw by inhibiting ice recrystallization, these molecules were promoting the proliferation and differentiation of HSCs into specific cell types and this was independent of cryopreservation. Thus, additional experiments in which unfrozen HSCs are incubated with IRIs and assessed for clonogenic potential are required.

Improved cryopreservation methods for UCB resulting in increased clonogenic potential would increase the effective dose of cells available in stored units and therefore allow greater access to UCB transplantation.¹¹⁴ Overall, the supplementation of the current cryoprotectant solution with small molecules capable of controlling ice growth and recrystallization resulted in an increase in CFU recovery and frequency of multipotent progenitors in UCB HSCs. Ultimately, this would reduce the percentage of engraftment failure and allow for a larger proportion of cord blood banks' inventory to provide an adequate dose for patients requiring transplants.

6.5 Experimental

All novel IRI active compounds (derivatives **501-503**, **509**, **517**, **520**, and **521**) used in this chapter for cytotoxicity and cryopreservation assays were prepared as described in the experimental section of Chapter 5. Cell culture protocols, MTT assay and cryopreservation protocols for TF-1 α cells are described below. Colony forming unit assays were done in collaboration with Priya Chandran (supervised by Prof. David Allan from the Faculty of Medicine, Biochemistry, Microbiology and Immunology at the University of Ottawa) and Suria Jahan (supervised by Prof. Nicolas Pineault, adjunct professor from the Faculty of Medicine, Biochemistry, Microbiology and Immunology at the University of Ottawa and Development Scientist at Canadian Blood Services). Suria Jahan also provided collaborative help with the

LTC-IC assays. Aldehyde dehydrogenase (ALDH) activity and apoptosis levels for HSCs cryopreserved with IRIs was measured by Suria Jahan.

Carboxyfluorescein (CF)-retention studies were performed by Prof. Willem F. Wolkers from Leibniz Universität Hannover. Experiments were performed as previously described.¹⁴⁵

6.5.1 *TF-1 α cell culture*

TF-1 α cells (human bone marrow erythroblast cells, ATCC, CRL-2451) were cultured in Rosewell Park Memorial Institute (RPMI) 1640 media supplemented with 10% FBS (fetal bovine serum) and 1% penicillin–streptomycin in 100 mm² culture dishes. Cells were incubated in a 37 °C incubator supplied with 5% CO₂. Cultures were maintained by replacement of medium by centrifugation and subsequent resuspension at 3x10⁵ viable cells/mL. Cell density was maintained between 3x10⁵ and 3x10⁶ viable cells/mL. Cell viability was determined by staining using the Trypan Blue exclusion test.¹⁴⁹

6.5.1.1 *Trypan blue exclusion test for cell viability*

The Trypan Blue exclusion test was used to obtain cell viabilities of cultured TF-1 α and HepG2 cells. Cell density was determined using a hemacytometer. 0.1 mL of a 0.4% trypan blue solution was added to 0.1 mL of cells. Using a hemacytometer the number of blue staining cells and the total number of cells was obtained. The cells which are stained blue are non-viable. Therefore the percent viable cells is obtained using the following equation:

$$\% \text{ viable cells} = \left[1.00 - \left(\frac{\text{number of blue cells}}{\text{number of total cells}} \right) \right] \times 100$$

Then, the number of viable cells is obtained using the following equation:

$$\text{Number of viable cells/mL} = (\text{number of total cells} - \text{number of blue cells}) \times 10^4 \times 2$$

Cultures with a cell viability < 95% were not used.

6.5.2 *TF-1 α cryopreservation and thawing*

TF-1 α cells were cultured and counted as described above. Aliquots containing 1×10^6 viable cells were added to 1.5 mL Eppendorf tubes and cells were pelleted by centrifugation for 10 min at 580 $\times$ g. The supernatant was removed and the cells were resuspended in 100 μ L RPMI-1640 supplemented with the desired compound and/or DMSO cryo-additives. Cell suspensions were transferred to 2 mL cryogenic vials and the subsequent steps depended on the desired freezing and/or storage and/or thawing conditions. For rate controlled freezing, cryogenic vials were placed in a “Mr. Frosty” freezing container. The container was placed in a -80 °C freezer for 16 hours to provide a cooling rate of 1 °C/min. Samples were then either stored in the -80 °C freezer or in the vapor phase of liquid nitrogen (-196 °C) for a minimum of 24 hours. For dump freezing conditions, cell suspensions were frozen by placing cryogenic vials directly in liquid nitrogen followed by storage in the vapor phase of liquid nitrogen (-196 °C) for a minimum of 24 hours. Following storage, samples were either rapidly thawed in a 37 °C water bath or slowly thawed by allowing thawing to occur at room temperature.

6.5.3 *Post-thaw TF-1 α viability, apoptosis and recovery*

After cryopreservation and thawing, cell suspensions were diluted with 900 μ L of 1 X Annexin V binding buffer (1/10 dilution of 10 X Annexin V binding buffer in distilled water). Cell viability and apoptosis were determined by flow cytometry and cell recovery was determined by hemacytometer.

6.5.3.1 *Flow cytometry*

An aliquot of 400 μ L of the cell suspension was transferred to a polypropylene flow cytometry tube. The remaining 600 μ L was kept for recovery analysis (described below). 10 μ L of each 7-AAD viability dye and Annexin V-FITC and samples were vortexed and incubated for 15 min in the dark. Following incubation, the samples were diluted to 1.0 mL with 1 X Annexin V binding buffer. Flow cytometry

analysis on 100,000 cells was carried out on a Beckman Coulter FC500 flow cytometer. Annexin V-FITC was measured with 525nm optical filter (FL-1), while 7-AAD was measured with 675 nm optical filter (FL-4). Viability was determined by the total number of 7-AAD- cells detected. Apoptosis was determined by the number of 7-AAD- cells that were Annexin V+. All samples were tested in triplicate.

6.5.3.2 *Post-thaw recovery*

Cell recovery was determined by hemacytometer. Cell recovery is calculated using the following equation:

$$\% \text{ Cell Recovery} = \left(\frac{\text{cell density post} - \text{thaw}}{\text{cell density pre} - \text{freeze}} \right) \times 100$$

6.5.4 *HepG2 cell culture*

HepG2 (human liver hepatocellular carcinoma cells, ATCC, HB-8065) were cultured in Eagle's minimum essential media (MEM) supplemented with 10% FBS (fetal bovine serum), 1% non-essential amino acids, 1 mM sodium pyruvate and 1% penicillin–streptomycin in 75 cm² Corning® flasks. Cells were incubated in a 37 °C incubator supplied with 5% CO₂. Cultures were maintained by replacement of medium. Cells were detached from the cell culture flask using 3 mL Accutase solution. Cells were resuspended at 5x10⁵ viable cells/mL. Cell density was maintained between 5x10⁵ and 2x10⁶ viable cells/mL. Cell viability was determined by staining using the Trypan Blue exclusion test as described in Section 6.5.1.1. Passages 8-11 were used in this study. All cells were removed from the plates using 3 mL Accutase solution for use in experiments.

6.5.5 *MTT assay with HepG2 cells*

The MTT assay was performed as described previously.^{94,150,151} HepG2 cells, cultured as described above, were plated in 96-well plates and allowed to reach confluency. Cells were then treated with 100 μ L of MEM supplemented with the compound of interest at various concentrations and incubated at 37 °C for 16 h with 5% CO₂. Cells incubated with MEM without supplement were used as a negative control, and cells supplemented with 1% Triton-X were used as a positive control. Following incubation, the supplemented media was removed and 200 μ L of fresh media and 50 μ L of MTT solution (5 mg/mL) in HBSS (Hank's balanced salt solution) were added and the plates were incubated at 37 °C with 5% CO₂ for 3 h. The plates were then centrifuged, the media aspirated and 200 μ L of MTT solubilization solution (10% Triton X-100, 0.1 N HCl in isopropanol) was added to each well. The plates were incubated at room temperature in the dark for 2-4 h and the absorbance of each well was then read at a wavelength of 570 nm with a multi-well plate reader (AD 34 °C Absorbance Detector, Beckman Coulter, Inc., Mississauga, ON). Each experiment was repeated on three separate plates in 10 consecutive wells for each condition. The average absorbance was calculated and compared to the control. Viability was reported as a percentage of the control.

6.5.6 Collection & processing of human umbilical cord blood

Umbilical cord blood was collected following healthy term delivery and informed consent from mothers, in accordance with institutional approval from the Research Ethics Board of The Ottawa Hospital (protocol 2006460-01H). The processing of UCB procedure was based upon Rubinstein's method for UCB processing.¹⁴ Whole UCB was stored at room temperature (15 – 25 °C) and processed within 48 h of collection. UCB was removed from the collection bag and spilt in equal parts into 50 mL Falcon tubes. UCB was diluted with 6% hetastarch (Hespan) to obtain a final concentration of 1% hetastarch and incubated for 10 minutes at room temperature. The tubes were then centrifuged at 50 g at 10 °C for 15-20 minutes, depending on volume. For total volumes up to 30 mL, 15-17 minute centrifugation was utilized and for total volumes of 30-35 mL, 17-20 minute centrifugation was utilized. The supernatant and buffy coat (plasma and leukocytes) were removed carefully (not collecting RBCs) from the tubes and collected

in a 50 mL Falcon tube. This tube was centrifuged at 400 g and 10 °C for 10 mins to pellet cells. Plasma was removed and kept on ice. The cell pellet was resuspended and combined in plasma to a total volume of 20 mL (leukocyte concentrate, LC). The LC was kept on ice for the duration of its use. Total mononuclear (CD45⁺) and CD34⁺ cell concentrations and viability were determined by flow cytometry using the ISHAGE gating strategy.¹⁰⁰

6.5.7 Cryopreservation of CD34⁺ cells

Aliquots of LC (50,000 CD34⁺ cells) were added to 2.0 mL cryovials kept on ice until placement in -80 °C freezer. Cryovials were centrifuged at 400 g and 10 °C for 5 minutes to pellet cells. After centrifugation, supernatant was removed by aspiration. Plasma was added and cells were suspended by pipetting. Pre-made cryosolutions were added and cells were mixed by pipetting. For specific volumes of plasma and cryosolutions, see Section 6.4.9. Cryovials were placed in a “Mr. Frosty” rate-controlled freezing container which was then placed in a -80 °C freezer for 24 hours. After 24 hours, the cryovials were transferred to a liquid nitrogen dewar for storage until thawed for analysis.

6.5.8 Cryosolutions for LCs

Cryosolutions were prepared in distilled autoclaved water supplemented with 0.9% saline and 5% dextran for cryopreservation experiments and diluted with different volumes of plasma in cryovial depending on desired concentration of ice recrystallization inhibitor. Compounds **502**, **504**, **537**, and **538** were prepared at 25 mM and compounds **508**, **509**, **516**, and **517** were prepared at 55 mM. 100, 50, 33, or 20 µL of 25 mM **502**, **504**, **537**, and **538** was added to cryovials containing 50,000 CD34⁺ cells suspended in 0, 50, 67, or 80 µL of plasma, respectively, for a final IRI concentration of 25, 12.5, 8, or 5 mM, respectively. 100, 50, 33, or 20 µL of 55 mM **508**, **509**, **516**, and **517** was added to cryovials containing 50,000 CD34⁺ cells

suspended in 0, 50, 67, or 80 μL of plasma, respectively, for a final IRI concentration of 55, 27.5, 18, or 11 mM, respectively.

6.5.9 Flow cytometry

Total mononuclear cell (CD45^+) and progenitor cell (CD34^+) viability was assessed by flow cytometry using ISHAGE (International Society of Hematotherapy and Graft Engineering) guidelines.¹⁰⁰

Fluorescently tagged antibodies for both CD45 and CD34 were employed to identify the population within the leukocytes which are HSCs using the ISHAGE gating strategy. HSCs are identified as being both CD45^+ and CD34^+ , by employing 7-aminoactinomycin D (7-AAD) in the analysis, cell viabilities of both total mononuclear cells and HSCs can be quantified. Countbright® counting beads were employed to obtain cell concentrations.

6.5.9.1 Pre-cryopreservation viability analysis and enumeration

Flow cytometry analysis was performed within 48 hours of UCB collection. 100 μL of the LC was diluted with 900 μL Dulbecco's phosphate buffered saline (DPBS). 200 μL of this cell suspension was added to a polypropylene flow cytometry tube. 8 μL of each CD45 fluorescein isothiocyanate (FITC) and CD34 phycoerythrin (PE) were added and incubated in the dark at room temperature for ten minutes. 8 μL of 7-AAD was added and cell suspension is allowed to incubate for an additional five minutes in the dark at room temperature. 20 μL of Countbright® counting beads was added and the suspension was diluted to 1 mL with 1X red blood cell lysis buffer. Samples were analyzed using a Beckman Coulter Gallios Flow Cytometer.

6.5.9.2 Post-thaw CD34^+ viability analysis and enumeration

For frozen UCB, flow cytometry analysis was performed within 1 hour of thawing. After thawing samples in a 37 °C water bath, the cell suspension was diluted with 900 μL of DPBS. 200 μL of this cell suspension was added to a polypropylene flow cytometry tube. 8 μL of each CD45 FITC and CD34 PE were added and incubated in the dark at room temperature for ten minutes. 8 μL of 7-AAD was added and cell suspension was allowed to incubate for an additional five minutes in the dark at room temperature. 20

μL of Countbright® counting beads was added and the suspension was diluted to 1 mL with 1X red blood cell lysis buffer. Samples were analyzed using a Beckman Coulter Gallios Flow Cytometer.

6.5.10 Colony forming unit (CFU) and long-term culture initiating cell (LTC-IC) assays

Cryovials were thawed in a 37 °C water bath and diluted with 900 μL of IMDM (10% FBS, 1% penicillin/streptomycin). Cryovials were placed on ice until washed. Samples were mixed by pipetting and 80 μL was transferred to a 15 mL Falcon tube. 5 mL of IMDM (10% FBS) was added and this mixture was centrifuged at 1100 rpm for six minutes. Supernatant was removed by aspiration and the cell pellet was resuspended in 1 mL IMDM (2% FBS). Suspension was mixed by pipetting. 150 μL of this cell suspension was added to 3 mL Methocult™ media and plating was performed as previously described.¹⁵² After two weeks of incubation, colonies were counted and scored according to Standardized Guide provided by manufacturer. Limiting dilution analysis (LDA) was used to measure the frequency of LTC-IC in thawed cryovials by plating decreasing doses of UCB TNC (250, 600, 1,200, 3,000, 6,000, 9,000, 18,000, and 30,000) were co-cultured for 5 weeks with the stromal cell line MS-5 in 0.1% gelatin pre-coated 96-well flat bottom culture plates in human long term culture medium (Myelocult MS 300) with hydrocortisone. Half medium change was done weekly. After 5 weeks, the entire well content was transferred to 1 mL of methylcellulose (MethoCult™ H4434) and plates scored for colony growth or not after 2 weeks. Each dose was tested in 4-6 replicates. LDA was carried out using the Extreme Limiting Dilution Analysis web-based app.¹⁵³ All reagents for progenitor assays were from StemCell Technologies. (StemCell™ Technologies, Vancouver, Canada)

6.5.11 CD43 and CD44 expression

For frozen UCB, flow cytometry analysis was performed within 1 hour of thawing. After thawing samples in a 37 °C water bath, the cell suspension was diluted with 900 μL of DPBS. 200 μL of this cell suspension was added to a polypropylene flow cytometry tube. 8 μL of each CD45 FITC, CD34 PE, CD43 APC-Alexa Fluor 750 and CD44 Pacific Blue were added and incubated in the dark at room

temperature for ten minutes. 8 μ L of 7-AAD was added and cell suspension is allowed to incubate for an additional five minutes in the dark at room temperature. 20 μ L of Countbright® counting beads was added and the suspension was diluted to 1 mL with 1X red blood cell lysis buffer. Samples were analyzed using a Beckman Coulter Gallios Flow Cytometer.

6.5.12 *Ice recrystallization inhibition (IRI) assay*

IRI activity was performed using the “splat cooling” method. The solution was prepared as described below (in Sample Preparation). A 10 μ L droplet of this solution was dropped onto a block of polished aluminum precooled on dry ice to approximately -80 °C through a two metre high plastic tube. The droplet froze instantly, forming a wafer on the polished aluminum block that was approximately 1 cm in diameter and 20 μ m thick. This wafer was then carefully removed from the surface of the block and transferred onto a circular glass coverslip and to a cryostage held at -6.4 °C for annealing. After a period of 30 min, the wafer was photographed between crossed polarizing filters using a digital camera (Nikon CoolPix 5000) fitted to the microscope. A total of three images were taken from each wafer and each solution was tested three times.

The initial ice crystals formed during flash freezing are relatively homogeneous in size and quite small. Recrystallization occurs during the 30 minute annealing cycle, resulting in an increase in ice crystal size. After the annealing process, images captured the frozen wafer and ice crystal sizes were obtained using a domain recognition software (DRS) program. This program allows a user to visually demarcate and store the vertices of ice domains in a digital micrograph. The data was then used to calculate the domain areas. A total of 12 domain areas were obtained for nine images (n = 108). All data were plotted and analyzed using Microsoft Excel. The mean grain (or ice crystal) size (MGS) of the sample was compared to the MGS of the control PBS solution for that same day of testing and IRI activity is reported as the percentage of the MGS (%MGS) relative to the PBS control. Therefore, small percentages represent small ice crystals and this is indicative of high IRI activity. The %MGS for each sample was plotted along with its standard error of the mean (SEM).

Sample Preparation:

TF-1 α cell solutions:

TF-1 α cells were cultured and counted as described in Section 6.5.2. Cells were harvested and counted. Aliquots of 3×10^6 cells were added to eppendorfs. The eppendorfs were centrifuged at 1000 rpm for five minutes. The supernatant was removed and the pellet was resuspended in RPMI media supplemented with **502** (5 mM), **509** (11 mM), **517** (11 mM) and **538** (5 mM). These solutions were assessed for IRI activity using the “splat cooling method” described in Section 6.5.13. Samples assessed were as follows:

- PBS (positive control for ice recrystallization)
- RPMI media
- RPMI media with TF-1 α cells (3×10^6 cells/mL)
- RPMI media supplemented with **502** (5 mM) and TF-1 α cells (3×10^6 cells/mL)
- RPMI media supplemented with **509** (11 mM) and TF-1 α cells (3×10^6 cells/mL)
- RPMI media supplemented with **517** (11 mM) and TF-1 α cells (3×10^6 cells/mL)
- RPMI media supplemented with **538** (5 mM) and TF-1 α cells (3×10^6 cells/mL)

HSC cell solutions:

Umbilical cord blood was collected and processed as described in Section 6.5.7. Viability was obtained by flow cytometry as described in Section 6.5.10. Solutions were prepared in distilled autoclaved water supplemented with 0.9% saline and 5% dextran for IRI experiments and diluted with different volumes of plasma in cryovial depending on desired concentration of ice recrystallization inhibitor. Compounds **502**, **504**, **537**, and **538** were prepared at 25 mM and compounds **508**, **509**, **516**, and **517** were prepared at 55 mM. 100, 50, 33, or 20 μ L of 25 mM **502**, **504**, **537**, and **538** were added to eppendorfs containing 50,000 CD34⁺ cells suspended in 0, 50, 67, or 80 μ L of plasma, respectively, for a final compound concentration of 25, 12.5, 8, or 5 mM, respectively. 100, 50, 33 or 20 μ L of 55 mM **508**, **509**, **516** and **517**

was added to eppendorfs containing 50,000 CD34⁺ cells suspended in 0, 50, 67, or 80 μ L of plasma, respectively, for a final compound concentration of 55, 22.5, 18 or 11 mM, respectively. These solutions were assessed for IRI activity using the “splat cooling method” described in Section 6.5.13. Samples assessed were as follows:

- PBS (positive control for ice recrystallization)
- Plasma
- Saline/dextran + plasma with HSCs (0.5×10^6 CD34⁺ cells/mL)
- Saline/dextran + plasma + **502** (5, 8, 12.5 or 25 mM) with HSCs (0.5×10^6 CD34⁺ cells/mL)
- Saline/dextran + plasma + **504** (5 or 25 mM) with HSCs (0.5×10^6 CD34⁺ cells/mL)
- Saline/dextran + plasma + **508** (11 or 55 mM) with HSCs (0.5×10^6 CD34⁺ cells/mL)
- Saline/dextran + plasma + **509** (11, 18, 27.5 or 55 mM) with HSCs (0.5×10^6 CD34⁺ cells/mL)
- Saline/dextran + plasma + **516** (11 or 55 mM) with HSCs (0.5×10^6 CD34⁺ cells/mL)
- Saline/dextran + plasma + **517** (11, 18, 27.5 or 55 mM) with HSCs (0.5×10^6 CD34⁺ cells/mL)
- Saline/dextran + plasma + **537** (5 or 25 mM) with HSCs (0.5×10^6 CD34⁺ cells/mL)
- Saline/dextran + plasma + **538** (5, 8, 12.5 or 25 mM) with HSCs (0.5×10^6 CD34⁺ cells/mL)

6.5.13 Dose-response curve and IC₅₀ calculations

In order to generate a dose-response curve and IC₅₀ values for IRIs, the ‘splat cooling method’ for IRI activity assessment was modified.¹¹⁷ This new method for the generation of a dose-response curve and IC₅₀ values for quantifying IRI activity was designed by Stephanie Abraham in collaboration with Prof. Jeffrey Keillor.¹¹⁷ Compounds were prepared at a minimum of five concentrations in PBS. A 10 μ L drop of each solution was dropped from a height of 2 m onto a polished aluminum block, which was precooled to -80 °C on dry ice, to form a frozen wafer. The wafer was then transferred to a cryostage and held at -6.4 °C for 5 min during which ice recrystallization occurs. Subsequently, the wafer was

photographed using a microscope equipped with a camera. ImageJ software was used to circle and calculate the areas (corrected for the appropriate magnification factor of the objective lens) of all crystals within the field of view. These crystals were then sorted into discrete bins based on size (area in mm²) using Excel. Bin sizes were assigned in increments of 0.001 mm², as it was observed that at time zero, all ice crystals could be just contained within this bin. Therefore, subsequent crystal growth would result in larger crystals moving out of bin 1 and into higher bins. The relative importance of each bin was determined by summing the area of each crystal within that bin and dividing by the sum of the areas of all crystals within the field of view. In this way, the proportionate area of each bin was calculated for every sample wafer. The change in the proportion of crystals in bin 1 over time allowed for the calculation of an initial rate of ice crystal growth.

Rate constants for the decrease of bin 1 were determined over 5 minutes in the presence of at least five concentrations of IRIs in PBS. The absence of inhibitor (0 mM) was also measured to define an upper plateau. For each sample concentration, triplicate wafers were prepared and analyzed as described above. The average rate constant measured in the absence of inhibitor (PBS alone) was used to normalize the rate constants measured in the presence of inhibitor. This provided, for each inhibitor, a set of normalized rate constants, k_{norm} , versus inhibitor concentration, $[I]$, whose log values were used in dose-response fitting according to the following equation:

$$k_{norm} = \frac{100}{1 + n + 10^{\log IC_{50} - \log [I]}}$$

In this equation, IC_{50} is the concentration of inhibitor that gives 50% antagonism ($k_{norm} = 50$) and n is the Hill slope.

Dose-response curves were generated for **502**, **509**, **517**, and **538**. Concentrations used for each compound and their corresponding IC_{50} values and Hill slopes are described in the table below.

Compound	Concentrations (mM)	IC_{50}	Hill slope
502	1, 1.5, 3, 5, 10, 20	4.07 mM	-2.22

509	1, 3, 5, 6.5, 8, 10, 20	5.06 mM	-2.55
517	1, 5, 10, 15, 20, 30	10.75 mM	-5.12
538	1, 10, 13, 17, 20	12.02 mM	-4.67

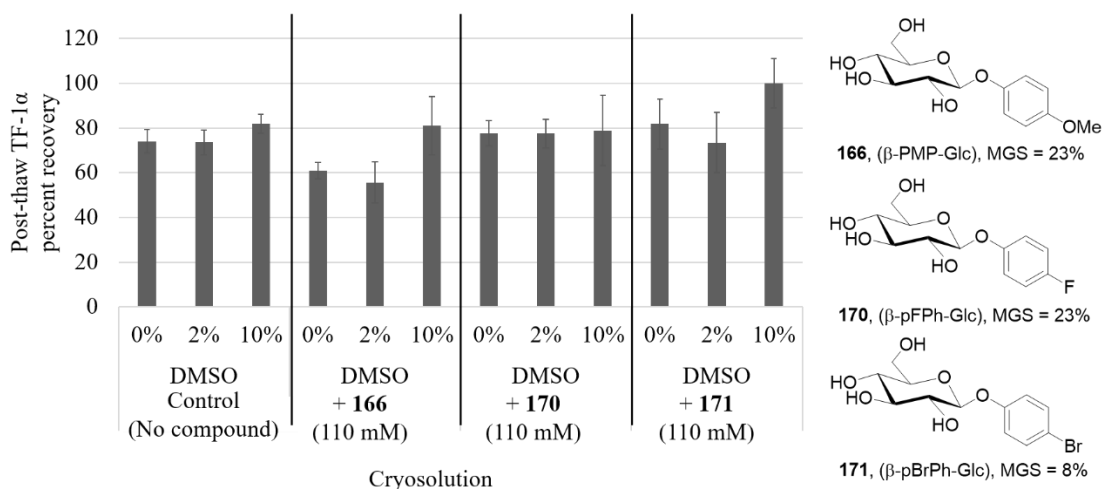
6.5.14 Calcein AM and Propidium Iodide (PI) fluorescence studies

The Calcein AM (CAM) assay is based on the conversion of the cell permeant non-fluorescent CAM to the fluorescent CAM by intracellular esterase activity in living cells.¹⁴⁶ Therefore living cells will fluoresce and absorbance can be detected. Excitation is at 490 nm and emission at 525 nm. The propidium iodide (PI) assay is based on the fact that intact cell membranes are impermeant to PI and therefore does not enter cells which have intact membranes.¹⁴⁶ If cells have compromised membranes, PI can enter the cell where it intercalates DNA and its fluorescence increases dramatically.¹⁴⁶ Therefore, cells with compromised membranes will fluoresce and absorbance is detected. Excitation is at 540 nm and emission at 620 nm.

TF-1 α cells were cultured and counted as described in Section 6.5.2. Cells were harvested and washed twice with Dulbecco's phosphate buffered saline (DPBS) and suspended at a concentration of 0.5 x 10⁶ cells/mL in DPBS. 100 μ L of this suspension was added to wells in a Costar black opaque 96-well plate. 100 μ L of test compound solution (prepared at twice the desired concentration) was added to the wells and the plate was incubated for 10 minutes at 37 °C. After incubation, 50 μ L of each dye solution (10 μ M CAM in DPBS and 22.5 μ M PI in DPBS) was added and the plated was incubated at 37° C for 30 minutes after which fluorescence was measured using a BioTek Synergy™ H4 2 multi-mode microplate fluorescence plate reader. Background fluorescence was corrected for using wells containing just DPBS and DPBS + dyes (no cells). Positive and negative controls were performed using cells in DPBS + dyes (with no treatment) and cells in DPBS treated with Triton X + dyes. Each treatment was assessed in six wells each on three separate plates. Fluorescence was averaged and normalized to the positive and negative controls.

6.6 Supplemental data and figures

6.6.1 TF-1 α post-thaw recovery



Supplemental Figure S6.1. Post-thaw percent recovery of TF-1 α cells cryopreserved with aryl glycosides, β -PMP-Glc (**166**), β -pFPh-Glc (**170**), and β -pBrPh-Glc (**171**). Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). No statistical significance ($p < 0.05$) was observed as assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test.

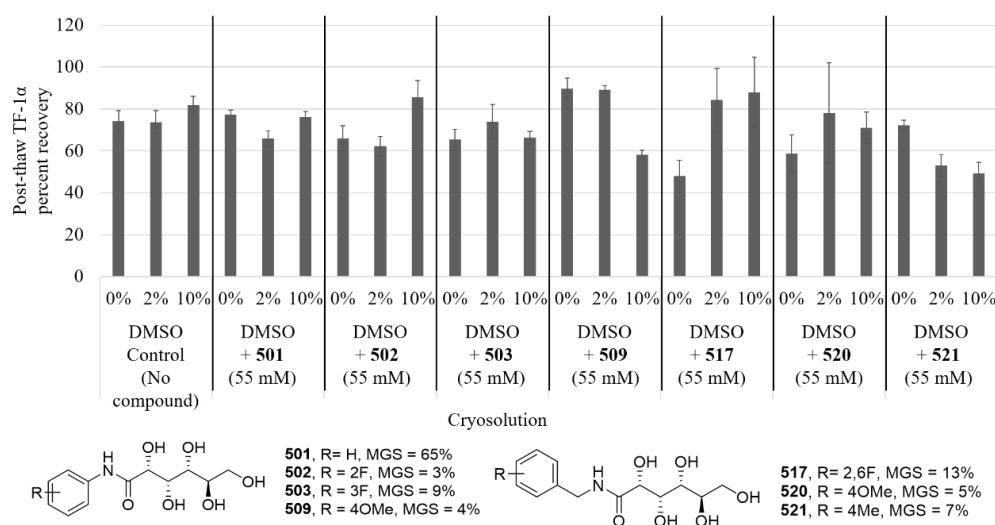
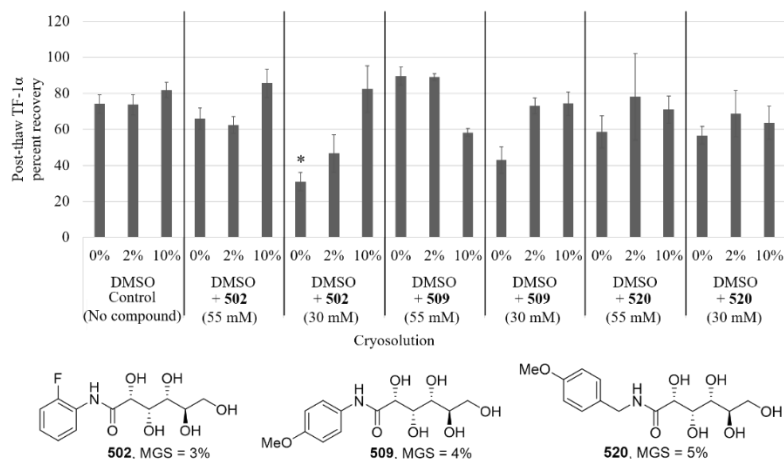
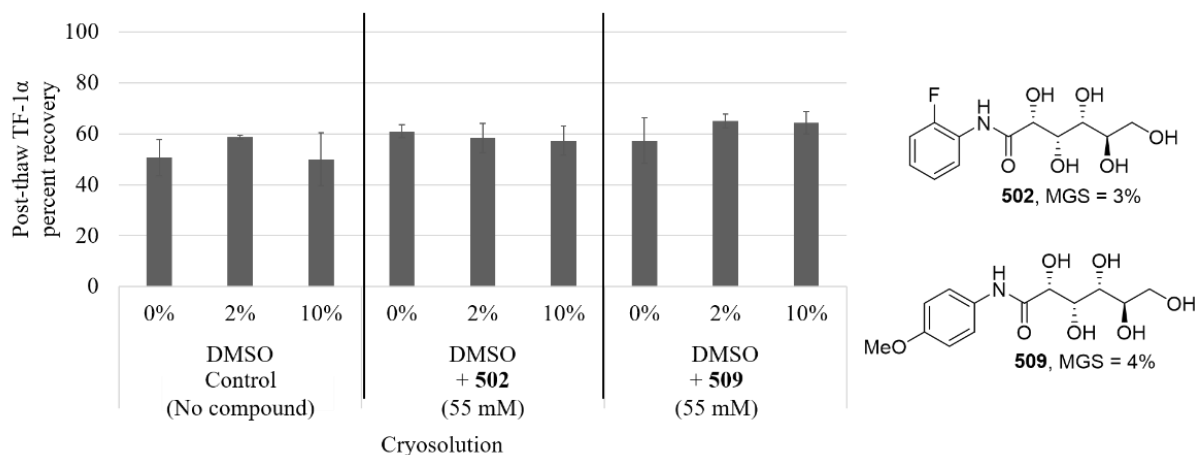


Figure S6.2. Post-thaw percent recovery of TF-1 α cells cryopreserved with aryl aldonamides, **501-503**, **509**, **517**, **520**, and **521**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed

quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). No statistical significance ($p < 0.05$) was observed as assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test.



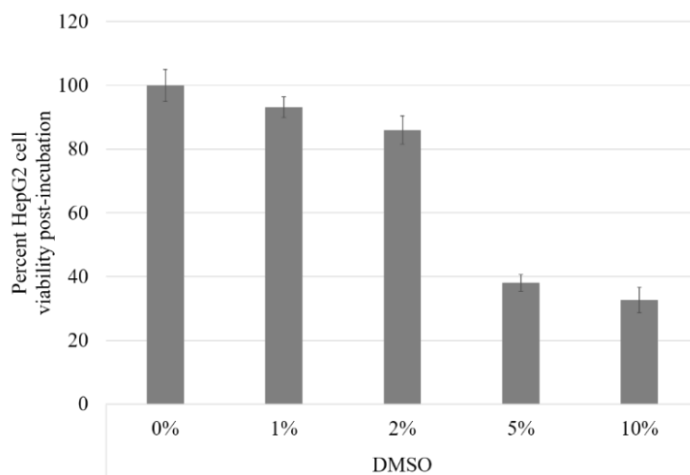
Supplemental Figure S6.3. Post-thaw percent recovery of TF-1 α cells cryopreserved with different concentrations of aryl aldonamides, **502**, **509**, and **520**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to no compound.



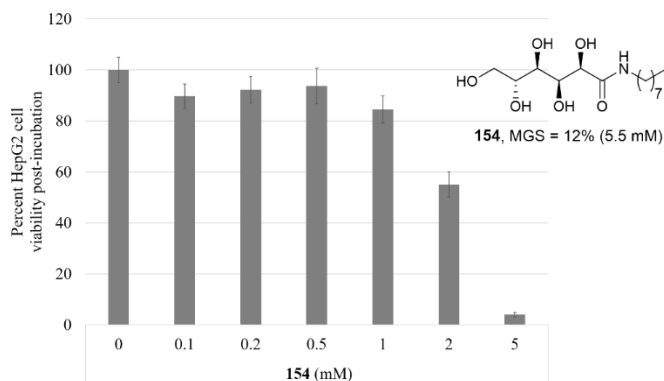
Supplemental Figure S6.4. Post-thaw percent recovery of TF-1 α cells cryopreserved with aryl aldonamides, **502** and **509**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were slowly at

room temperature. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test.

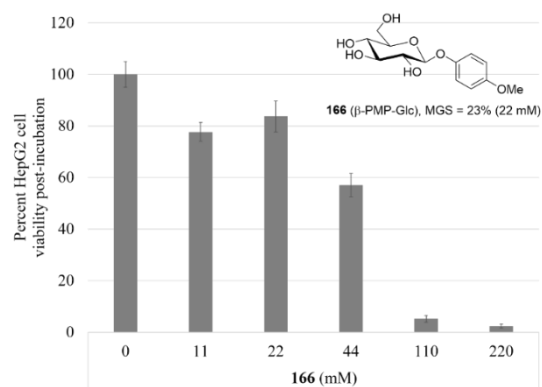
6.6.2 Cytotoxicity of DMSO and small molecule IRIs



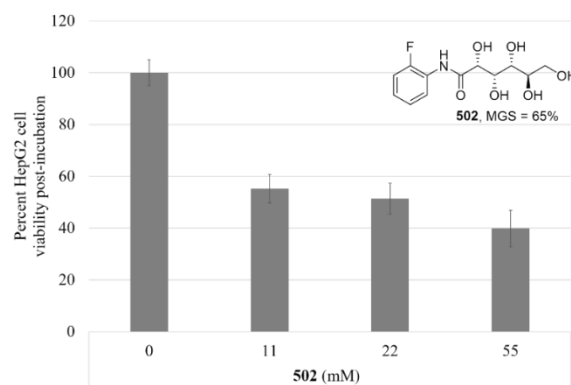
Supplemental Figure S6.5. Cytotoxicity analysis of DMSO. Percent HepG2 cell viability after 48 hours of incubation with DMSO at various concentrations.



