

Synthesis and *In Vitro* Applications of Ice Recrystallization Inhibitors

Jessica Poisson

B.Sc. Honours Biopharmaceutical Science,
Medicinal Chemistry (Co-op) – University of Ottawa, 2014

Thesis submitted
in partial fulfillment of the requirements
for the Doctorate in Philosophy degree in Chemistry

Department of Chemistry and Biomolecular Sciences
Faculty of Science
University of Ottawa

Candidate

Supervisor

Jessica Poisson

Robert N. Ben

Dedicated to my husband, Alex, and my family

Acknowledgements

I would first like to thank my supervisor Dr. Robert Ben for his invaluable guidance and assistance throughout the past five years. I am truly grateful for your helpful discussions and suggestions regarding my research and for teaching me to think “big picture” in research. My PhD has been an amazing experience and I thank you, not only for your tremendous academic support, but also for all of the opportunities you have given me.

I would also like to thank members of the Ben lab for their support, help and friendship throughout my graduate studies. Madeleine Adam (aka “Haddo”) and Julia Meyer (aka “Hulio”) have been wonderful friends both inside and outside of the lab, and amazing travel buddies and hotel roommates at several conferences and Edmonton research trips. I want to thank you both for your daily discussions about chemistry and life, and for making my time in the lab memorable. To Dr. Jennie Briard for training me on my first ever trip to Edmonton and for being a great friend. To Thomas Charlton (aka “Tchar”) for being a great deskmate and for all of his help in the lab. To the undergraduate students that I had the privilege to work with, Emily Zhang, Mary Lynn Cottee, Geneviève O’Keefe and Olivia Herzog, for your contributions. To other past and present Ben lab members that I had the pleasure to work with or alongside, Dr. Malay Doshi, Vanessa Musca, Stephanie Abraham, Kyle McClymont, Anna Ampaw, Karishma Chopra, Leah McMunn and Jatinder Singh, for making the lab a great workplace. I would like to give special thanks to Salma Alasmar for reading and editing my thesis.

I gratefully acknowledge the funding received in the last three years of my PhD from the Mitacs Accelerate fellowship and Canadian Blood Services Graduate fellowship. I would also like to thank Dr. Jason Acker and his research team (Tracey Turner, Angela Hill, Adele Hansen) at Canadian Blood Services in Edmonton, Alberta for all of their help with the red blood cell project. I greatly appreciate all of your pre-trip planning and preparation and I can’t thank you enough for all of your contributions to this thesis. I would also like to acknowledge all of the other collaborators that I have worked with including Dr. Brendan Wilkinson at the University of New England; Dr. Janelle Sauvageau at the National Research Council; Dr. Maxim Berezovski and Dr. Gleb

Mironov at the University of Ottawa and Dr. David Courtman and Liliana Davila at the Ottawa Hospital Research Institute.

I am grateful to my family for their constant love and support; thank you to my mom, dad and my sisters. Thanks for your understanding, your encouragement, and for always being there for me. Your support has meant more to me than you could possibly realize.

To my furry four-legged friend, thank you for keeping me company during the writing process and for being a good listener.

Finally, I would like to thank my husband, Alex, for his love, support and constant encouragement during the challenges of graduate school and life, and for always showing how proud he is of me. I can't thank you enough for all that you have done for me and I am so lucky to have you in my life.

Abstract

Recent advances in the clinical diagnosis and treatment of diseases using cell transplantation have emphasized the urgent need to cryopreserve many types of cells. In transfusion medicine, red blood cell (RBC) transfusions are used to treat anemia and inherited blood disorders, replace blood lost during or after surgery and treat accident victims and mass casualty events. In regenerative medicine, mesenchymal stem cell (MSC) therapy offers promising treatment for tissue injury and immune disorders. Current cryoprotective agents (CPAs) utilized for RBCs and MSCs are 40% glycerol and 10% dimethyl sulfoxide (DMSO), respectively. Although glycerol is required for successful cryopreservation of RBCs, it must be removed from RBCs post-thaw using costly and time-consuming deglycerolization procedures to avoid intravascular hemolysis. Unfortunately, while DMSO prevents cell damage and increases post-thaw MSC viability and recovery, recent reports have suggested that MSCs cryopreserved in DMSO display compromised function post-thaw. As a result, improvements to the current cryopreservation protocols such as reducing post-thaw RBC processing times and improving MSC function post-thaw are necessary in order to meet the increasing demands of emerging cellular therapies.

Ice recrystallization has been identified as a significant contributor to cellular injury and death during cryopreservation. Consequently, the ability to inhibit ice recrystallization is a very desirable property for an effective CPA, unlike the conventional CPAs such as DMSO and glycerol that function via a different mechanism and do not control or inhibit ice recrystallization. Over the past few years, our laboratory has reported several different classes of small molecules capable of inhibiting ice recrystallization such as lysine-based surfactants, non-ionic carbohydrate-based amphiphiles (alkyl and aryl aldonamides) and *O*-linked alkyl and aryl glycosides. The use of these small molecule ice recrystallization inhibitors (IRIs) as novel CPAs has become an important strategy to improve cell viability and function post-thaw.

With the overall goal to identify highly effective inhibitors of ice recrystallization, the first part of this thesis examines the IRI activity of three diverse classes of small molecules including carbohydrate-based surfactants bearing an azobenzene moiety, fluorinated aryl glycosides and phosphate sugars. While the majority of the carbohydrate-

based surfactants and fluorinated aryl glycosides were not effective inhibitors of ice recrystallization, this work revealed that monosaccharides possessing a phosphate group could be effective IRIs.

Our laboratory has previously demonstrated that small molecule IRIs β -PMP-Glc and β -pBrPh-Glc can protect human RBCs from cellular injury during freezing using reduced concentrations of glycerol (15% w/v). This was significant as reducing the concentration of glycerol can drastically decrease deglycerolization times. Consequently, structure-function studies were conducted on β -PMP-Glc and β -pBrPh-Glc to elucidate key structural features that further enhance their IRI activity and may increase their cryoprotective ability. In particular, the influence of an azido moiety on the IRI activity of β -PMP-Glc and β -pBrPh-Glc was investigated and it was determined that the position of the azide substituent on the pyranose ring is crucial for effective inhibition of ice recrystallization. Furthermore, the presence of an azido group at C-3 was found to increase the IRI activity of β -PMP-Glc and β -pBrPh-Glc.

Despite the discovery that β -PMP-Glc and β -pBrPh-Glc are beneficial additives for the freezing of RBCs, a significant amount of cellular damage occurred during deglycerolization, resulting in very low cell recoveries. Thus, IRI active azido aryl glucosides were explored for their cryopreservation potential in RBCs to determine whether they could function as effective additives that reduce cellular damage post-thaw and improve cell recovery. One of the most significant results of this thesis is the discovery that azido aryl glucosides can successfully cryopreserve RBCs in the presence of 15% glycerol with significantly improved cell recovery. This thesis also explores the use of small molecule IRIs to improve the cryopreservation of MSCs. In particular, the addition of an *N*-aryl-alDONamide (2FA) to the standard 10% DMSO solution was found to enhance the proliferative capacity of MSCs post-thaw.

Lastly, the ability of small molecule IRIs to cross the cell membrane and behave as permeating CPAs was evaluated in two different cell models, RBCs and human umbilical vein endothelial cells (HUVECs). These studies demonstrated that small molecule IRIs are capable of permeating the cell membrane and controlling intracellular ice recrystallization.

Table of Contents

Acknowledgements.....	iii
Abstract.....	v
Table of Contents.....	vii
List of Figures.....	xii
List of Tables.....	xvii
List of Schemes.....	xviii
List of Abbreviations.....	xix

Chapter 1: Introduction – Cryopreservation and Small Molecule Ice Recrystallization Inhibitors

1.1 Principles of Cryopreservation.....	1
1.1.1 Cellular Preservation Strategies.....	2
1.1.2 Cryopreservation – Cellular Injury and the Role of Ice Recrystallization.....	3
1.1.3 Cryoprotective Agents to Improve Post-Thaw Recovery.....	5
1.1.4 Long-Term Storage of Red Blood Cells.....	5
1.1.5 Cryopreservation of Mesenchymal Stem Cells.....	9
1.2 Controlling Ice Recrystallization During Cryopreservation: A Novel Approach ...	10
1.2.1 Structure of Ice.....	11
1.2.2 Recrystallization in Ice.....	12
1.2.3 Inhibiting Ice Recrystallization with Molecules Other than BAs.....	14
1.3 Small Molecule Ice Recrystallization Inhibitors.....	16
1.3.1 Simple Mono- and Disaccharides as Ice Recrystallization Inhibitors.....	17
1.3.2 Carbohydrate-Based Surfactants as Ice Recrystallization Inhibitors.....	19
1.3.3 Aryl Glycosides as Ice Recrystallization Inhibitors.....	20
1.4 Chapter Summary.....	21
1.5 References.....	22

Chapter 2: Goals and Objectives

2.1 Introduction.....	31
2.2 Objective 1 – Determining Structural Features Important for the IRI Activity of Small Molecules.....	34
2.2.1 Photoswitchable Surfactants.....	34
2.2.2 Fluorinated Aryl Glycosides.....	36
2.2.3 Phosphate Sugars.....	37
2.3 Objective 2 – Determining the Influence of an Azido Moiety on the IRI Activity of Aryl Glucosides.....	38

2.4 Objective 3 – Assessing the Cryopreservation Potential of Small Molecule Ice Recrystallization Inhibitors	39
2.5 Objective 4 – Investigating the Uptake and Internalization of Small Molecule Ice Recrystallization Inhibitors	42
2.6 References	43

Chapter 3: Determining Structural Features Important for the IRI Activity of Small Molecules

3.1 Introduction	46
3.2 Photoswitchable Surfactants as Ice Recrystallization Inhibitors	49
3.2.1 Synthesis of Photoswitchable Surfactants	51
3.2.2 IRI Activity of Photoswitchable Surfactants	52
3.3 Fluorinated Aryl Glycosides as Ice Recrystallization Inhibitors	60
3.3.1 Synthesis of Fluorinated Aryl Glycosides	62
3.3.2 IRI Activity of Fluorinated Aryl Glycosides	63
3.4 Phosphate Sugars as Ice Recrystallization Inhibitors	67
3.4.1 IRI Activity of Phosphate Sugars	68
3.5 Chapter Summary	71
3.6 Experimental Procedures and Characterization Data	72
3.6.1 Traditional Ice Recrystallization Inhibition (IRI) Assay	72
3.6.3 Thermal Hysteresis (TH) Assay.....	73
3.6.4 General Experimental	74
3.6.5 Compound Characterization	75
3.6.6 NMR Spectra	79
3.7 References	85

Chapter 4: Determining the Influence of an Azido Moiety on the IRI Activity of Aryl Glucosides

4.1 Introduction	87
4.2 Azido Aryl Glucosides as Ice Recrystallization Inhibitors	88
4.2.1 Structures and Synthesis of Azido Aryl Glucosides	89
4.2.2 IRI Activity of Azido Aryl Glucosides.....	94
4.2.3 Assessing Cytotoxicity of IRI Active Azido Aryl Glucosides	96
4.2.3.1 Measuring Apoptosis of IRI Active Azido Aryl Glucosides	98

4.3 Amino Aryl Glucosides as Ice Recrystallization Inhibitors	102
4.3.1 Structures and Synthesis of Amino Aryl Glucosides.....	102
4.3.2 IRI Activity of Amino Aryl Glucosides.....	104
4.4 Chapter Summary	107
4.5 Experimental Procedures and Characterization Data	108
4.5.1 Quantitative Analysis of Ice Recrystallization Inhibition (IRI) Activity.....	108
4.5.2 HepG2 Cell Culture	109
4.5.3 MTT Viability Assay with HepG2 Cells	109
4.5.4 Apo-ONE® Homogeneous Caspase-3/7 Assay with HepG2 Cells.....	110
4.5.5 General Experimental	110
4.5.6 Compound Characterization	111
4.5.7 NMR Spectra	133
4.6 References	170

Chapter 5: Assessing the Cryopreservation Potential of Small Molecule Ice Recrystallization Inhibitors

5.1 Introduction	173
5.2 Cryopreservation Ability of Small Molecule IRIs with Human RBCs	175
5.2.1 Introduction – Cryopreservation of Human RBCs	175
5.2.2 Cryopreservation of RBCs using β -PMP-Glc and β -pBrPh-Glc in the Presence of 15% Glycerol	177
5.2.2.1 Removal of Excess Cryosolution Pre-Freeze	180
5.2.3 Cryopreservation of RBCs using β -PMP-Glc and β -pBrPh-Glc in the Presence of Hydroxyethyl Starch	183
5.2.4 Azido Aryl Glucosides as Cryoprotectants for RBCs.....	188
5.2.4.1 <i>In Vitro</i> Toxicity of Azido Aryl Glucosides in RBCs.....	191
5.2.4.2 Freezing of RBCs using Azido Aryl Glucosides in the Presence of 15% Glycerol	197
5.2.4.3 Osmotic Tolerance of RBCs after Treatment with 15% Glycerol and Azido Aryl Glucosides	200
5.2.4.4 Post-Thaw Deglycerolization of RBCs in the Presence of 15% Glycerol and Azido Aryl Glucosides	204
5.2.4.5 Large-Scale Cryopreservation of RBCs in the Presence of 409 and 15% Glycerol.....	206
5.2.4.6 RBC Functional Analysis After Cryopreservation with 409 + 15% Glycerol	212
5.2.5 Summary for RBC Cryopreservation.....	218
5.3 Cryopreservation Ability of Small Molecule IRIs with MSCs	220
5.3.1 Introduction – Cryopreservation of MSCs.....	220

5.3.2 Cryopreservation of MSCs using β -pBrPh-Glc and 2FA in the Presence of DMSO	222
5.3.3 Summary for MSC Cryopreservation	227
5.4 Experimental Procedures	228
5.4.1 Blood Collection and Preparation	228
5.4.2 RBC Freezing and Thawing Experiments using 15% Glycerol	228
5.4.3 Post-Thaw Hemolysis	229
5.4.4 RBC Deglycerolization Experiments	229
5.4.5 Calculation of RBC Recovery	230
5.4.6 RBC Deglycerolization Experiment with Removal of Excess Cryosolution Pre-Freeze	230
5.4.7 RBC Freezing and Thawing Experiments using 11.5% HES	230
5.4.8 RBC <i>In Vitro</i> Toxicity (Hypothermic Storage)	231
5.4.9 RBC Indices	231
5.4.10 Hemoglobin Oxidation	231
5.4.11 Ektacytometric Analysis	231
5.4.12 RBC Osmotic Tolerance	231
5.4.13 RBC Freezing and Thawing Experiment using Different RBC:CPA Ratios	232
5.4.14 Large-Scale RBC Deglycerolization Experiment	232
5.4.15 Adenosine Triphosphate Concentration	233
5.4.16 2,3-Diphosphoglycerate Concentration	233
5.4.17 Oxygen Affinity (p50)	234
5.4.18 Extracellular K^+ and Na^+	234
5.4.19 MSC Culture	234
5.4.20 MSC Freezing and Thawing Experiments	234
5.4.21 MSC Viability	235
5.4.22 MSC Recovery	235
5.4.23 EdU Cell Proliferation Assay	235
5.4.24 MSC Population Doublings and Doubling Time	236
5.5 References	236
 Chapter 6: Investigating the Ability of Small Molecule IRIs to Mitigate Cell Lysis During Transient Warming	
6.1 Introduction	243
6.2 Controlling Ice Growth <i>In Vitro</i>	244
6.3 Exacerbating Cellular Injury from Ice Recrystallization	247
6.4 Chapter Summary	251
6.5 Experimental Procedures	252

6.5.1 Blood Collection and Preparation	252
6.5.2 RBC Cryomicroscopy	252
6.5.3 RBC Transient Warming Experiments	252
6.5.4 Post-Thaw Hemolysis	253
6.6 References	253
 Chapter 7: Investigating the Uptake and Internalization of Small Molecule Ice Recrystallization Inhibitors	
7.1 Introduction	255
7.2 Internalization Studies for the Freezing of RBCs in the Presence of IRIs	257
7.2.1 Interrupted Graded Freezing of RBCs	257
7.2.2 The Effect of Pre-Freeze Incubation on Post-Thaw Recovery of RBCs	262
7.3 Assessing the Uptake of Small Molecule IRIs by RBCs	266
7.4 Controlling Intracellular Ice Recrystallization in HUVECs	272
7.4.1 Assessing Cytotoxicity of β -pBrPh-Glc in HUVECs	279
7.5 Chapter Summary	282
7.6 Experimental Procedures	283
7.6.1 Blood Collection and Preparation	283
7.6.2 Interrupted Graded Freezing of RBCs	284
7.6.3 Post-Thaw Hemolysis	284
7.6.4 RBC Incubation Experiments	284
7.6.5 RBC Wet Preparation Microscopy	285
7.6.6 RBC Internalization Experiments	285
7.6.7 RBC Internalization Experiments in the Presence of GLUT-1 Inhibitor	286
7.6.8 Capillary Electrophoresis-Mass Spectrometry	286
7.6.9 HUVEC Culture for Cryomicroscopy Experiments	286
7.6.10 Freezing of HUVEC Monolayers and Visualization of Intracellular Ice	287
7.6.11 HUVEC Culture for Cytotoxicity Experiments	288
7.6.12 Alamar Blue Assay with HUVECs	288
7.7 References	288
 Conclusions	 291
Future Directions	294
Contributions to Original Research	296
Publications Resulting from this Thesis	296

List of Figures

Chapter 1:

Figure 1.1 The relationship between cell survival and cooling rate for various cell types	3
Figure 1.2 Schematic of physical events in cells during freezing.....	4
Figure 1.3 Hexagonal ice I_h lattice unit consisting of four axes a_1 , a_2 , a_3 , and c , and the basal, prism and pyramidal planes.....	11
Figure 1.4 Schematic of grain boundary migration where the shaded area represents the liquid layer between two curved grain boundaries.....	13
Figure 1.5 Ice crystal morphologies in the presence of various BAs: (A) AFGP-8; (B) WT <i>Lp</i> AFP; (C) Type I AFP; (D) Type III AFP at 1 mg/mL in water.....	14
Figure 1.6 Correlation between carbohydrate hydration index and IRI activity	18
Figure 1.7 Proposed mechanism for ice recrystallization inhibition	19
Figure 1.8 Structures of <i>n</i> -octyl- β -D-glycosides 101 and 102	20
Figure 1.9 General structure of the <i>O</i> -aryl glycosides and structures of effective ice recrystallization inhibitors in this class of molecules (103-105).....	21

Chapter 2:

Figure 2.1 Flow chart describing the screening and selection of molecules for cell cryopreservation	33
Figure 2.2 General structures of carbohydrate-based surfactants	36
Figure 2.3 General structure of carbohydrate-based fluorosurfactants.....	36
Figure 2.4 Structures of fluorinated aryl glycosides	37
Figure 2.5 Structures of monosaccharide phosphate sugars	38
Figure 2.6 Structures of β -PMP-Glc, β -pBrPh-Glc and proposed azide derivatives	39
Figure 2.7 Structures of β -PMP-Glc, β -pBrPh-Glc and β - <i>para</i> -azidophenyl-D-glucopyranoside.....	41
Figure 2.8 Structure of <i>N</i> -(2-fluorophenyl)-D-gluconamide (2FA).....	42

Chapter 3:

Figure 3.1 Structure of <i>n</i> -octyl- β -D-galactopyranoside 102	47
Figure 3.2 Structures of <i>O</i> -aryl glycosides 103-105	48
Figure 3.3 Reversible photoisomerization of azobenzene upon UV-vis irradiation.....	49
Figure 3.4 Structures of carbohydrate-based surfactants bearing an <i>n</i> -butyl azobenzene tail group and a variable mono- or disaccharide head group.....	51
Figure 3.5 Structures of carbohydrate-based fluorosurfactants possessing a variable mono- or disaccharide head group conjugated to a trifluoromethyl-substituted azobenzene tail group via a triazole linkage (311-322)	51
Figure 3.6 Ice recrystallization inhibition (IRI) activity of compounds 301-310	54

Figure 3.7 A dose-response curve for compound 303	56
Figure 3.8 Ice crystal morphology in the presence of compound 303 (0.5 mg mL ⁻¹) in water	56
Figure 3.9 Ice recrystallization inhibition (IRI) activity of compounds 311-322	57
Figure 3.10 Ice recrystallization inhibition (IRI) activity of compounds 301-303	59
Figure 3.11 Ice recrystallization inhibition (IRI) activity of compounds 311-313 , 316 , 319 and 322	60
Figure 3.12 Structures of β -PMP-Glc, β -pBrPh-Glc and β -pFPh-Glc	61
Figure 3.13 Structures of fluorinated aryl glycosides 323-328	62
Figure 3.14 A dose-response curve for compound 327	65
Figure 3.15 Dose-response curves for compounds 103-105	66
Figure 3.16 Structures of monosaccharide phosphate sugars 332-338 assessed for IRI activity	68
Figure 3.17 Dose-response curves for compounds 334-338	70

Chapter 4:

Figure 4.1 Structures of β -PMP-Glc (103) and β -pBrPh-Glc (104)	88
Figure 4.2 Structures of azido targets (401-409) based upon 103 and 104	89
Figure 4.3 Dose-response curves for azido aryl glucosides 405 , 406 and 409	96
Figure 4.4 Dose-response curves for azido aryl glucosides 405 , 406 and 409	98
Figure 4.5 The intrinsic (a) and extrinsic (b) apoptotic pathways	99
Figure 4.6 Cleavage of the non-fluorescent caspase substrate Z-DEVD-R110 by caspases-3/7 to create the fluorescent Rhodamine 110	100
Figure 4.7 Apo-ONE® Homogeneous Caspase-3/7 assay using HepG2 cells incubated with 405 (9 mM), 406 (3 mM) or 409 (12 mM) for 18 h at 37 °C	101
Figure 4.8 Structures of amino targets (441-445)	102
Figure 4.9 Dose-response curves for amino aryl glucosides 441-443	105
Figure 4.10 Dose-response curve for ammonium formate	106

Chapter 5:

Figure 5.1 Structures of IRI active aryl glucosides 103 (β -PMP-Glc) and 104 (β -pBrPh-Glc), azido aryl glucosides (405 , 406 and 409) and <i>N</i> -aryl-D-alidonamide 501 (2FA)	175
Figure 5.2 Percentage of intact RBCs post-thaw using 15% glycerol in combination with 103 (110 mM) or 104 (30 mM)	178
Figure 5.3 Percentage RBC recovery using 15% glycerol in combination with 103 (110 mM) or 104 (30 mM)	180
Figure 5.4 Percentage of intact RBCs post-process using 15% glycerol in combination with 103 (110 mM) or 104 (30 mM)	181

Figure 5.5 Percentage of hemoglobin lost post-thaw and in each wash step during the deglycerolization process when the excess cryosolution was removed prior to freezing	182
Figure 5.6 Percentage of intact RBCs post-thaw using HES (11.5% w/w in 30 mM NaCl) in combination with 103 (110 mM) or 104 (30 mM)	184
Figure 5.7 Percentage RBC recovery post-thaw using HES (11.5% w/w in 30 mM NaCl) in combination with 103 (110 mM) or 104 (30 mM)	185
Figure 5.8 Percentage of intact RBCs post-thaw after 48 hours of storage at 4 °C in a refrigerator	186
Figure 5.9 Percentage RBC recovery post-thaw after a simple wash procedure	187
Figure 5.10 Osmotic tolerance of RBCs incubated with SAGM or cryosolutions	190
Figure 5.11 Osmotic tolerance of RBCs after wash 1	190
Figure 5.12 Structures of azido aryl glucosides (405 , 406 and 409)	191
Figure 5.13 Percentage of RBC hemolysis after hypothermic storage (4 °C) with the indicated solution for up to 7 days	192
Figure 5.14 Mean cell volume after hypothermic storage (4 °C) with the indicated solution for up to 7 days	193
Figure 5.15 Percentage of methemoglobin after hypothermic storage (4 °C) with the indicated solution for up to 7 days	194
Figure 5.16 RBC deformability parameters ((A) EI_{max} and (B) K_{EI} after hypothermic storage (4 °C) with the indicated solution for up to 7 days.....	196
Figure 5.17 Percentage of intact RBCs post-thaw using 15% glycerol in combination with 405 (30 mM), 406 (10 mM) or 409 (20 mM).....	197
Figure 5.18 Percentage of intact RBCs post-thaw using 15% glycerol in combination with 409 at the indicated concentrations	199
Figure 5.19 Percentage of intact RBCs post-thaw using various concentrations of glycerol in combination with 409 (20 mM)	200
Figure 5.20 Osmotic tolerance of RBCs incubated with SAGM or cryosolutions	201
Figure 5.21 Osmotic tolerance of RBCs after wash 1	202
Figure 5.22 Comparison of osmotic tolerance profiles for RBCs suspended in the indicated solution after wash 1	203
Figure 5.23 Percentage of hemoglobin lost post-thaw and in each wash step during the deglycerolization process	205
Figure 5.24 Percentage RBC recovery after deglycerolization using 15% glycerol in combination with 405 (30 mM), 406 (10 mM) or 409 (20 mM)	206
Figure 5.25 Percentage of intact RBCs post-thaw following cryopreservation at different RBC:CPA ratios.....	208
Figure 5.26 Percentage of intact RBCs post-thaw using 15% glycerol in combination with 409 (20 mM).....	210
Figure 5.27 Percentage of hemoglobin lost in each wash step during the deglycerolization process	211

Figure 5.28 Percentage RBC recovery after deglycerolization using 15% glycerol in combination with 409 (20 mM)	212
Figure 5.29 Structures of 104 (β -pBrPh-Glc) and 501 (2FA).....	223
Figure 5.30 Cooling profiles for solutions of 104 (A) or 501 (B) in 5% HA-PLA and 10% DMSO using the optimized step-down program.....	224
Figure 5.31 Morphology of post-thaw MSCs after three days in culture	226

Chapter 6:

Figure 6.1 Structures of IRI active aryl glucosides 103 (β -PMP-Glc) and 104 (β -pBrPh-Glc) and azido aryl glucosides (405 , 406 and 409).....	244
Figure 6.2 Images of ice in the presence of (A) 15% glycerol; (B) 15% glycerol + RBCs; (C) 104 (30 mM) + 15% glycerol; (D) 104 (30 mM) + 15% glycerol + RBCs	245
Figure 6.3 Images of ice in the presence of RBCs with (A) 15% glycerol; (B) 104 (30 mM) + 15% glycerol.....	246
Figure 6.4 Images of ice in the presence of RBCs with (A1-A4) 15% glycerol; (B1-B4) 104 (30 mM) + 15% glycerol.....	247
Figure 6.5 A schematic representation of the transient warming experiment	248
Figure 6.6 Percentage of intact RBCs post-thaw after transient warming injury using 15% glycerol in combination with 103 (110 mM) or 104 (30 mM)	249
Figure 6.7 Percentage of intact RBCs post-thaw after transient warming injury using 15% glycerol in combination with 405 (30 mM), 406 (10 mM) or 409 (20 mM)	250

Chapter 7:

Figure 7.1 Interrupted graded freezing of RBCs with 103 (β -PMP-Glc)	256
Figure 7.2 Interrupted graded freezing of RBCs with 104 (β -pBrPh-Glc)	258
Figure 7.3 Interrupted graded freezing of RBCs with 409	260
Figure 7.4 The effect of pre-freeze incubation of cryosolutions on the percentage of intact RBCs post-thaw	263
Figure 7.5 The effect of pre-freeze incubation of cryosolutions on the percentage of intact RBCs post-thaw	264
Figure 7.6 The effect of incubation of cryosolutions on RBC morphology	265
Figure 7.7 Structures of <i>O</i> -aryl glucosides assessed in RBC uptake experiments	266
Figure 7.8 Possible model for sugar transport in the human red blood cell	268
Figure 7.9 Flow chart of the experimental protocol of the RBC uptake study	268
Figure 7.10 Schematic diagram of the interior of a capillary tube in the presence of a buffer solution	269

Figure 7.11 Values of area under the curve (AUC) for RBC samples treated with the indicated IRI in the presence (“+ inhibitor”) or absence (“- inhibitor”) of the GLUT1 inhibitor (cytochalasin B)	271
Figure 7.12 Intracellular ice formation at different warming rates.....	274
Figure 7.13 Mean grain size (mm ²) of ice crystals as a function of temperature (°C) under faster (25 °C/min) and slower thawing (2 °C/min) conditions	275
Figure 7.14 Intracellular ice formation at different warming rates in the presence of 110 mM 103 (β-PMP-Glc).....	276
Figure 7.15 Intracellular ice formation at different warming rates in the presence of 30 mM 104 (β-pBrPh-Glc).....	277
Figure 7.16 Correlation of intracellular ice crystal size (mm ²) as a function of temperature (°C) using a warming rate of 25 °C/min	278
Figure 7.17 Correlation of intracellular ice crystal size (mm ²) as a function of temperature (°C) using a warming rate of 2 °C/min	278
Figure 7.18 Percent cell viability of HUVECs treated with various concentrations of 104 (β-pBrPh-Glc) following incubation for 24 h at 37 °C as determined by Alamar Blue assay	280
Figure 7.19 Percent cell viability of HUVECs treated with 104 (β-pBrPh-Glc) and its individual components (Glc and pBrPh) following incubation for 24 h at 37 °C as determined by Alamar Blue assay	281

List of Tables

Chapter 3:

Table 3.1 Normalized rate constants (k_{norm}) for fluorinated aryl glycosides 323-328	65
Table 3.2 IC ₅₀ values for fluorinated aryl glycosides 323-328	65
Table 3.3 Normalized rate constants (k_{norm}) for phosphate sugars 332-338	69
Table 3.4 IC ₅₀ values for phosphate sugars 332-338	69

Chapter 4:

Table 4.1 Normalized rate constants (k_{norm}) for azido aryl glucosides 401-409	95
Table 4.2 IC ₅₀ values for azido aryl glucosides 401-409	95
Table 4.3 Normalized rate constants (k_{norm}) for amino aryl glucosides 441-445	105
Table 4.4 IC ₅₀ values for amino aryl glucosides 441-445	105

Chapter 5:

Table 5.1 Measured osmolalities (mOsm/kg) of NaCl solutions.....	189
Table 5.2 RBC quality measurements after 24 hours and 7 days of post-thaw storage	214
Table 5.3 Cell viability, recovery and proliferation post-thaw	225
Table 5.4 Cell viability, population doubling and doubling time after 3 days in culture post-cryopreservation	227

List of Schemes

Chapter 3:

Scheme 3.1 Synthesis of carbohydrate-based surfactants 301-304	52
Scheme 3.2 Synthesis of carbohydrate-based surfactants 305-322	52
Scheme 3.3 Synthesis of fluorinated aryl glycosides 323-328	63

Chapter 4:

Scheme 4.1 Synthesis of 6-azido-6-deoxy- β -D-glucopyranoside derivatives 401 and 402	90
Scheme 4.2 Synthesis of 4-azido-4-deoxy- β -D-glucopyranoside derivatives 403 and 404	91
Scheme 4.3 Synthesis of 3-azido-3-deoxy- β -D-glucopyranoside derivatives 405 and 406	92
Scheme 4.4 Synthesis of 2-azido-2-deoxy- β -D-glucopyranoside derivatives 407 and 408	93
Scheme 4.5 Synthesis of <i>p</i> -azidophenyl- β -D-glucopyranoside 409	94
Scheme 4.6 Synthesis of 6-amino-6-deoxy- β -D-glucopyranoside derivatives 442 (A) and 443 (B) from the corresponding azido aryl glucosides 401 and 402	103
Scheme 4.7 Synthesis of 3-amino-3-deoxy- β -D-glucopyranoside derivatives 444 (A) and 445 (B) from the corresponding azido aryl glucosides 405 and 406	104

List of Abbreviations

α	Alpha
β	Beta
δ	Delta
^1H	Proton
^{13}C	Carbon
$(\text{COCl})_2$	Oxalyl chloride
2,3-DPG	2,3-Diphosphoglycerate
3-OMG	3- <i>O</i> -Methyl-D-glucose
Ac	Acetyl
Ac_2O	Acetic anhydride
AcOH	Acetic acid
ACP	Automated cell processor
AFGP(s)	Antifreeze glycoprotein(s)
AFP(s)	Antifreeze protein(s)
AS-3	Additive solution-3
ATP	Adenosine triphosphate
AUC	Area under the curve
BA(s)	Biological antifreeze(s)
$\text{BF}_3 \cdot \text{OEt}_2$	Boron trifluoride diethyl etherate
BnNH_2	Benzylamine
BSA	Bovine serum albumin
Bz	Benzoyl
BzOCl	Benzoyl chloride
C-linked	<i>Carbon</i> -linked
Cbz	Carboxybenzyl
CbzCl	Benzyl chloroformate
CCB	Cytochalasin B
CCl_3CN	Trichloroacetonitrile
CDCl_3	Deuterated chloroform
CE	Capillary electrophoresis
CH_2Cl_2	Dichloromethane
CPA(s)	Cryoprotective agent(s)
CPD	Citrate phosphate dextrose
CuAAC	Copper(I)-catalyzed azide-alkyne cycloaddition
CuSO_4	Copper(II) sulfate
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	Copper(II) sulfate pentahydrate

d	Doublet
D ₂ O	Deuterium oxide
Da	Dalton
DAST	Diethylaminosulfur trifluoride
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
dd	Doublet of doublets
dddd	Doublet of doublet of doublet of doublets
DIS	Dynamic ice shaping
DISC	Death-inducing signaling complex
DMAP	4-Dimethylaminopyridine
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DRS	Domain recognition software
EdU	5-Ethynyl-2'-deoxyuridine
EGM	Endothelial growth medium
ESI	Electrospray ionization
Et ₃ N	Triethylamine
EtOH	Ethanol
FADD	FAS-associated protein with death domain
FBS	Fetal bovine serum
Glc	Glucose
GLUT(s)	Glucose transporter(s)
H ₂ SO ₄	Sulfuric acid
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
Hct	Hematocrit
HepG2	Human liver hepatocellular carcinoma cells
HES	Hydroxyethyl starch
Hgb	Hemoglobin
HgCl ₂	Mercuric chloride
HI	Hydration index
HSA	Human Serum Albumin
HSPC(s)	Hematopoietic stem and progenitor cell(s)
HUVEC(s)	Human umbilical vein endothelial cell(s)
Hz	Hertz
IC ₅₀	Half maximal inhibitory concentration
IIF	Intracellular ice formation

IRI	Ice recrystallization inhibition
IRI(s)	Ice recrystallization inhibitor(s)
K ₂ CO ₃	Potassium carbonate
kg	Kilogram
KI	Potassium iodide
LORCA	Laser assisted optical rotational cell analyzer
LRMS	Low resolution mass spectrometry
m	Multiplet
m/z	Mass-to-charge ratio
M ⁺	Parent molecular ion
MCV	Mean cell volume
MeCN	Acetonitrile
MEM	Minimum essential media
MeOD	Deuterated methanol
MeOH	Methanol
MetHb	Methemoglobin
MGS	Mean grain size
MgSO ₄	Magnesium sulfate
MHz	Mega hertz
mM	Millimolar
mmHg	Millimeter of mercury
mOsm	Milliosmole
MS	Molecular sieves
MS	Mass spectrometry
MSC(s)	Mesenchymal stem cell(s)
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaBH ₄	Sodium borohydride
NaCl	Sodium chloride
NaN ₃	Sodium azide
NaOAc	Sodium acetate
NaOMe	Sodium methoxide
NH ₄ HCO ₂	Ammonium formate
NMR	Nuclear magnetic resonance
O/N	Overnight
p50	Partial pressure of oxygen at which hemoglobin is 50% saturated
pBrPh	<i>para</i> -Bromophenol
PBS	Phosphate buffered saline
Pd(OH) ₂	Palladium hydroxide

PI	Propidium iodide
PLA	PlasmaLyte A
PSS	Photostationary state
PVA	Poly(vinyl alcohol)
PVP	Polyvinylpyrrolidone
py	Pyridine
q	Quartet
QLL	Quasi-liquid layer
quint	Quintet
RBC(s)	Red blood cell(s)
RFUs	Relative fluorescence units
RT	Room temperature
s	Singlet
SAGM	Saline-adenine-glucose-mannitol
SEM	Standard error of the mean
SMAC	Second mitochondrial activator of caspases
SO ₂ Cl ₂	Sulfuryl chloride
t	Triplet
<i>t</i> -BuOH	<i>tert</i> -Butanol
<i>t</i> -BuONO	<i>tert</i> -Butyl nitrite
TCA	Tricarboxylic acid
Tf ₂ O	Triflic anhydride
TFA	Trifluoroacetic acid
TfN ₃	Triflyl azide
TH	Thermal hysteresis
THF	Tetrahydrofuran
TMS-N ₃	Azidotrimethylsilane
TsCl	Tosyl chloride
TWE(s)	Transient warming event(s)
UCB	Umbilical cord blood
UV	Ultraviolet
w/v	weight/volume
WB	Whole blood
WBC(s)	White blood cell(s)

Chapter 1: Introduction – Cryopreservation and Small Molecule Ice Recrystallization Inhibitors

1.1 Principles of Cryopreservation

Cryopreservation is the process whereby biological samples such as cells and tissues are preserved by cooling to subzero temperatures (-78 °C or -196 °C).¹ Cryobiology, the study of life at low temperatures, is an emerging and rapidly expanding field. The recognition of the importance of cryopreservation to modern medicine is drastically increasing. Recent advances in the clinical diagnosis and treatment of diseases using cell and organ transplantation have accelerated cryobiology research and have emphasized the urgent need to cryopreserve and bank many types of living cells and tissues at low temperatures.^{2,3} In the past decade, the explosion of technologies (e.g. nanotechnology, sequencing, imaging and omics approaches) has facilitated an understanding of human physiology at the tissue and organ level, making it possible to dramatically accelerate progress in tissue and organ preservation.⁴

The need to cryopreserve cells is rapidly increasing due to the high demand of various cell types in clinical medicine such as red blood cells (RBCs) and mesenchymal stem cells (MSCs).⁵ In transfusion medicine, the demand for blood products has continually increased over the past decade. Due to the short shelf life of RBCs (i.e. 42 days),⁶ hundreds of units are discarded per year, resulting in significant waste.⁷ Furthermore, given the limited storage time of blood, local shortages are likely to occur and supply can become disrupted in the wake of mass casualty events.^{8,9} Therefore, RBC cryopreservation has a significant impact on blood supply as it can extend storage time and prevent frequent blood shortage and disposal of outdated units. In regenerative medicine, MSC therapy offers promising treatment for tissue injury and immune disorders, and consequently the number of registered clinical trials has been steadily increasing since 2004.¹⁰ Despite the potential of MSC therapy, these products are complex biological entities that introduce new challenges. One of the current major challenges is product storage, to transport cell therapy products between laboratories and hospitals and clinics at the required time, ensuring the delivery of a high quality, safe and efficacious product.^{11,12} Long-term cryopreservation of therapeutic cells is critical to the

ongoing development of cell therapy products as the success of its broad distribution and use depend largely on the ability to reliably store and transport these products.¹²

1.1.1 Cellular Preservation Strategies

There are many methods that are used to preserve cells and tissues; however, the three common strategies are hypothermic preservation, cryopreservation and vitrification. Hypothermic storage refers to the preservation of cells and tissues at temperatures ranging between 4 and 10 °C and is often used for short-term transportation.¹³ The hypothermia endured by cells at these temperatures reduces metabolism, energy storage degradation and oxygen demand.¹⁴ The use of preservation solutions enables cells to maintain ionic and osmotic balance, prevent free radical formation and provide energy substitutes.^{15,16} However, the temperature-dependent uncoupling of metabolic and biochemical processes results in the activation of numerous stress response mechanisms and cell death pathways such as apoptosis and necrosis that are detrimental to cell survival.^{14,15} As a result, hypothermic preservation is typically limited to a few days to weeks.¹⁵

Cryopreservation refers to the storage of cells and tissues at subzero temperatures ranging from -78 to -196 °C and extends storage time from months to years.¹⁷ While at these low temperatures all biological activities are stopped, the challenge of cryopreservation is to minimize the cellular injury and death that occurs during cooling and warming. Current strategies focus on reducing the detrimental effects of physicochemical stressors and membrane disruption that is associated with the formation of extra- and intracellular ice during the freeze-thaw process by employing cryoprotective agents (CPAs). The most effective CPA used in cryopreservation is dimethyl sulfoxide (DMSO); however, this solvent is toxic to both cells and patients^{18–20} and can necessitate its removal prior to clinical use. This process can greatly impact the quality of the post-thaw product.²¹ Given the toxic characteristics of DMSO,^{22,23} alternative non-toxic CPAs that afford the same protective ability are much needed.

Vitrification is an alternative approach to cryopreservation that enables cells and tissues to be stored at cryogenic temperatures in a glass-like state.²⁴ This approach avoids significant formation of extra- and intracellular ice,²⁵ thus reducing mechanical damage

from ice.²⁶ Although vitrification eliminates the requirement to find an optimal cooling and warming rate for each cell type, this process can only be achieved using a high concentration of CPA and ultra rapid cooling rates.^{26,27} The high concentration of CPA presents an even greater risk of toxicity. Furthermore, a greater challenge arises during the warming or thawing process of a vitrified sample where ice crystals are formed during warming. This is referred to as devitrification. Once these ice crystals are formed, they recrystallize causing cellular injury.²⁸ The rapid warming rate necessary to avoid devitrification is a major limitation since such rates cannot be achieved for larger samples such as tissues.²⁹ Overall, the challenges and implications encountered using these current preservation methods emphasize the need to improve CPAs and cryopreservation processes to meet the increasing demands of emerging cellular therapies.

1.1.2 Cryopreservation – Cellular Injury and the Role of Ice Recrystallization

Cellular injury resulting from the process of freezing and thawing in living cells is an issue that has been extensively studied.²⁸ The concept of cell and tissue preservation has led scientists to study the overall mechanisms underlying cellular injury that occur during exposure to cold temperatures for extended periods of time.³⁰ It is well known that the rate at which cells are cooled to low subzero temperatures significantly impacts the degree of survival. In cells that survive freezing, it was observed that plots of the percentage of survival versus cooling rate produced inverted U-shaped curves, shown in Figure 1.1.^{28,31} This demonstrated that there is an optimal cooling rate to ensure maximal cell survival for different cell types.^{28,31}

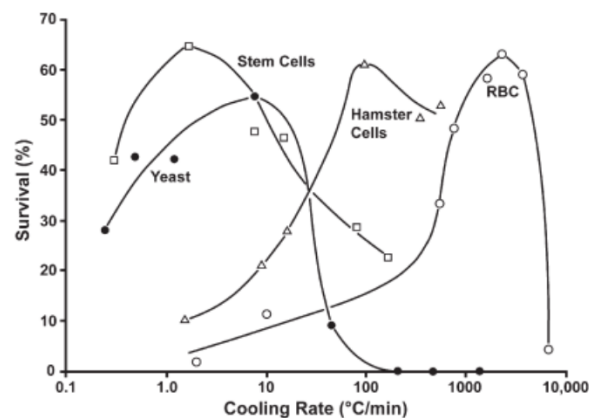


Figure 1.1 The relationship between cell survival and cooling rate for various cell types.³²

Mazur also demonstrated that the inverted U-shaped curve results from two opposing factors. This is known as Mazur's two-factor hypothesis and is depicted in Figure 1.2.^{33,34} Cells that are exposed to ice formation undergo extensive physical and chemical changes in their surrounding environment. The cooling of cells to approximately -5 °C causes the cells and extracellular medium to supercool and remain unfrozen. The formation of extracellular ice typically occurs spontaneously between the temperatures of -5 and -15 °C. During this time, the cellular contents remain supercooled and unfrozen, and water moves outside the cell in response to the formation of extracellular ice creating an osmotic gradient across the cell membrane.³¹ At this point, cells undergo two distinct physical events that are dependent on the rate of cooling. Under slow cooling conditions, the cells become severely dehydrated and experience significant volume shrinkage prior to freezing.³¹ Cellular injury typically results from excessive loss of volume and extended exposure to high concentrations of electrolytes as ice forms in the extracellular solution. This damaging effect was termed the “solute effect” and is lethal to cells.³⁵ Under fast cooling conditions, intracellular ice forms during freezing as the cells have insufficient time to dehydrate.^{33,36} The rate of water transport out of the cell is governed by the water permeability of the cell membrane, which is dependent on both temperature and cell type. Thus, each cell type has its own unique cooling rate to maximize post-thaw viability and recovery.³⁷

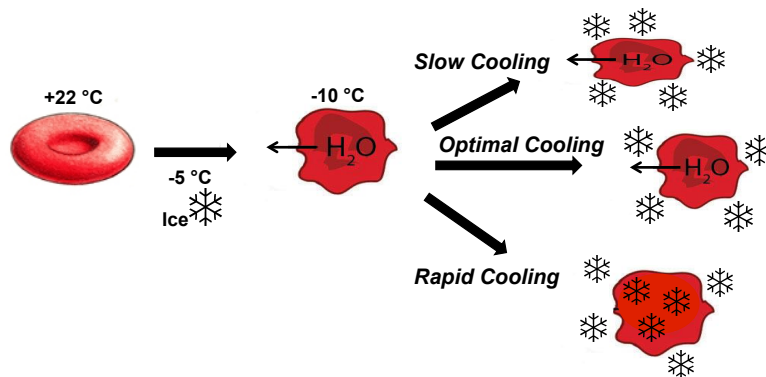


Figure 1.2 Schematic of physical events in cells during freezing.

As intracellular ice formation (IIF) is dependent upon the water content, cooling rates that result in IIF greatly vary among different cell types. While it is well documented that the formation of intracellular ice is detrimental to cell survival, more recent studies have shown that IIF itself is not necessarily damaging.^{37,38} The damaging effects of IIF are

attributed to the ice crystal size, whereby larger ice crystals are positively correlated with increased cell damage.³⁷ Nevertheless, while very rapid cooling rates can result in negligible cell dehydration and a large amount of intracellular ice with small crystals, the rate at which the cells are warmed has an even greater impact on the probability of cell survival.³⁹ Cellular damage and death associated with IIF is caused during the thawing cycle.^{30,40} During warming, ice recrystallization occurs; a process that results in the growth and formation of larger ice crystals at the expense of smaller ones. As previously mentioned, it has been reported that cellular damage is dependent upon ice crystal size,^{28,37} and thus the formation of larger ice crystals has a detrimental effect on the cell and its organelles.^{30,37} Consequently, recrystallization is recognized as a significant contributing factor to cellular injury and death.^{28,40}

1.1.3 Cryoprotective Agents to Improve Post-Thaw Recovery

To mitigate cellular damage and ensure the survival of cells during cryopreservation, specific chemicals are added to the cell solution prior to freezing known as cryoprotective agents (CPAs).⁴¹ CPAs are classified as permeating or non-permeating agents. Permeating CPAs such as dimethyl sulfoxide (DMSO) and glycerol have the ability to move freely across the cell membrane of several different cell types⁴² and offer protection against slow freezing injury. They function by replacing the cell water content, therefore, they prevent cell dehydration and reduce the concentration of damaging solutes.^{28,42,43} Non-permeating cryoprotectants such as polyvinylpyrrolidone (PVP), hydroxyethyl starch (HES), and dextran cannot cross the cell membrane and are limited to the exterior environment. These CPAs protect cells against rapid freezing injury by increasing the osmolality of the extracellular solution, thereby facilitating cell dehydration prior to freezing, and reducing the amount of intracellular ice.⁴² In addition, non-permeating CPAs also function by stabilizing cell membranes during cryopreservation.^{34,44} While non-permeating CPAs are typically less toxic than permeating agents,⁴³ permeating CPAs offer superior protection and are required for optimal cryopreservation.

1.1.4 Long-Term Storage of Red Blood Cells

One of the most common cell types that are cryopreserved are RBCs. This is because blood transfusions are one of the most common forms of cellular therapy and often the

only treatment option for a variety of conditions.⁴⁵ Blood transfusions are life-saving standard medical procedures used to treat anemia⁴⁶ and inherited blood disorders,⁴⁷ replace blood lost during or after surgery and treat accident victims and mass casualty events.⁴⁸ It is estimated that approximately 85 million RBC units are transfused annually worldwide.⁴⁹ While whole blood (WB) consists of several different components such as plasma, red blood cells, white blood cells (WBCs) and platelets, RBCs are the most frequently transfused blood component and thus, the need for RBCs is very high. RBC units are red cell concentrates that are prepared from whole blood collected in a citrate phosphate dextrose (CPD) anticoagulant.⁵⁰ RBCs are separated by centrifugation to remove plasma and filtered to reduce leukocytes.⁵¹ The final packed RBC component is resuspended in an additive solution such as saline-adenine-glucose-mannitol (SAGM) or additive solution-3 (AS-3) to provide nutrients for extended storage and additional volume.⁵²

RBC units are typically stored at 1 to 6 °C in a refrigerator for up to 42 days.^{53–55} Although the storage of RBC units has facilitated inventory management in hospitals and blood banks, the quality of stored RBCs deteriorates beyond this period. During RBC storage, well-defined biochemical and biomechanical changes occur known as “storage lesions” that impact both RBC viability and function.^{6,56,57} For example, levels of adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) decline over time,⁵⁸ resulting in morphological changes and loss of deformability which can impact tissue oxygen delivery.^{59,60} It has also been reported that during storage membrane lipid content decreases due to vesicle formation,^{61–63} free hemoglobin is released from lysed RBCs⁶² and cytokines are generated and accumulate over time.⁶⁴ In addition, stored RBCs can be a culture medium for bacterial contamination.^{6,65} At the time of collection, blood units can become contaminated with bacteria⁶⁶ and although most bacteria do not survive in cold storage, cold-tolerant bacteria have the ability to survive and grow at the temperatures used to preserve RBCs.⁶ The storage lesions associated with prolonged hypothermic storage of RBCs have raised concerns regarding the potential adverse consequences of transfusing prolonged stored blood. The clinical relevance of RBC storage lesions has been extensively studied and it has been shown that there is a relationship between the age of transfused blood and adverse clinical outcomes in

cardiac, trauma and critically ill patients.⁶⁷⁻⁷¹ For instance, it has been demonstrated that mild to moderately injured patients in the intensive care unit receiving units of blood stored beyond two weeks were associated with adverse outcomes such as pneumonia, renal failure and death.⁷²

Since refrigerated blood has a short shelf life of 42 days, this can lead to episodic shortages in blood supply and the disposal of outdated blood. While the use and supply of blood products must be constantly balanced, blood supply is always a significant issue in medicine. Cryopreservation of RBCs is the only current technology that can provide long-term product storage. This preservation method can extend storage time to at least 10 years^{73,74} and is primarily used to store RBCs for military combat casualty care and to maintain an inventory of autologous RBCs and RBCs with rare phenotypes.^{5,74,75} Glycerol is used to protect RBCs against freezing damage and the concentration of glycerol that is required depends on the cooling rate used during freezing and the storage temperature.⁷⁶ Currently, there are two methods that are clinically used for the cryopreservation of RBCs which are low concentrations of glycerol with rapid cooling rates^{77,78} and high concentrations of glycerol with slow cooling rates.^{79,80} In Europe, blood banks routinely freeze RBCs using low concentrations of glycerol (15-20% w/v) with rapid cooling rates (>100 °C/min) prior to storage in liquid nitrogen (-196 °C) or liquid nitrogen vapor (-165 °C). Using this method, RBCs are thawed rapidly in a 42 to 45 °C water bath. In Canada and the United States, RBCs are typically cryopreserved using high concentrations of glycerol (40% w/v) with slow cooling rates (1 °C/min) prior to storage at ≤-65 °C in a mechanical freezer. Using this method, RBCs are rapidly thawed in a 37 °C water bath. In each case, post-thaw processing must be performed to adequately remove the glycerol while ensuring minimal hemolysis and cell recovery of at least 80% after deglycerolization.⁵⁵ During the removal of glycerol, RBCs are exposed to decreasing concentrations of saline, which potentially results in damaging osmotic stress.⁵⁵ Therefore, to minimize the amount of hemolysis caused by osmotic stress, cells are washed step-wise with progressively decreasing concentrations of saline.^{79,81} This process, known as deglycerolization, reduces the glycerol content to less than 1% and is necessary to avoid intravascular hemolysis and adverse events after transfusion.^{55,75} Deglycerolization is typically performed using an automated cell processing system that

subjects the RBCs to multiple washing steps followed by centrifugation and supernatant removal. This process can be performed in an open system using the COBE 2991 cell processor; however, deglycerolized RBC units have a limited shelf life of 24 hours due to potential bacterial contamination.^{74,81} The introduction of a completely automated closed processing system using the Haemonetics ACP 215 device⁸² has enabled deglycerolized RBC units to be stored at 4 °C for up to a week in SAGM and up to 2-3 weeks in AS-3.^{83–86} The deglycerolization process for the low glycerol method is simpler and takes approximately 30 minutes to deglycerolize a unit.⁷³ In contrast, the high glycerol method requires more extensive washing and can take up to 60 minutes to deglycerolize a unit. As a result, frozen RBCs are not routinely used in the clinical setting due to the costly and time-consuming post-thaw processing.

Alternatively, RBCs have been cryopreserved using hydroxyethyl starch (HES) (a non-permeating CPA). In this method, RBCs are prepared with 11.5% (w/w) HES and frozen rapidly by immersion in liquid nitrogen. RBCs are stored in liquid nitrogen or liquid nitrogen vapor and thawed rapidly in a 48 °C water bath.^{87–89} Due to the biodegradable nature of HES, post-thaw RBCs can be directly transfused, thereby completely eliminating post-thaw processing.^{55,73} Additionally, since HES is commonly used as a volume expander,⁹⁰ it can prevent shock in cases of severe blood loss such as trauma or surgery. However, it was later demonstrated that the transfusion of HES cryopreserved RBCs resulted in significantly increased concentrations of plasma hemoglobin.⁹¹ As a result, HES is not clinically used for the cryopreservation of RBCs due to the inadequate data regarding the safety of HES in patients receiving repeated transfusions.^{55,75}

In summary, the cryopreservation of RBCs has the potential to address many of the current challenges encountered during hypothermic preservation such as limited storage time and blood shortages. While the standard protocol to cryopreserve RBCs in Canada uses a high concentration of glycerol (40% w/v) to prevent cellular damage during freezing and ensure a high RBC recovery, the glycerol must be removed after thawing prior to transfusion. The deglycerolization process is time-consuming and less-than-ideal in emergency transfusions where large quantities of RBC units are required in a short period of time. In addition, since glycerol is a permeating CPA, it only provides cellular

protection against slow freezing injury such as excessive dehydration and high concentrations of damaging solutes. As such, glycerol is not capable of protecting cells against lethal intracellular ice recrystallization that accounts for reduced recoveries post-thaw.^{33,92} As a result, improvements to the current protocol, such as reducing post-thaw processing times and protecting cells against injury by inhibiting ice recrystallization, are necessary in order to provide access to more readily available RBC units with improved quality. The routine use of cryopreservation could not only extend the shelf life of RBCs but could also prevent periodic blood shortages in order to meet patient demand.⁹³

1.1.5 Cryopreservation of Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are multipotent cells that possess the capacities of self-renewal and multilineage differentiation into osteoblasts, adipocytes, myocytes and chondrocytes.⁹⁴ MSCs exist in a variety of tissues and can be obtained primarily from bone marrow, umbilical cord tissue, and adipose tissue.⁹⁵ Due to the tissue repair properties of MSCs, these cells have recently gained a significant amount of interest in cell-based therapies for various inflammatory diseases,⁹⁶ cardiac repair,⁹⁷ musculoskeletal diseases,⁹⁸ and osteoarthritis.⁹⁹ Furthermore, the immunosuppressive properties of MSCs are advantageous compared to other cell types as it permits allogeneic transplantation without the need for immunosuppressive therapy.¹⁰⁰ For clinical applications, where large numbers of MSCs are required, the cells must be expanded in culture or pooled from multiple donors.¹⁰¹ However, not all MSCs can be cultured for long periods of time such as human amniotic membrane-derived MSCs.¹⁰² Additionally, long-term culture of MSCs introduces the possibility of contamination, genotypic drift, chromosomal abnormalities and phenotypic instability.^{95,103,104} To avoid such issues and enable the long-term storage of MSCs, cryopreservation remains the only viable method to preserve these cells for a significant period of time. This preservation method would allow therapeutic doses of MSCs to be stored on-site at hospitals and clinics, allowing off-the-shelf availability for the immediate treatment of acute conditions such as stroke and myocardial infarction.¹⁰⁵ While several cryopreservation techniques have been employed to preserve MSCs, the challenges lie in optimizing the freezing protocols to ensure that post-thaw MSCs maintain their viability and functional properties, and retain the characteristics of freshly

isolated MSCs. Conventional methods of MSC cryopreservation commonly use 10% DMSO in an electrolyte solution (ex. PlasmaLyte A, PLA) containing an added protein (ex. Human Serum Albumin, HSA). The cell solutions are typically frozen in a controlled rate freezer¹⁰⁶ or at a rate of 1 to 5 °C/min to approximately -100 °C at which point the cells are stored in liquid nitrogen.¹⁰⁷ Although these protocols yield high post-thaw viability and recovery,¹⁰⁶ DMSO is cytotoxic at the concentrations used to preserve cells, therefore complicating the direct clinical use of frozen-thawed cells. While the most common DMSO related toxicity includes nausea and vomiting,¹⁰⁸ DMSO has been shown to possess a wide spectrum of toxicities and adverse effects on the central nervous system,^{109,110} respiratory,^{111,112} hemolytic,¹¹³ gastrointestinal,²⁰ hepatic,¹¹³ cardiovascular^{111,114} and renal systems.²⁰ As a result, this can necessitate the removal of DMSO from thawed cells prior to use; however, this process is both complex and time-consuming¹⁰⁰ and has been shown to be associated with damaging osmotic shock,²¹ resulting in cell loss. Therefore, while cryopreservation of MSCs simplifies logistics of cell therapy, DMSO remains the only effective CPA and its toxicity has resulted in the need for development of alternative cryopreservation medium.

In recent years, there have been several conflicting reports examining the impact of cryopreservation on MSC functionality, particularly on its immunomodulatory functions. While some groups have reported no significant decline in post-thaw functionality,^{105,115–117} others have shown that cryopreserved MSCs display impaired immunomodulatory and blood regulatory properties directly after thawing.^{95,118–120} These results highlight a possible cause for the failure of clinical trials on MSC-based immunotherapy using cryopreserved products immediately post-thaw.^{95,120,121} Consequently, the impact of cryopreservation on MSC function must be well understood in order to advance MSC-based therapies.

1.2 Controlling Ice Recrystallization During Cryopreservation: A Novel Approach

Given that ice recrystallization is a major contributor to cellular injury and death during cryopreservation (Section 1.1.2), an understanding of the structure of ice and the mechanism of ice recrystallization is required prior to a discussion on inhibitors of ice recrystallization. Importantly, nature's biological solvent, water, exists as the primary

component in all living organisms and is highly influential on the survival of organisms after exposure to subzero temperatures. Liquid water is transformed to its solid state, ice, at temperatures of 0 °C, which is well above the temperatures employed for cryopreservation, making ice formation inevitable in biological systems. Although the concept of freezing is straightforward, the physical process is rather complex. The unique physicochemical properties of water are essential for cell function and cryobiological manipulations have a large impact on the different physical states of water. Consequently, water plays a central role in cryopreservation and understanding the properties of ice is essential to the field of cryobiology.

1.2.1 Structure of Ice

Ice exists in numerous structural forms depending on ambient pressure and temperature. At atmospheric pressure and temperatures below 0 °C, ordinary hexagonal ice I_h is the most common form.^{122,123} Within the ice I_h crystal lattice, each oxygen atom is bonded to two hydrogen atoms and surrounded by four other oxygen atoms in a tetrahedral arrangement. The approximate distance between the oxygen atoms is 2.76 Å.¹²⁴ This arrangement of water molecules produces a crystalline structure having hexagonal symmetry. The hexagonal ice I_h lattice unit is identified by four axes labeled a_1 , a_2 , a_3 , and c , and can be visualized as a hexagonal prism that consists of two basal faces, which are normal to the c -axis, and six prism faces as shown in Figure 1.3.^{122,123,125,126} Once a nucleation event occurs, a hexagonal ice crystal can grow in any direction, but the growth rate is dependent on the surrounding water temperature and the extent of cooperative hydration.¹²⁷ Normally, at 0 °C and atmospheric pressure, hexagonal ice crystals grow rapidly along the a axis (basal plane).^{122,123,125,128}

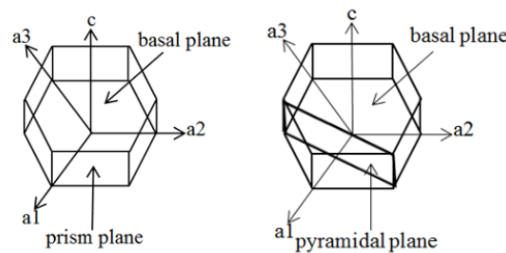


Figure 1.3 Hexagonal ice I_h lattice unit consisting of four axes a_1 , a_2 , a_3 , and c , and the basal, prism and pyramidal planes.

Studies have shown that the surface of ice is characterized by a disordered state (quasi-liquid) at temperatures close to its melting point.^{129–131} This is known as the quasi-liquid layer (QLL) and is a thin liquid-like layer that is formed by surface melting and exists between the ice crystal lattice and bulk water phase.^{126,132–139} The thickness of this layer has shown to be temperature dependent^{135–138} and estimated to be approximately 10-15 Å thick.^{135,136} While the nature of the QLL has been widely investigated and remains a topic of debate,^{130,140–143} its importance in the recrystallization of ice and cryopreservation is well acknowledged.^{129,130,144}

1.2.2 Recrystallization in Ice

The phenomenon of ice recrystallization has been extensively studied over the past 40 years. The recrystallization of ice is a thermodynamically driven process that results in the growth and formation of larger ice crystals at the expense of smaller ones.²⁸ This process has been shown to occur through either grain boundary migration or Ostwald ripening.^{145–147} An ice I_h grain is the ordered arrangement of water molecules to produce a crystalline structure having hexagonal symmetry. The growth and formation of larger ice grains at the expense of smaller ones can be explained through grain boundary migration, which refers to the movement of the interface boundary between two ice crystal grains. Hence, grain boundaries are the interfaces between differently orientated ice crystal grains.^{145,146} The boundary between an actively growing ice grain and a neighboring grain exhibits a certain degree of curvature, which is related to the size of the grain. The boundaries of large grains tend to be concave (bulge inward) and have a lower surface energy. In contrast, the boundaries of small grains are more convex (bulge outward) and have a higher surface energy.¹⁴⁶ Grain boundaries tend to migrate towards their centers of curvature such that the degree of curvature is reduced. As a result, large grains with concave boundaries grow larger while smaller grains with convex boundaries grow smaller and eventually disappear (Figure 1.4).^{148,149} This process of grain growth due to grain boundary migration is driven by the reduction in surface energy associated with the decrease in grain boundary curvature.^{148,149}

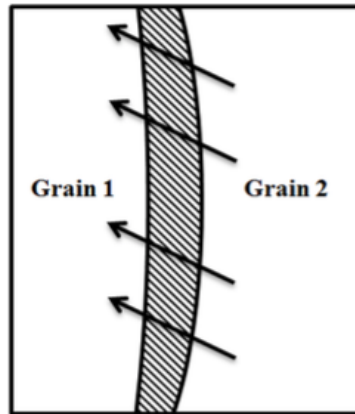


Figure 1.4 Schematic of grain boundary migration where the shaded area represents the liquid layer between two curved grain boundaries. The movement of the interface boundary between two grains occurs such that large grains with concave boundaries (grain 2) grow larger while small grains with convex boundaries grow smaller (grain 1), as indicated by the arrows.¹⁵⁰

The theory of grain boundary migration is strictly limited to ice at temperatures of -10 °C, where it assumes that grain growth in ice occurs due to the direct transfer of water molecules from one grain to another.¹⁴⁸ However, at temperatures above approximately -10 °C, the behavior of the system changes due to changes in the thickness of the QLL. To account for this, the recrystallization of ice can be explained by an Ostwald ripening process where large ice crystals grow at the expense of smaller ones at a constant total ice volume and as a result, the average ice crystal size increases and the total number of ice crystals decreases.¹⁵¹ In this process, the driving force is the decrease in total energy of the ice crystal/bulk water interface.^{151–153} In an ice crystal, an ordered arrangement of water molecules exists through hydrogen bonding. Water molecules that reside at the bulk water/ice crystal interface exhibit a lesser extent of hydrogen bonding and thus are higher in energy than water molecules within the ice crystal lattice.^{152,153} Smaller ice crystals possess a higher surface-area-to-volume ratio and are less stable than larger ice crystals.^{152,153} Ostwald ripening suggests that water molecules on the surface of small ice crystals migrate to the surface of larger ice crystals through the QLL and bulk water, resulting in a decrease in the total surface-area-to-volume ratio.^{152,153} Although the exact mechanism by which ice recrystallization occurs is still a matter of controversy, the benefits of inhibiting the recrystallization of ice were recognized hundreds of years ago and have attracted widespread academic and commercial interest.

1.2.3 Inhibiting Ice Recrystallization with Molecules Other than BAs

Naturally occurring biological antifreezes (BAs), such as antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs), are found in organisms inhabiting sub-zero environments and provide protection against injury associated with freezing by controlling the growth of ice.^{154–156} While the antifreeze activity exhibited by BAs has been investigated for more than 40 years, the exact mechanism of action is still poorly understood. Several theoretical models of ice growth inhibition by BAs have been proposed;^{154,157,158} however, the most widely accepted is the adsorption-inhibition model, whereby native AFPs and AFGPs inhibit ice growth and recrystallization by irreversibly binding to certain ice crystal surfaces.^{154,156,159–162} The binding of a BA to an interface of a growing ice crystal results in curvature on the ice surface between bound BAs. The increased surface area and curvature make the addition of water molecules along the ice surface energetically unfavourable, resulting in inhibition of further ice growth (Kelvin Effect).^{160,163–165} This is accompanied by a change in ice crystal morphology and a localized freezing point depression referred to as thermal hysteresis (TH).^{154,163,164,166–168} When the temperature is lowered below this freezing point, the growth of ice occurs and is characterized by distinct burst patterns as supercooled water rapidly joins the ice lattice.^{169,170} The ice shaping effect (depicted in Figure 1.5) and TH activity stem directly from the ability of a BA to bind to ice and these properties appear to be intertwined such that proteins that exhibit TH activity also demonstrate an ice shaping effect.^{154,171}

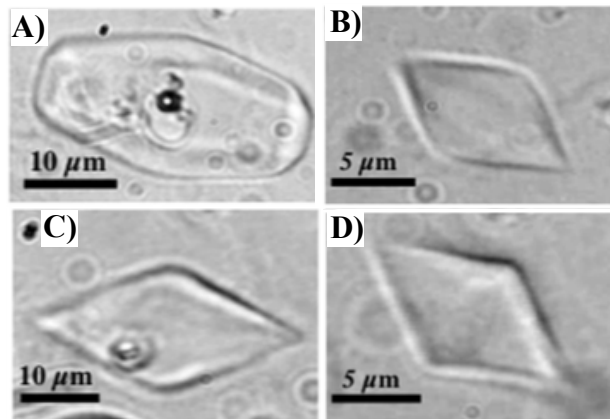


Figure 1.5 Ice crystal morphologies in the presence of various BAs: (A) AFGP-8; (B) WT *Lp*AFP; (C) Type I AFP; (D) Type III AFP at 1 mg/mL in water.¹⁷²

While TH and ice shaping are correlated, the ability to inhibit ice recrystallization appears to function independently and does not necessarily correlate to TH activity.¹⁷³ For instance, AFPs such as *Tm*AFP, type I-hyp AFP from winter flounder and *Mp*AFP are hyperactive AFPs that are very effective at TH but exhibit relatively low ice recrystallization inhibition (IRI) activity. On the contrary, AFGP and winter flounder type I AFP exhibit moderate TH activity but high IRI activity.¹⁶⁹ The lack of correlation between TH and IRI activity suggests that multiple mechanisms may be present.^{173,174} In a recent study, Wu *et al.* discovered an ion-specific effect on ice recrystallization whereby the average size of ice crystals after recrystallization could be regulated by changing the type of ion in the aqueous solution.¹⁷⁵ The ion-specific effect on IRI activity was investigated with various anions and cations and molecular dynamics simulations demonstrated that the ability of the ion to be incorporated into the ice lattice plays an important role in the size of recrystallized ice crystals and thus its ability to inhibit ice growth.¹⁷⁵ Consequently, while binding to ice crystal surfaces is required for TH activity,^{154,163,164,166–168} this study suggests that it may not be necessary for IRI activity and alternative mechanisms for inhibiting ice recrystallization may exist.

Inspired by the unique function of BAs to inhibit ice growth and recrystallization, a number of synthetic polymers and materials have recently been developed to control ice formation and growth. These include ice recrystallization inhibitors such as poly (vinyl alcohol) (PVA),^{176–180} block copolymers,¹⁸¹ nylon-3 polymers,¹⁸² zirconium acetate,¹⁸³ graphene oxide,¹⁸⁴ and amphipathic metalloheliices.¹⁸⁵ Among these polymers and materials, PVA has been extensively studied and remains one of the most IRI active synthetic polymers to date.^{176–180,186} Inada *et al.* showed that the effectiveness of PVA in inhibiting ice recrystallization increased as the molar concentration and molecular weight of PVA increased.¹⁷⁸ High molecular weight PVA was found to possess comparable IRI activity to a type I AFP at similar molar concentrations. However, due to the large difference in molecular weights, the quantity of PVA required to exhibit this IRI activity was significantly higher than the AFP.¹⁷⁸ The molecular weight dependence of PVA on IRI activity was re-examined by Gibson *et al.* and they demonstrated that high molecular weight (115,000 Da) PVA was an effective inhibitor of ice recrystallization at a concentration of 5 mg/mL.¹⁸⁷ It was proposed that the IRI activity exhibited by PVA is

attributed to its ability to interact with the ice crystal lattice. But PVA exhibits very slight TH activity¹⁸⁶ and ice shaping effects, suggesting adsorption to specific ice crystal surfaces.¹⁷⁹ Koop *et al.* hypothesized that the spacing of the PVA hydroxyl groups closely match those of the prism planes of the ice crystal lattice, allowing PVA molecules to adsorb to these planes.¹⁷⁹ However, this speculation was based on a two-dimensional, straight-line configuration. Recently, Toner *et al.* conducted molecular dynamics simulations at the ice-PVA interfaces to explicitly show the geometrical match between the PVA hydroxyl groups and the ice lattice and demonstrated that PVA adsorbs to both the basal and prism planes of ice and short-chain PVA is incorporated into the ice lattice.^{188,189} Furthermore, it is hypothesized that the PVA chain length (i.e. the number of hydroxyl groups) has an effect on IRI activity.¹⁸⁸ Despite this, the exact mechanism by which PVA interacts with ice and inhibits ice recrystallization is not fully understood.^{188–190}

Unlike high molecular weight polymers, our laboratory was the first to report that low molecular weight (< 340 Da) molecules could inhibit ice recrystallization.^{191–198} Small molecules are attractive alternatives to high molecular weight polymers since they can be efficiently synthesized and are amenable to large-scale preparations required for various applications. In addition, small molecules are less likely to elicit immunogenic responses than polymers in biological systems.¹⁹⁹

1.3 Small Molecule Ice Recrystallization Inhibitors

Ice recrystallization has been identified as a significant contributing factor to cellular injury and death during cryopreservation.^{28,40} Given that many cryopreservation protocols are suboptimal, interest has arisen in designing novel inhibitors of ice recrystallization for their use as cryoprotective agents. Over the past decade, our laboratory has studied several different classes of naturally occurring biological antifreezes (BAs) found in organisms inhabiting sub-zero environments.²⁰⁰ The biological purpose of BAs is to control ice growth and protect organisms against freezing damage.^{201–205} Although BAs possess the desirable property of ice recrystallization inhibition,¹⁴⁶ they cannot be used effectively as CPAs due to the detrimental effects of TH.^{206–209} This can be explained by the morphological changes in ice crystal habit that occur as a result of BA binding to the

ice crystal surface. At temperatures well below the TH gap (i.e. cryopreservation temperatures), ice crystal morphology changes from hexagonal shaped to needle-like structures (spicules)^{154,210} that penetrate cell membranes and cause cell damage.²¹¹

Early work led to the development of *carbon*-linked (*C*-linked) AFGP analogues that possess “custom-tailored” antifreeze activity.^{191,193,212,213} These analogues exhibit the ability to inhibit ice recrystallization without the detrimental effects of TH.^{191,200,212} Extensive structure-function studies of the *C*-linked AFGPs led to the discovery that small molecules could be effective inhibitors of ice recrystallization. Subsequent to this, our laboratory has reported several different classes of small molecules capable of inhibiting ice recrystallization such as lysine-based surfactants, non-ionic carbohydrate-based amphiphiles (alkyl and aryl aldonamides) and *O*-linked alkyl and aryl glycosides.^{195–198,214}

1.3.1 Simple Mono- and Disaccharides as Ice Recrystallization Inhibitors

In 2008, our laboratory reported that simple carbohydrates (mono- and disaccharides) can exhibit moderate IRI activity.¹⁹² Previous work on a series of *C*-linked AFGP analogues demonstrated that the relative stereochemistry of the hydroxyl groups of the carbohydrate moiety influenced IRI activity.¹⁹¹ It was shown that the presence of a highly hydrated carbohydrate residue on the glycopeptide resulted in more IRI activity than that of a less hydrated carbohydrate.¹⁹¹ Consequently, this work suggested that the hydration of a carbohydrate is important for IRI activity.¹⁹¹ To further investigate the correlation between carbohydrate hydration and IRI activity, several simple mono- and disaccharides were assessed for their ability to inhibit ice recrystallization and correlated to their respective hydration parameters.¹⁹² Among the monosaccharides examined in this study, D-galactose exhibited the most IRI activity at a concentration of 22 mM. Meanwhile, D-glucose and D-mannose possessed weak IRI activity and D-talose displayed no IRI activity.¹⁹² In the disaccharides, D-melibiose possessed the most IRI activity, while D-lactose and D-trehalose displayed weak IRI activity and D-maltose and D-sucrose showed no IRI activity (Figure 1.6).

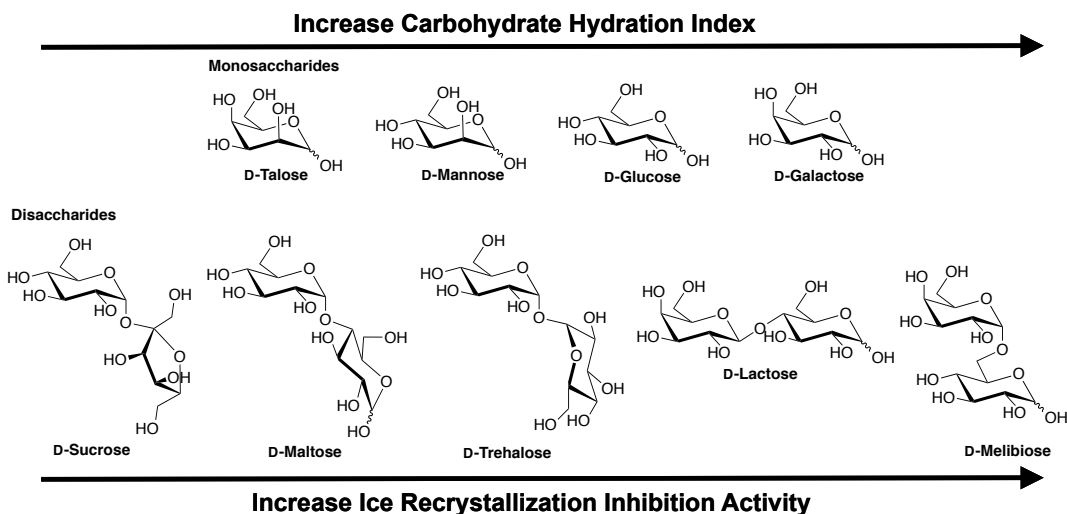


Figure 1.6 Correlation between carbohydrate hydration index and IRI activity.

The hydration layer of a carbohydrate refers to the number of tightly bound water molecules that surround the carbohydrate.^{215–217} The ability of the sugar to “fit” in or to be compatible with the three-dimensional hydrogen-bonded network of water depends on the relative stereochemistry of the hydroxyl groups.^{215–218} More specifically, it was shown that the C-2 and C-4 hydroxyl groups are important for the fit of a carbohydrate in water.²¹⁹ A new parameter termed the hydration index (HI) was developed and this value corresponds to the hydration number divided by the partial molar volume of the carbohydrate.¹⁹² It was shown that mono- and disaccharides displayed a correlation between IRI activity and hydration index (Figure 1.6).¹⁹² Consequently, mono- and disaccharides possessing higher hydration indices were more IRI active.

Since none of these carbohydrates exhibit TH activity or ice shaping effects, it was unlikely that their IRI activity was due to interactions with the ice lattice. This led to an alternative proposed mechanism for ice recrystallization inhibition based upon the compatibility of the carbohydrate within the network of bulk water molecules, which is linked to hydration.¹⁹² As mentioned in Section 1.2.1, a quasi-liquid layer (QLL) exists between the ice crystal lattice and bulk water phase. During ice recrystallization, there is a movement of bulk water molecules to the QLL then to the growing ice lattice.^{220,221} It was proposed that simple carbohydrates localize at the bulk water-QLL interface and inhibit ice recrystallization by disrupting the pre-ordering of water.¹⁹² Highly hydrated carbohydrates, such as D-galactose and D-melibiose, fit poorly into the three-dimensional

hydrogen-bonded network of bulk water and cause greater disruption on the pre-ordering of water molecules.^{215–217} This increases the energy associated with the transfer of bulk water molecules to the QLL and makes this process energetically unfavourable, resulting in ice recrystallization inhibition. In contrast, less hydrated carbohydrates, such as D-talose and D-sucrose, fit well into the three-dimensional hydrogen-bonded network of bulk water and disrupt the pre-ordering of bulk water molecules less. As a result, they exhibit little to no IRI activity.¹⁹² The proposed mechanism for ice recrystallization inhibition is depicted in Figure 1.7.

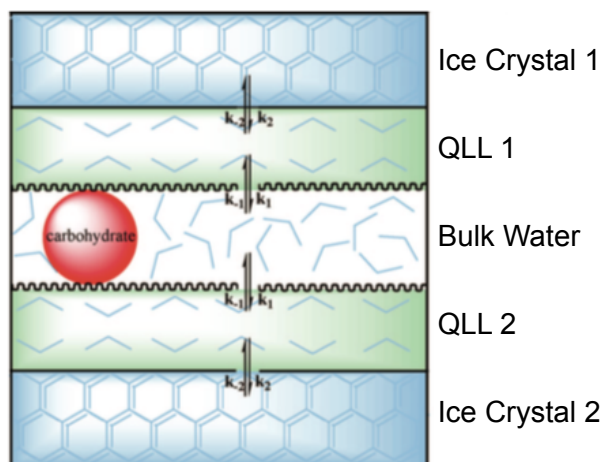


Figure 1.7 Proposed mechanism for ice recrystallization inhibition. A carbohydrate, represented as a red sphere, localized at the bulk water-QLL (quasi-liquid layer) interface between adjacent ice lattices. The hydration of a carbohydrate is correlated to IRI activity.¹⁹²

While this proposed mechanism is related to the hydration of a carbohydrate and hypothetically explains how simple carbohydrates (mono- and disaccharides) exhibit IRI activity, it is crucial to emphasize that the exact mechanism by which small molecules inhibit ice recrystallization is not known.

1.3.2 Carbohydrate-Based Surfactants as Ice Recrystallization Inhibitors

Given that the proposed mechanism for ice recrystallization inhibition of simple carbohydrates was based on their ability to disrupt the structure of bulk water,¹⁹² this led to the discovery of carbohydrate-based surfactants as IRIs.¹⁹⁵ These molecules possess a hydrophilic carbohydrate head group and a hydrophobic tail; thus, they can self-assemble to form micelles/hydrogels in solution.^{222–224} Carbohydrate-based surfactants such as *n*-

octyl- β -D-glycosides are commonly used detergents for membrane protein solubilization, purification and crystallization.^{225,226} However, their ability to inhibit ice recrystallization had not been previously explored. As such, *n*-octyl- β -D-glycosides **101** and **102** were synthesized and assessed for IRI activity (Figure 1.8).¹⁹⁵ The glucose analogue **101** showed very little IRI activity at 22 mM, similar to that of D-glucose at the same concentration. Interestingly, when assessed at 22 mM, the galactose analogue **102** displayed significantly more IRI activity than D-galactose.¹⁹⁵ The results from this study suggested that hydrophobic moieties such as long alkyl chains at the C-1 position of the pyranose ring are important for IRI activity.^{195,196}

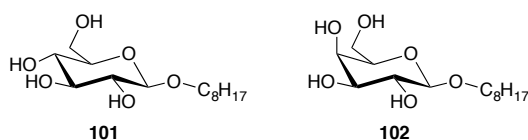


Figure 1.8 Structures of *n*-octyl- β -D-glycosides **101** and **102**.

1.3.3 Aryl Glycosides as Ice Recrystallization Inhibitors

Despite the discovery that carbohydrate-based surfactants such as *n*-octyl- β -D-galactoside (**102**) are effective inhibitors of ice recrystallization, their surfactant-like properties impair their ability to function as novel CPAs since they solubilize cell membranes and are detrimental to cell survival.^{227,228} Thus, to maintain the amphiphilic nature of these molecules but eliminate the deleterious effects of long alkyl chains on membrane structural integrity, other hydrophobic substituents were explored. More specifically, the hydrophobic alkyl chains at the C-1 position were replaced with hydrophobic aryl moieties. A structurally diverse class of *O*-linked aryl glycosides was synthesized and assessed for IRI activity.¹⁹⁸ The general structure is shown in Figure 1.9, as well as structures of certain effective inhibitors of ice recrystallization in this class of molecules (**103-105**). Various functional groups were substituted at the *ortho*-, *meta*- and *para*-positions of the aryl ring; however, it was shown that *ortho*- and *meta*-substituted analogues were significantly less IRI active than *para*-substituted analogues and highlighted the importance of having a substituent at the *para*-position of the aryl ring.²²⁹ Given that *n*-octyl- β -D-galactoside **102** was shown to be an effective inhibitor of ice recrystallization, *O*-aryl galactosides were also explored. However, the *O*-aryl

galactosides displayed a very different activity profile than their glucoside counterparts and exhibited less IRI activity overall.¹⁹⁸ Within the class of *O*-aryl glucosides, analogues **103-105** bearing a methoxy group (-OCH₃) or a halogen (-Br and -F) at the *para*-position of the aryl ring are some of the most effective inhibitors of ice recrystallization.¹⁹⁸

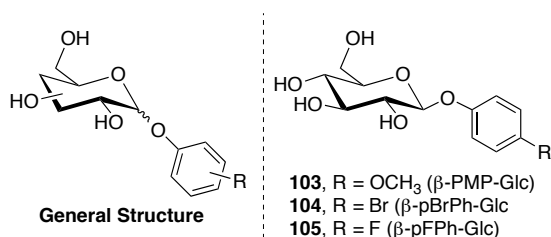


Figure 1.9 General structure of the *O*-aryl glycosides and structures of effective ice recrystallization inhibitors in this class of molecules (**103-105**).

Extensive structure-function studies have revealed critical features that influence the IRI activity of these molecules including the relative orientation of the hydroxyl groups on the pyranose ring, the stereochemistry and nature of the C-1 glycosidic bond, and the substituent on the aromatic ring and its regiochemistry.¹⁹⁸ The *O*-aryl glycosides represent a promising class of small molecule IRIs for use in cellular applications due to their inability to solubilize cell membranes and synthetic simplicity that is amenable to large-scale preparations required for *in vitro* studies. Furthermore, the *O*-aryl glycosides have recently been shown to be effective cryo-additives for the freezing of human RBCs.²³⁰

1.4 Chapter Summary

Cryopreservation remains the only viable method to preserve cell types, such as RBCs and MSCs, for a significant period of time for transfusion medicine and regenerative medicine applications. Current CPAs such as DMSO and glycerol prevent cellular damage and increase post-thaw viability and recovery; however, the toxicity associated with DMSO remains a key-limiting factor. Although the CPA glycerol is required for successful cryopreservation of RBCs, it must be removed from RBCs post-thaw to avoid intravascular hemolysis. The high concentration of glycerol (40% w/v) that is typically utilized presents a significant limitation due to the costly and time-consuming deglycerolization process. Consequently, current cryopreservation protocols are

suboptimal, and the rapidly emerging fields of cell bioprocessing and cellular therapy have elicited a rising demand for improved protocols that reduce or eliminate the use of DMSO or glycerol. Due to the fact that ice recrystallization is a major contributor to cellular injury and death during cryopreservation, compounds that have the ability to inhibit this process are very attractive candidates as alternative CPAs. Over the years, several different classes of small molecule IRIs have been discovered. Extensive structure-function studies have revealed the importance of hydrophobic moieties for IRI activity. While carbohydrate-based surfactants possessing long alkyl chains were shown to be effective inhibitors of ice recrystallization, their surfactant-like properties impaired their ability to function as novel CPAs due to their solubilizing effect on cell membranes. The subsequent discovery of the *O*-aryl glycosides represents a novel class of small molecule IRIs with great potential as CPAs in cryopreservation applications. Overall, the design and study of IRI active small molecules will aid in identifying additional structural features that are necessary for effective inhibition of ice recrystallization and facilitate a better understanding of the mechanisms responsible for inhibiting ice recrystallization. In addition, further development of small molecule IRIs for their use as novel CPAs can significantly improve current cryopreservation protocols.

1.5 References

- (1) Stacey, G. N.; Masters, J. R. *Nat. Protoc.* **2008**, 3 (12), 1981.
- (2) Baust, J. G.; Gao, D.; Baust, J. M. *Organogenesis* **2009**, 5 (3), 90.
- (3) Jang, T. H.; Park, S. C.; Yang, J. H.; Kim, J. Y.; Seok, J. H.; Park, U. S.; Choi, C. W.; Lee, S. R.; Han, J. *Integr. Med. Res.* **2017**, 6, 12.
- (4) Giwa, S.; Lewis, J. K.; Alvarez, L.; Langer, R.; Roth, A. E.; Church, G. M.; Markmann, J. F.; Sachs, D. H.; Chandraker, A.; Wertheim, J. A.; Rothblatt, M.; Boyden, E. S.; Eidbo, E.; Lee, W. P. A.; Pomahac, B.; Brandacher, G.; Weinstock, D. M.; Elliott, G.; Nelson, D.; Acker, J. P.; Uygun, K.; Schmalz, B.; Weegman, B. P.; Tocchio, A.; Fahy, G. M.; Storey, K. B.; Rubinsky, B.; Bischof, J.; Elliott, J. A. W.; Woodruff, T. K.; Morris, G. J.; Demirci, U.; Brockbank, K. G. M.; Woods, E. J.; Ben, R. N.; Baust, J. G.; Gao, D.; Fuller, B.; Rabin, Y.; Kravitz, D. C.; Taylor, M. J.; Toner, M. *Nat. Biotechnol.* **2017**, 35 (6), 530.
- (5) Asghar, W.; El Assal, R.; Shafiee, H.; Anchan, R. M.; Demirci, U. *Biotechnol. J.* **2014**, 9 (7), 895.
- (6) Hess, J. R. *Transfus. Apher. Sci.* **2010**, 43 (1), 51.
- (7) Ramirez-Arcos, S.; Perkins, H.; Kou, Y.; Mastronardi, C.; Kumaran, D.; Taha, M.; Yi, Q. L.; McLaughlin, N.; Kahwash, E.; Lin, Y.; Acker, J. *Vox Sang.* **2013**, 105, 100.

- (8) Dann, E. J.; Bonstein, L.; Arbov, L.; Kornberg, A.; Rahimi-Levene, N. *Transfus. Med.* **2007**, *17*, 135.
- (9) Shander, A.; Goodnough, L. T. *Crit. Care Clin.* **2009**, *25*, 261.
- (10) Wei, X.; Yang, X.; Han, Z. P.; Qu, F. F.; Shao, L.; Shi, Y. F. *Acta Pharmacol. Sin.* **2013**, *34*, 747.
- (11) Fuller, B.; Gonzalez-Molina, J.; Erro, E.; Mendonca, J. De; Chalmers, S.; Poirier, A.; Selden, C. *Cell Gene Ther. Insights* **2017**, *3* (5), 359.
- (12) Woods, E. J.; Thirumala, S.; Badhe-Buchanan, S. S.; Clarke, D.; Mathew, A. J. *Cytotherapy* **2016**, *18* (6), 697.
- (13) Correia, C.; Koshkin, A.; Carido, M.; Espinha, N.; Saric, T.; Lima, P.; Serra, M.; Alves, P. *Stem Cells Transl. Med.* **2016**, *5*, 658.
- (14) Rubinsky, B. *Heart Fail. Rev.* **2003**, *8*, 277.
- (15) Baust, J. M. *Bioprocess Int.* **2005**, 46.
- (16) Mathew, A. J.; Baust, J. M.; Van Buskirk, R. G.; Baust, J. G. *Tissue Eng.* **2004**, *10* (11–12), 1662.
- (17) Buskirk, R. G. Van; Baust, J. M.; Snyder, K. K.; Mathew, A. J.; Baust, J. G. *Bioprocess Int.* **2004**, 42.
- (18) Zenhausem, R.; Tobler, A.; Leoncini, L.; Hess, O. M.; Ferrari, P. *Ann. Hematol.* **2000**, *79* (9), 523.
- (19) Brockbank, K. G. M. *MOJ Cell Sci. Rep.* **2016**, *3* (4), 3.
- (20) Berz, D.; Colvin, G. Cryopreservation of Hematopoietic and Non-Hematopoietic Stem Cells – A Review for the Clinician. In: T. Demirer, editor. *New Advances in Stem Cell Transplantation*. New York: InTech; 2012. p 231–266.
- (21) Wood, E. J.; Pollok, K. E.; Byers, M. A.; Perry, B. C.; Purttman, J.; Heimfeld, S.; Gao, D. *Transfus. Med. Hemotherapy* **2007**, *34*, 276.
- (22) Fahy, G. M. *Cryobiology* **1986**, *23*, 1.
- (23) Fahy, G. M.; Lilley, J. T. H.; Linsdell, H.; Douglas, M. S. J.; Meryman, H. T. *Cryobiology* **1990**, *27*, 247.
- (24) Wowk, B. *Cryobiology* **2010**, *60* (1), 11.
- (25) Rall, W. F.; Fahy, G. *Nature* **1985**, *313*, 573.
- (26) Fahy, G. M.; Wowk, B. Principles of Cryopreservation by Vitrification. In: Wolkers, W.; Oldenhof, H., eds. *Cryopreservation and Freeze-Drying Protocols. Methods in Molecular Biology*. New York: Springer; 2015. p 21–82.
- (27) Kasai, M.; Mukaida, T. *Reprod. Biomed.* **2004**, *9* (2), 164.
- (28) Mazur, P. Principles of Cryobiology. In: Fuller, B.; Lane, N.; Benson, E., eds. *Life in the Frozen State*. Boca Raton, FL: CRC Press; 2004.
- (29) Fuller, B. J.; Grout, B. W. W. *Clinical Applications of Cryobiology*. Boca Raton, FL: CRC Press; 1991.
- (30) Fowler, A.; Toner, M. *Ann. N. Y. Acad. Sci.* **2005**, *1066*, 119.
- (31) Gao, D.; Critser, J. K. *ILAR J.* **2000**, *41* (4), 187.
- (32) Mazur, P.; Leibo, S. P.; Farrant, J.; Chu, E. H. Y.; Hanna, M. G.; Smith, L. H. Interactions of Cooling Rate, Warming Rate and Protective Additive on the Survival of Frozen Mammalian Cells. In: Wolstenholme, G.E.W.; O'Connor, M., eds. *The Frozen Cell*. London: J & A Churchill; 1970. p 69–88.
- (33) Mazur, P.; Leibo, S. P.; Chu, E. H. *Exp. Cell Res.* **1972**, *71* (2), 345.
- (34) Mazur, P. *Am. J. Physiol.* **1984**, *247* (3 Pt 1), C125.

- (35) Lovelock, J. E. *Proc. R. Soc. Lond., B, Biol. Sci.* **1957**, 147 (929), 427.
- (36) Mazur, P. J. *Gen. Physiol.* **1963**, 47, 347.
- (37) Mullen, S. F.; Critser, J. K. *Cancer Treat Res.* **2007**, 138, 83.
- (38) Shimada, K.; Asahina, E. *Cryobiology* **1975**, 12 (3), 209.
- (39) Bank, H. *Cryobiology* **1973**, 10 (2), 157.
- (40) Farrant, J. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **1977**, 278 (959), 191.
- (41) Meryman, H. T. *Transfusion* **2007**, 47, 935.
- (42) Fuller, B. J. *CryoLetters* **2004**, 25 (6), 375.
- (43) Elliott, G. D.; Wang, S.; Fuller, B. J. *Cryobiology* **2017**, 76, 74.
- (44) McGann, L. E. *Cryobiology* **1978**, 15 (4), 382.
- (45) Li, Z.; Liu, D. *Exp. Hematol. Oncol.* **2016**, 5 (26), 1.
- (46) Schrijvers, D. *Oncologist* **2011**, 16 (suppl 3), 12.
- (47) Weatherall, D. J.; Clegg, J. B. *Bull. World Health Organ.* **2001**, 79, 704.
- (48) Kaur, P.; Basu, S.; Kaur, G.; Kaur, R. *J. Emerg. Trauma Shock* **2011**, 4 (1), 103.
- (49) Takei, T.; Amin, N. A.; Schmid, G.; Dhingra-Kumar, N.; Rugg, D. J. *Acquir. Immune Defic. Syndr.* **2009**, 52 (Suppl 2), S127.
- (50) Marti-Carvajal, A. J.; Simancas-Racines, D.; Pena-Gonzalez, B. S. *Cochrane Database Syst. Rev.* **2015**, No. 7, 1.
- (51) Klein, H. G.; Spahn, D. R.; Carson, J. L.; Johnson, R. W. *Lancet* **2007**, 270, 415.
- (52) Amici, G. M. D.; Mirasole, C.; Alessandro, A. D.; Yoshida, T.; Larry, J.; Zolla, L. *Blood Transfus.* **2012**, 10 (Suppl 2), S46.
- (53) Yap, C.; Lau, L.; Krishnaswamy, M.; Gaskell, M.; Yui, M. *Ann. Thorac. Surg.* **2008**, 86, 554.
- (54) Hebert, P. C.; Chin-Yee, I.; Fergusson, D.; Blajchman, M.; Martineau, R.; Clinch, J.; Olberg, B. *Anesth. Analg.* **2005**, 100, 1433.
- (55) Scott, K. L.; Lecak, J.; Acker, J. P. *Transfus. Med. Rev.* **2005**, 19 (2), 127.
- (56) Hess, J. R. *J. Proteomics* **2010**, 73 (3), 368.
- (57) Kanas, T.; Acker, J. P. *FEBS J.* **2010**, 277, 343.
- (58) Wolfe, L. C. *Transfusion* **1985**, 25, 185.
- (59) Fitzgerald, R. D.; Martin, C. M.; Dietz, G. E.; Al., E. *Crit. Care Med.* **1997**, 25, 726.
- (60) Marik, P. E.; Sibbald, W. J. *JAMA* **1993**, 269, 3024.
- (61) Greenwalt, T. J.; Bryan, D. J.; Dumaswala, U. J. *Vox Sang.* **1984**, 47, 261.
- (62) Latham, J. T. J.; Bove, J. R.; Weirich, F. L. *Transfusion* **1982**, 22, 158.
- (63) Greenwalt, T. J.; Zehner Sostok, C.; Dumaswala, U. J. *Vox Sang.* **1990**, 58, 90.
- (64) Smith, K. J.; Sierra, E. R.; Nelson, E. J. *Transfusion* **1993**, 33, 53S.
- (65) Wagner, S. J. *Vox Sang.* **2004**, 86, 157.
- (66) Yomtovian, R. A.; Palavecino, E. L.; Dysktra, A. H.; Downes, K. A.; Morrissey, A. M.; Bajaksouzian, S.; Pokorny, M. A.; Lazarus, H. M.; Jacobs, M. R. *Transfusion* **2006**, 46, 719.
- (67) Cholette, J. M.; Pietropaoli, A. P.; Henrichs, K. F.; Al., E. *Pediatr. Crit. Care Med.* **2015**, 16, 227.
- (68) Min, J. J.; Bae, J. Y.; Kim, T. K.; Al., E. *BMC Anesth.* **2014**, 14, 95.
- (69) Eikelboom, J. W.; Cook, R. J.; Liu, Y.; Heddle, N. M. *Am. Hear. J.* **2010**, 159, 737.
- (70) Murrell, Z.; Haukoss, J. S.; Putnam, B.; Klein, S. R. *Am. Surg.* **2005**, 71, 781.

- (71) Weinberg, J. A.; McGwin, G. J.; Vandromme, M. J.; Al., E. *J. Trauma* **2010**, *69*, 1427.
- (72) Weinberg, J. A.; McGwin, G.; Marques, M. B.; Cherry, S. A.; Reiff, D. A.; Kerby, J. D.; Rue, L. W. *J. Trauma* **2008**, *65*, 794.
- (73) Bohoněk, M. Cryopreservation of Blood. In: P. Kochhar, editor. *Blood Transfusion in Clinical Practice*. Rijeka, HR: InTech; 2012. P 233–242.
- (74) Henkelman, S.; Noorman, F.; Badloe, J. F.; Lagerberg, J. W. M. *Vox Sang.* **2015**, *108*, 103.
- (75) Hess, J. R. *Transfus. Med.* **2004**, *14* (1), 1.
- (76) Rowe, A. W. *Transfus. Med. Hemotherapy* **2002**, *29*, 25.
- (77) Krijnen, H. W.; De Wit, J. J.; Kuivenhoven, A. C.; Loos, J. A.; Prins, H. K. *Vox Sang.* **1964**, *9*, 559.
- (78) Rowe, A. W.; Eyster, E.; Kellner, A. *Cryobiology* **1968**, *5* (2), 119.
- (79) Meryman, H. T., Hornblower, M. *Transfusion* **1972**, *12* (3), 145.
- (80) Tullis, J. L.; Ketchel, M. M.; Pyle, H. M.; B, P. R.; Gibson, J. G.; Tinch, R. J.; Driscoll, S. G. *JAMA* **1958**, *168* (4), 399.
- (81) Turner, T.; Hansen, A.; Kurach, J.; Acker, J. P. *Transfus. Med. Hemotherapy* **2016**, *44*, 30.
- (82) Valeri, C. R.; Ragno, G.; Houten, P. Van; Rose, L.; Rose, M.; Egozy, Y. *Transfusion* **2005**, *45* (10), 1621.
- (83) Bandarenko, N.; Hay, S. N.; Holmberg, J.; Al., E. *Transfusion* **2004**, *44*, 1656.
- (84) Bohonek, M.; Petras, M.; Turek, I.; Al., E. *Transfusion* **2010**, *50*, 1007.
- (85) Lagerberg, J. W. M.; Truijens-de Lange, R.; de Korte, D.; Al., E. *Transfusion* **2007**, *47*, 2242.
- (86) Sen, A.; Khetarpal, A. *Med. J. Armed Forces India* **2013**, *69*, 345.
- (87) Sputtek, A. Cryopreservation of Red Blood Cells and Platelets. In: Day, J. G.; Stacey, G. N., eds. *Cryopreservation and Freeze-Drying Protocols. Methods in Molecular Biology*. Totowa, NJ: Humana Press; 2007. p 283–301.
- (88) Sputtek, A.; Rau, G. *Infusionsther Transfusionsmed.* **1992**, *19* (6), 269.
- (89) Sputtek, A.; Singbartl, G.; Langer, R.; Schleinzner, W.; Henrich, H. A.; Kuhn, P. *CryoLetters* **1995**, *16*, 283.
- (90) Hightower, C. M.; Salazar Vazquez, B. Y.; Cabrales, P.; Tsai, A. G.; Al., E. *Transfusion* **2012**, *53* (1), 49.
- (91) Horn, E.-P.; Sputtek, A.; Standl, T.; Rudolf, B.; Kuhn, P.; Schulte am Esch, J. *Anesth. Analg.* **1997**, *85* (4), 739.
- (92) Muldrew, K.; McGann, L. E. *Biophys. J.* **1990**, *57* (3), 525.
- (93) Henkelman, S.; Noorman, F.; Badloe, J. F.; Lagerberg, J. W. M. *Vox Sang.* **2015**, *108* (2), 103.
- (94) Miyagi-Shiohira, C.; Kurima, K.; Kobayashi, N.; Saitoh, I.; Watanabe, M. *Cell Med.* **2015**, *8*, 3.
- (95) Marquez-Curtis, L. A.; Janowska-Wieczorek, A.; McGann, L. E.; Elliott, J. A. W. *Cryobiology* **2015**, *71* (2), 181.
- (96) Zhang, J.; Huang, X.; Wang, H.; Liu, X.; Zhang, T.; Wang, Y.; Hu, D. *Stem Cell Res. Ther.* **2015**, *6* (234), 1.
- (97) Thakker, R.; Yang, P. *Curr. Treat. Options Cardiovasc. Med.* **2014**, *16* (7), 1.
- (98) Bashir, J.; Sherman, A.; Lee, H.; Kaplan, L.; Hare, J. M. *PM&R* **2014**, *6* (1), 61.

- (99) Freitag, J.; Bates, D.; Boyd, R.; Shah, K.; Barnard, A.; Huguenin, L.; Tenen, A. *BMC Musculoskelet. Disord.* **2016**, *17* (230), 1.
- (100) Thirumala, S.; Goebel, W. S.; Woods, E. J. *Organogenesis* **2009**, *5* (3), 143.
- (101) Yong, K. W.; Safwani, W.; Xu, F.; Abas, W.; Choi, J.; Pingguan-Murphy, B. *Biopreserv. Biobank.* **2015**, *13* (4), 231.
- (102) Moon, J. H.; Lee, J. R.; Jee, B. C.; Suk Suh, C.; Hyun Kim, S.; Lim, H. J.; Kim, H. K. *Hum. Reprod.* **2008**, *23* (8), 1760.
- (103) Vacanti, V.; Kong, E.; Suzuki, G.; Sato, K.; Canty, J. M.; Lee, T. J. *Cell. Physiol.* **2005**, *205* (2), 194.
- (104) Ben-David, U.; Mayshar, Y.; Benvenisty, N. *Cell Stem Cell* **2011**, *9* (2), 97.
- (105) Gramlich, O. W.; Burand, A. J.; Brown, A. J.; Deutsch, R. J.; Kuehn, M. H.; Ankrum, J. A. *Sci. Rep.* **2016**, *6*, 1.
- (106) Pollock, K.; Sumstad, D.; Kadidlo, D.; McKenna, D. H.; Hubel, A. *Cytotherapy* **2015**, *17* (1), 38.
- (107) Haack-Sørensen, M.; Kastrup, J. *Methods Mol. Biol.* **2011**, *698*, 161.
- (108) Zambelli, A.; Poggi, G.; Da Prada, G.; Pedrazzoli, P.; Cuomo, A.; Miotti, D.; Periotti, C.; Preti, P.; Robustelli della Cuna, G. *Anticancer Res.* **1998**, *18* (6B), 4705.
- (109) Rowley, S.; MacLeod, B.; Heimfeld, S.; Holmberg, L.; Bensinger, W. *Cytotherapy* **1999**, *1* (4), 311.
- (110) Windrum, P.; Morris, T. C. *Bone Marrow Transplant.* **2003**, *31* (4), 315.
- (111) Windrum, P.; Morris, T. C.; Drake, M. B.; Niederwieser, D.; Ruutu, T. *Bone Marrow Transplant.* **2005**, *36* (7), 601.
- (112) Benekli, M.; Anderson, B.; Wentling, D.; Bernstein, S.; Czuczman, M.; McCarthy, P. *Bone Marrow Transplant.* **2000**, *25* (12), 1299.
- (113) Yi, X.; Liu, M.; Luo, Q.; Zhuo, H.; Cao, H.; Wang, J.; Han, Y. *FEBS Open Bio* **2017**, *7*, 485.
- (114) Davis, J. M.; Rowley, S. D.; Braine, H. G.; Piantadosi, S.; Santos, G. W. *Blood* **1990**, *75* (3), 781.
- (115) Farance, I.; Lock, L. T.; Baraniak, P.; Rowley, J. A. *Cytotherapy* **2015**, *17* (67–68).
- (116) Luetzkendork, J.; Nerger, K.; Hering, J.; Moegel, A.; Hoffmann, K.; Hoefers, C.; Mueller-Tidow, C.; Mueller, L. P. *Cytotherapy* **2015**, *17*, 186.
- (117) Cruz, F. F.; Borg, Z. D.; Goodwin, M.; Sokocevic, D.; Wagner, D.; McKenna, D. H.; Rocco, P. R.; Weiss, D. J. *Stem Cells Transl. Med.* **2015**, *4*, 615.
- (118) Moll, G.; Alm, J. J.; Davies, L. C.; von Bahr, L.; Heldring, H.; Stenbeck-Funke, L.; Hamad, O. A.; Hinsch, R.; Ignatowicz, L.; Locke, M.; Lonnie, H.; Lambris, J. D.; Termura, Y.; Nilsson-Ekdahl, K.; Nilsson, B.; Le Blanc, K. *Stem Cells* **2015**, *32* (9), 2430.
- (119) Francois, M.; Copland, I. B.; Yuan, S.; Romieu-Mourez, R.; Waller, E. K.; Galipeau, J. *Cytotherapy* **2012**, *14*, 147.
- (120) Galipeau, J. *Cytotherapy* **2013**, *15*, 2.
- (121) Galipeau, J. *ISBT Sci. Ser.* **2013**, *8*, 100.
- (122) Fletcher, N. H. Chapter 2 – Structure and Energy of Ordinary Ice. In: *The Chemical Physics of Ice* (1st ed.). London: Cambridge University Press; 1970. p 23–48.

- (123) Devries, A. L.; Yuan, L. *Biochim. Biophys. Acta* **1977**, 495, 388.
- (124) Owston, P. G.; Lonsdale, K. *J. Glaciol.* **1930**, 1 (3), 118.
- (125) Harding, M. M.; Ward, L. G.; Haymet, A. D. J. *FEBS J.* **1999**, 264 (3), 653.
- (126) Hayward, J. A.; Haymet, A. D. J. *J. Chem. Phys.* **2001**, 114 (8), 3713.
- (127) Shokr, M.; Sinha, N. Polycrystalline Ice Structure. In: *Sea Ice: Physics and Remote Sensing*. Hoboken, NJ: John Wiley & Sons, Inc.; 2015. p 139–193.
- (128) Harding, M. M.; Anderberg, P. I.; Haymet, A. D. *Eur. J. Biochem.* **2003**, 270 (7), 1381.
- (129) Dash, J. G.; Rempel, A. W.; Wettlaufer, J. S. *Rev. Mod. Phys.* **2006**, 78, 695.
- (130) Sazaki, G.; Zepeda, S.; Nakatsubo, S.; Yokomine, M.; Furukawa, Y. *Proc. Natl. Acad. Sci.* **2012**, 109 (4), 1052.
- (131) Sazaki, G.; Asakawa, H.; Nagashima, K.; Nakatsubo, S.; Furukawa, Y. *Cryst. Growth Des.* **2013**, 13 (4), 1761.
- (132) Nenow, D. *Prog. Cryst. Growth Charact.* **1984**, 9 (3–4), 185.
- (133) Fletcher, N. H. *Philos. Mag.* **1962**, 7 (74), 255.
- (134) Fletcher, N. H. *Philos. Mag.* **1968**, 18 (156), 1287.
- (135) Karim, O. A.; Haymet, A. D. J. *J. Chem. Phys. Lett.* **1987**, 138 (6), 531.
- (136) Karim, O.; Haymet, A. D. J.; Diego, S. *J. Chem. Phys.* **1988**, 89 (11), 6889.
- (137) Furukawa, Y.; Yamamoto, M.; Kuroda, T. *J. Cryst. Growth* **1987**, 82 (4), 665.
- (138) Furukawa, Y.; Ishikawa, I. *J. Cryst. Growth* **1993**, 128 (1–4 (Part 2)), 1137.
- (139) Beaglehole, D.; Wilson, P. *J. Phys. Chem.* **1993**, 97 (42), 11053.
- (140) Li, Y.; Somorjai, G. A. *J. Phys. Chem. C* **2007**, 111 (27), 9631.
- (141) Bartels-Rausch, T.; Jacobi, H. W.; Kahan, T. F.; Thomas, J. L.; Al., E. *Atmos. Chem. Phys.* **2014**, 14, 1587.
- (142) Asakawa, H.; Sazaki, G.; Nagashima, K.; Nakatsubo, S.; Furukawa, Y. *Proc. Natl. Acad. Sci.* **2016**, 113 (7), 1749.
- (143) Sánchez, M. A.; Kling, T.; Ishiyama, T.; van Zadel, M.-J.; Bisson, P. J.; Mezger, M.; Jochum, M. N.; Cyran, J. D.; Smit, W. J.; Bakker, H. J.; Shultz, M. J.; Morita, A.; Donadio, D.; Nagata, Y.; Bonn, M.; Backus, E. H. G. *Proc. Natl. Acad. Sci.* **2017**, 114 (2), 227.
- (144) Kuroda, T. *J. Cryst. Growth* **1982**, 56, 189.
- (145) Knight, C. A. *J. Appl. Phys.* **1966**, 37 (2), 568.
- (146) Knight, C. A.; Wen, D.; Laursen, R. A. *Cryobiology* **1995**, 32 (1), 23.
- (147) Pronk, P.; Infante Ferreira, C. A.; Witkamp, G. J. J. *J. Cryst. Growth* **2005**, 275, e1355.
- (148) Alley, R. B.; Perepezko, J. H.; Bentley, C. R. *J. Glaciol.* **1986**, 32 (112), 415.
- (149) Alley, R. B.; Perepezko, J. H.; Bentley, C. R. *J. Glaciol.* **1986**, 32 (112), 425.
- (150) Capicciotti, C. J.; Doshi, M.; Ben, R. N. Ice Recrystallization Inhibitors: From Biological Antifreezes to Small Molecules. In: P. Wilson, editor. *Recent Developments in the Study of Recrystallization* ed. New York: InTech; 2013. p 177–224.
- (151) Budke, C.; Heggemann, C.; Koch, M.; Sewald, N.; Koop, T. *J. Phys. Chem. B* **2009**, 113, 2865.
- (152) Sutton, R. L.; Lips, A.; Piccirillo, G.; Sztehlo, A. *J. Food Sci.* **1996**, 61 (4), 741.
- (153) Hagiwara, T.; Hartel, R. W.; Matsukawa, S. *Food Biophys.* **2006**, 1 (2), 74.
- (154) Raymond, J. A.; Devries, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, 74 (6), 2589.