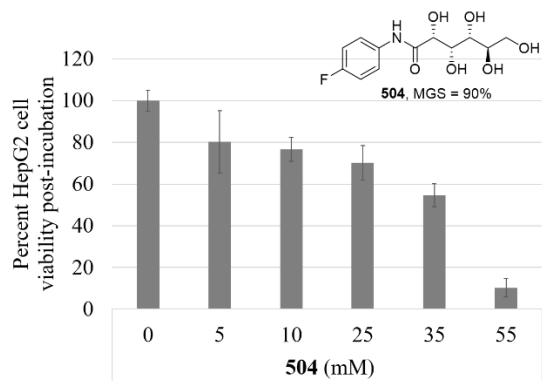
Supplemental Figure S6.6. Cytotoxicity analysis of **154**. Percent HepG2 cell viability after 48 hours of incubation with **154** at various concentrations.



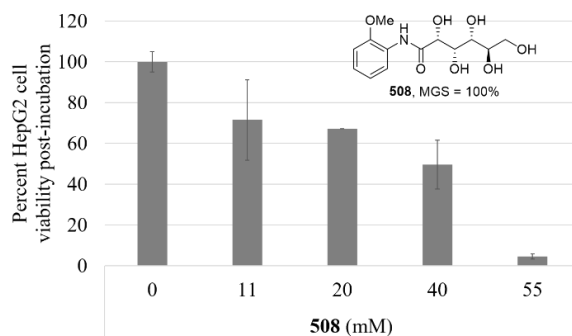
Supplemental Figure S6.7. Cytotoxicity analysis of **166**. Percent HepG2 cell viability after 48 hours of incubation with **166** at various concentrations.



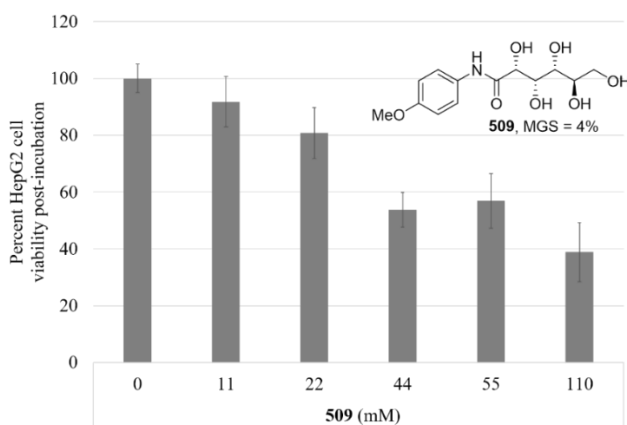
Supplemental Figure S6.8. Cytotoxicity analysis of **502**. Percent HepG2 cell viability after 48 hours of incubation with **502** at various concentrations.



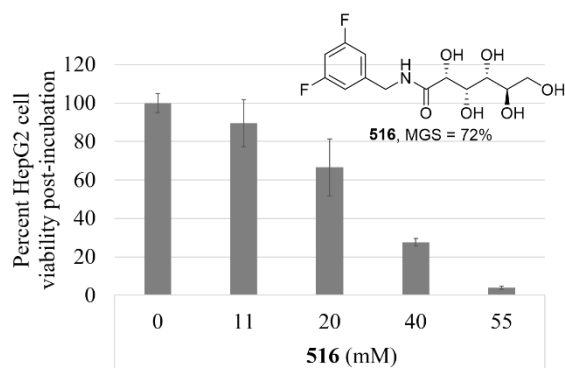
Supplemental Figure S6.9. Cytotoxicity analysis of **504**. Percent HepG2 cell viability after 48 hours of incubation with **504** at various concentrations.



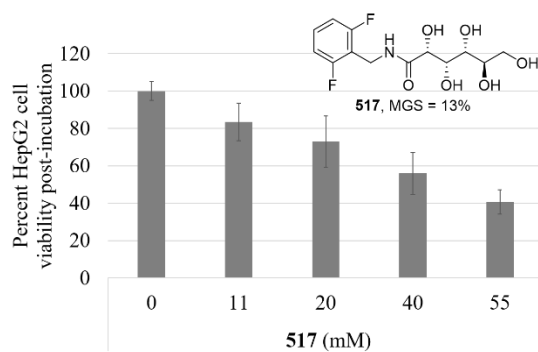
Supplemental Figure S6.10. Cytotoxicity analysis of **508**. Percent HepG2 cell viability after 48 hours of incubation with **508** at various concentrations.



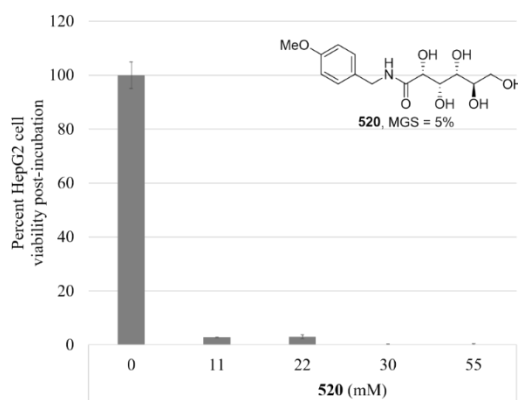
Supplemental Figure S6.11. Cytotoxicity analysis of **509**. Percent HepG2 cell viability after 48 hours of incubation with **509** at various concentrations.



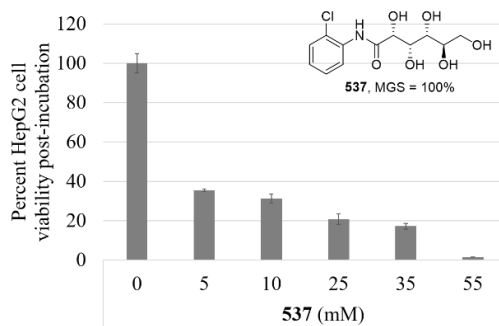
Supplemental Figure S6.12. Cytotoxicity analysis of **516**. Percent HepG2 cell viability after 48 hours of incubation with **516** at various concentrations.



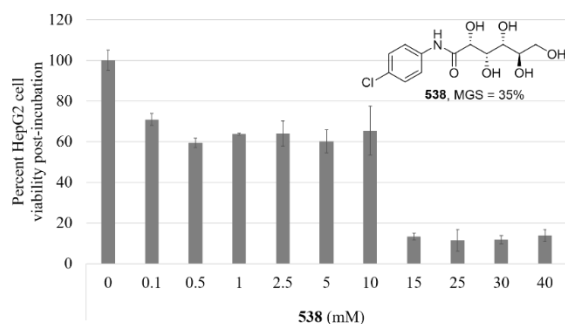
Supplemental Figure S6.13. Cytotoxicity analysis of **517**. Percent HepG2 cell viability after 48 hours of incubation with **517** at various concentrations.



Supplemental Figure S6.14. Cytotoxicity analysis of **520**. Percent HepG2 cell viability after 48 hours of incubation with **520** at various concentrations.

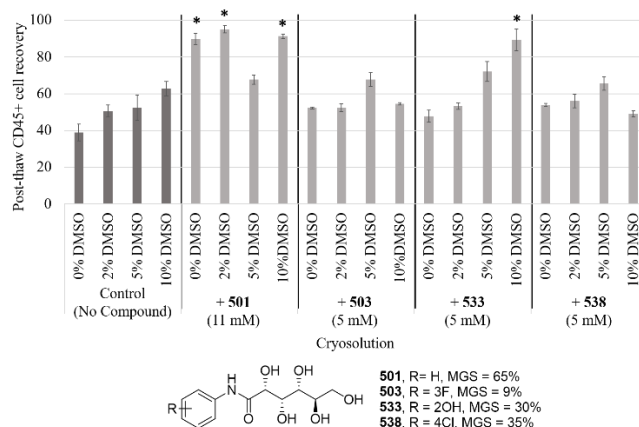


Supplemental Figure S6.15. Cytotoxicity analysis of **537**. Percent HepG2 cell viability after 48 hours of incubation with **537** at various concentrations.

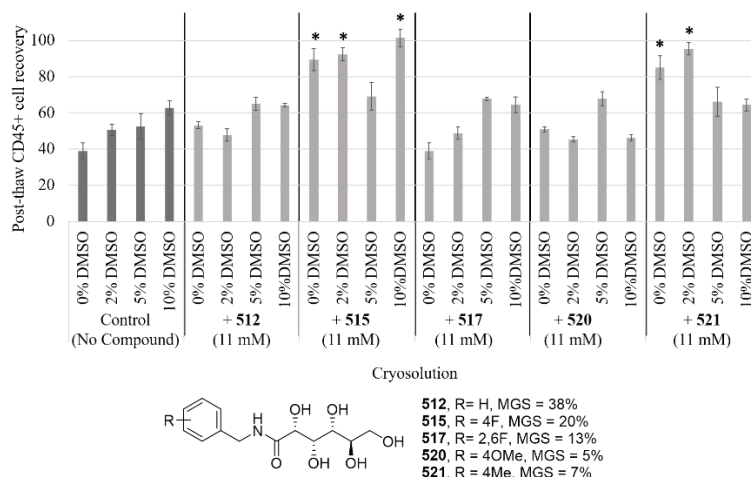


Supplemental Figure S6.16. Cytotoxicity analysis of **538**. Percent HepG2 cell viability after 48 hours of incubation with **538** at various concentrations.

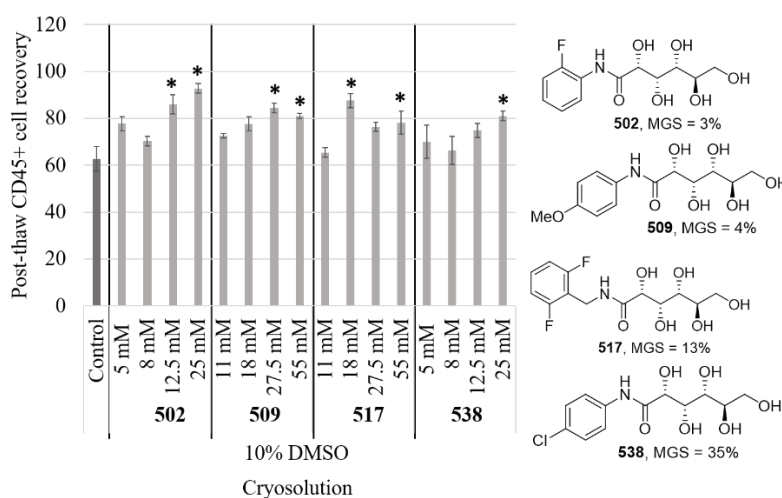
6.6.3 CD45⁺ post-thaw recovery



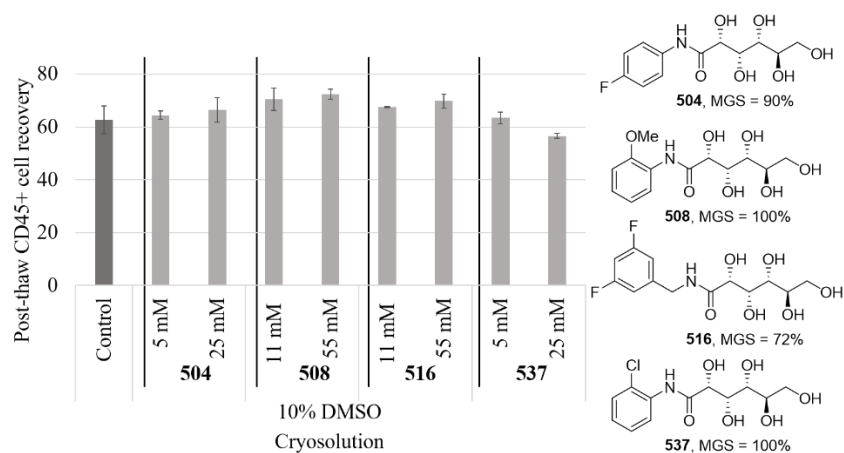
Supplemental Figure S6.17. Post-thaw percent recovery of CD45⁺ cells from UCB cryopreserved with *N*-aryl-alidonamides **501**, **503**, **533** and **538**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control.



Supplemental Figure S6.18. Post-thaw percent recovery of CD45⁺ cells from UCB cryopreserved with *N*-phenylaldonamides **512**, **515**, **517**, **520**, and **521**. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control.



Supplemental Figure S6.19. Post-thaw percent recovery of CD45⁺ cells from UCB cryopreserved with *N*-arylaldonamides **502**, **509**, **517** and **538** at various concentrations. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Statistical significance ($p < 0.05$) was assessed using a one-way analysis of variance (ANOVA) with a Tukey post-test. Asterisks indicate significant difference compared to control.



Supplemental Figure S6.20. Post-thaw percent recovery of CD45⁺ cells from UCB cryopreserved with *N*-arylaldonamides **504**, **508**, **516** and **537** at various concentrations. Cells were frozen at a rate of 1 °C/min to -80 °C before being stored at -196 °C. Samples were thawed quickly in a 37 °C water bath. Error bars are reported as the standard error of the mean (SEM). Asterisks indicate significant difference (p < 0.05) compared to the controls as assessed by the Student's unpaired T-test for comparison to control.

6.7 References

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Chapter 7: Assessing the potential of IRI active small molecules to improve the cryopreservation of human red blood cells

7.1 Introduction

The most commonly transfused blood product is red blood cells (RBCs). Transfusions of RBCs are used to treat patients with anemias, low oxygen carrying capacities resulting from extreme blood loss (traumas or surgeries), and certain diseases and infections such as sickle cell disease and malaria.¹ The most widely used method for the storing of RBCs is by hypothermic storage at 4 °C. Blood is collected into an anticoagulant solution and leukoreduced to remove plasma and leukocytes.² The resulting red cell concentrate is stored at 4 °C in a stabilizing solution, such as AS-3 (additive solution 3) or SAGM (sodium, adenine, glucose and mannitol) and can be stored for up to 42 days.² The major drawback associated with the hypothermic storage of RBCs is that this relatively short storage period can result in shortages of blood supplies during emergencies.^{3,4} In order to mitigate this, hospitals and blood banks keep RBC units on hand in anticipation of a potential need; however, many RBC units remain unused and are wasted when expired. This method also relies on routine blood donations from the public to keep a consistent stock. Furthermore, there is a risk of inadequate supplies of RBC units in the event of an emergency when large amounts of RBC units are required. Another major drawback includes molecular disruptions that affect RBCs during storage, referred to as hypothermic storage lesions. Hypothermic storage does not fully suppress biological activity and as a result, hypothermic storage lesions occur over the storage period.^{1-3,5-8} Elements of storage lesions include morphological changes, slowed metabolism, acidosis, loss of function in ion channels, apoptotic changes, and membrane vesiculation.^{2,9,10} Some of these changes occur within hours, but many occur over the course of several days or weeks.¹⁰ Hypothermic storage lesions compromise the safety and efficacy of the blood product and have been correlated to a reduced oxygen carrying capacity and increased toxicity.^{1,3,6,7,11}

Cryopreservation of RBCs would extend the storage life up to 10 years; however, it is not yet a routine practice.^{1,12,13} This is due to the current cryopreservation protocols required for RBCs. There are two

main methods for the cryopreservation of RBCs. These employ either “High Glycerol/Slow Freeze” or “Low Glycerol/Rapid Freeze” conditions. A third method for the cryopreservation of RBCs which is not used clinically employs “Hydroxyethyl Starch/Rapid Freeze” conditions. In North America, clinical cryopreservation protocols utilize “High Glycerol/Slow Freeze” preservation conditions developed by Meryman. This method employs slow cooling rates (1 °C/min) and storage at -80 °C using high concentrations of glycerol (40% v/v) as a cryoprotectant.¹ Consequently, it can take 1-1.5 hours to remove the required amount of glycerol from a thawed RBC unit (400-500 mL) to prevent intravascular hemolysis.^{1,11,12,14} Furthermore, the RBCs must be transfused within 24 hours after deglycerolization due to risk of infection.¹⁵

In certain areas of Europe, clinical protocols utilize “Low Glycerol/Rapid Freeze” preservation conditions. These conditions, optimized by Rowe *et al.*, utilize lower concentrations of glycerol (20% v/v), reducing the deglycerolization time.¹⁶⁻¹⁹ The “Hydroxyethyl Starch/Rapid Freeze” method is another approach which uses extracellular additives, such as hydroxyethyl starch (HES). HES does not enter cells and therefore significantly facilitates the removal after thawing. Furthermore, in the case of hypovolemia (severe blood loss resulting in low blood volume), HES can be transfused directly and used as a blood volume substitute. RBCs experience osmotic stress during the addition and removal of glycerol which can be evaded by extracellular additives, like HES, that do not permeate the cell membrane. Sputcsek *et al.* reported the successful transfusion of RBCs cryopreserved with HES (11.5% w/w) without post-thaw CPA removal.^{20,21} However, to ensure high recovery of RBCs, ultra-fast cooling rates and extremely low storage temperatures (-165 °C or -195 °C) are necessary for all low-glycerol procedures, requiring the use of liquid nitrogen for freezing and storage.¹⁶⁻²¹

To summarize, cryopreservation of RBCs is currently not a routine practice because either large amounts of glycerol or ultra-fast cooling rates are required. Research on improving RBC cryopreservation has focused on methods to improve deglycerolization procedures to either allow faster access to RBC units post-thaw or to increase the storage time after thawing and subsequent deglycerolization.

Additionally, alternative additive solutions and cryosolutions have been explored. These alternatives to clinical RBC cryopreservation procedures will be described in Section 7.1.1.

7.1.1 Alternatives to clinical RBC cryopreservation procedures

Over the past several years there has been an interest in improving the cryopreservation of RBCs to allow for more readily available units. Research on improving the cryopreservation of RBCs has focused on a variety of different areas of the cryopreservation protocol. A major area has been on improving current deglycerolization procedures either to allow for faster deglycerolization times or to increase the storage time post-thaw.^{11,15,22–26} For example, Valeri and coworkers designed a closed system (the ACP 215 Automated Cell Processor) which is able to both glycerolize RBCs pre-freeze and deglycerolize RBCs post-thaw.¹¹ The ACP is now the standard system for the addition and removal of glycerol in RBC units in the USA. Using this system, the length of time RBCs can be safely stored in AS-3 at 4 °C post-thaw was increased from 24 hours to 15 days.¹¹ AS-3 is an FDA-approved additive solution, containing an anticoagulant, glucose and various salts.²⁷ However, the deglycerolization process still requires about one hour per unit. Recently, Lusianti *et al.* used a mathematical model of cell membrane transport to design an optimal procedure for glycerol addition and removal.²⁶ The optimal procedure allowed for the deglycerolization of 0.6-1.1 mL RBCs in several minutes. However, this procedure was not amenable to large scale as it requires upwards of 30 litres of saline solutions to deglycerolize a full unit of RBCs. Recent research in this area involves the design of microfluidic devices to rapidly deglycerolize full units of RBCs.²⁵

Alternative cryoprotectants have also been explored. These have included various non-penetrating polymers such as HES, polyvinylpyrrolidone (PVP) and dextran.^{20,21,28–30} Recently, Gibson *et al.* demonstrated that the use of both polymers HES and poly(vinyl)alcohol (PVA) resulted in high RBCs recoveries post-thaw without the need for glycerol.^{31,32} However, extremely fast cooling rates required the use of liquid nitrogen for freezing and storage. Simple carbohydrates have also been investigated as

alternative cryoprotectants for RBCs. The use of trehalose as a cryoprotectant and methods to internalize trehalose using liposomes is an active area of research.^{33,34}

The use of ice recrystallization inhibitors (IRIs) as cryoprotectants for several cell types has been studied extensively in our laboratory.^{35–37} Recently, Capicciotti *et al.* demonstrated that the amount of glycerol required for RBC cryopreservation could be reduced from 40% to 15% with the addition of small-molecule IRIs to the cryosolution while using current clinically used freezing and thawing rates.³⁸ This work was performed in collaboration with Dr. Jason Acker, Tracey R. Turner and Jayme D. R. Kurach from Canadian Blood Services (CBS) Edmonton. The details of this work are described in Section 7.1.2.

7.1.2 IRIs as novel cryoprotectants for RBCs

Previous work from the Ben laboratory determined **166** (110 mM) and **171** (30 mM) (Figure 7.1) to be effective cryoprotectants for RBCs using reduced amounts of glycerol (15% Glyc).^{38,39} This section is a brief overview of the previous work performed by Dr. Chantelle Capicciotti in collaboration with Dr. Jason Acker and research technicians at Canadian Blood Services (CBS) Edmonton.³⁹ RBC freezing was carried out using a two-step interrupted freezing protocol in which RBCs, suspended in cryopreservation solution, were cooled to -5 °C at which point ice nucleation was induced.^{40,41} The samples were then frozen at a rate of 1 °C/min to -40 °C before being rapidly cooled to -80 °C by plunging into dry ice. Samples were thawed under fast thawing conditions in a 37 °C water bath after which RBC hemolysis was measured. Remarkably, the percentage of intact RBCs post-thaw doubled when **166** (110 mM) or **171**

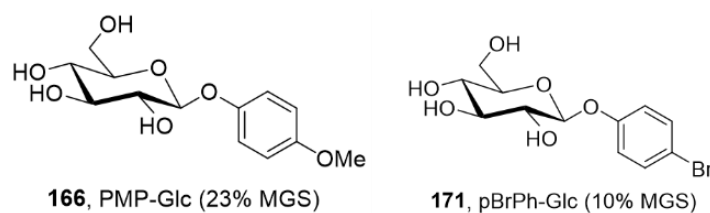


Figure 7.1. Chemical structure and IRI activity of aryl glycosides **166** and **171**.

(55 mM) was added to the 15% glycerol cryosolution (49% and 51% intact RBCs with **166** and **171**, respectively, compared to 25% intact RBCs with 15% glycerol control). A concentration scan was performed to identify the optimal concentrations for IRIs **166** and **171**. Prior to this result, low glycerol cryosolutions under slow cooling conditions resulted in negligible RBC recoveries.

The two-step interrupted freezing protocol was then exploited to investigate the mechanism of action for the cryoprotection afforded by **166**.^{40,41} The two-step interrupted freezing protocol was repeated in which samples were cooled to sub-zero temperatures between -5 to -50 °C and either thawed immediately from the defined sub-zero temperature or rapidly cooled and stored at -80 °C prior to thawing (Figure 7.2). The addition of **166** resulted in a significant increase in RBC integrity at lower sub-zero cooling

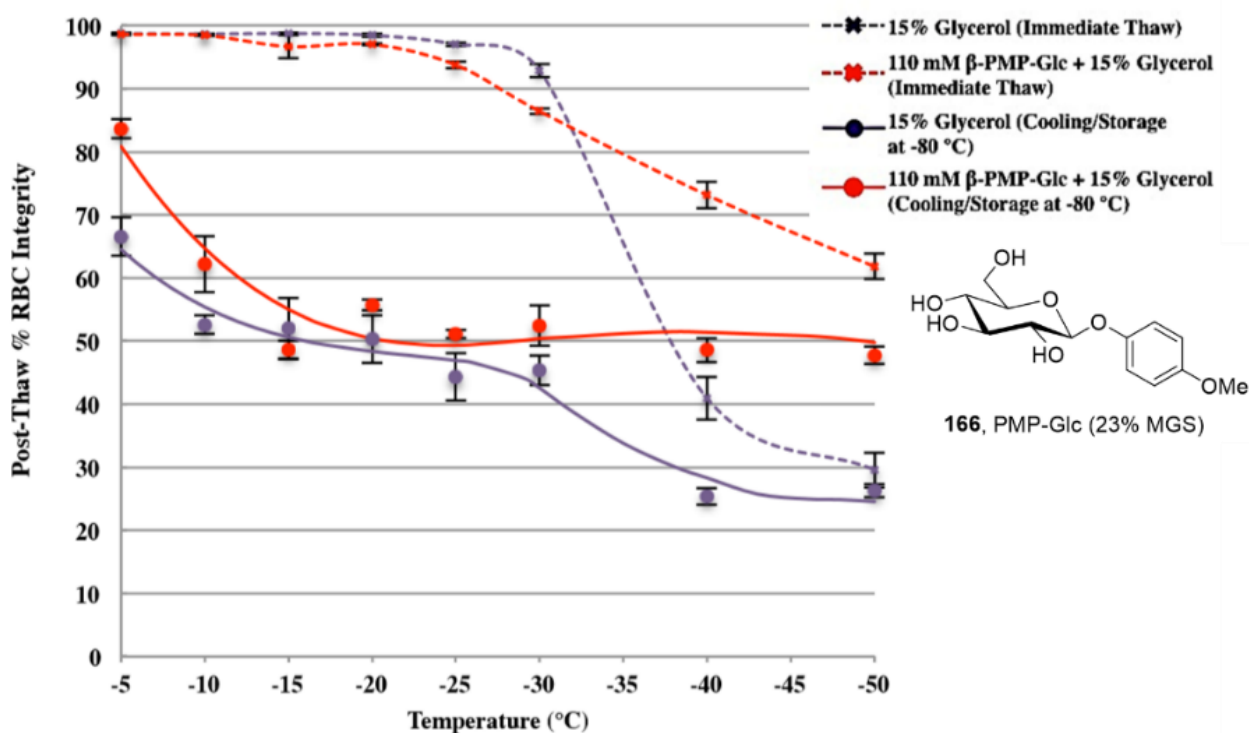


Figure 7.2. The effect of 15% glycerol cryosolutions with or without 110 mM β-PMP-Glc (**166**) on post-thaw RBC integrity. Samples were slow cooled (1 °C/min) to sub-zero temperatures then either immediately thawed (“cooling” curves, dashed lines) or rapidly cooled and stored at -80 °C prior to thawing at 37 °C (“cooling/storage” curves, solid lines). This figure was adapted from Dr. Chantelle Capicciotti’s PhD thesis.³⁹

temperatures suggesting cryoprotection was occurring during the slow cooling of RBCs. This was consistent with that of a cell-permeating cryoprotectant.⁴⁰ It was therefore hypothesized that **166** is affording cryoprotection either as a cell permeating cryoprotectant or is interacting in a synergistic effect extracellularly with glycerol. In order to investigate whether or not **166** was acting as a cell-permeating cryoprotectant, **166** was incubated with RBCs for different amounts of time at room temperature or at 0 °C prior to freezing. Glucose is readily transported across RBC membranes through glucose transporters (GLUTS).^{42,43} However, the rate of glucose transport is considerably slowed at 0 °C.⁴⁴ It was found that incubation of **166** for 5-10 minutes at room temperature prior to freezing resulted in the highest RBC integrities. Interestingly, incubations at 0 °C resulted in low post-thaw integrities, probably because **166** was not able to permeate into the cell. This was further evidence that **166** was functioning like a permeating cryoprotectant and that it may be entering RBCs through GLUTS.

Another aryl glycoside, β -pBrPh-Glc (**171**), resulted in similar post-thaw RBC integrities as **166** at half the concentration (55 mM compared to 110 mM). A subsequent concentration scan revealed the concentration could be lowered to 30 mM without sacrificing RBC integrity post-thaw. Remarkably, using freezing conditions that closely mimic those used for the clinically-used “High Glycerol/Slow Cooling” method, post-thaw RBC integrities using 15% glycerol with **171** (30 mM) were 70% - almost triple that of 15% glycerol alone (25% intact RBCs post-thaw).

Next, Dr. Chantelle Capicciotti investigated how quickly these samples can be deglycerolized. As a starting point, washing solutions utilized for the “Low Glycerol/Rapid Freeze” method were chosen (Wash 1 (W1) = 16% mannitol in 0.9% saline; W2 = 0.9% saline; W3 = 0.9% saline). Samples were rapidly frozen by plunging in liquid nitrogen or dry ice. Unfortunately, high levels of hemolysis were experienced during the deglycerolization. However, upon analysis of the amount of hemoglobin lost at each washing step, RBCs frozen with 15% glycerol + **171** (30 mM) lost considerably less hemoglobin compared to RBCs frozen with 15% glycerol alone immediately after thawing, indicating a lower degree of hemolysis. This suggested that modifications to the washings might allow for improved recoveries.

Wash 2 (W2) was modified because a high percentage of hemoglobin was lost at this stage. This was attributed to the large difference in osmolality between W1 (16% mannitol in 0.9% saline) and W2 (0.9% saline). Therefore, W2 was modified to provide a more gradual step down in osmolality (10% mannitol in 0.9% saline). This modification resulted in less hemoglobin lost at W2 but increased the losses at W3. Therefore, further optimization of the deglycerolization washing solutions was required.

Osmotic tolerances of RBCs in the presence of cryosolutions were measured and optimal saline concentrations for the washes were determined.^{45,46} Briefly, osmotic tolerances were measured by transferring aliquots of RBCs premixed with cryosolutions into different concentrations of saline solutions and determining the amount of hemolysis. For 15% glycerol and 15% glycerol + **166** (110 mM), optimal washes were as follows: W1 = 5% saline, W2 = 1.5% saline and W3 = 0.9% saline. For 15% glycerol + **171** (30 mM), the optimal washes were determined to be: W1 = 5% saline, W2 = 3% saline and W3 = 2% saline. With the addition of **171** to the cryosolution, RBCs cannot return to isotonic conditions after three washes. A fourth wash allowed a step down in saline concentration to 1.5% saline. Using these washing conditions, the recovery was 35% for the glycerol control. The recovery was increased to 77% and 45% when **166** (110 mM) and **171** (30 mM) were present. While there was an increase in post-deglycerolization RBC recovery, these experiments were performed using rapid freezing conditions by plunging the samples in liquid nitrogen. Further experiments were required to optimize the deglycerolization washing solutions and to transition to slower freezing rates utilized clinically.

7.1.3 Goals and objectives

Previous work revealed that when using slow cooling conditions, the amount of glycerol required to cryopreserve RBCs could be reduced from 40% to 15% with the addition of small-molecule IRIs **166** and **171**. However, deglycerolization of the RBCs post-thaw was problematic and resulted in high levels of hemolysis. Thus, it was hypothesized that the addition of **166** and **171** to the alternative hydroxyethyl starch (HES) cryosolution could allow for facilitated deglycerolization. The “Hydroxyethyl Starch/Rapid Freeze” method is a “Rapid Freeze” approach involving the use of HES. HES does not enter cells and

therefore significantly facilitates the removal after thawing. RBCs experience osmotic stress during the addition and removal of glycerol which can be evaded by extracellular additives, like HES, that do not permeate the cell membrane. Thus, it was hypothesized that the addition of **166** or **171** to the HES solution could permit high RBC recoveries post-thaw using slow cooling conditions. Furthermore, it was hypothesized that this new HES cryosolution supplemented with **166** or **171** would be more readily removed post-thaw.

Another approach to improve the deglycerolization process post-thaw is to remove the excess cryosolution prior to freezing. During slow cooling, ice forms first extracellularly.⁴⁷ Solutes are excluded from the ice and thus the solute concentration outside the cell gradually increases. As a result, water flows osmotically out of the cell. In the event IRIs are present on the outside of the cell, this would increase the extracellular solute concentration and cause more water to leave the cell during cooling. This may impair the osmotic tolerances of the RBCs post-thaw. While it is unknown whether this is occurring and what result this would have on the RBCs, it was hypothesized that removing excess cryosolution prior to freezing could result in facilitated CPA removal post-thaw. It was previously found that incubation of RBCs with 15% glycerol + **166** (110 mM) for 10 minutes prior to cooling resulted in the highest RBC integrity post-thaw. It was hypothesized that 10 minutes was sufficient for **166** to permeate the cell membrane. Thus, RBCs will be incubated for 10 minutes in cryosolution at room temperature. The RBCs will then be centrifuged and excess cryosolution removed prior to freezing. The effect of removal of cryosolution pre-freeze on post-thaw deglycerolization will be investigated.

Chapter 6 reported that several *N*-aryl-aldonamides were effective at improving the cryopreservation of hematopoietic stem cells (HSCs). Therefore, the ability of this new class of small-molecule IRIs, as well as other IRI active aryl glycosides, to be effective cryoprotectants for RBCs will be assessed. Deglycerolization on RBCs cryopreserved with these new molecules will also be assessed. Furthermore, the ability to deglycerolize a full unit of RBCs cryopreserved with novel IRIs will be investigated.

Finally, RBCs will be stored hypothermically in the presence of IRIs to determine if there are any cytotoxic effects to RBCs exposed to these molecules.

The results from this Chapter were recently published in *Scientific Reports* entitled “Small molecule ice recrystallization inhibitors mitigate red blood cell lysis during freezing, transient warming and thawing”⁴⁸ and in an article recently submitted to *RSC Advances* entitled “Ice recrystallization inhibitors with hydroxyethyl starch improve cryopreservation of human red blood cells with slow freezing rates”.⁴⁹

7.2 Cryopreservation of human RBCs with IRIs using hydroxyethyl starch

β -PMP-Glc (**166**) and β -pBrPh-Glc (**171**) were assessed for their cryoprotective ability with human RBCs using the “Hydroxyethyl Starch/Rapid Freeze” method for the cryopreservation of RBCs. The original protocol for HES cryopreservation of small RBC samples proposed by Sputtek *et al.* was utilized.⁵⁰ RBCs suspended in cryoprotectant solution were rapidly frozen by either plunging in liquid nitrogen (115 °C/min) or plunging in dry ice (5 °C/min). Additional samples were frozen slowly by placement of samples in a -80 °C freezer (1.8 °C/min). A thermocouple was inserted into a glycerol control sample for temperature measurements at 1 s intervals to obtain freezing rates. Cryosolutions assessed were HES (11.5%) and HES supplemented with **166** (110 mM) or **171** (30 mM). Samples were subsequently thawed in a 48 °C water bath and diluted with 0.9% saline. It was hypothesized that all samples frozen rapidly will result in high post-thaw recoveries and integrities. When samples are frozen more slowly, it was hypothesized that recovery and integrity will be decreased. Supplementation of **166** or **171** should improve the recovery and integrity of the RBCs under slower cooling conditions. After thawing, RBC recovery and integrity was measured (Figure 7.3). Hemolysis was quantified utilizing Drabkin’s assay,^{51,52} which is the standard assay used to determine RBC hemolysis by comparing total and supernatant hemoglobin quantities.

Figure 7.3 shows that highest RBC integrities and recoveries were obtained when the samples were frozen rapidly by submersion in liquid nitrogen (“Rapid Freeze -196 °C”). This is not surprising because

the use of HES as a cryoprotectant requires ultra-fast freezing rates. When the samples were frozen by submersion in dry ice (“Rapid Freeze -80 °C”) or by placement in a -80 °C freezer (“Freezer -80 °C”), the freezing rate is significantly slower (from 115 °C/min to 5 °C/min and 1.8 °C/min, respectively). There is a reduction in recovery and RBC integrity when slower cooling rates are used with HES or with HES + **166** (110 mM). However, when HES is supplemented with **171** (30 mM), there is an increase in both recovery and integrity compared to HES alone ($p < 0.001$, 0.01 or 0.05). Interestingly, **171** is more effective at preserving RBC integrity and recovery during slower cooling conditions than **166** and it is approximately three times as IRI active (8% MGS vs 23% MGS).

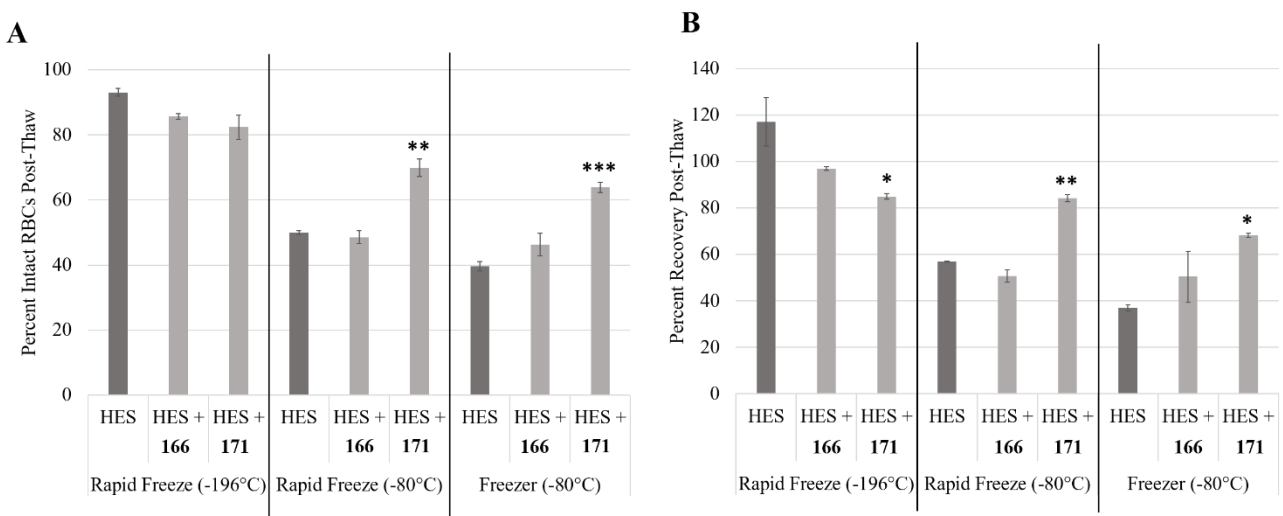


Figure 7.3. (A) Post-thaw RBC integrity and (B) Post-thaw RBC recovery of RBCs cryopreserved with HES and IRIs **166** and **171**. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons with a 99.9% ($p < 0.001$, ***), 99.0% ($p < 0.01$, **) or 95% ($p < 0.05$, *) confidence level.

Next, we wanted to assess how the removal of the cryosolution post-thaw would impact the integrity and recovery of the RBCs. In the previous experiment, the supernatant hemoglobin level, required for the hemolysis analysis, was obtained by centrifugation of the samples at 500 x g for 15 minutes and poor separation was observed. Therefore, faster centrifugation speeds were utilized. Both 16,000 x g for 5 minutes and 2,200 x g for 10 minutes were assessed. RBCs suspended in cryosolution

were frozen using the same three freezing conditions using previously (“Rapid Freeze -196 °C”, “Rapid Freeze -80 °C” and “Freezer -80 °C”). After thawing, samples were diluted with 0.9% saline and centrifuged at either 16,000 x g for 5 minutes or 2,200 x g for 10 minutes. The supernatant was removed and the samples were resuspended in 0.9% saline. Percent intact RBCs and recoveries post-wash are shown in Figure 7.4 (for 2,200 x g centrifugation) and Figure 7.5 (for 16,000 x g centrifugation). RBC integrities and recoveries are highest when the samples were frozen more rapidly (“Rapid Freeze -196 °C”) for both centrifugation conditions. Interestingly, when RBCs are cryopreserved with HES alone, a reduction in RBC integrity and recovery was observed after washing. This reduction did not occur when **171** was added to the cryosolution. Therefore, because there was no reduction in RBC integrity and recovery after washing when **171** was present, this could suggest that **171** is helping to mitigate osmotic stress during CPA removal. Low post-wash integrities were experienced when slower cooling rates are utilized (“Rapid Freeze -80 °C” or “Freezer -80 °C”). However, higher post-wash integrities and recoveries were observed when a centrifugation speed of 16,000 x g was used and therefore, these conditions were utilized for the remainder of this study.

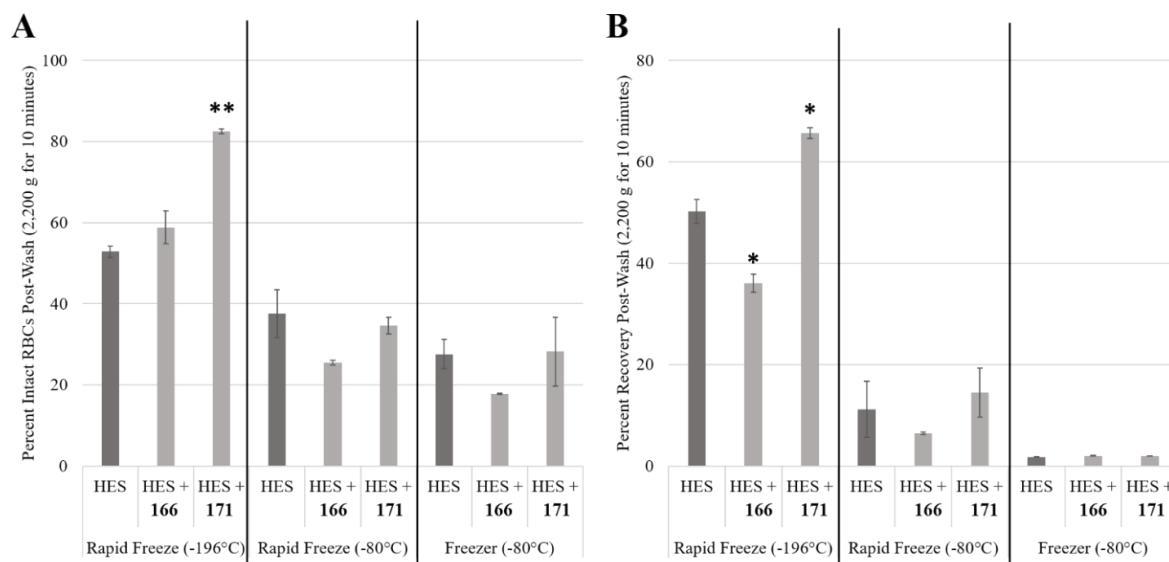


Figure 7.4. (A) Post-wash RBC integrity and (B) Post-wash RBC recovery of RBCs cryopreserved with HES and IRIs **166** and **171** using centrifugation conditions of 2,200 g for 10 minutes. Statistical significance was assessed by

one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons with a 99.0% ($p < 0.01$, **) or 95% ($p < 0.05$, *) confidence level. Asterisks indicate significant difference compared to control (HES).

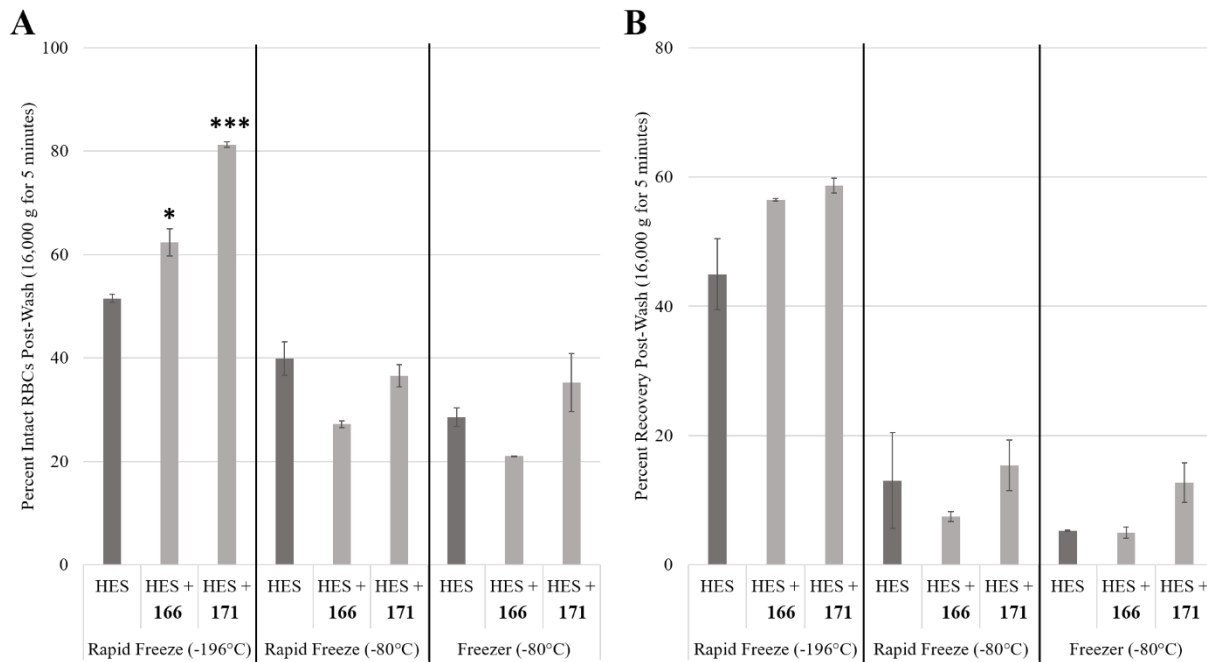


Figure 7.5. (A) Post-wash RBC integrity and (B) Post-wash RBC recovery of RBCs cryopreserved with HES and IRIs **166** and **171** using centrifugation conditions of 16,000 g for 5 minutes. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons with a 99.9% ($p < 0.001$, ***) or 95% ($p < 0.05$, *) confidence level. Asterisks indicate significant difference compared to control (HES).

Supplementation of IRIs **166** or **171** to the HES cryosolution does not permit slower cooling conditions which are used clinically in North America. However, using the typical protocol for “HES/Rapid freeze” conditions (“Rapid Freeze -196 °C”), higher post-wash RBC integrity and recovery are observed when the HES cryosolution is supplemented with **171** (30 mM) ($p < 0.05$). Therefore, the effect of post-thaw storage times on RBC integrity using this cryosolution was investigated.

RBCs suspended in cryosolution were frozen using “Rapid Freeze -196 °C” conditions. After thawing, the samples were treated in one of two ways: (1) samples were either diluted with 0.9% saline or with the clinically used additive solution, AS-3 (“Dilute”); or (2) diluted with 0.9% saline, centrifuged at

16,000 g for 5 minutes and resuspended in either 0.9% saline or AS-3 (“Resuspend”). Samples were then stored at 4 °C and RBC integrity was measured immediately and at 24 and 48 hours post-thaw. The results are shown in Table 7.1.

Percent Intact RBCs					
			Rapid Freeze (-196 °C)		
			Immediately Post-Thaw	24 Hours Post-Thaw	48 Hours Post-Thaw
Dilute	0.9% saline	HES	73	72	43
		HES + 171 (30 mM)	83	75	76
	AS-3	HES	78	87	63
		HES + 171 (30 mM)	84	59*	75
Resuspend	0.9% saline	HES	48	41	47
		HES + 171 (30 mM)	59	44	35
	AS-3	HES	64	50	52
		HES + 171 (30 mM)	65	50	46

Table 7.1. Percent intact RBCs after dilution or resuspension in 0.9% saline or AS-3 immediately post-thaw or 24 and 48 hours post-thaw. Statistical significance was assessed by unpaired Student’s T-test for comparison to the control (HES) with a 95% ($p < 0.05$, *) confidence level.

Overall, integrity was higher post-thaw without resuspension (“Dilute”) (assessed by unpaired Student’s T-test, $p < 0.05$). In other words, removal of the CPA resulted in a reduction of intact RBCs for both cryosolutions. Integrity was also reduced after resuspension over time (after storage for 24 and 48 hours). For example, cryopreservation of RBCs with HES and subsequent post-thaw resuspension in AS-3 resulted in 64% intact RBCs, which was further reduced to 50% after 24 hours of storage. However, resuspension in AS-3 resulted in higher integrity compared to 0.9% saline. This could be because AS-3 contains anticoagulant, glucose and salts that may be required by RBCs during storage. The most interesting result is with 24 and 48 hour “Dilute” conditions. With HES, when the samples are diluted with 0.9% saline or AS-3, a reduction in RBC integrity was observed over time. Integrity was reduced

from 73% immediately post-thaw to 43% after 48 hours of storage after dilution with 0.9% saline. Integrity is also reduced, but to a lesser extent when the RBCs are diluted with AS-3 (78% to 63% after 48 hours). This again could be a result of the anticoagulant, glucose and salts present in this solution. However, when RBCs are cryopreserved with HES + **171** (30 mM) and diluted with either 0.9% saline or AS-3, there was only a slight reduction in integrity even after 48 hours post-thaw. Conversely, reductions in integrity were observed when RBCs were cryopreserved with **171** but resuspended post-thaw. In other words, removal of **171** post-thaw resulted in a reduction in RBC integrity over time. This could suggest that **171** is somehow preventing RBC hypothermic storage lesion post-thaw. This result is significant as it suggests that RBCs could be stored for up to 48 hours post-thaw prior to transfusion, whereas RBCs cryopreserved with HES alone should be transfused immediately. The optimization of post-thaw storage of RBCs will be crucial for clinical applications. Whether or not the RBCs can be stored longer post-thaw is something that should be investigated in future work.

7.3 Cryopreservation of human RBCs with removal of excess cryosolution pre-freeze

As described in Section 7.1.2, RBC integrity is preserved using cryoprotectant solutions with greatly reduced glycerol concentrations by supplementation of small-molecule IRIs **166** and **171** using slow cooling conditions. However, deglycerolization of the RBCs post-thaw was problematic and resulted in high levels of hemolysis. The optimal saline concentrations for deglycerolization were previously determined by measuring the osmotic tolerances of RBCs in the presence of **166** and **171**. Using these optimal washing conditions, the recovery was 35% for the glycerol control. The recovery was increased to 77% and 45% when **166** (110 mM) and **171** (30 mM) were present, respectively.

In order to improve the post-thaw deglycerolization process, the excess cryosolution was removed prior to freezing. It was hypothesized that excess cryosolution removal pre-freeze would help to mitigate osmotic imbalances during freezing. If excess IRI in solution is present extracellularly, this would result in more extensive dehydration during slow cooling. This is because during slow cooling, ice forms first extracellularly. Solutes are excluded from the ice and therefore the solute concentration outside of the cell

gradually increases causing water to flow osmotically out of the cell. It was hypothesized that removal of excess cryosolution prior to freezing would mitigate this and facilitate CPA removal post-thaw. Previous results indicate the IRIs are functioning as permeating cryoprotectants. Therefore, removal of any extracellular IRI should not impact RBC integrity post-thaw.

RBCs suspended in cryosolution were incubated for 10 minutes. It was previously found that incubation of RBCs with 15% glycerol + **166** (110 mM) for 10 minutes prior to cooling resulted in the highest RBC integrity post-thaw.³⁹ Therefore, it was hypothesized that 10 minutes was sufficient for **166** to permeate the cell membrane. The RBCs were then centrifuged and excess cryosolution was removed prior to freezing using “Rapid Freeze -196 °C” and slower cooling “Rapid Freeze -80 °C” conditions. As a control, samples were frozen which did not have excess cryosolution removed. After thawing, RBCs were deglycerolized using the optimal washing conditions determined previously (W1 = 5% saline, W2 = 1.5% saline, and W3 = 0.9% saline). The percentage of intact RBCs after deglycerolization is shown in Figure 7.6. Samples in which excess cryosolution was not removed prior to freezing are indicated as “Cryosolution Removed Post-Thaw” and samples in which excess cryosolution was removed prior to freezing are indicated as “Cryosolution Removed Pre-Freeze”. As shown in Figure 7.6, using “Rapid Freeze -196 °C” conditions, there is no difference in RBC integrity when the excess cryosolution is removed prior to freezing. However, decreased RBC integrity when IRIs **166** and **171** are present is experienced for both freezing conditions. Interestingly, when slower cooling conditions are used (“Rapid Freeze -80 °C”), there is a decrease in RBC integrity with **166** when the cryosolution is removed prior to freezing. In other words, higher RBC integrity was observed after deglycerolization when extracellular **166** was not removed prior to freezing. This could suggest that **166** is functioning as both a permeating and non-permeating cryoprotectant and that it must be present on both sides of the cell membrane to be effective. The percent hemoglobin (%Hgb) lost (Eq. 7.1) at each washing step is shown in Figure 7.7

$$\%Hgb\ lost = \frac{\text{Supernatant Hgb (at various stages of deglycerolization)}}{\text{Total Hgb in RBCs prior to freezing}} \times 100\% \quad \text{Equation 7.1}$$

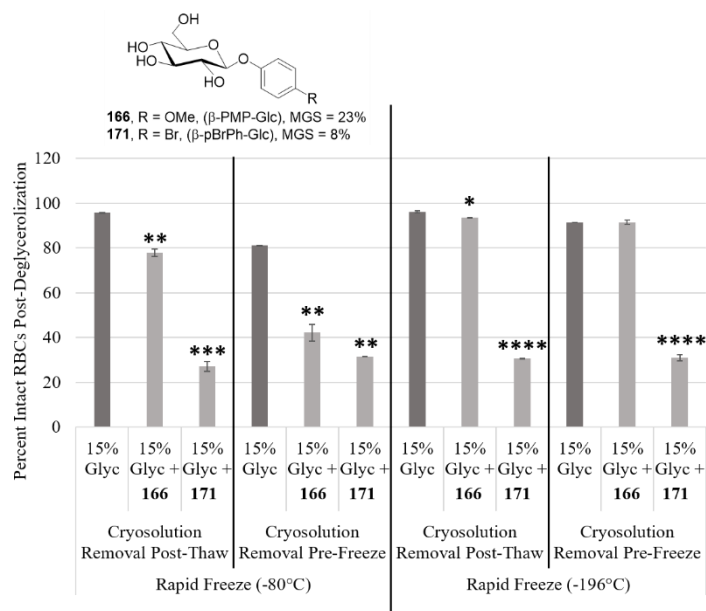


Figure 7.6. Percent intact RBCs post-deglycerolization when excess cryosolution is removed prior to freezing. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons with a 99.99% ($p < 0.0001$, ****), 99.9% ($p < 0.001$, ***), 99.0% ($p < 0.01$, **) or 95% ($p < 0.05$, *) confidence level.

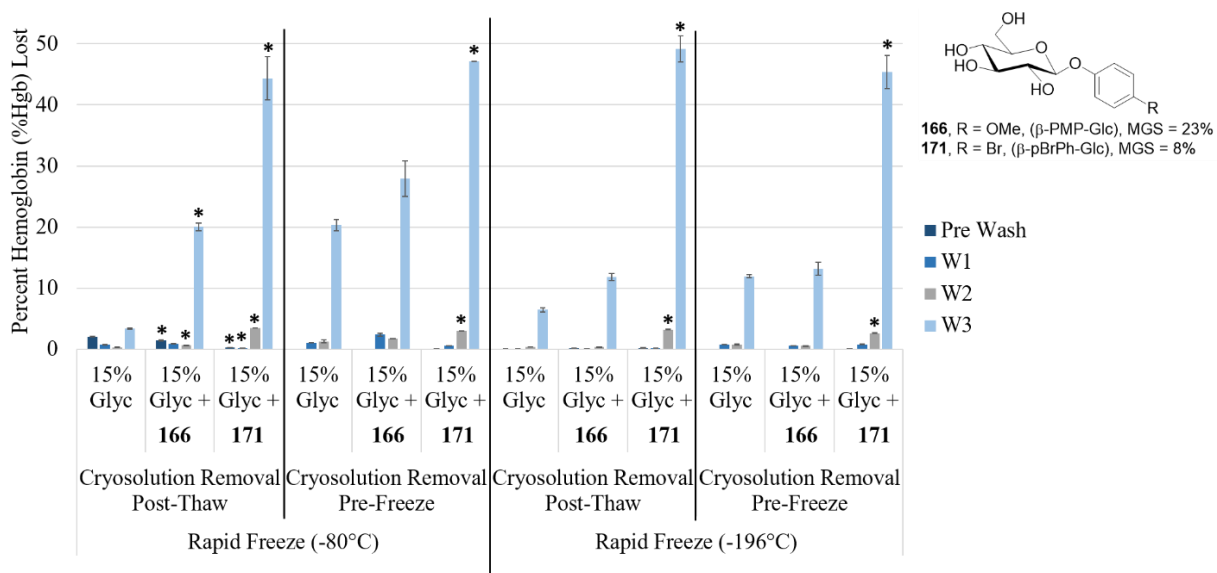


Figure 7.7. Percent hemoglobin (%Hgb) lost at each wash (relative to RBC Hgb prior to freezing) during deglycerolization when excess cryosolution is removed prior to freezing. Statistical significance was assessed by unpaired Student's T-test for comparison to the control with a 95% ($p < 0.05$, *) confidence level.

Percent hemoglobin (%Hgb) is the amount of Hgb in the supernatant at various stages of the deglycerolization process (PreWash, W1, W2 or W3) compared to the total amount of Hgb in the sample prior to freezing. Less than 5% hemoglobin (Hgb) is lost for all freezing conditions and cryosolutions post-thaw (PreWash), W1 and W2. It is after W3 that significant amounts of Hgb are lost.

Overall, removal of excess cryosolution prior to freezing does not facilitate post-thaw deglycerolization. A reduction in RBC integrity was experienced with **166** when the cryosolution was removed pre-freeze, which suggests that **166** is required both intra- and extracellularly. For all conditions with **166** and **171**, the highest percentage of hemoglobin was lost after W3 indicating the difference in osmolality is too great between W2 and W3 causing RBC hemolysis. To mitigate this, a fourth wash could be incorporated; however, this would increase the deglycerolization time. Unfortunately, deglycerolization of the RBCs cryopreserved with **166** and **171** post-thaw is problematic and results in high levels of hemolysis. Thus, other small-molecule IRIs were investigated.

7.4 *N*-Aryl-aldonamides as cryoprotectants for RBCs

As described in Section 7.1.2, RBC integrity is preserved using cryoprotectant solutions with greatly reduced glycerol concentrations by supplementation of small-molecule IRIs **166** and **171** using slow cooling conditions.³⁹ However, efforts to effectively deglycerolize the RBCs post-thaw were not successful and resulted in high amounts of hemolysis. Therefore, other small-molecule IRIs were investigated for their ability to effectively cryopreserve RBCs. Chapter 6 revealed that several *N*-aryl-aldonamides were effective at improving the cryopreservation of hematopoietic stem cells (HSCs). Therefore, the ability of this new class of small-molecule IRIs to be effective cryoprotectants for RBCs was assessed. Furthermore, the galactose derivatives of aryl glycosides **166** and **171**, β -PMP-Gal (**171**) and β -pBrPh-Gal (**173**), were assessed to investigate the influence of the carbohydrate moiety on cryopreservation ability. β -PMP-Gal (**172**) and β -pBrPh-Gal (**173**) have IRI activities of 78% and 12%, respectively.

RBCs suspended in cryosolutions containing reduced glycerol (15%) and IRIs **172**, **173**, **501**, **509**, **512**, **515**, **521**, **532**, **533** and **538** were frozen using rate controlled freezing conditions (1 °C/min) to -40 °C and rapid freezing conditions. The chemical structures and IRI activities are shown in Figure 7.8. Samples were thawed using fast thawing conditions by submersion in a 37 °C water bath. After thawing, RBC integrity was measured using the Drabkin's assay. Under rapid freezing conditions, post-thaw integrities were above 80% (with the exception of **173** at 110 mM) because high cooling rates are required for low glycerol conditions (Supplemental Figures S7.1 & S7.2). Post-thaw RBC integrities for RBCs cryopreserved with 15% glycerol and small-molecule IRIs using slow cooling conditions are shown in Figure 7.8. *N*-Aryl-aldonamides were substantially less soluble than the aryl glycosides and therefore were assessed at much lower concentrations. Under slow cooling conditions, 15% glycerol resulted in 40% intact RBCs post-thaw. Addition of **172** (110 mM) or **173** (55 mM) resulted in an increased RBC integrity (60% intact, $p < 0.05$). Interestingly, lower concentrations of **172** (55 mM) were less effective whereas lower concentrations of **173** (55 mM) were more effective. Remarkably, *N*-aryl-aldonamide **538** resulted in over 80% intact RBCs post-thaw at only 20 mM.

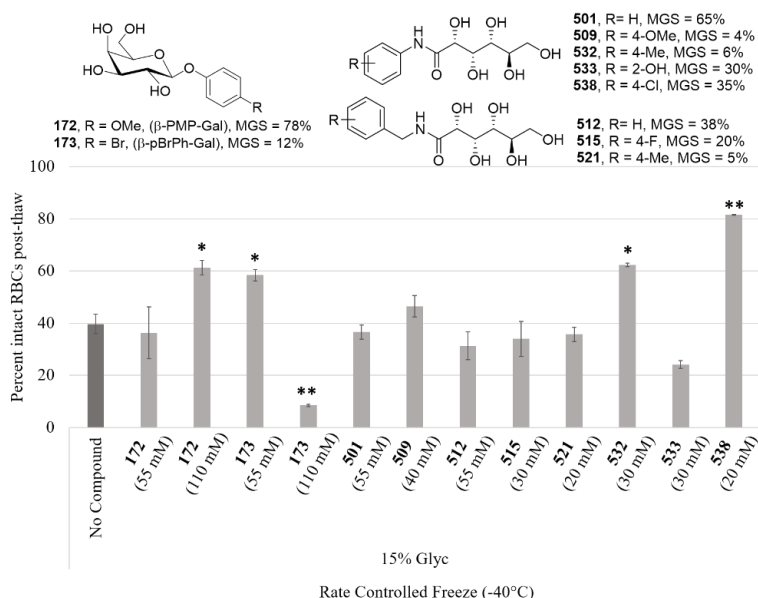


Figure 7.8. Percent intact RBCs post-thaw of RBCs frozen with small-molecule IRIs. RBC samples in glycerol solutions with or without IRIs were slow cooled (1 °C/min) to -40 °C and thawed 37 °C. Statistical significance was

assessed by one-way analysis of variance (ANOVA) with a Dunnett's post-test for comparison to the control (no compound, 15% Glyc) with a 99% ($p < 0.01$, **) or 95% ($p < 0.05$, *) confidence level.

Next, a concentration scan on **173** and **538** was performed to identify the lowest effective concentration of IRI. **172** was more effective at 110 mM than 55 mM; however, higher concentrations were not tested due to solubility limits. RBCs were suspended in cryosolutions containing 15% glycerol and various concentrations of **173** and **538**. Samples were frozen and thawed as described in the previous experiment using rate controlled freezing conditions (1 °C/min) to -40 °C and fast thawing (37 °C water bath). After thawing, RBC integrity was measured using the Drabkin's assay (Figures 7.9 & 7.10). For compound **173**, post-thaw RBC integrity is highest at concentrations between 20 and 40 mM (Figure 7.9).

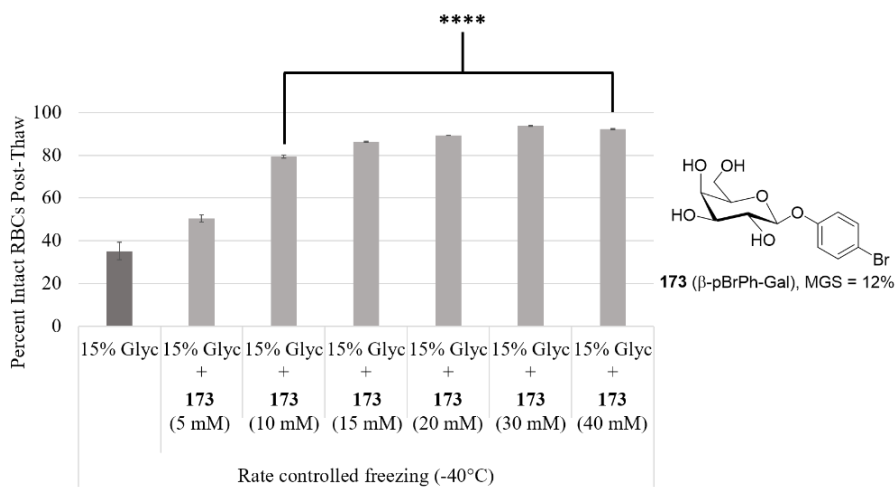


Figure 7.9. The effect of various concentrations of **173** on post-thaw RBC integrity using reduced concentrations of glycerol. RBC samples in glycerol solutions were slow cooled (1 °C/min) to -40 °C and thawed 37 °C. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Dunnett's post-test for comparison to the control (15% Glyc) with a 99.99% ($p < 0.0001$, ****) confidence level.

At lower and higher concentrations, integrity is reduced. This is likely due to the fact that at lower concentrations, there is not enough IRI to be effective, whereas at higher concentrations, too much IRI results in toxic effects. For compound **538**, as low as 5 mM is effective at maintaining high levels of intact RBCs post-thaw (Figure 7.10). It is interesting to note that **173** was effective at 20 mM and was IRI

active (12% MGS) at a similar concentration of 22 mM. Compound **538** was also assessed for IRI activity at 22 mM (35% MGS) but was effective at maintaining RBC integrity post-thaw at only 5 mM.

Therefore, the IRI activity of **538** was reassessed at 5 mM and was found to possess moderate IRI activity of 50% (Supplemental Figure S7.3). This could suggest one of two things: **538** exerts cryoprotective effects that are not related to inhibition of ice recrystallization; or inhibiting ice crystals to 50% their size is sufficient to mitigate the cryoinjury associated with ice recrystallization. This was investigated further and the results are described later in this chapter.

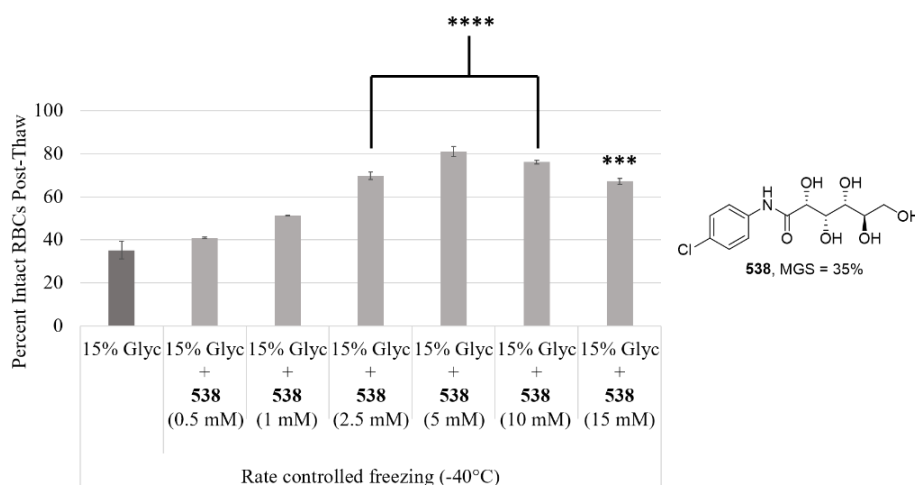


Figure 7.10. The effect of various concentrations of **538** on post-thaw RBC integrity using reduced concentrations of glycerol. RBC samples in glycerol solutions were slow cooled (1 °C/min) to -40 °C and thawed at 37 °C. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Dunnett’s post-test for comparison to the control (15% Glyc) with a 99.99% ($p < 0.0001$, ****) or 99.9% ($p < 0.001$, ***) confidence level.

Next, we investigated whether the glycerol concentration could be further reduced from 15% to 10% or 5% by using IRIs **173** or **538**. Optimal concentrations identified above were utilized as well as one higher concentration. A higher concentration was also assessed because it was hypothesized that if the glycerol concentration is reduced, perhaps a higher concentration of IRI would be required to compensate. RBCs were cooled and thawed as described in the previous experiment. After thawing, RBC integrity was measured using the Drabkin’s assay (Figures 7.11 & 7.12). Even if using higher

concentrations of IRIs, the glycerol concentration cannot be reduced to 10% or 5%. Thus, the optimal cryosolutions resulting in high post-thaw RBC integrities are 15% glycerol supplemented with **173** (20 mM) or **538** (5 mM).

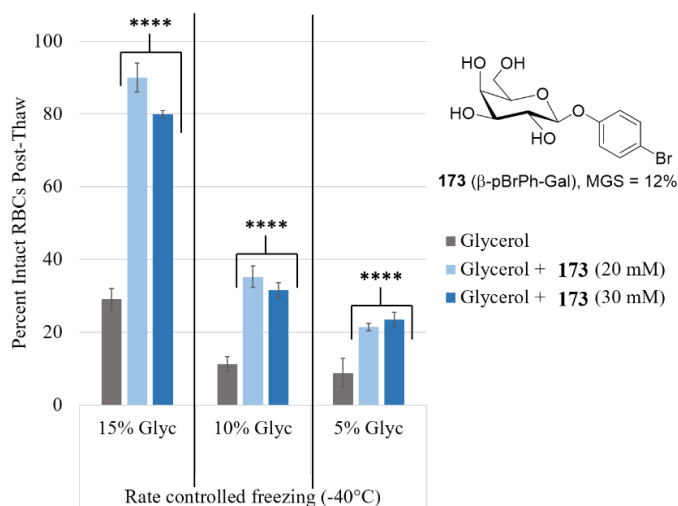


Figure 7.11. The effect of various concentrations of glycerol on post-thaw RBC integrity of RBCs frozen with **172** (20 and 30 mM). RBC samples in glycerol solutions were slow cooled (1 °C/min) to -40 °C and thawed at 37 °C. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons with a 99.99% ($p < 0.0001$, ****) confidence level.

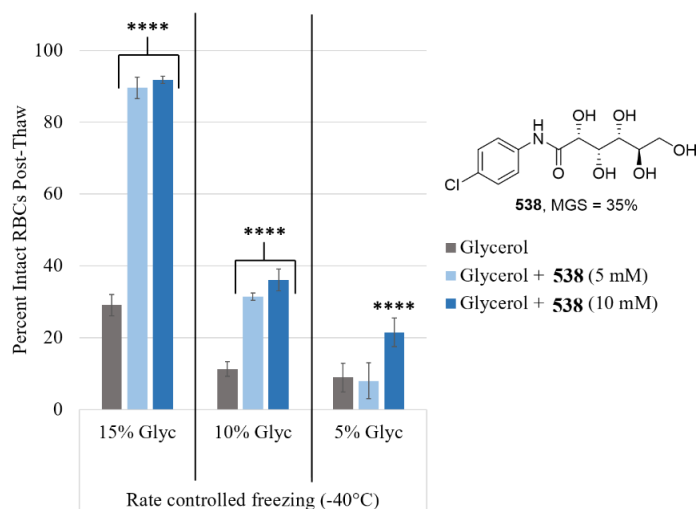


Figure 7.12. The effect of various concentrations of glycerol on post-thaw RBC integrity of RBCs frozen with **538** (5 and 10 mM). RBC samples in glycerol solutions were slow cooled (1 °C/min) to -40 °C and thawed at 37 °C.

Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons with a 99.99% ($p < 0.0001$, ****) confidence level.

The two-step interrupted freezing protocol was then exploited to obtain information with respect to the mechanisms of cryoinjury and more importantly how the cryoprotectants, **172**, **173** and **538** are functioning. To accomplish this, the two-step interrupted freezing protocol was repeated at sub-zero temperatures between -5 and -50 °C and samples were either thawed immediately from the defined sub-zero temperature (see “cooling” profiles depicted by solid lines in Figures 7.13-15) or rapidly cooled and stored at -80 °C prior to thawing (see “cooling/storage” profiles depicted by dashed lines in Figures 7.13-15). The results from this study are presented in Figures 7.13 (**172**), 7.14 (**173**) & 7.15 (**538**).

The “cooling” profile (Figure 7.13, solid lines) for 15% glycerol is typical of that observed with a cell permeating cryoprotectant.⁴⁰ RBC integrity progressively decreased as the temperature to which the samples were cooled was decreased. Higher post-thaw integrities were observed at temperatures above -30 °C and lower integrities were observed upon slow cooling to lower sub-zero temperatures.⁴⁰ The higher integrities obtained above -30 °C could be due to the colligative effects of glycerol, decreasing the amount of ice at any sub-zero temperature.^{40,47} The lower RBC integrities below -30 °C are likely attributed to cellular damage due to concentrating solutes, consistent with Mazur’s two-factor hypothesis of freezing injury (described in Chapter 1, Section 1.2.2).⁴⁷ A similar cooling profile was observed for β -PMP-Gal (**172**); however, **172** resulted in a significant increase in RBC integrity at lower sub-zero cooling temperatures (Figure 7.13). At -40 °C and -50 °C, RBC integrity frozen with 15% glycerol is 51% and 31% respectively and this is increased by 20% at both temperatures (70% and 50%) with the addition of 110 mM β -PMP-Gal (**172**). This “cooling” profile is almost identical to β -PMP-Glc (**166**) shown in Figure 7.2.^{38,39}

Different profiles were obtained with the “cooling/storage” conditions of the RBC samples frozen in the same cryosolutions (Figure 7.13, dashed lines). For the 15% glycerol cryosolution, as the temperature to which the samples were slow-cooled was decreased, integrities also decreased prior to

rapid cooling and storage at -80 °C. The higher post-thaw integrities at temperatures above -30 °C is likely due to the initiation of rapid cooling at higher sub-zero temperatures creating an overall faster cooling rate to -80 °C. This is consistent with the need for a rapid cooling rate for the low glycerol/rapid freeze cryopreservation method of RBCs.^{16,53,54} When 110 mM β -PMP-Gal (**172**) was added to 15% glycerol, post-thaw RBC integrities were 8 - 15% higher than 15% glycerol alone at sub-zero cooling temperatures of -40 °C and -50 °C. Taken together, these results indicate that β -PMP-Gal (**172**) confers protection during the slow-cooling stage of the freezing protocol.