- (155) Yeh, Y.; Feeney, R. E. *Chem. Rev.* **1996**, *96* (2), 601.
- (156) Nada, H.; Furukawa, Y. *Polym. J.* **2012**, *44*, 690.
- (157) Knight, C. A.; Devries, A. L. *Science* **1989**, *245* (4917), 505.
- (158) Sander, L. M.; Tkachenko, A. V. *Phys. Rev. Lett.* **2004**, *93* (12), 128102.
- (159) Nada, H.; Furukawa, Y. *J. Phys. Chem. B* **2008**, *112* (23), 7111.
- (160) Celik, Y.; Drori, R.; Pertaya-Braun, N.; Altan, A.; Barton, T.; Bar-Dolev, M.; Groisman, A.; Davies, P. L.; Braslavsky, I. *Proc. Natl. Acad. Sci.* **2013**, *110* (4), 1309.
- (161) Kuiper, M. J.; Morton, C. J.; Abraham, S. E.; Gray-Weale, A. *Elife* **2015**, *4*, e05142.
- (162) Meister, K.; DeVries, A. L.; Bakker, H. J.; Drori, R. *J. Am. Chem. Soc.* **2018**, *140*, 9365.
- (163) Knight, C. A.; Cheng, C.-H.; DeVries, A. L. *Biophys. J.* **1991**, *59*, 409.
- (164) Wilson, P. W. *CryoLetters* **1993**, *14*, 31.
- (165) Voets, I. K. *Soft Matter* **2017**, *13*, 4808.
- (166) Knight, C. A.; Devries, A. L. *J. Cryst. Growth* **1994**, *143* (3–4), 301.
- (167) Knight, C. A.; Wierzbicki, A. *Cryst. Growth Des.* **2001**, *1* (6), 439.
- (168) Knight, C. A.; Driggers, E.; Devries, A. L. *Biophys. J.* **1993**, *64*, 252.
- (169) Scotter, A. J.; Marshall, C. B.; Graham, L. A.; Gilbert, J. A.; Garnham, C. P.; Davies, P. L. *Cryobiology* **2006**, *53* (2), 229.
- (170) Lorv, J. S. H.; Rose, D. R.; Glick, B. R. *Scientifica* **2014**, *2014*, 1.
- (171) Davies, P. L. *Trends Biochem. Sci.* **2014**, *39* (11), 548.
- (172) Capicciotti, C. J.; Poisson, J. S.; Boddy, C. N.; Ben, R. N. *Cryobiology* **2015**, *70*, 79.
- (173) Sidebottom, C.; Buckley, S.; Pudney, P.; Twigg, S.; Jarman, C.; Holt, C.; Telford, J.; McArthur, A.; Worrall, D.; Hubbard, R.; Lillford, P. *Nature* **2000**, *406*, 256.
- (174) Biggs, C. I.; Bailey, T. L.; Ben Graham; Stubbs, C.; Fayter, A.; Gibson, M. I. *Nat. Commun.* **2017**, *8* (1546), 1.
- (175) Wu, S.; Zhu, C.; He, Z.; Xue, H.; Fan, Q.; Song, Y.; Francisco, J. S.; Zeng, X. C.; Wang, J. *Nat. Commun.* **2017**, *8* (15154).
- (176) Gibson, M. I. *Polym. Chem.* **2010**, *1* (8), 1141.
- (177) Congdon, T.; Notman, R.; Gibson, M. I. *Biomacromolecules* **2013**, *14*, 1578.
- (178) Inada, T.; Lu, S.-S. *Cryst. Growth Des.* **2003**, *3* (5), 747.
- (179) Budke, C.; Koop, T. *ChemPhysChem* **2006**, *7*, 2601.
- (180) Deller, R. C.; Vatish, M.; Mitchell, D. A.; Gibson, M. I. *Nat. Commun.* **2014**, *5* (3244).
- (181) Mastai, Y.; Rudloff, J.; Cölfen, H.; Antonietti, M. *ChemPhysChem* **2002**, *3* (1), 119.
- (182) Macdonald, M. J.; Cornejo, N. R.; Gellman, S. H. *ACS Macro Lett.* **2017**, *6*, 695.
- (183) Mizrahy, O.; Bar-Dolev, M.; Guy, S.; Braslavsky, I. *PLoS One* **2013**, *8* (3), 1.
- (184) Geng, H.; Liu, X.; Shi, G.; Bai, G.; Ma, J.; Chen, J.; Wu, Z.; Song, Y.; Fang, H.; Wang, J. *Angew. Chem. Int. Ed.* **2017**, *56*, 997.
- (185) Mitchell, D. E.; Clarkson, G.; Fox, D. J.; Vipond, R. A.; Scott, P.; Gibson, M. I. *J. Am. Chem. Soc.* **2017**, *139*, 9835.
- (186) Inada, T.; Lu, S.-S. *Chem. Phys. Lett.* **2004**, *394*, 361.
- (187) Gibson, M. I.; Barker, C. A.; Spain, S. G.; Albertin, L.; Cameron, N. R.

- Biomacromolecules* **2009**, *10*, 328.
- (188) Weng, L.; Stott, S. L.; Toner, M. *Langmuir* **2018**, *34*, 5116.
- (189) Naullage, P. M.; Lupi, L.; Molinero, V. *J. Phys. Chem. C* **2017**, *121* (48), 26949.
- (190) Burkey, A. A.; Riley, C. L.; Wang, L. K.; Hatridge, T. A.; Lynd, N. A. *Biomacromolecules* **2018**, *19*, 248.
- (191) Czechura, P.; Tam, R. Y.; Dimitrijevic, E.; Murphy, A. V.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 2928.
- (192) Tam, R. Y.; Ferreira, S. S.; Czechura, P.; Chaytor, J. L.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 17494.
- (193) Tam, R. Y.; Rowley, C. N.; Petrov, I.; Zhang, T.; Afagh, N. A.; Woo, T. K.; Ben, R. N. *J. Am. Chem. Soc.* **2009**, *131*, 15745.
- (194) Balcerzak, A. K.; Ferreira, S. S.; Trant, J. F.; Ben, R. N. *Bioorg. Med. Chem. Lett.* **2012**, *22* (4), 1719.
- (195) Capicciotti, C. J.; Leclère, M.; Perras, F. A.; Bryce, D. L.; Paulin, H.; Harden, J.; Liu, Y.; Ben, R. N. *Chem. Sci.* **2012**, *3*, 1408.
- (196) Balcerzak, A. K.; Febbraro, M.; Ben, R. N. *RSC Adv.* **2013**, *3* (10), 3232.
- (197) Briard, J. G.; Fernandez, M.; De Luna, P.; Woo, T. K.; Ben, R. N. *Sci. Rep.* **2016**, *6*, 26403.
- (198) Capicciotti, C. J.; Mancini, R. S.; Turner, T. R.; Koyama, T.; Alteen, M. G.; Doshi, M.; Inada, T.; Acker, J. P.; Ben, R. N. *ACS Omega* **2016**, *1* (4), 656.
- (199) Li, B.; Yuan, Z.; Hung, H. C.; Ma, J.; Jain, P.; Tsao, C.; Xie, J.; Zhang, P.; Lin, X.; Wu, K.; Jiang, S. *Angew. Chem. Int. Ed.* **2018**, *57*, 13873.
- (200) Eniade, A.; Purushotham, M.; Ben, R. N.; Wang, J. B.; Horwath, K. *Cell Biochem. Biophys.* **2003**, *38* (2), 115.
- (201) Scholander, P. F.; van Dam, L.; Kanwisher, J. W.; Hammel, H. T.; Gordon, M. S. *J. Cell. Physiol.* **1957**, *49* (1), 5.
- (202) Gordon, M. S.; Amdur, B. H.; Scholander, P. F. *Biol. Bull.* **1962**, *122* (1), 52.
- (203) DeVries, A. L.; Wohlschlag, D. E. *Science* **1969**, *163* (3871), 1073.
- (204) DeVries, A. L.; Komatsu, S. K.; Feeney, R. E. *J. Biol. Chem.* **1970**, *245* (11), 2901.
- (205) DeVries, A. L. *Science* **1971**, *172* (3988), 1152.
- (206) Carpenter, J. F.; Hansen, T. N. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89* (19), 8953.
- (207) Chao, H.; Davies, P. L.; Carpenter, J. F. *J. Exp. Biol.* **1996**, *199* (Pt 9), 2071.
- (208) Rubinsky, B.; Arav, A.; Devries, A. L. *Cryobiology* **1992**, *29* (1), 69.
- (209) Wang, T.; Zhu, Q.; Yang, X.; Layne, J. R. J.; Devries, A. L. *Cryobiology* **1994**, *31* (2), 185.
- (210) DeVries, A. L. *Comp. Biochem. Physiol. Part B Comp. Biochem.* **1988**, *90* (3), 611.
- (211) Bar-Dolev, M.; Celik, Y.; Wettlaufer, J. S.; Davies, P. L.; Braslavsky, I. *J. R. Soc. Interface* **2012**, *9*, 3249.
- (212) Liu, S.; Ben, R. N. *Org. Lett.* **2005**, *7* (12), 2385.
- (213) Capicciotti, C. J.; Trant, J. F.; Lecl, M.; Ben, R. N. *Bioconjug. Chem.* **2011**, *22*, 605.
- (214) Balcerzak, A.; Capicciotti, C. J.; Briard, J. G.; Ben, R. N. *RSC Adv.* **2014**, *4* (80), 42682.
- (215) Galema, S. A.; Engberts, J. B. F. N.; Høiland, H.; Førland, G. M. *J. Phys. Chem.*

- 1993**, 97 (26), 6885.
- (216) Galema, S. A.; Høiland, H. *J. Phys. Chem.* **1991**, 95 (13), 5321.
 - (217) Galema, S. A.; Howard, E.; Engberts, J. B. F. N.; Grigera, J. R. *Carbohydr. Res.* **1994**, 265 (2), 215.
 - (218) Danford, M. D.; Levy, H. A. *J. Am. Chem. Soc.* **1962**, 84 (20), 3965.
 - (219) Galema, S. A.; Blandamer, M. J.; Engberts, J. B. F. N. *J. Org. Chem.* **1992**, 57 (7), 1995.
 - (220) Bilgram, J. H. *Prog. Cryst. Growth Charact.* **1993**, 26, 99.
 - (221) Halter, P. U.; Bilgram, J. H.; Kanzig, W. *J. Chem. Phys.* **1988**, 89 (5), 2622.
 - (222) Estroff, L. A.; Hamilton, A. D. *Chem. Rev.* **2004**, 104 (3), 1201.
 - (223) De Loos, M.; Feringa, B. L.; Van Esch, J. H. *European J. Org. Chem.* **2005**, 2005 (17), 3615.
 - (224) Lombardo, D.; Kiselev, M. A.; Magazù, S.; Calandra, P. *Adv. Condens. Matter Phys.* **2015**, 2015, 1.
 - (225) Gutmann, D. A. P.; Mizohata, E.; Newstead, S.; Ferrandon, S.; Postis, V.; Xia, X.; Henderson, P. J. F.; Van Veen, H. W.; Byrne, B. *Protein Sci.* **2007**, 16, 1422.
 - (226) Stetsenko, A.; Guskov, A. *Crystals* **2017**, 7 (7), 1.
 - (227) Lorber, B.; Bishop, J. B.; DeLucas, L. J. *Biochim. Biophys. Acta* **1990**, 1023, 254.
 - (228) Michel, H.; Oesterhelt, D. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, 77 (3), 1283.
 - (229) Capicciotti, C. J. The Rational Design of Potent Ice Recrystallization Inhibitors for Use as Novel Cryoprotectants, University of Ottawa, 2014.
 - (230) Capicciotti, C. J.; Kurach, J. D. R.; Turner, T. R.; Mancini, R. S.; Acker, J. P.; Ben, R. N. *Sci. Rep.* **2015**, 5, 9692.

Chapter 2: Goals and Objectives

2.1 Introduction

The rapidly emerging field of cellular therapeutics has highlighted the importance of cryopreservation for reliable storage, transport and banking of products.¹ To date, cryopreservation remains the only viable method to preserve various cell types such as red blood cells (RBCs) and mesenchymal stem cells (MSCs) for a significant period of time; however, major limitations of this technique still exist. As stated in the introduction to this thesis, cryoprotective agents (CPAs) are key to the success of these cryopreservation methods. Permeating CPAs such as dimethyl sulfoxide (DMSO) and glycerol are commonly used to mitigate cellular damage during cryopreservation and ensure high post-thaw cell viability and recovery. However, the concentration of DMSO used to preserve cells is toxic to both cells and patients,²⁻⁴ therefore complicating the direct clinical use of frozen-thawed cells. While DMSO can be removed from thawed cells prior to use, the process is complex and time-consuming and it has been shown to be associated with damaging osmotic shock, resulting in cell loss.^{5,6} Although the CPA glycerol is required for successful cryopreservation of RBCs, it must be removed from RBCs post-thaw to avoid intravascular hemolysis.^{7,8} The high concentration of glycerol (40% w/v) that is typically utilized presents a significant drawback due to the costly and time-consuming deglycerolization process. Consequently, the limitations highlighted above emphasize the need to improve current CPAs and cryopreservation processes to reduce or eliminate the use of conventional CPAs such as DMSO and glycerol.

It is well documented in the literature that a significant amount of cells experience cellular injury during cryopreservation due to ice formation and recrystallization.^{9,10} This process occurs as large ice crystals grow larger at the expense of smaller ones. Compounds with the ability to inhibit this process are known as ice recrystallization inhibitors (IRIs). Consequently, the ability to inhibit ice recrystallization is a very desirable property of an effective CPA, unlike the conventional CPAs such as DMSO and glycerol that function by a different mechanism and **do not control** or **inhibit** ice recrystallization. From the discovery of biological antifreezes (BAs) possessing IRI and TH activity¹¹⁻¹⁵ to the first report of “custom-tailored” AFGP analogues exhibiting IRI activity only,^{16,17} these significant advancements have not only provided a better

understanding of the structural features necessary for IRI activity but have led to the design of several different classes of small molecule IRIs.^{18–22} The first class of small molecules reported to exhibit effective inhibition of ice recrystallization were carbohydrate-based surfactants possessing long hydrophobic alkyl chains.^{18,19} However, the surfactant-like nature of these compounds prevented their use in cellular applications due to their solubilizing effect on cell membranes.^{23,24} Therefore, research efforts focused on designing a new class of carbohydrate-based small molecules containing hydrophobic aryl moieties that are more compatible with cell membranes since they are not capable of membrane solubilization.²² The *O*-aryl glycosides represent a novel class of small molecules that have the ability to inhibit ice recrystallization and were recently shown to enable the freezing of human RBCs with greatly reduced concentrations of glycerol.²⁵ These small molecule IRIs constitute a new class of CPAs that function by a different mechanism than conventional CPAs. This would significantly improve upon current cryopreservation protocols and further investigation is required in the development of small molecule IRIs as novel CPAs.

In this thesis, candidate IRIs for cryopreservation applications are identified using the following screening approach. Small molecules are first assessed for their ability to inhibit ice recrystallization using a modified “splat-cooling” assay that quantifies IRI activity using an IC_{50} value.²⁶ This value represents the concentration of IRI that is required to achieve half of its maximum inhibitory capability. Ideally, these molecules should be effective inhibitors of ice recrystallization at minimal concentrations to limit the quantity that could potentially be used to cryopreserve various cell types. An IC_{50} threshold of 50 mM was used to distinguish effective from ineffective inhibitors of ice recrystallization. Thus, compounds with IC_{50} values greater than 50 mM were considered ineffective inhibitors. Compounds exhibiting effective inhibition of ice recrystallization (i.e. $IC_{50} < 50$ mM) then underwent *in vitro* cytotoxicity screening in eukaryotic cells to determine the IC_{50} value (Figure 2.1). This value refers to the concentration of IRI that reduces cell viability by 50%. Ideally, these molecules should be non-toxic at maximal concentrations. Compounds with IC_{50} values less than 1 mM were considered highly toxic. By examining the IC_{50} values for IRI activity and cytotoxicity, candidate compounds for cell cryopreservation were selected and their ability to protect cells

against injury associated with ice recrystallization was explored utilizing an appropriate cell model. It is important to emphasize that the cytotoxicity assessment is cell type-dependent and served as the first round of toxicity screening. Thus, compounds that pass through this initial screen (i.e. $IC_{50} > 1$ mM) will undergo further toxicity testing in the cell type to be cryopreserved. For cryopreservation studies, candidate compounds were assessed within their safety window, which refers to the concentration of IRI that can function as a beneficial cryo-additive without causing toxic effects.

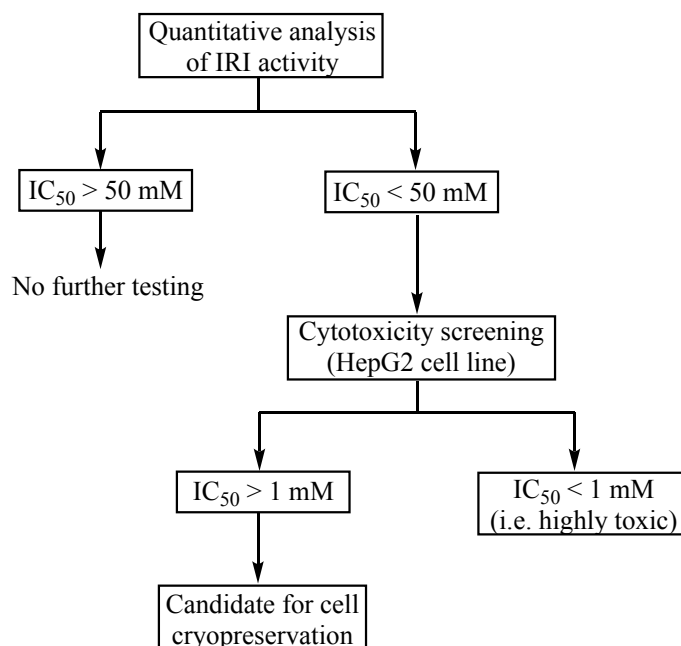


Figure 2.1 Flow chart describing the screening and selection of molecules for cell cryopreservation.

Although several structure-function studies have been done over the years to elucidate key structural features that are important for the IRI activity of small molecules, few small molecules have shown to be effective inhibitors of ice recrystallization. The goal of this thesis is to identify novel small molecules that are effective inhibitors of ice recrystallization and determine additional structural features that are important for the IRI activity of these molecules. The design and study of IRI active small molecules will facilitate a better understanding of the underlying mechanisms responsible for inhibiting ice recrystallization and will aid in the rational design of small molecule IRIs for cryopreservation applications. Furthermore, the cryopreservation potential of small molecule IRIs will be explored in model systems including RBCs and MSCs to determine

if these molecules can be effective cryo-additives and reduce the quantities of glycerol or DMSO or even eliminate their use. Lastly, recent studies have suggested that the cryoprotective effects exhibited by the *O*-aryl glycosides may be due to their ability to permeate the cell membrane and internalize into cells.²⁵ As such, the internalization of small molecule IRIs will be investigated to determine if these molecules are crossing the cell membrane and behaving as permeating CPAs. The objectives of this thesis are discussed below.

2.2 Objective 1 – Determining Structural Features Important for the IRI Activity of Small Molecules

2.2.1 Photoswitchable Surfactants

Over the years, several different classes of small molecule IRIs have been identified and structure-function studies have provided a better understanding of the structural features necessary for IRI activity. It has been shown that the moderate IRI activity exhibited by simple carbohydrates (mono- and disaccharides) is correlated to the degree of hydration, thus, highly hydrated carbohydrates are more IRI active.²⁷ Since carbohydrate hydration is related to the ability of the sugar to “fit” within the three-dimensional network of water, carbohydrates that are highly hydrated disrupt the pre-ordering of water molecules to a greater extent than less hydrated carbohydrates.^{27–30} Based upon this, it was suggested that the compatibility of the carbohydrate within the three-dimensional hydrogen-bonded network of water is inversely related to IRI activity. Since the proposed mechanism for ice recrystallization inhibition exhibited by these simple carbohydrates was based on their ability to disrupt the structure of bulk water,²⁷ this led to the discovery of carbohydrate-based surfactants, such as *n*-octyl- β -D-galactopyranoside, as a new class of small molecule IRIs.¹⁸ These molecules possess a hydrophilic carbohydrate head group and a hydrophobic tail. Structure-function studies revealed that long hydrophobic alkyl chains at C-1 are important for IRI activity and a more hydrated carbohydrate moiety (i.e. galactose) resulted in better IRI activity as it further disrupts the pre-ordering of water molecules.¹⁸ However, the hydration characteristics of these molecules are not known. Therefore, since a direct correlation

between carbohydrate hydration and IRI activity could not be made, it is not known whether these molecules are inhibiting ice recrystallization by a similar mechanism.

Organic photochromic molecular switches represent a fascinating class of molecules that allow molecular structure and function to be controlled with light.^{31,32} Many different organic photochromic molecules are known in photochemistry; however, azobenzenes are the most widely used photochromic group due to their reversible photoisomerization between *cis* and *trans* isomers.^{33–35} The *cis* and *trans* isomers of functionalized azobenzenes have a different spatial arrangement of the aromatic moieties, and thus, these two isomers exhibit drastically different physical and chemical properties.^{33,36} In particular, *trans-cis* photoisomerization of functionalized azobenzenes induces a change in molecular geometry and hydrophobicity of these molecules.^{35,37} While the photoisomerization of azobenzene has been known for decades, this process has only been recently applied to biological systems for the photo-control of biomolecules.^{33,35} Azobenzene functionalization of a molecule with biological activity, such as a protein, can permit spatiotemporal control of various biological processes by regulating enzymatic activity using light.^{35,38,39} Thus, light-sensitive biologically active molecules offer the possibility to study the dynamics of biological processes. Towards this end, it would be desirable to selectively switch the activity of an IRI active molecule off and on by light to study the dynamic mechanisms of ice recrystallization inhibition. This could potentially be accomplished by the introduction of a photosensitive group such as azobenzene onto the molecule of interest. Consequently, carbohydrate-based surfactants bearing an azobenzene moiety were investigated as ice recrystallization inhibitors.

Various carbohydrate-based surfactants incorporating an *n*-butyl azobenzene tail group were assessed for their ability to inhibit ice recrystallization (Figure 2.2). The carbohydrate head group was modified with various mono- and disaccharides to determine its influence on IRI activity as previous studies have suggested that the identity of the carbohydrate moiety has an impact on IRI activity.^{18,27} A triethylene glycolate spacer was incorporated to promote water solubility. In addition, there was interest in investigating the nature of the linker at the C-1 position. Consequently, the carbohydrate moiety was conjugated to the *n*-butyl azobenzene tail group through an *O*-glycosidic linkage and a triazole linkage.

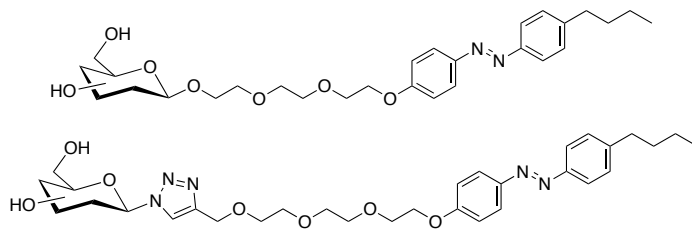


Figure 2.2 General structures of carbohydrate-based surfactants.

Since the IRI activity of amphiphilic molecules has been shown to be highly sensitive to changes in the hydrophilic-hydrophobic balance,¹⁹ the hydrophobicity of the azobenzene tail was modified by replacing the *n*-butyl group with a trifluoromethyl group ($-\text{CF}_3$). It was hypothesized that modifying the hydrophilic-hydrophobic balance may affect the IRI activity of these molecules. The general structure of the carbohydrate-based fluorosurfactants is shown in Figure 2.3. These molecules possessed a variable mono- or disaccharide head group conjugated to a hydrophobic fluoroalkyl-substituted azobenzene tail group via a triazole linkage. The azobenzene ring was substituted with the trifluoromethyl group in the *ortho*-, *meta*- or *para*-positions relative to the azo group to examine the effect of regiochemistry on IRI activity. Lastly, *trans-cis* photoisomerization of the azobenzene tail group was investigated to determine if the isomeric state of the azobenzene tail group has an effect on IRI activity. This will provide information in regards to additional structural features that are necessary for the IRI activity of these molecules.

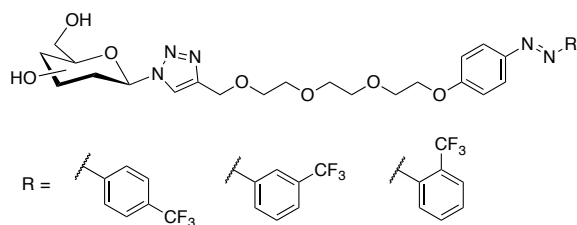


Figure 2.3 General structure of carbohydrate-based fluorosurfactants.

2.2.2 Fluorinated Aryl Glycosides

The *O*-aryl glycosides represent a novel class of small molecules with the ability to effectively inhibit ice recrystallization.²² Extensive structure-function studies have revealed critical features that influence the IRI activity of these molecules.⁴⁰ These include the relative orientation of the hydroxyl groups on the pyranose ring, the stereochemistry and nature of the glycosidic bond, and the substituent on the aromatic

ring and its regiochemistry. Within this class, some of the most effective inhibitors of ice recrystallization were shown to possess a D-glucose moiety, a β -glycosidic bond, and either a methoxy substituent (-OCH₃) or a halogen (-Br or -F) at the *para*-position of the aryl ring.²² Although many modifications to the D-glucose moiety have been explored,⁴⁰ the presence of a fluorine atom on the carbohydrate moiety has not yet been investigated for its influence on IRI activity. Therefore, a small class of fluorinated aryl glycosides was synthesized and assessed for IRI activity (Figure 2.4). Three *para*-substituted *O*-aryl glycosides that are effective inhibitors of ice recrystallization were selected for further investigation. Specifically, the hydroxyl group at C-6 was replaced with a fluorine atom to assess the importance of the C-6 hydroxyl group of aryl glycosides for IRI activity. In addition, due to synthetic simplicity, difluorinated aryl glycosides were also synthesized in which the C-6 and C-3 hydroxyl groups were replaced with fluorine atoms.

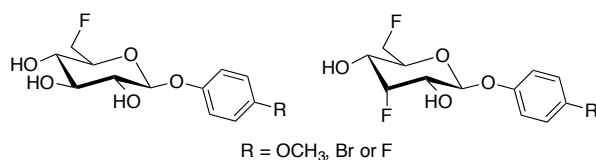


Figure 2.4 Structures of fluorinated aryl glycosides.

2.2.3 Phosphate Sugars

The phosphate group is a biologically important functional group in a variety of different ways.⁴¹ For instance, it is an important structural component of nucleotides, which are the building blocks of nucleic acids (DNA and RNA).⁴² Phosphate groups are also a component of energy-rich molecules such as adenosine triphosphate (ATP),^{41,42} and are bound to coenzymes, such as NADP⁺ and NADPH, involved in anabolic reactions such as lipid and nucleic acid synthesis.⁴³ Additionally, the phosphate group comprises the hydrophilic portion of phospholipids in biological membranes.⁴⁴ Throughout evolution, phosphorylation has become the most important post-translational modification owing to its versatility and reversibility.⁴¹ In phosphorylated proteins, the unique properties of the phosphate group can be exploited to control important biological functions.⁴¹ In the context of small molecules, the phosphorylation of glucose generates glucose-6-phosphate, which is an important intermediate in various metabolic processes including glycolysis.⁴⁵ Despite the importance of phosphate groups in biological systems,

small molecules bearing biocompatible phosphate groups have not yet been investigated as ice recrystallization inhibitors. Thus, as a preliminary study, a small diverse class of monosaccharides possessing a phosphate group was explored for their ability to inhibit ice recrystallization (Figure 2.5). This will provide preliminary information regarding the ability of phosphate sugars to inhibit ice recrystallization and further investigation will likely be required.

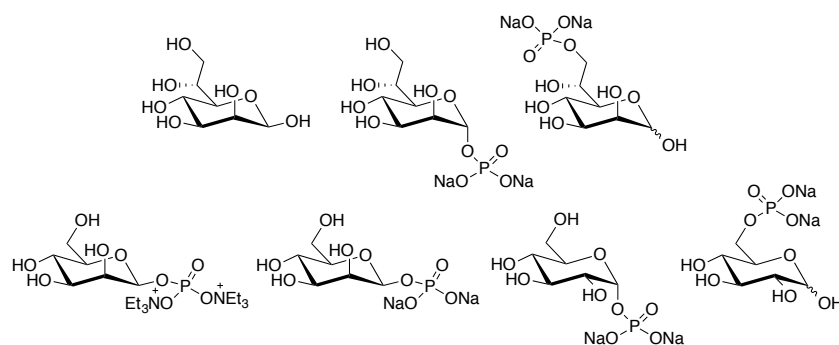


Figure 2.5 Structures of monosaccharide phosphate sugars.

2.3 Objective 2 – Determining the Influence of an Azido Moiety on the IRI Activity of Aryl Glucosides

The *O*-aryl glycosides represent a class of small molecules that are effective inhibitors of ice recrystallization.²² Structure-function analysis has revealed several structural features that are essential for the IRI activity of these molecules such as the relative orientation of the hydroxyl groups on the pyranose ring, the stereochemistry and nature of the glycosidic bond, and the substituent on the aromatic ring and its relative position. Within this class, two of the most effective inhibitors of ice recrystallization are β -PMP-Glc and β -pBrPh-Glc (Figure 2.6). Furthermore, these small molecule IRIs were recently shown to be effective cryo-additives for the freezing of human RBCs with the ability to increase post-thaw RBC integrity in the presence of reduced concentrations of glycerol (15% w/v) and slow cooling rates.²⁵ This was significant as reducing the concentration of glycerol can drastically decrease deglycerolization times. This work also highlighted the potential of small molecule IRIs to function as novel CPAs for the cryopreservation of RBCs. To aid in the development of these small molecule IRIs as novel CPAs for the cryopreservation of RBCs, we sought to further investigate the structural features that are important for the IRI activity of β -PMP-Glc and β -pBrPh-Glc.

Despite the plethora of interesting functional groups in organic chemistry, the azide (N_3) is the most popular functionality since it possesses unique reactivity and selectivity.^{46,47} In the field of bioconjugation, azides have become a powerful tool and are the most widely used bioorthogonal group.^{47–50} The inertness, small size and stability of the azide moiety in physiological conditions have permitted azide-derivatized metabolic precursors to “hijack” the biosynthetic pathways for various biomolecules, and thus, has enabled azide functionalities to be metabolically incorporated into biomolecules.^{51–53} These azide-containing biomolecules provide potential targets for azide-specific bioorthogonal labeling.^{54,55} For instance, this method has been utilized to incorporate azide-bearing unnatural monosaccharides into proteins to label proteins of interest.^{49,56} Evidently, the azide is an appealing moiety due to its bioorthogonal and biocompatible nature. Consequently, we sought to explore whether chemically modifying β -PMP-Glc and β -pBrPh-Glc with an azide functional group could increase their IRI activity. An azide substituent was incorporated at various positions on the pyranose ring as well as the *para*-position of the aryl ring to determine the relationship between the position of the azide and IRI activity. The structures are shown in Figure 2.6.

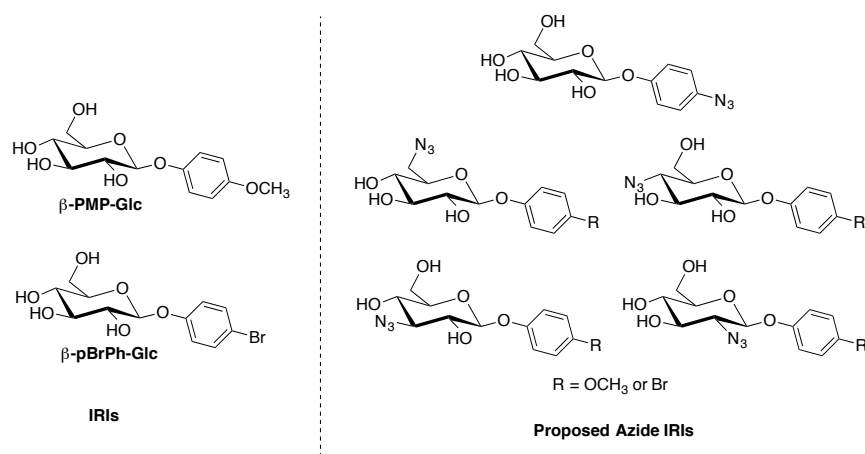


Figure 2.6 Structures of β -PMP-Glc, β -pBrPh-Glc and proposed azide derivatives.

2.4 Objective 3 – Assessing the Cryopreservation Potential of Small Molecule Ice Recrystallization Inhibitors

Cryopreservation remains the only viable method to preserve cell types such as RBCs and MSCs for a significant period of time for transfusion medicine and regenerative medicine applications. Traditional CPAs such as glycerol and DMSO prevent cellular

damage and increase post-thaw cell viability and recovery; however, the lengthy post-thaw RBC processing times and the toxicity associated with DMSO remain limiting factors. Consequently, current cryopreservation protocols are suboptimal, and the rapidly emerging fields of cellular therapy have elicited a rising demand for improved cryopreservation protocols that reduce or eliminate the use of glycerol or DMSO. As ice recrystallization is a major contributing factor to cellular injury and death during cryopreservation, compounds that have the ability to inhibit this process are very attractive candidates as novel CPAs.

To explore the cryopreservation potential of small molecule IRIs, anucleate human RBCs were utilized as a suitable *in vitro* model for assessing post-thaw integrity. Previous work by our laboratory has demonstrated that small molecule IRIs β -PMP-Glc and β -pBrPh-Glc (Figure 2.7) are effective additives for the freezing of human RBCs with the ability to increase post-thaw RBC integrity in the presence of reduced concentrations of glycerol (15% w/v) and slow cooling rates.²⁵ This discovery was significant since post-thaw processing times would be greatly reduced with low glycerol concentrations, and would significantly improve the cryopreservation of RBCs. Given these promising results, RBCs containing β -PMP-Glc or β -pBrPh-Glc in 15% glycerol were subjected to deglycerolization. However, it was shown that a significant amount of cell damage occurs during deglycerolization, resulting in very low cell recoveries. While efforts to optimize the deglycerolization conditions for these novel RBC cryo-solutions remained unsuccessful, these studies were crucial as they provided insight into the possible cause of failure. The low RBC recoveries observed in the presence of β -PMP-Glc or β -pBrPh-Glc were hypothesized to be the result of membrane interactions during freezing, causing the RBCs to be more susceptible to osmotic stress during deglycerolization. Thus, improvements to the deglycerolization process of RBCs frozen with *O*-aryl glycosides were explored. Specifically, it was postulated that the deglycerolization process may be improved using IRI active azide derivatives since these molecules may be less likely to interact with the RBC membrane during freezing and thus, facilitate their removal during deglycerolization. Thus, the cryopreservation potential of IRI active azide derivatives (Figure 2.7) was assessed to determine their

ability to function as beneficial cryo-additives that reduce cellular damage post-thaw and improve RBC recovery.

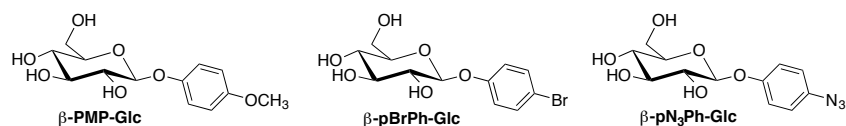


Figure 2.7 Structures of β -PMP-Glc, β -pBrPh-Glc and β -*para*-azidophenyl-D-glucopyranoside.

Relying on post-thaw integrity alone, however, is not sufficient because cryopreserved cells must be functional post-thaw in order to be clinically useful. In contrast to RBCs, stem cells such as MSCs are nucleated cells that possess the ability to differentiate into a variety of cell types.⁵⁷ Thus, MSCs provide an appropriate *in vitro* model for studying the effect of IRIs on cell proliferation and differentiation and ultimately, provide an assessment of functional capacity.

Given that β -pBrPh-Glc is an effective additive for the freezing of human RBCs,²⁵ its cryopreservation potential was assessed in MSCs. Conversely, IRI active azide derivatives were not explored in MSCs due to their lengthy chemical syntheses that are not amenable to large-scale preparations necessary for cryopreservation applications. In addition to β -pBrPh-Glc, *N*-(2-fluorophenyl)-D-gluconamide (2FA, Figure 2.8) was also explored for the cryopreservation of MSCs. It has been previously shown that this class of novel small molecules, *N*-aryl-D-alidonamides, possess the ability to inhibit ice recrystallization and function as beneficial additives to the standard 10% DMSO solution to improve the post-thaw function of hematopoietic stem and progenitor cells (HSPCs) from umbilical cord blood (UCB).⁵⁸ Specifically, it was demonstrated that 2FA was the most effective inhibitor of ice recrystallization and resulted in a significant increase in the number of post-thaw committed progenitors and CD34+ cell numbers.⁵⁸ This highlighted the ability of 2FA supplemented with 10% DMSO to increase the post-thaw function and potency of HSPCs from UCB. Consequently, interest arose in examining the ability of 2FA to protect other cellular products. Thus, small molecule IRIs, β -pBrPh-Glc and 2FA, were assessed for their ability to function as beneficial additives for the cryopreservation of MSCs to improve post-thaw function.

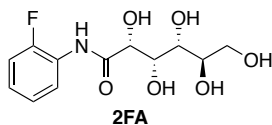


Figure 2.8 Structure of *N*-(2-fluorophenyl)-D-gluconamide (2FA).

Overall, these results will determine the capacity of small molecule IRIs to function as additives for the cryopreservation of RBCs and MSCs to improve upon current protocols.

2.5 Objective 4 – Investigating the Uptake and Internalization of Small Molecule Ice Recrystallization Inhibitors

Although the *O*-aryl glycosides represent a novel class of small molecule IRIs with the ability to protect RBCs against cryoinjury during freezing in the presence of 15% glycerol, it is not known whether their cryoprotective ability is the result of internalization into RBCs. Previous studies have speculated that the increase in post-thaw RBC integrity observed with β -PMP-Glc may be due to its ability to interact with the RBC membrane and/or permeate the cell.²⁵ These postulations were based on data from a two-step interrupted freezing experiment in which the “cooling” profile for β -PMP-Glc (110 mM) in 15% glycerol, where RBCs were thawed immediately after slow cooling to defined sub-zero temperatures, was typical of a cell permeating CPA.^{25,59} Furthermore, incubation studies of β -PMP-Glc in 15% glycerol with RBCs prior to freezing were shown to yield higher post-thaw integrity with a longer incubation (10 minutes) compared to no incubation (immediate freezing), suggesting that this molecule may be internalized into RBCs.²⁵ However, the results from these studies are merely supporting pieces of data, thus, they do not provide conclusive evidence for internalization. Therefore, further investigation was performed to determine if these molecules are crossing the cell membrane and behaving as permeating CPAs. IRI permeation was evaluated in two different cell models, RBCs and human umbilical vein endothelial cells (HUVECs). In RBCs, glucose is readily transported across cell membranes through facilitated diffusion. This process is mediated by glucose transporters (GLUTs) that are highly expressed on the RBC surface and are substrate specific.^{60,61} The transport of glucose and its analogues is highly dependent on the stereochemistry of the hydroxyl groups on the pyranose ring and ultimately the shape of the molecule.^{62,63} Consequently, the uptake of *O*-aryl glycosides was assessed in RBCs to determine whether these

molecules are being internalized and if their internalization mechanism is mediated by GLUTs. This will provide additional information to support the hypothesis of internalization. Additionally, internalization was assessed in HUVECs to investigate the ability of small molecule IRIs to permeate into cells and inhibit intracellular ice recrystallization. Ultimately, the effect of IRIs on the intracellular growth of ice crystals was explored.

2.6 References

- (1) Woods, E. J.; Thirumala, S.; Badhe-Buchanan, S. S.; Clarke, D.; Mathew, A. J. *Cytotherapy* **2016**, *18* (6), 697.
- (2) Zenhausem, R.; Tobler, A.; Leoncini, L.; Hess, O. M.; Ferrari, P. *Ann. Hematol.* **2000**, *79* (9), 523.
- (3) Brockbank, K. G. M. *MOJ Cell Sci. Rep.* **2016**, *3* (4), 3.
- (4) Berz, D.; Colvin, G. Cryopreservation of Hematopoietic and Non-Hematopoietic Stem Cells – A Review for the Clinician. In: T. Demirer, editor. *New Advances in Stem Cell Transplantation*. New York: InTech; 2012. p 231–266.
- (5) Thirumala, S.; Goebel, W. S.; Woods, E. J. *Organogenesis* **2009**, *5* (3), 143.
- (6) Wood, E. J.; Pollok, K. E.; Byers, M. A.; Perry, B. C.; Purttman, J.; Heimfeld, S.; Gao, D. *Transfus. Med. Hemotherapy* **2007**, *34*, 276.
- (7) Scott, K. L.; Lecak, J.; Acker, J. P. *Transfus. Med. Rev.* **2005**, *19* (2), 127.
- (8) Hess, J. R. *Transfus. Med.* **2004**, *14* (1), 1.
- (9) Mazur, P. Principles of Cryobiology. In: Fuller, B.; Lane, N.; Benson, E., eds. *Life in the Frozen State*. Boca Raton, FL: CRC Press; 2004.
- (10) Farrant, J. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **1977**, *278* (959), 191.
- (11) Scholander, P. F.; van Dam, L.; Kanwisher, J. W.; Hammel, H. T.; Gordon, M. S. *J. Cell. Physiol.* **1957**, *49* (1), 5.
- (12) Gordon, M. S.; Amdur, B. H.; Scholander, P. F. *Biol. Bull.* **1962**, *122* (1), 52.
- (13) DeVries, A. L.; Wohlschlag, D. E. *Science* **1969**, *163* (3871), 1073.
- (14) DeVries, A. L.; Komatsu, S. K.; Feeney, R. E. *J. Biol. Chem.* **1970**, *245* (11), 2901.
- (15) DeVries, A. L. *Science* **1971**, *172* (3988), 1152.
- (16) Liu, S.; Ben, R. N. *Org. Lett.* **2005**, *7* (12), 2385.
- (17) Czechura, P.; Tam, R. Y.; Dimitrijevic, E.; Murphy, A. V.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 2928.
- (18) Capicciotti, C. J.; Leclère, M.; Perras, F. A.; Bryce, D. L.; Paulin, H.; Harden, J.; Liu, Y.; Ben, R. N. *Chem. Sci.* **2012**, *3*, 1408.
- (19) Balcerzak, A. K.; Febbraro, M.; Ben, R. N. *RSC Adv.* **2013**, *3* (10), 3232.
- (20) Balcerzak, A.; Capicciotti, C. J.; Briard, J. G.; Ben, R. N. *RSC Adv.* **2014**, *4* (80), 42682.
- (21) Briard, J. G.; Fernandez, M.; De Luna, P.; Woo, T. K.; Ben, R. N. *Sci. Rep.* **2016**, *6*, 26403.
- (22) Capicciotti, C. J.; Mancini, R. S.; Turner, T. R.; Koyama, T.; Alteen, M. G.; Doshi, M.; Inada, T.; Acker, J. P.; Ben, R. N. *ACS Omega* **2016**, *1* (4), 656.

- (23) Lorber, B.; Bishop, J. B.; DeLucas, L. J. *Biochim. Biophys. Acta* **1990**, *1023*, 254.
- (24) Michel, H.; Oesterhelt, D. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77* (3), 1283.
- (25) Capicciotti, C. J.; Kurach, J. D. R.; Turner, T. R.; Mancini, R. S.; Acker, J. P.; Ben, R. N. *Sci. Rep.* **2015**, *5*, 9692.
- (26) Abraham, S.; Keillor, K.; Capicciotti, C. J.; Perley-Robertson, G. E.; Keillor, J. W.; Ben, R. N. *Cryst. Growth Des.* **2015**, *15* (10), 5034.
- (27) Tam, R. Y.; Ferreira, S. S.; Czechura, P.; Chaytor, J. L.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 17494.
- (28) Galema, S. A.; Engberts, J. B. F. N.; Høiland, H.; Førland, G. M. *J. Phys. Chem.* **1993**, *97* (26), 6885.
- (29) Galema, S. A.; Høiland, H. *J. Phys. Chem.* **1991**, *95* (13), 5321.
- (30) Galema, S. A.; Howard, E.; Engberts, J. B. F. N.; Grigera, J. R. *Carbohydr. Res.* **1994**, *265* (2), 215.
- (31) Katsonis, N.; Lubomska, M.; Pollard, M. M.; Feringa, B. L.; Rudolf, P. *Prog. Surf. Sci.* **2007**, *82*, 407.
- (32) Helmy, S.; Leibfarth, F. A.; Oh, S.; Poelma, J. E.; Hawker, C. J.; Alaniz, J. R. De. *J. Am. Chem. Soc.* **2014**, *136*, 8169.
- (33) Beharry, A.; Woolley, A. *Chem. Soc. Rev.* **2011**, *40* (8), 4422.
- (34) Mourot, A.; Kienzler, M. A.; Banghart, M. R.; Fehrentz, T.; Huber, F. M. E.; Stein, M.; Kramer, R. H.; Trauner, D. *ACS Chem. Neurosci.* **2011**, *2*, 536.
- (35) Merino, E.; Ribagorda, M. *Beilstein J. Org. Chem.* **2012**, *8*, 1071.
- (36) Bandara, H. M. D.; Friss, T. R.; Enriquez, M. M.; Isley, W.; Incarvito, C.; Frank, H. A.; Gascon, J.; Burdette, S. J. *J. Org. Chem.* **2010**, *75* (14), 4817.
- (37) Klajn, R. *Pure Appl. Chem.* **2010**, *82* (12), 2247.
- (38) Westmark, P. R.; Kelly, J. P.; Smith, B. D. *J. Am. Chem. Soc.* **1993**, *115* (9), 3416.
- (39) Schierling, B.; Noël, A.-J.; Wende, W.; Hien, L. T.; Volkov, E.; Kubareva, E.; Oretskaya, T.; Kokkinidis, M.; Rompp, A.; Spengler, B.; Pingoud, A. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107* (4), 1361.
- (40) Capicciotti, C. J. The Rational Design of Potent Ice Recrystallization Inhibitors for Use as Novel Cryoprotectants, University of Ottawa, 2014.
- (41) Hunter, T. *Phil. Trans. R. Soc. B* **2012**, *367*, 2513.
- (42) Westheimer, F. H. *Science* **1987**, *235* (4793), 1173.
- (43) Agledal, L.; Niere, M.; Ziegler, M. *Redox Rep.* **2010**, *15* (1), 2.
- (44) Ohlrogge, J.; Browse, J. *Plant Cell* **1995**, *7*, 957.
- (45) Cole, J. T.; Kean, W. S.; Pollard, H. B.; Verma, A.; Watson, W. D. *Front. Mol. Neurosci.* **2012**, *5*, 1.
- (46) Brase, S.; Gil, C.; Knepper, K.; Zimmermann, V. *Angew. Chem. Int. Ed.* **2005**, *44* (33), 5188.
- (47) Debets, M. F.; van der Doelen, C. W. J.; Rutjes, F. P. J. T.; van Delft, F. L. *ChemBioChem* **2010**, *11*, 1168.
- (48) Ma, Y.; Zhang, H.; Gruzdy, V.; Sun, X.-L. *Langmuir* **2011**, *27* (21), 13097.
- (49) Kim, E. J. *Molecules* **2018**, *23* (10), E2411.
- (50) Chakraborty, P. J. *Mol. Genet. Med.* **2018**, *12* (1), 332.
- (51) Kiick, K. L.; Saxon, E.; Tirrell, D. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99* (1), 19.
- (52) Dube, D. H.; Bertozzi, C. R. *Curr. Opin. Chem. Biol.* **2003**, *7* (5), 616.

- (53) Weller, R. L.; Rajske, S. R. *Org. Lett.* **2005**, 7 (11), 2141.
- (54) Lang, K.; Chin, J. W. *ACS Chem. Biol.* **2014**, 9 (1), 16.
- (55) Chakraborty, A.; Wang, D.; Ebright, Y. W.; Ebright, R. H. *Methods Enzym.* **2010**, 472, 19.
- (56) Vocadlo, D. J.; Hang, H. C.; Kim, E.-J.; Hanover, J. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, 100 (16), 9116.
- (57) Han, I.; Kwon, B.-S.; Park, H.-K.; Kim, K. S. *Int. Neurol. J.* **2017**, 21 (Suppl 1), S24.
- (58) Briard, J. G.; Jahan, S.; Chandran, P.; Allan, D.; Pineault, N.; Ben, R. N. *ACS Omega* **2016**, 1, 1010.
- (59) McGann, L. E. *Cryobiology* **1978**, 15 (4), 382.
- (60) Carruthers, A.; DeZutter, J.; Ganguly, A.; Devaskar, S. U. *Am. J. Physiol. Endocrinol. Metab.* **2009**, 297 (4), E836.
- (61) Concha, I. I.; Velasquez, F. V.; Martinez, J. M.; Angulo, C.; Droppelmann, A.; Reyes, A. M.; Slebe, J. C.; Vera, J. C.; Golde, D. W. *Blood* **1997**, 89 (11), 4190.
- (62) Barnett, J. E. G.; Holman, G. D.; Chalkley, R. A.; Munday, K. A. *Biochem. J.* **1975**, 145, 417.
- (63) Garcia-Alvarez, I.; Garrido, L.; Fernandez-Mayoralas, A. *ChemMedChem* **2007**, 2 (4), 496.

Chapter 3: Determining Structural Features Important for the IRI Activity of Small Molecules

3.1 Introduction

Our laboratory has previously reported that simple carbohydrates (mono- and disaccharides) exhibit moderate IRI activity.¹ As described in the introduction, the IRI activity exhibited by these molecules was correlated to their compatibility within the network of bulk water molecules, which is linked to carbohydrate hydration.¹ It has been hypothesized that highly hydrated carbohydrates such as D-galactose and D-melibiose, concentrated at the bulk water-quasi liquid layer (QLL) interface, fit poorly into the three-dimensional hydrogen-bonded network of bulk water and cause greater disruption on the pre-ordering of water molecules.²⁻⁴ For ice recrystallization to occur, there is a movement of bulk water molecules to the QLL then to the growing ice lattice.^{5,6} Therefore, a greater disruption on the pre-ordering of water molecules increases the energy associated with the transfer of bulk water molecules to the QLL and makes this an energetically unfavourable process, resulting in inhibition of ice recrystallization. In contrast, less hydrated carbohydrates, such as D-talose and D-sucrose, fit well into the three-dimensional hydrogen-bonded network of bulk water and disrupt the pre-ordering of bulk water molecules less. As a result, no inhibition of ice recrystallization is observed.¹

Given that the proposed mechanism for inhibition of ice recrystallization by simple carbohydrates was attributed to their ability to disrupt or disorder the structure of bulk water,¹ carbohydrate-based molecules that are known to alter and/or sequester the structure of bulk water by self-assembling in solution were assessed for IRI activity. Such compounds included surfactants, organogelators and hydrogelators.⁷ These molecules possess a hydrophilic carbohydrate head group and a hydrophobic alkyl tail. In particular, carbohydrate-based non-ionic surfactants such as *n*-octyl- β -D-galactopyranoside (**102**, Figure 3.1) were shown to be effective inhibitors of ice recrystallization. This highlighted the importance of hydrophobic moieties such as long alkyl chains at the C-1 position of the pyranose ring for IRI activity.⁷ This study also revealed the importance of having a more hydrated carbohydrate moiety (i.e. galactose) since substituting this moiety with a less hydrated D-glucose moiety resulted in decreased IRI activity.⁷ However, the

hydration characteristics for *n*-octyl- β -D-galactopyranoside are not known; therefore, a direct correlation between hydration and IRI activity could not be made. Consequently, the mechanism by which carbohydrate-based surfactants inhibit ice recrystallization is not known.

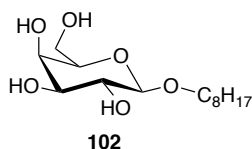


Figure 3.1 Structure of *n*-octyl- β -D-galactopyranoside **102**.

Despite the IRI activity of carbohydrate-based surfactants possessing long alkyl chains (> 8 carbons in length), the surfactant nature of these compounds is detrimental to cells due to their solubilizing effect on cell membranes.^{8,9} As a result, this prevents their use in cellular applications such as cryopreservation. Although carbohydrate-based surfactants are capable of solubilizing cell membranes, this study provided insight into the importance of hydrophobic moieties at C-1 for ice recrystallization inhibition. Furthermore, it was previously demonstrated that surfactant-like molecules possessing shorter alkyl chains (i.e. 4-6 carbons in length) were less cytotoxic than those possessing longer alkyl chains.¹⁰ Thus, to eliminate the deleterious effects of long alkyl chains on membrane structural integrity, the hydrophobic alkyl chain at C-1 was replaced with a 6-carbon cyclic ring, in particular an aryl moiety. It was hypothesized that an aromatic ring would maintain the required hydrophobicity while their cyclic nature would minimize their ability to solubilize membranes. A variety of *O*-aryl glycosides were previously synthesized and assessed for IRI activity.^{11,12} Within this class, some of the most effective inhibitors of ice recrystallization were shown to possess a D-glucose moiety, a β -glycosidic bond, and either a methoxy substituent ($-\text{OCH}_3$) or a halogen ($-\text{Br}$ or $-\text{F}$) at the *para*-position of the aryl ring (Figure 3.2).¹² This study revealed critical features that influence the IRI activity of the *O*-aryl glycosides including the relative orientation of the hydroxyl groups on the pyranose ring, the stereochemistry and nature of the glycosidic bond, and the substituent on the aromatic ring and its regiochemistry.^{11,12} Furthermore, some of the *O*-aryl glycosides were recently shown to enable the freezing of human RBCs with greatly reduced concentrations of glycerol.^{12,13}

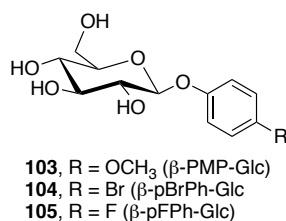


Figure 3.2 Structures of *O*-aryl glycosides **103-105**.

Although extensive structure-function studies have been performed on many molecules to determine key structural features that are important for the IRI activity of small molecules, few small molecules have been shown to be effective inhibitors of ice recrystallization. As such, the structural features responsible for the IRI activity of small molecules have not been definitively determined and the exact mechanism by which small molecules inhibit ice recrystallization is not known. To facilitate the design of small molecules that are effective inhibitors of ice recrystallization, further structure-function studies were conducted to determine additional structural features that are important for IRI activity. In this chapter, three diverse classes of small molecules were investigated as ice recrystallization inhibitors with the overall goal to identify novel small molecules that are effective inhibitors of ice recrystallization. Thus, this chapter will describe the following:

1. Previous work demonstrated that carbohydrate-based surfactants could be effective inhibitors of ice recrystallization, however, the precise mechanism by which these molecules inhibit ice recrystallization remains unknown. Since the isomerization of azobenzene can provide spatiotemporal control over molecular structure and properties, it would be desirable to employ this strategy to photo-modulate IRI activity. This approach could potentially be used as a tool to study the mechanism of ice recrystallization inhibition. As such, carbohydrate-based (fluoro)surfactants bearing an azobenzene moiety were examined as ice recrystallization inhibitors. The results from these studies are discussed in Section 3.2.
2. Given the unique properties of fluorine and its important role in medicinal chemistry, fluorinated aryl glycosides were investigated as ice recrystallization inhibitors. In particular, β -PMP-Glc, β -pBrPh-Glc and β -pFPh-Glc were selected

for further investigation. The results from these studies are discussed in Section 3.3.