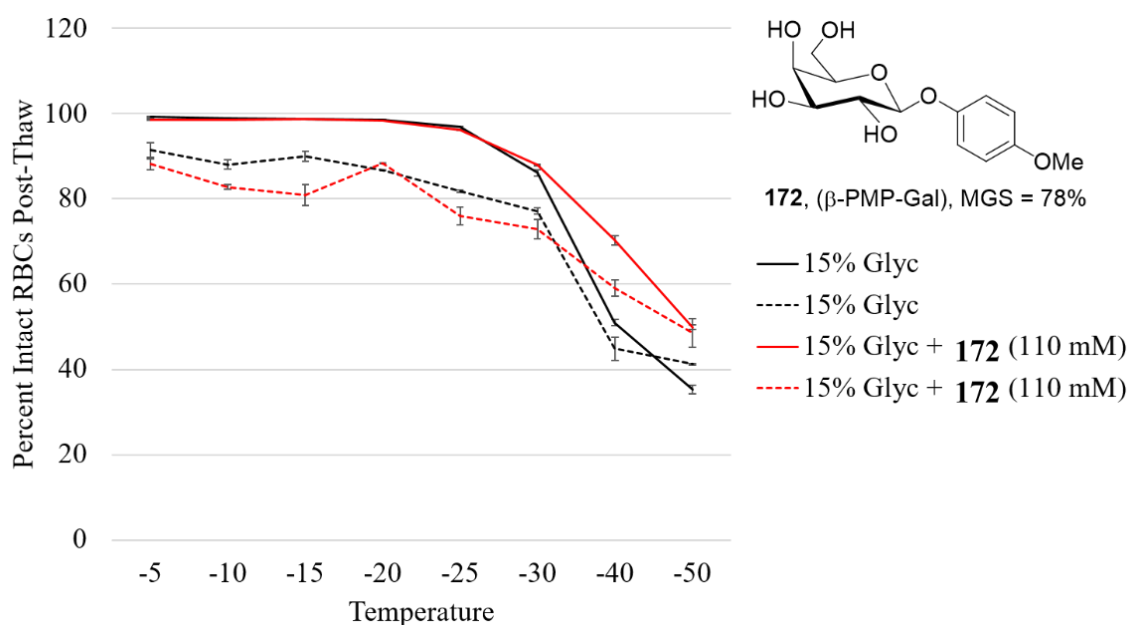


Figure 7.13. The effect of 15% glycerol cryosolutions with or without 110 mM β -PMP-Gal (**172**) on post-thaw RBC integrity. Samples were slow cooled (1 °C/min) to sub-zero temperatures then either immediately thawed (“cooling” curves, solid lines) or rapidly cooled and stored at -80 °C prior to thawing at 37 °C (“cooling/storage” curves, dashed lines).

The “cooling” and “cooling/storage” profiles (Figure 7.14 & 7.15) for 15% glycerol + **173** (20 mM) and 15% glycerol + **538** (5 mM) were very similar. Remarkably, RBC integrities were above 87% for all temperatures with **173** (20 mM) and above 70% with **538** (5 mM). The most impressive result is at the -40 °C and -50 °C temperatures. For both “cooling” and “cooling/storage”, 15% glycerol resulted in

40-50% intact RBCs and with the addition of **172** or **538**, RBC integrity was increased to 87-93% and 70-86%, respectively.

The fact that the addition of **172**, **173** and **538** to the 15% glycerol freezing solution resulted in increased integrities (reduced hemolysis) under the slow-cooling/immediate thaw conditions is consistent with the profile of a cell permeating cryoprotectant.⁴⁰ This suggests that these molecules are being internalized into the RBCs. A second reasonable explanation for this reduced hemolysis is a synergistic effect between the internalized glycerol and IRI activity in the extracellular environment by **172**, **173**, or **538**. Regardless of the mechanism of action, these results clearly demonstrate that these molecules are providing protection during the slow cooling phase of the protocol which is significant as these freezing conditions mimic RBC clinical cryopreservation protocols that utilize slow freezing rates but high glycerol concentrations.¹

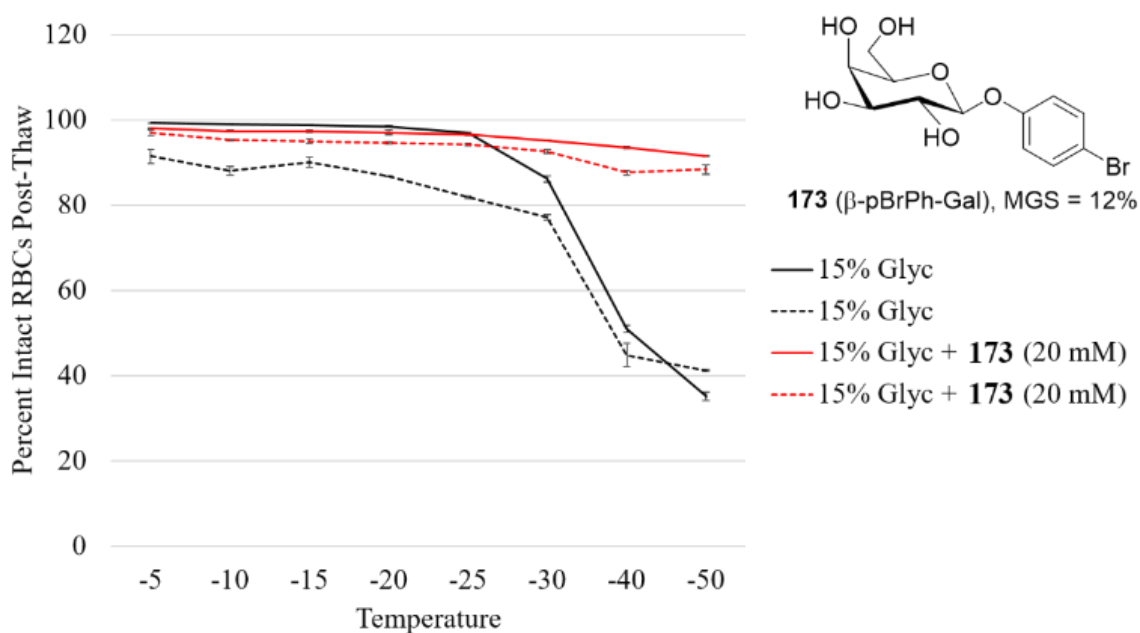


Figure 7.14. The effect of 15% glycerol cryosolutions with or without 20 mM β -pBrPh-Gal (**173**) on post-thaw RBC integrity. Samples were slow cooled (1 °C/min) to sub-zero temperatures then either immediately thawed (“cooling” curves, solid lines) or rapidly cooled and stored at -80 °C prior to thawing at 37 °C (“cooling/storage” curves, dashed lines).

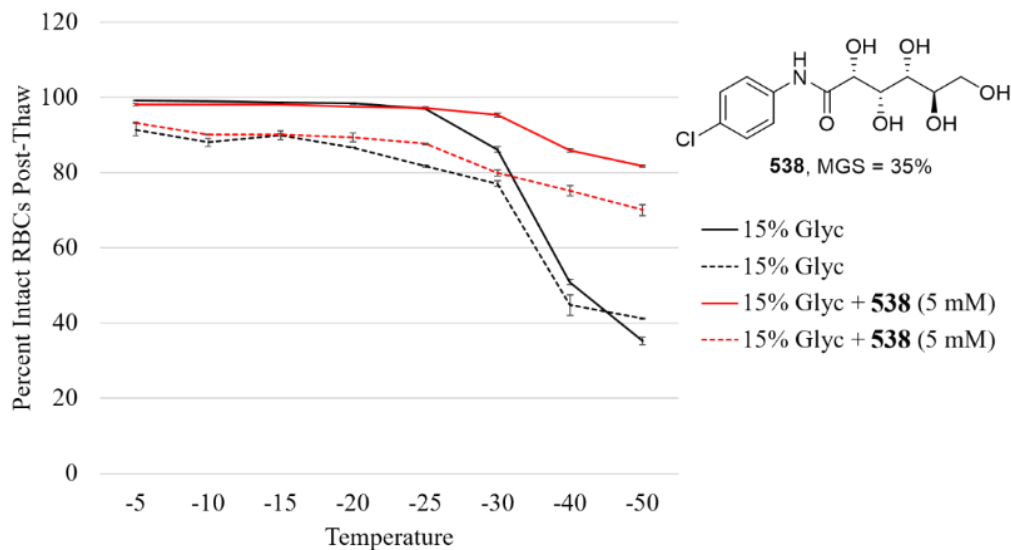


Figure 7.15. The effect of 15% glycerol cryosolutions with or without 5 mM **538** on post-thaw RBC integrity. Samples were slow cooled (1 °C/min) to sub-zero temperatures then either immediately thawed (“cooling” curves, solid lines) or rapidly cooled and stored at -80 °C prior to thawing at 37 °C (“cooling/storage” curves, dashed lines).

Overall, **172**, **173** and **538** are conferring protection during the slow cooling phase of this freezing protocol. RBC integrities are above 87% for all conditions with **173** (20 mM) and above 70% with **538** (5 mM). It is interesting that compounds **173** and **538** are better at preserving RBC integrities during slow cooling to lower sub-zero temperatures than **172**. This can be attributed to their greater IRI activities (12% and 35% for **172** and **538** respectively compared to 78% MGS). The fact that these molecules preserve RBC integrity upon slow cooling is either because these molecules are functioning as permeating cryoprotectants or they are acting in a synergistic manner with glycerol by inhibiting ice recrystallization in the extracellular environment. This was investigated and the results are described later this chapter. Next, the ability to deglycerolize RBCs frozen with compounds **173** and **538** was investigated, described in the following section.

7.5 Recovery of RBCs after deglycerolization

In the previous section, **173** and **538** were identified as effective cryoprotectants for RBCs. Under slow cooling conditions (1 °C/min), **173** (20 mM) and **538** (5 mM) resulted in over 80% intact RBCs

post-thaw compared to 35% intact RBCs for the 15% glycerol control. We next wanted to assess how easily these samples could be deglycerolized. Three washing conditions were utilized and are described in Table 7.2: (1) washing solutions determined previously for 15% glycerol and β -PMP-Glc (**166**) represented as “Washing Protocol 1”; (2) washing solutions utilized for low glycerol conditions established by Rowe *et al.* represented as “Washing Protocol 2”^{16–19}; and (3) modified low glycerol conditions determined previously by Dr. Capicciotti represented as “Washing Protocol 3”.

	Washing Protocol 1	Washing Protocol 2	Washing Protocol 3
W1	5.0% saline	16% mannitol in 0.9% saline	16% mannitol in 0.9% saline
W2	1.5% saline	0.9% saline	10% mannitol in 0.9% saline
W3	0.9% saline	0.9% saline	0.9% saline

Table 7.2. Washing solutions used for Washing Protocols 1, 2 & 3.

RBCs were suspended in cryosolutions of 15% glycerol, 15% glycerol + **173** (20 mM) or 15% glycerol + **538** (5 mM) and frozen using “Rapid Freeze -80 °C” or “Freezer -80 °C” conditions. RBC samples with 15% glycerol and 110 mM β -PMP-Glc (**166**) or 30 mM β -pBrPh-Glc (**171**) were included for comparison. After thawing, samples were deglycerolized using the three washing conditions described above (Table 7.2). Post-deglycerolization recovery is shown in Figure 7.16. Post-thaw recovery was between 20-30% with 15% glycerol under “Rapid Freeze -80 °C” conditions compared to 2-3% using “Freezer -80 °C”. This reduction in recovery can be attributed to the difference in freezing rates (5 °C/min vs 0.7 °C/min) and is consistent with the need for rapid cooling rates with low glycerol cryosolutions. Freezing rate curves are shown in Supplemental Figure S7.4.

Under “Rapid Freeze -80 °C” conditions (Figure 7.16A), addition of **166** or **173** resulted in increased recovery compared with 15% glycerol alone when Washing Protocol 3 was utilized ($p < 0.01$ and 0.05). Addition of **171** shows a reduction in recovery under all washing conditions compared to the control ($p < 0.05$) which is consistent with previous results, indicating the post-thaw deglycerolization of RBCs cryopreserved with **171** is problematic and results in high levels of hemolysis, particularly at W2 and W3.

Interestingly, addition of **538** allowed for an increase in recovery after deglycerolization with all washing conditions compared to the 15% glycerol control (38-43% recovery compared to 20-30%).

When “Freezer -80 °C” conditions are used (Figure 7.16B), the cooling rate is decreased. This resulted in a large decrease in recovery for the 15% glycerol control (20-30% to 2-3%). Addition of all compounds resulted in an increase in recovery to 11-23% when Washing Protocol 2 or 3 washing conditions were used. Interestingly, when Washing Protocol 1 conditions were used, **538** resulted in the highest recovery (18% compared to 3% for the control).

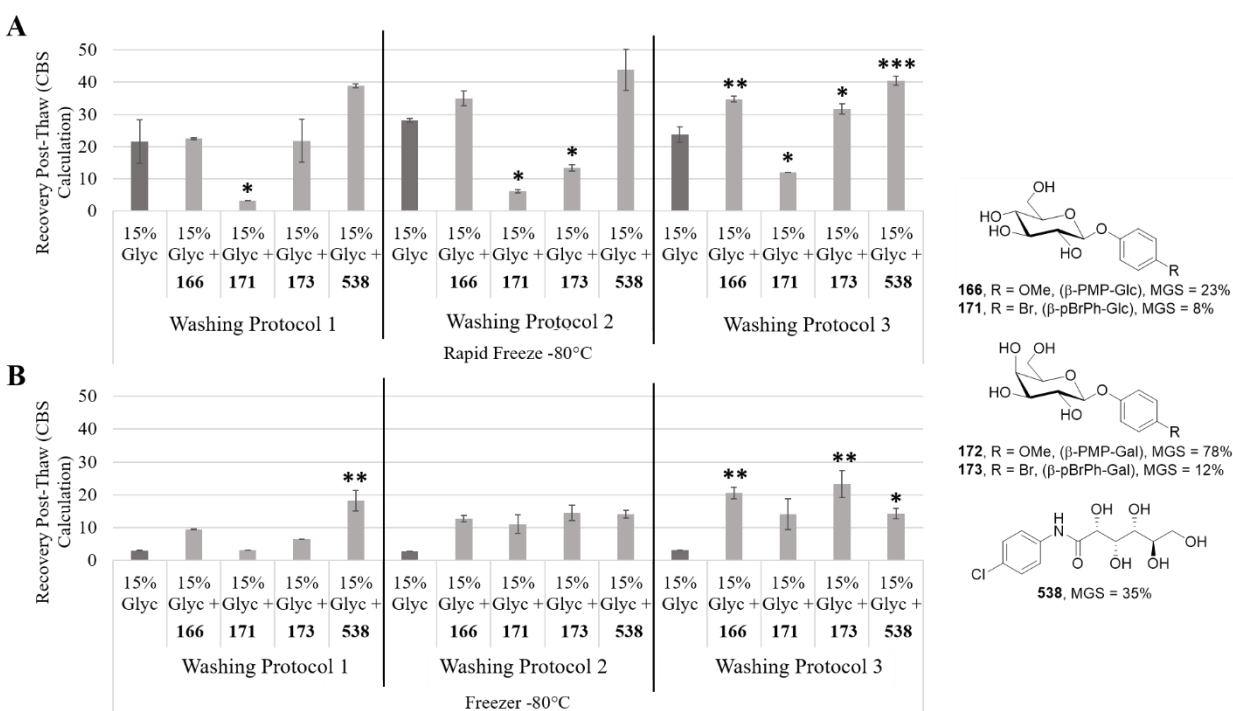


Figure 7.16. Recovery of RBCs frozen with 15% glycerol containing IRIs **166**, **171**, **173** and **538** under (A) “Rapid Freeze -80 °C” or (B) “Freezer -80 °C” conditions. Samples were deglycerolized using Washing Protocol 1, 2 or 3 deglycerolization conditions. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Dunnett’s post-test for comparison to the control (no compound) with a 99.9% ($p < 0.001$, ***), 99.0% ($p < 0.01$, **) or 95% ($p < 0.05$, *) confidence level.

The percentage of hemoglobin (%Hgb) lost at each wash for “Rapid Freeze -80 °C” was measured and the results are summarized in Figure 7.17. These data for “Freezer -80 °C” were not included because post-deglycerolization recoveries were very low. For 15% glycerol, the majority of the hemoglobin was lost immediately post-thaw (60%). This is a result of extensive cryoinjury to the RBCs as a result of low glycerol concentrations and relatively low cooling rate (5 °C/min). Subsequent washes result in <10% hemoglobin loss for all washing conditions. When 15% glycerol is supplemented with aryl glycosides **171** or **173**, less than 10% hemoglobin is lost immediately post-thaw. However, compounds **166** and **538** result in 35-45% hemoglobin lost from RBCs immediately post-thaw. This may be explained by the differences in IRI activities. Compounds **171** and **173** are highly IRI active (8% and 12% MGS) and provide better protection to RBCs during slow cooling under reduced glycerol conditions. In contrast, compounds **166** and **538** are less IRI active (23% and 35% MGS) and therefore do not provide the same

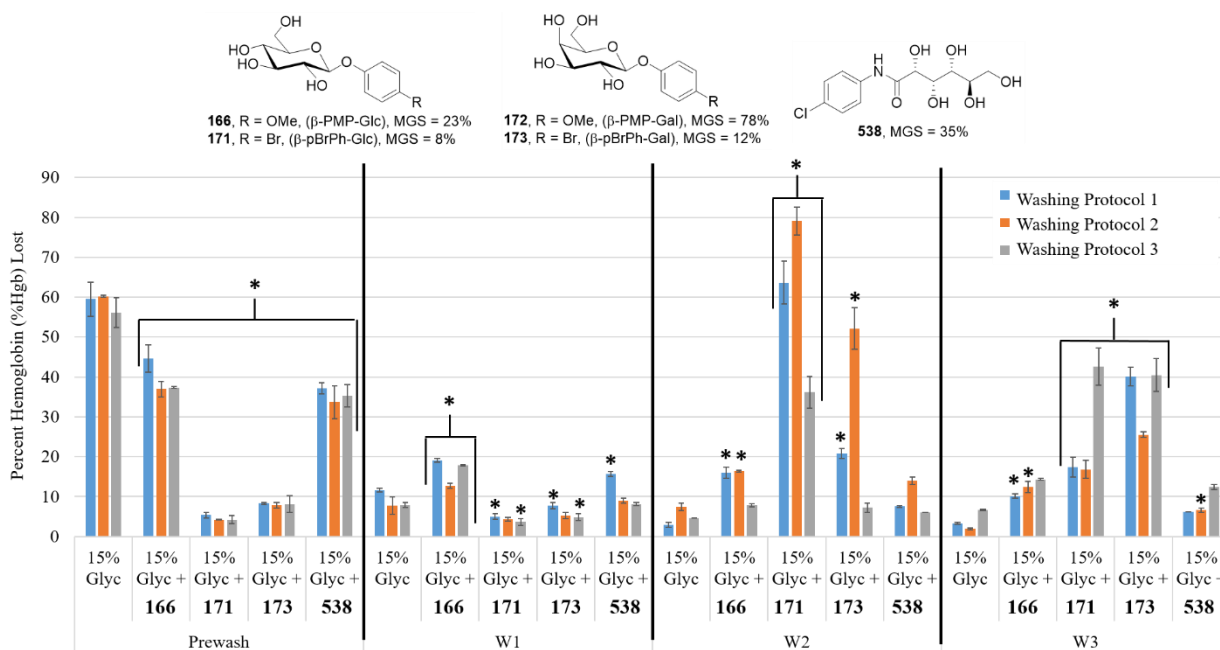


Figure 7.17. Percent hemoglobin lost during deglycerolization washes relative to RBC Hgb prior to freezing using Washing Protocol 1, 2 or 3 deglycerolization conditions. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Dunnett’s post-test for comparison to the control (15% Glyc) with a 95% ($p < 0.05$, *) confidence level.

level of protection resulting in more hemolysis during cryopreservation and higher percentages of hemoglobin lost immediately post-thaw. However, **171** and **173** are more difficult to remove during the deglycerolization process and result in high amounts of RBC hemolysis during deglycerolization, particularly during W2 and W3. Meanwhile, RBCs frozen with **166** and **538** are more readily deglycerolized and therefore exhibit higher post-deglycerolization recoveries compared to **171** and **173**.

One explanation for this could be that **171** and **173** are interacting with or integrating into RBC membranes. This could explain the increased hemolysis during the washing protocol. This is also consistent with the fact that RBCs frozen with **171** and **173** are more sensitive to different washing solutions compared to **166** and **538**. For example, with **171**, if W2 is 0.9% saline (Washing Protocol 2), nearly 80% of RBC hemoglobin is lost whereas if W2 is 10% mannitol in 0.9% saline (Washing Protocol 3), only 35% of RBC hemoglobin is lost. This is similar to **173** where significantly more hemoglobin is lost at W2 when Washing Protocol 2 conditions are used compared to Washing Protocol 3 (52% vs 7% Hgb lost).

The percent intact RBCs post-deglycerolization for “Rapid Freeze -80 °C” is shown in Figure 7.18. For 15% glycerol and 15% glycerol with **166**, approximately 80% of RBCs are intact after all deglycerolization conditions. Aryl glycoside **171** shows reduced RBC integrity post-deglycerolization for all washing conditions ($p < 0.0001$) which is consistent with previous results.³⁹ Interestingly, aryl glycoside **173** shows reduced RBC integrity with Washing Protocol 2 and Washing Protocol 3 deglycerolization conditions ($p < 0.0001$) whereas with Washing Protocol 1 deglycerolization conditions, 95% of RBCs are intact post-deglycerolization. This is the opposite of what is observed for **538**, where RBC integrity is reduced after Washing Protocol 1 compared to Washing Protocol 2 and Washing Protocol 3.

To summarize these results, RBC recovery post-deglycerolization is highly dependent on the freezing conditions and washing solutions utilized. For all cryosolutions, faster cooling rates are more

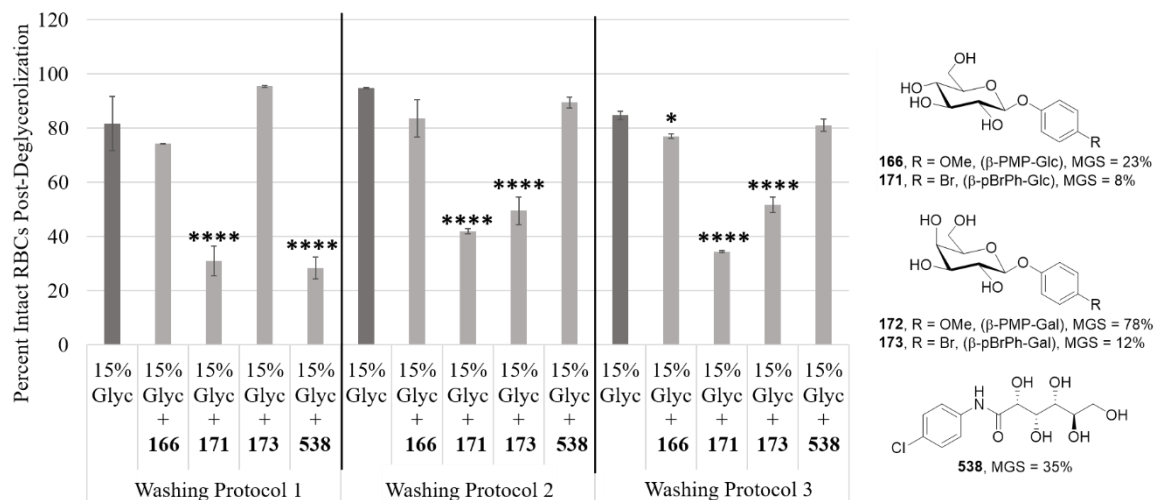


Figure 7.18. Percent intact RBCs post-deglycerolization using Washing Protocol 1, 2, or 3 deglycerolization conditions. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Dunnett's post-test for comparison to the control (15% Glyc) with a 99.99% ($p < 0.0001$, ****) or 95% ($p < 0.05$, *) confidence level.

favourable which is consistent with the need for rapid freezing rates using reduced glycerol concentrations.¹⁶⁻²¹ For aryl glycoside **171**, low recoveries were experienced for all conditions which is consistent with previous results.³⁹ Aryl glycosides **166** and **173** result in higher recoveries when Washing Protocol 2 or Washing Protocol 3 conditions are utilized for both freezing conditions. The most interesting result is with aryl aldonamides **538** which is the only compound to consistently result in higher recoveries compared to the 15% glycerol control for all freezing and washing conditions assessed. However, overall low recoveries were experienced with the highest recoveries approximately 40% (**538** under "Rapid Freezing -80 °C" conditions). Analysis of post-deglycerolization intact RBCs revealed that for **538**, RBC integrity is reduced after Washing Protocol 1 compared to Washing Protocol 2 and Washing Protocol 3. A possible remedy for this would be to resuspend the RBCs in a stabilizing solution such as SAGM after deglycerolization.

A possible reason for low recoveries could be the difficulty of supernatant removal after washes. The interface between packed RBCs and supernatants was difficult to distinguish. This could be a result

of the use of small volumes. For our standard deglycerolization experiments, 0.5 mL of RBCs is mixed with 0.5 mL cryosolution for a final volume of 1 mL. To assess the effect of volume on RBC recovery, 10 mL samples were utilized: 5 mL RBCs mixed with 5 mL cryosolution. It was hypothesized that larger volumes would be more easily handled, resulting in better separation between packed RBCs and supernatant during washes and therefore higher recoveries. RBCs suspended in cryosolution (15% glycerol or 15% glycerol + **538**) were frozen using “Rapid Freeze -80 °C” and “Freezer -80 °C” conditions. The cooling rates for 1 mL and 10 mL volumes are shown in Supplemental Figure S7.4. After thawing, RBC samples were deglycerolized using CBS washing conditions (Washing Protocol 1). Washing Protocol 1 conditions utilize 50, 15, and 9 g/L saline for W1, W2 and W3, respectively (Table 7.2). In the previous experiment with 1 mL sample volumes, a high degree of hemolysis was observed with **538**. To remedy this, RBCs were resuspended in the stabilizing solution SAGM post-deglycerolization.

As shown in Figure 7.19, for 15% glycerol, 8-10% of RBCs were recovered after deglycerolization. Meanwhile, 33-38% of RBCs were recovered when **538** was added, which is similar to

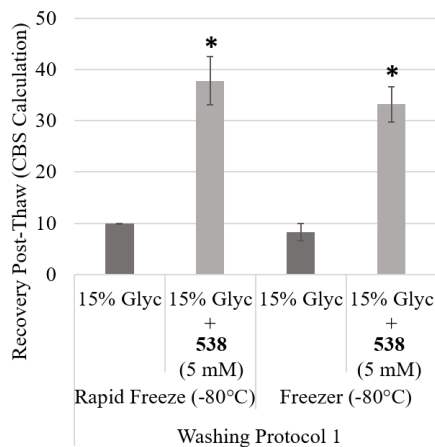


Figure 7.19. Recovery of RBCs frozen with 15% glycerol containing **538** under “Rapid Freeze -80 °C” or “Freezer -80 °C” conditions. Samples were deglycerolized using CBS washing conditions (“Washing Protocol 1”) and resuspended in SAGM. Statistical significance was assessed by unpaired Student’s T-test for comparison to the control (15% Glyc) with a 95% ($p < 0.05$, *) confidence level.

recoveries observed with 1 mL sample volumes. Therefore, there is no difference in recovery when sample volumes are increased from 1 mL to 10 mL. However, resuspension in SAGM significantly improves RBC integrity post-deglycerolization (Figure 7.20): over 98% of RBCs were intact after deglycerolization and suspension in SAGM.

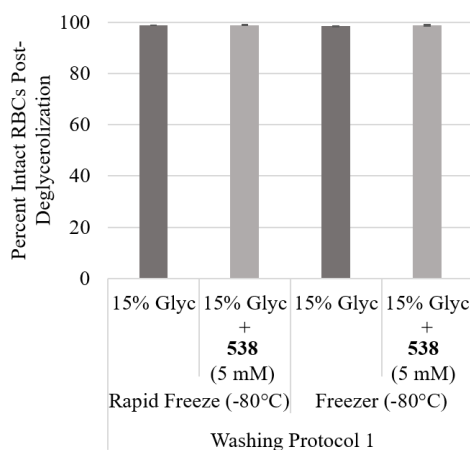


Figure 7.20. Percent intact RBCs post-deglycerolization using CBS washing conditions (Washing Protocol 1) and resuspension in SAGM. Statistical significance was assessed by unpaired Student’s T-test for comparison to the control (15% Glyc) with a 95% ($p < 0.05$, *) confidence level.

To summarize, it was hypothesized that recovery would be increased when larger sample volumes were utilized due to the facilitated removal of supernatants after washings. With 15% glycerol, RBC recovery was reduced from 20% to 10%. This could be attributed to the difference in cooling rate upon transition to larger sample volumes (Supplemental Figure S7.4). The 1 mL sample volumes possess a cooling rate of 5 °C/min for “Rapid Freeze -80 °C” conditions whereas 10 mL sample volumes cool more slowly (2 °C/min). Interestingly, there was no difference in recovery when sample volumes were increased from 1 mL to 10 mL with the 15% glycerol + **538** cryosolution even with the decrease in cooling rate. This suggests that slower cooling rates are better tolerated with the addition of **538** to the 15% glycerol cryosolution. This is significant because slower freezing rates are more desirable for clinical protocols. Subsequent to this, the ability of **538** to be effective at cryopreserving an entire unit of RBCs was investigated.

7.6 Full scale cryopreservation of RBC unit with 538

In the previous section, **538** (Figure 7.21) was found to be an effective cryoprotectant for RBCs. Furthermore, RBCs cryopreserved with **538** were more readily deglycerolized post-thaw compared to other IRIs assessed, resulting in higher recoveries post-deglycerolization. When the sample volume was increased from 1 mL to 10 mL, recovery was reduced with 15% glycerol cryosolution which was attributed to the slower cooling rate. However, recovery was not impacted when **538** was present in the cryosolution. This result suggested that slower cooling rates were tolerated with **538**. To investigate this, full units of RBCs were cryopreserved with 15% glycerol + **538** and recoveries were assessed.

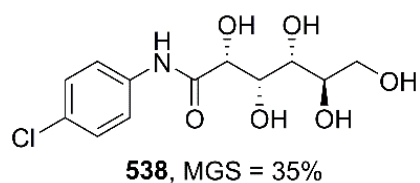


Figure 7.21. Chemical structure and IRI activity of **538**.

RBC units (270 mL) were suspended in an equal volume of cryosolution (15% glycerol + 5 mM **538**). Units were frozen using “Rapid Freeze -80 °C” or “Freezer -80 °C” condition. After thawing, units were deglycerolized using CBS deglycerolization (Washing Protocol 1) conditions. Post-deglycerolization recoveries are shown in Figure 7.22. Recoveries were 42% for “Rapid Freeze -80 °C” and 33% for “Freezer -80 °C” conditions. These recoveries are remarkably similar to the recoveries obtained with 1 mL and 10 mL sample volumes (38-43% and 33-38% recovery, respectively). The freezing rate curves for the freezing of a full RBC unit are shown in Supplemental Figure S7.5. Cooling rates were decreased when the sample volume was increased. For 1 mL, 10 mL, and RBC unit (540 mL) volumes, under “Rapid Freeze -80 °C” conditions, the cooling rates were 5, 2, and 1 °C/min respectively. However, these differences in cooling rates do not affect post-deglycerolization recoveries for RBCs cryopreserved with **538**.

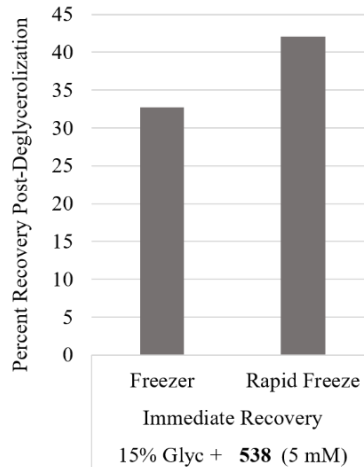


Figure 7.22. Recovery of full unit of RBCs frozen with 15% glycerol containing **538** under “Rapid Freeze -80 °C” or “Freezer -80 °C” conditions. Samples were deglycerolized using CBS washing conditions (Washing Protocol 1).

After deglycerolization, the units were split and resuspended in either 0.9% saline or SAGM. 24 hour recoveries were measured (Supplemental Figure S7.6) and were higher when RBCs were resuspended in 0.9% saline. Percent hemoglobin (%Hgb) lost during washes was measured and the results are shown in Supplemental Figure S7.7. Similar to the other sample volumes, the majority of RBC Hgb is lost immediately post-thaw. During subsequent washes, less than 10% Hgb is lost (with the exception of W1, “Freezer -80 °C” which resulted in 16% Hgb lost). RBC integrity was measured immediately after deglycerolization and 24 hours and 7 days after deglycerolization. RBC integrity is >80% for both freezing conditions and all time points (Supplemental Figure S7.8).

To summarize, it was hypothesized that larger volumes could be deglycerolized without an effect on recovery. To test this, full units of RBCs were cryopreserved with 15% glycerol + **538** and recoveries were assessed. Similar recoveries (approximately 30-40%) between volumes ranging from 1 mL to full RBC units were observed. This is significant because previously, only negligible recoveries were observed using low glycerol concentrations and slow cooling rates. Washing conditions that had been optimized for use with other IRIs were utilized in these studies. It is impressive that such high recoveries were observed even though osmotic tolerances of RBCs in the presence of **538** were not established.

However, higher recoveries are required because typical recoveries for “High Glycerol/Slow Freezing” conditions are above 80%.¹ Therefore, the osmotic tolerance of RBCs in the presence of **538** were determined to obtain the optimal washing conditions for deglycerolization of RBCs frozen with **538**.

7.7 Osmotic tolerance of RBCs after treatment with 538

In the previous experiment, RBC units cryopreserved with **538** showed 33-42% RBC recovery after deglycerolization. While this result is significant because previously, only negligible recoveries were observed for low glycerol concentrations under slow cooling conditions, higher recoveries are required. The previous experiment utilized washes for deglycerolization that had been optimized for other IRIs and not for **538**. Therefore, it was hypothesized that the recovery of RBCs cryopreserved with **538** could be improved if the washes for deglycerolization were optimized for RBCs treated with **538**.

The RBC osmotic tolerance experiments were conducted as described previously.^{45,46} RBCs were first incubated at room temperature with solutions of 40% glycerol, 15% glycerol or 15% glycerol + **538** (5 mM). As a control, RBCs suspended in SAGM was also examined in the osmotic tolerance assay as this is the isotonic solution RBCs are typically stored in. Following incubation with these solutions, samples were centrifuged, the supernatants were removed and the packed RBCs were resuspended in saline solutions ranging in concentration and the percent hemolysis obtained from resuspension in these solutions was quantified spectrophotometrically. The osmolalities of the saline solutions are provided in Table 7.3. Figure 7.23 shows the percent hemolysis observed after resuspension in the saline solutions following incubation with each cryosolution.

Immediately following incubation with the different cryosolutions, the RBCs exhibited different osmotic tolerance profiles (Figure 7.23). The profiles indicate that after incubation with 15% glycerol and 15% glycerol + **538**, the RBCs can be suspended in saline solutions ranging from 5.0-7.5% saline (50-75 g/L) corresponding to 1498-2216 mOsm/kg (Table 7.3) without causing extensive hemolysis and damage to the RBCs. Therefore, after thawing, the RBC samples should be able to be successfully washed with

Concentration (g/L)	Osmolality (mOsm/kg)	Concentration (g/L)	Osmolality (mOsm/kg)
130	No Read (NR)	35	1074
125	NR	30	929
120	NR	25	774
115	NR	20	623
110	NR	15	475
105	NR	10	317
100	NR	9.0	285
95	NR	7.5	240
90	NR	6.5	208
85	NR	6.0	193
80	2355	5.5	176
75	2216	5.0	161
70	2064	4.0	131
65	1903	3.5	114
60	1760	3.0	99
55	1628	2.0	64
50	1498	1.0	33
45	1374	dH ₂ O	
40	1235		

Table 7.3. Measured osmolalities (mOsm/kg) of NaCl solutions. Osmolalities greater than 3000 mOsm/kg were not able to be accurately read and are recorded as NR (no reading).

saline solutions at concentrations as low as 5.0% without causing extensive cell damage resulting in hemolysis of the RBCs. However, after incubation with 40% glycerol, the RBCs can only be suspended in 11.5% saline (115 g/L) without causing hemolysis. Therefore, the optimal wash 1 (W1) for 15% glycerol and 15% glycerol + **538** was determined to be 50 g/L NaCl, and for 40% glycerol was determined to be 115 g/L NaCl.