3. Considering the importance of phosphate groups in biological systems, a small diverse class of monosaccharide phosphate sugars were examined for their ability to inhibit ice recrystallization. This study will generate preliminary data and the results from these studies are discussed in Section 3.4.

3.2 Photoswitchable Surfactants as Ice Recrystallization Inhibitors

Photochromic molecular switches are an attractive class of molecules that permit the control of molecular structure and function when irradiated with light.^{14,15} Members of this class include azobenzenes, which are the most widely used photochromic group owing to their reversible photoisomerization between *cis* and *trans* isomers.^{16–18} The isomerization of the N=N double bond from the *trans* to *cis* isomer occurs readily upon near-UV irradiation and is accompanied by a change in molecular geometry and dipole moment.^{16,19} The reverse isomerization is triggered when the *cis* isomer is irradiated with visible light or heated.¹⁸ Figure 3.3 illustrates the two geometric isomers of azobenzene: the *trans* (or *E*) isomer and the *cis* (or *Z*) isomer. The *trans* isomer is thermodynamically more stable than the *cis* isomer and exhibits a smaller dipole moment.^{18,20,21} The change in dipole moment between the *trans* and *cis* isomers ultimately determines the hydrophobic and hydrophilic nature of these isomers.¹⁴ Since the *cis* isomer has a larger dipole moment, it is more polar, and thus more hydrophilic than the *trans* isomer.^{18,20,21}

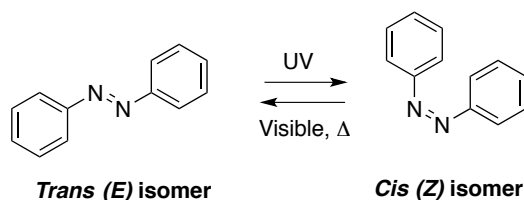


Figure 3.3 Reversible photoisomerization of azobenzene upon UV-vis irradiation.

The isomerization of azobenzene has been applied as molecular switches to provide spatiotemporal control over molecular structure and properties.^{16,18,20,21} This strategy is very attractive and has been used as a synthetic tool to control supramolecular assemblies, to study biomolecular interactions, and to control macroscopic properties of functional materials.^{18,21,22} In biological systems, this process has been applied for the

photo-control of biomolecules.^{16,18} For instance, azobenzene-functionalized proteins have been prepared to photo-control enzymatic activity, thus permitting spatiotemporal control of various biological processes.^{18,23,24} Consequently, light-sensitive biologically active molecules offer the possibility to study the dynamics of biological processes. Towards this end, it would be desirable to selectively switch the activity of an IRI active molecule off and on using light to study the dynamic mechanisms of ice recrystallization inhibition. This could potentially be accomplished by introducing a photosensitive group such as azobenzene onto the molecule of interest. Given that the exact mechanism by which carbohydrate-based surfactants inhibit ice recrystallization is not known, we sought to examine whether carbohydrate-based surfactants bearing an azobenzene moiety could be effective inhibitors of ice recrystallization. This could present promising opportunities for the design of light-responsive tools for studying the mechanism of ice recrystallization inhibition.

The carbohydrate-based surfactants assessed for IRI activity are shown in Figures 3.4 and 3.5. Their structures consist of a hydrophilic carbohydrate head group and a hydrophobic azobenzene tail. The carbohydrate head group was modified with various mono- and disaccharides to determine its influence on IRI activity as previous studies have suggested that the identity of the carbohydrate moiety greatly affects activity.¹ A triethylene glycolate spacer was incorporated to promote water solubility. The carbohydrate-based surfactants shown in Figure 3.4 contain an *n*-butyl azobenzene tail group conjugated to a carbohydrate moiety through an *O*-glycosidic linkage (**301-304**) or a triazole linkage (**305-310**) to investigate the nature of the linker at C-1. Since the IRI activity of amphiphilic molecules has been shown to be highly sensitive to changes in the hydrophilic-hydrophobic balance of a molecule,²⁵ the hydrophobicity of the azobenzene tail was modified by replacing the *n*-butyl group with a trifluoromethyl group (-CF₃). The carbohydrate-based fluorosurfactants (**311-322**) shown in Figure 3.5 contain a trifluoromethyl-substituted azobenzene tail group conjugated to a carbohydrate moiety through a triazole linkage due to synthetic simplicity. The azobenzene ring was substituted with a trifluoromethyl group in the *ortho*-, *meta*- or *para*-position to examine the effect of regiochemistry on IRI activity.

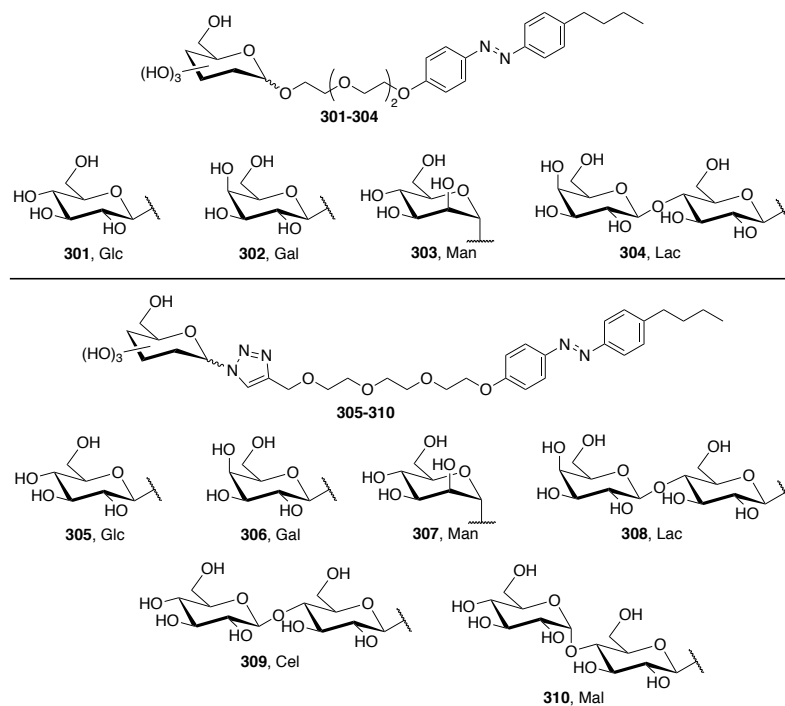


Figure 3.4 Structures of carbohydrate-based surfactants bearing an *n*-butyl azobenzene tail group and a variable mono- or disaccharide head group. Structures **301-304** contain an *O*-glycosidic linkage and structures **305-310** contain a triazole linkage.

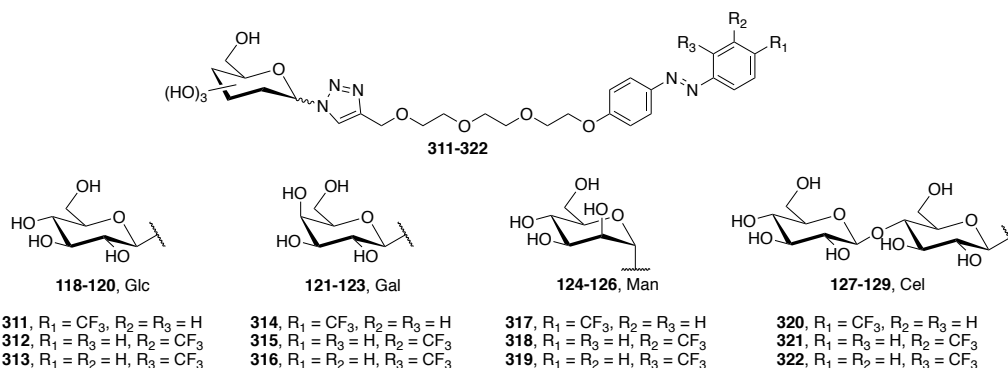
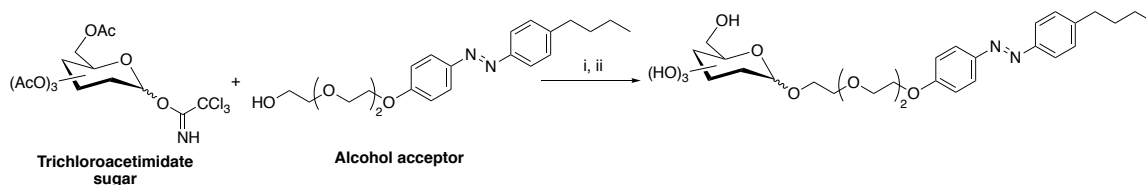


Figure 3.5 Structures of carbohydrate-based fluorosurfactants possessing a variable mono- or disaccharide head group conjugated to a trifluoromethyl-substituted azobenzene tail group via a triazole linkage (**311-322**). The substituent ($-CF_3$) was attached to the azobenzene ring at the *ortho*-, *meta*- or *para*-position.

3.2.1 Synthesis of Photoswitchable Surfactants

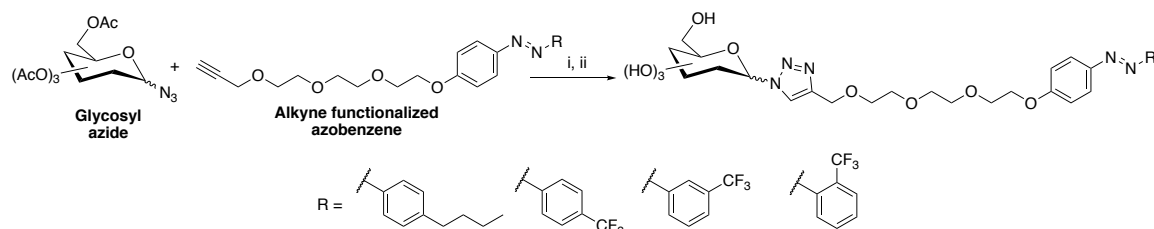
This work was part of a collaboration and compounds **301-322** were synthesized and provided by Dr. Brendan Wilkinson from the School of Science and Technology at the

University of New England. For compounds **301-304**, their strategy employed Lewis acid-promoted glycosidation of the corresponding trichloroacetimidate sugars with *n*-butyl azobenzene as the alcohol acceptor.²⁶ Following this, the compounds were deprotected using sodium methoxide in methanol (Scheme 3.1).²⁷



Scheme 3.1 Synthesis of carbohydrate-based surfactants **301-304**. Conditions: i) $\text{BF}_3 \cdot \text{OEt}_2$, CH_2Cl_2 , 0 °C. ii) NaOMe, MeOH.

The synthesis of compounds **305-310**, bearing a triazole linkage, was achieved using the well-established copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction between the corresponding glycosyl azide and alkyne functionalized azobenzene fragment.²⁸⁻³⁰ Base-catalyzed deprotection afforded the final compounds. Compounds **311-322** were synthesized in the same manner using the CuAAC reaction (Scheme 3.2).³¹



Scheme 3.2 Synthesis of carbohydrate-based surfactants **305-322**. Conditions: i) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, sodium ascorbate, *t*-BuOH/ H_2O , 40 °C. ii) NaOMe, MeOH.

3.2.2 IRI Activity of Photoswitchable Surfactants

IRI activity is typically measured using the “splat-cooling” assay where ice recrystallization is observed by a change in ice crystal size.^{32,33} In this assay, compounds are dissolved in a phosphate buffered saline (PBS) solution and a 10 μL aliquot of the solution is dropped through a two-meter high plastic tube, having a 10 cm diameter, onto a polished aluminum block cooled to approximately -80 °C with dry ice. The ice wafer is separated from the surface of the block and transferred to a cryostage, held at -6.4 °C, for 30 minutes. During this time, recrystallization occurs where the ice crystals dramatically increase in size. The ice crystal wafer is then photographed using a digital camera. Using a domain recognition software,³³ the area of 12 randomly selected ice crystals is

measured and IRI activity is quantified by calculating the percent mean grain size (% MGS) of ice crystals relative to the positive PBS control for ice recrystallization. Larger MGS values indicate larger ice crystal size and thus, less inhibition of ice recrystallization, whereas, smaller MGS values indicate smaller ice crystal size and greater IRI activity. Thus, molecules that were assessed for IRI activity using this method are categorized as inactive, moderately active or highly active based upon their % MGS relative to PBS. The cut-off values for categorization were chosen such that molecules with a MGS > 80% are inactive, molecules with a MGS < 30% are highly active and molecules with a MGS between 30 and 80% are moderately active. Although measuring the % MGS provides a general indication for the degree of IRI activity, this previously employed method has several limitations and only provides a semi-quantitative measurement. The reasons for this are that the analysis of 12 randomly selected ice crystals does not take into account the heterogeneity of ice crystal size within a population.³⁴ Additionally, molecules are assessed at a single time point of 30 minutes and a typical concentration of 22 mM and thus the effects of both time and concentration on IRI activity are neglected.³⁴ To account for these limitations, our laboratory has recently implemented a more accurate method of quantifying IRI activity based on IC₅₀ values (half maximal inhibitory concentration). In this assay, the compound is assessed at various concentrations in a PBS solution as opposed to a single concentration. Our laboratory also demonstrated that ice recrystallization primarily occurs within the first 5-10 minutes of annealing,^{34,35} thus, ice wafers are annealed at -6.4 °C for 5 minutes as opposed to 30 minutes. Within the field of view, the area of individual ice crystals are measured using ImageJ software and the ice crystal areas are sorted into discrete bins based on size and the proportionate area of each bin is measured as a function of time.³⁴ The rate of depletion of the smallest bin size is used to measure the initial rate of ice crystal growth. Thus, for each inhibitor concentration, rate constants are determined and normalized to PBS. A dose-response curve is constructed by plotting the normalized rate constants (k_{norm}) for each concentration as a function of the corresponding log values of the concentration. To obtain the IC₅₀ value, a two-parameter sigmoidal curve is fit to the data. This type of kinetic analysis provides a more quantitative measurement of IRI activity, compared to the % MGS formerly used, because it takes into account the time

and concentration dependence of ice recrystallization as well as the heterogeneity of ice crystal size.³⁵ Our laboratory implemented this kinetic approach for the screening of new compounds for their IRI activity in 2015 and it is currently the standard protocol for screening new compounds.

However, in the case of compounds **301-322**, they were initially assessed for IRI activity prior to implementation of the kinetic approach. As a result, these compounds were assessed for IRI activity utilizing the former “splat-cooling” assay,^{32,33} where ice crystal size (area) was quantified at a single concentration after an annealing period of 30 minutes at -6.4 °C, and thus their IRI activity is represented as a % MGS relative to PBS. The IRI activity of carbohydrate-based surfactants **301-310**, bearing an *n*-butyl azobenzene tail group and a variable mono- or disaccharide head group conjugated via an *O*-glycosidic linkage (**301-304**) or a triazole linkage (**305-310**), is presented in Figure 3.6. All compounds were assessed at a concentration of 22 mM in PBS with the exception of compounds **302** and **304**, which were insoluble at 22 mM, thus, they were assessed at their maximum solubility of 5 μ M and 1 mM in PBS, respectively.

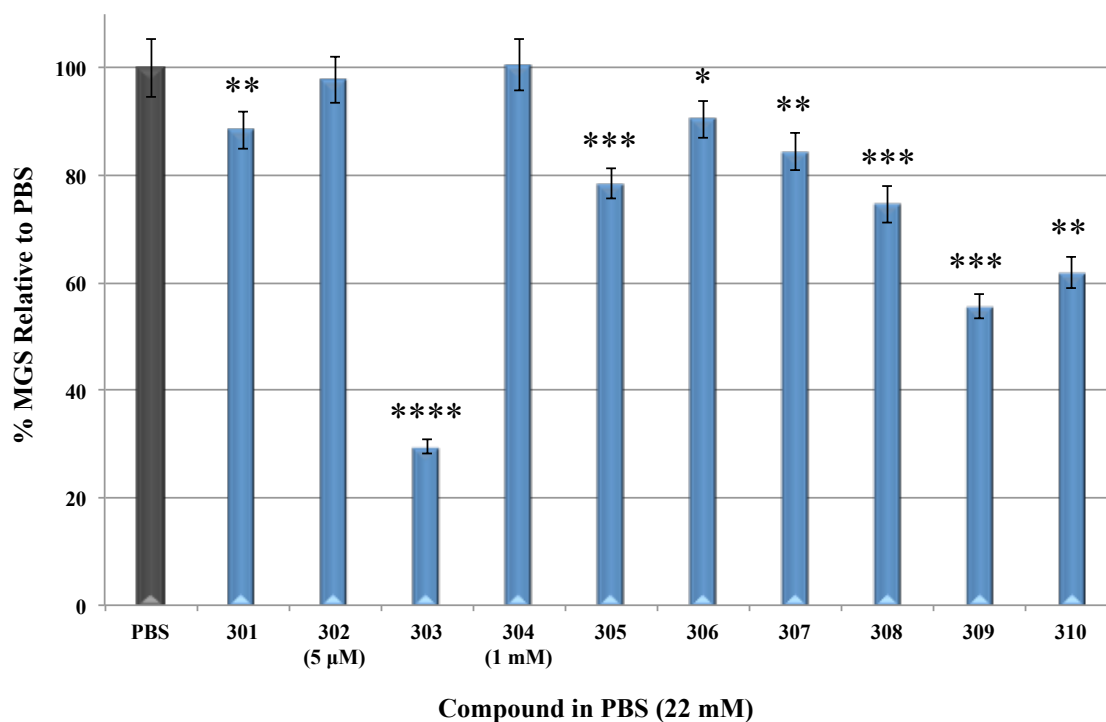


Figure 3.6 Ice recrystallization inhibition (IRI) activity of compounds **301-310**. Compounds are measured as % MGS (mean grain size) of ice crystals relative to a PBS positive control. Error bars represent the standard error of the mean (SEM). Asterisks

indicate significant difference relative to the PBS control as determined by the unpaired Student's T-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Similar to the PBS control, compounds **301**, **302** and **304**, bearing an *O*-glycosidic linkage, exhibited weak to no IRI activity. This was surprising since previous studies have shown that carbohydrate-based surfactants possessing a more hydrated carbohydrate moiety (i.e. galactose) resulted in higher IRI activity.^{1,7} In this case, compound **302** possessing a galactose moiety exhibited no IRI activity. Presumably, the lack of IRI activity may be due to its poor solubility in PBS (5 μ M). Remarkably, the D-mannose derivative **303** was highly IRI active with a MGS of 30% relative to PBS.²⁷ This was surprising given that **301** and **303** are structurally similar and possess carbohydrate moieties with comparable hydration indices (D-glucose versus D-mannose).^{1,36} Previous studies have shown that D-glucose analogues exhibited greater IRI activity than D-mannose analogues,³⁶ however, in the case of compounds **301** and **303**, the trend is reversed. This further emphasizes that changes in head group stereochemistry and polarity greatly affect IRI activity. In the case of the triazole-linked compounds, those possessing a monosaccharide head group (**305-307**) exhibited similar IRI activity and all possessed weak activity relative to the PBS control. Surprisingly, compound **307**, which only differs from compound **303** in its linkage, did not retain its IRI activity, as the incorporation of a triazole linkage was detrimental. Compounds possessing a disaccharide head group (**308-310**) exhibited weak to moderate IRI activity relative to PBS. It has been previously shown that D-lactose is more hydrated than D-maltose and D-cellobiose,³ thus, exhibits greater IRI activity.¹ Interestingly, in this case, compounds **308**, **309** and **310**, possessing a D-lactose, D-cellobiose and D-maltose head group, respectively, all exhibited IRI activities that were not statistically different from one another ($p > 0.15$, two-tailed Student's T-test).

Given that the D-mannose derivative **303** was the most IRI active in this series, a kinetic analysis was performed using the improved "splat-cooling" assay described above to quantify IRI activity by an IC₅₀ value.³⁵ The dose-response curve for **303** is shown in Figure 3.7 and it was found to have an IC₅₀ value of 7 mM. This kinetic analysis demonstrates the ability of the D-mannose derivative **303** to effectively inhibit ice recrystallization at low millimolar concentrations.²⁷

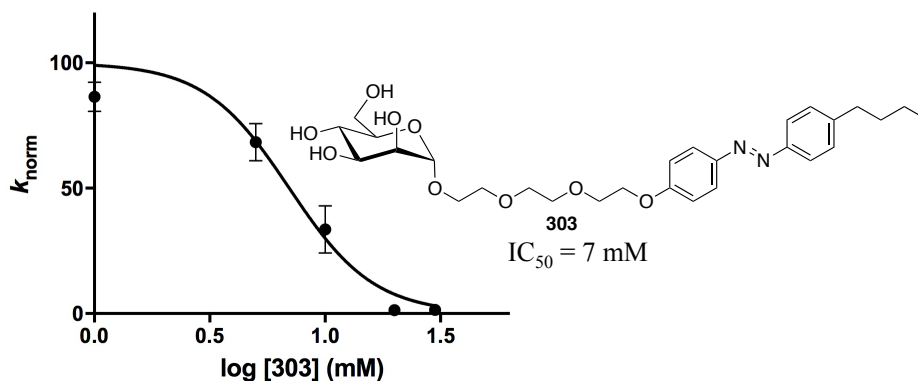


Figure 3.7 A dose-response curve for compound **303**. Rate constants (k_{norm}) were normalized to a PBS control and measured in triplicate. Error bars represent the standard error of the mean (SEM). A two-parameter sigmoidal curve was fit to the data.

Since compound **303** was shown to be an effective inhibitor of ice recrystallization, it was also assessed for thermal hysteresis (TH) activity and dynamic ice shaping (DIS) capability to determine whether this compound was binding to ice in a similar manner as AFGPs. The TH activity of compound **303** was assessed utilizing nanoliter osmometry.³⁷ Previously investigated C-linked AFGP analogues and carbohydrate-based surfactants were assessed for TH activity at a concentration of 10 mg mL⁻¹.^{1,7} However, due to the amphiphilic nature of compound **303**, it was assessed at a lower concentration (0.5 mg mL⁻¹) than what is typically used. Thus, at this concentration, compound **303** did not possess TH activity or DIS capability (Figure 3.8), suggesting that this compound does not interact with the ice lattice.²⁷ This result is consistent with previous studies that reported the lack of TH activity for IRI active carbohydrate- and amino acid-based surfactants and hydrogelators.^{7,25}

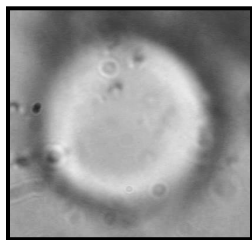


Figure 3.8 Ice crystal morphology in the presence of compound **303** (0.5 mg mL⁻¹) in water.

The IRI activity of carbohydrate-based fluorosurfactants **311-322**, possessing a variable mono- or disaccharide head group conjugated to a trifluoromethyl-substituted

azobenzene tail group via a triazole linkage, is presented in Figure 3.9. The substituent (CF_3) was attached to the azobenzene ring at the *para*-, *meta*- or *ortho*-position relative to the azo group to examine the effect of regiochemistry on IRI activity. All compounds were assessed at a concentration of 22 mM in PBS with the exception of the *para*-substituted compounds (**311**, **314**, **317** and **320**) and compound **318**, which were insoluble at 22 mM, thus, they were assessed at their maximum solubility of 5 μM and 0.5 mM in PBS, respectively. Compounds **317-319** were assessed for IRI activity by my colleague Madeleine Adam in the Ben laboratory.

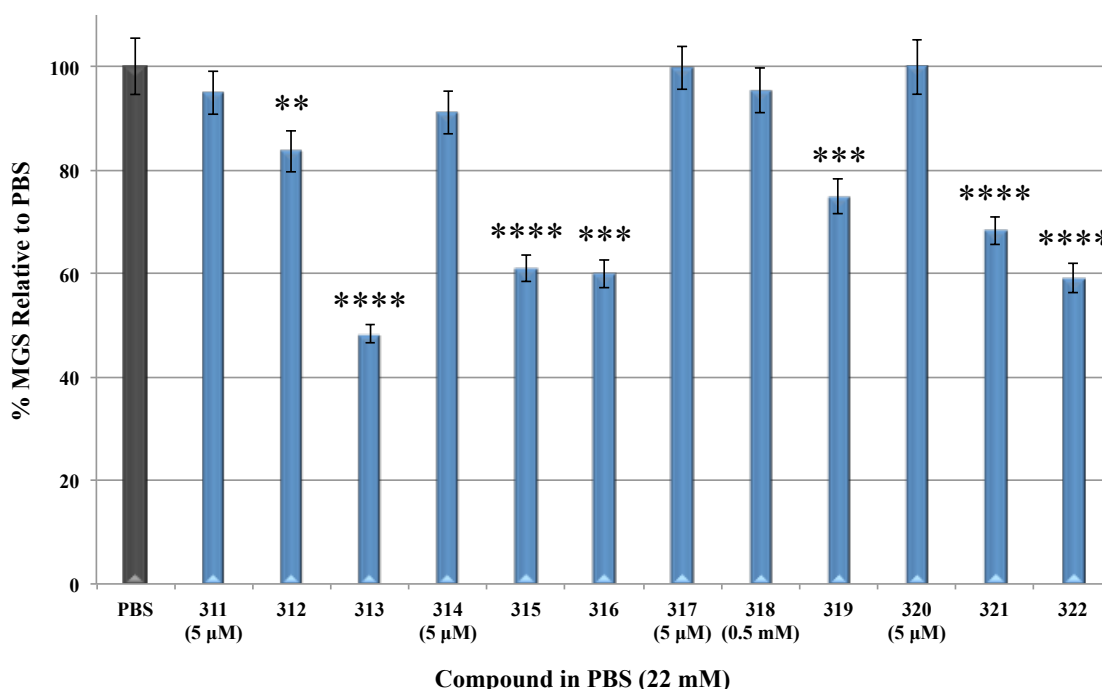


Figure 3.9 Ice recrystallization inhibition (IRI) activity of compounds **311-322**. Compounds are measured as % MGS (mean grain size) of ice crystals relative to a PBS positive control. Error bars represent the standard error of the mean (SEM). Asterisks indicate significant difference relative to the PBS control as determined by the unpaired Student's T-test: ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Relative to the PBS control, the *para*-substituted compounds (**311**, **314**, **317** and **320**) exhibited weak to no IRI activity. The lack of any appreciable IRI activity may be due to their poor solubility in PBS. Furthermore, it is evident that replacing the *n*-butyl group with a trifluoromethyl group at the *para*-position greatly reduced solubility (22 mM versus 5 μM). Interestingly, in each series, a trend was observed where the most IRI active compounds were those possessing a trifluoromethyl substituent at the *ortho*-

position relative to the azo group (**313**, **316**, **319** and **322**).²⁹ In most cases, IRI activity progressively decreased when the trifluoromethyl substituent was placed at the *meta*-position and finally at the *para*-position. This highlights the importance of trifluoromethyl group regiochemistry for the IRI activity of these carbohydrate-based fluorosurfactants.

A comparison of the *ortho*-substituted compounds demonstrated that compound **313**, bearing a D-glucose head group, was the most IRI active. Meanwhile, compounds **316**, **319** and **322**, bearing a D-galactose, D-mannose and D-cellobiose head group, respectively, possessed weaker IRI activity.²⁹ This result is not consistent with previous observations where compound **303**, bearing a D-mannose head group and an *n*-butyl azobenzene tail group, was the most IRI active compared to its corresponding D-glucose- and D-galactose-based compounds (**301** and **302**). Additionally, previous studies have shown that D-galactose analogues exhibited greater IRI activity than D-glucose analogues due to the higher hydration index (HI) of galactose.^{1,7} A comparison of the *meta*-substituted compounds showed that compound **312** exhibited weaker IRI activity than compounds **315** and **321**, whereas compound **318** showed reduced solubility in PBS (0.5 mM). These results demonstrate that the IRI activity of these compounds is highly sensitive to changes in head group stereochemistry and tail group hydrophobicity.²⁹

The results discussed above are based on compounds **301-322** in their resting *trans*-dominated photostationary state (PSS). To affect *trans-cis* photoisomerization, aqueous solutions of the compounds in their *trans* states were converted into their corresponding *cis* states by irradiation with UV light (361 nm) for 10 minutes.^{27,29} Select compounds were assessed for IRI activity after *trans-cis* photoisomerization to examine whether the isomeric state of the azobenzene tail group has an effect on IRI activity. The compounds selected for the photoisomerization experiments exhibited varying degrees of IRI activity in their *trans* states to establish whether photo-modulation of activity was possible. The photoisomerization experiments and subsequent IRI testing of the *cis* isomers were performed by my colleague Madeleine Adam in the Ben laboratory.

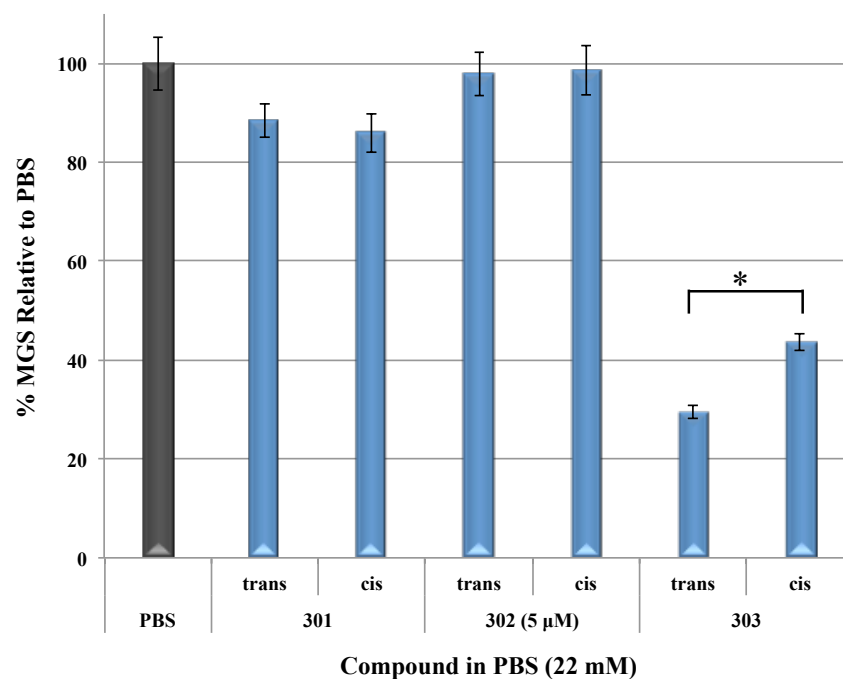


Figure 3.10 Ice recrystallization inhibition (IRI) activity of compounds **301-303**. Compounds are measured as % MGS (mean grain size) of ice crystals relative to a PBS positive control. The *cis* isomers are compared to their corresponding *trans* isomers. Error bars represent the standard error of the mean (SEM). Asterisk indicates significant difference as determined by the unpaired Student's T-test (* $p < 0.05$).

The IRI activity of compounds **301-303**, in their *cis* states, was compared to their corresponding *trans* isomers to determine whether the *trans-cis* photoisomerization had an effect on activity (Figure 3.10). The *trans* and *cis* isomers of compounds **301** and **302** both showed nearly identical IRI activity, indicating that photo-modulation of IRI activity was not observed.²⁷ Interestingly, *trans-cis* photoisomerization of the D-mannose derivative **303** resulted in decreased IRI activity, which may be due to the reduced hydrophobic nature of the azobenzene tail group. The IRI activity of compounds **311-313**, **316**, **319** and **322**, in their *cis* states, was compared to their corresponding *trans* isomers (Figure 3.11). The *trans* and *cis* isomers of compounds **311-313** exhibited nearly identical IRI activity, whereas *trans-cis* photoisomerization of compounds **316**, **319** and **322** significantly reduced their IRI activity.²⁹ It is hypothesized that the decrease in IRI activity observed upon *trans-cis* photoisomerization is the result of increased hydrophilicity of the tail group. However, in this case, the photoisomerization studies demonstrated that only some carbohydrate-based (fluoro)surfactants are affected by the

change of the isomeric state of the azobenzene tail group. This suggests that some molecules may be more sensitive to changes in amphiphilicity than others and reiterates the importance of having carefully balanced hydrophilic and hydrophobic groups for IRI activity.

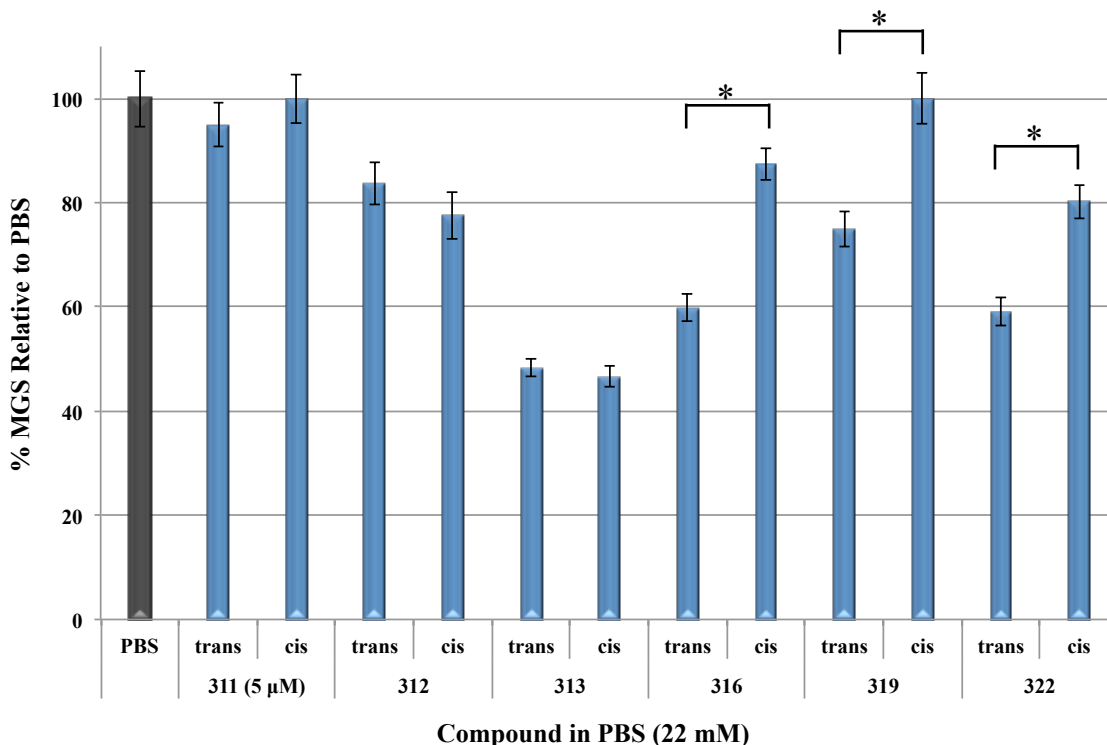


Figure 3.11 Ice recrystallization inhibition (IRI) activity of compounds **311-313**, **316**, **319** and **322**. Compounds are measured as % MGS (mean grain size) of ice crystals relative to a PBS positive control. The *cis* isomers are compared to their corresponding *trans* isomers. Error bars represent the standard error of the mean (SEM). Asterisk indicates significant difference as determined by the unpaired Student's T-test ($*p < 0.05$).

3.3 Fluorinated Aryl Glycosides as Ice Recrystallization Inhibitors

Recently, *O*-aryl glycosides have been shown to be effective inhibitors of ice recrystallization.¹² Previous structure-function studies have revealed critical features that influence the IRI activity of these molecules including the relative orientation of the hydroxyl groups on the pyranose ring, the stereochemistry and nature of the glycosidic bond, and the substituent on the aromatic ring and its regiochemistry.^{11,12} Specifically, some of the most effective inhibitors of ice recrystallization in this class were shown to possess a D-glucose moiety, a β -glycosidic bond, and either a methoxy substituent (-

OCH₃) or a halogen (-Br or -F) at the *para*-position of the aryl ring.¹² The structures of these highly IRI active small molecules, β -PMP-Glc, β -pBrPh-Glc and β -pFPh-Glc, are shown in Figure 3.12.

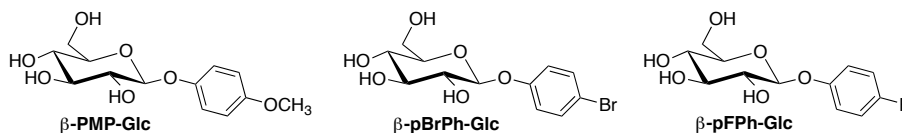


Figure 3.12 Structures of β -PMP-Glc, β -pBrPh-Glc and β -pFPh-Glc.

Although many modifications to the D-glucose moiety have been explored,¹¹ the presence of a fluorine atom on the carbohydrate moiety has not yet been investigated for its influence on IRI activity. The small size and electronegativity of fluorine make it a unique atom that has a profound effect when bound to carbon in small molecules.³⁸ Fluorinated compounds have attracted considerable attention in the fields of pharmaceuticals, agrochemicals, and materials science.^{39,40} In the pharmaceutical field, fluorinated analogues of natural products have become useful tools for pharmacological and physiological studies since the introduction of fluorine can result in significant changes in physical and biological properties.^{38,41} Consequently, fluorine plays an important role in medicinal chemistry where installation into a therapeutic small molecule candidate can enhance biological activity and improve chemical or metabolic stability.³⁸

Our laboratory previously correlated IRI activity to carbohydrate hydration and thus it is hypothesized that the IRI activity of *O*-aryl glycosides is attributed to their ability to disrupt the structure of bulk water. The hydration of fluorine atoms has been studied and it was observed that fluorine atoms behave like hydrocarbons in that they cause water molecules to become more ordered around themselves.⁴² Fluorine has also been reported to be more hydrophobic than oxygen and therefore it may have an effect on hydration.⁴³ For instance, it was shown that 4-hydroxyproline was more effectively hydrated than 4-fluoroproline, suggesting that the formation of hydrogen bonds to the fluorine atoms are unlikely.⁴³ Generally, compounds that cause a greater disruption of water molecules are well hydrated, whereas those that tend to order water molecules are poorly hydrated. In the case of fluorine atoms, they behave as hydrophobic groups and cause minimal disruption of water molecules within the hydration shell and are thus poorly hydrated.

Given the unique properties of fluorine, a small class of fluorinated aryl glycosides was synthesized and assessed for IRI activity. Three *para*-substituted *O*-aryl glycosides (β -PMP-Glc, β -pBrPh-Glc and β -pFPh-Glc) that are effective inhibitors of ice recrystallization were selected for further investigation. The structures of the fluorinated aryl glycosides are shown in Figure 3.13. Specifically, the hydroxyl group at C-6 was replaced with a fluorine atom to assess the importance of the C-6 hydroxyl group of aryl glycosides for IRI activity (**323-325**). By substituting the C-6 hydroxyl group for a fluorine atom, this may alter the hydration of these aryl glycosides and subsequently, may affect their IRI activity. Due to synthetic simplicity, difluorinated aryl glycosides were also synthesized in which the C-6 and C-3 hydroxyl groups were replaced with fluorine atoms. This synthesis employed a nucleophilic fluorinating agent; therefore, inversion of stereochemistry at the C-3 position furnished difluorinated aryl glycosides, which possess a D-allose moiety rather than a D-glucose moiety. As a result, the allopyranoside derivatives (**326-328**) were not compared to their non-fluorinated counterparts; however, this will further elucidate structural features that are important for the IRI activity of these *O*-aryl glycosides. The synthesis and IRI testing of these compounds were performed with assistance from two undergraduate students, Geneviève O'Keefe and Olivia Herzog, both supervised by myself.

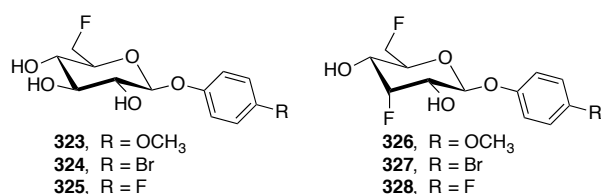
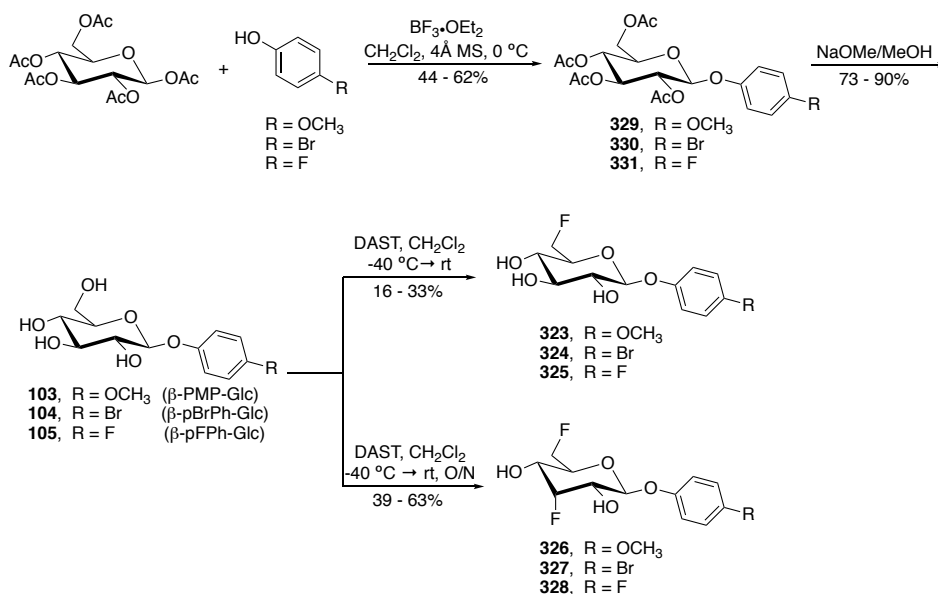


Figure 3.13 Structures of fluorinated aryl glycosides **323-328**.

3.3.1 Synthesis of Fluorinated Aryl Glycosides

The synthesis of fluorinated aryl glycosides **323-328** (Scheme 3.3) began with β -D-glucose pentaacetate. Lewis-acid catalyzed activation of the β -linked anomeric acetate with boron trifluoride diethyl etherate (BF₃•OEt₂) allowed for glycosidation with the appropriate *para*-substituted phenol (R = OCH₃, Br or F) to afford the β -linked aryl glycosides exclusively (**329-331**). Deacetylation of compounds **329-331** under Zemplén conditions afforded the intermediates **103-105**,¹² which were selectively fluorinated using

(diethylamino)sulfur trifluoride (DAST).⁴⁴ It has been previously shown that DAST can react with β -glucosides to prepare monofluorinated products when short reaction times are employed. Thus, β -glucosides **103-105** were treated with DAST, using short reaction times (< 30 min), to afford the desired monofluorinated products **323-325**, in which the C-6 hydroxyl group was replaced with a fluorine atom. It is well known in the literature that β -glucosides also undergo rapid fluorination at the C-3 position of the pyranose ring to afford 3-fluoroallopysanosides.⁴⁴ Since DAST is a nucleophilic fluorinating agent,⁴⁵ the reaction of DAST with β -glucosides installs fluorine atoms selectively at the less sterically hindered C-3 and C-6 positions when longer reaction times are employed (> 24 h).⁴⁴ Thus, β -glucosides **103-105** were treated with DAST overnight to afford the desired difluorinated products **326-328**, in which the C-6 and C-3 hydroxyl groups were replaced with fluorine atoms. Using this synthetic approach, allopysanoside derivatives **326-328** were obtained, whereby the equatorial C-3 hydroxyl group was converted to an axial fluorine substituent.⁴⁶



Scheme 3.3 Synthesis of fluorinated aryl glycosides **323-328**.

3.3.2 IRI Activity of Fluorinated Aryl Glycosides

To quantify the IRI activity of compounds **323-328**, the kinetic approach was utilized as this is the standard protocol that is now employed in our laboratory for screening new compounds (previously described in Section 3.2.2).³⁵ Using this method, IRI activity was quantified by an IC₅₀ value. Briefly, in this assay, the compound was dissolved at various

concentrations in a PBS solution and the areas of ice crystals were measured after a 5-minute annealing period at -6.4 °C. For each concentration, rate constants for ice crystal growth were determined and normalized to the positive PBS control for ice recrystallization. A dose-response curve was generated by plotting the normalized rate constants (k_{norm}) for each concentration as a function of the corresponding log values of the concentration. To obtain the IC_{50} value, a two-parameter sigmoidal curve was fit to the data. The characteristic dose-response curve that is typically observed shows the response as a function of the logarithm of concentrations and forms a sigmoidal shape. These curves contain several data points that fit to a sigmoidal shape: one at the top plateau (100%), one at the bottom plateau (0%), one at the inflection point, and one each at the upper and lower ends of the inflection point. Two important properties of IRIs can be determined from a dose-response curve. The first is efficiency, which refers to the maximal inhibition of ice recrystallization that a compound can achieve. In other words, it refers to the ability of a compound to fully inhibit ice crystal growth ($k_{\text{norm}} = 0\%$) at its maximum solubility in PBS. The second property that can be determined from a dose-response curve is efficacy, which refers to the concentration of compound necessary to inhibit ice recrystallization. Thus, compounds can be compared in terms of their efficiency and efficacy.

The majority of the fluorinated aryl glycosides were not effective inhibitors of ice recrystallization. With the exception of compound **327**, the remaining compounds (**323-326** and **328**) failed to achieve full inhibition of ice crystal growth (i.e. $k_{\text{norm}} \neq 0\%$) when assessed at their maximum solubility in PBS (Table 3.1). This may be due to their very poor solubility in aqueous solution. For example, the monofluorinated derivatives **323-325** were only able to achieve a k_{norm} of 72.67%, 78.33% and 46.64%, respectively (Table 3.1). When assessed at their maximum solubility of 5 mM, difluorinated derivatives **326** and **328** were only able to achieve a k_{norm} of 30.58% and 92.46%, respectively (Table 3.1). As a result, the data was not sufficient to construct a proper sigmoidal curve (data points only on the upper half) and an IC_{50} value could not be determined for compounds **323-326** and **328**.

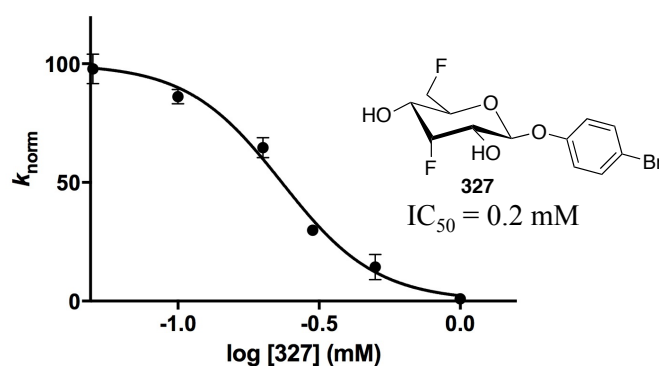
Table 3.1 Normalized rate constants (k_{norm}) for fluorinated aryl glycosides **323-328**.

Compound	Maximum Concentration (mM)	Normalized Rate Constant (k_{norm})
323	5	$72.67 \pm 4.38\%$
324	10	$78.33 \pm 4.71\%$
325	40	$46.64 \pm 4.35\%$
326	5	$30.58 \pm 5.23\%$
327	1	$0.99 \pm 0.99\%$
328	5	$92.46 \pm 0.80\%$

Table 3.2 IC_{50} values for fluorinated aryl glycosides **323-328**.

Compound	IC_{50} Value (mM)
323	N/A
324	N/A
325	N/A
326	N/A
327	0.2
328	N/A

Remarkably, compound **327** was capable of fully inhibiting ice crystal growth at its maximum solubility of 1 mM. A dose-response curve was constructed (Figure 3.14) and compound **327** was found to have an IC_{50} value of 0.2 mM (Table 3.2). This demonstrates that compound **327** is an efficient and efficacious inhibitor of ice recrystallization.

**Figure 3.14** A dose-response curve for compound **327**. Rate constants (k_{norm}) were normalized to a PBS control and measured in triplicate. Error bars represent the standard error of the mean (SEM). A two-parameter sigmoidal curve was fit to the data.

To assess the importance of the C-6 hydroxyl group of aryl glycosides for IRI activity, compounds **323-325** were directly compared to their non-fluorinated counterparts (**103-105**). As mentioned previously, compounds **326-328** cannot be compared to these non-fluorinated derivatives because their carbohydrate moiety consists of D-allose rather than D-glucose. Previously identified IRI active molecules, β -PMP-Glc (**103**) and β -pBrPh-Glc (**104**), were found to have IC_{50} values of 16 mM and 15 mM, respectively (Figure 3.15).³⁵ A dose-response curve was generated for β -pFPh-Glc (**105**) and it was found to have an IC_{50} value of 51 mM (Figure 3.15).

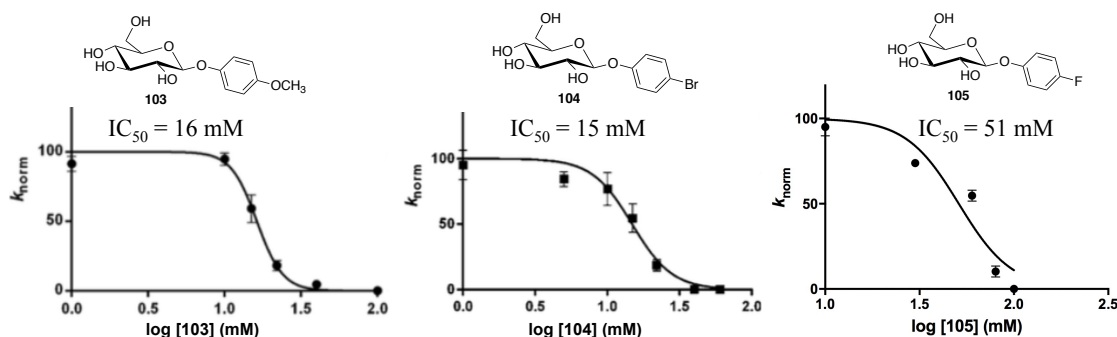


Figure 3.15 Dose-response curves for compounds **103-105**. Rate constants (k_{norm}) were normalized to a PBS control and measured in triplicate. Error bars represent the standard error of the mean (SEM). A two-parameter sigmoidal curve was fit to the data.³⁵

Amongst the non-fluorinated compounds, it is evident that β -pFPh-Glc (**105**) is less efficacious than β -PMP-Glc (**103**) and β -pBrPh-Glc (**104**) since it requires a considerably higher concentration to elicit an inhibitory response. In comparison, monofluorinated derivatives **323-325** failed to achieve full inhibition of ice crystal growth (Table 3.1). This demonstrates that replacing the C-6 hydroxyl group with a fluorine atom decreases the IRI activity of the aryl glycosides **103-105**. It also shows that replacement of the C-6 hydroxyl group with a fluorine atom significantly reduces solubility in aqueous solution. This is not surprising since fluorine is more hydrophobic than oxygen and cannot form hydrogen bonds.⁴⁷ Ultimately, the solubility constraints may account for the lack of IRI activity exhibited by these compounds. However, more importantly, fluorine atoms behave as hydrophobic groups and cause water molecules to become more ordered around them and are therefore poorly hydrated. As a result, it is not surprising that the majority of the fluorinated aryl glucosides are ineffective at inhibiting ice recrystallization. These results suggest that these compounds are poorly hydrated.

Although our laboratory previously correlated IRI activity to carbohydrate hydration, the hydration of fluorinated aryl glucosides has not been reported. Consequently, it is not known whether the fluorinated aryl glucosides are less hydrated than their corresponding non-fluorinated derivatives and ultimately, a correlation cannot be made.

3.4 Phosphate Sugars as Ice Recrystallization Inhibitors

Simple monosaccharides such as D-glucose and D-mannose were previously shown to be moderate inhibitors of ice recrystallization.¹ In search of a novel class of small molecule IRIs, we sought to commence with examining the ability of small structural subunits (i.e. monosaccharides) modified with unique functional groups to inhibit ice recrystallization. In particular, we sought to investigate the ability of monosaccharides possessing a phosphate group to inhibit ice recrystallization.

The phosphate group is ubiquitously found in biological systems either in its inorganic form or incorporated into biomolecules where it has many essential roles in biological processes.⁴⁸ For instance, phosphate is an important structural component of nucleotides, which are the building blocks of nucleic acids (DNA and RNA).⁴⁹ It is also a component of energy-rich molecules such as adenosine triphosphate (ATP),^{48,49} and is bound to coenzymes, such as NADP⁺ and NADPH, that are involved in anabolic reactions including lipid and nucleic acid synthesis.⁵⁰ Furthermore, the phosphate group is found in phospholipids in biological membranes where it comprises the hydrophilic portion.⁵¹ Phosphorylation has evolved as the most important post-translational modification owing to its versatility and reversibility.⁴⁸ In phosphorylated proteins, the unique properties of the phosphate group can be exploited to control important biological functions.⁴⁸ Within the context of small molecules, the phosphorylation of glucose generates an important intermediate that is involved in various metabolic processes including glycolysis.⁵² Despite the importance of phosphate groups in biological systems, small molecules bearing a phosphate group have not yet been investigated as ice recrystallization inhibitors and represent a novel opportunity to develop pyranoses with unique functional groups as IRIs. As a preliminary study, a small class of monosaccharides possessing a phosphate group was explored for their ability to inhibit ice recrystallization. The structures of the phosphate sugars **332-338** assessed for IRI

activity are shown in Figure 3.16. This work was part of a collaboration and compounds **332-338** were synthesized and provided by Dr. Janelle Sauvageau from the Department of Human Health Therapeutics at the National Research Council (NRC). The compounds assessed for IRI activity are structurally diverse, thus allowing for the generation of preliminary screening data. Compound **332** is a D-glycero- β -D-manno-heptose sugar lacking a phosphate group. The structures of compounds **333** and **334** are D-glycero-D-manno-heptose-based in which the hydrogen of the hydroxyl group at C-1 (**333**) or C-7 (**334**) is substituted with a phosphate group bearing sodium counterions. Compounds **335** and **336** are D-mannose-based derivatives possessing a β -linked phosphate group, which contains either triethylamine or sodium counterions. Compounds **337** and **338** are D-glucose-based derivatives containing an α -linked phosphate group and a C-6 phosphate group each containing sodium counterions, respectively.

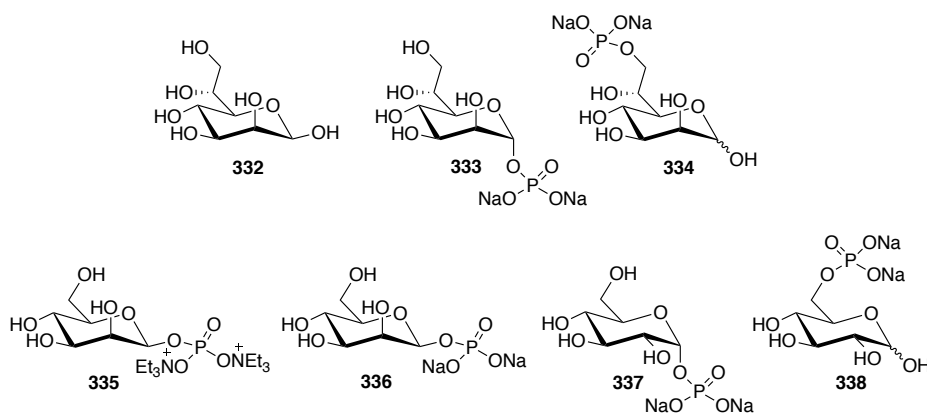


Figure 3.16 Structures of monosaccharide phosphate sugars **332-338** assessed for IRI activity.

3.4.1 IRI Activity of Phosphate Sugars

The IRI activity of compounds **332-338** was quantified by performing kinetic analyses utilizing the modified “splat-cooling” assay (previously described in Sections 3.2.2 and 3.3.2).³⁵ Using this kinetic approach, the IRI activities were quantified by an IC_{50} value. The majority of the phosphate sugars were effective inhibitors of ice recrystallization with the exception of compounds **332** and **333**, which failed to achieve full inhibition of ice crystal growth (i.e. $k_{norm} \neq 0\%$) when assessed at their maximum solubility in PBS (Table 3.3). For instance, D-glycero- β -D-manno-heptose (**332**) and D-

glycero-D-manno-heptose derivative possessing an α -linked phosphate group (**333**) had normalized rate constants (k_{norm}) comparable to that of the PBS control (103.53% and 94.53%, respectively). These compounds were poorly soluble in PBS and could only be assessed at a maximum concentration of 2 mM, which may account for the lack of IRI activity. Substitution of the hydrogen of the C-7 hydroxyl group with a phosphate group (**334**) drastically increased solubility. Compound **334** was shown to have a maximum solubility of 150 mM in PBS and was capable of achieving full inhibition of ice crystal growth (i.e. $k_{\text{norm}} = 0\%$). Thus, a complete sigmoidal dose-response curve was constructed (Figure 3.17) and compound **334** was found to have an IC_{50} value of 60 mM (Table 3.4).

Table 3.3 Normalized rate constants (k_{norm}) for phosphate sugars **332-338**.

Compound	Maximum Concentration (mM)	Normalized Rate Constant (k_{norm})
332	2	$103.53 \pm 5.28\%$
333	2	$94.53 \pm 2.43\%$
334	150	$0.93 \pm 0.93\%$
335	100	$5.62 \pm 1.50\%$
336	80	$2.39 \pm 1.19\%$
337	200	$1.74 \pm 0.88\%$
338	150	$0.00 \pm 0.00\%$

Table 3.4 IC_{50} values for phosphate sugars **332-338**.

Compound	IC_{50} Value (mM)
332	N/A
333	N/A
334	60
335	28
336	29
337	53
338	35

Compounds **335-337** were also quite soluble; however, they were only capable of achieving a k_{norm} of 5.62%, 2.39% and 1.74%, respectively, when assessed at their maximum solubility (Table 3.3). Although **335-337** did not achieve full inhibition of ice crystal growth at these concentrations, it can be seen from the dose-response curves (Figure 3.17) that k_{norm} is very close to zero. As such, these compounds still exhibit a

characteristic sigmoidal dose-response curve with identifiable inflection points, enabling the calculation of an IC_{50} value. Thus, the IC_{50} values for **335-337** were found to be 28 mM, 29 mM and 53 mM, respectively (Table 3.4). Lastly, compound **338** was shown to have a maximum solubility of 150 mM in PBS and was capable of achieving full inhibition of ice crystal growth. A complete sigmoidal dose-response curve was generated (Figure 3.17) and compound **338** was found to have an IC_{50} value of 35 mM.

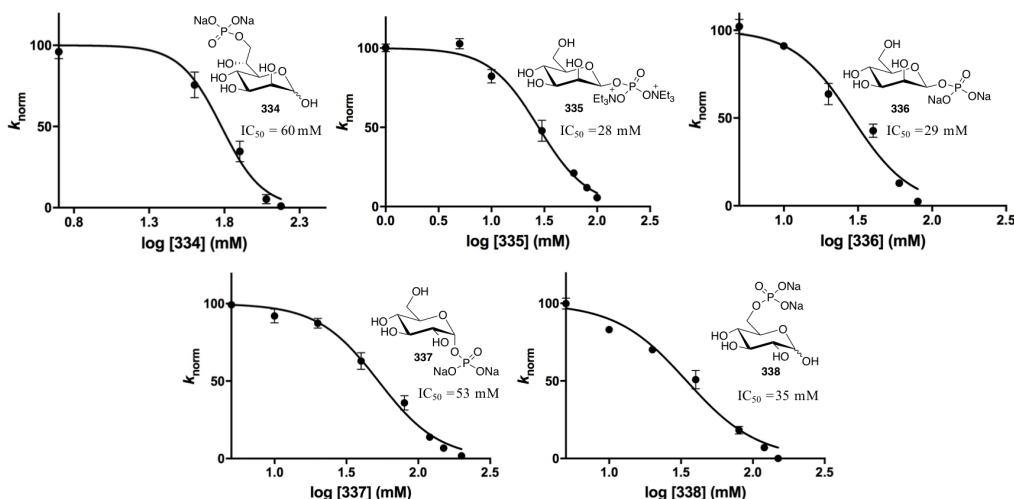


Figure 3.17 Dose-response curves for compounds **334-338**. Rate constants (k_{norm}) were normalized to a PBS control and measured in triplicate. Error bars represent the standard error of the mean (SEM). A two-parameter sigmoidal curve was fit to the data.

Overall, IC_{50} values could be determined for the majority of the phosphate sugars examined. It is evident that the D-mannose-based derivatives **335** and **336**, possessing a β -linked phosphate group (either triethylamine or sodium counterions), are the most efficacious inhibitors of ice recrystallization within this set of compounds. The D-glucose-based derivative **338** containing a C-6 phosphate group is slightly less efficacious than **335** and **336** (IC_{50} value of 35 mM versus 28 mM). However, the D-glycero-D-manno-heptose-based derivative **334** and D-glucose-based derivative **337** are the least efficacious since they require considerably higher concentrations to elicit an effective inhibitory response. This preliminary study demonstrates that some phosphate sugars can be effective inhibitors of ice recrystallization and highlights the need for further investigation including extensive structure-function studies.

3.5 Chapter Summary

Our laboratory has reported several different classes of small molecules capable of inhibiting ice recrystallization. To date, very few of these small molecules are effective inhibitors of ice recrystallization and the structural features responsible for IRI activity have not been definitively determined. In this chapter, we sought to investigate three diverse classes of small molecules as ice recrystallization inhibitors to determine additional structural features that are important for IRI activity with the overall goal to identify highly effective inhibitors of ice recrystallization. Previous work demonstrated that carbohydrate-based surfactants could be effective inhibitors of ice recrystallization, however, the precise mechanism by which these molecules inhibit ice recrystallization remains unknown. Since the isomerization of azobenzene can provide spatiotemporal control over molecular structure and properties, it would be desirable to employ this strategy to photo-modulate IRI activity. This approach could potentially be used as a tool to study the mechanism of ice recrystallization inhibition. The first class of small molecules investigated as ice recrystallization inhibitors were carbohydrate-based (fluoro)surfactants bearing an azobenzene moiety. These surfactant molecules were assessed for IRI activity utilizing the former “splat-cooling” assay, in which activity is reported as the % MGS relative to PBS. The majority of the carbohydrate-based (fluoro)surfactants were weak to moderate inhibitors of ice recrystallization, with the exception of the D-mannose derivative **303**. By performing a kinetic analysis, this surfactant molecule was shown to be an effective inhibitor of ice recrystallization with an IC_{50} value of 7 mM. This study reaffirmed the importance of the identity of the carbohydrate moiety for IRI activity. In the series of carbohydrate-based fluorosurfactants, the most active compounds were those possessing a trifluoromethyl substituent at the *ortho*-position, highlighting the importance of regiochemistry for the IRI activity of these molecules. The *trans-cis* photoisomerization studies demonstrated that some carbohydrate-based (fluoro)surfactants can exhibit significantly reduced IRI activity in their *cis* states, signifying the importance of the isomeric state of the azobenzene tail group.

The second class of small molecules investigated as ice recrystallization inhibitors were fluorinated aryl glycosides. In particular, three *para*-substituted *O*-aryl glycosides β -

PMP-Glc, β -pBrPh-Glc and β -pFPh-Glc have been previously shown to be effective inhibitors of ice recrystallization. Given the unique properties of fluorine and its important role in medicinal chemistry, fluorinated derivatives of β -PMP-Glc, β -pBrPh-Glc and β -pFPh-Glc were assessed for IRI activity by performing kinetic analyses. Most of the fluorinated aryl glycosides were not effective inhibitors of ice recrystallization, with the exception of the difluorinated derivative **327** which had an IC_{50} value of 0.2 mM. A comparative analysis demonstrated that the replacement of the C-6 hydroxyl group with a fluorine atom decreased the IRI activity and solubility of β -PMP-Glc, β -pBrPh-Glc and β -pFPh-Glc in all cases.

Lastly, a small diverse class of phosphate sugars were investigated as ice recrystallization inhibitors given the importance of phosphate groups in biological systems. This study served to generate preliminary data. Most of the phosphate sugars examined were effective inhibitors of ice recrystallization with IC_{50} values ranging from 28-60 mM, with the exception of the D-glycero-D-manno-heptose derivatives **332** and **333**. This preliminary study demonstrated that some phosphate sugars can be effective inhibitors of ice recrystallization and further investigation is required.

3.6 Experimental Procedures and Characterization Data

3.6.1 Traditional Ice Recrystallization Inhibition (IRI) Assay

To assess ice recrystallization inhibition (IRI) activity, the “splat cooling” method was used.^{32,33} The sample was dissolved in a phosphate buffered saline (PBS) solution. Using a micropipette, a 10 μ L droplet of the solution was dropped through a two-meter high plastic tube, having a 10 cm diameter, onto a polished aluminum block cooled to approximately -80 °C with dry ice. The droplet froze immediately upon contact with the surface of the aluminum block, creating a wafer approximately 1 cm in diameter and 20 μ m thick. Using precooled tools, the wafer was separated from the surface of the block and transferred to a cryostage, kept at -6.4 °C. The wafer was annealed for 30 minutes, and then photographed between crossed polarizing filters using a digital camera (Nikon CoolPix 5000) fitted to a microscope. A programmable Peltier unit (S3 Series 800 temperature controller, Alpha Omega Instruments) was used to maintain the temperature of the cryostage. Three images were taken from each wafer. During flash freezing, small

ice crystals from the solution are formed very quickly, and during annealing, the ice crystals dramatically increase in size due to recrystallization. In order to quantitatively measure the difference in recrystallization inhibition of two compounds, the difference in the dynamics of ice crystal size distribution was examined. This was done using a novel domain recognition software (DRS)22 program to analyze the image of the ice wafer. From this, domain areas from each image were calculated and data was inputted into Microsoft Excel. Using Microsoft Excel, the data was then plotted and analyzed. The ice crystal mean grain size (MGS) of the sample was compared to the MGS of the PBS control for the same day of testing and IRI activity was reported as the percent mean grain size (% MGS). Each compound was performed in triplicate.

3.6.2 Quantitative Analysis of Ice Recrystallization Inhibition (IRI) Activity

To quantify IRI activity, a modified “splat cooling” method was used.³⁵ The sample was dissolved in a phosphate buffered saline (PBS) solution at various concentrations. A 10 μ L droplet of the solution was dropped through a two-meter high plastic tube onto a polished aluminum block cooled to approximately -80 °C. The wafer was separated from the surface of the block and transferred to a cryostage, kept at -6.4 °C, to anneal for 5 minutes and then photographed using a digital camera fitted to the microscope. Using ImageJ, ice crystals with well-defined boundaries within the image were circled and the area of each crystal was calculated. Using Excel, the ice crystal areas were sorted into discrete bins based on size (bin size increases in increments of 0.001 mm²) and the proportionate area of each bin was calculated by adding the area of each crystal within a bin and dividing by the sum of the areas of all crystals in the image. For each inhibitor concentration, rate constants were determined and normalized to the positive PBS control for ice recrystallization. Using GraphPad, a dose-response curve was generated by plotting the normalized rate constants for each concentration as a function of the corresponding log values of the concentration. To obtain the half maximal inhibitory concentration (IC₅₀), a two-parameter sigmoidal curve was fit to the data. Each compound concentration was performed in triplicate.

3.6.3 Thermal Hysteresis (TH) Assay

Nanoliter osmometry, using a Clifton nanoliter osmometer (Clifton Technical

Physics, Hartford, NY), was performed in order to assess TH activity and dynamic ice shaping (DIS) ability.³⁷ In this assay, a single droplet of an aqueous solution (milli q water) of compound was enclosed within an oil-filled well in a sample holder. Using a thermoelectrically-controlled microscope stage, the sample was frozen and then slowly thawed until a single ice crystal remained. At this point, the morphology of the single ice crystal was monitored, as the temperature of the sample was gradually decreased, through a Leitz compound microscope equipped with an Olympus 20X objective, a Leitz Periplan 32X photo eyepiece, and a Hitachi KPM2U CCD camera connected to a Toshiba MV13K1 TV/VCR system. Non-uniform ice crystal growth is indicative of DIS and TH activity is measured as the depression of the freezing point of the solution in relation to a static melting point.

3.6.4 General Experimental

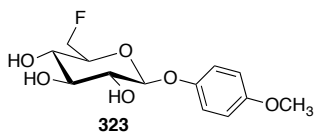
All anhydrous reactions were performed in flame-dried glassware under a positive pressure of dry argon. Air or moisture-sensitive reagents and anhydrous solvents were transferred with oven-dried syringes or cannulae. All flash chromatography was performed with E. Merck silica gel 60 (230-400 mesh). All solution phase reactions were monitored using analytical thin layer chromatography (TLC) with 0.2 mm pre-coated silica gel aluminum plates 60 F254 (E. Merck). Components were visualized by illumination with a short-wavelength (254 nm) ultra- violet light and/or staining (ceric ammonium molybdate, potassium permanganate, or phosphomolybdate stain solution). All solvents used for anhydrous reactions were distilled. Tetrahydrofuran (THF) was distilled from sodium/benzophenone under nitrogen. Dichloromethane (DCM) was distilled from calcium hydride. *N,N*-dimethylformamide (DMF) was stored over activated 4Å molecular sieves under argon. ¹H (300 or 400 MHz) and ¹³C (100 or 125 MHz) spectra were recorded at ambient temperature on a Bruker Avance 300 or Bruker Avance 400. Deuterated chloroform (CDCl₃) or water (D₂O) were used as NMR solvents, unless otherwise stated. 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 **301-322** were generously provided by Dr. Brendan Wilkinson from the School of Science and Technology at the University of New England. The synthesis and characterization data for these compounds are reported in the *RSC Advances* and *Carbohydrate Research* manuscripts published in 2016 and 2017, respectively.^{27,29} Aryl glycosides **329-331** and **103-105** were previously reported by our laboratory.¹² Compounds **332-338** were generously provided by Dr. Janelle Sauvageau from the Department of Human Health Therapeutics at the National Research Council (NRC). NMR spectra for novel compounds and final compounds assessed for IRI activity are provided below.

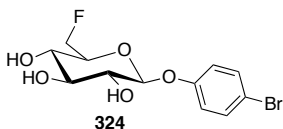
3.6.5 Compound Characterization

4-Methoxyphenyl-6-fluoro-6-deoxy- β -D-glucopyranoside (**323**)



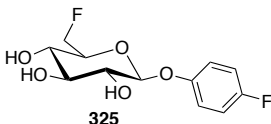
4-Methoxyphenyl- β -D-glucopyranoside (0.1 g, 0.22 mmol) was added to a flame-dried flask under a constant flow of argon and suspended in anhydrous CH_2Cl_2 (1.5 mL). The solution was cooled to $-40\text{ }^\circ\text{C}$ and DAST (0.17 mL, 1.32 mmol) was added dropwise. The cooling bath was removed and the mixture was stirred at room temperature until the solution became clear. The solution was cooled to $-40\text{ }^\circ\text{C}$, quenched via dropwise addition of methanol and concentrated under reduced pressure. Column chromatography (9.5:0.5 CH_2Cl_2 /methanol) afforded **323** as a white powder (16 mg, 25% yield). ^1H NMR (400 MHz; Acetone- d_6): δ 7.04-7.00 (m, 2H), 6.86-6.82 (m, 2H), 4.87 (d, J = 7.6 Hz, 1H), 4.77-4.52 (m, 3H), 4.46 (dd, J = 21.9, 4.3 Hz, 2H), 3.74 (s, 3H), 3.68 (dddd, J = 24.4, 9.8, 5.1, 1.6 Hz, 1H), 3.56-3.50 (m, 1H), 3.46-3.40 (m, 2H). ^{13}C NMR (101 MHz; Acetone- d_6): δ 156.1, 152.8, 118.9, 115.2, 103.0, 83.3 (d, J = 171.3 Hz), 77.9, 75.9 (d, J = 17.9 Hz), 74.7, 70.0 (d, J = 7.4 Hz), 55.8. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{17}\text{FNaO}_6$ $[\text{M}+\text{Na}]^+$ 311.1; found, 311.1.

4-Bromophenyl-6-fluoro-6-deoxy- β -D-glucopyranoside (**324**)



4-Bromophenyl-β-D-glucopyranoside (0.3 g, 0.89 mmol) was added to a flame-dried flask under a constant flow of argon and suspended in anhydrous CH₂Cl₂ (5 mL). The solution was cooled to -40 °C and DAST (0.71 mL, 5.37 mmol) was added dropwise. The cooling bath was removed and the mixture was stirred at room temperature until the solution became clear. The solution was cooled to -40 °C, quenched via dropwise addition of methanol and concentrated under reduced pressure. Column chromatography (9.5:0.5 CH₂Cl₂/methanol) afforded **324** as a white powder (47 mg, 16% yield). ¹H NMR (400 MHz; Acetone-*d*₆): δ 7.46-7.42 (m, 2H), 7.06-7.02 (m, 2H), 5.03 (d, *J* = 7.6 Hz, 1H), 4.77-4.62 (m, 2H), 4.57-4.51 (m, 1H), 4.47-4.46 (m, 1H), 3.75 (dddd, *J* = 24.5, 9.8, 4.9, 1.7 Hz, 1H), 3.59-3.42 (m, 3H). ¹³C NMR (101 MHz; Acetone-*d*₆): δ 158.0, 133.0, 119.5, 114.8, 101.8, 83.1 (d, *J* = 171.3 Hz), 77.7, 76.0 (d, *J* = 17.9 Hz), 74.5, 69.8 (d, *J* = 7.2 Hz). LRMS (ESI): *m/z* calcd. for C₁₂H₁₄BrFNaO₅ [M+Na]⁺ 359.0; found, 359.0.

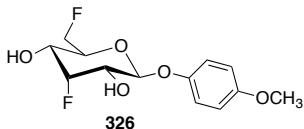
4-Fluorophenyl-6-fluoro-6-deoxy-β-D-glucopyranoside (325)



4-Fluorophenyl-β-D-glucopyranoside (0.26 g, 0.95 mmol) was added to a flame-dried flask under a constant flow of argon and suspended in anhydrous CH₂Cl₂ (5 mL). The solution was cooled to -40 °C and DAST (0.75 mL, 5.69 mmol) was added dropwise. The cooling bath was removed and the mixture was stirred at room temperature until the solution became clear. The solution was cooled to -40 °C, quenched via dropwise addition of methanol and concentrated under reduced pressure. Column chromatography (9.5:0.5 CH₂Cl₂/methanol) afforded **325** as a white powder (85 mg, 33% yield). ¹H NMR (300 MHz; Acetone-*d*₆): δ 7.12-7.02 (m, 4H), 4.96 (d, *J* = 7.5 Hz, 1H), 4.80-4.61 (m, 2H), 4.55-4.43 (m, 2H), 3.73 (dddd, *J* = 24.3, 9.7, 5.0, 1.8 Hz, 1H), 3.58-3.41 (m, 3H). ¹³C NMR (101 MHz; Acetone-*d*₆): δ 158.9 (d, *J* = 239.0 Hz), 155.0 (d, *J* = 2.5 Hz), 119.0 (d, *J* = 8.1 Hz), 116.4 (d, *J* = 23.0 Hz), 102.5, 83.2 (d, *J* = 171.4 Hz), 77.8, 76.0 (d, *J* =

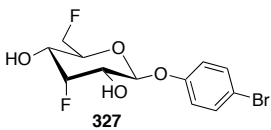
17.9 Hz), 74.6, 69.9 (d, $J = 7.3$ Hz). LRMS (ESI): m/z calcd. for $C_{12}H_{14}F_2NaO_5$ $[M+Na]^+$ 299.1; found, 299.1.

4-Methoxyphenyl-3,6-dideoxy-3,6-difluoro- β -D-allopyranoside (**326**)



4-Methoxyphenyl- β -D-glucopyranoside (0.3 g, 1.05 mmol) was added to a flame-dried flask under a constant flow of argon and suspended in anhydrous CH_2Cl_2 (6 mL). The solution was cooled to -40 °C and DAST (0.83 mL, 6.29 mmol) was added dropwise. The cooling bath was removed and the mixture was stirred overnight at room temperature. The solution was cooled to -40 °C, quenched via dropwise addition of methanol and concentrated under reduced pressure. Column chromatography (1:1 Hexanes/ethyl acetate) afforded **326** as a white powder (120 mg, 39% yield). 1H NMR (400 MHz; Acetone- d_6): δ 7.06-7.02 (m, 2H), 6.87-6.83 (m, 2H), 5.12 (dd, $J = 8.0, 1.0$ Hz, 1H), 5.02 (dd, $J = 53.2, 0.9$ Hz, 1H), 4.83 (d, $J = 6.0$ Hz, 1H), 4.79-4.57 (m, 3H), 4.03-3.93 (m, 1H), 3.86-3.68 (m, 5H). ^{13}C NMR (101 MHz; Acetone- d_6): δ 155.3, 151.8, 118.0, 114.3, 100.0 (d, $J = 3.4$ Hz), 93.5 (d, $J = 179.0$ Hz), 82.4 (d, $J = 171.6$ Hz), 73.0 (dd, $J = 17.9, 3.1$ Hz), 70.0 (d, $J = 16.9$ Hz), 65.7 (dd, $J = 18.4, 7.9$ Hz), 54.9. LRMS (ESI): m/z calcd. for $C_{13}H_{16}F_2NaO_5$ $[M+Na]^+$ 313.1; found, 313.1.

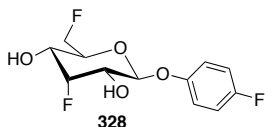
4-Bromophenyl-3,6-dideoxy-3,6-difluoro- β -D-allopyranoside (**327**)



4-Bromophenyl- β -D-glucopyranoside (0.3 g, 0.89 mmol) was added to a flame-dried flask under a constant flow of argon and suspended in anhydrous CH_2Cl_2 (6 mL). The solution was cooled to -40 °C and DAST (0.71 mL, 5.37 mmol) was added dropwise. The cooling bath was removed and the mixture was stirred overnight at room temperature. The solution was cooled to -40 °C, quenched via dropwise addition of methanol and concentrated under reduced pressure. Column chromatography (1:1 Hexanes/ethyl acetate) afforded **327** as a white powder (190 mg, 63% yield). 1H NMR (400 MHz; Acetone- d_6): δ 7.47-7.43 (m, 2H), 7.09-7.05 (m, 2H), 5.26 (dd, $J = 8.0, 0.8$ Hz, 1H), 5.03

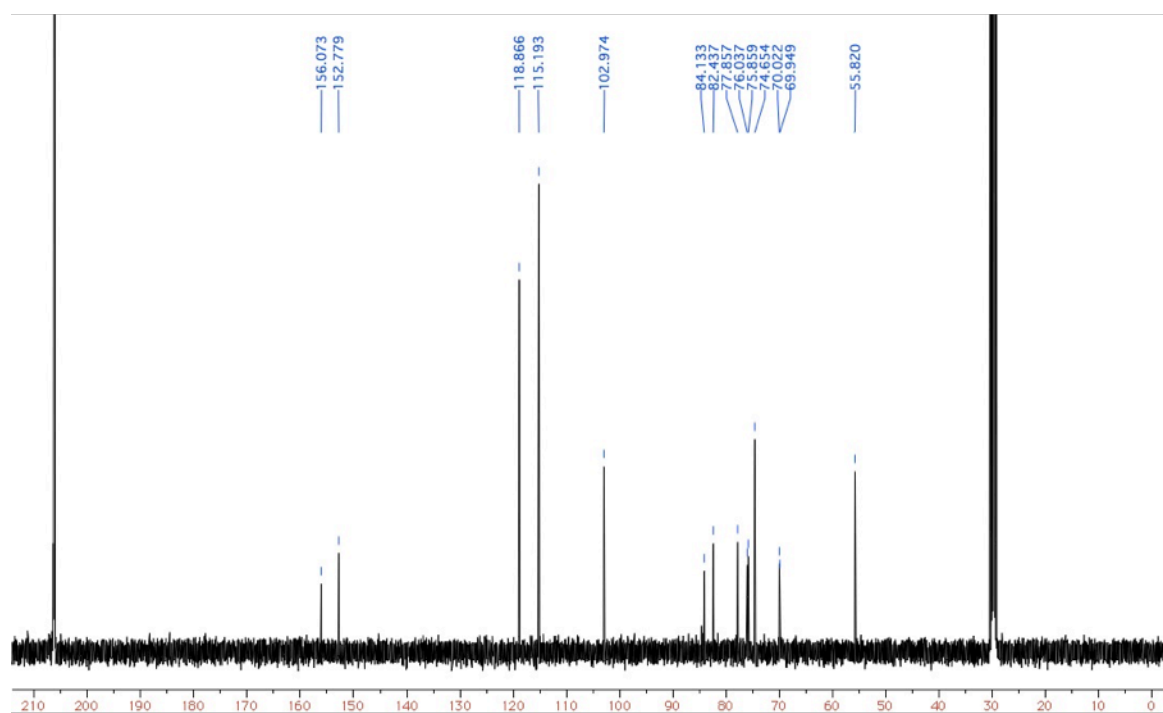
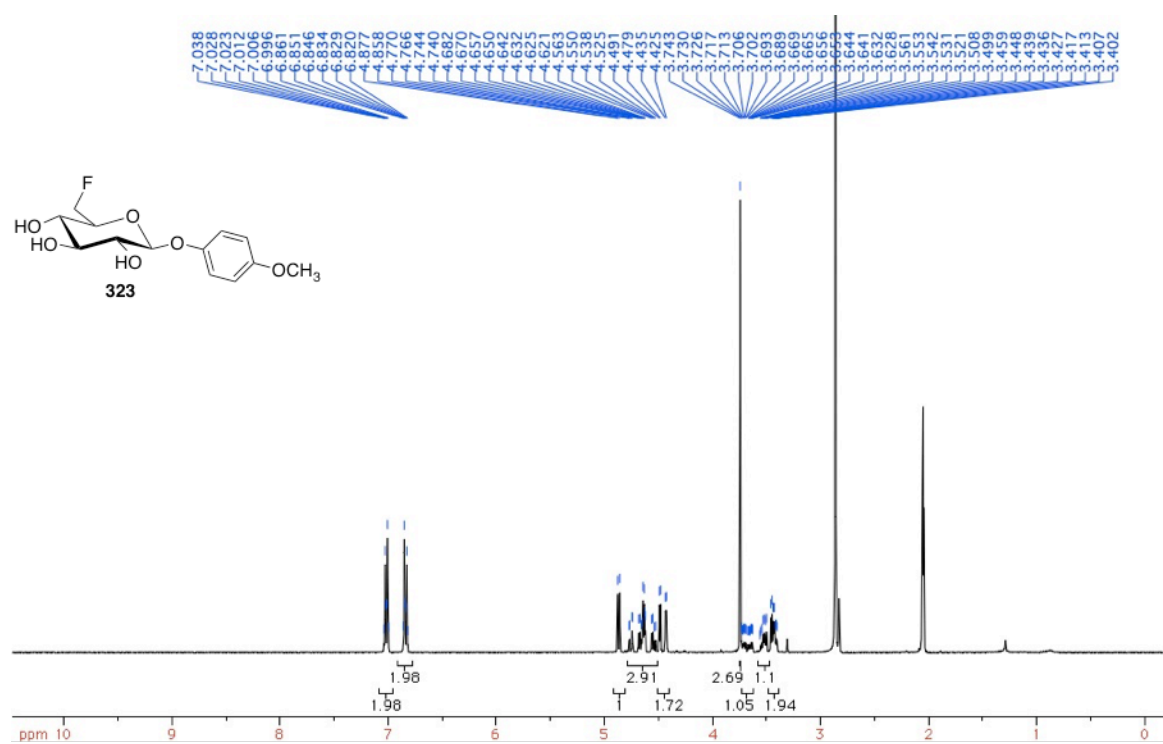
(dd, $J = 53.4, 0.5$ Hz, 1H), 4.92 (d, $J = 6.2$ Hz, 1H), 4.79-4.57 (m, 3H), 4.05 (ddd, $J = 25.7, 10.4, 4.3$ Hz, 1H), 3.89-3.73 (m, 2H). ^{13}C NMR (101 MHz; Acetone- d_6): δ 157.9, 133.1, 119.6, 114.9, 99.8, 94.3 (d, $J = 178.1$ Hz), 83.1 (d, $J = 171.9$ Hz), 73.9 (dd, $J = 17.9, 3.1$ Hz), 70.7 (d, $J = 16.9$ Hz), 66.4 (dd, $J = 18.4, 7.6$ Hz). LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{13}\text{BrF}_2\text{NaO}_4$ $[\text{M}+\text{Na}]^+$ 361.0; found, 360.9.

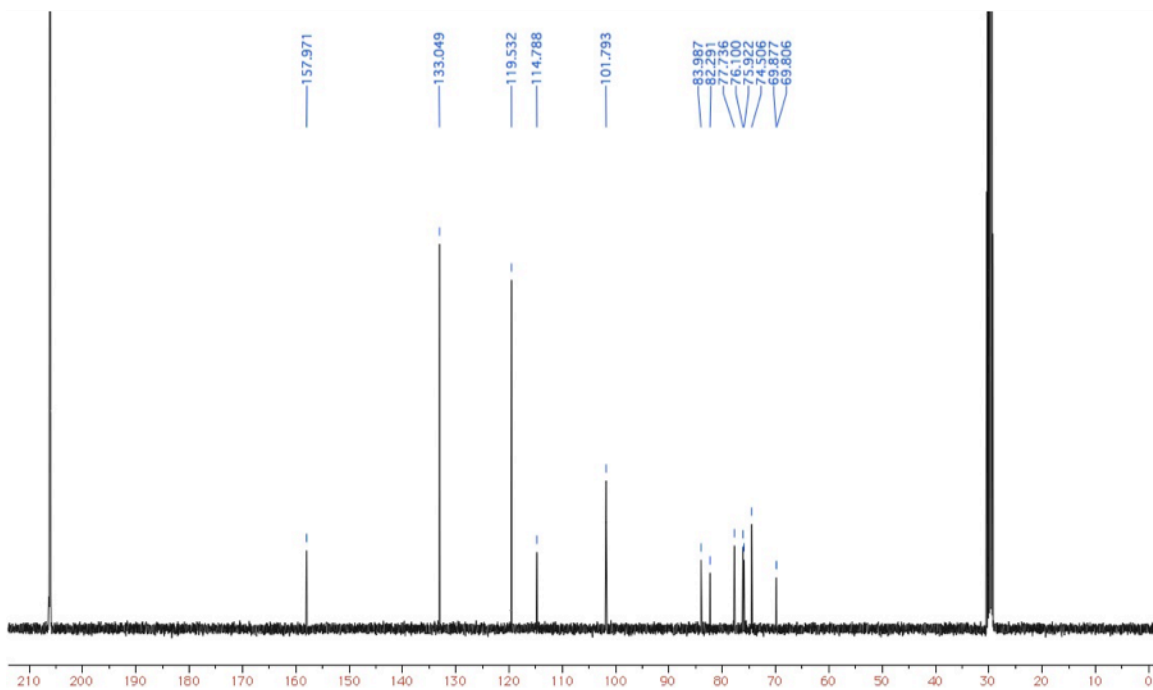
4-Fluorophenyl-3,6-dideoxy-3,6-difluoro- β -D-allopyranoside (**328**)

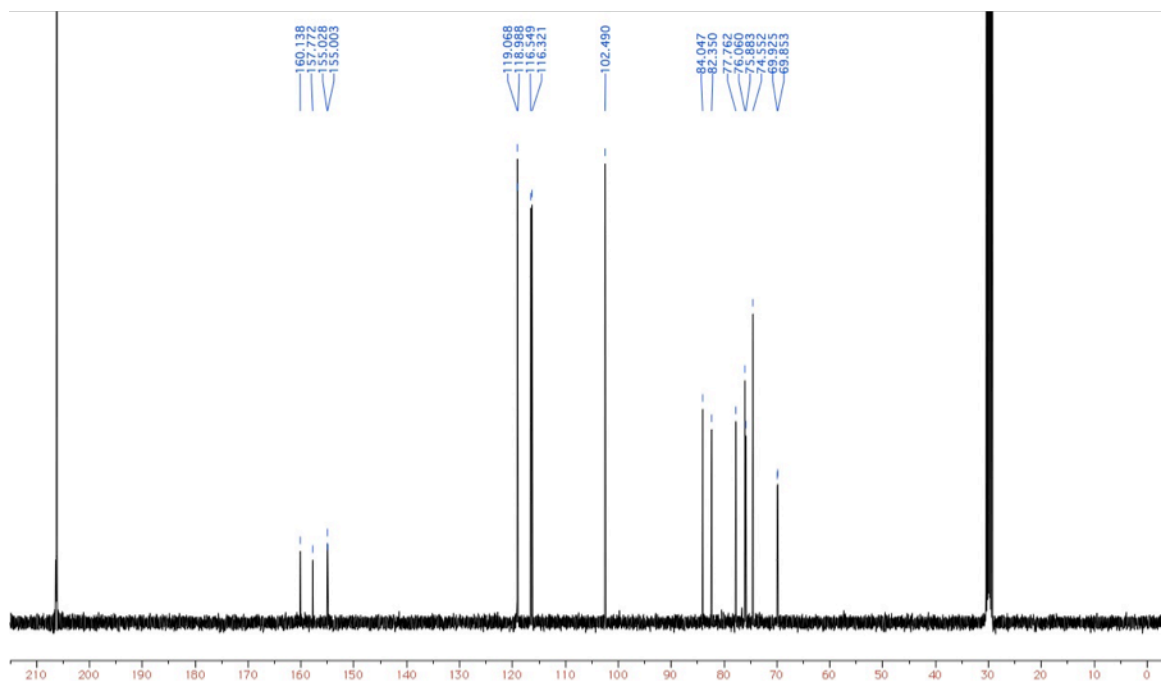
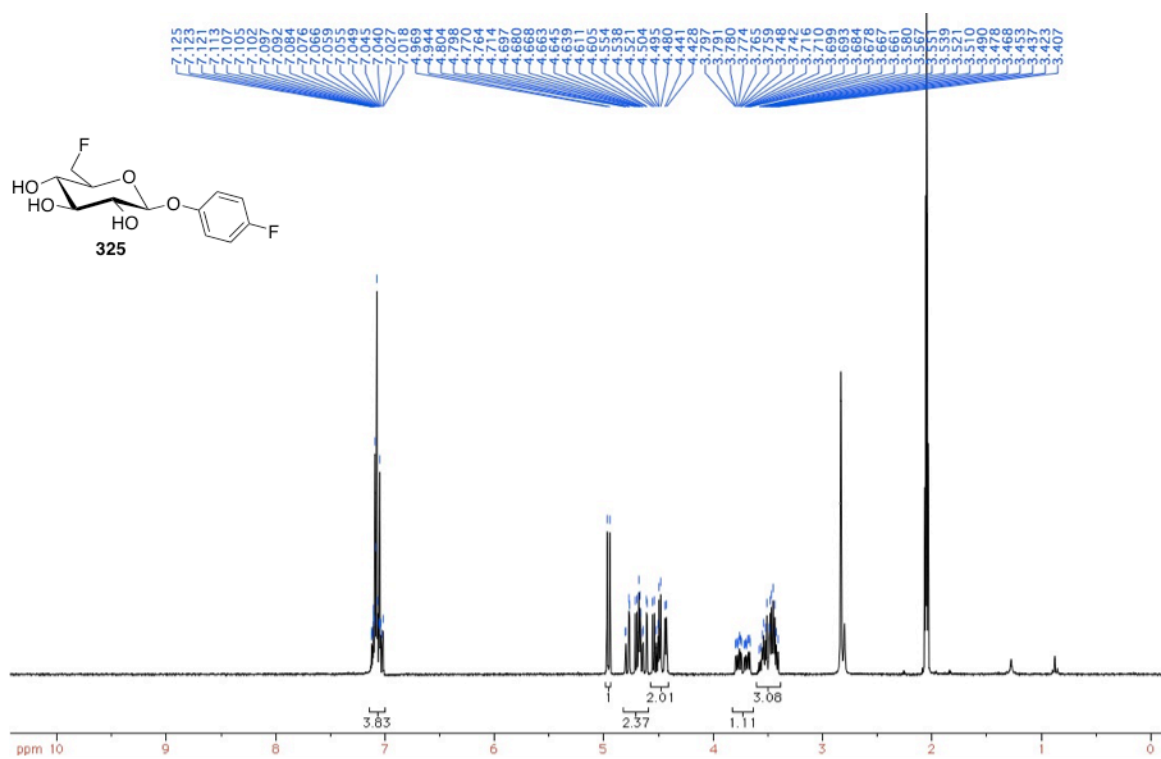


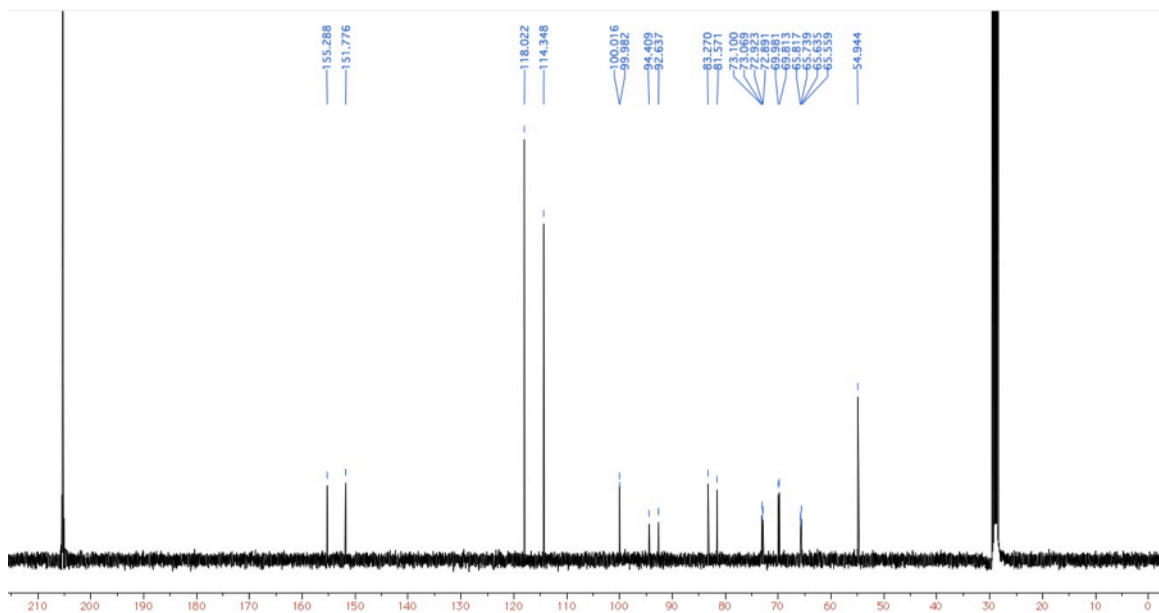
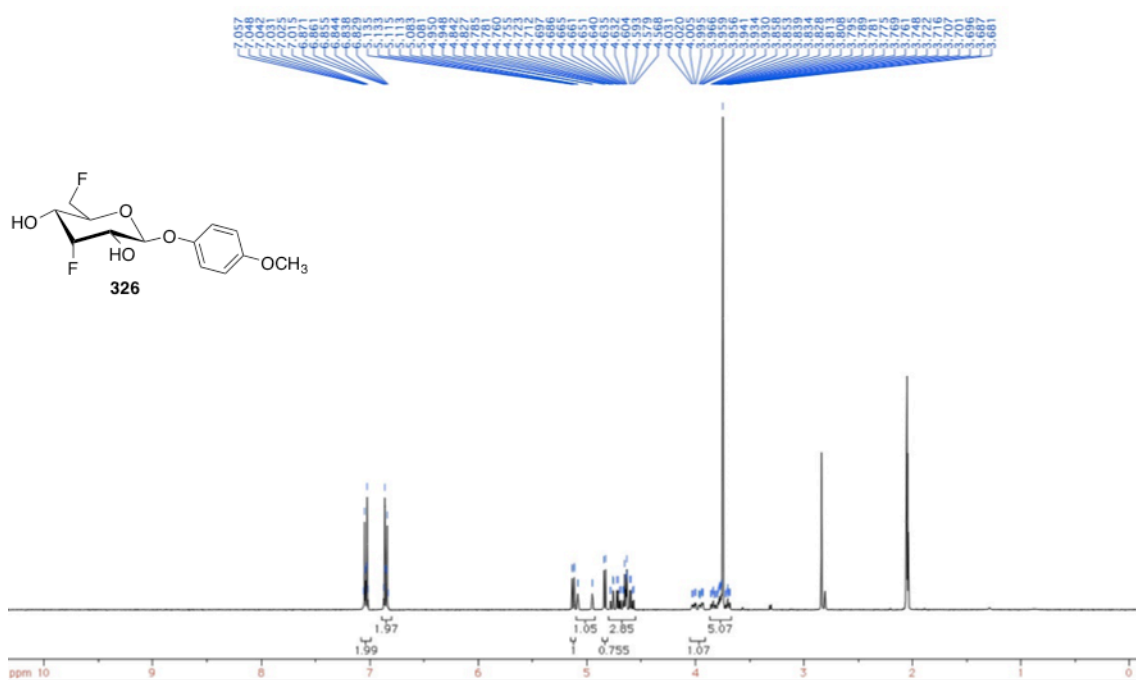
4-Fluorophenyl- β -D-glucopyranoside (0.5 g, 1.82 mmol) was added to a flame-dried flask under a constant flow of argon and suspended in anhydrous CH_2Cl_2 (10 mL). The solution was cooled to -40 $^\circ\text{C}$ and DAST (1.45 mL, 10.94 mmol) was added dropwise. The cooling bath was removed and the mixture was stirred overnight at room temperature. The solution was cooled to -40 $^\circ\text{C}$, quenched via dropwise addition of methanol and concentrated under reduced pressure. Column chromatography (1:1 Hexanes/ethyl acetate) afforded **328** as a white powder (212 mg, 42% yield). ^1H NMR (400 MHz; Acetone- d_6): δ 7.15-7.03 (m, 4H), 5.20 (dd, $J = 8.0, 0.8$ Hz, 1H), 5.03 (dd, $J = 53.3, 0.6$ Hz, 1H), 4.92 (d, $J = 6.1$ Hz, 1H), 4.79-4.58 (m, 3H), 4.08-3.98 (m, 1H), 3.88-3.72 (m, 2H). ^{13}C NMR (101 MHz; Acetone- d_6): δ 159.0 (d, $J = 239.0$ Hz), 154.9 (d, 2.2 Hz), 119.1 (d, $J = 8.1$ Hz), 116.5 (d, $J = 23.1$ Hz), 100.4, 94.3 (d, $J = 178.1$ Hz), 83.2 (d, $J = 171.6$ Hz), 73.9 (dd, $J = 18.0, 3.2$ Hz), 70.7 (d, $J = 16.9$ Hz), 66.4 (dd, $J = 18.3, 7.9$ Hz). LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{13}\text{F}_3\text{NaO}_4$ $[\text{M}+\text{Na}]^+$ 301.1; found, 301.0.

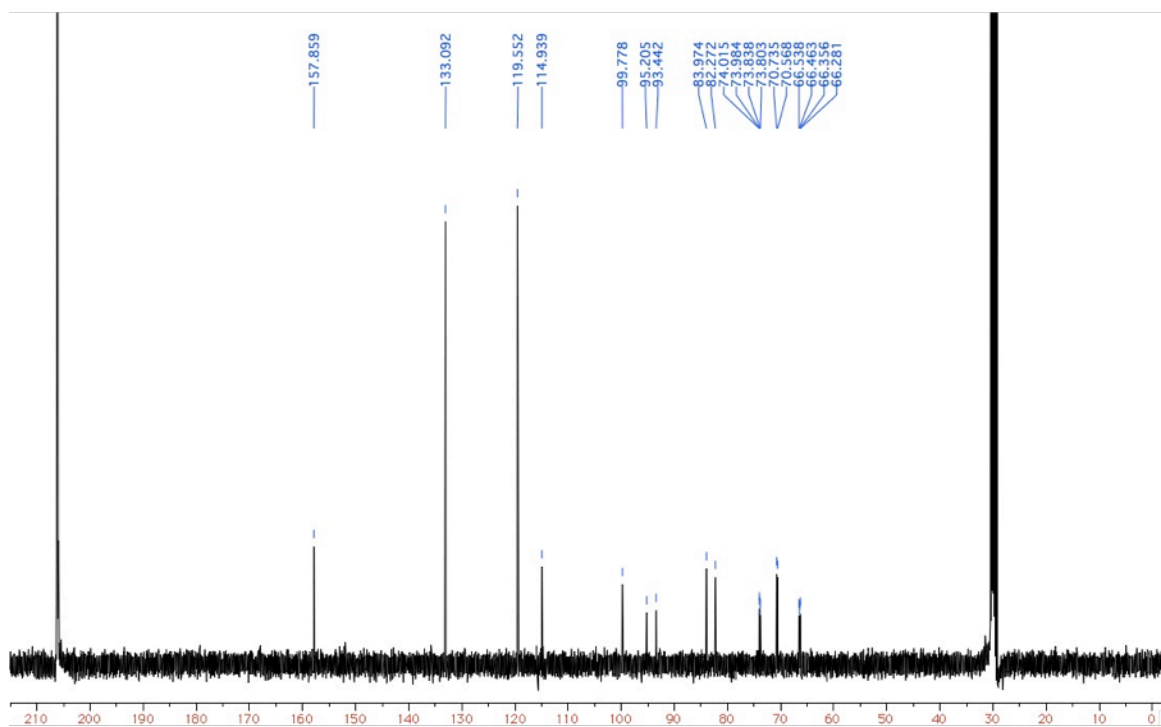
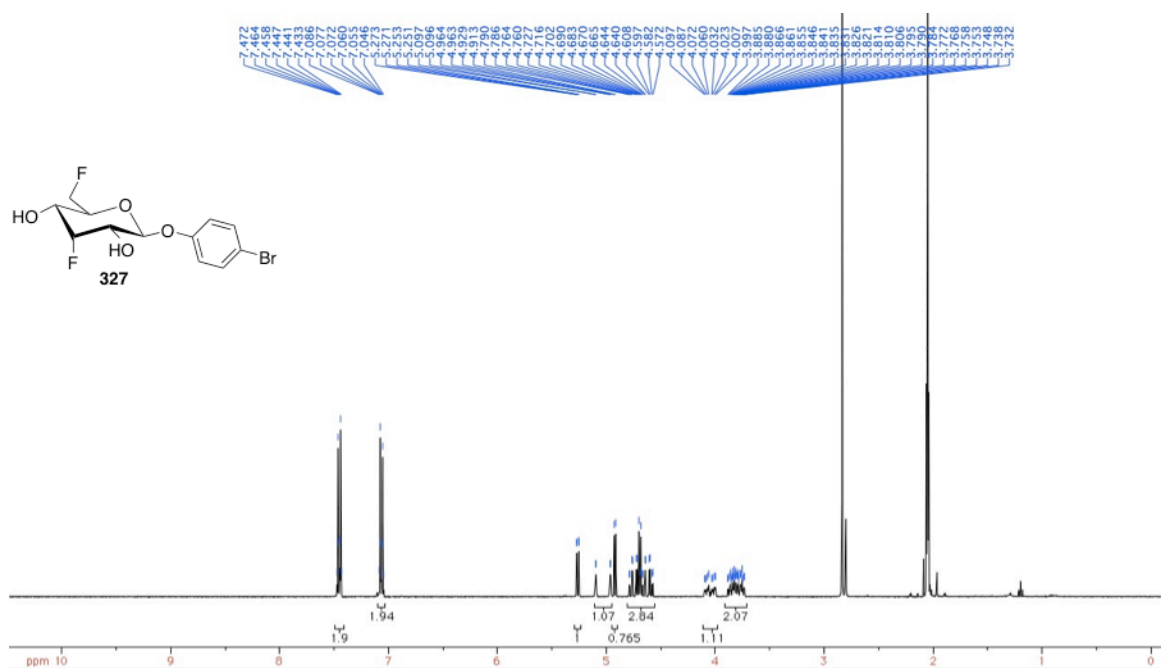
3.6.6 NMR Spectra

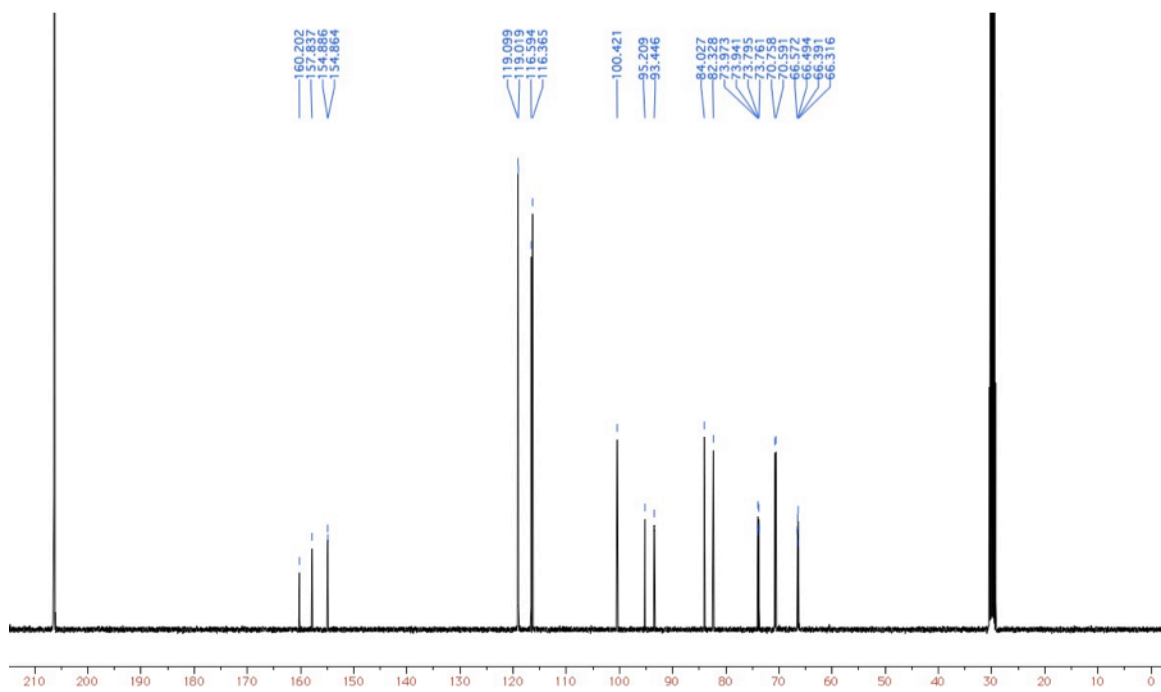
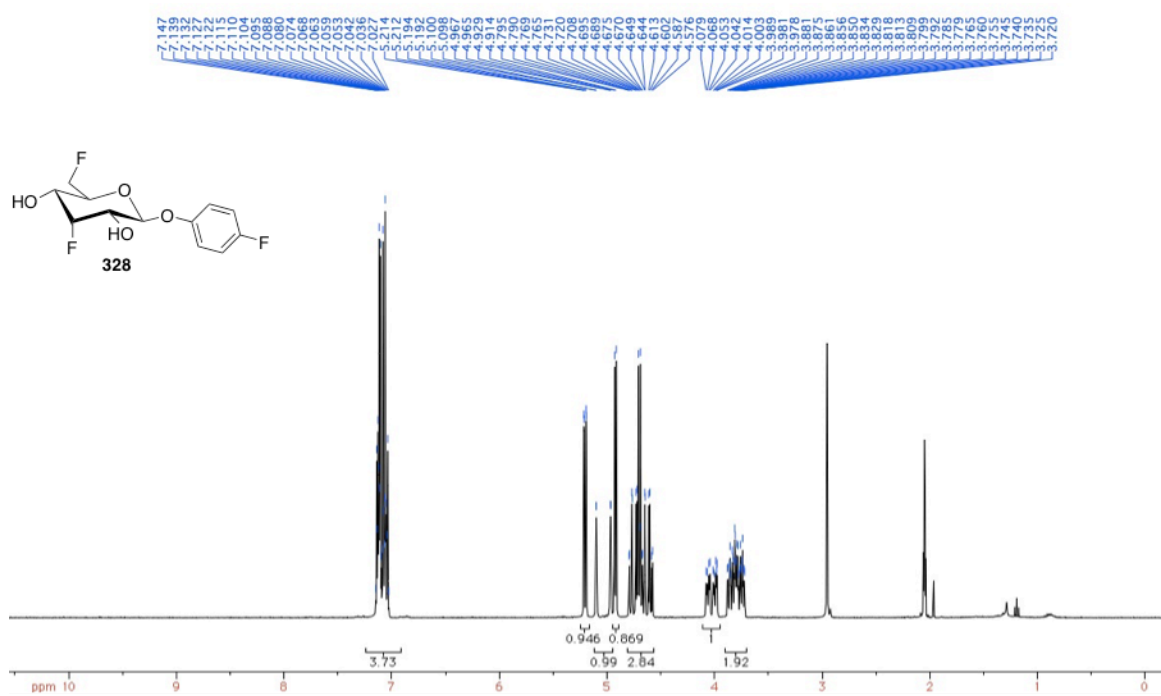












3.7 References

- (1) Tam, R. Y.; Ferreira, S. S.; Czechura, P.; Chaytor, J. L.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 17494.
- (2) Galema, S. A.; Engberts, J. B. F. N.; Høiland, H.; Førland, G. M. *J. Phys. Chem.* **1993**, *97* (26), 6885.
- (3) Galema, S. A.; Høiland, H. *J. Phys. Chem.* **1991**, *95* (13), 5321.
- (4) Galema, S. A.; Howard, E.; Engberts, J. B. F. N.; Grigera, J. R. *Carbohydr. Res.* **1994**, *265* (2), 215.
- (5) Bilgram, J. H. *Prog. Cryst. Growth Charact.* **1993**, *26*, 99.
- (6) Halter, P. U.; Bilgram, J. H.; Kanzig, W. *J. Chem. Phys.* **1988**, *89* (5), 2622.
- (7) Capicciotti, C. J.; Leclère, M.; Perras, F. A.; Bryce, D. L.; Paulin, H.; Harden, J.; Liu, Y.; Ben, R. N. *Chem. Sci.* **2012**, *3*, 1408.
- (8) Lorber, B.; Bishop, J. B.; DeLucas, L. J. *Biochim. Biophys. Acta* **1990**, *1023*, 254.
- (9) Michel, H.; Oesterhelt, D. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77* (3), 1283.
- (10) Balcerzak, A. K. Elucidating the Key Structural Features of Carbohydrates and Surfactants Necessary for Inhibiting Ice Recrystallization, University of Ottawa, 2014.
- (11) Capicciotti, C. J. The Rational Design of Potent Ice Recrystallization Inhibitors for Use as Novel Cryoprotectants, University of Ottawa, 2014.
- (12) Capicciotti, C. J.; Mancini, R. S.; Turner, T. R.; Koyama, T.; Alteen, M. G.; Doshi, M.; Inada, T.; Acker, J. P.; Ben, R. N. *ACS Omega* **2016**, *1* (4), 656.
- (13) Capicciotti, C. J.; Kurach, J. D. R.; Turner, T. R.; Mancini, R. S.; Acker, J. P.; Ben, R. N. *Sci. Rep.* **2015**, *5*, 9692.
- (14) Katsonis, N.; Lubomska, M.; Pollard, M. M.; Feringa, B. L.; Rudolf, P. *Prog. Surf. Sci.* **2007**, *82*, 407.
- (15) Helmy, S.; Leibfarth, F. A.; Oh, S.; Poelma, J. E.; Hawker, C. J.; Alaniz, J. R. De. *J. Am. Chem. Soc.* **2014**, *136*, 8169.
- (16) Beharry, A.; Woolley, A. *Chem. Soc. Rev.* **2011**, *40* (8), 4422.
- (17) Mourot, A.; Kienzler, M. A.; Banghart, M. R.; Fehrentz, T.; Huber, F. M. E.; Stein, M.; Kramer, R. H.; Trauner, D. *ACS Chem. Neurosci.* **2011**, *2*, 536.
- (18) Merino, E.; Ribagorda, M. *Beilstein J. Org. Chem.* **2012**, *8*, 1071.
- (19) Bandara, H. M. D.; Friss, T. R.; Enriquez, M. M.; Isley, W.; Incarvito, C.; Frank, H. A.; Gascon, J.; Burdette, S. *J. Org. Chem.* **2010**, *75* (14), 4817.
- (20) Hamon, F.; Djedaini-Pilard, F.; Barbot, F.; Len, C. *Tetrahedron* **2009**, *65* (49), 10105.
- (21) Hu, Y.; Tabor, R. F.; Wilkinson, B. L. *Org. Biomol. Chem.* **2015**, *13*, 2216.
- (22) Russew, M.; Hecht, S. *Adv. Mater.* **2010**, *22* (31), 3348.
- (23) Westmark, P. R.; Kelly, J. P.; Smith, B. D. *J. Am. Chem. Soc.* **1993**, *115* (9), 3416.
- (24) Schierling, B.; Noël, A.-J.; Wende, W.; Hien, L. T.; Volkov, E.; Kubareva, E.; Oretskaya, T.; Kokkinidis, M.; Rompp, A.; Spengler, B.; Pingoud, A. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107* (4), 1361.
- (25) Balcerzak, A. K.; Febbraro, M.; Ben, R. N. *RSC Adv.* **2013**, *3* (10), 3232.
- (26) Billamboz, M.; Mangin, F.; Drillaud, N.; Chevrin-Villette, C.; Banaszak-Le, E. *J. Org. Chem.* **2014**, *79*, 493.
- (27) Adam, M. K.; Poisson, J. S.; Hu, Y.; Prasannakumar, G.; Pottage, M. J.; Ben, N.; Wilkinson, B. L. *RSC Adv.* **2016**, *6*, 39240.