We next investigated the solution osmolalities that would be tolerated after W1. Following incubation with the cryosolutions and removal of the supernatant, the packed RBCs were resuspended in W1 solutions. The samples were centrifuged, the supernatants were removed and the packed RBCs were resuspended in saline solutions ranging in concentration and the percent hemolysis obtained from resuspension in these solutions was quantified spectrophotometrically. The osmotic tolerance profiles

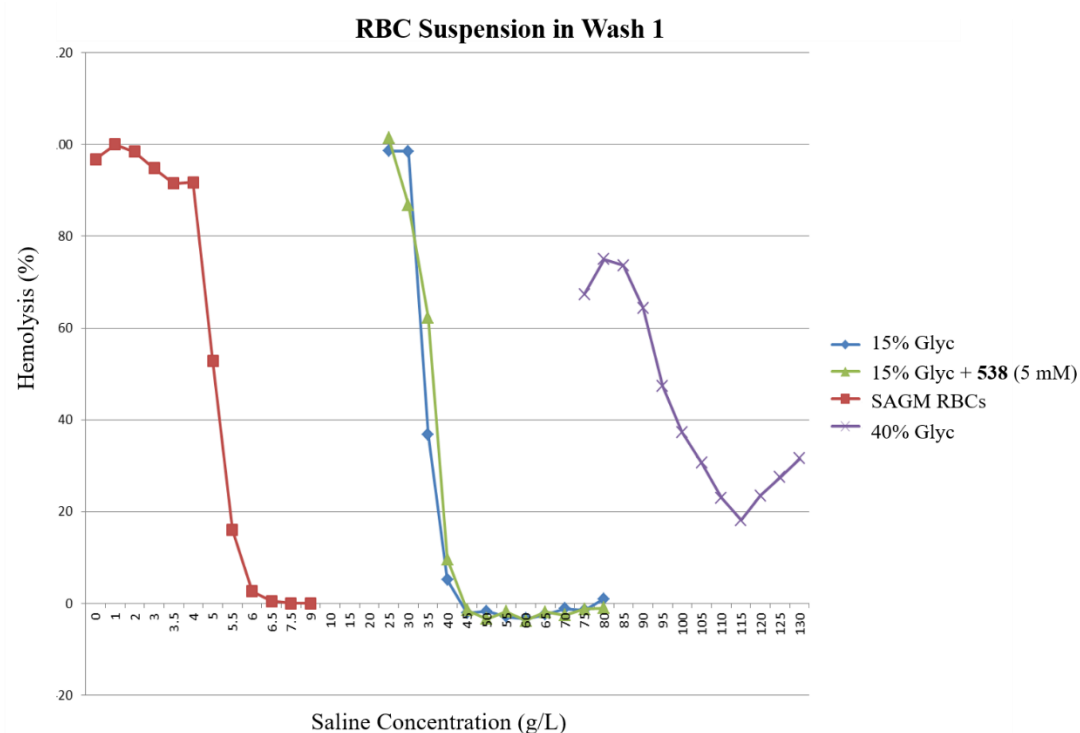


Figure 7.23. Osmotic tolerance of RBCs incubated with SAGM or cryosolutions. RBCs incubated in SAGM were resuspended in various NaCl solutions and assessed for % hemolysis to determine the NaCl solution to be utilized for W1 during deglycerolization.

following W1 are presented in Figure 7.24. 15% glycerol and 15% glycerol + **538** could be resuspended into a 15 g/L saline solution following W1 condition and minimal hemolysis was observed (Figure 7.21). With the 40% glycerol cryosolution, resuspension into 60 g/L saline was the lowest saline concentration tolerated without extensive RBC hemolysis. As shown in Figure 7.21, high levels of hemolysis were observed when the RBCs were incubated with 40% glycerol. This is because saline solutions with lower osmolality were introduced in a step-wise fashion. When thawed RBC units are deglycerolized clinically, a gradual reduction in osmolality is achieved by introducing less concentrated saline solutions continuously rather than in a step-wise fashion. Therefore, in this experiment the RBCs were exposed to more dramatic differences in osmolality causing increased hemolysis. The optimal W2 for 15% glycerol and 15% glycerol + **538** was determined to be 20 g/L NaCl and for 40% glycerol was determined to be 60 g/L NaCl. This is different than the previously used W2 solution of 15 g/L.

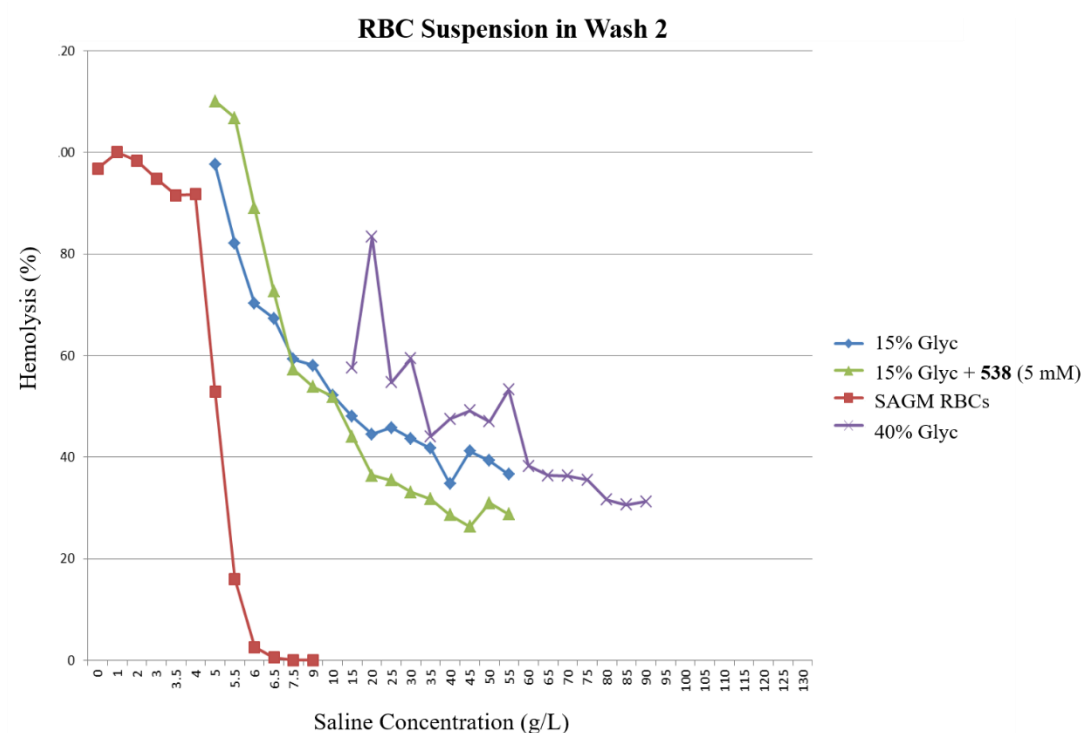


Figure 7.24. Osmotic tolerance of RBCs after wash 1. RBCs incubated in SAGM were resuspended in W1, centrifuged and resuspended in various NaCl solutions and assessed for % hemolysis to determine the NaCl solution to be utilized for W2 during deglycerolization.

We next investigated the solution osmolalities that would be tolerated after W2. RBCs were incubated with the cryosolutions, centrifuged, the supernatant removed, resuspended in W1 solution, centrifuged and after removal of the supernatant, RBCs were resuspended in W2 solution. The samples were then centrifuged again, the supernatants were removed and the packed RBCs were resuspended in saline solutions ranging in concentration and the percent hemolysis obtained from resuspension in these solutions. The osmotic tolerance profiles following W2 is presented in Figure 7.25. 15% glycerol and 15% glycerol + **538** could be resuspended into a 9 g/L saline solution and returned to isotonic conditions following W2 with minimal hemolysis observed (Figure 7.25). With the 40% glycerol cryosolution, resuspension into 35 g/L saline was the lowest saline concentration tolerated without extensive RBC hemolysis. Therefore, the optimal W3 for 15% glycerol and 15% glycerol + **538** was determined to be 9 g/L NaCl and for 40% glycerol was determined to be 35 g/L NaCl. Since the 40% glycerol cryosolution

could not be returned to isotonic conditions after three washes without causing extensive hemolysis, two additional washes were utilized. RBCs with 40% glycerol were returned to isotonic conditions with five washes of 115, 60, 35, 15 and 9 g/L saline.

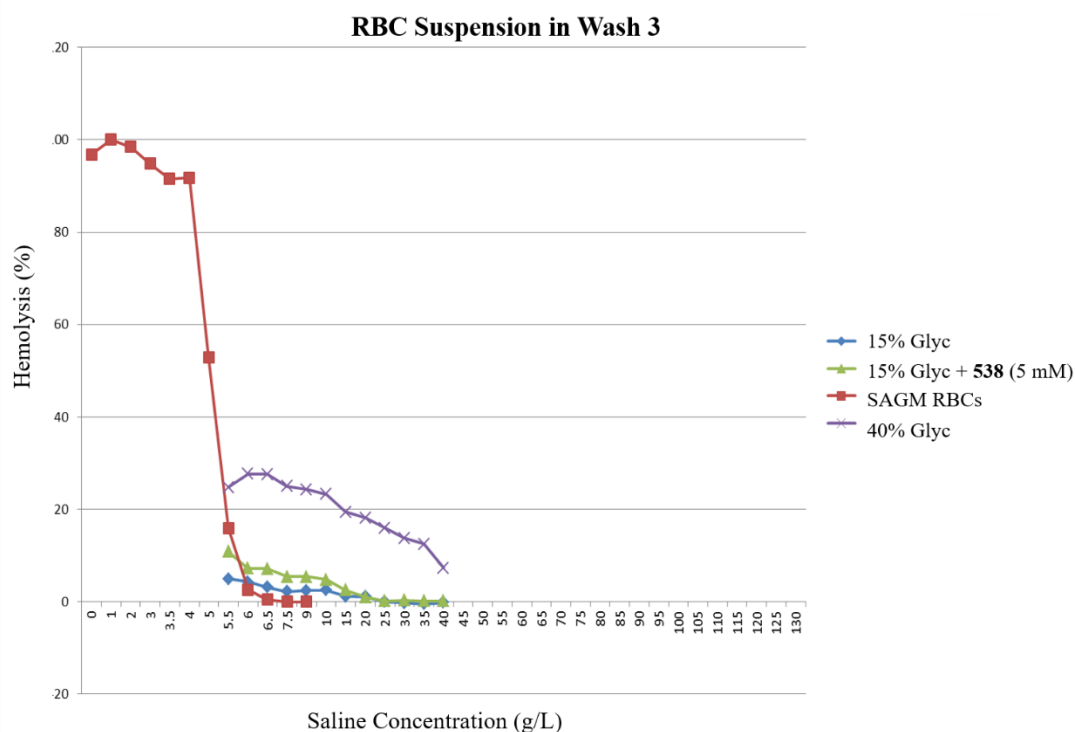


Figure 7.25. Osmotic tolerance of RBCs after wash 2. RBCs incubated in SAGM were resuspended in W1, centrifuged, resuspended in W2, centrifuged and resuspended in various NaCl solutions and assessed for % hemolysis to determine the NaCl solution to be utilized for W3 during deglycerolization.

The optimal deglycerolization washing conditions for all cryosolutions are shown in Table 7.4. It was found that the optimal washes for the deglycerolization of RBCs frozen with 15% glycerol + 538 (5 mM) possessed a different saline concentration for W2 than what was used previously. The optimal saline concentration was found to be 20 g/L rather than 15 g/L used previously. Therefore, the recovery after deglycerolization using this new W2 was determined. This new washing procedure was referred to as “Washing Protocol 4”. RBCs suspended in cryosolution were frozen using “Rapid Freeze -80 °C” and “Freezer -80 °C” conditions. After thawing, samples were deglycerolized using Washing Protocol 1

	15% Glyc	15% Glyc + 538 (5 mM)	40% Glyc
W1	50 g/L	50 g/L	115 g/L
W2	20 g/L	20 g/L	60 g/L
W3	9 g/L	9 g/L	35 g/L
W4			15 g/L
W5			9 g/L

Table 7.4. Optimal deglycerolization washing conditions as determined by osmotic tolerance experiments.

(Table 7.2) and Washing Protocol 4 (new W2, Table 7.4) conditions and resuspended in SAGM.

Recovery was determined and the results are shown in Figure 7.26. There was no difference in recovery using Washing Protocol 4 washing conditions ($p < 0.05$ as determined by one-way ANOVA with Tukey post-test for multiple comparisons). Furthermore, there was no difference in %Hgb lost between any washes, including W2 between Washing Protocol 1 and the Washing Protocol 4 conditions (Supplemental Figure S7.9). After deglycerolization, RBCs were suspended in SAGM and RBC integrity was above 97% for all conditions (Supplemental Figure S7.10). Thus, the modification of W2 from 15 g/L to 20 g/L did not significantly influence recovery and both washing conditions (Washing Protocol 1 and Washing Protocol 4) were sufficient for deglycerolization of RBCs cryopreserved with 15% glycerol + **538**.

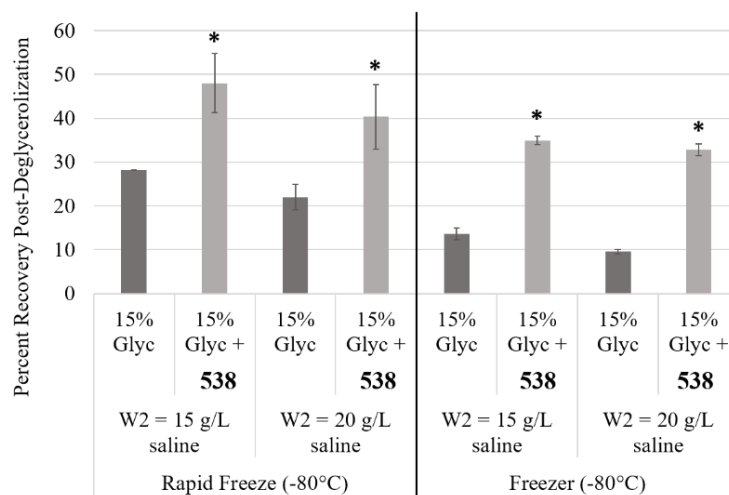


Figure 7.26. Recovery of RBCs frozen with 15% glycerol + **538** using Washing Protocol 1 and Washing Protocol 4 conditions. Statistical significance was assessed by unpaired Student's T-test for comparison to the control (no compound) with a 95% ($p < 0.05$, *) confidence level.

Since the optimal washing solutions were found to be the same as those previously used, it does not appear that recoveries can be increased by modification to the deglycerolization procedure. It was hypothesized that recovery could be increased by mixing of wash solutions during deglycerolization. It was thought that this would provide a more gradual step down of saline concentrations and therefore result in increased recoveries. This was performed as follows: upon thawing of the RBCs, W1 was added to the RBCs and allowed to incubate for five minutes. Next, W2 was added and mixed thoroughly. The RBCs were centrifuged, supernatants removed and RBCs resuspended in W2, followed by addition of W3. The RBCs would again be centrifuged and RBCs resuspended in SAGM. To assess if this different deglycerolization procedure would improve RBC recoveries, full RBC units were frozen with 40% glycerol, 15% glycerol and 15% glycerol + **538** and deglycerolized using these deglycerolization conditions. Post-deglycerolization functional assays were performed on the RBCs to determine the quality of the RBCs after cryopreservation and subsequent deglycerolization using 15% glycerol with IRI **538** (5 mM).

RBC units suspended in cryosolution were frozen using “Freezer -80 °C” conditions which are currently used in the RBC banks for cryopreservation of RBCs using 40% glycerol.¹ Recoveries were determined after deglycerolization and are shown in Figure 7.27. For the full unit, cryopreservation with 40% glycerol resulted in 89% recovery after deglycerolization. This is similar to what is typically observed for recoveries of RBCs cryopreserved with 40% glycerol. Recovery was reduced to 32% and 24% recovery with 15% glycerol and 15% glycerol + **538** respectively. RBC integrity was greater than 97% after deglycerolization and resuspension in SAGM for all cryosolutions. The the unit frozen with 15% glycerol + **538**, the recovery was reduced from 33% (observed for the previous RBC unit frozen with 15% glycerol + **538** in Section 7.6) to 24% resulting from this trial. This could be a result of the new mixing procedure utilized in the deglycerolization process. Furthermore, the large volumes acquired upon mixing washing solutions for deglycerolization required the splitting of thawed units into two separate bags. Thus, several transfers between different collection bags were performed and could have resulted in

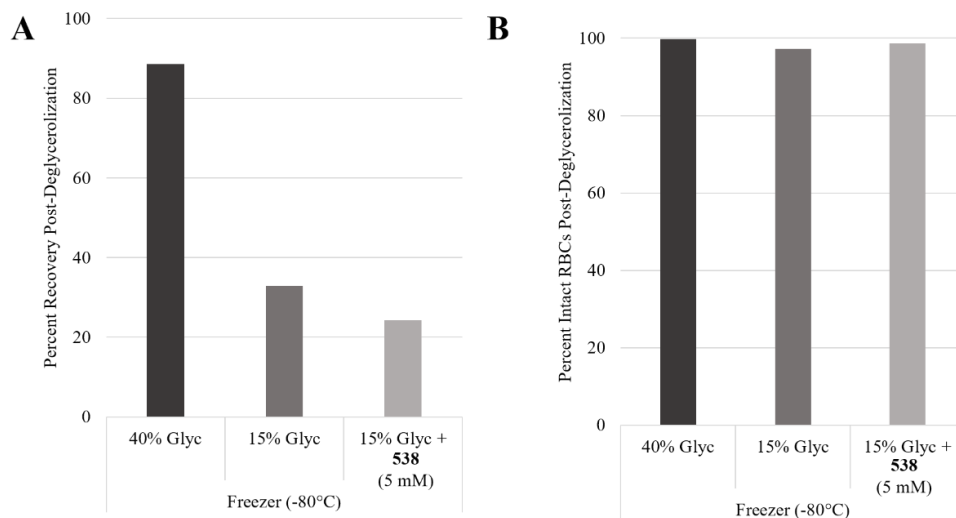


Figure 7.27. (A) Recovery of RBCs using Washing Protocol 1 conditions; (B) intact RBCs after deglycerolization.

a decrease in recovery. Another possible explanation could be that exposing the thawed RBCs to a mixture of both W1 and W2 resulted in increased hemolysis due to a larger difference in osmolality. However, upon examination of the percent hemoglobin (%Hgb) lost at each washing step (Figure 7.28), there does not appear to be larger than usual %Hgb lost using the new washing procedure (W1 = 50 g/L + 15 g/L saline and W2 = 15 g/L + 9 g/L saline).

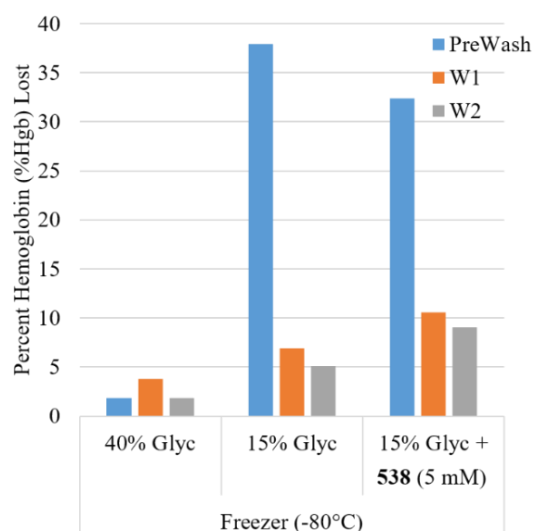


Figure 7.28. Percent hemoglobin (%Hgb) lost (relative to RBC Hgb prior to freezing) during deglycerolization of RBC units.

Next, the refractive index (RI) of the supernatant from the deglycerolized RBC units was measured. In the clinic, thawed RBC units must be deglycerolized to less than 1% glycerol prior to transfusion. The RI of the RBC supernatant is indicative of the residual glycerol. If the RI is less than 40, this indicates that there is less than 1% glycerol remaining. The RIs of the supernatants after deglycerolization are shown in Table 7.5. All are less than 40 indicating that the deglycerolization procedure was successful at removing the necessary amounts of glycerol required for transfusion.

Cryosolution	Immediate Refractive Index	24 Hour Refractive Index
40% Glyc	32	30
15% Glyc	33	32
15% Glyc + 538 (5 mM)	33	33

Table 7.5. Refractive index (RI) of supernatants from deglycerolized RBC units frozen with 40% glycerol, 15% glycerol and 15% glycerol + **538** (5 mM).

7.7.1 RBC functional analysis after cryopreservation with 538

During storage, RBCs experience storage lesions. Storage lesions that occur during hypothermic storage include morphological changes, slowed metabolism, acidosis, loss of function in ion channels, apoptotic changes, and membrane vesiculation.^{2,9,10} These storage lesions compromise the safety and efficacy of the blood product and have been correlated to a reduced oxygen carrying capacity and increased toxicity.^{1,3,6,7,11} Several of these unfavorable effects of storage can be measured including adenosine triphosphate (ATP) levels, methemoglobin (MetHgb) levels, 2,3-diphosphoglycerate (2,3-DPG) deficiency, p50 levels, and an increased rigidity of the red cells.⁵⁵⁻⁵⁹ These RBC qualities were measured for the RBC units cryopreserved with 15% glycerol + **538** (5 mM) and compared to RBC units cryopreserved with 40% glycerol and 15% glycerol.

The levels of ATP in the deglycerolized units were measured 24 hour after deglycerolization and storage at 4 °C in SAGM. This measurement has become a routine approach for evaluating the storage lesions in RBCs.⁵⁵ A close association has been established between the ATP content of stored red cells and their viability.⁵⁵ When ATP levels drop below a certain level, RBCs lose their capacity to

phosphorylate glucose and therefore to obtain the energy required for survival.⁵⁵ RBCs stored hypothermically for 1 day possess ATP levels of $3.5 (\pm 0.4) \mu\text{mol/g Hgb}$.⁵⁵ The levels of ATP for RBC units frozen with 40% glycerol, 15% glycerol and 15% glycerol + **538** (5 mM) are shown in Figure 7.29. RBC units frozen with 40% glycerol possess normal ATP levels after deglycerolization ($3.4 \mu\text{mol/g Hgb}$). However, below normal ATP levels are experienced when RBCs are frozen with 15% glycerol ($2.7 \mu\text{mol/g Hgb}$). Interestingly, the addition of **538** (5 mM) to the 15% glycerol cryosolution prevented this reduction in ATP concentration and possessed normal ATP levels ($3.6 \mu\text{mol/g Hgb}$).

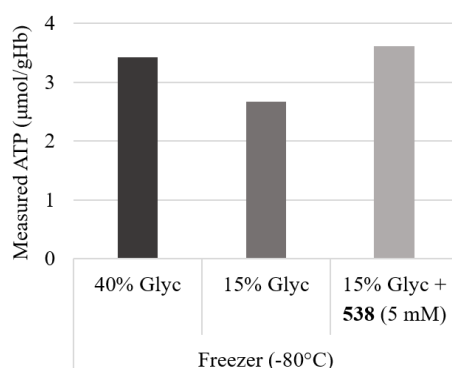


Figure 7.29. Post-deglycerolization ATP levels for RBCs frozen with 40% glycerol, 15% glycerol and 15% glycerol + **538** (5 mM).

Several other RBC properties were measured at 24 hours post-deglycerolization and these measurements are listed in Table 7.6. RBC integrity 24 hours after deglycerolization was above 96% for all cryosolutions. The percentage of methemoglobin (MetHgb) was also measured. During the life span of RBCs, exposure to multiple oxidizing agents results in the oxidation of Hgb to MetHgb.⁵⁶ However, RBCs are capable of reducing MetHgb back to Hgb through the use of reducing enzymes.⁵⁶ Efficient human RBCs accumulate less than 0.6% MetHgb and therefore the evaluation of the methemoglobin concentration is an important marker of biological processes of oxidative damage.⁵⁶ The percentage of MetHgb 24 hours post-deglycerolization for all cryosolutions is shown in Table 7.6. RBCs frozen with 40% glycerol possess 0.3% MetHgb 24 hours post-deglycerolization. However, RBCs frozen with 15% glycerol possess significantly higher MetHgb levels (12.5%) indicative of oxidative damage.

Interestingly, RBCs frozen with 15% glycerol supplemented with **538** (5 mM) resulted in normal MetHgb levels (0.6%) 24 hours after deglycerolization. This could suggest that ice recrystallization is inducing pathways resulting in oxidative damage to RBCs and that the addition of IRI **538** prevents this oxidative damage.

Mean cell volumes (MCVs) were also measured (Table 7.6). Normal RBC MCV is 80-100 fL.⁶⁰ MCVs < 80 fL or > 100 fL are indicative of either (1) RBC shrinking or swelling as a result of osmotic imbalances; or (2) abnormal RBC abnormal morphologies.⁶⁰ RBCs frozen with 40% glycerol return to a normal MCV after deglycerolization whereas both 15% glycerol and 15% glycerol + **538** (5 mM) have larger than normal MCVs. 15% glycerol has a significantly higher than normal MCV. RI measurements described earlier indicate that less than 1% glycerol remains after deglycerolization. Therefore, the RBCs could be swollen for an alternative reason. Measurement of ATP levels indicate that RBCs frozen with 15% glycerol suffer a reduction in ATP levels after deglycerolization (Figure 7.29). ATP is required for the active transport of sodium and potassium ions across the cell membrane.² Na⁺/K⁺-ATPase is responsible for transporting three sodium ions out of the cell and two potassium ions into the cell per ATP molecule.² Thus, the reduction in ATP levels could reduce the activity of this enzyme resulting in an accumulation of sodium ions inside the cell. In turn, this would cause water to flow osmotically inside the cell and thus result in larger than normal MCVs. Alternatively, RBCs could be experiencing morphological changes after cryopreservation with 15% glycerol and 15% glycerol + **538** (5 mM) resulting in larger than normal MCVs.

The release of oxygen from hemoglobin in RBCs is controlled by 2,3-diphosphoglycerate (2,3-DPG).⁶¹ 2,3-DPG binds to deoxygenated hemoglobin with greater affinity compared to oxygenated hemoglobin and decreases the affinity of hemoglobin for oxygen.⁶¹ As such, 2,3-DPG allosterically promotes the release of oxygen from hemoglobin and enhances the ability of RBCs to release oxygen into tissues.⁶¹ However, during storage 2,3-DPG levels are reduced.^{55,61} As a result, the ability of RBCs to efficiently oxygenate tissues is diminished.⁶¹ Reduced 2,3-DPG levels cause an increase in the affinity of

hemoglobin for oxygen and this lowers the p50.⁶¹ The p50 is the partial pressure of oxygen (PO₂) at which Hgb is 50% saturated.⁶¹ Therefore, lower p50 values correspond to a decreased ability of RBCs to release oxygen and deliver it to where it is needed.⁶¹ The p50 pressures were evaluated 24 hours post-deglycerolization and are shown in Table 7.6. For comparison, p50 pressures were measured for untreated and unfrozen RBCs suspended in SAGM. RBCs frozen with 40% glycerol had p50 pressures of 17.3 mmHg, similar to untreated RBCs (18.1 mmHg). In contrast, RBCs frozen with 15% glycerol resulted in a reduced p50 pressure of 13.7 mmHg indicating that these RBCs have a reduced capacity to deliver oxygen. Interestingly, when **538** is added to the 15% glycerol cryosolution, the p50 pressure is near-normal (16.4 mmHg) and therefore these RBCs can carry and deliver oxygen better than RBCs cryopreserved with 15% glycerol alone.

RBC deformability was measured by ektacytometry using the laser-assisted optical rotation cell analyzer (LORCA).^{55,58} Over the course of RBC storage, RBCs become less deformable and more rigid. This is thought to be a result of decreased ATP levels.^{55,58,59} Decreased RBC deformability has been correlated to impaired microvascular oxygenation following transfusion.⁵⁷ Deformability is measured by subjecting the RBCs to different levels of shear stress by rotation at different speeds causing the RBCs to elongate.⁵⁹ By shining a laser beam through the RBC suspension, the shape and diffraction pattern of the refracted laser beam can be used to calculate the EI_{MAX} and K_{EI}.⁵⁹ The EI_{MAX} is the maximum theoretical elongation index and the K_{EI} is the shear stress required to obtain half of the EI_{MAX}.⁵⁹ A high EI_{MAX} indicates the RBCs are very deformable whereas a high K_{EI} indicates the RBCs are very rigid and thus require a high degree of shear stress to reach half of the EI_{MAX}.⁵⁹ EI_{MAX} and K_{EI} values 24 hours post-deglycerolization are shown in Table 7.6. Normal EI_{MAX} and K_{EI} values are 0.56-0.58 and 2.40-2.94 respectively.⁵⁹ Table 7.6 shows that RBCs frozen with 40% glycerol possess normal deformability values after deglycerolization. However, a significant decrease in deformability is observed when RBCs are frozen with 15% glycerol (K_{EI} = 0.77). This decrease is partially remedied by the addition of **538** to the 15% glycerol cryosolution (K_{EI} = 1.28) suggesting that these RBCs will have improved microvascular oxygenation following transfusion.

This result is consistent with p50 values indicating that RBCs frozen with 15% glycerol + **538** (5 mM) are better at carrying and delivering oxygen than RBCs frozen with 15% glycerol alone.

	% Intact	% MetHgb	MCV (fL)	p50 (mmHg)	EI _{MAX}	K _{EI}
Unfrozen				18.1		
40% Glyc	99.7	0.3	96.7	17.3	0.6	2.1
15% Glyc	96.1	12.5	138.8	13.7	0.4	0.8
15% Glyc + 538 (5 mM)	97.5	0.6	124.8	16.4	0.5	1.3

Table 7.6. RBC quality measurements 24 hours after deglycerolization.

Microparticles (MPs) are membrane vesicles (0.15 μ m) which are released by RBCs in response to stress.⁶² The release of MPs is triggered by different stimuli including pro-apoptotic stimulation, shear stress, or damage.^{62,63} While MP release is a part of the normal RBC aging process, elevated MP levels are associated with various pathological conditions including cardiovascular disease and sickle cell anemia.^{62,63} Furthermore, MPs are released during storage of RBCs and are considered a type of hypothermic storage lesion.^{2,62,63} It was recently found that MPs released during RBC storage are progressively enriched with oxidized proteins.⁶² During storage, oxidative stress results in the formation of reactive oxygen species (ROS) which react with and damage proteins and lipids.^{2,62} MP formation is a way to eliminate these proteins and lipids that have been altered by oxidative stress.^{2,62}

MP formation was measured on RBC units frozen with 40% glycerol, 15% glycerol and 15% glycerol + **538** (5 mM) 24 hours post-deglycerolization. MPs were measured using flow cytometry. RBC samples were stained with CD325a-fluorescein isothiocyanate (CD325a-FITC) and Annexin V-allophycocyanin (Annexin V-APC). CD325a (Glycophorin A) is a cell membrane sialoglycoprotein present on erythrocytes and was used to identify the erythrocyte population in the sample.⁶⁴ Annexin V is used to detect cells which are expressing phosphatidylserine (PS) on the extracellular membrane.⁶⁵ PS is present on the intracellular membrane in healthy cells and upon apoptotic activation, PS becomes expressed on the extracellular membrane.⁶⁵ Therefore, cells which express PS are considered apoptotic.⁶⁵ The flow cytometry results are presented in Figure 7.30. Figure 7.30A depicts the percent composition of

the RBC samples frozen with different cryosolutions after deglycerolization. Percent composition was calculated by taking the ratio of the number of RBCs, RBC MPs or RBC ghosts compared to the total number of events. RBC ghosts are RBCs which possess intact membranes but which are devoid of all internal contents.^{66,67} Most notably, RBC ghosts lack hemoglobin and therefore appear as white or colourless RBCs.^{66,67} Because RBC ghosts do not possess hemoglobin, RBC ghosts are not functioning RBCs.⁶⁶ The three populations (RBCs, RBC MPs & RBC ghosts) were distinguished by their size and morphology (Supplemental Figure S7.11). The RBC unit which was frozen with 40% glycerol is comprised of 94% RBCs, 2% MPs and 4% ghosts. The RBC unit which was frozen with 15% glycerol is comprised of much higher levels of RBC MPs and ghosts (29% and 68% respectively) and only 3% RBCs. When the 15% glycerol cryosolution is supplemented with **538** (5 mM), a greater proportion of the sample is RBCs (39%) and less is MPs and ghosts (12% and 49% respectively) compared to 15% glycerol alone. This suggests that the addition of **538** is preventing the high degree of MP and ghost formation observed when the RBCs are cryopreserved with 15% glycerol alone. In other words, storage lesions resulting in MP formation occur to a lesser extent in the presence of **538**. This could suggest that during cryopreservation, ice recrystallization injury causes MP and ghost formation and that this process is

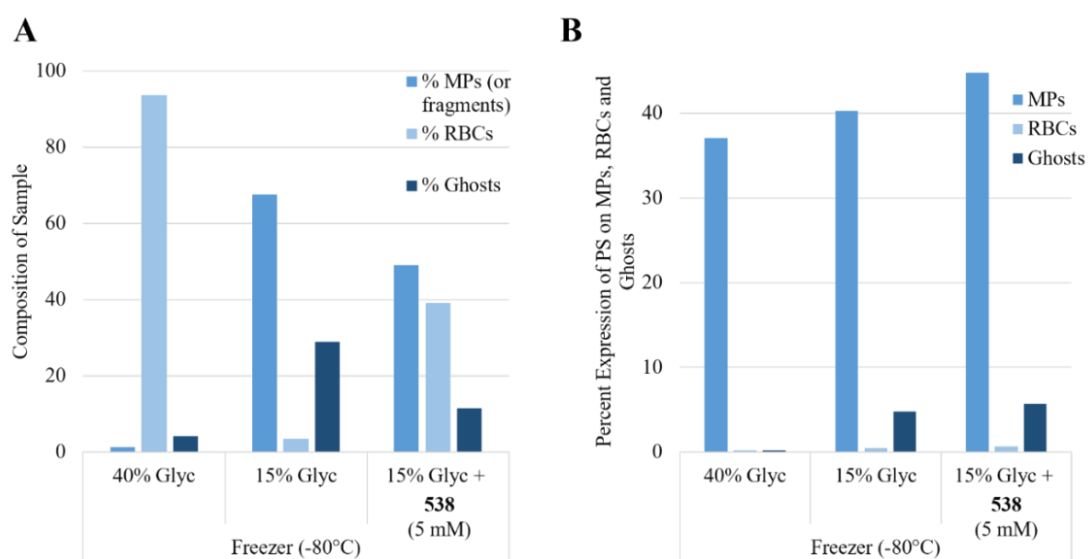


Figure 7.30. (A) Percent composition of RBC samples post-thaw and; (B) Expression of PS on RBC populations.

inhibited by the addition of an IRI to the cryosolution. Ice recrystallization could be initiating apoptosis and as a result, MP formation occurs. Previously, the Ben lab demonstrated a reduction in apoptotic CD34⁺ cells from cord blood with samples cryopreserved with IRI active carbohydrates.³⁶ The expression of PS on different RBC populations is shown in Figure 7.30B. Upon apoptosis activation, PS becomes exposed on the outer surface of the cell, causing membrane blebbing and the release of MPs.⁶⁸ Therefore, PS expression is highest on MPs (Figure 7.30B). Less than 10% of RBCs and RBC ghosts express PS.

To summarize this section, RBC units were cryopreserved with 40% glycerol, 15% glycerol and 15% glycerol + **538** (5 mM). Current clinical protocols for the cryopreservation of RBC units were utilized which include slow freezing by placement of the unit directly into a -80 °C freezer and thawing quickly in a 37 °C water bath. Upon thawing, the units were deglycerolized using saline solutions with different concentrations that were optimized to limit RBC hemolysis. In order to provide a more gradual step down in osmolality, thawed RBCs were diluted with W1, allowed to incubate for five minutes followed by addition of W2. The units were then centrifuged, supernatant removed and RBCs were resuspended in W2 followed by addition of W3. The units were centrifuged again, the supernatant was removed, and the RBCs were resuspended in SAGM. Removal of glycerol to < 1% was confirmed by measurement of supernatant RI. RBC recovery was determined to be > 80% for RBCs frozen with 40% glycerol which is what is observed clinically. Recovery was reduced to 32% and 24% for RBCs frozen with 15% glycerol and 15% glycerol + **538** (5 mM) respectively.