- (28) Tabor, R. F.; Tan, D. D.; Han, S. S.; Young, S. A.; Seeger, Z. L. E.; Pottage, M. J.; Garvey, C. J.; Wilkinson, B. L. *Chem. - A Eur. J.* **2014**, *20*, 13881.
- (29) Adam, M. K.; Hu, Y.; Poisson, J. S.; Pottage, M. J.; Ben, R. N.; Wilkinson, B. L. *Carbohydr. Res.* **2017**, *439*, 1.
- (30) Wilkinson, B. L.; Bornaghi, L. F.; Poulsen, S.; Houston, T. A. *Tetrahedron* **2006**, *62*, 8115.
- (31) Hu, Y.; Marlow, D. J. B.; Ramanathan, D. R.; Zou, W.; Tiew, B. H. G.; Pottage, C. M. J.; Bansal, A. V.; Tabor, R. F.; A, B. L. W. *Aust. J. Chem* **2015**, *68*, 1880.
- (32) Knight, C. A.; Hallett, J.; Devries, A. L. *Cryobiology* **1988**, *25* (1), 55.
- (33) Jackman, J.; Noestheden, M.; Moffat, D.; Pezacki, J. P.; Findlay, S.; Ben, R. N. *Biochem. Biophys. Res. Commun.* **2007**, *354*, 340.
- (34) Abraham, S. Development and Implementation of a Kinetic Quantitative Analysis of Novel Small Molecule Ice Recrystallization Inhibitors, University of Ottawa, 2015.
- (35) Abraham, S.; Keillor, K.; Capicciotti, C. J.; Perley-Robertson, G. E.; Keillor, J. W.; Ben, R. N. *Cryst. Growth Des.* **2015**, *15* (10), 5034.
- (36) Czechura, P.; Tam, R. Y.; Dimitrijevic, E.; Murphy, A. V.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 2928.
- (37) Chakrabarty, A.; Hew, C. L. *Eur. J. Biochem.* **1991**, *202*, 1057.
- (38) Shah, P.; Westwell, A. D. *J. Enzyme Inhib. Med. Chem.* **2007**, *22* (5), 527.
- (39) Filler, R.; Kobayashi, Y.; Yagupolskii, L. *Organofluorine Compounds in Medicinal Chemistry and Biomedical Applications*. Amsterdam: Elsevier; 1993.
- (40) Liebman, J.; Greenberg, A.; Dolbier, W. *Fluorine-Containing Molecules: Structure, Reactivity, Synthesis, and Applications*. New York: VCH; 1988.
- (41) Martin, G. V.; Caldwell, J. H.; Graham, M. M.; Grierson, J. R.; Kroll, K.; Cowan, M. J.; Lewellen, T. K.; Rasey, J. S.; Casciari, J. J.; Krohn, K. A. *J. Nucl. Med.* **1992**, *33* (12), 2202.
- (42) Biffinger, J. C.; Kim, H. W.; DiMagno, S. G. *ChemBioChem* **2004**, *5* (5), 622.
- (43) Nishi, Y.; Uchiyama, S.; Doi, M.; Nishiuchi, Y.; Nakazawa, T.; Ohkubo, T.; Kobayashi, Y. *Biochemistry* **2005**, *44* (16), 6034.
- (44) Card, P. J.; Reddy, G. S. *J. Org. Chem.* **1983**, *48*, 4734.
- (45) Kirk, K. L. *Org. Process Res. Dev.* **2008**, *12* (2), 305.
- (46) Kumar, V.; Banala, A. K.; Garcia, E. G.; Cao, J.; Keck, T. M.; Bonifazi, A.; Deschamps, R.; Newman, A. H. *ACS Med. Chem. Lett.* **2014**, *5*, 647.
- (47) Dunitz, J. D.; Taylor, R. *Chem. - A Eur. J.* **1997**, *3* (1), 89.
- (48) Hunter, T. *Phil. Trans. R. Soc. B* **2012**, *367*, 2513.
- (49) Westheimer, F. H. *Science* **1987**, *235* (4793), 1173.
- (50) Agledal, L.; Niere, M.; Ziegler, M. *Redox Rep.* **2010**, *15* (1), 2.
- (51) Ohlrogge, J.; Browse, J. *Plant Cell* **1995**, *7*, 957.
- (52) Cole, J. T.; Kean, W. S.; Pollard, H. B.; Verma, A.; Watson, W. D. *Front. Mol. Neurosci.* **2012**, *5*, 1.

Chapter 4: Determining the Influence of an Azido Moiety on the IRI Activity of Aryl Glucosides

4.1 Introduction

Previous work by our laboratory has demonstrated that simple monosaccharides are moderate inhibitors of ice recrystallization.¹ It was hypothesized that the moderate activity exhibited by these molecules is attributed to their ability to disrupt the structure of bulk water.¹ Based upon this hypothesis, carbohydrate-based surfactants that have the ability to disrupt the structure of bulk water or sequester bulk water were investigated as inhibitors of ice recrystallization. It was demonstrated that carbohydrate-based surfactants possessing long alkyl chains at C-1, such as *n*-octyl- β -D-galactoside, are effective inhibitors of ice recrystallization.² Due to the fact that the hydration characteristics for *n*-octyl- β -D-galactoside are not known, a direct correlation between hydration and IRI activity cannot be made. However, the results from this study indicated that long alkyl side chains counter balanced with a hydrophilic moiety increased IRI activity.^{2,3} Although these molecules inhibited ice recrystallization, they also possessed surfactant-like properties that are detrimental to cells since they solubilize membranes,^{4,5} thus precluding their use in cellular applications.

Given the importance of hydrophobic moieties at C-1, our laboratory sought to employ an aryl ring instead of a long alkyl chain. A structurally diverse class of *O*-aryl glycosides was synthesized and assessed for their ability to inhibit ice recrystallization.^{6,7} Various functional groups were substituted at the *ortho*-, *meta*- and *para*-position of the aryl ring; however, it was demonstrated that *ortho*- and *meta*-substituted analogues were significantly less IRI active than *para*-substituted analogues, emphasizing the importance of having a substituent at the *para*-position of the aryl ring.⁶ Since *n*-octyl- β -D-galactoside was shown to be an effective inhibitor of ice recrystallization,² *O*-aryl galactosides were also investigated. However, the majority of the *O*-aryl galactosides were less IRI active than their glucoside counterparts.⁷ Of the *O*-aryl glucosides assessed, the *para*-methoxy (**103**, β -PMP-Glc) and *para*-bromo (**104**, β -pBrPh-Glc) derivatives were shown to be very effective inhibitors of ice recrystallization (Figure 4.1). By assessing the kinetics of ice recrystallization inhibition of β -PMP-Glc and β -pBrPh-Glc, IC₅₀ values were found to be 16 mM and 15 mM, respectively.⁸

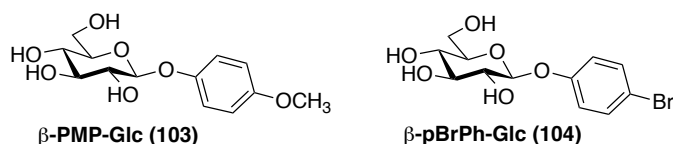


Figure 4.1 Structures of β -PMP-Glc (**103**) and β -pBrPh-Glc (**104**).

Since β -PMP-Glc and β -pBrPh-Glc are effective inhibitors of ice recrystallization, our laboratory previously assessed the ability of these molecules to protect human RBCs against cellular injury during cryopreservation. It was demonstrated that β -PMP-Glc and β -pBrPh-Glc are beneficial additives for the freezing of human RBCs with the ability to increase the post-thaw integrity in the presence of reduced concentrations of glycerol (15% versus 40%) and slow cooling rates.⁹ Specifically, in the absence of β -PMP-Glc or β -pBrPh-Glc, the post-thaw RBC integrity with a 15% glycerol solution was approximately 20-30%.⁹ Meanwhile, the addition of β -PMP-Glc (110 mM) or β -pBrPh-Glc (30 mM) to a solution of 15% glycerol significantly increased the post-thaw RBC integrity to approximately 50% and 70%, respectively.⁹ This result was significant as reducing the quantity of glycerol can decrease post-thaw processing times, enabling frozen RBCs units to be more readily accessible. This work also highlighted the potential of small molecule IRIs to function as novel CPAs and protect human RBCs against freezing injury. Consequently, structure-function analyses of β -PMP-Glc and β -pBrPh-Glc may elucidate key structural features that further enhance their IRI activity and increase their cryoprotective ability.

4.2 Azido Aryl Glucosides as Ice Recrystallization Inhibitors

Despite the plethora of possible functional groups that we could introduce into an ice recrystallization inhibitor, the azide (N_3) is one of the most popular functionalities due to its unique reactivity and selectivity.^{10,11} Numerous methods have been developed for the introduction of an azide functional group at different positions of sugars; however, one of the most common methods is nucleophilic substitution of an appropriate leaving group with an azide ion.^{12,13} In carbohydrate synthesis, an azide functionality serves as a useful synthon for amines since they can be easily reduced to amines.¹²⁻¹⁴ Given that azides are reasonably stable against various reaction conditions, a useful property of azides is that they can serve as “masked” amines during synthesis.^{12,13,15} Furthermore, the diazo

transfer reaction permits the use of azides not only as amine synthons but also as protecting groups for amines.¹³

During the last decade, azides have become a powerful tool in the field of bioconjugation and are the mostly widely used bioorthogonal group.^{11,16–18} The development of two azide-based bioorthogonal reactions^{19,20} (copper-catalyzed azide-alkyne [3+2] cycloaddition and the Staudinger ligation) offer the ability to selectively label and manipulate biomolecules via incorporation of azide tags into biomolecules including proteins and glycans.^{21–25} For instance, azide-tagged sugar moieties have been introduced into glycans using the cell's biosynthetic machinery and subsequently labeled with a detectable probe.^{26–28} Evidently, the azide is an appealing moiety due to its bioorthogonal and biocompatible nature. Consequently, we sought to explore whether chemically modifying β -PMP-Glc and β -pBrPh-Glc with an azide functional group could increase their IRI activity.

4.2.1 Structures and Synthesis of Azido Aryl Glucosides

The structures of the azido aryl glucosides (**401-409**) are based on the parent compounds *p*-methoxyphenyl- β -D-glucopyranoside (**103**, β -PMP-Glc) and *p*-bromophenyl- β -D-glucopyranoside (**104**, β -pBrPh-Glc). Specifically, an azide substituent was incorporated at various positions (C-6, C-4, C-3 and C-2) on the pyranose ring as well as the *para*-position of the aryl ring to determine the relationship between the position of the azide and IRI activity.

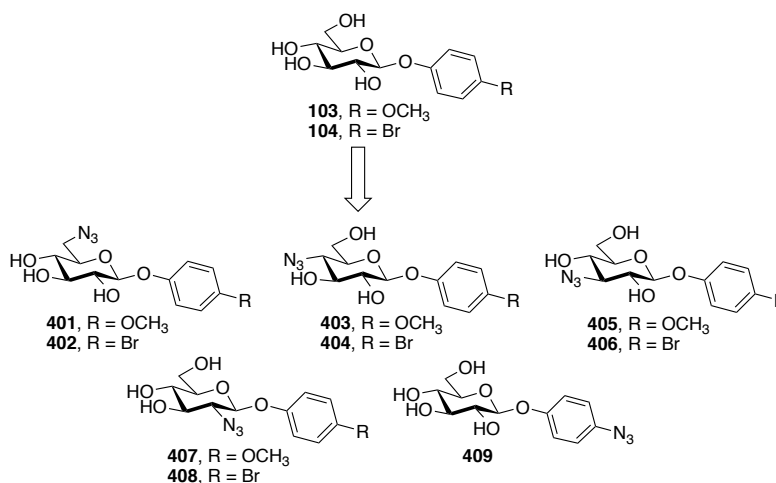
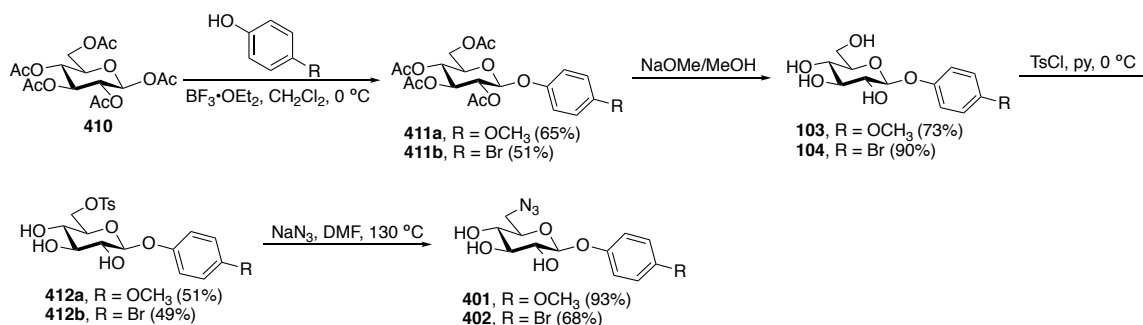


Figure 4.2 Structures of azido targets (**401-409**) based upon **103** and **104**.

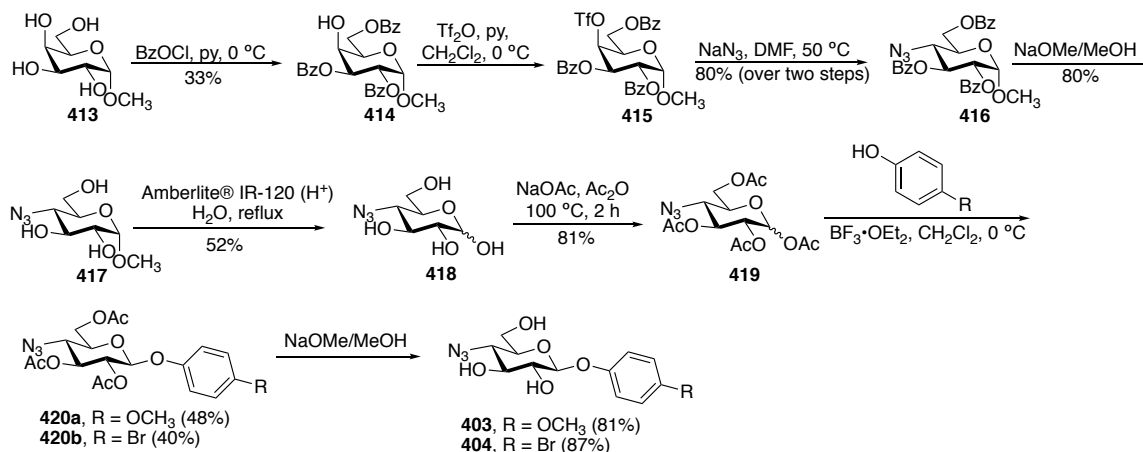
The azide functional group was specifically introduced at the *para*-position of the aryl ring because it has been previously demonstrated that *para*-substituted analogues are significantly more IRI active than *ortho*- and *meta*-substituted analogues.⁶ The structures are shown in Figure 4.2. The azido aryl glucosides **401-409** were synthesized using conventional procedures. The introduction of an azide at the primary carbon of a carbohydrate is efficiently carried out by an S_N2 reaction. The 6-azido-6-deoxy-β-D-glucopyranoside derivatives **401** and **402** were prepared from β-D-glucose pentaacetate (**410**) as indicated in Scheme 4.1. Lewis acid catalyzed activation of the β-linked anomeric acetate with boron trifluoride diethyl etherate (BF₃•OEt₂) allowed for glycosidation with the appropriate *para*-substituted phenol (R = OCH₃ or Br) to provide the β-linked aryl glycosides **411a** and **411b** exclusively. Deacetylation of **411a** and **411b** under Zemplén conditions afforded the intermediates **103** and **104**.⁷ Conversion of the C-6 primary hydroxyl group to a good leaving group (i.e. tosylate) was selectively achieved, without the need for protection of the secondary hydroxyl groups, to afford the intermediates **412a** and **412b**. Subsequent displacement with sodium azide (NaN₃) at elevated temperatures afforded the desired 6-azido aryl glucosides **401** and **402** in an overall yield of 23% and 15%, respectively.



Scheme 4.1 Synthesis of 6-azido-6-deoxy-β-D-glucopyranoside derivatives **401** and **402**.

The introduction of azido groups at the sugar ring system can also be carried out by an S_N2 reaction. Although this approach is successful, it can sometimes lead to the formation of products with incorrect stereochemical configuration or elimination products.¹³ In contrast to substitutions at primary carbons, S_N2 reactions at secondary carbons of the sugar ring are more complicated since they typically require several synthetic manipulations to ensure correct stereochemical configuration of the desired

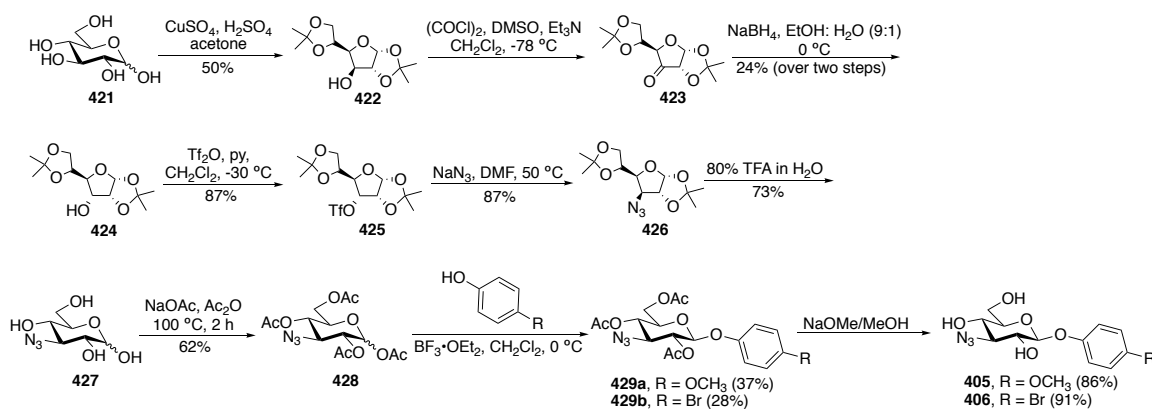
product. The synthesis of the 4-azido-4-deoxy- β -D-glucopyranoside derivatives **403** and **404** is described in Scheme 4.2. Methyl α -D-galactopyranoside (**413**) was selectively benzoylated to give the 2,3,6-tri-*O*-benzoate **414** due to the lower reactivity of the axial C-4 hydroxyl compared to C-2, C-3 and C-6.^{29,30} Conversion of the C-4 hydroxyl group to a good leaving group (i.e. triflate) was achieved using triflic anhydride (Tf₂O) to afford intermediate **415**, which then underwent an S_N2 reaction with sodium azide to yield intermediate **416**. Removal of the benzoyl protecting groups with sodium methoxide resulted in intermediate **417**, which was subsequently hydrolyzed with acidic cation exchange resin (Amberlite® IR-120 (H⁺)) to afford 4-azido-4-deoxy-D-glucose (**418**).³¹ Acetylation of **418** followed by Lewis acid catalyzed glycosidation of glucose tetraacetate derivative **419** with the appropriate *para*-substituted phenol (R = OCH₃ or Br) yielded intermediates **420a** and **420b**. Zemplén deacetylation of **420a** and **420b** afforded the desired 4-azido aryl glucosides **403** and **404** in an overall yield of 4% and 3%, respectively.



Scheme 4.2 Synthesis of 4-azido-4-deoxy- β -D-glucopyranoside derivatives **403** and **404**.

The synthesis of the 3-azido-3-deoxy- β -D-glucopyranoside derivatives **405** and **406** is shown in Scheme 4.3. The synthesis began with the preparation of diacetone-D-glucose (**422**) from D-glucose (**421**), in which the C-3 position can be directly manipulated. Swern oxidation of **422** followed by diastereoselective reduction at C-3 using sodium borohydride (NaBH₄) afforded diacetone-D-allose (**424**), which was then converted to the azido derivative **426** via a triflation/azide displacement sequence.³² The furanose diacetonide derivative **426** was treated with aqueous trifluoroacetic acid (TFA) followed

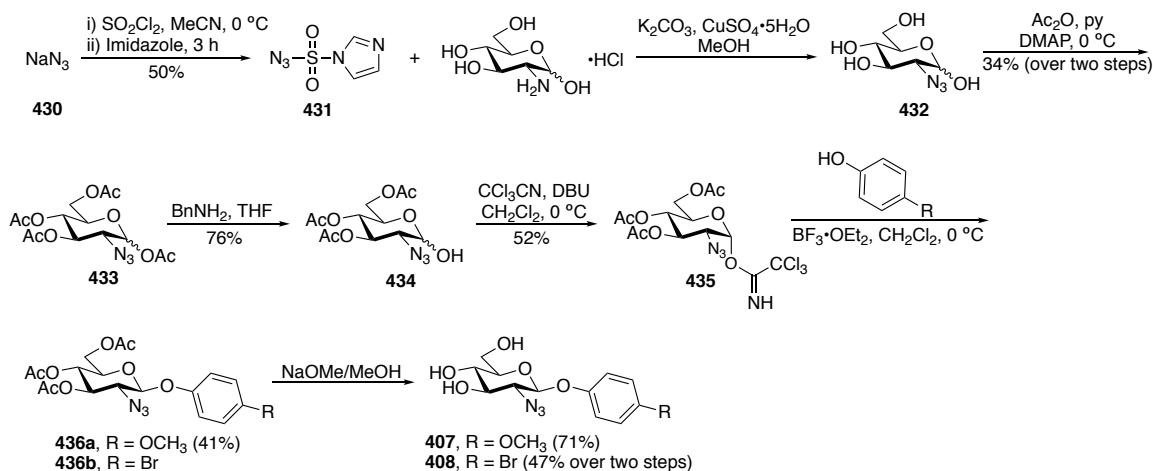
by acetylation to furnish the glucose tetraacetate derivative **428**. Lewis acid catalyzed glycosidation of glucose tetraacetate derivative **428** with the appropriate *para*-substituted phenol (R = OCH₃ or Br) yielded intermediates **429a** and **429b**. Zemplén deacetylation of **429a** and **429b** afforded the desired 3-azido aryl glucosides **405** and **406** in each an overall yield of 1%.



Scheme 4.3 Synthesis of 3-azido-3-deoxy-β-D-glucopyranoside derivatives **405** and **406**.

Another method for the synthesis of azides is the use of the diazo transfer reaction.³³ In contrast to the synthetic methods described above where an entire azido group is incorporated into the molecule, the diazo transfer reaction converts amines to azides by transferring a diazo group (-N₂) onto an existing amine, resulting in retention of configuration.^{13,33} This method was employed for the preparation of 2-azido aryl glucosides **407** and **408** as well as *p*-azidophenyl-β-D-glucopyranoside (**409**). The synthetic scheme for the preparation of 2-azido-2-deoxy-β-D-glucopyranoside derivatives **407** and **408** is displayed in Scheme 4.4. The synthesis began with the preparation of the diazo transfer reagent imidazole-1-sulfonyl azide (**431**), which is a shelf-stable and efficient alternative to the formerly used “diazo donor” triflyl azide (TfN₃).³⁴ D-glucosamine hydrochloride was treated with **431** under typical diazo transfer conditions to give the glucose tetraacetate derivative **433** following acetylation.³⁴ The anomeric acetate was selectively removed using benzylamine (BnNH₂) followed by activation with trichloroacetonitrile (CCl₃CN) in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as the base to provide the α-trichloroacetimidate **435**.^{35,36} Trichloroacetimidates are useful since the stereochemistry of the resulting glycoside can be controlled.^{35,37} For instance, under reversible conditions in the presence of DBU as base, the α-

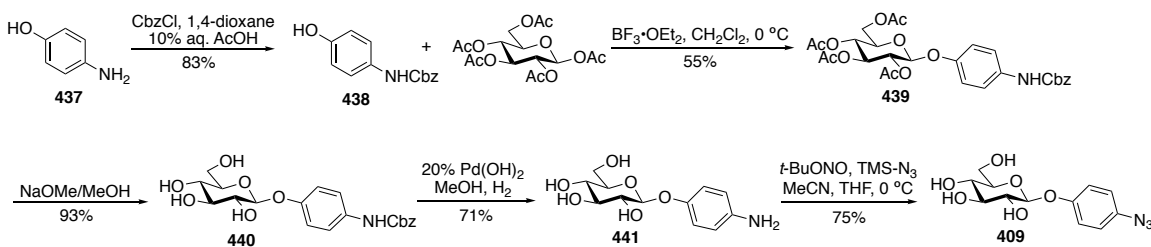
trichloroacetimidate is the most stable form and is formed preferentially upon quenching (thermodynamic product). Conversely, under irreversible conditions in the presence of K_2CO_3 as base, the β -trichloroacetimidate would be formed (kinetic product).^{35,37} In a glycosidation reaction, α - or β -glycosides can be formed from an α -trichloroacetimidate depending on the conditions.³⁵ The use of a strong promoter (e.g. TMSOTf) results in the formation of an oxocarbenium ion intermediate, and thus a mixture of α - and β -glycosides is possible, particularly in the absence of a participating group at C-2. In contrast, the use of a mild promoter (e.g. $BF_3 \cdot OEt_2$) results in the selective formation of the β -glycoside through an S_N2 -like pathway.^{35,38} Thus, the α -trichloroacetimidate moiety of **435** was displaced by the appropriate *para*-substituted phenol ($R = OCH_3$ or Br) using $BF_3 \cdot OEt_2$ to afford β -glycoside intermediates **436a** and **436b**. Zemplén deacetylation of **436a** and **436b** furnished the desired 2-azido aryl glucosides **407** and **408** in an overall yield of 2% and 3%, respectively.



Scheme 4.4 Synthesis of 2-azido-2-deoxy- β -D-glucopyranoside derivatives **407** and **408**.

The synthesis of *p*-azidophenyl- β -D-glucopyranoside (**409**) is described in Scheme 4.5. Firstly, 4-aminophenol (**437**) was treated with benzyl chloroformate (CbzCl) to install a carboxybenzyl protecting group. Lewis acid catalyzed glycosidation of β -D-glucose pentaacetate with the Cbz-protected derivative **438** followed by deacetylation afforded the intermediate **440**. Removal of the carboxybenzyl protecting group by hydrogenation furnished the *p*-aminophenyl- β -D-glucoside (**441**) in which the aromatic amine was converted to an aromatic azide using *tert*-butyl nitrite (*t*-BuONO) and

azidotrimethylsilane (TMS-N₃).³⁹ The desired product **409** was synthesized in an overall yield of 23%.



Scheme 4.5 Synthesis of *p*-azidophenyl-β-D-glucopyranoside **409**.

4.2.2 IRI Activity of Azido Aryl Glucosides

The IRI activity of the azido aryl glucosides **401-409** was assessed utilizing the modified “splat-cooling” assay.⁸ Using this method, the IC₅₀ value of each compound was determined. Briefly, in this assay, the compound was dissolved at various concentrations in a PBS solution and the areas of ice crystals were measured after a 5-minute annealing period at -6.4 °C. For each concentration, rate constants for ice crystal growth were determined and normalized to the positive PBS control for ice recrystallization. A dose-response curve was generated by plotting the normalized rate constants (k_{norm}) for each concentration as a function of the corresponding log values of the concentration. To obtain the IC₅₀ value, a two-parameter sigmoidal curve was fit to the data.

The screening approach used for the identification of candidate small molecule IRIs for cryopreservation applications was previously described in Chapter 2 (Section 2.1). The majority of the azido aryl glucosides were not effective inhibitors of ice recrystallization. With the exception of the 3-azido aryl glucosides **405** and **406** and *p*-azidophenyl-β-D-glucoside (**409**), all of the compounds (**401-404** and **407, 408**) failed to achieve full inhibition of ice crystal growth (i.e. $k_{\text{norm}} \neq 0\%$) when assessed at their maximum solubility in PBS (Table 4.1). This is likely due to their very poor solubility in aqueous solution, particularly the 4-azido aryl glucosides **403** and **404**, which possessed a maximum solubility of 50 μM. For instance, the 6-azido aryl glucosides **401** and **402** were only able to achieve a k_{norm} of 89.35% and 74.48%, respectively (Table 4.1). The 4-azido aryl glucosides **403** and **404** had normalized rate constants comparable to that of the PBS control (102.24% and 89.40%, respectively). Similarly, the 2-azido aryl glucosides **407** and **408** could only achieve a k_{norm} of 78.57% and 74.35%, respectively

(Table 4.1). As a result, a complete sigmoidal dose-response curve could not be constructed for compounds **401-404** and **407, 408** and an IC_{50} value could not be determined (Table 4.2).

Table 4.1 Normalized rate constants (k_{norm}) for azido aryl glucosides **401-409**.

Compound	Maximum Concentration (mM)	Normalized Rate Constant (k_{norm})
401	2	$89.35 \pm 2.55\%$
402	10	$74.48 \pm 2.28\%$
403	0.05	$102.24 \pm 2.42\%$
404	0.05	$89.40 \pm 3.87\%$
405	30	$0.00 \pm 0.00\%$
406	10	$0.00 \pm 0.00\%$
407	10	$78.57 \pm 2.89\%$
408	5	$74.35 \pm 1.09\%$
409	100	$0.85 \pm 0.85\%$

Table 4.2 IC_{50} values for azido aryl glucosides **401-409**.

Compound	IC_{50} Value (mM)
401	N/A
402	N/A
403	N/A
404	N/A
405	6
406	0.7
407	N/A
408	N/A
409	26

In contrast, the 3-azido aryl glucosides **405** and **406** and *p*-azidophenyl- β -D-glucoside (**409**) were capable of achieving full inhibition of ice crystal growth (i.e. $k_{\text{norm}} = 0\%$). Thus, complete sigmoidal dose-response curves were generated (Figure 4.3) and compounds **405**, **406** and **409** were found to have IC_{50} values of 6 mM, 0.7 mM and 26 mM, respectively (Table 4.2). Amongst these compounds, it is evident that the 3-azido aryl glucosides **405** and **406** are more efficacious inhibitors of ice recrystallization than **409** since they require considerably lower concentrations to elicit a response (IC_{50} values

of 6 mM and 0.7 mM versus 26 mM). Nevertheless, the 3-azido aryl glucoside **406** (R = Br) is the most efficacious within this class of ice recrystallization inhibitors.

In comparison to the parent compounds β -PMP-Glc (**103**) and β -pBrPh-Glc (**104**) which possess IC_{50} values of 16 mM and 15 mM, respectively,⁸ it can be seen that the incorporation of an azido group at C-6, C-4 and C-2 reduced their IRI activity. Furthermore, the azide functionality generally decreased solubility in aqueous solution, which may account for the lack of activity. Interestingly, the incorporation of an azido group at C-3 increased the IRI activity, as the 3-azido aryl glucosides **405** and **406** were more efficacious than their corresponding parent compounds (IC_{50} value of 6 mM versus 16 mM and IC_{50} value of 0.7 mM versus 15 mM). Although replacing the methoxy (-OCH₃) or bromo (-Br) substituent at the *para*-position of the aryl ring with an azido group (-N₃) resulted in a less efficacious inhibitor compared to **103** and **104** (IC_{50} value of 26 mM versus 16 mM and 15 mM), compound **409** is nevertheless an effective inhibitor of ice recrystallization.

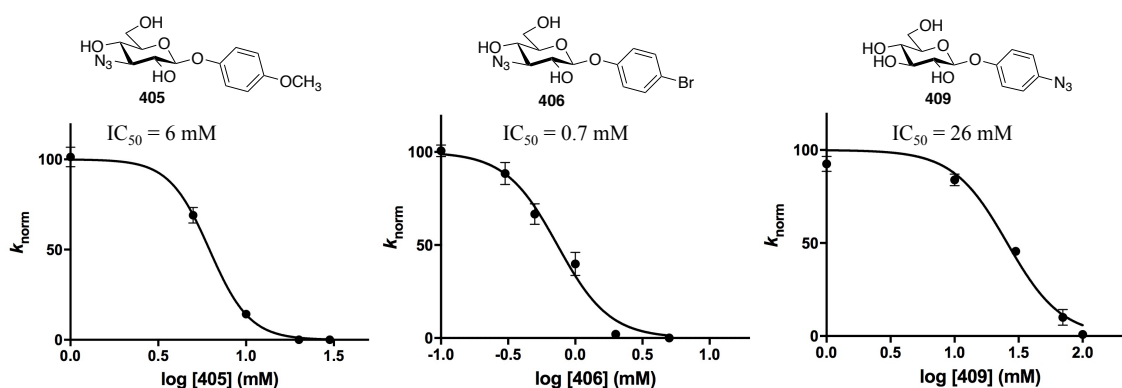


Figure 4.3 Dose-response curves for azido aryl glucosides **405**, **406** and **409**. Rate constants (k_{norm}) were normalized to a PBS control and measured in triplicate. Error bars represent the standard error of the mean (SEM). A two-parameter sigmoidal curve was fit to the data.

4.2.3 Assessing Cytotoxicity of IRI Active Azido Aryl Glucosides

Prior to utilizing the IRI active azido aryl glucosides **405**, **406** and **409** for cryopreservation applications, these molecules were assessed for cytotoxic effects in an immortalized cell line (HepG2 cells) using the conventional MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide).^{40–42} This is a colorimetric assay that measures cellular metabolic activity as a means of assessing cell viability. It is based

on the ability of mitochondrial and cytosolic dehydrogenase enzymes in metabolically active cells to reduce the water-soluble yellow tetrazolium dye MTT to its insoluble purple formazan.⁴³ An isopropanol solubilization solution is added to dissolve the insoluble formazan, allowing the absorbance of the coloured solution to be measured using a spectrophotometer to quantify cell viability. Thus, an intense purple colour represents viable, metabolically active cells whereas a decrease in the intensity of the purple colour signifies reduced cell number and thus cytotoxicity of a given compound.

Cytotoxic effects of **405**, **406** and **409** were determined by assessing cell viability in HepG2 cells at various concentrations. A dose-response curve was generated by plotting the average percent cell viability for each concentration as a function of the corresponding log values of the concentration. A two-parameter sigmoidal curve was fit to the data. This allowed cytotoxicity to be quantified by an IC₅₀ value, which refers to the concentration of compound required to reduce cell viability by 50%.^{44–48} Thus, the lower the IC₅₀ value, the higher the cytotoxic effect of the compound. The 3-azido aryl glucoside **405** was assessed at concentrations ranging from 1 mM to 60 mM, whereas the 3-azido aryl glucoside **406** was assessed at lower concentrations of 0.1 mM to 15 mM. In contrast, the *p*-azidophenyl-β-D-glucoside (**409**) was assessed at concentrations ranging from 1 mM to 80 mM. The dose-response curves for **405**, **406** and **409** are shown in Figure 4.4. The azido aryl glucosides **405**, **406** and **409** caused significant loss of HepG2 cell viability after incubation for 18 h at 37 °C, displaying IC₅₀ values of 9 mM, 3 mM and 12 mM, respectively. While the cytotoxicity profiles (i.e. IC₅₀ values) for **405** and **409** are similar, **406** had a significantly different profile and was more toxic than **405** and **409**. The cytotoxic results of **405** and **406** show that at the concentration required to inhibit ice recrystallization by 50% (i.e. IC₅₀ value (IRI activity) of 6 mM and 0.7 mM), these compounds exhibit moderate to low toxicity (cell viabilities of ~61% and 96%, respectively). In contrast, **409** is highly toxic to HepG2 cells at its IC₅₀ value for IRI activity (26 mM) as it resulted in cell viabilities of 0% at concentrations of 20 mM or higher.

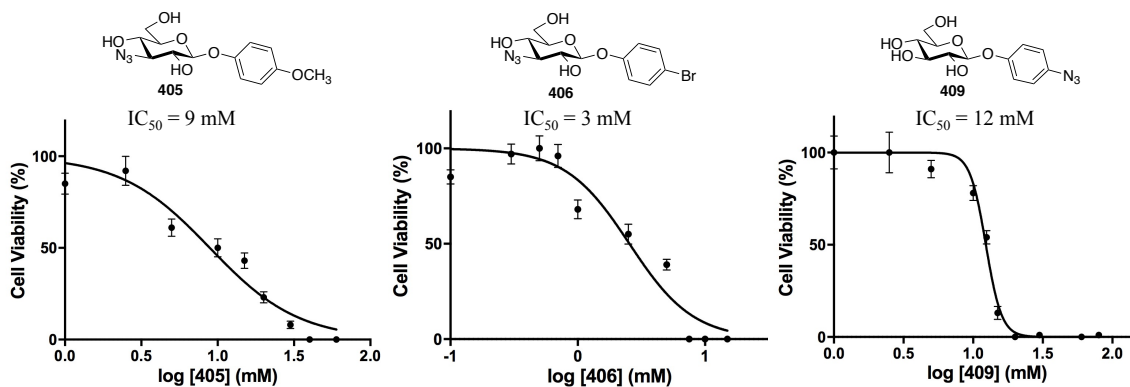


Figure 4.4 Dose-response curves for azido aryl glucosides **405**, **406** and **409**. Cell viability of HepG2 cells was calculated as a percentage of the control following incubation for 18 h at 37 °C as determined by the MTT assay. Error bars represent the standard error of the mean (SEM). A two-parameter sigmoidal curve was fit to the data.

Although this assessment provides cytotoxicity data for compounds **405**, **406** and **409** in a nucleated cell line (HepG2 cells) under extended incubation times (i.e. 18 h), these results may not necessarily translate to anucleated cells such as RBCs. Furthermore, since the IC_{50} value is dependent on both the type of nucleated cell line and the incubation time, these values may not be the same for different nucleated cell lines and incubation times (e.g. shorter incubation). Nevertheless, this assay served to provide first round toxicity screening and further toxicity testing will be performed prior to cryopreservation studies depending on the cell type of interest.

4.2.3.1 Measuring Apoptosis of IRI Active Azido Aryl Glucosides

Although the azido aryl glucosides **405**, **406** and **409** were capable of causing cell death in HepG2 cells, the process by which they were achieving this was not known. Therefore, we sought to investigate whether compounds **405**, **406** and **409** were acting selectively by initiating apoptosis. There are several different forms of cell death; however, necrosis and apoptosis are the most studied and the best characterized mechanisms of cell death.⁴⁹ Necrotic cell death typically involves swelling of mitochondria and cell membrane damage leading to early plasma membrane rupture and release of intracellular contents into the surrounding medium.^{49,50} Conversely, apoptosis is a form of programmed cell death that involves the activation of a group of cysteine aspartic acid-specific proteases known as caspases and an energy-dependent cascade of

molecular events.^{50,51} There are two main apoptotic pathways: the intrinsic or the extrinsic pathway (Figure 4.5).

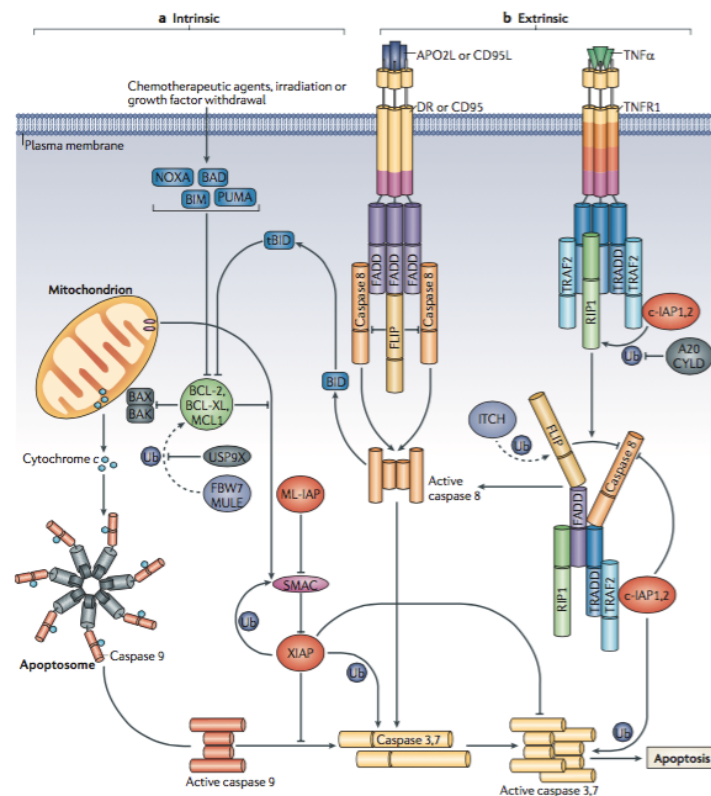


Figure 4.5 The intrinsic (a) and extrinsic (b) apoptotic pathways.⁵¹

The intrinsic pathway is triggered by stress or cell damage and includes diverse stimuli such as chemotherapeutic agents, irradiation or growth factor withdrawal.⁵¹ In response to stress or cell damage, a set of proteins called “pro-apoptotic BH3-only proteins” are activated which leads to the activation of BAX and BAK proteins, resulting in disruption of mitochondrial integrity. This causes the release of cytochrome *c* and second mitochondrial activator of caspases (SMAC).^{50,51} Cytochrome *c* in the cytoplasm prompts the formation of an apoptosome complex that leads to activation of initiator caspase-9. This in turn activates executioner caspases-3 and -7, leading to the cleavage of protein substrates and cell death. Contrarily, the extrinsic pathway is initiated by the binding of death ligands to receptors, triggering receptor aggregation.^{50,51} The aggregation of receptors recruits an adaptor protein, known as FAS-associated DEATH domain protein (FADD), which in turn recruits initiator caspase-8 to form the receptor-associated death-inducing signaling complex (DISC). Consequently, caspase-8 is activated and the death

signal is amplified by the subsequent activation of caspases-3 and -7, resulting in apoptotic cell death.^{50,51} Ultimately, whether apoptosis occurs via the intrinsic or extrinsic pathway, the general mechanism of activation is similar such that an apoptotic stimulus initiates a protein cascade triggering the activation of initiator caspases (caspases-8 and -9) which subsequently results in the activation of caspases-3 and -7 that are the main executors of apoptosis.

Thus, to understand if compounds **405**, **406** and **409** were causing cell death by initiating apoptosis, a caspase-3/7 activation assay was conducted. Specifically, the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega) was used for the evaluation of protease activity of caspases-3 and -7. This assay is composed of a cell lysis buffer and a caspase -3/7 profluorescent substrate rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110). Cleavage of the DEVD (Asp-Glu-Val-Asp) peptides by active caspases-3 and -7 creates the fluorescent rhodamine 110 which can be measured spectroscopically (Figure 4.6).

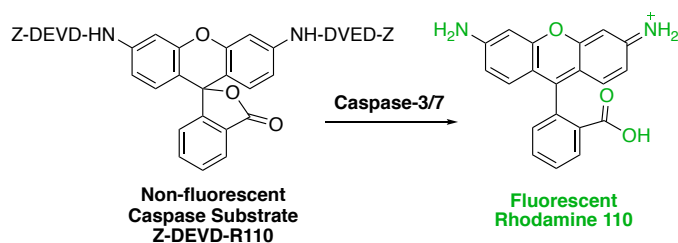


Figure 4.6 Cleavage of the non-fluorescent caspase substrate Z-DEVD-R110 by caspases-3/7 to create the fluorescent Rhodamine 110.

To perform this assay, HepG2 cells were cultured and treated with **405**, **406** and **409** for 18 h at 37 °C at their IC₅₀ concentrations for cytotoxicity. Staurosporine, a known inducer of apoptosis, was used as a positive control.⁵² Cells containing culture medium were used as a negative control and served to quantify caspase-3/7 activity in normal cells as a function of time. DMSO (1%) was required to solubilize staurosporine and therefore was added to all wells. The blank control wells contained culture medium without cells and were used to measure background fluorescence associated with the culture system and Apo-ONE® Caspase-3/7 Reagent. The blank control was subtracted from experimental values. Fluorescence measurements were taken over the course of a 48-hour time period with appropriate time increments to capture peak apoptotic activity and are reported as

relative fluorescence units (RFUs). Thus, high or low RFUs represent activated or depressed caspase-3/7 activity, respectively. Figure 4.7 shows the results of the analysis of caspase-3/7 activity in HepG2 cells treated with **405**, **406** or **409**. Apoptosis in HepG2 cells treated with and without **405**, **406** or **409** was not detectable before 24 hours of treatment. The results revealed that caspase-3/7 levels in HepG2 cells treated with **405**, **406** or **409** did not increase relative to the untreated negative control. This suggests that executioner caspases-3 and -7 are not activated upon exposure to compounds **405**, **406** or **409** and an apoptotic process is not being initiated. Consequently, the cytotoxic effects of **405**, **406** and **409** in HepG2 cells may be due to necrosis; however, additional studies would be required to support the occurrence of a non-apoptotic mechanism of cell death.

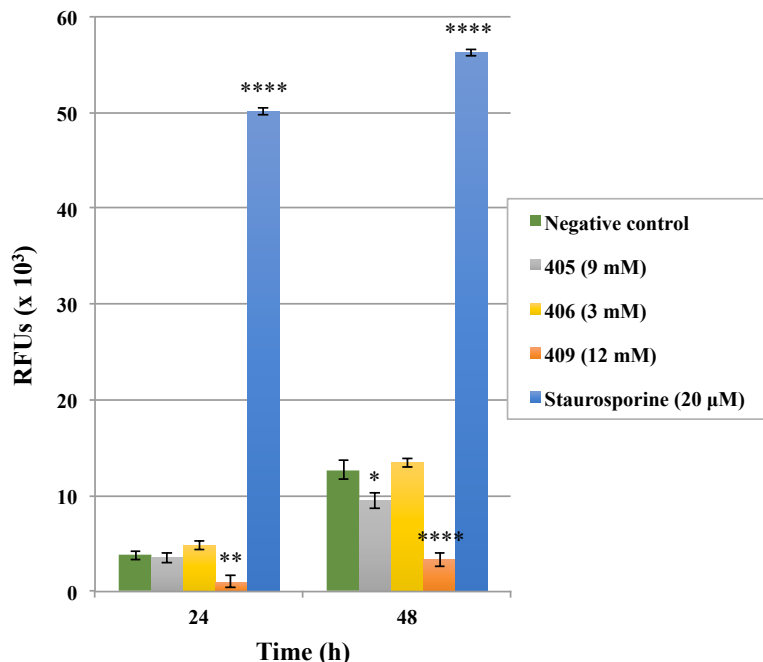


Figure 4.7 Apo-ONE® Homogeneous Caspase-3/7 assay using HepG2 cells incubated with **405** (9 mM), **406** (3 mM) or **409** (12 mM) for 18 h at 37 °C. HepG2 cells incubated with vehicle treatment (1% DMSO in MEM) served as the negative control. The positive control consisted of HepG2 cells incubated with 20 µM staurosporine. Caspase activity was measured by fluorescence at the indicated time points and reported as relative fluorescence units (RFUs). Error bars represent the standard error of the mean (SEM). Asterisks indicate significant difference relative to the negative control as determined by the unpaired Student's T-test: * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

4.3 Amino Aryl Glucosides as Ice Recrystallization Inhibitors

Given that some of the azido aryl glucosides were effective inhibitors of ice recrystallization, we sought to investigate whether reducing the azide to an amine could increase their IRI activity. Furthermore, an amine functionality should increase solubility in aqueous solution and may circumvent the poor solubility of the majority of the azido aryl glucosides. Azides can be easily converted to amines by catalytic hydrogenation or by reaction with thiols or complex hydrides.^{10,12} In carbohydrate chemistry, a widely applied method is the Staudinger reaction using triaryl- or trialkylphosphines.⁵³ Thus, amino derivatives of IRI active azido aryl glucosides (**405**, **406** and **409**) were synthesized and assessed for their ability to inhibit ice recrystallization. In addition, amino derivatives of the 6-azido aryl glucosides **401** and **402** were also prepared due to synthetic simplicity. The remaining azido aryl glucosides were not reduced to their corresponding amino derivatives due to their complex and lengthy synthesis that is not amenable to large-scale preparation.

4.3.1 Structures and Synthesis of Amino Aryl Glucosides

The structures of the amino derivatives **441-445** are shown in Figure 4.8. The majority of the amino aryl glucosides were prepared from their corresponding azido aryl glucosides and their syntheses are described below.

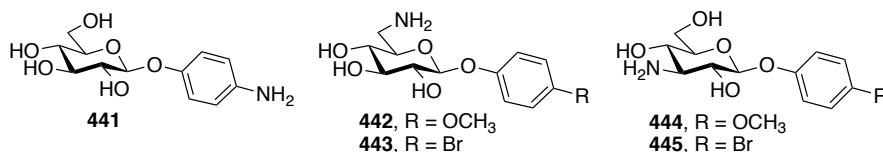
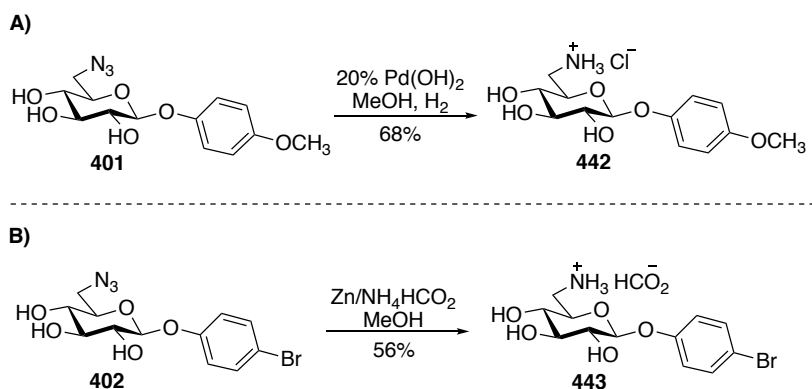


Figure 4.8 Structures of amino targets (**441-445**).

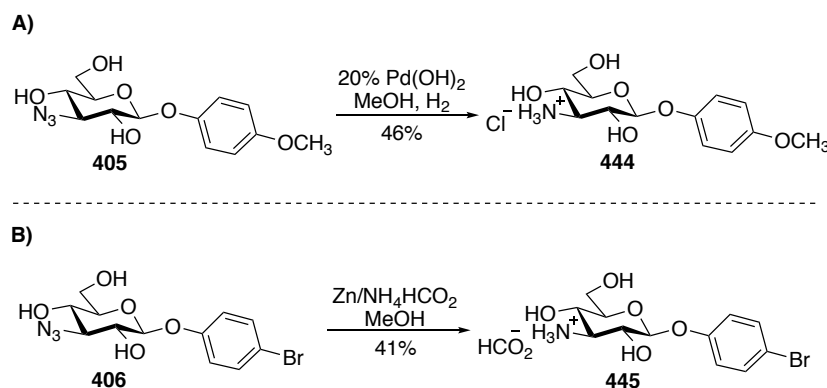
The synthesis of *p*-aminophenyl- β -D-glucoside (**441**) was previously described in Scheme 4.5 (Section 4.2.1). The 6-amino-6-deoxy- β -D-glucopyranoside derivatives **442** and **443** were prepared from their corresponding 6-azido aryl glucosides **401** and **402** (previously described in Scheme 4.1) as indicated in Scheme 4.6. Reduction of the azide (**401**) to an amine by palladium-catalyzed hydrogenation afforded the 6-amino aryl glucoside **442** as a hydrochloride salt upon treatment with 10% HCl. However, the same synthetic approach could not be used for the preparation of the 6-amino aryl glucoside **443** because the aryl C–Br bond would be cleaved by palladium-catalyzed hydrogenation,

resulting in the loss of the bromine substituent at the *para*-position.^{54,55} Consequently, we sought to explore alternative approaches to reduce the azide to an amine without cleaving the aryl C–Br bond. Another possibility was the Staudinger reaction, which was successful in converting the azide to an amine; however, removal of the phosphine oxide by-product from the final product proved to be very difficult and could not be achieved. Thus, a different synthetic route was attempted using catalytic transfer hydrogenation with ammonium formate as the hydrogen source in the presence of zinc dust.⁵⁶ Under these reaction conditions, several other functionalities including aryl halides were shown to be well tolerated.⁵⁶ The azide derivative (**402**) was treated with zinc and ammonium formate to give the corresponding 6-amino aryl glucoside **443** as a formate salt.



Scheme 4.6 Synthesis of 6-amino-6-deoxy-β-D-glucopyranoside derivatives **442** (A) and **443** (B) from the corresponding azido aryl glucosides **401** and **402**.

The 3-amino-3-deoxy-β-D-glucopyranoside derivatives **444** and **445** were also prepared from their corresponding 3-azido aryl glucosides **405** and **406** (previously described in Scheme 4.3) as indicated in Scheme 4.7. As described above, the 3-amino aryl glucoside **444** was prepared as a hydrochloride salt by palladium-catalyzed hydrogenation and subsequent acidic (10% HCl) workup. In contrast, the 3-amino aryl glucoside **445** was prepared as a formate salt using the zinc/ammonium formate method to avoid cleavage of the aryl C–Br bond that would otherwise occur utilizing palladium-catalyzed hydrogenation.⁵⁶



Scheme 4.7 Synthesis of 3-amino-3-deoxy-β-D-glucopyranoside derivatives **444** (A) and **445** (B) from the corresponding azido aryl glucosides **405** and **406**.

4.3.2 IRI Activity of Amino Aryl Glucosides

The IRI activity of the amino aryl glucosides **441-445** was assessed utilizing the modified “splat-cooling” assay.⁸ Using this method, the IC₅₀ value of each compound was determined. As previously described in Section 4.2.2, the compound was dissolved at various concentrations in a PBS solution and the areas of ice crystals were measured after a 5-minute annealing period at -6.4 °C. For each concentration, rate constants for ice crystal growth were determined and normalized to the positive PBS control for ice recrystallization. A dose-response curve was generated by plotting the normalized rate constants (k_{norm}) for each concentration as a function of the corresponding log values of the concentration. To obtain the IC₅₀ value, a two-parameter sigmoidal curve was fit to the data.

The amino aryl glucosides **441-443** were effective inhibitors of ice recrystallization, capable of achieving full inhibition of ice crystal growth (i.e. $k_{\text{norm}} = 0\%$) at their maximum solubility in PBS (Table 4.3). In contrast, the 3-amino aryl glucoside **444** (R = OCH₃) could only achieve a k_{norm} of 14.55% when assessed at its maximum concentration of 100 mM (Table 4.3). The 3-amino aryl glucoside **445** (R = Br) was poorly soluble in PBS (maximum concentration of 2.5 mM) and possessed a k_{norm} comparable to that of the PBS control (89.22%). As a result, complete sigmoidal dose-response curves could only be constructed for compounds **441-443** (Figure 4.9). The IC₅₀ values for compounds **441-443** were found to be 39 mM, 40 mM and 16 mM, respectively (Table 4.4).

Table 4.3 Normalized rate constants (k_{norm}) for amino aryl glucosides **441-445**.

Compound	Maximum Concentration (mM)	Normalized Rate Constant (k_{norm})
441	200	$0.00 \pm 0.00\%$
442	200	$0.00 \pm 0.00\%$
443	150	$0.00 \pm 0.00\%$
444	100	$14.55 \pm 1.47\%$
445	2.5	$89.22 \pm 0.96\%$

Table 4.4 IC_{50} values for amino aryl glucosides **441-445**.

Compound	IC_{50} Value (mM)
441	39
442	40
443	16
444	N/A
445	N/A

Based on the IC_{50} values (Table 4.4), it is evident that the 6-amino aryl glucoside **443** (R = Br) is a more efficacious inhibitor of ice recrystallization than **441** and **442** since it requires a lower concentration to produce an effective response.

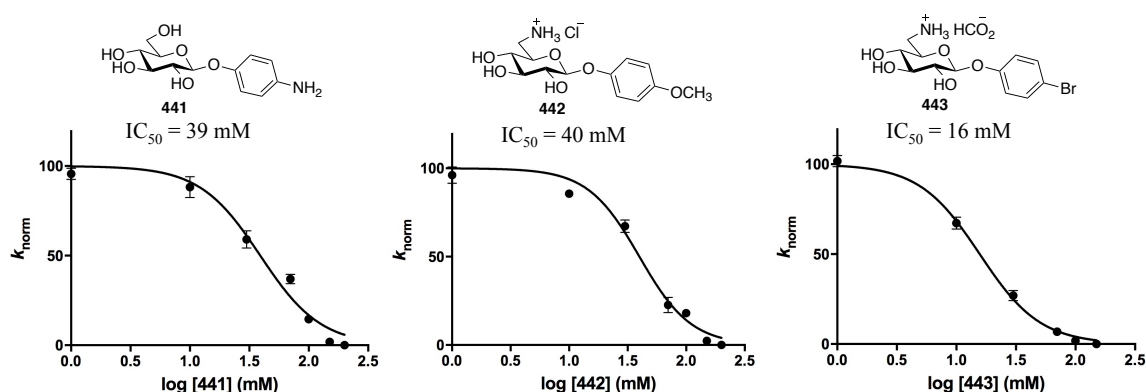


Figure 4.9 Dose-response curves for amino aryl glucosides **441-443**. Rate constants (k_{norm}) were normalized to a PBS control and measured in triplicate. Error bars represent the standard error of the mean (SEM). A two-parameter sigmoidal curve was fit to the data.

To simplify the purification process, the 6-amino aryl glucosides **442** and **443** were prepared as salts (a hydrochloride salt and a formate salt, respectively) to produce a crystalline compound that can be recrystallized. The importance of the counterion to IRI activity has been previously investigated by our laboratory and it was shown that the

nature of the counterion can affect IRI activity and should be taken into consideration if the compound is a salt.^{3,57} However, these previous studies were limited to anionic (chloride, bromide, and iodide) and cationic (lithium, sodium, and potassium) counterions and thus, the effect of a formate counterion on IRI activity is not known. As a preliminary investigation, ammonium formate (NH_4HCO_2) was assessed for IRI activity at various concentrations. Interestingly, ammonium formate was found to be IRI active with an IC_{50} value of 3 mM (Figure 4.10).

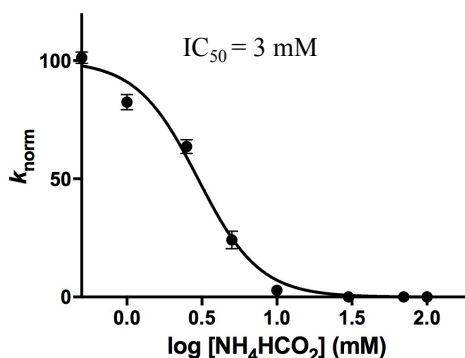


Figure 4.10 Dose-response curve for ammonium formate. Rate constants (k_{norm}) were normalized to a PBS control and measured in triplicate. Error bars represent the standard error of the mean (SEM). A two-parameter sigmoidal curve was fit to the data.

Given that the 6-amino aryl glucoside **443** possessed an IC_{50} value of 16 mM suggests that the formate counterion increases IRI activity. In fact, the IRI activity of **443** may be solely due to the formate counterion and the differences in IRI activity (i.e. IC_{50} values) between ammonium formate and **443** may be exclusively due to structural differences in the cationic component. As a result, further investigation is required and the formate counterion should be exchanged for a chloride counterion to determine the effect of a formate counterion on the IRI activity of **443**. Ultimately, the nature and effect of the negative counterion for amino aryl glucosides should be examined in the future.

In comparison to their azido counterparts, the *p*-aminophenyl- β -D-glucoside (**441**) was a less efficacious inhibitor of ice recrystallization (IC_{50} value of 39 mM versus 26 mM), whereas the 6-amino aryl glucosides **442** ($\text{R} = \text{OCH}_3$) and **443** ($\text{R} = \text{Br}$) possessed significantly increased IRI activity. On the other hand, the 3-amino aryl glucosides **444** ($\text{R} = \text{OCH}_3$) and **445** ($\text{R} = \text{Br}$) exhibited substantially decreased IRI activity compared to their corresponding azido derivatives. While it is difficult to establish whether reducing

the azide to an amine results in increased IRI activity due to the small number of compounds examined and the absence of any well-defined trend, this study demonstrated that some amino aryl glucosides can be effective inhibitors of ice recrystallization. In addition, high aqueous solubility is one of the desired properties for an ice recrystallization inhibitor for product development as a novel CPA. In this study, the majority of the amino aryl glucosides were considerably more soluble in aqueous solution (at least a two-fold increase) than their azido counterparts. Consequently, IRI active amino aryl glucosides are desirable additives for the cryopreservation of cells and should be assessed for cytotoxic effects in HepG2 cells in the future prior to their utilization as potential CPAs.

4.4 Chapter Summary

Previous studies have shown that aryl glucosides β -PMP-Glc and β -pBrPh-Glc are effective inhibitors of ice recrystallization with the ability to protect human RBCs against freezing injury. Thus, structure-function analyses of β -PMP-Glc and β -pBrPh-Glc were performed to elucidate key structural features that further enhance their IRI activity. In particular, since the azide is an appealing moiety owing to its bioorthogonal and biocompatible nature, we sought to explore whether chemically modifying β -PMP-Glc and β -pBrPh-Glc with an azide functional group had an influence on IRI activity. A class of azido aryl glucosides was synthesized whereby an azide substituent was incorporated at various positions (C-6, C-4, C-3 and C-2) on the pyranose ring as well as the *para*-position of the aryl ring to determine the relationship between the position of the azide and IRI activity. The majority of the azido aryl glucosides were ineffective inhibitors of ice recrystallization, with the exception of 3-azido aryl glucosides **405** (R = OCH₃) and **406** (R = Br) and *p*-azidophenyl- β -D-glucoside (**409**). By performing kinetic analyses, these molecules were shown to be effective inhibitors of ice recrystallization with IC₅₀ values of 6 mM, 0.7 mM and 26 mM, respectively. A comparative analysis demonstrated that the incorporation of an azido group at C-3 increased the IRI activity of β -PMP-Glc and β -pBrPh-Glc. *In vitro* cytotoxicity screening in HepG2 cells using the MTT assay revealed that **405**, **406** and **409** caused significant loss of cell viability after incubation for 18 h at 37 °C, displaying IC₅₀ values of 9 mM, 3 mM and 12 mM, respectively. To

understand if compounds **405**, **406** and **409** were causing cell death by initiating apoptosis, a caspase-3/7 activation assay was conducted and it was demonstrated that executioner caspases-3 and -7 were not activated upon exposure to **405**, **406** or **409**, and thus an apoptotic process was not being initiated.

Since some of the azido aryl glucosides were effective inhibitors of ice recrystallization, we also sought to investigate whether reducing the azide to an amine could increase IRI activity. For this study, *p*-aminophenyl- β -D-glucoside (**441**), 6-amino aryl glucosides **442** and **443**, and 3-amino aryl glucosides **444** and **445** were prepared and assessed for IRI activity. Amino aryl glucosides **441-443** were shown to be effective inhibitors of ice recrystallization with IC₅₀ values of 39 mM, 40 mM and 16 mM, respectively. With the exception of **441**, all of the compounds were prepared as salts (hydrochloride salt or formate salt). While the counterion effects were not thoroughly investigated in this study due to time restraints, the nature and effect of the negative counterion for amino aryl glucosides should be examined in the future. In comparison to their azido derivatives, it was difficult to establish whether reducing the azide to an amine resulted in increased IRI activity due to the small number of compounds examined and the absence of any well-defined trend. Nonetheless, this study demonstrated that some amino aryl glucosides can be effective inhibitors of ice recrystallization and their high aqueous solubility is a desirable property for an effective CPA. Thus, IRI active amino aryl glucosides should be assessed for cytotoxic effects in HepG2 cells in the future prior to assessing their cryopreservation potential.

4.5 Experimental Procedures and Characterization Data

4.5.1 Quantitative Analysis of Ice Recrystallization Inhibition (IRI) Activity

To quantify IRI activity, a modified “splat cooling” method was used.⁸ The sample was dissolved in a phosphate buffered saline (PBS) solution at various concentrations. A 10 μ L droplet of the solution was dropped through a two-meter high plastic tube onto a polished aluminum block cooled to approximately -80 °C. The wafer was separated from the surface of the block and transferred to a cryostage, kept at -6.4 °C, to anneal for 5 minutes and then photographed using a digital camera fitted to the microscope. Using ImageJ, ice crystals with well-defined boundaries within the image were circled and the

area of each crystal was calculated. Using Excel, the ice crystal areas were sorted into discrete bins based on size (bin size increases in increments of 0.001 mm²) and the proportionate area of each bin was calculated by adding the area of each crystal within a bin and dividing by the sum of the areas of all crystals in the image. For each inhibitor concentration, rate constants were determined and normalized to the positive PBS control for ice recrystallization. Using GraphPad, a dose-response curve was generated by plotting the normalized rate constants for each concentration as a function of the corresponding log values of the concentration. To obtain the half maximal inhibitory concentration (IC₅₀), a two-parameter sigmoidal curve was fit to the data. Each compound concentration was performed in triplicate.

4.5.2 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% penicillin-streptomycin, 1% non-essential amino acids and 1 mM sodium pyruvate in 75 cm² Corning® flasks. Cells were incubated in a 37 °C incubator supplied with 5% CO₂ and cultures were maintained by changing media every second day until 70-80% confluent. Once confluent, the cells were removed from the flasks using 5 mL Accutase solution for use in experiments. Passages 8-11 were used in this study.

4.5.3 MTT Viability Assay with HepG2 Cells

The MTT assay was performed as described previously.⁴⁰⁻⁴² HepG2 cells were plated in 96-well plates (~40,000 cells/well) and incubated overnight at 37 °C with 5% CO₂ to allow cells to adhere. Cells were then treated with 100 µL of MEM supplemented with the test compound at various concentrations and incubated at 37 °C with 5% CO₂ for 18 h. 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 µL of fresh MEM and 50 µL of MTT solution (5 mg/mL) in HBSS (Hank's balanced salt solution) was added and the plates were incubated at 37 °C with 5% CO₂ for 4 h. The plates were then centrifuged, the media was removed and 200 µL of MTT solubilization solution (10% Triton X-100, 0.1 N HCl in isopropanol) was added to each well. The plates were incubated for 1-3 h at

room temperature in the dark and the absorbance of each well was read at a wavelength of 570 nm with a multi-well plate reader (AD 340C Absorbance Detector, Beckman Coulter, Inc., Mississauga, ON). Each experiment was repeated at least three times in 10 consecutive wells for each condition. Cell viability was calculated as a percentage of the control. Using GraphPad, a dose-response curve was generated by plotting the average percent cell viability for each concentration as a function of the corresponding log values of the concentration. To obtain the IC₅₀ value (concentration required to reduce cell viability by 50%), a two-parameter sigmoidal curve was fit to the data.

4.5.4 Apo-ONE® Homogeneous Caspase-3/7 Assay with HepG2 Cells

HepG2 cells were plated in black 96-well plates (~40,000 cells/well) and incubated overnight at 37 °C with 5% CO₂ to allow cells to adhere. Cells were then treated with 100 µL of MEM supplemented with the test compound containing 1% DMSO and incubated at 37 °C with 5% CO₂ for 18 h. Cells incubated with MEM without supplement containing 1% DMSO were used as a negative control and cells supplemented with staurosporine (20 µM) were used as a positive control. Following incubation, the substrate/buffer solution was prepared according to the procedure supplied by Promega® and 100 µL was added to each well containing cells for a final volume of 200 µL. The plates were covered, placed on a shaker to gently mix the contents of the wells and incubated at room temperature. The fluorescence of each well was measured at 4, 6, 10, 24 and 48 hours using a fluorescence plate reader with an excitation at 499 nm (emission at 521 nm).⁴² Each experiment was repeated at least two times in 6 consecutive wells for each condition.

4.5.5 General Experimental

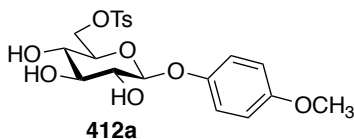
All anhydrous reactions were performed in flame-dried glassware under a positive pressure of dry argon. Air or moisture-sensitive reagents and anhydrous solvents were transferred with oven-dried syringes or cannulae. All flash chromatography was performed with E. Merck silica gel 60 (230-400 mesh). All solution phase reactions were monitored using analytical thin layer chromatography (TLC) with 0.2 mm pre-coated silica gel aluminum plates 60 F254 (E. Merck). Components were visualized by illumination with a short-wavelength (254 nm) ultra-violet light and/or staining (ceric

ammonium molybdate, potassium permanganate, or phosphomolybdate stain solution). All solvents used for anhydrous reactions were distilled. Tetrahydrofuran (THF) was distilled from sodium/benzophenone under nitrogen. Dichloromethane (DCM) was distilled from calcium hydride. *N,N*-dimethylformamide (DMF) was stored over activated 4Å molecular sieves under argon. ^1H (300 or 400 MHz) and ^{13}C (100 or 125 MHz) spectra were recorded at ambient temperature on a Bruker Avance 300 or Bruker Avance 400. Deuterated chloroform (CDCl_3) or water (D_2O) were used as NMR solvents, unless otherwise stated. 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).

Aryl glycosides **411a/b**, **103** and **104** were previously reported by our laboratory.⁷ NMR spectra for novel compounds and final compounds assessed for IRI activity are provided below.

4.5.6 Compound Characterization

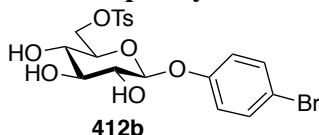
4-Methoxyphenyl-6-*O*-toluenesulfonyl- β -D-glucopyranoside (**412a**)



4-Methoxyphenyl- β -D-glucopyranoside (1.0 g, 3.5 mmol) was added to a flame-dried flask under a constant flow of argon and dissolved in anhydrous pyridine (15 mL). The reaction mixture was stirred at 0 °C for 20 minutes and 4-toluenesulfonylchloride (1.3 g, 7.0 mmol) was added to the solution. The reaction mixture was stirred overnight, diluted with CH_2Cl_2 and extracted with saturated aqueous sodium bicarbonate (2x). The organic layer was dried over MgSO_4 , filtered and concentrated under reduced pressure. Column chromatography (98:2 CH_2Cl_2 /methanol) afforded **412a** as a white powder (0.79 g, 51% yield). ^1H NMR (300 MHz; $\text{DMSO}-d_6$): δ 7.75-7.71 (m, 2H), 7.38 (m, 2H), 6.94-6.89 (m, 2H), 6.86-6.82 (m, 2H), 5.37 (d, J = 4.9 Hz, 1H), 5.32 (d, J = 5.4 Hz, 1H), 5.19 (d, J = 4.8 Hz, 1H), 4.72 (d, J = 7.5 Hz, 1H), 4.24 (dd, J = 10.6, 1.7 Hz, 1H), 4.05 (dd, J = 10.6, 6.4

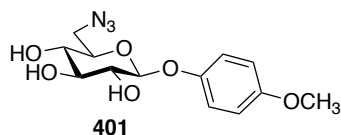
Hz, 1H), 3.72 (s, 3H), 3.55 (ddd, $J = 9.6, 6.4, 1.9$ Hz, 1H), 3.26-3.04 (m, 3H), 2.38 (s, 3H). ^{13}C NMR (101 MHz; DMSO- d_6): δ 154.5, 151.2, 144.9, 132.2, 130.1, 127.6, 117.8, 114.4, 101.2, 76.2, 73.26, 73.11, 69.9, 69.3, 55.4, 21.1. LRMS (ESI): m/z calcd. for $\text{C}_{20}\text{H}_{24}\text{NaO}_9\text{S}$ $[\text{M}+\text{Na}]^+$ 463.1; found, 463.3.

4-Bromophenyl-6-*O*-toluenesulfonyl- β -D-glucopyranoside (**412b**)



4-Bromophenyl- β -D-glucopyranoside (0.5 g, 1.5 mmol) was added to a flame-dried flask under a constant flow of argon and dissolved in anhydrous pyridine (8 mL). The reaction mixture was stirred at 0 °C for 20 minutes and 4-toluenesulfonylchloride (570 mg, 3.0 mmol) was added to the solution. The reaction mixture was stirred overnight, diluted with CH_2Cl_2 and extracted with saturated aqueous sodium bicarbonate (2x). The organic layer was dried over MgSO_4 , filtered and concentrated under reduced pressure. Column chromatography (9.5:0.5 CH_2Cl_2 /methanol) afforded **412b** as a white powder (0.36 g, 49% yield). ^1H NMR (400 MHz; DMSO- d_6): δ 7.71-7.69 (m, 2H), 7.45-7.41 (m, 2H), 7.34-7.32 (m, 2H), 6.94-6.91 (m, 2H), 5.43 (d, $J = 5.0$ Hz, 1H), 5.35 (d, $J = 5.4$ Hz, 1H), 5.23 (d, $J = 4.8$ Hz, 1H), 4.89 (d, $J = 7.5$ Hz, 1H), 4.24 (dd, $J = 10.5, 1.3$ Hz, 1H), 4.06 (dd, $J = 10.7, 6.3$ Hz, 1H), 3.65-3.61 (m, 1H), 3.28-3.07 (m, 3H), 2.36 (s, 3H). ^{13}C NMR (101 MHz; DMSO- d_6): δ 156.3, 144.8, 132.1, 132.0, 130.0, 127.5, 118.5, 113.5, 99.8, 76.0, 73.2, 72.9, 69.7, 69.1, 21.1. LRMS (ESI): m/z calcd. for $\text{C}_{19}\text{H}_{21}\text{BrNaO}_8\text{S}$ $[\text{M}+\text{Na}]^+$ 511.0; found, 511.2.

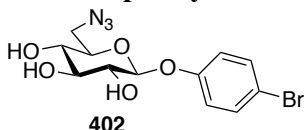
4-Methoxyphenyl-6-azido-6-deoxy- β -D-glucopyranoside (**401**)



Compound **412a** (0.48 g, 1.1 mmol) was added to a flame-dried flask under a constant flow of argon and dissolved in anhydrous DMF (30 mL). Sodium azide (0.24 g, 3.7 mmol) was added and the reaction mixture was stirred at 130 °C overnight. The resulting solution was concentrated under reduced pressure and column chromatography (9.5:0.5 CH_2Cl_2 /methanol) afforded **401** as a white powder (315 mg 93% yield). ^1H NMR (300

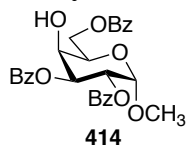
MHz; D₂O): δ 7.13-7.08 (m, 2H), 7.00-6.94 (m, 2H), 5.03 (d, J = 7.6 Hz, 1H), 3.79 (s, 3H), 3.74-3.67 (m, 1H), 3.63 (m, 1H), 3.57-3.43 (m, 4H). ¹³C NMR (101 MHz; D₂O): δ 154.8, 150.7, 118.3, 115.0, 101.1, 75.3, 74.8, 72.9, 70.3, 55.8, 50.9. LRMS (ESI): m/z calcd. for C₁₃H₁₇N₃NaO₆ [M+Na]⁺ 334.1; found, 334.0.

4-Bromophenyl-6-azido-6-deoxy- β -D-glucopyranoside (**402**)



Compound **412b** (0.3 g, 0.61 mmol) was added to a flame-dried flask under a constant flow of argon and dissolved in anhydrous DMF (20 mL). Sodium azide (0.14 g, 2.1 mmol) was added and the reaction mixture was stirred at 130 °C overnight. The resulting solution was concentrated under reduced pressure and column chromatography (9.5:0.5 CH₂Cl₂/methanol) afforded **402** as a white powder (0.15 g 68% yield). ¹H NMR (400 MHz; D₂O): δ 7.55-7.51 (m, 2H), 7.09-7.05 (m, 2H), 5.14 (d, J = 7.6 Hz, 1H), 3.78 (ddd, J = 9.5, 6.8, 2.6 Hz, 1H), 3.68 (dd, J = 13.6, 2.5 Hz, 1H), 3.63-3.46 (m, 4H). ¹³C NMR (101 MHz; D₂O): δ 155.5, 132.6, 118.5, 115.1, 100.0, 75.2, 74.9, 72.8, 70.3, 50.9. LRMS (ESI): m/z calcd. for C₁₂H₁₄BrN₃NaO₅ [M+Na]⁺ 382.0; found, 382.1.

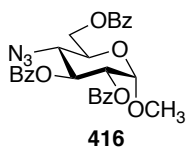
Methyl 2,3,6-tri-*O*-benzoyl- α -D-galactopyranoside (**414**)



Methyl- α -D-galactopyranoside (2.0 g, 10.3 mmol) was added to a flame-dried flask under a constant flow of argon and dissolved in anhydrous pyridine (40 mL). The solution was cooled to 0 °C and benzoyl chloride (4.2 mL, 36.1 mmol) was added dropwise. The solution was allowed to warm to room temperature slowly and stirred overnight, then diluted with CH₂Cl₂, washed with 10% aqueous HCl, saturated aqueous sodium bicarbonate, H₂O, dried over MgSO₄, filtered and concentrated under reduced pressure. Column chromatography (7:3 hexanes/ethyl acetate) afforded **414** as a white powder (1.7 g, 33% yield). ¹H NMR (300 MHz; CDCl₃): δ 8.08-8.04 (m, 2H), 8.02-7.97 (m, 4H), 7.61-7.34 (m, 9H), 5.76 (dd, J = 10.7, 3.0 Hz, 1H), 5.68 (dd, J = 10.7, 3.5 Hz, 1H), 5.22 (d, J = 3.5 Hz, 1H), 4.69 (dd, J = 11.4, 6.0 Hz, 1H), 4.56 (dd, J = 11.4, 6.7 Hz, 1H), 4.40-

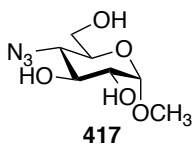
4.33 (m, 2H), 3.45 (s, 3H). ^{13}C NMR (101 MHz; CDCl_3): δ 166.6, 166.2, 165.9, 133.56, 133.43, 133.40, 129.99, 129.93, 129.86, 129.78, 129.51, 129.43, 128.62, 128.53, 97.7, 70.9, 69.0, 68.3, 67.8, 63.4, 55.7. LRMS (ESI): m/z calcd. for $\text{C}_{28}\text{H}_{26}\text{NaO}_9$ $[\text{M}+\text{Na}]^+$ 529.1; found, 529.1.

Methyl 4-azido-4-deoxy-2,3,6-tri-*O*-benzoyl- α -D-glucopyranoside (416)



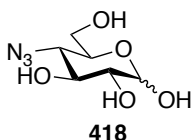
Compound **414** (1.47 g, 2.9 mmol) was added to a flame-dried flask under a constant flow of argon and dissolved in anhydrous CH_2Cl_2 (20 mL). Anhydrous pyridine (5.8 mL, 72.6 mmol) was added and the solution was cooled to 0 °C. Triflic anhydride (4.8 mL, 28.4 mmol) was added slowly and the solution was stirred at 0 °C for 30 minutes, then at room temperature for 45 minutes. The reaction mixture was quenched with methanol, washed with H_2O (2x), dried over MgSO_4 , filtered and concentrated under reduced pressure to afford a crude triflate **415**, which was dissolved in anhydrous DMF (100 mL). Sodium azide (0.97 g, 14.9 mmol) was added and the solution was stirred at 50 °C overnight. The solution was allowed to cool to room temperature and concentrated under reduced pressure. The resulting residue was partitioned between CH_2Cl_2 and H_2O , dried over MgSO_4 , filtered and concentrated under reduced pressure to afford **416** as a yellow oil (1.23 g, 80% yield over two steps). Characterization data is consistent with that previously reported.^{58,59} ^1H NMR (400 MHz; CDCl_3): δ 8.13-8.10 (m, 2H), 8.02-7.96 (m, 4H), 7.64-7.60 (m, 1H), 7.54-7.48 (m, 4H), 7.41-7.36 (m, 4H), 6.01 (t, J = 9.8 Hz, 1H), 5.22-5.17 (m, 2H), 4.71 (dd, J = 12.2, 2.3 Hz, 1H), 4.64 (dd, J = 12.2, 4.6 Hz, 1H), 4.07 (ddd, J = 10.5, 4.5, 2.3 Hz, 1H), 3.87 (t, J = 10.1 Hz, 1H), 3.44 (s, 3H). ^{13}C NMR (101 MHz; CDCl_3): δ 166.3, 166.0, 165.7, 133.59, 133.56, 133.47, 130.08, 129.92, 129.87, 129.79, 129.20, 129.03, 128.68, 128.59, 128.58, 97.3, 72.0, 71.2, 68.1, 63.5, 61.2, 55.8. LRMS (ESI): m/z calcd. for $\text{C}_{28}\text{H}_{25}\text{N}_3\text{NaO}_8$ $[\text{M}+\text{Na}]^+$ 554.2; found, 554.0.

Methyl 4-azido-4-deoxy- α -D-glucopyranoside (417)



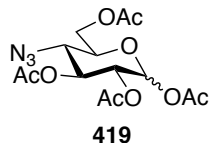
Compound **416** (1.23 g, 2.3 mmol) was dissolved in a solution of sodium methoxide in methanol and stirred for two hours at room temperature. The solution was then neutralized with Amberlite® IR-120 (H⁺) ion-exchange resin, filtered and concentrated under reduced pressure to afford **417** as a white powder (0.4 g, 80% yield). ¹H NMR (300 MHz; D₂O): δ 4.83 (d, J = 3.8 Hz, 1H), 3.89-3.76 (m, 3H), 3.65-3.58 (m, 2H), 3.42 (dd, J = 10.5, 9.6 Hz, 1H), 3.40 (s, 3H). ¹³C NMR (101 MHz; D₂O): δ 99.3, 72.2, 71.1, 70.1, 61.7, 60.7, 55.1. LRMS (ESI): *m/z* calcd. for C₇H₁₃N₃NaO₅ [M+Na]⁺ 242.1; found, 242.0.

4-Azido-4-deoxy-D-glucopyranoside (**418**)



Compound **417** (0.29 g, 1.3 mmol) was suspended in H₂O (10 mL) and Amberlite® IR-120 (H⁺) ion-exchange resin (4.0 g) was added. The reaction mixture was refluxed overnight and the solution was allowed to cool to room temperature, filtered to remove the resin and concentrated under reduced pressure. Column chromatography (8.5: 1.5 CH₂Cl₂/methanol) afforded **418** as a mixture of anomers (0.14 g, 52% yield). ¹H NMR (400 MHz; D₂O): δ 5.24 (d, J = 3.7 Hz, 0.6 H), 4.61 (d, J = 8.0 Hz, 1H), 3.90-3.73 (m, 5H), 3.65-3.57 (m, 2H), 3.47-3.40 (m, 3H), 3.29 (dd, J = 9.4, 8.0 Hz, 1H). ¹³C NMR (101 MHz; D₂O): δ 95.9, 92.2, 75.1, 74.4, 74.2, 71.9, 71.4, 70.0, 61.88, 61.76, 60.93, 60.82. LRMS (ESI): *m/z* calcd. for C₆H₁₁N₃NaO₅ [M+Na]⁺ 228.1; found, 228.1.

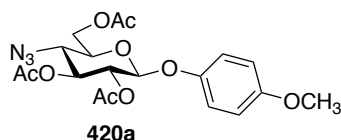
1,2,3,6-Tetra-*O*-acetyl-4-azido-4-deoxy-D-glucopyranoside (**419**)



Compound **418** (0.15 g, 0.7 mmol) and sodium acetate (0.16 g, 1.9 mmol) were suspended in acetic anhydride (0.46 g, 4.5 mmol) and the reaction mixture was refluxed at 100 °C for 2 hours. The solution was allowed to cool to room temperature and dissolved in CH₂Cl₂, extracted with H₂O (2x), dried over MgSO₄, filtered and concentrated under reduced pressure. Column chromatography (7:3 hexanes/ethyl acetate) afforded **419** as a mixture of anomers (0.22 g, 81% yield). ¹H NMR (400 MHz;

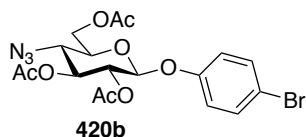
CDCl₃): δ 6.24 (d, J = 3.7 Hz, 1H), 5.65 (d, J = 8.3 Hz, 1H), 5.43 (t, J = 10.0 Hz, 1H), 5.19 (t, J = 9.6 Hz, 1H), 5.03-4.99 (m, 2H), 4.36-4.30 (m, 2H), 4.26-4.22 (m, J = 1.5 Hz, 2H), 3.84 (ddd, J = 10.6, 3.6, 2.4 Hz, 1H), 3.69-3.63 (m, 2H), 3.58 (ddd, J = 10.4, 4.2, 2.1 Hz, 1H), 2.13 (s, 2H), 2.09 (s, 3H), 2.08 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 1.99 (s, 3H), 1.98 (s, 2H). ¹³C NMR (101 MHz; CDCl₃): δ 170.47, 170.45, 169.86, 169.81, 169.6, 168.82, 168.79, 91.6, 89.2, 73.6, 72.9, 70.48, 70.43, 70.2, 69.3, 62.48, 62.41, 59.89, 59.71, 20.92, 20.81, 20.78, 20.77, 20.73, 20.65, 20.57, 20.49. LRMS (ESI): m/z calcd. for C₁₄H₁₉N₃NaO₉ [M+Na]⁺ 396.1; found, 396.0.

4-Methoxyphenyl-2,3,6-tri-*O*-acetyl-4-azido-4-deoxy- β -D-glucopyranoside (**420a**)



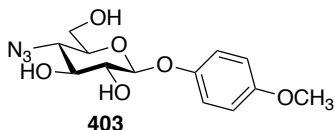
Compound **419** (107 mg, 0.29 mmol) was added to a flame-dried flask (with 4 Å MS) under a constant flow of argon and dissolved in anhydrous CH₂Cl₂ (5 mL). 4-Methoxyphenol (43 mg, 0.34 mmol) was added and the reaction mixture was stirred at 0 °C, slowly adding boron trifluoride diethyl etherate (0.32 mL, 2.6 mmol). The reaction mixture was stirred overnight, then diluted with CH₂Cl₂ and quenched with saturated aqueous sodium bicarbonate. The solution was filtered through Celite® and extracted with CH₂Cl₂. The organic layer was washed with saturated aqueous sodium bicarbonate, H₂O, saturated brine, then dried over MgSO₄, filtered and concentrated under reduced pressure. Column chromatography (8:2 hexanes/ethyl acetate) afforded **420a** as a clear oil (60 mg, 48% yield). ¹H NMR (400 MHz; CDCl₃): δ 6.94-6.89 (m, 2H), 6.82-6.78 (m, 2H), 5.25-5.12 (m, 2H), 4.91 (d, J = 7.8 Hz, 1H), 4.42 (dd, J = 12.2, 2.3 Hz, 1H), 4.28 (dd, J = 12.2, 4.9 Hz, 1H), 3.74-3.71 (m, 1H), 3.55 (ddd, J = 10.3, 4.9, 2.3 Hz, 1H), 2.12 (s, 3H), 2.10 (s, 3H), 2.07 (s, 3H). ¹³C NMR (101 MHz; CDCl₃): δ 170.5, 170.0, 169.7, 156.0, 150.9, 118.9, 114.7, 100.4, 73.8, 72.3, 71.6, 62.8, 60.1, 55.7, 20.84, 20.76, 20.74. LRMS (ESI): m/z calcd. for C₁₉H₂₃N₃NaO₉ [M+Na]⁺ 460.1; found, 460.1.

4-Bromophenyl-2,3,6-tri-*O*-acetyl-4-azido-4-deoxy- β -D-glucopyranoside (**420b**)



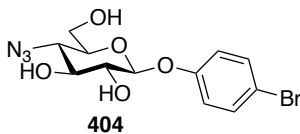
Compound **419** (97 mg, 0.26 mmol) was added to a flame-dried flask (with 4 Å MS) under a constant flow of argon and dissolved in anhydrous CH₂Cl₂ (5 mL). 4-Bromophenol (54 mg, 0.31 mmol) was added and the reaction mixture was stirred at 0 °C, slowly adding boron trifluoride diethyl etherate (0.29 mL, 2.3 mmol). The reaction mixture was stirred overnight, then diluted with CH₂Cl₂ and quenched with saturated aqueous sodium bicarbonate. The solution was filtered through Celite® and extracted with CH₂Cl₂. The organic layer was washed with saturated aqueous sodium bicarbonate, H₂O, saturated brine, then dried over MgSO₄, filtered and concentrated under reduced pressure. Column chromatography (8.5: 1.5 hexanes/ethyl acetate) afforded **420b** as a clear oil (51 mg, 40% yield). ¹H NMR (400 MHz; CDCl₃): δ 7.41-7.37 (m, 2H), 6.87-6.83 (m, 2H), 5.27-5.14 (m, 2H), 5.00 (d, J = 7.7 Hz, 1H), 4.43 (dd, J = 12.2, 2.3 Hz, 1H), 4.28 (dd, J = 12.2, 5.1 Hz, 1H), 3.74 (dd, J = 9.9, 9.9 Hz, 1H), 3.59 (ddd, J = 10.3, 5.1, 2.3 Hz, 1H), 2.13 (s, 3H), 2.10 (s, 3H), 2.06 (s, 3H). ¹³C NMR (101 MHz; CDCl₃): δ 170.5, 170.0, 169.7, 155.9, 132.7, 119.0, 116.2, 99.2, 73.7, 72.5, 71.4, 62.8, 60.0, 20.85, 20.79, 20.75. LRMS (ESI): *m/z* calcd. for C₁₈H₂₀BrN₃NaO₈ [M+Na]⁺ 508.0; found, 508.0.

4-Methoxyphenyl-4-azido-4-deoxy-β-D-glucopyranoside (**403**)



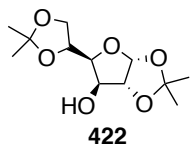
Compound **420a** (60 mg, 0.14 mmol) was dissolved in a solution of sodium methoxide in methanol and stirred for two hours at room temperature. The solution was neutralized with Amberlite® IR-120 (H⁺) ion-exchange resin, filtered and concentrated under reduced pressure to afford **403** as a white powder (35 mg, 81% yield). ¹H NMR (400 MHz; D₂O): δ 7.12-7.08 (m, 2H), 6.99-6.95 (m, 2H), 4.98 (d, J = 7.9 Hz, 1H), 3.90 (dd, J = 12.6, 1.7 Hz, 1H), 3.80 (s, 3H), 3.79-3.70 (m, 2H), 3.62-3.50 (m, 3H). ¹³C NMR (101 MHz; D₂O): δ 154.8, 150.8, 118.2, 115.0, 101.1, 74.9, 74.5, 72.9, 61.5, 60.7, 55.8. LRMS (ESI): *m/z* calcd. for C₁₃H₁₇N₃NaO₆ [M+Na]⁺ 334.1; found, 334.1.

4-Bromophenyl-4-azido-4-deoxy-β-D-glucopyranoside (**404**)



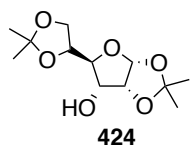
Compound **420b** (51 mg, 0.11 mmol) was dissolved in a solution of sodium methoxide in methanol and stirred for two hours at room temperature. The solution was neutralized with Amberlite® IR-120 (H⁺) ion-exchange resin, filtered and concentrated under reduced pressure. Column chromatography (9.5:0.5 CH₂Cl₂/methanol) afforded **404** as a white powder (33 mg, 87% yield). ¹H NMR (400 MHz; DMSO-*d*₆): δ 7.48-7.44 (m, 2H), 7.00-6.97 (m, 2H), 5.78 (d, *J* = 5.9 Hz, 1H), 5.61 (d, *J* = 5.2 Hz, 1H), 4.95 (d, *J* = 7.7 Hz, 1H), 4.87 (t, *J* = 5.8 Hz, 1H), 3.62-3.41 (m, 5H), 3.33-3.26 (m, 1H). ¹³C NMR (101 MHz; DMSO-*d*₆): δ 156.4, 132.1, 118.4, 113.4, 99.8, 75.2, 74.3, 73.2, 61.5, 60.3. LRMS (ESI): *m/z* calcd. for C₁₂H₁₄BrN₃NaO₅ [M+Na]⁺ 382.0; found, 382.0.

1,2:5,6-Di-*O*-isopropylidene- α -D-glucofuranose (**422**)



Compound was prepared as previously described.⁶⁰ All spectral data was consistent with that reported in the literature.⁶¹

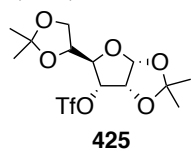
1,2:5,6-Di-*O*-isopropylidene- α -D-allofuranose (**424**)



A flame dried flask was charged with anhydrous CH₂Cl₂ (350 mL) and cooled to -78 °C. Freshly distilled oxalyl chloride (8.9 g, 69.9 mmol) was added followed by anhydrous DMSO (10.5 g, 134.5 mmol) and the mixture was allowed to stir for 10 minutes where compound **422** (14.0 g, 53.8 mmol, dissolved in anhydrous CH₂Cl₂) was cannulated in down the side of the flask. The cloudy white mixture was allowed to stir for 15 minutes before triethylamine (20.7 g, 204.4 mmol) was added. The mixture was allowed to stir for 20 minutes at -78 °C before being warmed to room temperature. The reaction mixture was quenched with saturated aqueous ammonium chloride and the organic phase was extracted with brine (3x), then dried over MgSO₄, filtered and concentrated under reduced pressure. The resulting mixture was resuspended in diethyl ether and filtered through Celite® to remove oxalic acid. The crude ketone **423** (11.4 g, 44.1 mmol) was

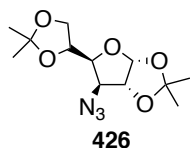
dissolved in a 9:1 mixture of ethanol: water. The solution was cooled to 0 °C in an ice bath and sodium borohydride (4.3 g, 114.8 mmol) was added. The resulting mixture was stirred at 0 °C for 1 hour. Saturated aqueous ammonium chloride was added to destroy unreacted borohydride and the resulting mixture was concentrated under reduced pressure. The residue was extracted with CH₂Cl₂ (3x) and the combined extracts were washed with H₂O (2x), dried over MgSO₄, filtered and concentrated under reduced pressure. Column chromatography (7:3 hexanes/ethyl acetate) afforded **424** as white crystals (3.3 g, 24% yield over two steps). ¹H NMR (300 MHz; CDCl₃): δ 5.81 (d, J = 3.8 Hz, 1H), 4.61 (dd, J = 5.1, 4.0 Hz, 1H), 4.31 (td, J = 6.5, 4.7 Hz, 1H), 4.11-3.99 (m, 3H), 3.81 (dd, J = 8.5, 4.7 Hz, 1H), 2.54 (d, J = 8.3 Hz, 1H), 1.58 (s, 3H), 1.46 (s, 3H), 1.38 (s, 3H), 1.37 (s, 3H). ¹³C NMR (101 MHz; CDCl₃): δ 113.0, 110.0, 104.1, 79.9, 79.1, 75.8, 72.7, 66.0, 26.7, 26.7, 26.5, 25.4. LRMS (ESI): *m/z* calcd. for C₁₂H₂₀O₆ [M+Na]⁺ 283.1; found, 284.2.

1,2:5,6-Di-*O*-isopropylidene-3-*O*-trifluoromethanesulphonyl- α -D-allofuranose (425**)**



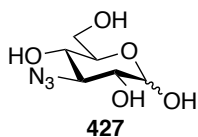
Compound **424** (3.3 g, 12.7 mmol) was added to a flame-dried flask under a constant flow of argon and dissolved in anhydrous CH₂Cl₂ (30 mL) containing pyridine (2 mL). The solution was cooled to -30 °C and freshly distilled triflic anhydride (4.3 g, 15.3 mmol) was added dropwise over 15 minutes. The solution was stirred at -30 °C for a further 30 minutes and quenched with methanol. The clear solution was allowed to warm to room temperature and washed with H₂O (2x), dried over MgSO₄, filtered and concentrated under reduced pressure to afford **425** as a light yellow oil (4.3 g, 87% yield). ¹H NMR (300 MHz; CDCl₃): δ 5.83 (d, J = 3.8 Hz, 1H), 4.90 (dd, J = 7.0, 5.3 Hz, 1H), 4.76 (dd, J = 5.2, 3.9 Hz, 1H), 4.23-4.08 (m, 3H), 3.92-3.88 (m, 1H), 1.58 (s, 3H), 1.44 (s, 3H), 1.38 (s, 3H), 1.34 (s, 3H). ¹³C NMR (76 MHz; CDCl₃): δ 120.6, 114.5, 110.4, 104.3, 83.0, 77.9, 77.8, 75.3, 66.4, 27.0, 26.6, 26.4, 24.9. LRMS (ESI): *m/z* calcd. for C₁₃H₁₉F₃O₈S [M+Na]⁺ 415.0; found, 414.2.

1,2:5,6-Di-*O*-isopropylidene-3-azido-3-deoxy- α -D-glucofuranose (426**)**



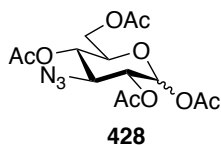
Compound **425** (4.3 g, 11.1 mmol) was added to a flame-dried flask under a constant flow of argon and dissolved in anhydrous DMF. Sodium azide (1.4 g, 22.1 mmol) was added and the mixture was stirred at 50 °C overnight. The solution was allowed to cool to room temperature and the solvent was evaporated under reduced pressure. The residue was partitioned between CHCl₃ and H₂O, dried over MgSO₄, filtered and concentrated under reduced pressure to afford **426** as a light yellow oil (2.8 g, 87% yield). Characterization data is consistent with that previously reported.⁶² ¹H NMR (300 MHz; CDCl₃): δ 5.78 (d, J = 3.6 Hz, 1H), 4.55 (d, J = 3.6 Hz, 1H), 4.21-4.14 (m, 1H), 4.09-4.00 (m, 3H), 3.90 (dd, J = 8.6, 4.8 Hz, 1H), 1.44 (s, 3H), 1.36 (s, 3H), 1.30 (s, 3H), 1.25 (s, 3H). ¹³C NMR (101 MHz; CDCl₃): δ 112.5, 109.7, 105.2, 83.6, 80.6, 73.2, 67.8, 66.5, 27.0, 26.8, 26.3, 25.3. LRMS (ESI): *m/z* calcd. for C₁₂H₁₉N₃O₅ [M+Na]⁺ 308.1; found, 308.2.

3-Azido-3-deoxy-D-glucopyranoside (**427**)



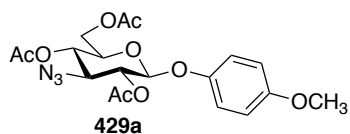
Compound **426** (2.7 g, 9.7 mmol) was dissolved in 80% TFA in H₂O and stirred at room temperature for 1 hour. The solvent was evaporated under reduced pressure and the crude product was dissolved in toluene (3x) and volatiles were evaporated to remove residual H₂O as an azeotrope. Column chromatography (9:1 CH₂Cl₂/MeOH) afforded **427** as a mixture of anomers (1.4 g, 73% yield). ¹H NMR (300 MHz; D₂O): δ 5.20 (d, J = 3.6 Hz, 1H), 4.68 (d, J = 7.9 Hz, 1H), 3.89-3.83 (m, 2H), 3.80-3.64 (m, 4H), 3.56-3.41 (m, 5H), 3.27-3.21 (m, 1H). ¹³C NMR (76 MHz; D₂O): δ 95.9, 91.5, 76.5, 72.8, 71.2, 70.3, 68.62, 68.50, 65.8, 60.57, 60.41. LRMS (ESI): *m/z* calcd. for C₆H₁₁N₃O₅ [M+Na]⁺ 228.1; found, 228.1.

1,2,4,6-Tetra-*O*-acetyl-3-azido-3-deoxy-D-glucopyranoside (**428**)



Compound **427** (1.4 g, 7.0 mmol) and sodium acetate (1.5 g, 18.3 mmol) were suspended in acetic anhydride (4.4 g, 42.8 mmol) and refluxed at 100 °C for 2 hours. The resulting mixture was dissolved in CH₂Cl₂ and extracted with H₂O (2x), dried over MgSO₄, filtered and concentrated under reduced pressure to afford **428** as a mixture of anomers (1.6 g, 62% yield). ¹H NMR (300 MHz; CDCl₃): δ 6.30 (d, J = 3.6 Hz, 0.5H), 5.67 (d, J = 8.3 Hz, 1H), 5.07-4.99 (m, 2H), 4.95 (dd, J = 10.7, 3.6 Hz, 0.5H), 4.23 (ddd, J = 12.6, 8.0, 4.6 Hz, 1H), 4.12-4.01 (m, 2H), 3.96 (t, J = 10.4 Hz, 0.5H), 3.78 (ddd, J = 9.9, 4.7, 2.3 Hz, 1H), 3.68 (t, J = 10.1 Hz, 1H), 2.18 (s, 1H), 2.13 (s, 1H), 2.13 (s, 3H), 2.12 (s, 3H), 2.11 (s, 3H), 2.09 (s, 1H), 2.09 (s, 1H), 2.08 (s, 3H). ¹³C NMR (101 MHz; CDCl₃): δ 170.7, 170.6, 169.4, 169.2, 169.1, 169.1, 169.0, 168.6, 91.9, 88.8, 73.6, 70.2, 70.1, 70.0, 67.9, 67.8, 64.3, 61.6, 61.5, 60.9, 20.9, 20.8, 20.7, 20.6, 20.6, 20.5. LRMS (ESI): *m/z* calcd. for C₁₄H₁₉N₃O₉ [M+Na]⁺ 396.1; found, 396.2.

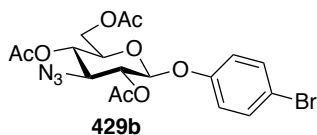
4-Methoxyphenyl-2,4,6-tri-*O*-acetyl-3-azido-β-D-glucopyranoside (**429a**)



Compound **428** (0.77 g, 2.1 mmol) was added to a flame-dried flask (with 4 Å MS) under a constant flow of argon and dissolved in anhydrous CH₂Cl₂ (20 mL). 4-Methoxyphenol (0.3 g, 2.5 mmol) was added and the reaction mixture was stirred at 0 °C, slowly adding boron trifluoride diethyl etherate (1.5 mL, 12.4 mmol). The reaction mixture was stirred overnight, then diluted with CH₂Cl₂ and quenched with saturated aqueous sodium bicarbonate. The solution was filtered through Celite® and extracted with CH₂Cl₂. The organic layer was washed with saturated aqueous sodium bicarbonate, H₂O, saturated brine, then dried over MgSO₄, filtered and concentrated under reduced pressure. Column chromatography (7:3 hexanes/ethyl acetate) afforded **429a** as a white powder (0.3 g, 37% yield). ¹H NMR (300 MHz; CDCl₃): δ 6.96-6.91 (m, 2H), 6.84-6.78 (m, 2H), 5.16 (dd, J = 10.2, 7.9 Hz, 1H), 5.06 (dd, J = 9.9, 9.9 Hz, 1H), 4.89 (d, J = 7.9 Hz, 1H), 4.26 (dd, J = 12.3, 5.3 Hz, 1H), 4.15 (dd, J = 12.3, 2.7 Hz, 1H), 3.77 (s, 3H), 3.79-3.73 (m, 1H), 3.69 (dd, J = 10.1, 10.1 Hz, 1H), 2.16 (s, 3H), 2.14 (s, 3H), 2.08 (s, 3H). ¹³C NMR (101 MHz; CDCl₃): δ 170.8, 169.3, 169.1, 156.0, 151.0, 118.8, 114.7, 100.7, 73.0, 71.2, 68.6, 64.4,

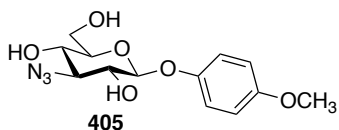
62.1, 55.8, 20.9, 20.8, 20.8. LRMS (ESI): m/z calcd. for $C_{19}H_{23}N_3O_9$ $[M+Na]^+$ 460.0; found, 460.3.

4-Bromophenyl-2,4,6-tri-*O*-acetyl-3-azido- β -D-glucopyranoside (**429b**)



Compound **428** (0.9 g, 2.5 mmol) was added to a flame-dried flask (with 4 Å MS) under a constant flow of argon and dissolved in anhydrous CH_2Cl_2 (20 mL). 4-Bromophenol (0.5 g, 3.0 mmol) was added and the reaction mixture was stirred at 0 °C, slowly adding boron trifluoride diethyl etherate (1.9 mL, 15.1 mmol). The reaction mixture was stirred overnight, then diluted with CH_2Cl_2 and quenched with saturated aqueous sodium bicarbonate. The solution was filtered through Celite® and extracted with CH_2Cl_2 . The organic layer was washed with saturated aqueous sodium bicarbonate, H_2O , saturated brine, then dried over $MgSO_4$, filtered and concentrated under reduced pressure. Column chromatography (7:3 hexanes/ethyl acetate) afforded **429b** as a white powder (0.3 g, 28% yield). 1H NMR (300 MHz; $CDCl_3$): δ 7.42-7.37 (m, 2H), 6.89-6.84 (m, 2H), 5.17 (dd, J = 10.1, 7.8 Hz, 1H), 5.05 (dd, J = 9.9, 9.9 Hz, 1H), 4.97 (d, J = 7.8 Hz, 1H), 4.24 (dd, J = 12.3, 5.5 Hz, 1H), 4.15 (dd, J = 12.3, 2.6 Hz, 1H), 3.80 (ddd, J = 9.9, 5.5, 2.6 Hz, 1H), 3.71 (dd, J = 10.0, 10.0 Hz, 1H), 2.14 (s, 6H), 2.08 (s, 3H). ^{13}C NMR (101 MHz; $CDCl_3$): δ 170.6, 169.3, 169.0, 156.0, 132.6, 118.9, 116.1, 99.4, 73.1, 71.0, 68.4, 64.2, 62.1, 20.8, 20.7, 20.7. LRMS (ESI): m/z calcd. for $C_{18}H_{20}BrN_3O_8$ $[M+Na]^+$ 508.0; found, 508.1.

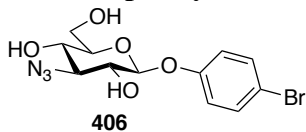
4-Methoxyphenyl-3-azido- β -D-glucopyranoside (**405**)



Compound **429a** (0.3 g, 0.8 mmol) was dissolved in a solution of sodium methoxide in methanol and stirred for two hours at room temperature. The solution was neutralized with Amberlite® IR-120 (H^+) ion-exchange resin, filtered and concentrated under reduced pressure to afford **405** as a white powder (0.2 g, 86% yield). 1H NMR (300 MHz; D_2O): δ 7.11-7.06 (m, 2H), 6.98-6.92 (m, 2H), 5.03 (d, J = 6.4 Hz, 1H), 3.88 (dd, J =

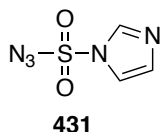
12.4, 1.9 Hz, 1H), 3.78 (s, 3H), 3.72 (dd, $J = 12.4, 5.2$ Hz, 1H), 3.65-3.49 (m, 4H). ^{13}C NMR (101 MHz; D_2O): δ 154.8, 150.8, 118.2, 115.0, 101.2, 76.6, 71.6, 68.4, 68.3, 60.3, 55.8. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{17}\text{N}_3\text{O}_6$ $[\text{M}+\text{Na}]^+$ 334.1; found, 334.2.

4-Bromophenyl-3-azido-3-deoxy- β -D-glucopyranoside (**406**)



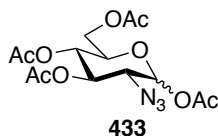
Compound **429b** (0.3 g, 0.7 mmol) was dissolved in a solution of sodium methoxide in methanol and stirred for two hours at room temperature. The solution was neutralized with Amberlite® IR-120 (H^+) ion-exchange resin, filtered and concentrated under reduced pressure to afford **406** as a white powder (0.2 g, 91% yield). ^1H NMR (400 MHz; $\text{DMSO}-d_6$): δ 7.49-7.45 (m, 2H), 7.01-6.99 (m, 2H), 5.96 (d, $J = 5.8$ Hz, 1H), 5.64 (d, $J = 6.4$ Hz, 1H), 4.99 (d, $J = 7.7$ Hz, 1H), 4.66 (t, $J = 5.6$ Hz, 1H), 3.68-3.64 (m, 1H), 3.50-3.40 (m, 3H), 3.31-3.21 (m, 2H). ^{13}C NMR (101 MHz; $\text{DMSO}-d_6$): δ 156.4, 132.2, 118.6, 113.5, 100.1, 77.5, 71.6, 69.7, 67.9, 60.2. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{14}\text{BrN}_3\text{O}_5$ $[\text{M}-\text{H}]^-$ 358.0; found, 358.0.