Following deglycerolization, several functional assays were performed. Hemolysis was less than 3% even after 48 hours of storage at 4 °C post-deglycerolization for all cryosolutions. The RBC unit frozen with 40% glycerol possessed normal ATP and MetHgb levels, had a regular p50 pressure, and normal deformability and composition of RBC populations. On the other hand, the RBC unit frozen with 15% glycerol possessed a lower than usual ATP concentration, a high MetHgb level, a reduced p50 pressure, a significant decrease in deformability and a skewed composition of RBC populations with the majority of the sample being comprised of RBC MPs and ghosts. These results indicate that while 33% of RBCs are

recovered, the RBCs are functionally impaired. When the 15% glycerol cryosolution was supplemented with **538**, nearly all functional assay results returned to normal (or near-normal) levels indicating that **538** is capable of protecting the RBCs against the storage lesion and cryoinjury experienced by RBCs cryopreserved with 15% glycerol alone.

7.8 Hypothermic storage

We next wanted to assess any toxic effects of our small-molecule IRIs in RBCs. RBCs suspended in solutions containing IRIs were assessed for hemolysis and functional changes after incubation for 24 and 48 hours. RBCs incubated with saline/dextrose (SD) (0.9%/0.2%) were used as a control. RBC integrity after incubation with **166** (110 mM), **171** (30 mM), **173** (20 mM) and **538** (5 mM) is shown in Figure 7.31. Even after 48 hours, less than 3% hemolysis was observed, indicating that these IRIs are not causing extensive hemolysis upon incubation with RBCs.

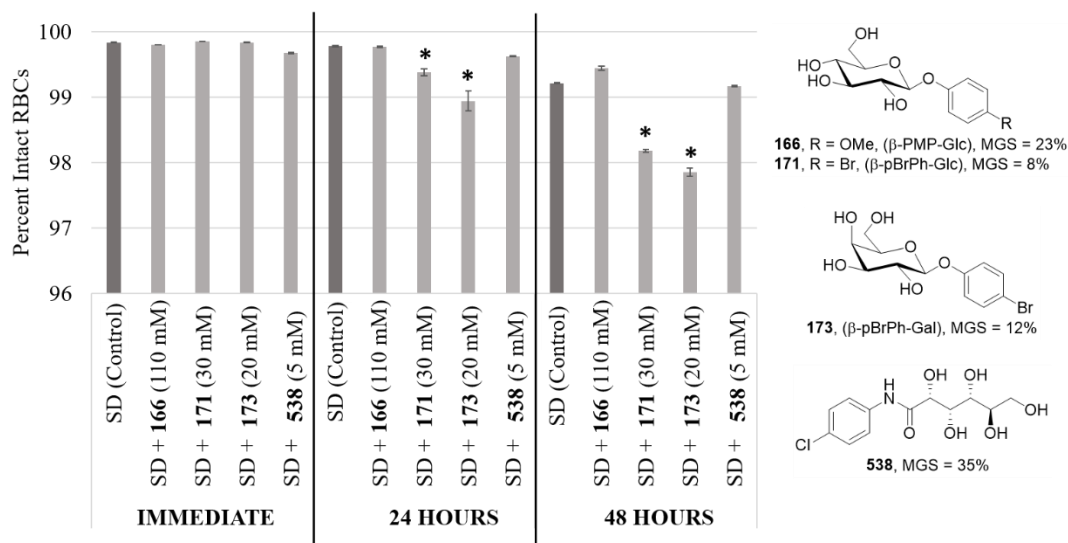


Figure 7.31. RBC integrity after incubation with IRIs for up to 48 hours. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons with a 95% ($p < 0.05$) confidence level. Asterisks indicate significant difference ($p < 0.05$) compared to the control (saline/dextrose, SD).

Mean cell volumes (MCVs) were also measured after incubation of RBCs with IRIs (Figure 7.32). Normal MCV ranges from 80-100 fL. RBCs incubated in SD with **538** possessed normal MCVs for

all time points assessed. Incubation with **166**, **171** and **173** resulted in larger than normal RBC MCVs. This could suggest one of two things: (1) that these molecules are causing morphological changes resulting in increased MCVs; or (2) that these molecules are being internalized and thus increasing the solute concentration intracellularly causing the RBCs to swell. Interestingly, **166**, **171** and **173** are all from the aryl glycoside class of small-molecule IRIs indicating that perhaps molecules with this same generic structure are internalized into RBCs whereas *N*-aryl-aldonamides are not. It is also interesting to note that the MCV of RBCs in the presence of **166** increases over time. This might indicate that internalization occurs gradually over time and **166** accumulates intracellularly. It is important to note that internalization cannot be determined by MCV alone and therefore the internalization of these molecules was investigated further and is discussed in Section 7.10.

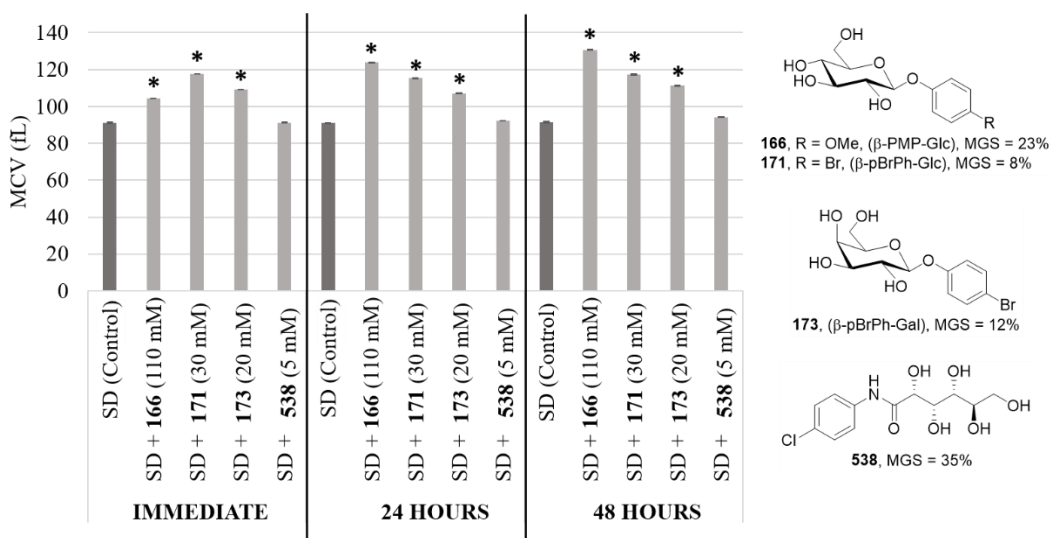


Figure 7.32. RBC MCV after incubation with IRIs for up to 48 hours. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons with a 95% ($p < 0.05$) confidence level. Asterisks indicate significant difference ($p < 0.05$) compared to the control (saline/dextrose, SD).

The percentage of methemoglobin (MetHgb) was also measured (Figure 7.33). As described in the previous section, during the life span of RBCs, exposure to multiple oxidizing agents results in the oxidation of Hgb to MetHgb.⁵⁶ However, RBCs are capable of reducing MetHgb back to Hgb through the use of reducing enzymes.⁵⁶ The evaluation of the MetHgb concentration is an important marker of

biological processes of oxidative damage.⁵⁶ RBCs incubated in SD have MetHgb percentages ranging from 2-4%. MetHgb is increased when RBCs are incubated with **166**, **172** and **538**. This is interesting because it was previously found that **538** prevents increases in MetHgb during cryopreservation when used to supplement the 15% glycerol cryosolution (Section 7.7). However, in the previous experiment, MetHgb was measured after deglycerolization when the RBCs had been washed several times. Therefore, we assessed whether MetHgb percentages or RBC MCVs would return to normal after washing. RBCs were incubated with RBCs for 24 hours before being centrifuged and resuspended in SD prior to MetHgb and RBC MCV measurements. The results are shown in Figure 7.34. After incubation with IRIs **166**, **171** and **173** for 24 hours, MCV were increased to above normal levels. After washing, the RBCs return to normal (or near-normal) MCVs indicating that RBCs can be returned to their normal size by simply washing. MetHgb was also measured after 24 hours of incubation and subsequent washing. MetHgb levels are increased the most with addition of **173** and **538**. After washing, the MetHgb levels are

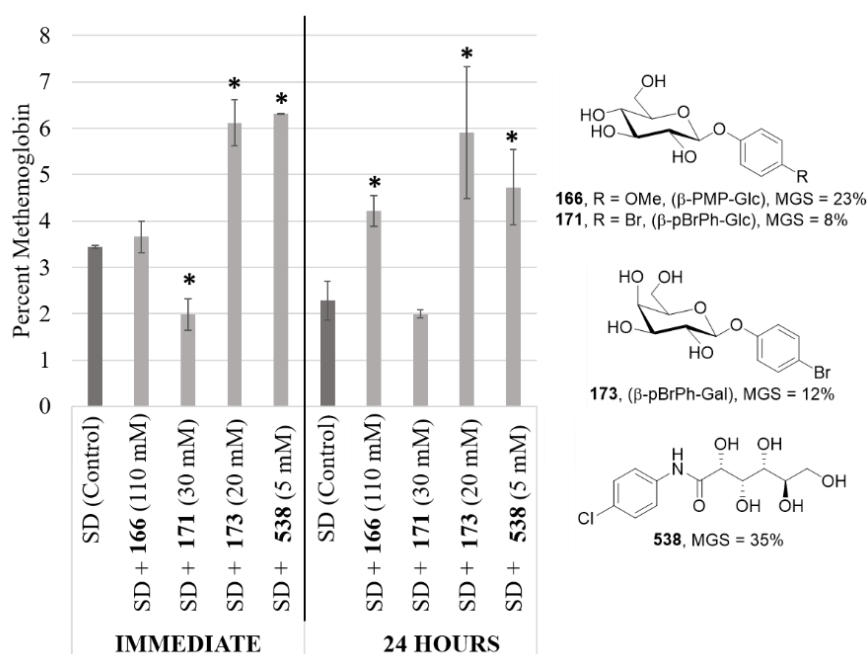


Figure 7.33. RBC %MetHgb after incubation with IRIs for up to 24 hours. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons with a 95% ($p < 0.05$) confidence level. Asterisks indicate significant difference ($p < 0.05$) compared to the control (saline/dextrose, SD).

reduced to normal levels with **538** which is consistent with previous results (Section 7.7). However, washing increased the MetHgb percentage of RBCs incubated with **173**.

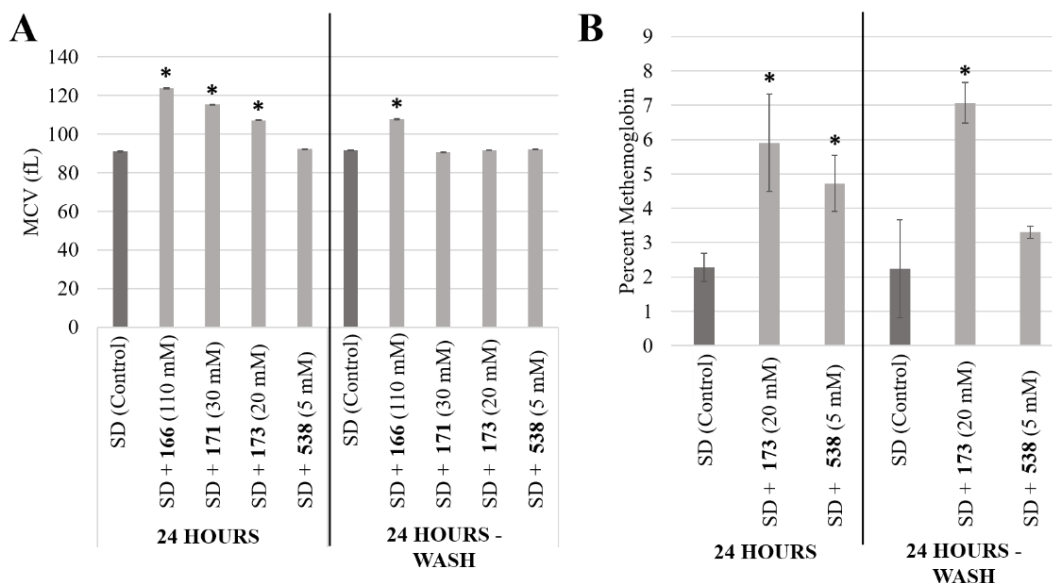


Figure 7.34. (A) RBC MCV after incubation with IRIs and subsequent washing; (B) RBC %MetHgb after incubation with IRIs and subsequent washing. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Tukey post-test for multiple comparisons with a 95% ($p < 0.05$) confidence level. Asterisks indicate significant difference ($p < 0.05$) compared to the control (saline/dextrose, SD).

Finally, flow cytometry analysis was performed on RBC samples incubated with IRIs for 48 hours to measure the degree of MP formation. MP formation and PS expression was measured by flow cytometry using the gating strategy depicted in Supplemental Figure S7.11. As described in the previous section, MPs are released by RBCs in response to stress.⁶² The release of MPs is triggered by different stimuli including pro-apoptotic stimulation, shear stress, or damage.^{62,63} MPs released during storage of RBCs is considered a type of hypothermic storage lesion.^{2,62,63} The percentage of RBC samples which are comprised of MPs after incubation with IRIs is shown in Figure 7.35A. The expression levels of PS on RBCs and MPs is shown in Figure 7.35B. Figure 7.35A shows increased MP formation when RBCs are incubated with **171** and **173**. This could be indicating that **171** and **173** are interacting with and disrupting

the cell membrane and causing MP formation. Alternatively, **171** and **173** could be causing RBCs to undergo apoptosis and thus release MPs in response. This is consistent with the results shown in Figure 7.35B which demonstrate larger levels of PS expression on RBC populations of RBC samples incubated with **171** and **173** compared to the SD control.

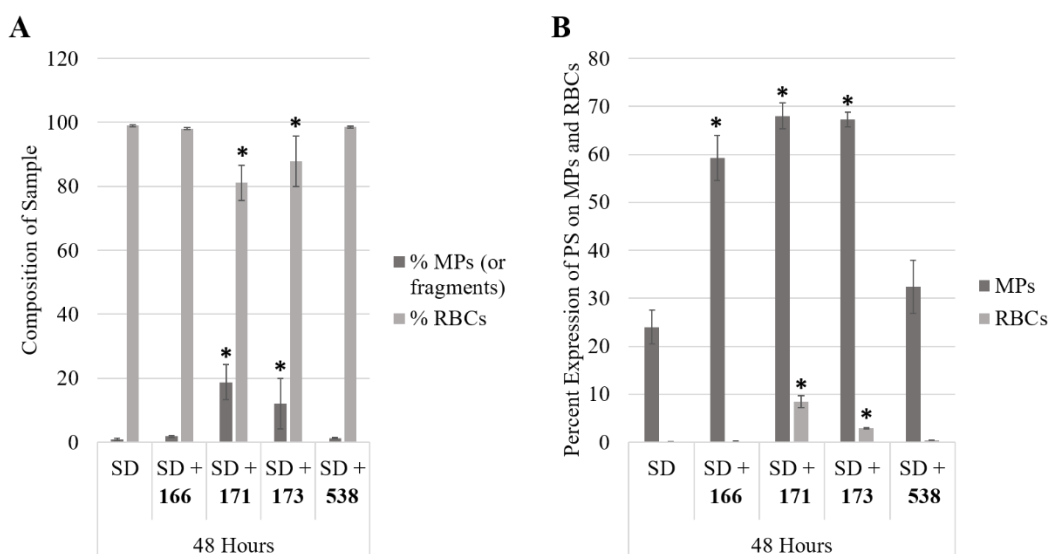


Figure 7.35. (A) Percent composition of RBC samples after 48 hour incubation with IRIs and; (B) Expression of PS on RBC populations. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Dunnett's post-test for comparison to the control (saline/dextrose, SD) with a 95% ($p < 0.05$, *) confidence level.

To summarize this section, hypothermic storage studies indicate little to no toxicity is associated with incubation of RBCs with IRIs. Less than 3% hemolysis is observed even after 48 hours of incubation with IRIs. Aryl glycosides **166**, **171** and **173** resulted in increased RBC MCV after incubation, which could be attributed to internalization. This potential internalization is investigated further in Section 7.10. MCV can be returned to normal after centrifugation and resuspension in SD. MetHgb levels were increased after incubation with IRIs **173** and **538** indicating some oxidative damage is occurring. However, with **538**, this can be remedied by centrifugation and resuspension in SD. Finally, increased MP formation is observed with **171** and **173** which was attributed to apoptosis activation confirmed by the increased levels of PS on the RBC population in RBCs incubated with **171** and **173**.

7.9 IRIs preserve human RBC integrity during transient warming events

In Section 7.4 it was determined that the addition of small-molecule IRIs increased post-thaw RBC integrity and allowed for a reduction in the amount of glycerol. Therefore, we hypothesized that ice crystals in frozen RBC samples should be noticeably smaller in size in the presence of an IRI. To investigate this, RBCs were frozen using a Linkam Cryostage and the ice was imaged in the presence of RBCs. The results from these experiments were recently published in *Scientific Reports* entitled “Small molecule ice recrystallization inhibitors mitigate red blood cell lysis during freezing, transient warming and thawing”.⁴⁸ RBCs suspended in 15% glycerol or 15% glycerol + **171** (30 mM) were cooled to -40 °C (25 °C/min). Initially, **171** was chosen because of its high IRI activity (8% MGS). The samples were then warmed to -10 °C (10 °C/min) and held for 10 minutes. Images of ice crystal morphologies are shown in Figure 7.36. It is clear in Figure 7.36 that the percentage of ice stays constant even in the presence of RBCs indicating that the presence of RBCs does not influence the amount of ice in the frozen sample. However, less ice is observed in samples (with and without RBCs) when **171** (30 mM) is present compared to the 15% glycerol control. The percentage of frozen fraction is small in these experiments because -10 °C is close to the colligative freezing point depression of the 15% glycerol solution (-4 °C) resulting in a large fraction being unfrozen.

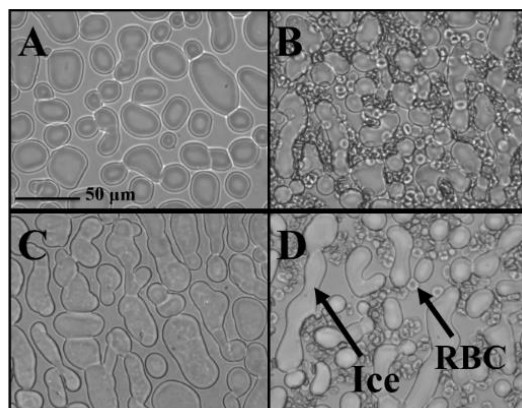


Figure 7.36. Images of ice in presence of A) 15% glycerol, B) 15% glycerol + RBCs, C) 15% glycerol + 30 mM **171** and D) 15% glycerol + 30 mM **171** + RBCs.

The IRI activity of **171** was assessed in conditions with high amounts of ice present. Therefore, the experiment was repeated at a lower holding temperature to ensure higher ice volume. Figure 7.37 shows images of ice crystal size when RBCs were cooled to -40 °C (25 °C/min) and then warmed to -20 °C and held for 10 minutes at this temperature. A domain recognition software⁶⁹ was used to calculate the percentage of ice and average size of ice crystals in the frozen samples. In the presence of IRIs **171** and **538**, the percentage of ice is reduced to 86% and 70% respectively compared to 92% in the absence of any IRI. Furthermore, the ice crystals were determined to be four times smaller in the presence of **171** (30 mM) than with 15% glycerol alone (Figure 7.37B & A).

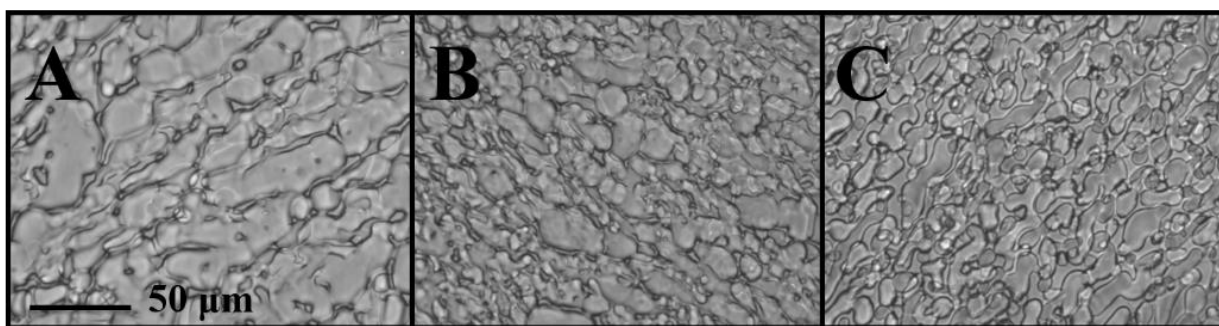


Figure 7.37. Images of ice in presence of A) 15% glycerol + RBCs, B) 15% glycerol + **171** (30 mM) + RBCs, C) 15% glycerol + 5 mM **538** + RBCs.

Cryopreserved RBCs are routinely thawed under fast thawing conditions to prevent injury associated with ice recrystallization.⁷⁰⁻⁷² It was hypothesized that if frozen RBCs were thawed under conditions that would exacerbate ice recrystallization injury, the presence of IRIs in the cryosolution would be beneficial resulting in less hemolysis compared to RBCs cryopreserved in solutions without IRIs. In order to assess this, RBCs were frozen with 15% glycerol and 15% glycerol supplemented with IRIs **166** and **171**. The thawing conditions were modified in order to increase ice recrystallization injury. To do this, samples were held at 0 °C for 20 or 40 minutes prior to thawing in a 37 °C water bath. Samples were also thawed under slow thawing conditions which involved thawing at room temperature. For comparison, RBC

samples were thawed under normal fast thawing conditions by thawing in a 37 °C water bath. After thawing, RBC integrity was measured and the results are shown in Figure 7.38.

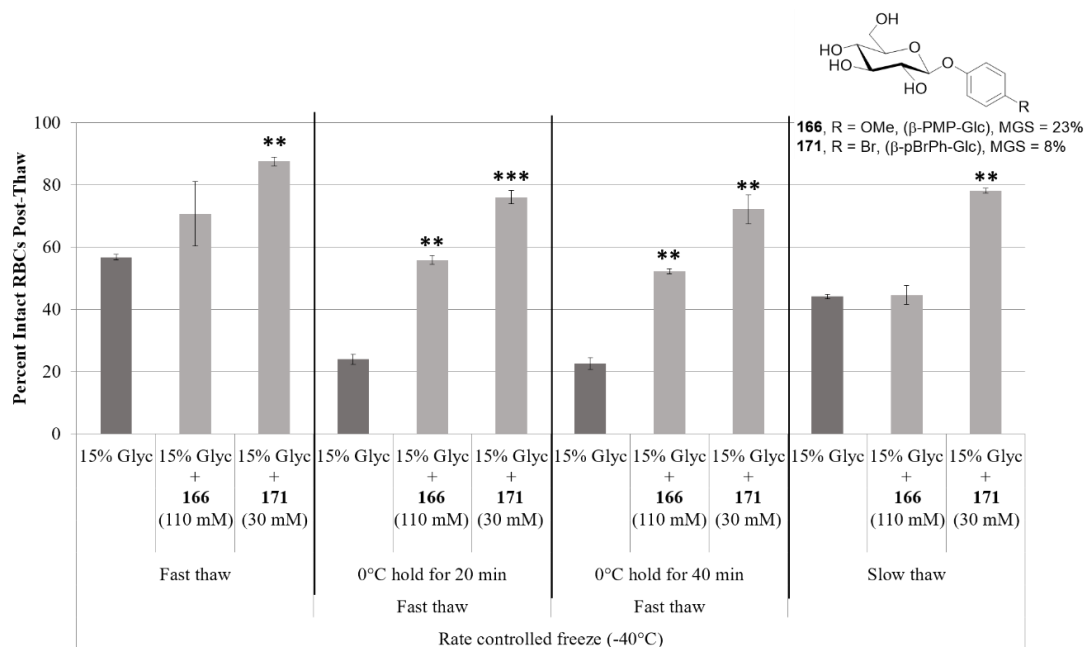


Figure 7.38. RBC integrity post-thaw using thawing conditions which exacerbate ice recrystallization injury. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Dunnett's post-test for comparison to the control (SD) with a 99.9% ($p < 0.001$, ***) or 99.0 ($p < 0.01$, **) confidence level. Asterisks indicate significant difference compared to control (15% Glyc).

Under normal fast thawing conditions, the 15% glycerol cryosolution resulted in 57% intact RBCs. Supplementation of the cryosolution with IRIs **166** and **171** resulted in 71% and 88% intact RBCs respectively. When samples were thawed by holding in a 0 °C for 20 or 40 minutes prior to thawing in a 37 °C water bath, a reduction in RBC integrity for RBCs cryopreserved with 15% glycerol was observed resulting in only 24% intact RBCs. This can be attributed to the increase in ice recrystallization cryoinjury during thawing. In the presence of IRIs **166** and **171**, RBC integrity is significantly increased 2 to 3 times ($p < 0.01$ or 0.001), resulting in 56% and 76% intact RBCs respectively (compared to 24% with 15% glycerol alone). When the samples were thawed slowly by thawing at room temperature, RBCs cryopreserved with 15% glycerol show reduced RBC integrity post-thaw compared to fast thawing

conditions (44% intact RBCs compared to 57%). Again, this was attributed to the increase in ice recrystallization injury during thawing. Supplementation of the cryosolution with **171** resulted in increased intact RBCs (78% intact, $p < 0.01$) during slow thawing conditions attributed to the IRI activity of **171**. However, supplementation of the cryosolution with **166** resulted in similar RBC integrities to the 15% glycerol control ($p > 0.05$). It is not surprising that **171** is better at protecting against ice recrystallization injury because it is approximately three times more IRI active as **166** (8% vs 23% MGS at 22 mM in PBS).

IRI active compounds, **166** and **171** were capable of protecting RBCs from thawing conditions that exacerbate ice recrystallization injury. We therefore hypothesized that these compounds can protect cells against injury resulting from ice recrystallization during transient warming events (TWEs). TWEs have been recognized as a significant contributor to reduced post-thaw viabilities in sperm,⁷³ placental cord blood,^{74,75} peripheral blood mononuclear cells^{76,77} and tissue allografts.⁷⁸ In order to investigate this, an experiment was designed to mimic transient warming. RBCs suspended in cryosolution were frozen by “Rapid Freeze -80 °C” conditions followed by partial thawing to -20 °C by placement in a cold ethanol bath. “Rapid Freeze -80 °C” conditions were chosen to maximize RBC integrity when using only 15% glycerol in order to more easily observe a reduction in integrity after temperature cycling. After stabilization at -20 °C, the sample was cooled again to -80 °C. This process was repeated for several cycles (1, 3 or 5 times) before being thawed. A schematic representation of this cycling process is shown in Figure 7.39. The temperature profile for cycling is shown in Supplemental Figure S7.12.

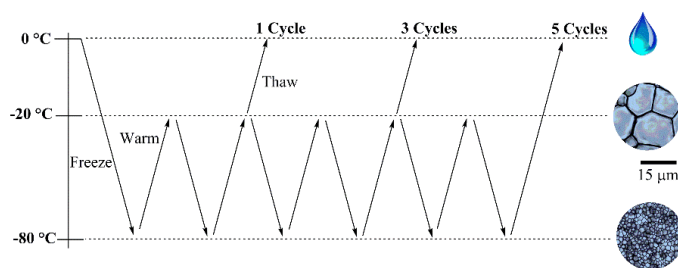


Figure 7.39. Illustration of how to exacerbate ice recrystallization related injury and transient warming effects. Each -80 °C to -20 °C to -80 °C transition represents one cycle of transient warming.

Post-thaw RBC integrities for RBCs frozen with **166** (110 mM) and **171** (30 mM) after 1, 3 and 5 cycles of TWEs were measured (Figure 7.40). One cycle of transient warming resulted in >70% RBC integrity for all cryosolutions. After 3 and 5 cycles of transient warming, the integrity of RBCs frozen with 15% glycerol is reduced from 79% to 59% and 43% respectively. This can be attributed to the increase in ice recrystallization experienced during transient warming. A similar reduction in RBC integrity is observed when the 15% glycerol cryosolution is supplemented with **166** (110 mM). However, >80% RBC integrity is observed when the 15% glycerol cryosolution is supplemented with **171** (30 mM) even after 5 cycles of transient warming.

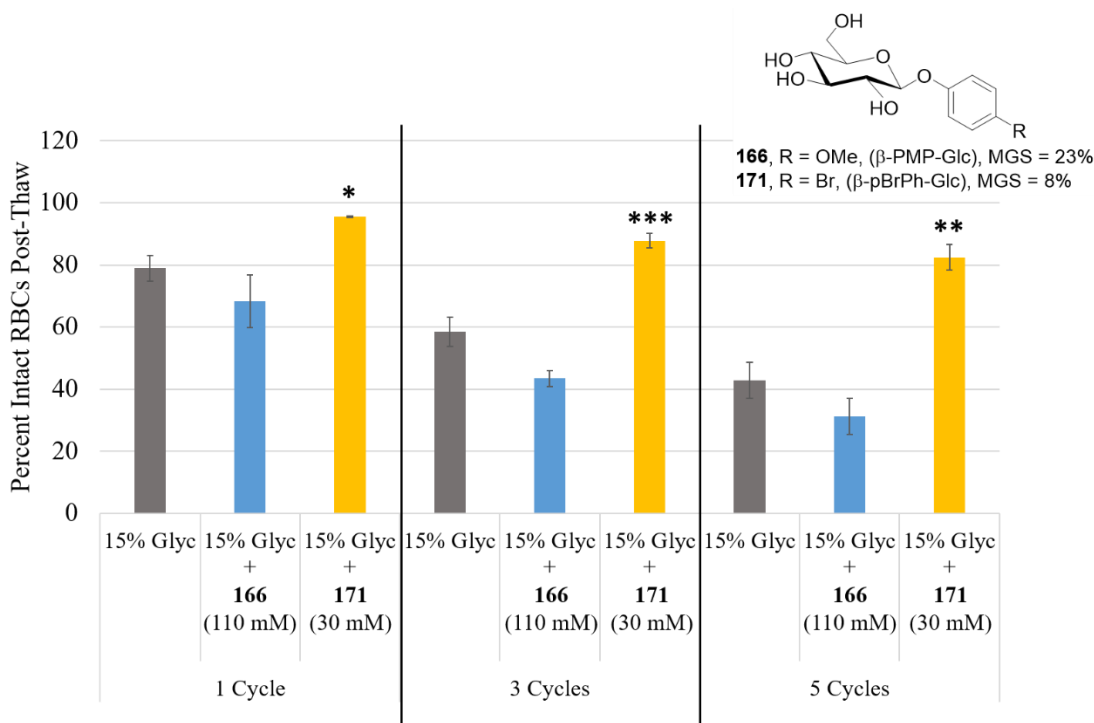


Figure 7.40. Percentage of intact RBCs after transient warming injury utilizing different cryosolutions. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Dunnett's post-test for comparison to the control (15% Glyc) with a 99.9% ($p < 0.001$, ***), 99.0 ($p < 0.01$, **) or 95% ($p < 0.05$, *) confidence level.

Post-thaw RBC integrities for RBCs frozen with **172** (110 mM), **173** (20 mM) and **538** (5 mM) after 1, 3 and 5 cycles of transient warming are shown in Figure 7.41. One cycle of transient warming resulted in >70% RBC integrity for all cryosolutions, with the exception of **172** (110 mM). Hemolysis is

actually increased in the presence of **172** compared to the 15% glycerol control. An explanation for this could be that **172** is toxic to RBCs. As described in the previous results, 3 and 5 cycles of transient warming resulted in a decrease in RBC integrity for RBCs frozen with 15% glycerol is reduced from 79% to 59% and 43% respectively. RBC integrities were higher when **538** (5 mM) was present compared to the control but were reduced after progressive TWEs. Remarkably, >80% RBC integrity is observed when the 15% glycerol cryosolution is supplemented with **173** (20 mM) even after 5 cycles of transient warming. It is interesting to note that the two most active IRIs, **171** and **173** (8% and 12% MGS respectively), were better able to protect RBC integrity during TWEs.

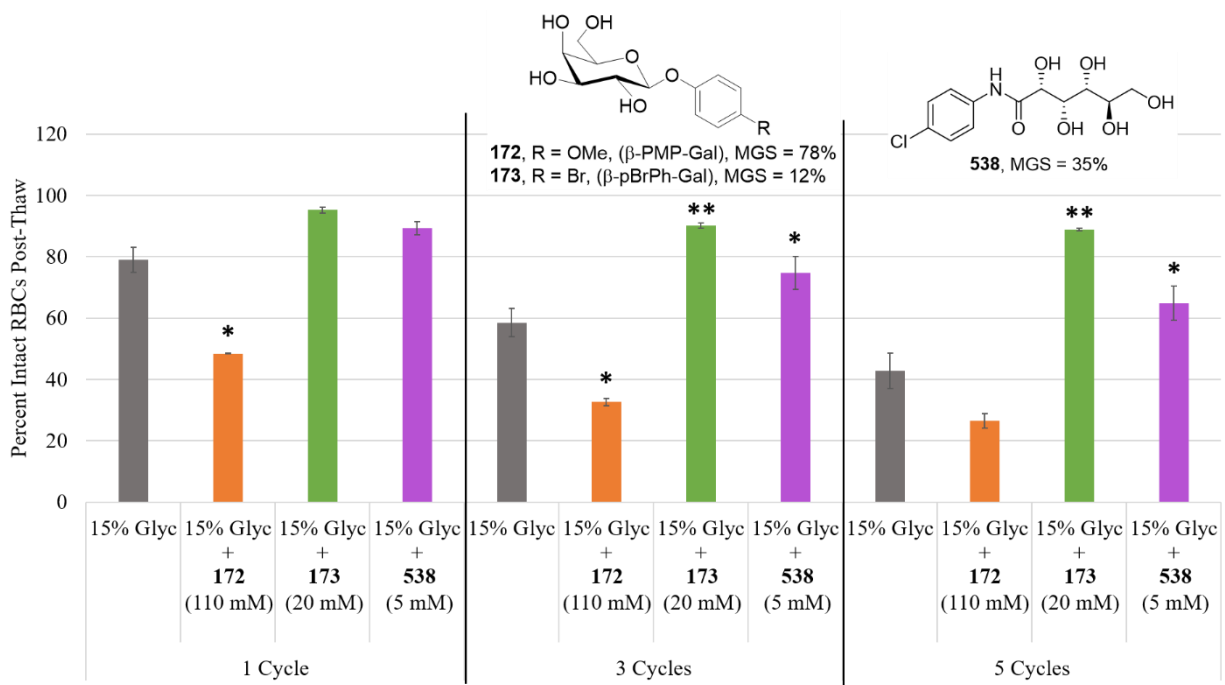


Figure 7.41. Percentage of intact RBCs after transient warming injury utilizing different cryosolutions. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Dunnett's post-test for comparison to the control (15% Glyc) with a 99.0 ($p < 0.01$, **) or 95% ($p < 0.05$, *) confidence level.

To summarize, it was previously found that small-molecule IRIs were effective cryoprotectants for RBCs resulting in high RBC integrities post-thaw using greatly reduced glycerol concentrations and slow cooling conditions (Section 7.4). These molecules were also found to be inhibitors of ice recrystallization

in the presence of RBCs and resulted in a decrease in the percentage of ice in frozen RBC samples. This validated the positive correlation between inhibiting the process of ice recrystallization and increased post-thaw integrity of RBCs. Finally, compounds **171** and **173**, the most effective inhibitors of ice recrystallization in this study, were shown to prevent cellular injury due to ice recrystallization during TWEs further demonstrating the utility of these novel small-molecule ice recrystallization inhibitors as cryoprotectants.

7.10 Controlling intracellular ice recrystallization

Previous results have hinted at the possibility that several small-molecule IRIs are being internalized. For example, it was previously determined that RBC integrity post-thaw was reduced if **166** was not allowed to incubate with RBCs at room temperature for at least 10 minutes prior to freezing.³⁹ Incubation of RBCs with **166** at 0 °C resulted in a decrease in post-thaw intact RBCs. This was hypothesized to be a result of the GLUT receptors being inactive at 0 °C and thus **166** was not able to be internalized. Recent work from our laboratory has demonstrated that several aryl glycosides are crossing the cell membranes of RBCs and are being internalized.⁷⁹

In order to investigate this, a method was developed to observe intracellular ice formation (IIF). This method was developed by Prof. Robert Ben and Dr. Jason Acker from CBS and was based on previously identified methods for visualizing intracellular ice.⁸⁰ For this study, human umbilical vein endothelial cells (HUVEC) were cultured on coverslips. HUVEC cells were utilized for this study because they possess a cell nucleus. A droplet of 0.9% NaCl containing IRIs **166** or **171** and SYTO13 dyes was added to the HUVEC cells on the coverslip. SYTO13 is a cell permeable dye that becomes fluorescent when bound to nucleic acid.⁸¹ After addition of the droplet containing **166** or **171** and SYTO13, the coverslips were incubated for five minutes. The coverslips were then transferred to a Linkham cryostage and cooled to -40 °C (25 °C/min) followed by thawing. Samples were thawed using either fast thawing (25 °C/min) or slow thawing (2 °C/min) conditions. During cooling and thawing, images were taken at five second

intervals. Due to the localization of SYTO13 within the cell nuclei, we were able to image intracellular ice formation (IIF) and recrystallization. This is because solutes (including SYTO13) are excluded from growing ice crystals and therefore, ice crystals in the nucleus were identified as dark spots.

Images of IIF in HUVEC nuclei for fast thawing conditions are shown in Figure 7.42. Initially, there were many ice crystals in the nuclei after rapid cooling to -40°C . In the absence of an IRI, the ice crystals recrystallize and become larger during thawing. It is evident that the presence of IRIs **166** and **171** results in an inhibition of ice recrystallization and the crystals remain very small upon thawing. These results are significant because they indicate that these IRIs are being internalized within the cell and are capable of inhibiting ice recrystallization of intracellular ice.

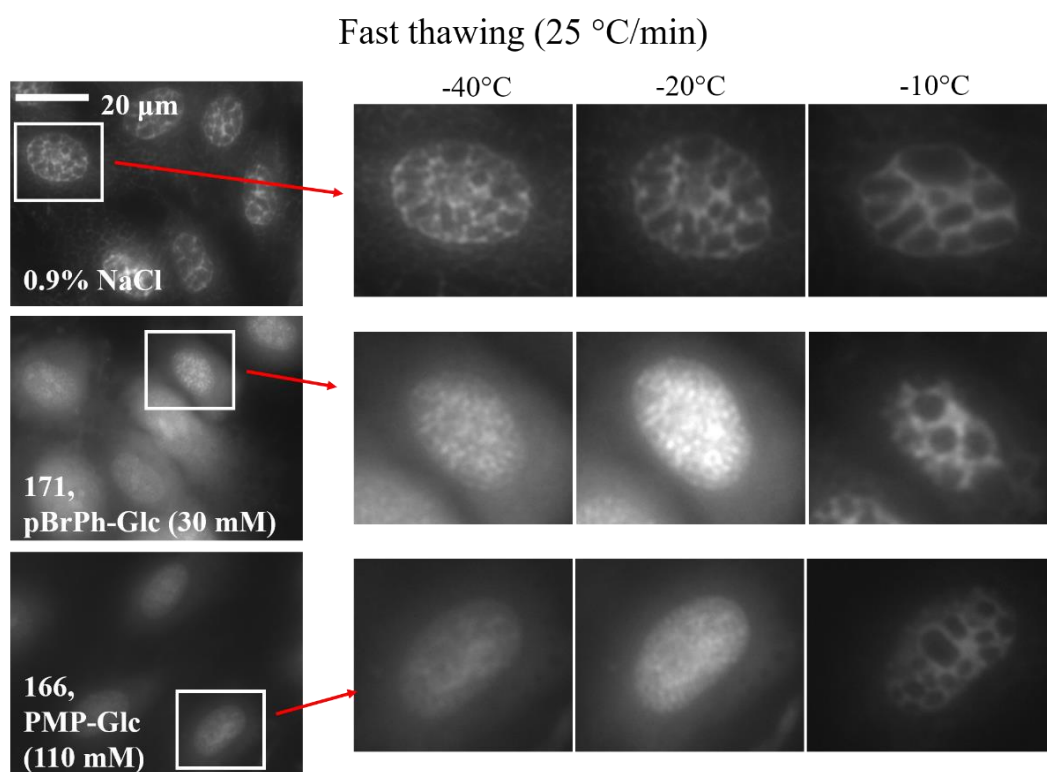


Figure 7.42. Images of ice crystals during fast thawing inside HUVEC nuclei in the presence of IRIs **166** or **171**.

This is even more apparent when analyzing ice crystals in the nuclei of HUVEC samples thawed slowly (Figure 7.43). During slow thawing, ice recrystallization is exacerbated. In the absence of an IRI,

the ice crystals recrystallized into one large ice crystal, approximately 50% of the size of the nuclei. When IRIs **166** and **171** were present, ice recrystallization was inhibited resulting in significantly smaller crystals upon thawing. It is important to note that addition of **171** resulted in a greater degree of inhibition of ice recrystallization compared to **166**. This can be attributed to its greater IRI activity as assessed by the splat-cooling assay (8% MGS for **171** vs 23% MGS for **166**).

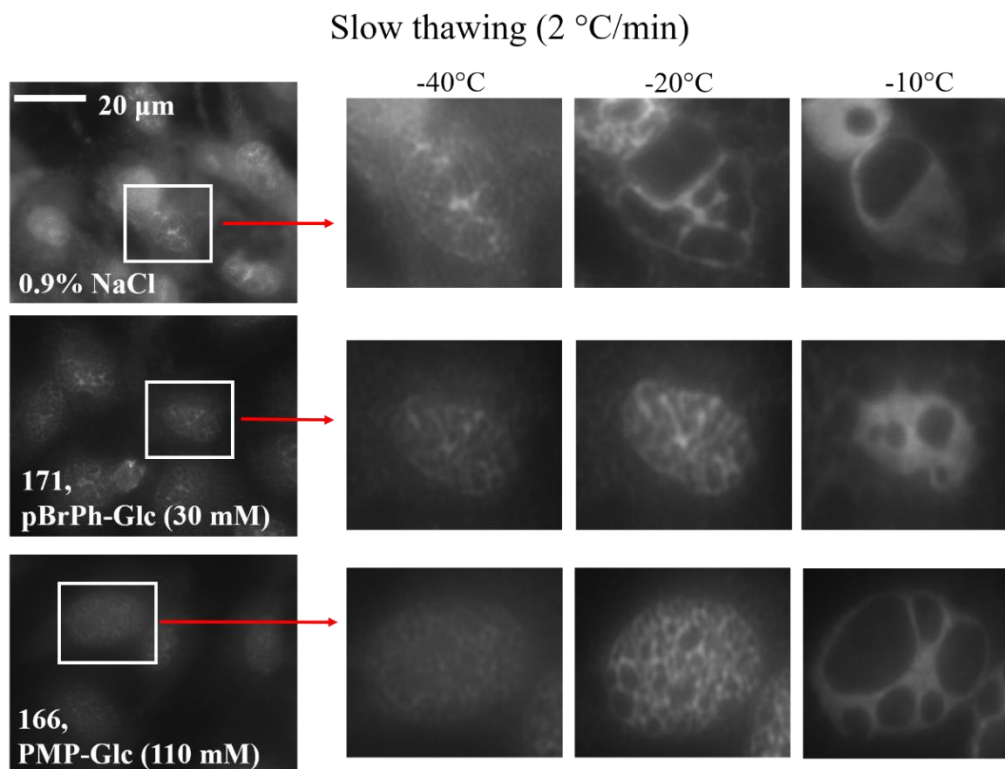


Figure 7.43. Images of ice crystals during slow thawing inside HUVEC nuclei in the presence of IRIs **166** or **171**.

Next, we quantified the size of ice crystals in the nuclei. Image J analysis software was employed to obtain the average ice crystal sizes at different time points during thawing. The average ice crystal size with respect to time (or temperature) during thawing was plotted and several non-linear regression curves were investigated. The data was found to fit an exponential growth curve ($R^2 > 0.92$). The curves for fast thawing conditions are shown in Figure 7.44. It is clear that ice crystal growth is significantly decelerated in the presence of IRIs. The endpoint resulted in significantly smaller ice crystals with the addition of **166**

or **171**. Additionally, ice crystals were also significantly smaller upon initial cooling to $-40\text{ }^{\circ}\text{C}$ in the presence of **166** or **171**. To summarize, when IRIs were present, there were significantly smaller ice crystals during the entire cooling and thawing process. It is important to note that while ice crystals changed in size throughout thawing, the percentage of ice in the nuclei remained constant. The number of ice crystals per nuclei changed during thawing (shown in Table 7.7).

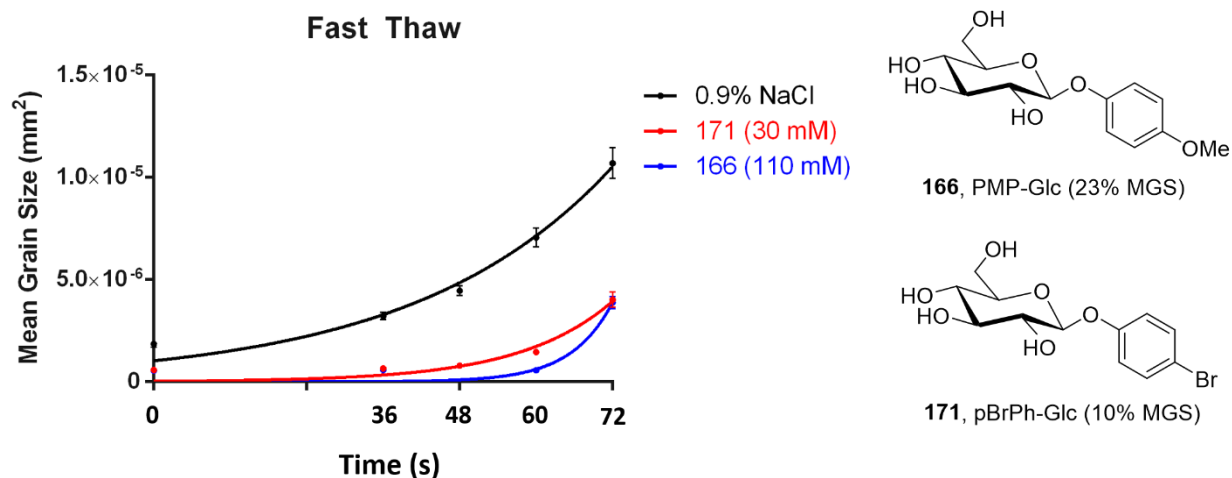


Figure 7.44. Exponential growth curves for ice crystals in HUVEC nuclei from frozen HUVEC cells thawed quickly ($25\text{ }^{\circ}\text{C}/\text{min}$).

		Subzero temperature				
		$-40\text{ }^{\circ}\text{C}$	$-25\text{ }^{\circ}\text{C}$	$-20\text{ }^{\circ}\text{C}$	$-15\text{ }^{\circ}\text{C}$	$-10\text{ }^{\circ}\text{C}$
Number of ice crystals per nucleus	0.9% NaCl (fast thaw)	57	32	23	11	10
	0.9% NaCl (slow thaw)	59	20	10	7	3
	171 (30 mM) (fast thaw)	104	117	96	58	21

Table 7.7. Average number of ice crystals per nuclei at different times during thawing.

The curves for slow thawing conditions are shown in Figure 7.45. Again, it is obvious that ice crystal growth is significantly decelerated in the presence of IRIs. At the endpoint, HUVEC cells frozen with **171** have significantly smaller ice crystals. Interestingly, the endpoint ice crystal size for HUVEC cells frozen with **166** is similar to the 0.9% NaCl control. This could be attributed to the weaker IRI activity of **166** compared to **171**.

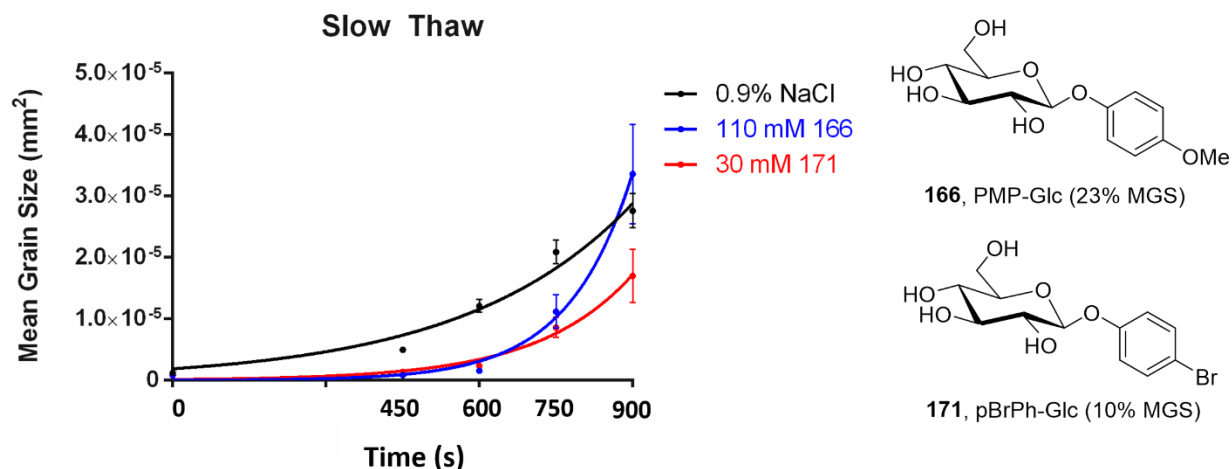


Figure 7.45. Exponential growth curves for ice crystals in HUVEC nuclei from frozen HUVEC cells thawed slowly (2 °C/min).

Finally, post-thaw cell viability was determined qualitatively. For this, HUVEC cells were stained with SYTO13 prior to freezing. Figure 7.46A shows HUVEC cells treated with 0.9% NaCl or with 0.9% NaCl supplemented with **166** (110 mM) or **171** (30 mM) and stained with SYTO13 (unfrozen). Next, the cells were cooled to -40 °C (90 °C/min) (Figure 7.46B) for visualization of IIF. This was followed by thawing and staining with ethidium bromide (EtBr) (Figure 7.46C). EtBr also binds to nucleic acid but in contrast to SYTO13, EtBr is known to be excluded from intact cells and only stains nucleic acids of cells with damaged membranes.⁸² In this fashion, necrotic cells can be identified after thawing by treatment with EtBr by red staining (Figure 7.46C). As shown in Figure 7.46C, all of the cells stained red indicating dead cells after thawing when the cells were treated with 0.9% NaCl. Remarkably, after treatment with β -PMP-Glc (**166**) or β -pBrPh-Glc (**171**), the cells did not stain red after thawing indicating that these cells are viable. This is significant because this is the first example that demonstrates the ability of small molecule IRIs to permeate the cell membrane of HUVEC cells and inhibit intracellular ice recrystallization resulting in viable cells after thawing.

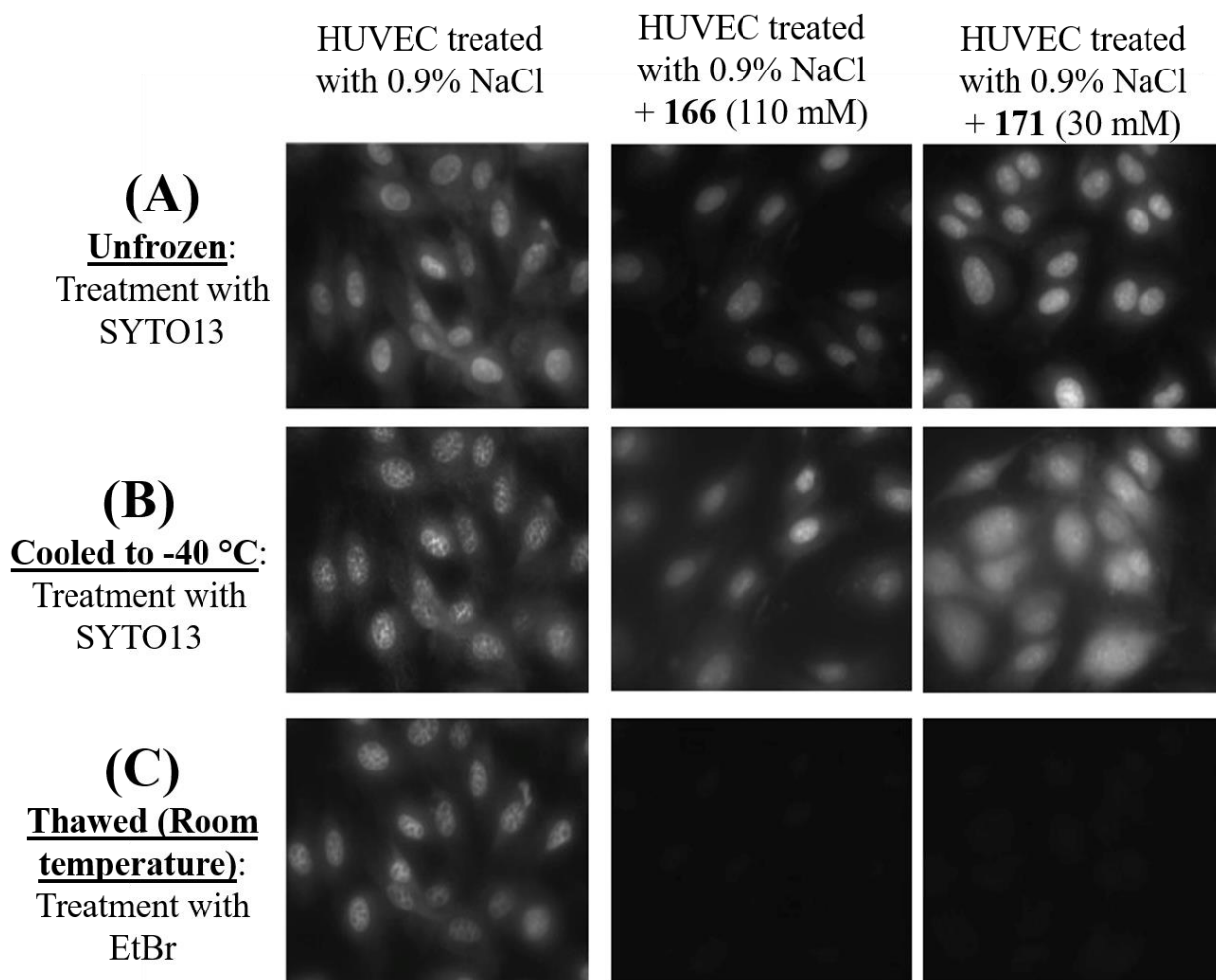


Figure 7.46. Intracellular ice formation in HUVEC treated with 0.9% NaCl, 0.9% NaCl + **166** (110 mM) or 0.9% NaCl + **171** (30 mM). Images taken when (A) cells were unfrozen and stained with SYTO13, (B) cells were cooled to -40 °C and stained with SYTO13; and (C) cells were thawed and treated with EtBr. Cells were cooled at 90 °C/min to -40 °C in 0.9% NaCl with SYTO13 in the absence or presence of IRIs **166** or **171**. Fast warming rates were 25 °C/min. Cells were treated with EtBr (ethidium bromide) post-thaw and incubated for 10 minutes prior to imaging. Ice crystals appear as black spots.

To summarize, it was hypothesized that **166** and **171** were being internalized in RBCs and therefore functioning as cell permeating cryoprotectants. This was confirmed by imaging ice crystals in HUVEC nuclei which were frozen in the presence of IRIs **166** and **171**. A clear reduction in ice crystal size was

observed at all points during thawing compared to when no IRIs were present. This indicates that **166** and **171** are being internalized and present inside the cell nuclei where they are able to prevent the growth of small ice crystals into larger ones. The ability of these molecules to permeate the cell membrane and inhibit intracellular ice recrystallization was also shown to result in viable cells after thawing. As such, these molecules have tremendous potential as cryoprotectants for the preservation of cells and tissues at high subzero temperatures.

7.11 Chapter summary

Overall, this chapter has highlighted the potential for small-molecule ice recrystallization inhibitors to be utilized as novel cryoprotectants for the cryopreservation of RBCs. The aryl glycoside **173** and *N*-aryl-aldoamide **538** were discovered to be beneficial additives for the freezing of RBCs. The addition of **173** and **538** to reduced glycerol concentrations (15%) significantly increased post-thaw RBC integrity. This is important because decreased glycerol concentrations could result in faster deglycerolization methods for quicker access to cryopreserved RBC units.

The ability of these small molecules to increase RBC integrity post-thaw with reduced glycerol concentrations was correlated with their ability to inhibit ice recrystallization. Cryomicroscopy revealed that there is less ice and smaller ice crystals when RBCs are in the presence of IRIs. Furthermore, when RBCs were frozen and thawed under conditions to exacerbate ice recrystallization (slow thawing and transient warming), a significant increase in RBC integrity post-thaw was observed.

The removal of the glycerol post-thaw was found to be problematic, especially for the aryl glycosides, resulting in poor post-deglycerolization recoveries. However, analysis of several RBC properties after deglycerolization indicated that RBCs frozen in the presence of **538** (5 mM) experience less oxidative damage and are better able to carry and deliver oxygen compared to RBCs frozen with 15% glycerol alone.

Previous results indicated that aryl glycosides are exerting their cryoprotective effects by being internalized and functioning as permeating cryoprotectants in RBCs. To investigate this, an experiment was designed to image intracellular ice using HUVEC cells. It was found that ice formed inside the HUVEC nuclei. Upon thawing, the presence of IRIs **166** and **171** prevented recrystallization of the ice inside the nuclei therefore indicating that these molecules are in fact being internalized.

Collectively, the results from this chapter have established that small-molecule IRIs have tremendous potential for improving upon current cryopreservation protocols for RBCs.

7.12 Experimental

7.12.1 RBC Blood Collection and Preparation:

Human whole blood was collected from healthy volunteers into vacutainer tubes containing EDTA anticoagulant using standard phlebotomy. The sample was centrifuged at 2200 g for 10 min at 4 °C. Plasma supernatant and buffy coat were removed and the RBC pellet was washed twice with 0.2%/0.9% dextrose/saline (SD) buffer. After the last wash, RBCs were resuspended in an equal volume of packed RBCs of 0.2%/0.9% SD buffer to a final hematocrit (Hct) of 0.50 L/L. RBC samples in buffer solution were stored refrigerated (4–6 °C) for up to 1 week until needed.

7.12.2 RBC freezing experiments for post-thaw RBC integrity:

An equal volume of cryosolution in 0.2%/0.9% SD buffer was added to 150 µL of leukoreduced RBCs in 0.2%/0.9% SD buffer for a final volume of 300 µL. The final concentrations of all freezing solutions are as indicated in the text. RBC suspensions were transferred to cryo-tubes and incubated at room temperature for 10 min prior to immersion in dry ice (“Rapid Freeze -80 °C”) or liquid nitrogen (“Rapid Freeze -196 °C”) or by immersion in a methanol bath and cooling to -5 °C (“Rate Controlled Freeze”). A thermocouple was inserted into a glycerol control sample for temperature measurements at 1 s intervals. For the “Rate Controlled Freeze” conditions, once the internal solution reached -5 °C, ice nucleation was induced by touching the cryo-tubes with pre-cooled (to -196 °C) forceps. RBC samples were then held at

-5 °C for 5 min. Samples were then slow-cooled at a rate of 1 °C/min to defined subzero temperatures (see text), then either thawed immediately from this temperature (“cooling” profiles) or plunged and stored into dry ice (-80 °C) or liquid nitrogen (-196 °C) for 30 min (“cooling/storage” profiles).

7.12.3 RBC freezing experiments for post-thaw RBC recovery:

An equal volume of cryosolution in 0.2%/0.9% SD buffer was added to 500 µL of leukoreduced RBCs in 0.2%/0.9% SD buffer for a final volume of 1.0 mL (unless indicated in the text). The final concentrations of all freezing solutions are as indicated in the text. RBC suspensions were transferred to cryo-tubes and incubated at room temperature for 10 min prior to immersion in dry ice (“Rapid Freeze -80 °C”) or liquid nitrogen (“Rapid Freeze -196 °C”) or by immersion in a methanol bath and cooling to -5 °C (“Rate Controlled Freeze”). A thermocouple was inserted into a glycerol control sample for temperature measurements at 1 s intervals. For the “Rate Controlled Freeze” conditions, once the internal solution reached -5 °C, ice nucleation was induced by touching the cryo-tubes with pre-cooled (in liquid nitrogen) forceps. RBC samples were then held at -5 °C for 5 min. Samples were then slow-cooled at a rate of 1 °C/min to defined -40 °C, then thawed immediately from this temperature.

7.12.4 RBC Thawing and Post-Thaw Hemolysis:

RBC samples were thawed under fast-thaw conditions in a 37 °C water bath unless otherwise indicated in the text. Post-thaw Hcts and percent hemolysis were determined for all freezing experiments by comparing the supernatant hemoglobin concentration to total hemoglobin concentration using the cyanmethemoglobin Drabkin’s method.^{51,52} The Drabkin’s solution oxidizes the ferrous ions (Fe^{2+}) of hemoglobin (Hgb) to ferric ions (Fe^{3+}) to form methemoglobin (MetHgb). MetHgb then reacts with cyanide ions in the Drabkin’s solution to produce cyanmethemoglobin. The amount of cyanmethemoglobin can be quantified spectrophotometrically to determine Hgb in the sample by comparison to known standards of Hgb.^{51,52,83} Percent hemolysis is calculated using Equation 7.2 using the hematocrit (Hct) and the measured supernatant Hgb (Hgb_s) and the total Hgb in the RBC sample

(Hgb_T). Results are reported as a percentage of intact RBCs (100 - % hemolysis), which represents the percentage of RBCs that were not hemolyzed during cryopreservation and thawing. Recovery was calculated using the CBS method to calculate RBC recovery (Equation 7.3).

$$\% \text{ Hemolysis} = \frac{((100 - \text{Hct}) \times \text{Hgb}_S)}{\text{Hgb}_T} \quad \text{Equation 7.2}$$

$$\text{Recovery} = \frac{\text{Hct}_{\text{Post-Process}} \text{Mass}_{\text{Post-Process}}^{1/\text{Density}_{\text{Post-Process}}}}{\text{Hct}_{\text{Pre-Process}} \text{Mass}_{\text{Pre-Process}}^{1/\text{Density}_{\text{Pre-Process}}}} \times 100\% \quad \text{Equation 7.3}$$

Percent post-thaw RBC integrity was calculated using the measured percent hemolysis values according Equation 7.4.

$$\% \text{ Post Thaw RBC Integrity} = 100 - \% \text{ Hemolysis} \quad \text{Equation 7.4}$$

7.12.5 RBC Deglycerolization and Post-Thaw Recovery:

RBC samples were thawed under fast-thaw conditions in a 37 °C water bath unless otherwise indicated in the text. Deglycerolization washing solutions and corresponding saline concentrations are as indicated in the text. RBC samples were centrifuged at 2,200 g for 5 minutes at 4 °C, supernatant removed and resuspended in W1 solution. RBC samples were mixed thoroughly, centrifuged at 2,200 g for 5 minutes at 4 °C, supernatant removed and resuspended in W2 solution. RBC samples are mixed thoroughly, centrifuged at 2,200 g for 5 minutes at 4 °C, supernatant removed and resuspended in W3 solution. Finally, RBC samples are mixed thoroughly, centrifuged at 2,200 g for 5 minutes at 4 °C, supernatant removed and resuspended in either 0.9% saline or SAGM. Supernatant hemoglobin is measured at each washing step (PREWASH, W1, W2 and W3) and compared to the amount of hemoglobin present prior to freezing. Recovery is calculated using the following equation:

$$\text{Recovery} = \frac{\text{Hct}_{\text{Post-Process}} \text{Mass}_{\text{Post-Process}}^{1/\text{Density}_{\text{Post-Process}}}}{\text{Hct}_{\text{Pre-Process}} \text{Mass}_{\text{Pre-Process}}^{1/\text{Density}_{\text{Pre-Process}}}} \times 100\%$$

7.12.6 RBC Osmotic Tolerance:

Osmotic tolerance was determined using methods that were previously described.^{45,46} RBC samples in buffer (500 µL) were suspended with cryosolutions (500 µL) and incubated at room temperature for 10 mins. Following incubation, 10 µL of the RBC/cryosolution mixture was added to 1 mL of saline solution (concentrations indicated in the text) and the absorbance of the solution was measured at 540 nm. Hemolysis was calculated by comparing the ratio of the measured absorbance of the sample to the measured absorbance of a positive control for RBC hemolysis (SAGM/RBC solution added to 0% Saline (water)) that results in 100% hemolysis. For the osmotic tolerances after washes, following incubation with cryosolution or wash solution, samples were centrifuged at 2,200 g for 10 min at 4 °C and the supernatant was removed. Packed RBCs were then resuspended in the desired wash solution (wash 1 after cryosolution incubation; wash 2 after wash 1 incubation; wash 3 after wash 2 incubation) and incubated at room temperature for 10 min. Following incubation, 10 µL of the RBC/wash solution mixture was added to 1 mL of saline solution (concentrations indicated in the text) and the absorbance of the solution was measured at 540 nm. Hemolysis was calculated as described above.

7.12.7 Refractive index measurement:

Refractive index measurements were performed using a handheld refractometer. A small drop of supernatant was placed on the measuring prism of the refractometer and the refractive index was noted.

7.12.8 RBC indices:

Mean cell volume (MCV) was measured using a hematology analyzer (Coulter AcT 8, Beckman Coulter, Inc., Fullerton, CA).

7.12.9 Adenosine triphosphate concentration:

Adenosine triphosphate (ATP) concentrations were determined using methods that were previously described.⁸⁴ RBC samples were deproteinized on ice in 10 or 12% trichloroacetic acid at 5:6 or 1:1

dilution, respectively, centrifuged ($3000 \times g$, 10 min, 4 °C) to obtain a clear supernatant and ATP was measured using a commercial kit (ATP Hexokinase FS, DiaSys Diagnostic Systems GmbH, Holzheim, Germany) adapted to a microplate method from the manufacturer's protocol. Absorbance was read at 340 nm which measured the reduction of NAD⁺ to NADH. The increase in NADH was directly proportional to the concentration of ATP, which was converted to $\mu\text{mol/g Hb}$. A commercial standard (ATP Standard FS, DiaSys Diagnostic Systems GmbH or Rolf Greiner BioChemica) was run with every batch as a control for the assay.

7.12.10 Oxygen affinity (p50):

Oxygen affinity was determined using methods that were previously described.⁸⁴ RBCs (50 μL) were added to 5 mL of buffer solution (Hemox, TCS Scientific Corp., New Hope, PA), 10 μL of antifoaming agent (TCS Scientific), and 20 μL of 22% bovine serum albumin. The p50 value was determined using an automated blood oxygen analyzer (Hemox, Model B, TCS Scientific) following the manufacturer's protocol. Briefly, dual-wavelength spectrophotometry was used to measure Hgb at 560 and 570 nm, and a Clark electrode was used to measure the partial pressure of oxygen. Samples were oxygenated with air and equilibrated to 37 °C, deoxygenated with nitrogen gas, and then an oxygen equilibrium curve was plotted. A tonometered blood gas control (QC 463 Equil Plus Level 2, RNA Medical, Devens, MA) was used for QC.

7.12.11 RBC deformability:

RBC deformability was measured using methods that were previously described.^{58,59} Briefly, RBC deformability was analyzed via ektacytometry using the laser-assisted optical rotational cell analyzer (LORCA, Mechatronics, Zwaag, The Netherlands). Deformability is measured by subjecting the RBCs to different levels of shear stress by rotation at different speeds causing the RBCs to elongate.⁵⁹ By shining a laser beam through the RBC suspension, the shape and diffraction pattern of the refracted laser beam can be used to calculate the EI_{MAX} and K_{EI} .⁵⁹ The EI_{MAX} is the maximum theoretical elongation index and the

K_{EI} is the shear stress required to obtain half of the EI_{MAX} .⁵⁹ A high EI_{MAX} indicates the RBCs are very deformable whereas a high K_{EI} indicates the RBCs are very rigid and thus require a high degree of shear stress to reach half of the EI_{MAX} .⁵⁹

7.12.12 Flow cytometry analysis of MP and ghost formation:

MP and ghost formation were measured by flow cytometry using methods that have been previously described.⁵⁸ RBC samples were diluted with Annexin V binding buffer (1:5650). An aliquot of 985 μ L was then labelled with 5 μ L of each CD235a-FITC (FITC-conjugated anti-glycophorin A) and Annexin V-APC and incubated for 15 minutes at room temperature in the dark. Samples were then analyzed using a FACSCalibur flow cytometer (BD Biosciences). Previously established gates were employed to identify RBCs, MPs and ghosts.⁵⁸ Only events that were positive for CD235a were considered to be of RBC origin and all other events were discarded. CD235a+ events were further analyzed for PS expression (Annexin V+). The data obtained by flow cytometry was used to calculate the percent composition of the sample and to determine the expression of PS on different RBC populations.

7.12.13 Hypothermic storage:

An equal volume of IRI dissolved in 0.2%/0.9% SD buffer was added to 3.5 mL of leukoreduced RBCs in 0.2%/0.9% SD buffer for a final volume of 7.0 mL. The final concentrations of all IRI solutions are as indicated in the text. RBC properties were measured immediately or after 24 or 48 hours of incubation at 4 °C as indicated in the text.

7.12.14 HUVEC intracellular ice imaging:

Human umbilical vein endothelial cells (HUVEC) were graciously isolated, cultured and transferred to coverslips by Prof. Locksley McGann at the University of Alberta. HUVEC cells were plated on coverslips at 100,000-200,000 cells/mL in appropriate growth medium. IRIs were dissolved in 0.9% NaCl (at concentrations indicated in the text) containing dyes SYTO13 and ethidium bromide (1:100 dilution). A droplet of this solution was added onto coverslips containing HUVEC cells and allowed to incubate for

five minutes. The coverslips were transferred to a Linkam FDCS cryostage with temperature controllers that were fitted to a Nikon 80i microscope. Samples were frozen to -40 °C (25 °C/min) before being thawed quickly (25 °C/min) or slowly (2 °C/min). Images were taken every 5 seconds throughout the cooling and thawing of samples.

7.12.15 Cryomicroscopy:

Cryomicroscopy was used to image frozen RBCs and HUVEC cells in the presence of different solutions containing IRIs. Cryomicroscopy was performed using a Linkam FDCS cryostage and temperature controllers that were fitted to a Nikon 80i microscope. A small volume (10 µL) of sample was added to a quartz crucible, placed on the cryostage and cooled at 25 °C/min to a pre-determined temperature as indicated in the text. The freezing and thawing of samples was imaged in real time using a Hamamatsu Photonics ORCA II camera and Nikon NIS-Elements software. Ice crystal areas were assessed using Image J analysis software.

7.13 Supplemental figures

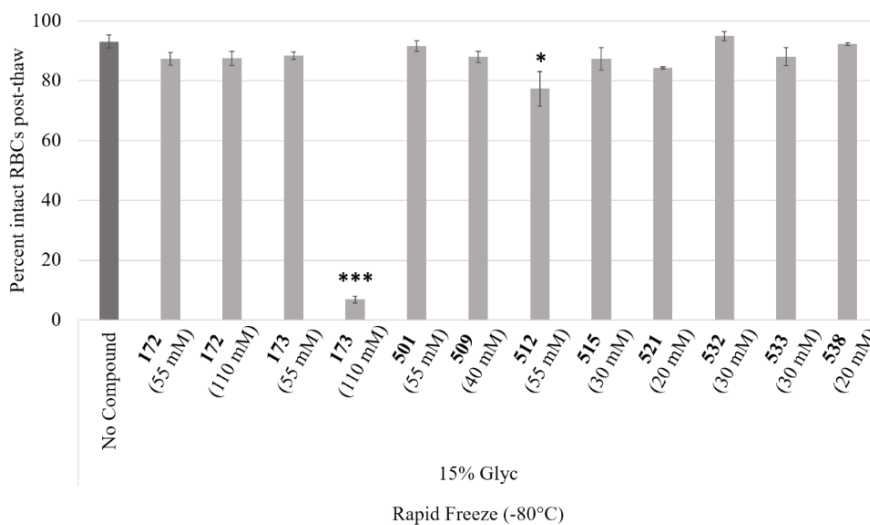


Figure S7.1. The effect of small-molecule IRIs on post-thaw RBC integrity using reduced concentrations of glycerol. RBC samples in glycerol solutions with or without IRIs were rapidly cooled to -80 °C by submersion in dry ice and thawed 37 °C. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a

Dunnett's post-test for comparison to the control (no compound) with a 99.9% ($p < 0.001$, ***) or 95% ($p < 0.05$, *) confidence level.

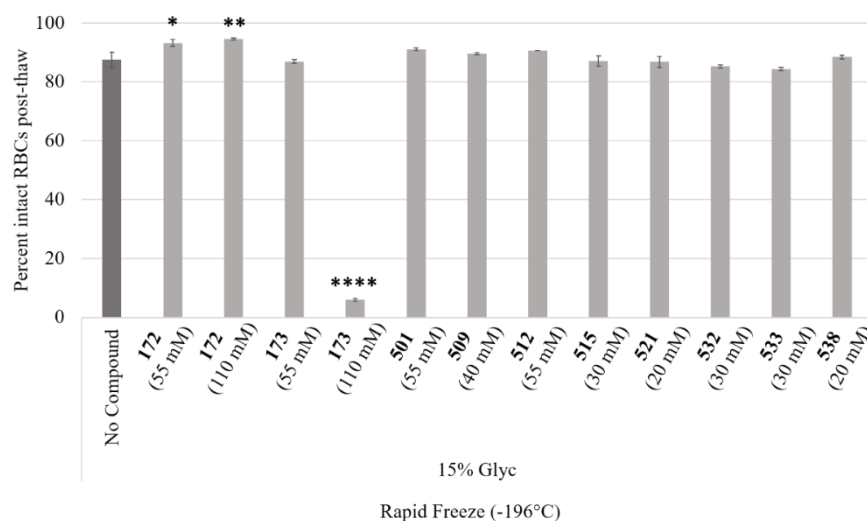


Figure S7.2. The effect of small-molecule IRIs on post-thaw RBC integrity using reduced concentrations of glycerol. RBC samples in glycerol solutions with or without IRIs were rapidly cooled to -196 °C by submersion in liquid nitrogen and thawed at 37 °C. Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Dunnett's post-test for comparison to the control (no compound) with a 99.99% ($p < 0.0001$, ****), 99.0% ($p < 0.01$, **) or 95% ($p < 0.05$, *) confidence level.

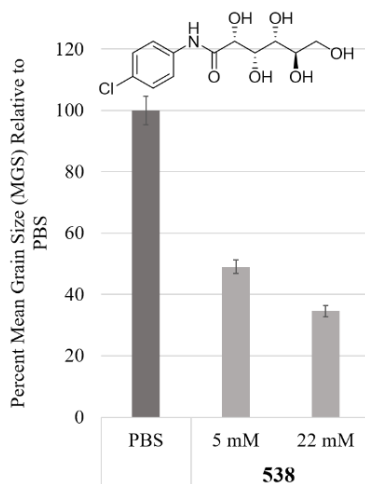


Figure S7.3. IRI activity of **538** at 5 mM and 22 mM.

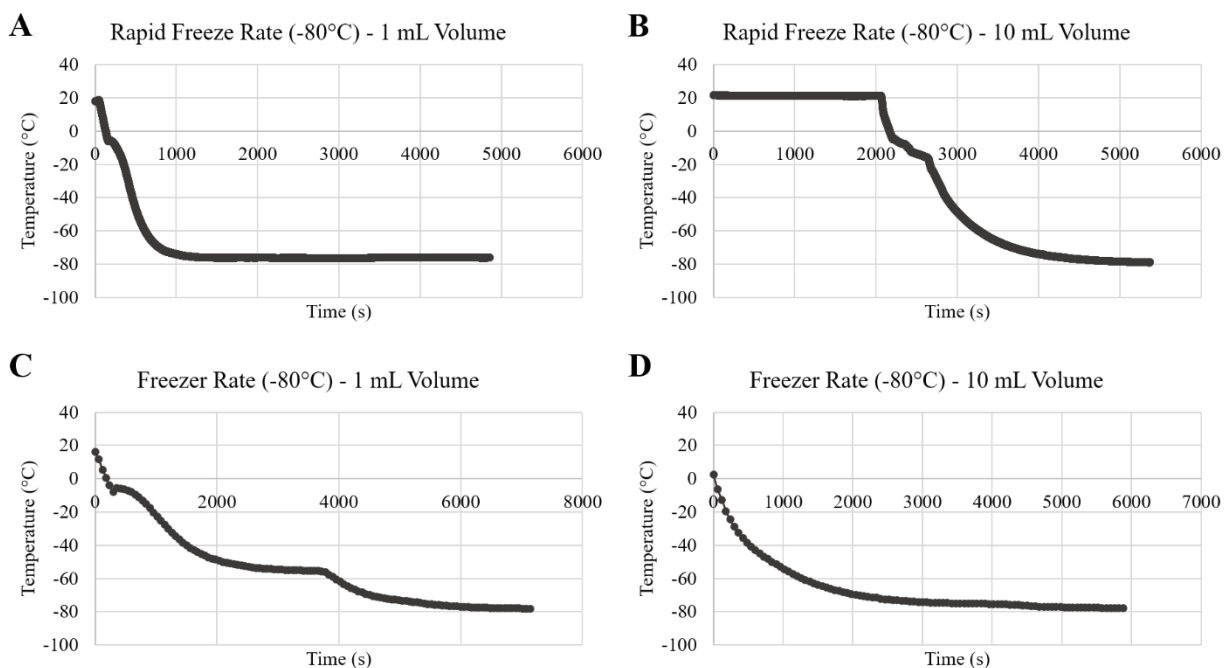


Figure S7.4. Freezing rate curves for “Rapid Freeze -80 °C” and “Freezer -80 °C” conditions with 1 mL and 10 mL volumes. Freezing rates for all conditions were taken between -20 °C and -75 °C. (A) “Rapid Freeze -80 °C” with 1 mL (5 °C/min); (B) “Rapid Freeze -80 °C” with 10 mL (2 °C/min); (C) “Freezer -80 °C” with 1 mL (0.7 °C/min); (D) “Freezer -80 °C” with 10 mL (0.7 °C/min).

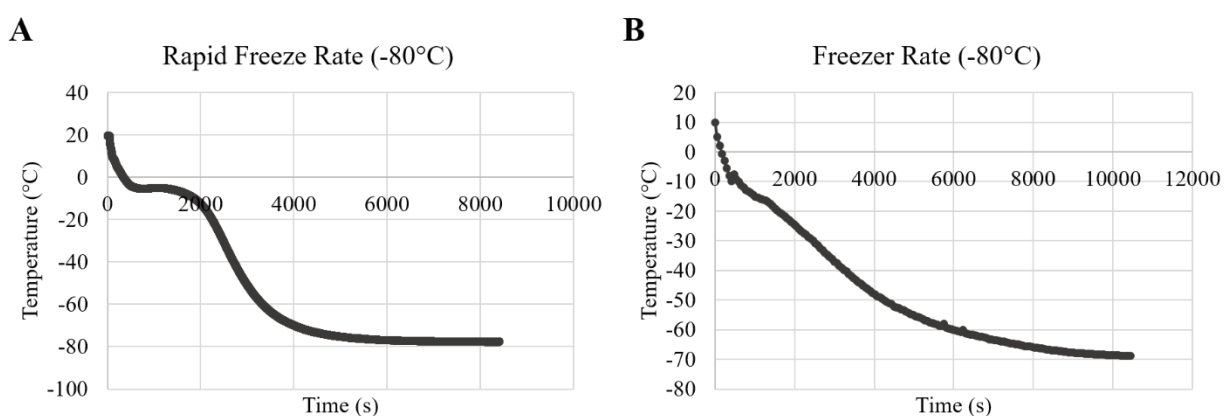


Figure S7.5. Freezing rate curves for “Rapid Freeze -80 °C” and “Freezer -80 °C” conditions with full RBC unit volumes (540 mL). (A) Freezing rates for “Rapid Freeze -80 °C” condition were taken between -20 °C and -75 °C (1 °C/min); and (B) for “Freezer -80 °C” condition were taken between -20 °C and -65 °C (0.43 °C/min).

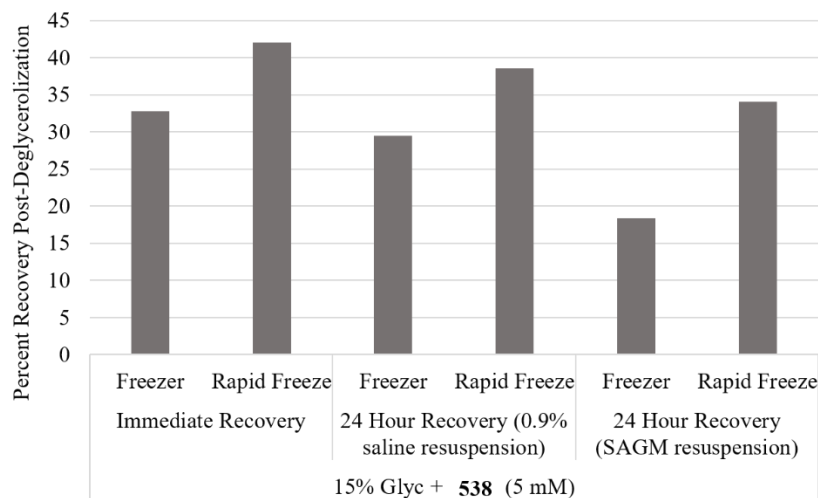


Figure S7.6. Recovery of full unit of RBCs frozen with 15% glycerol containing 538 under “Rapid Freeze -80 °C” or “Freezer -80 °C” conditions immediately post-thaw and 24 hour post-thaw. Samples were deglycerolized using CBS washing conditions and resuspended in 0.9% saline or SAGM.

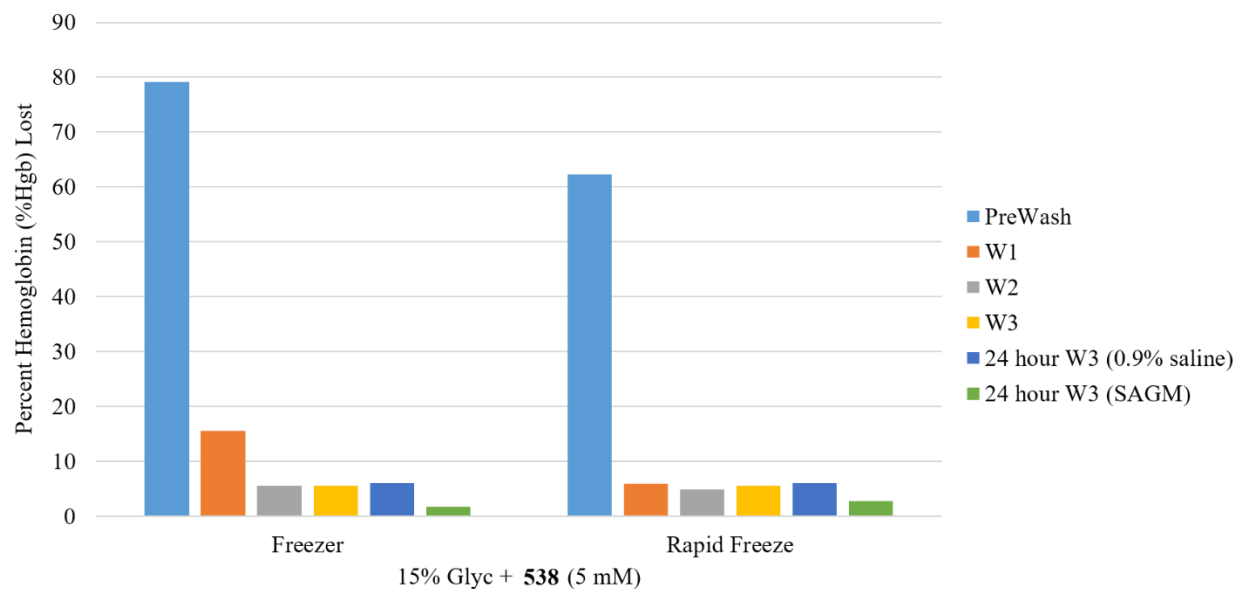


Figure S7.7. Percent hemoglobin (%Hgb) lost during deglycerolization washes relative to RBC Hgb prior to freezing using Washing Protocol 1 deglycerolization conditions.

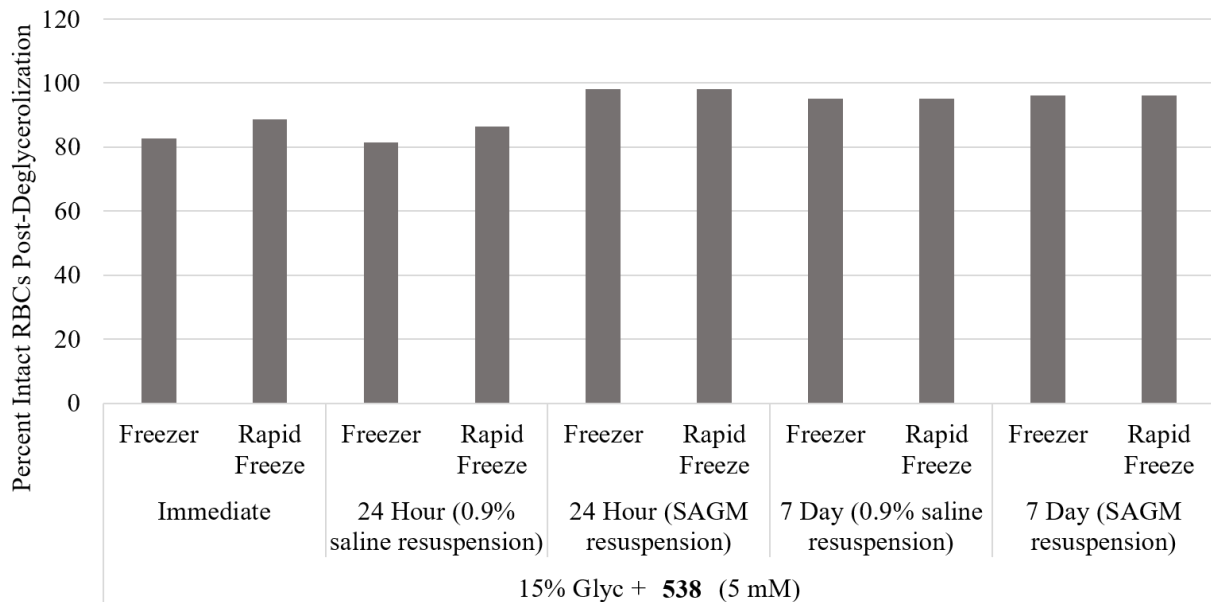


Figure S7.8. Percent intact RBCs post-deglycerolization using Washing Protocol 1 conditions and resuspension in SAGM or 0.9% saline immediately after deglycerolization, 24 hours and 7 days after deglycerolization.

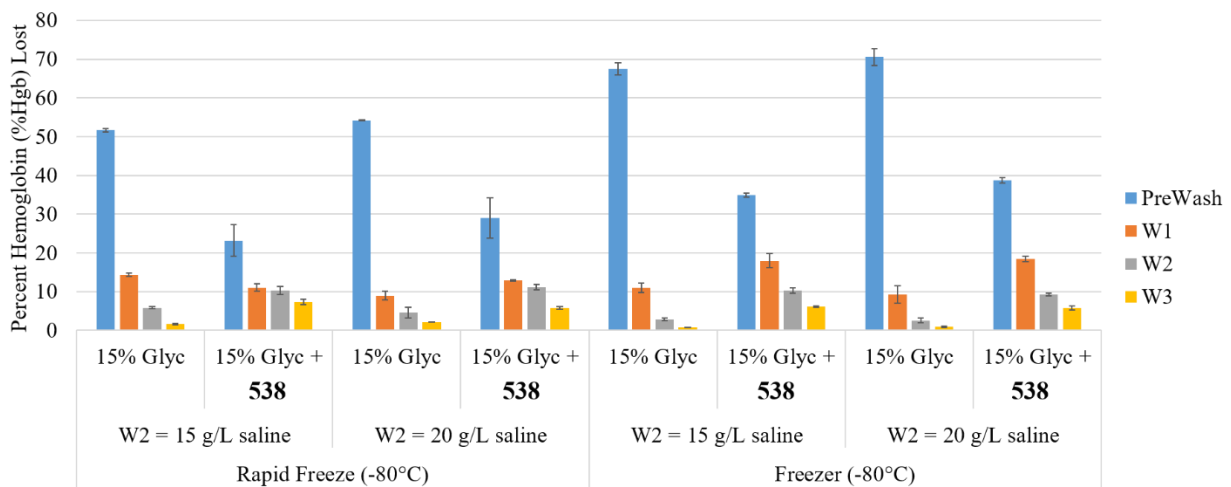


Figure S7.9. Percent hemoglobin (%Hgb) lost during deglycerolization washes relative to RBC Hgb prior to freezing using Washing Protocol 1 (W2 = 15 g/L NaCl) and Washing Protocol 2 (W2 = 20 g/L NaCl). Statistical significance was assessed by unpaired Student's T-test for comparison between %Hgb lost at W2 with a 95% ($p < 0.05$, *) confidence level.

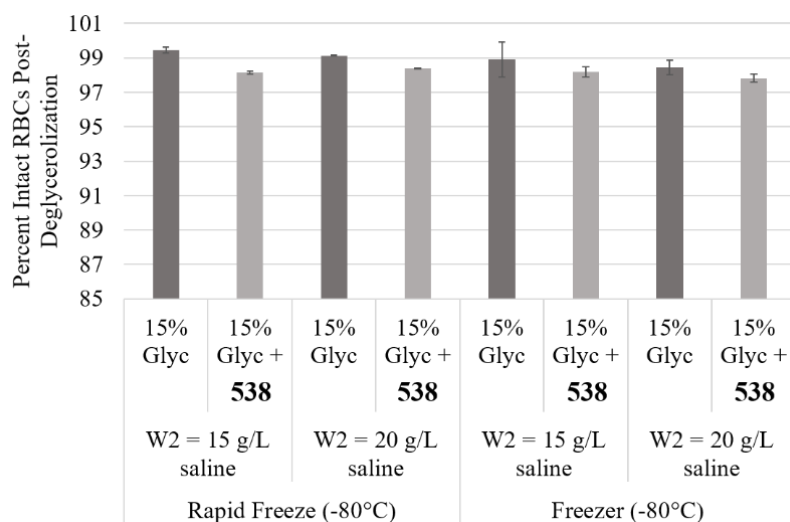


Figure S7.10. Percent intact RBCs after deglycerolization using Washing Protocol 1 (W2 = 15 g/L NaCl) and Washing Protocol 2 (W2 = 20 g/L NaCl). Statistical significance was assessed by unpaired Student's T-test for comparison to the control (no compound) with a 95% ($p < 0.05$, *) confidence level.

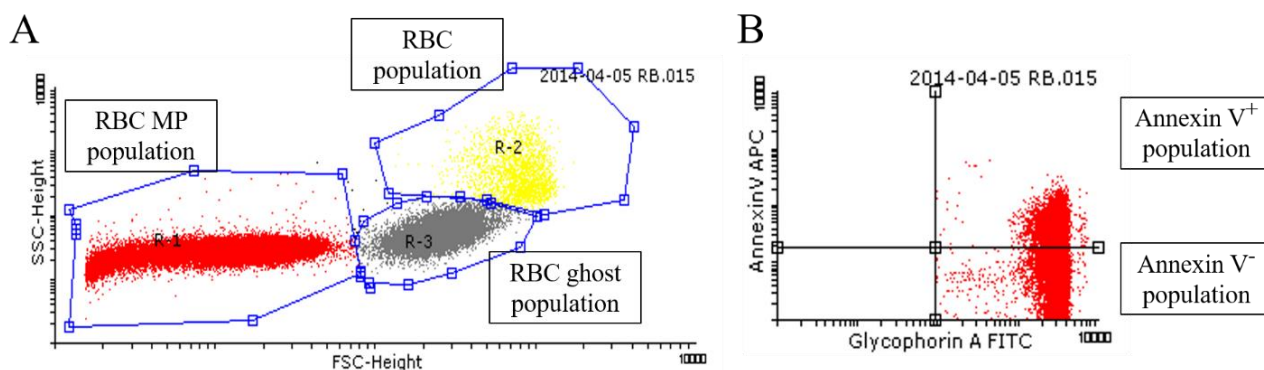


Figure S7.11. Flow cytometry gating strategy to identify RBC populations and PS expression (Annexin V).

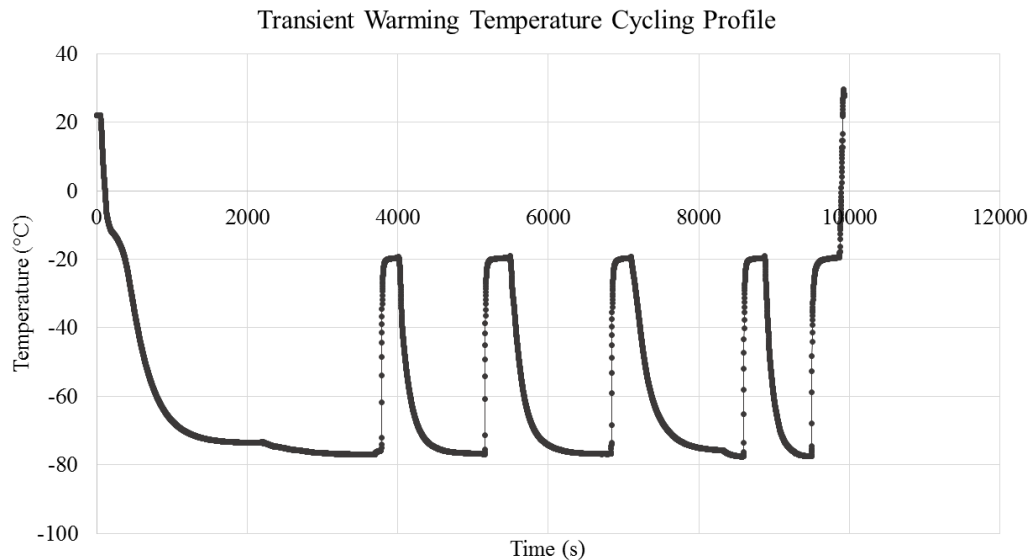


Figure S7.12. Transient warming temperature cycling profile.

7.14 References

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Conclusions

The focus of this thesis was to rationally design small-molecule ice recrystallization inhibitors with low cytotoxicity. The development of new cryoprotectants which are non-toxic and capable of controlling ice recrystallization could dramatically improve current cryopreservation protocols. Therefore, the overall objectives of this thesis were: (1) to design novel IRI active molecules that are less toxic which feature aromatic rings instead of alkyl chains; (2) to determine the relationship between IRI activity and amphiphilicity; and (3) to explore the cryopreservation potential of IRI active molecules for HSCs and RBCs.

The first objective was achieved by synthesizing truncated AFGP analogues in which the alkyl chain has been replaced with an aromatic ring (Chapter 3) as well as *N*-alkyl-aldonamide analogues in which the alkyl chain has been replaced with an aromatic ring (Chapter 5). Truncated AFGP analogues with long alkyl chains were previously found to be highly IRI active but their surfactant structure impeded their ability to act as novel cryoprotectants. It was hypothesized that replacement of the alkyl chain in the truncated AFGPs with an aryl ring could result in a new class of small molecules with potent IRI activity. *N*-aryl-D-pyranosyl-acetamides were rationally designed and assessed for IRI activity. Overall, the *N*-aryl-D-pyranosyl-acetamides possessed moderate to weak IRI activity. *N*- α -aryl-D-galactosyl-acetamide derivatives were more highly IRI active than *N*- α -aryl-D-glucosyl-acetamide derivatives. This may be attributed to the fact that D-galactose is more highly hydrated and more IRI active than D-glucose. However, none of these compounds possessed IRI activity greater than D-galactose and therefore were considered weakly active or inactive. Collectively, these results indicate that the replacement of the alkyl chain in the truncated AFGPs with an aromatic ring is not tolerated and resulted in weakly active molecules.

Chapter 5 described the rational design of the *N*-aryl-aldonamide class of small-molecules. These molecules are *N*-alkyl-aldonamides that feature an aromatic ring with various substituents in place of the alkyl chain. Since the replacement of the alkyl chain with an aromatic ring in the β -alkyl-glycosides

resulted in the discovery of potent IRIs, it was hypothesized that the same replacement in the *N*-alkyl-gluconamides could also result in the discovery of potent IRIs. Thirty derivatives of this new class of compounds, referred to as *N*-aryl-gluconamides, were synthesized and assessed for IRI activity. This resulted in several very potent IRIs being discovered. Unfortunately, structure-function analyses of these derivatives did not result in the identification of key structural attributes required for inhibition of ice recrystallization. A 3D-QSAR model was designed and used to pre-screen compounds for IRI potency to minimize labour-intensive chemical synthesis. The model was able to successfully predict the activity of molecules which ultimately accelerated the discovery process.

The second objective was achieved by establishing a metric to quantify amphiphilicity to investigate the relationship between amphiphilicity and IRI activity. Amphiphilicity was quantified by CH_n/OH ratio for *N*-alkyl-aldonamides. It was found that IRI activity was not correlated to amphiphilicity. However, while these experiments did not establish a robust trend between the CH_n/OH ratio and IRI activity, some key properties required for IRI activity were observed: the hydrophobic region must contain at least six carbons ($\text{CH}_n \geq 6$), and the hydrophilic moiety must contain at least four hydroxyl groups ($\text{CH}_n/\text{OH} \geq 4$, *N*-alkyl-gluconamides or -arabonamides). Shortening the hydrophilic moiety to three hydroxyl groups (*N*-alkyl-erythronamides) resulted in an inactive or weakly active compound, regardless of the alkyl chain length. The results from this study reaffirm the understanding that molecules with longer alkyl chains possess increased IRI activity compared to those with shorter alkyl chains. However, IRI activity cannot be simply related to amphiphilicity. Furthermore, these surfactant-like molecules are not useful for cellular applications due to their propensity to interact with and solubilize cell membranes.

The final objective was accomplished by investigating the ability of small-molecule IRIs to improve the cryopreservation of HSCs (Chapter 6) and RBCs (Chapter 7). Several *N*-aryl-aldonamides were found to be beneficial additives to the conventional 10% DMSO cryosolution for HSCs from UCB. TF-1 α cells were used as a model cell line to pre-screen different cryosolutions containing IRIs. Unfortunately, this cell line did not accurately predict the cryopreservation outcome for HSCs from UCB,

requiring the screening of IRI active molecules in UCB HSCs. The supplementation of the conventional 10% DMSO cryosolution with IRI active *N*-aryl-aldonamides did not increase post-thaw viability but resulted in an increase in HSC clonogenic potential post-thaw. Furthermore, these solutions resulted in less apoptosis post-thaw and increased ALDH levels. This is significant because HSCs expressing signs of apoptosis were found to not engraft into animal models and the expression of high ALDH activity in UCB is indicative of a progenitor cell population that is enriched with long-term culture-initiating cells. Overall, the supplementation of the current cryoprotectant solution with small molecules capable of controlling ice growth and recrystallization resulted in an increase in CFU recovery and frequency of multipotent progenitors in UCB HSCs. Ultimately, this would reduce the percentage of engraftment failure and allow for a larger proportion of cord blood banks' inventory to provide an adequate dose for patients requiring transplants.

Several small molecule IRIs were also found to be effective cryoprotectants for RBCs with greatly reduced amounts of glycerol. This is important because decreased glycerol concentrations could result in faster deglycerolization methods for quicker access to cryopreserved RBC units. The ability of these small molecules to increase RBC integrity post-thaw with reduced glycerol concentrations was correlated with their ability to inhibit ice recrystallization. Post-deglycerolization analysis of several RBC properties indicated that RBCs frozen in the presence of **538** (5 mM) experience less oxidative damage and are better able to carry and deliver oxygen compared to RBCs frozen with 15% glycerol alone. Furthermore, it was found that small-molecule IRIs are being internalized during cryopreservation. It was found that ice formed inside frozen HUVEC nuclei and that upon thawing, the presence of IRIs prevented recrystallization of the ice inside the nuclei.

Collectively, the results presented in this thesis have established that small-molecule IRIs have tremendous potential for improving upon current cryopreservation protocols for important cellular therapy products such as HSCs and RBCs.

Future Directions

Collectively, the results from this thesis have established that small-molecule IRIs have tremendous potential for improving upon current cryopreservation protocols for cellular therapy products like HSCs and RBCs. It is evident that the use of IRIs as either alternatives or additives to the current clinically used cryoprotectants improves the cryopreservation of HSCs and RBCs. However, substantial work lies ahead to determine the exact mechanism of action of these molecules. While it is important to understand the mechanism of ice recrystallization inhibition, it is more important to first understand how these molecules are acting as cryoprotectants. It is unclear whether they are preserving cell viability and functionality through inhibition of ice recrystallization, by acting as cell permeating cryoprotectants or by providing protective interactions with cell membranes. Understanding the mechanism of cryoprotection will facilitate the design of future molecules capable of improving the cryopreservation of HSCs and RBCs.

HSCs frozen with IRIs **502** and **509** were shown to have improved functionality post-thaw compared to 10% DMSO alone. However, it is unclear how these molecules are functioning. Preliminary studies suggest these types of molecules could be interacting with the cell membrane. Whether or not these molecules are being internalized is something that should be investigated. Furthermore, it is unclear whether these molecules are providing protection of clonogenic potential due to their ability to inhibit ice recrystallization. Compounds that were inactive but structurally similar did not improve the clonogenic potential of HSCs post-thaw. This suggested that the improved post-thaw clonogenic potential of HSCs frozen with IRI active molecules was correlated to IRI activity. However, IRI active molecules, **502**, **509**, and **517** did not preserve functionality when frozen HSC samples were subjected to transient warming. Interestingly, the composition of colonies formed post-thaw was altered with the addition of IRIs to the cryosolution. It is therefore possible that rather than improving functionality post-thaw by inhibiting ice recrystallization, these molecules were promoting the proliferation and differentiation of HSCs into specific cell types and this was independent of cryopreservation. Thus, additional experiments in which unfrozen HSCs are incubated with IRIs and assessed for clonogenic potential are required.

HSCs frozen with IRIs **502** and **509** were found to have decreased levels of apoptosis post-thaw. Several studies have found that damage to the mitochondria during cryopreservation causes the release of caspases that subsequently results in cell losses due to apoptosis. Caspase expression occurs early in the apoptosis cascade and is therefore an earlier marker of damage compared to the traditionally used apoptosis marker, Annexin V. Furthermore, caspase expression has been associated with freeze-thaw damage. Therefore, future work should include the measurement of caspase activity in HSC samples frozen with 10% DMSO supplemented with IRIs.

The ability of small molecule IRIs to increase RBC integrity post-thaw with reduced glycerol concentrations was correlated with their ability to inhibit ice recrystallization. Cryomicroscopy revealed that there is less ice and smaller ice crystals when RBCs are in the presence of IRIs. Furthermore, when RBCs were frozen and thawed under conditions to exacerbate ice recrystallization (slow thawing and transient warming), a significant increase in RBC integrity post-thaw was observed.

The removal of the glycerol post-thaw was found to be problematic, especially for the aryl glycosides, resulting in poor post-deglycerolization recoveries. Therefore, this is an area which requires further optimization. Analysis of several RBC properties after deglycerolization indicated that RBCs frozen in the presence of **538** (5 mM) experience less oxidative damage and are better able to carry and deliver oxygen compared to RBCs frozen with 15% glycerol alone. It is unclear how **538** is able to improve these RBC properties during cryopreservation. Therefore, future work should include investigation into the mechanism of action of **538** in order to facilitate the rational design of future molecules with enhanced cryoprotective ability.

Contributions to Original Research

1. Synthesis of *N*-aryl-D-pyranosyl-acetamides and analysis of the ice recrystallization inhibition (IRI) activity of derivatives **321-330**, **333** and **334** to elucidate key structural motifs important for potent IRI activity as described in chapter 3.

2. Investigating the relationship between IRI activity and amphiphilicity in carbohydrate-based surfactants. Synthesis of *N*-(cyclo)alkyl-aldonamides and analysis of the IRI activity of derivatives **411-417**.
3. Synthesis of *N*-aryl-gluconamides and analysis of the IRI activity of derivatives **501-543** as described in chapter 5.
4. Analysis of cytotoxicity and cryopreservation potential of small molecule ice recrystallization inhibitors with a TF-1 α cells, HSCs obtained from UCB, and RBCs obtained from peripheral blood.

Publications, Presentations & Patents Resulting from this Thesis

Publications

1. Briard, J.G., Davis, K.A. & Ben, R.N., “Structure activity relationships of small-molecule amphiphilic ice recrystallization inhibitors”, Submitted, June 2016.
2. Poisson, J.S., Briard, J.G., Turner, T.R., Acker, J.P. & Ben, R.N., “Ice recrystallization inhibitors with hydroxyethyl starch improve cryopreservation of human red blood cells with slow freezing rates”, Submitted, June 2016.
3. Briard, J.G., Chandran, P., Jahan, S., Allan, D., Pineault, N. & Ben, R.N., “Improving the post-thaw functionality of hematopoietic stem cells (HSCs) from umbilical cord blood using novel ice recrystallization inhibitors (IRIs)”, Submitted, June 2016.
4. Briard, J.G., Fernandez, M., De Luna, P., Woo, T.K. & Ben, R.N. “QSAR Accelerated Discovery of Potent Ice Recrystallization Inhibitors”, *Scientific Reports*. Accepted, May 2016.
5. Briard, J.G., Poisson, J.P., Turner, T.R., Capicciotti, C.J., Acker, J.P. & Ben, R.N., “Small molecule ice recrystallization inhibitors mitigate red blood cell lysis during freezing, transient warming and thawing”, *Scientific Reports*. **2016**. 6, DOI:10.1038/srep23619

6. Briard, J.G., Shiao, T.C. & Ben, R.N. "Synthesis of *N*-(4-Methoxybenzyl)-2-(α -D-glucopyranosyl) acetamide" *Carbohydrate Chemistry: Proven Synthetic Methods*. **2015**. Volume 3, Chapter 33, 257-266.
7. Balcerzak, A.K, Capicciotti, C.J., Briard, J.G. & Ben, R.N. "Designing ice recrystallization inhibitors: from antifreeze (glyco)proteins to small molecules" *RSC Advances*. **2014**. 4, 42682-42696.

Conference Presentations

1. **Jennie G. Briard**, Jessica S. Poisson, Tracey R. Turner, Jason P. Acker & Robert N. Ben, "Carbohydrate-Based Small Molecule Ice Recrystallization Inhibitors as Cryopreservatives for Red Blood Cells" Cryo2015, Ostrava, Czech Republic, July 26-29, 2015. ***This presentation was awarded the Peter L. Steponkus Crystal Award.**
2. **Jennie G. Briard**, Jessica S. Poisson, Tracey R. Turner, Jason P. Acker & Robert N. Ben. "Ice Recrystallization Inhibitors – Novel Cryoprotectants for Hematopoietic Stem Cells and Red Blood Cells" CSC2015, Ottawa, ON, June 13-17, 2015.
3. **Jennie G. Briard**, Emily Gardiner, Melody Afagh & Robert N. Ben, "The Design of Small Molecule Ice Recrystallization Inhibitors for use as Cryopreservatives" 2014 World Forum on Biology, Savannah, Georgia, USA, May 31 – June 4.
4. **Jennie G. Briard**, Melody Afagh & Robert N. Ben, "Investigating the Effect of Fluorine on Ice Recrystallization Inhibition (IRI) Activity" CSC 2013. Quebec City, Quebec, May 26 – 30, 2013.

Patents

1. Ben, R.N., Capicciotti, C.J. & Briard, J.G. "Small molecule ice recrystallization inhibitors and methods of use thereof." Patent publication Number: US 2015/0373968 A1. December 31, 2015.