Imidazole-1-sulfonyl azide (**431**)



Compound was prepared as previously described.³⁴ All spectral data was consistent with that reported in the literature.³⁴

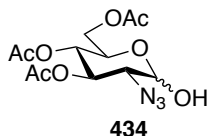
1,3,4,6-Tetra-*O*-acetyl-2-azido-2-deoxy-D-glucopyranoside (**433**)



D-Glucosamine hydrochloride (3.0 g, 13.9 mmol), potassium carbonate (2.9 g, 20.9 mmol) and copper (II) sulfate pentahydrate (35 mg, 0.14 mmol) were dissolved in methanol (75 mL). Imidazole-1-sulfonyl azide **431** (2.9 g, 16.7 mmol) was added and the mixture was stirred at room temperature for 2 hours. The mixture was concentrated under reduced pressure and co-evaporated with toluene (2x). The crude residue **432** was

dissolved in anhydrous pyridine (70 mL) and the solution was cooled to 0 °C in an ice bath. Acetic anhydride (70 mL) and a crystal of DMAP were added and the reaction mixture was stirred overnight. The solution was diluted with CH₂Cl₂, washed with 10% aqueous HCl (2x) and the combined aqueous layers were extracted with CH₂Cl₂ (3x). The combined organic layers were washed with saturated aqueous sodium bicarbonate, saturated brine, dried over MgSO₄, filtered and concentrated under reduced pressure. Column chromatography (7.5:2.5 hexanes/ethyl acetate) afforded **433** as a mixture of anomers (1.79 g, 34% yield over two steps). Characterization data is consistent with that previously reported.⁶³ ¹H NMR (400 MHz; CDCl₃): δ 6.27 (d, J = 3.6 Hz, 1H), 5.54 (d, J = 8.6 Hz, 1H), 5.43 (dd, J = 10.2, 9.7 Hz, 1H), 5.11-4.99 (m, 3H), 4.27 (dt, J = 12.5, 4.0 Hz, 2H), 4.04 (td, J = 12.4, 2.0 Hz, 2H), 3.79 (ddd, J = 9.6, 4.4, 2.1 Hz, 1H), 3.64 (td, J = 7.2, 3.0 Hz, 1H), 2.17 (s, 5H), 2.08 (s, 2H), 2.07 (s, 3H), 2.05 (s, 5H), 2.02 (s, 2H), 2.00 (s, 3H). ¹³C NMR (101 MHz; CDCl₃): δ 170.6, 170.1, 169.83, 169.68, 169.59, 168.62, 168.56, 92.6, 90.0, 72.78, 72.75, 70.8, 69.8, 67.95, 67.85, 62.6, 61.49, 61.47, 60.4, 20.99, 20.95, 20.76, 20.74, 20.69, 20.62. LRMS (ESI): *m/z* calcd. for C₁₄H₁₉N₃NaO₉ [M+Na]⁺ 396.1; found, 395.9.

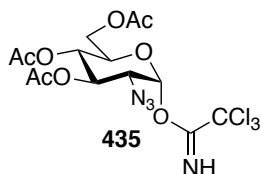
3,4,6-Tri-*O*-acetyl-2-azido-2-deoxy-D-glucopyranoside (**434**)



Compound **433** (1.64 g, 4.4 mmol) was added to a flame-dried flask under a constant flow of argon and dissolved in anhydrous THF (55 mL). Benzyl amine (0.72 mL, 6.6 mmol) was added and the reaction mixture was stirred at room temperature overnight. The solution was diluted with ice-cold H₂O and extracted with CH₂Cl₂ (3x). The organic layer was washed with ice-cold 10% aqueous HCl, saturated aqueous sodium bicarbonate, saturated brine, dried over MgSO₄, filtered and concentrated under reduced pressure. Column chromatography (6:4 hexanes/ethyl acetate) afforded **434** as a mixture of anomers (1.11 g, 76% yield). ¹H NMR (400 MHz; CDCl₃): δ 5.52 (dd, J = 10.5, 9.3 Hz, 1H), 5.39 (d, J = 3.4 Hz, 1H), 5.08-4.98 (m, 2H), 4.73 (d, J = 8.0 Hz, 1H), 4.29-4.20 (m, 3H), 4.15-4.08 (m, 2H), 3.73-3.69 (m, J = 2.3 Hz, 1H), 3.50-3.46 (m), 3.42 (dd, J = 10.5, 3.4 Hz, 1H), 2.09 (s, 2H), 2.08 (s, 5H), 2.04 (s, 3H), 2.02 (s, 1H). ¹³C NMR (101

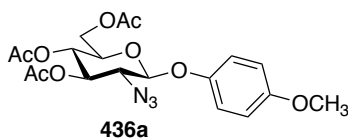
MHz; CDCl₃): δ 171.02, 170.98, 170.29, 170.23, 169.97, 169.88, 96.3, 92.2, 72.7, 72.1, 70.6, 68.7, 68.5, 67.7, 65.0, 62.1, 61.6, 20.87, 20.83, 20.8, 20.74, 20.7. LRMS (ESI): m/z calcd. for C₁₂H₁₇N₃NaO₈ [M+Na]⁺ 354.1; found, 354.0.

3,4,6-Tri-*O*-acetyl-2-azido-2-deoxy- α -D-glucosyl trichloroacetimidate (**435**)



Compound **434** (1.11 g, 3.4 mmol) was added to a flame-dried flask under a constant flow of argon, dissolved in anhydrous CH₂Cl₂ (60 mL) and cooled to 0 °C in an ice bath. Trichloroacetonitrile (3.4 mL, 33.5 mmol) and DBU (0.25 mL, 1.68 mmol) were added and the reaction mixture was stirred for 2 hours at 0 °C. The solution was concentrated under reduced pressure and column chromatography (8:2 hexanes/ethyl acetate) afforded **435** as a white solid (0.83 g, 52% yield). ¹H NMR (400 MHz; CDCl₃): δ 8.83 (s, 1H), 6.48 (d, J = 3.6 Hz, 1H), 5.51 (dd, J = 10.5, 9.4 Hz, 1H), 5.15 (dd, J = 10.2, 9.4 Hz, 1H), 4.27 (dd, J = 12.3, 4.3 Hz, 1H), 4.21 (ddd, J = 10.2, 4.3, 1.9 Hz, 1H), 4.09 (dd, J = 12.3, 2.1 Hz, 1H), 3.77 (dd, J = 10.5, 3.6 Hz, 1H), 2.11 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H). ¹³C NMR (101 MHz; CDCl₃): δ 170.6, 169.95, 169.77, 160.7, 94.2, 90.7, 70.8, 70.3, 68.1, 61.5, 60.8, 20.81, 20.78, 20.72. LRMS (ESI): m/z calcd. for C₁₄H₁₇Cl₃N₄NaO₈ [M+Na]⁺ 497.0; found, 496.9.

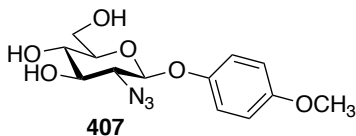
4-Methoxyphenyl-3,4,6-tri-*O*-acetyl-2-azido-2-deoxy- β -D-glucopyranoside (**436a**)



Compound **435** (0.32 g, 0.67 mmol) was added to a flame-dried flask (with 4 Å MS) under a constant flow of argon and dissolved in anhydrous CH₂Cl₂ (15 mL). 4-Methoxyphenol (0.1 g, 0.81 mmol) was added and the solution was cooled to 0 °C in an ice bath. Boron trifluoride diethyl etherate (0.17 mL, 1.34 mmol) was added dropwise and the reaction mixture was stirred for 2 hours. Triethylamine was added and the resulting mixture was filtered through Celite® and concentrated under reduced pressure.

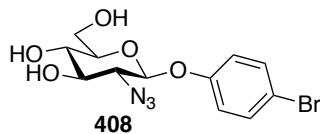
Column chromatography (9.5:0.5 toluene/acetone) afforded **436a** (0.12 g, 41% yield). ^1H NMR (400 MHz; CDCl_3): δ 7.05-7.01 (m, 2H), 6.86-6.82 (m, 2H), 5.10-5.02 (m, 2H), 4.81 (d, J = 8.1 Hz, 1H), 4.30 (dd, J = 12.3, 5.3 Hz, 1H), 4.14 (dd, J = 12.3, 2.4 Hz, 1H), 3.78 (s, 3H), 3.79-3.74 (m, 2H), 2.11 (s, 3H), 2.09 (s, 3H), 2.03 (s, 3H). ^{13}C NMR (101 MHz; CDCl_3): δ 170.7, 170.1, 169.7, 156.1, 150.9, 119.0, 114.8, 101.8, 72.5, 72.1, 68.5, 63.7, 62.1, 55.8, 20.84, 20.83, 20.72. LRMS (ESI): m/z calcd. for $\text{C}_{19}\text{H}_{23}\text{N}_3\text{NaO}_9$ $[\text{M}+\text{Na}]^+$ 460.1; found, 460.1.

4-Methoxyphenyl-2-azido-2-deoxy- β -D-glucopyranoside (**407**)



Compound **436a** (0.12 g, 0.27 mmol) was dissolved in a solution of sodium methoxide in methanol and stirred for two hours at room temperature. The solution was neutralized with Amberlite® IR-120 (H^+) ion-exchange resin, filtered and concentrated under reduced pressure. Column chromatography (9.5:0.5 CH_2Cl_2 /methanol) afforded **407** as a white powder (60 mg, 71% yield). ^1H NMR (400 MHz; D_2O): δ 7.13-7.09 (m, 2H), 6.98-6.94 (m, 2H), 5.03 (d, J = 7.7 Hz, 1H), 3.90 (dd, J = 12.3, 1.2 Hz, 1H), 3.79 (s, 3H), 3.75 (dd, J = 12.3, 4.5 Hz, 1H), 3.62-3.48 (m, 4H). ^{13}C NMR (101 MHz; D_2O): δ 154.9, 150.5, 118.3, 115.1, 100.5, 76.1, 74.1, 69.3, 65.5, 60.4, 55.8. LRMS (ESI): m/z calcd. for $\text{C}_{13}\text{H}_{17}\text{N}_3\text{NaO}_6$ $[\text{M}+\text{Na}]^+$ 334.1; found, 334.1.

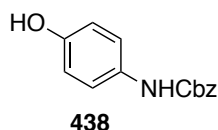
4-Bromophenyl-2-azido-2-deoxy- β -D-glucopyranoside (**408**)



Compound **435** (0.27 g, 0.57 mmol) was added to a flame-dried flask (with 4 Å MS) under a constant flow of argon and dissolved in anhydrous CH_2Cl_2 (15 mL). 4-Bromophenol (0.12 g, 0.68 mmol) was added and the solution was cooled to 0 °C in an ice bath. Boron trifluoride diethyl etherate (0.14 mL, 1.14 mmol) was added dropwise and the reaction mixture was stirred for 2 hours. Triethylamine was added and the resulting mixture was filtered through Celite® and concentrated under reduced pressure.

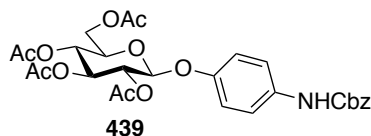
Column chromatography (8:2 hexanes/ethyl acetate) afforded **436b** containing an impurity which was removed in the next step by dissolving the crude compound in a solution of sodium methoxide in methanol and stirring for two hours at room temperature. The solution was neutralized with Amberlite® IR-120 (H⁺) ion-exchange resin, filtered and concentrated under reduced pressure. Column chromatography (9.5:0.5 CH₂Cl₂/methanol) afforded **408** as a white powder (95 mg, 47% yield over two steps). ¹H NMR (400 MHz; DMSO-*d*₆): δ 7.51-7.47 (m, 2H), 7.03-6.99 (m, 2H), 5.66 (d, *J* = 5.8 Hz, 1H), 5.30 (d, *J* = 5.5 Hz, 1H), 5.07 (d, *J* = 8.0 Hz, 1H), 4.65 (t, *J* = 5.8 Hz, 1H), 3.68 (ddd, *J* = 11.8, 5.6, 1.6 Hz, 1H), 3.50-3.34 (m, 4H), 3.25-3.19 (m, 1H). ¹³C NMR (101 MHz; DMSO-*d*₆): δ 155.9, 132.2, 118.5, 113.9, 98.3, 77.1, 74.6, 69.6, 66.0, 60.4. LRMS (ESI): *m/z* calcd. for C₁₂H₁₄BrN₃NaO₅ [M+Na]⁺ 382.0; found, 382.0.

4-(Benzyloxycarbonyl)aminophenol (**438**)



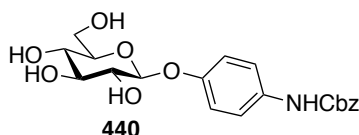
4-Aminophenol (7.0 g, 64.2 mmol) was dissolved in 150 mL of 10% aqueous AcOH and stirred for 5 minutes. A solution of benzyl chloroformate (16.4 g, 96.2 mmol) in 1,4-dioxane (140 mL) was added dropwise and the mixture was stirred at room temperature for 24 hours. The mixture was quenched with H₂O and extracted with CH₂Cl₂ (3x). The combined organic extracts were washed with saturated aqueous sodium bicarbonate, brine, dried over MgSO₄, filtered and concentrated under reduced pressure to afford **438** as white crystals (13.0 g, 83% yield). ¹H NMR (300 MHz; MeOD): δ 7.41-7.26 (m, 5H), 7.20 (d, *J* = 8.6 Hz, 2H), 6.73-6.68 (m, 2H), 5.14 (s, 2H). ¹³C NMR (101 MHz; MeOD): δ 156.33, 154.62, 138.21, 131.91, 129.47, 129.02, 128.88, 122.18, 116.27, 67.40. LRMS (ESI): *m/z* calcd. for C₁₄H₁₃NO₃ [M+Na]⁺ 266.1; found, 266.1.

4-(Benzyloxycarbonyl)aminophenyl-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**439**)



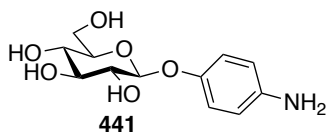
1,2,3,4,6-Penta-*O*-acetyl- β -D-glucopyranoside (10.0 g, 25.6 mmol) was added to a flame-dried flask (with 4 Å MS) under a constant flow of argon and dissolved in anhydrous CH₂Cl₂ (200 mL). Compound **438** (6.9 g, 28.2 mmol) was added and the reaction mixture was stirred at 0 °C, slowly adding boron trifluoride diethyl etherate (79 mL, 640.5 mmol). The reaction mixture was stirred overnight, then diluted with CH₂Cl₂ and quenched with saturated aqueous sodium bicarbonate. The solution was filtered through Celite® and extracted with CH₂Cl₂. The organic layer was washed with saturated aqueous sodium bicarbonate, H₂O, saturated brine, then dried over MgSO₄, filtered and concentrated under reduced pressure. Column chromatography (7:3 hexanes/ethyl acetate) afforded **439** as a white powder (8.1 g, 55% yield). Characterization data is consistent with that previously reported.⁷ ¹H NMR (300 MHz; CDCl₃): δ 7.42-7.33 (m, 5H), 7.30 (d, *J* = 8.9, 2H), 6.97-6.92 (m, 2H), 6.62 (br s, 1H), 5.32-5.21 (m, 2H), 5.19-5.12 (m, 3H), 5.00 (d, *J* = 7.7 Hz, 1H), 4.28 (dd, *J* = 12.3, 5.3 Hz, 1H), 4.16 (dd, *J* = 12.3, 2.4 Hz, 1H), 3.82 (ddd, *J* = 9.9, 5.3, 2.4 Hz, 1H), 2.08 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H). ¹³C NMR (101 MHz; CDCl₃): δ 170.7, 170.4, 169.5, 169.4, 153.6, 153.1, 136.1, 133.6, 128.7, 128.5, 128.4, 120.4, 118.0, 99.8, 72.8, 72.2, 71.3, 68.4, 67.2, 62.1, 20.8, 20.7, 20.7, 20.7. LRMS (ESI): *m/z* calcd. for C₂₈H₃₁NO₁₂ [M+Na]⁺ 596.2; found, 596.2.

4-(Benzyloxycarbonyl)aminophenyl- β -D-glucopyranoside (**440**)



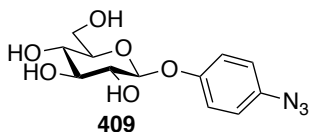
Compound **439** (8.0 g, 13.9 mmol) was dissolved in a solution of sodium methoxide in methanol and stirred for two hours at room temperature. The solution was neutralized with Amberlite® IR-120 (H⁺) ion-exchange resin, filtered and concentrated under reduced pressure to afford **440** as a white powder (5.2 g, 93% yield). Characterization data is consistent with that previously reported.⁷ ¹H NMR (300 MHz; MeOD): δ 7.42-7.27 (m, 7H), 7.07-7.02 (m, 2H), 5.16 (s, 2H), 4.83 (d, *J* = 7.5 Hz, 1H), 3.89 (dd, *J* = 11.9, 1.7 Hz, 1H), 3.69 (dd, *J* = 11.9, 5.1 Hz, 1H), 3.46-3.37 (m, 4H). ¹³C NMR (101 MHz; MeOD): δ 156.1, 155.1, 138.2, 129.5, 129.1, 128.9, 121.3, 118.3, 102.9, 78.1, 77.9, 74.9, 71.4, 67.5, 62.5. LRMS (ESI): *m/z* calcd. for C₂₀H₂₃NO₈ [M+Na]⁺ 428.1; found, 428.2.

4-Aminophenyl- β -D-glucopyranoside (**441**)



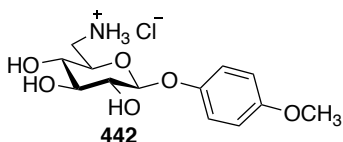
Compound **440** (5.2 g, 12.8 mmol) was dissolved in methanol and 20% palladium (II) hydroxide on carbon (0.9 g, 1.28 mmol) was added, the atmosphere was removed from the flask using a water aspirator and a positive pressure of hydrogen gas was added. The mixture was stirred overnight at room temperature, after which the mixture was filtered over a bed of Celite® and concentrated under reduced pressure to afford **441** as light brown crystals (2.5 g, 71% yield). Characterization data is consistent with that previously reported.⁷ ¹H NMR (300 MHz; D₂O): δ 7.02-6.97 (m, 2H), 6.84-6.79 (m, 2H), 4.95 (d, J = 7.5 Hz, 1H), 3.91 (dd, J = 12.4, 2.1 Hz, 1H), 3.74 (dd, J = 12.4, 5.3 Hz, 1H), 3.61-3.44 (m, 4H). ¹³C NMR (101 MHz; D₂O): δ 150.1, 141.7, 118.2, 117.5, 101.3, 76.0, 75.6, 73.0, 69.4, 60.5. LRMS (ESI): m/z calcd. for C₁₂H₁₇NO₆ [M+Na]⁺ 294.1; found, 294.1.

4-Azidophenyl- β -D-glucopyranoside (**409**)



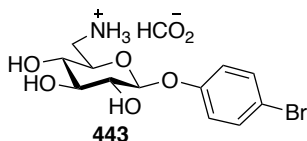
Compound **441** (2.5 g, 9.2 mmol) was added to a flame-dried flask and suspended in a mixture of anhydrous acetonitrile (50 mL) and tetrahydrofuran (50 mL). The solution was cooled to 0 °C in an ice bath. To this stirred mixture, *tert*-butyl nitrite (3.4 mL, 28.7 mmol) was added followed by azidotrimethylsilane (TMS-N₃) (3.0 mL, 23.0 mmol) dropwise. The mixture was stirred at room temperature and concentrated under reduced pressure. Column chromatography (9:1 CH₂Cl₂: MeOH) afforded **409** as brown crystals (2.1 g, 75% yield). ¹H NMR (300 MHz; D₂O): δ 7.16-7.11 (m, 2H), 7.09-7.04 (m, 2H), 5.06 (d, J = 7.4 Hz, 1H), 3.91 (dd, J = 12.4, 2.1 Hz, 1H), 3.73 (dd, J = 12.4, 5.6 Hz, 1H), 3.63-3.44 (m, 4H). ¹³C NMR (101 MHz; D₂O): δ 153.8, 134.6, 120.2, 118.1, 100.6, 76.1, 75.5, 72.9, 69.4, 60.5. LRMS (ESI): m/z calcd. for C₁₂H₁₅N₃O₆ [M+Na]⁺ 320.1; found, 320.1.

4-Methoxyphenyl-6-amino-6-deoxy- β -D-glucopyranoside hydrochloride (**442**)



Compound **401** (0.15 g, 0.48 mmol) was dissolved in methanol and 20% palladium (II) hydroxide on carbon (34 mg, 0.048 mmol) was added, the atmosphere was removed from the flask using a water aspirator and a positive pressure of hydrogen gas was added. The reaction mixture was stirred at room temperature until no starting material remained as per TLC. The mixture was filtered over a bed of Celite® and concentrated under reduced pressure. The crude product was dissolved in methanol and neutralized with 10% HCl, concentrated under reduced pressure and recrystallized by dissolving in a minimum amount of methanol and adding diethyl ether until a precipitate formed to afford **442** as a white powder (106 mg, 68% yield). ¹H NMR (400 MHz; D₂O): δ 7.15-7.11 (m, 2H), 7.03-6.99 (m, 2H), 5.09 (d, J = 7.5 Hz, 1H), 3.83 (s, 3H), 3.81-3.78 (m, 1H), 3.65-3.56 (m, 2H), 3.53-3.42 (m, 2H), 3.20 (dd, J = 13.5, 8.7 Hz, 1H). ¹³C NMR (101 MHz; D₂O): δ 154.9, 150.5, 118.3, 115.1, 101.1, 75.0, 72.7, 71.8, 71.1, 55.8, 40.3. LRMS (ESI): *m/z* calcd. for C₁₃H₂₀NO₆ [M+H]⁺ 286.1; found, 286.0.

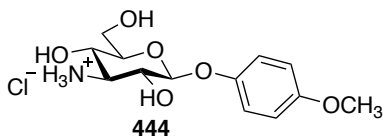
4-Bromophenyl-6-amino-6-deoxy-β-D-glucopyranoside hydroformate (**443**)



Compound **402** (0.1 g, 0.28 mmol) was added to a flame-dried flask under a constant flow of argon and dissolved in anhydrous methanol (2 mL). Ammonium formate (35 mg, 0.56 mmol) and zinc dust (36 mg, 0.56 mmol) was added and the reaction mixture was stirred at room temperature overnight and concentrated under reduced pressure. The residue was dissolved in water, filtered over a bed of Celite® and concentrated under reduced pressure. The crude product was recrystallized by dissolving in a minimum amount of methanol and adding diethyl ether until a precipitate formed to afford **443** as a white powder (59 mg, 56% yield). ¹H NMR (400 MHz; D₂O): δ 8.43 (s, 1H), 7.55-7.51 (m, 2H), 7.06-7.02 (m, 2H), 5.17-5.15 (m, 1H), 3.81 (ddd, J = 9.7, 8.7, 3.2 Hz, 1H), 3.64-3.56 (m, 2H), 3.48-3.41 (m, 2H), 3.14 (dd, J = 13.6, 8.7 Hz, 1H). ¹³C NMR (101 MHz;

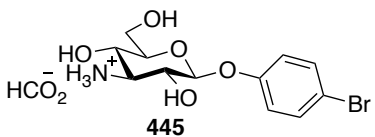
D₂O): δ 171.0, 155.5, 132.6, 118.5, 115.2, 100.0, 75.0, 72.6, 72.1, 71.0, 40.4. LRMS (ESI): m/z calcd. for C₁₂H₁₇BrNO₅ [M+H]⁺ 334.0; found, 334.1.

4-Methoxyphenyl-3-amino-3-deoxy- β -D-glucopyranoside hydrochloride (**444**)



Compound **405** (0.2 g, 0.64 mmol) was dissolved in methanol and 20% palladium (II) hydroxide on carbon (45 mg, 0.064 mmol) was added, the atmosphere was removed from the flask using a water aspirator and a positive pressure of hydrogen gas was added. The reaction mixture was stirred at room temperature until no starting material remained as per TLC. The mixture was filtered over a bed of Celite® and concentrated under reduced pressure. The crude product was dissolved in methanol and neutralized with 10% HCl, concentrated under reduced pressure and recrystallized by dissolving in a minimum amount of methanol and adding diethyl ether until a precipitate formed to afford **444** as a white powder (97 mg, 46% yield). ¹H NMR (400 MHz; D₂O): δ 7.15-7.11 (m, 2H), 7.01-6.97 (m, 2H), 5.08 (d, J = 7.7 Hz, 1H), 3.94 (dd, J = 12.5, 2.0 Hz, 1H), 3.82 (s, 3H), 3.81-3.66 (m, 4H), 3.29 (t, J = 10.0 Hz, 1H). ¹³C NMR (101 MHz; D₂O): δ 154.9, 150.8, 118.3, 115.1, 101.5, 76.9, 69.9, 66.2, 60.0, 57.6, 55.8. LRMS (ESI): m/z calcd. for C₁₃H₂₀NO₆ [M+H]⁺ 286.1; found, 285.9.

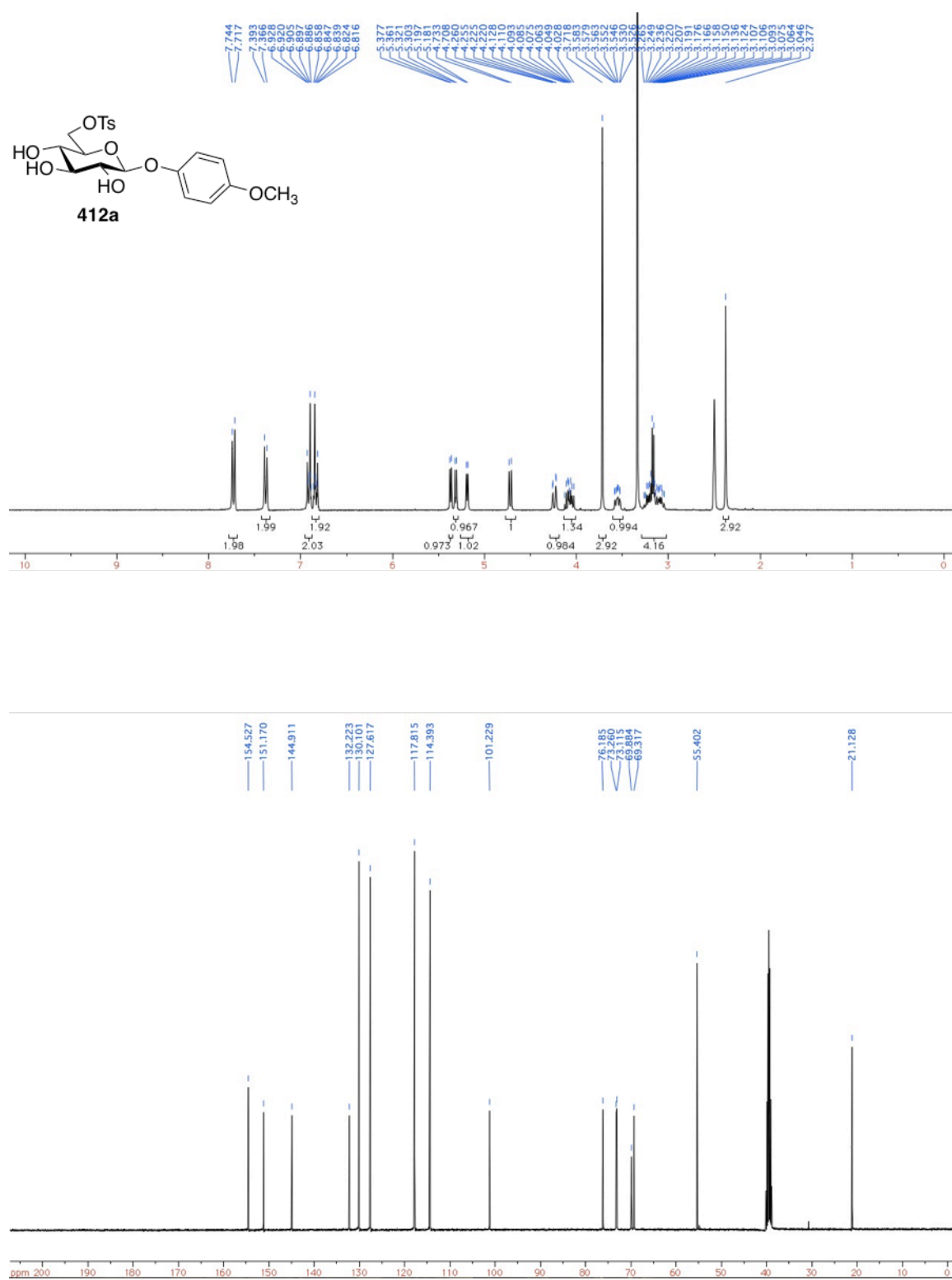
4-Bromophenyl-3-amino-3-deoxy- β -D-glucopyranoside hydroformate (**445**)

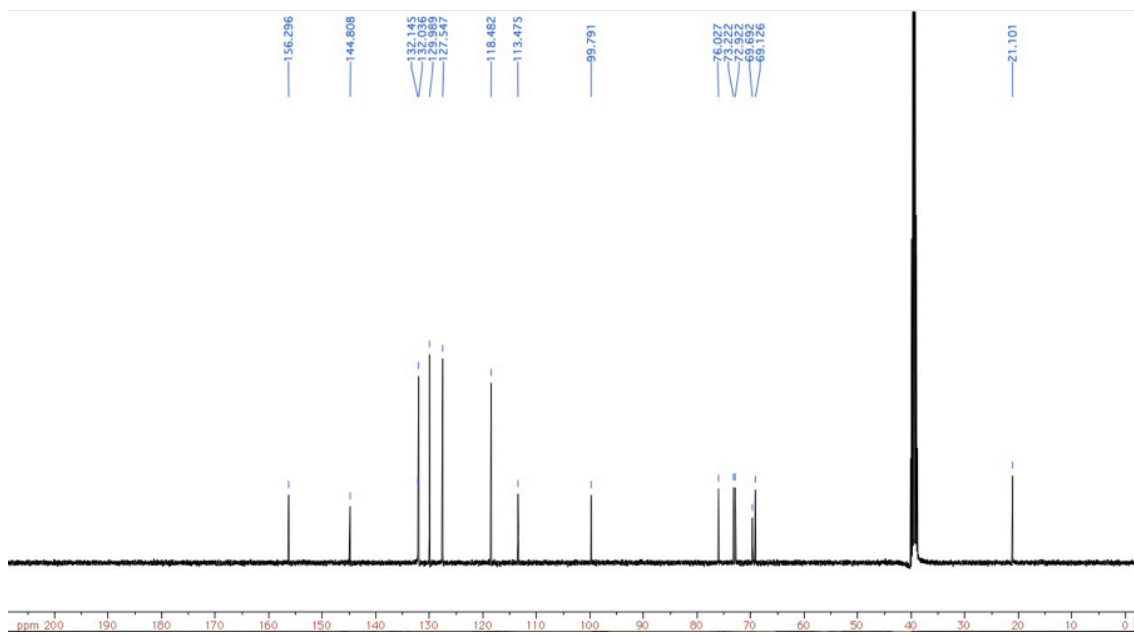
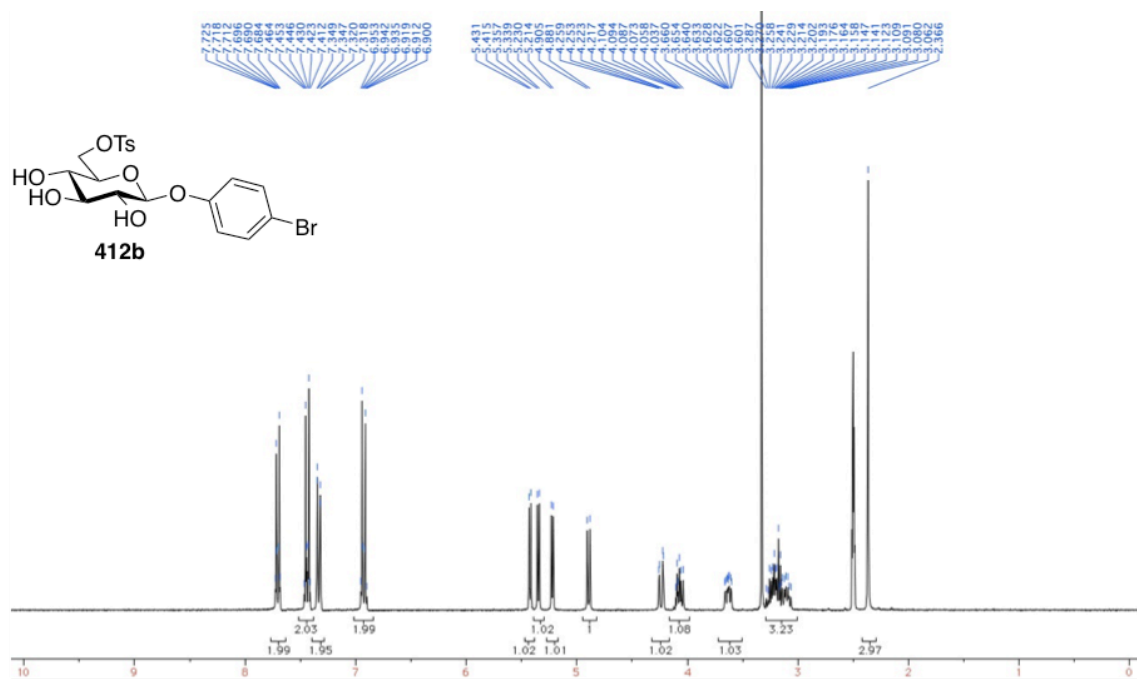


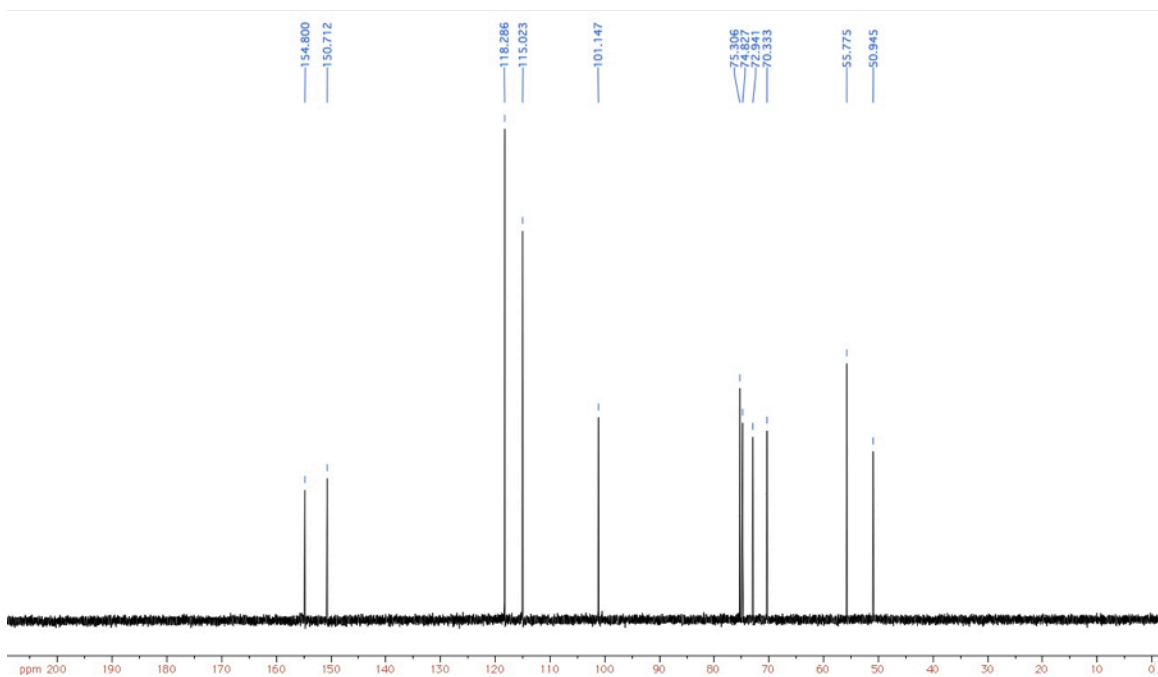
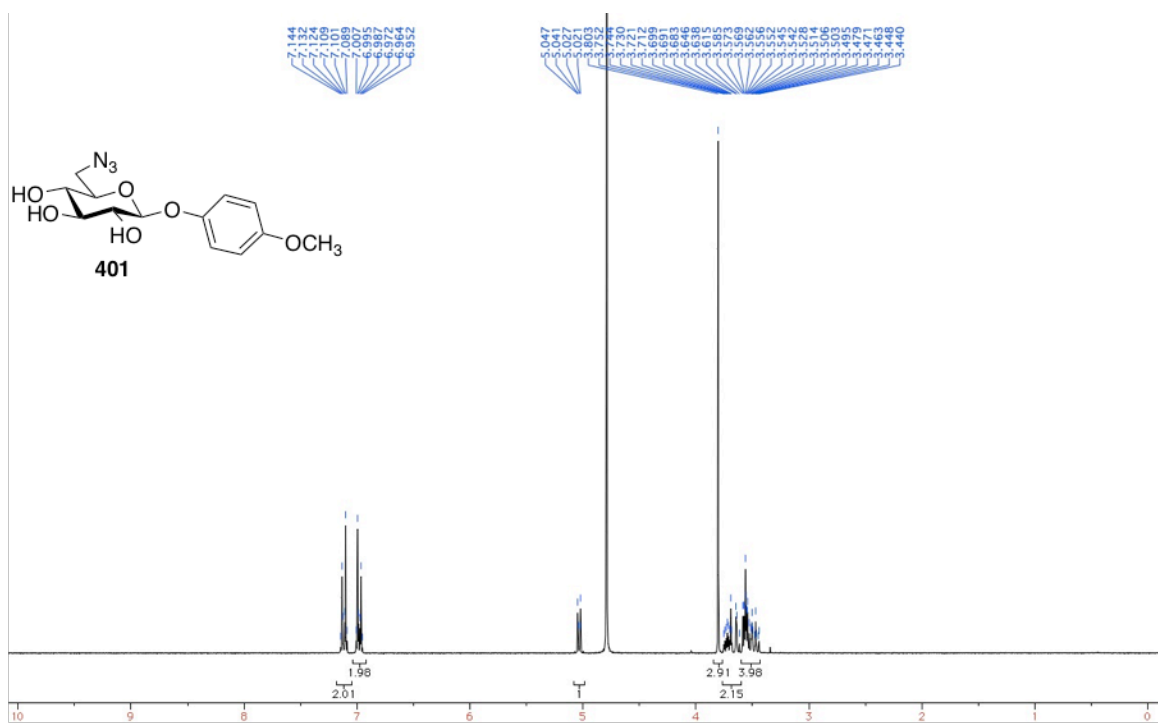
Compound **406** (0.15 g, 0.42 mmol) was added to a flame-dried flask under a constant flow of argon and dissolved in anhydrous methanol (3 mL). Ammonium formate (53 mg, 0.83 mmol) and zinc dust (54 mg, 0.83 mmol) was added and the reaction mixture was stirred at room temperature overnight and concentrated under reduced pressure. The residue was dissolved in water, filtered over a bed of Celite® and concentrated under reduced pressure. The crude product was recrystallized by dissolving in a minimum amount of methanol and adding diethyl ether until a precipitate formed to afford **445** as a

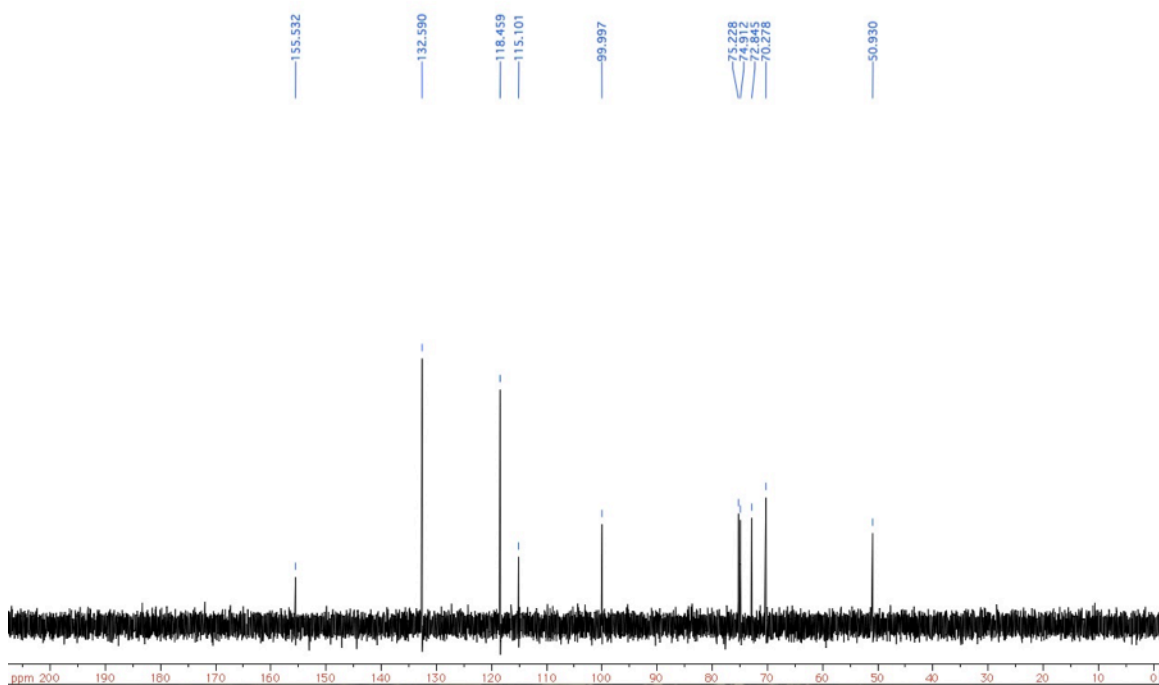
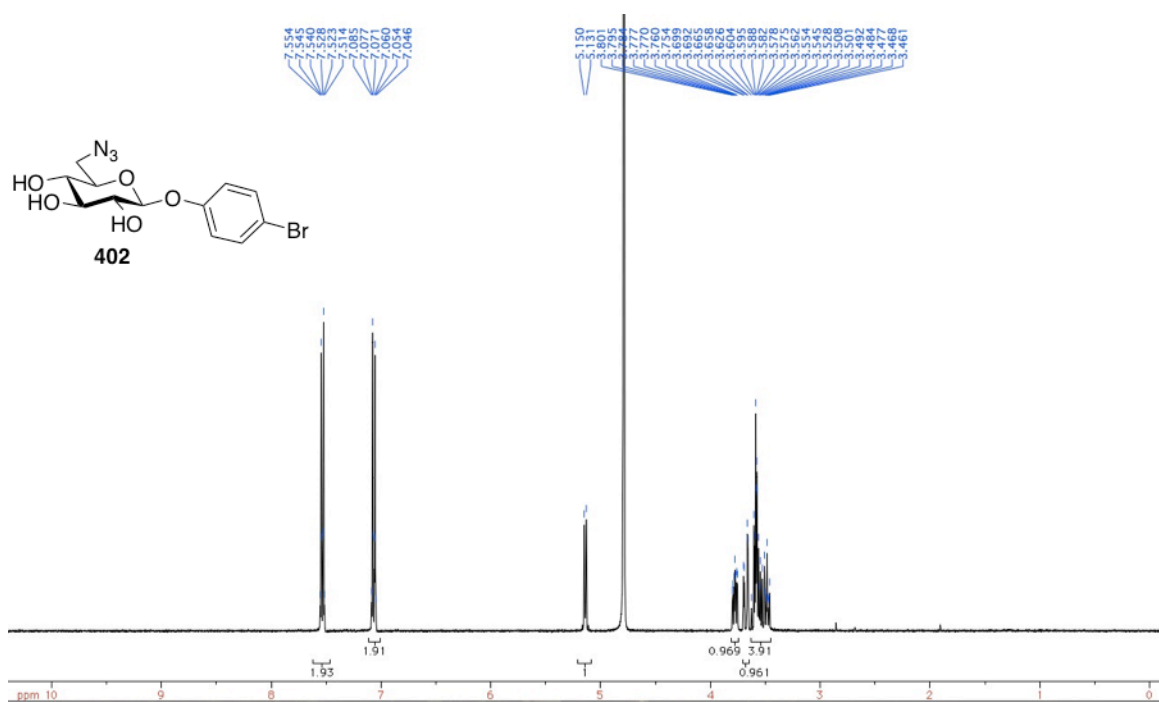
white powder (64 mg, 41% yield). ^1H NMR (400 MHz; D_2O): δ 8.43 (s, 1H), 7.53-7.49 (m, 2H), 7.07-7.03 (m, 2H), 5.15 (d, $J = 7.7$ Hz, 1H), 3.92 (dd, $J = 12.3, 1.5$ Hz, 1H), 3.79-3.68 (m, 4H), 3.32-3.27 (m, 1H). ^{13}C NMR (101 MHz; D_2O): δ 171.0, 155.7, 132.6, 118.5, 115.2, 100.5, 76.9, 69.6, 66.0, 59.9, 57.6. LRMS (ESI): m/z calcd. for $\text{C}_{12}\text{H}_{17}\text{BrNO}_5$ $[\text{M}+\text{H}]^+$ 334.0; found, 334.0.

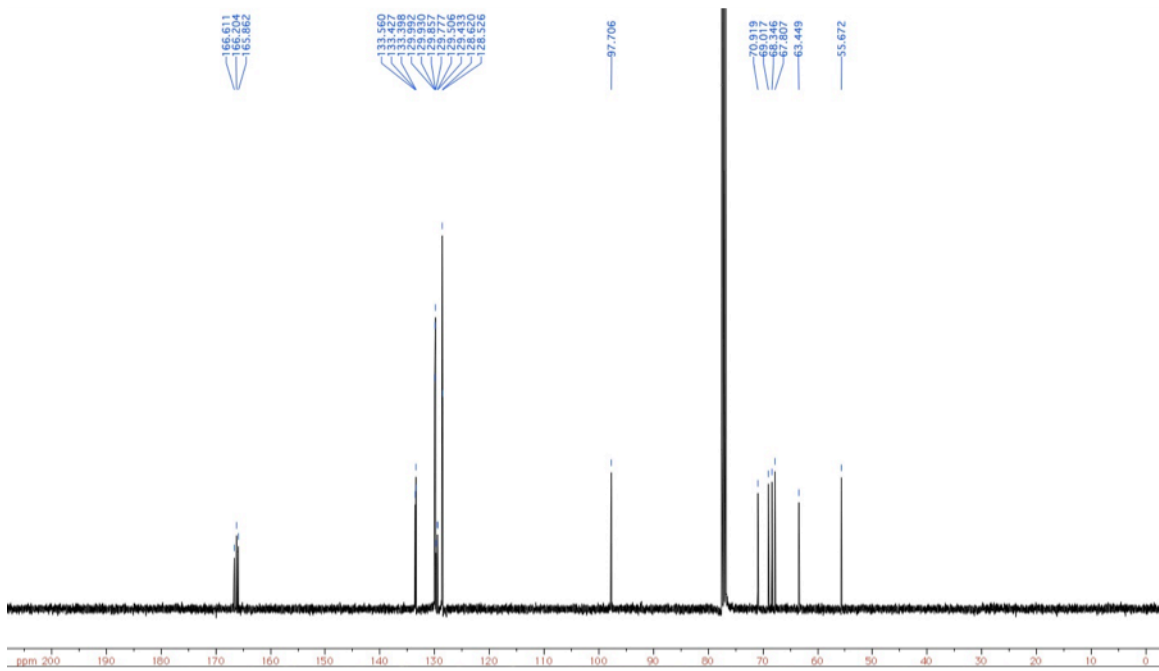
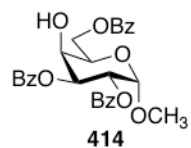
4.5.7 NMR Spectra

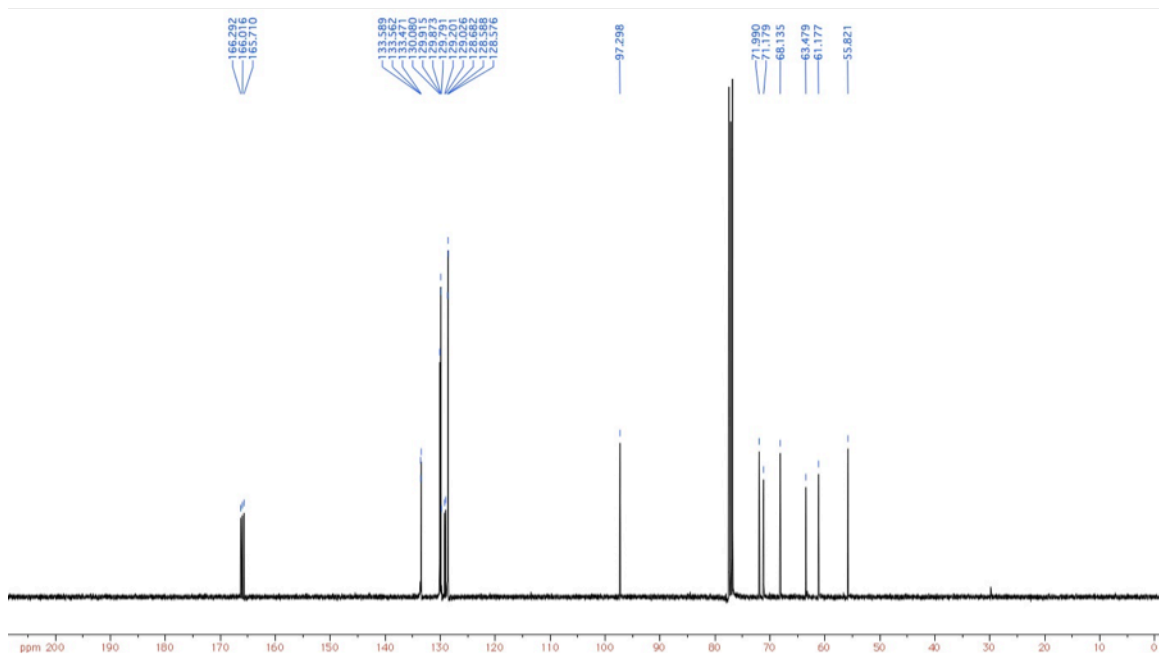
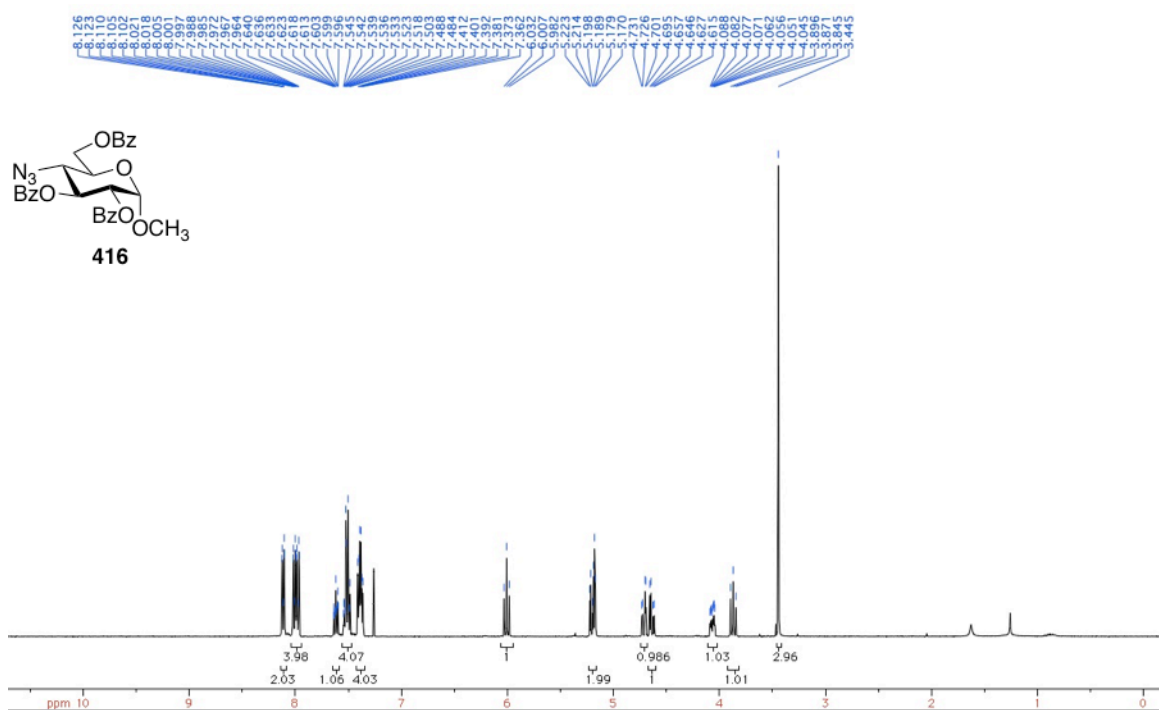


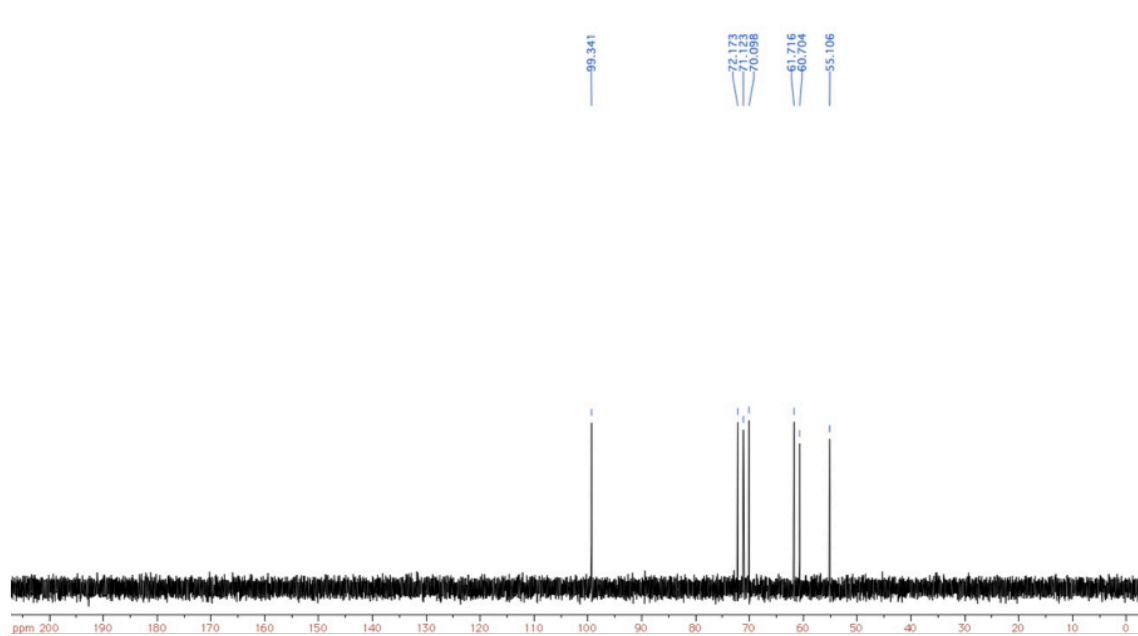
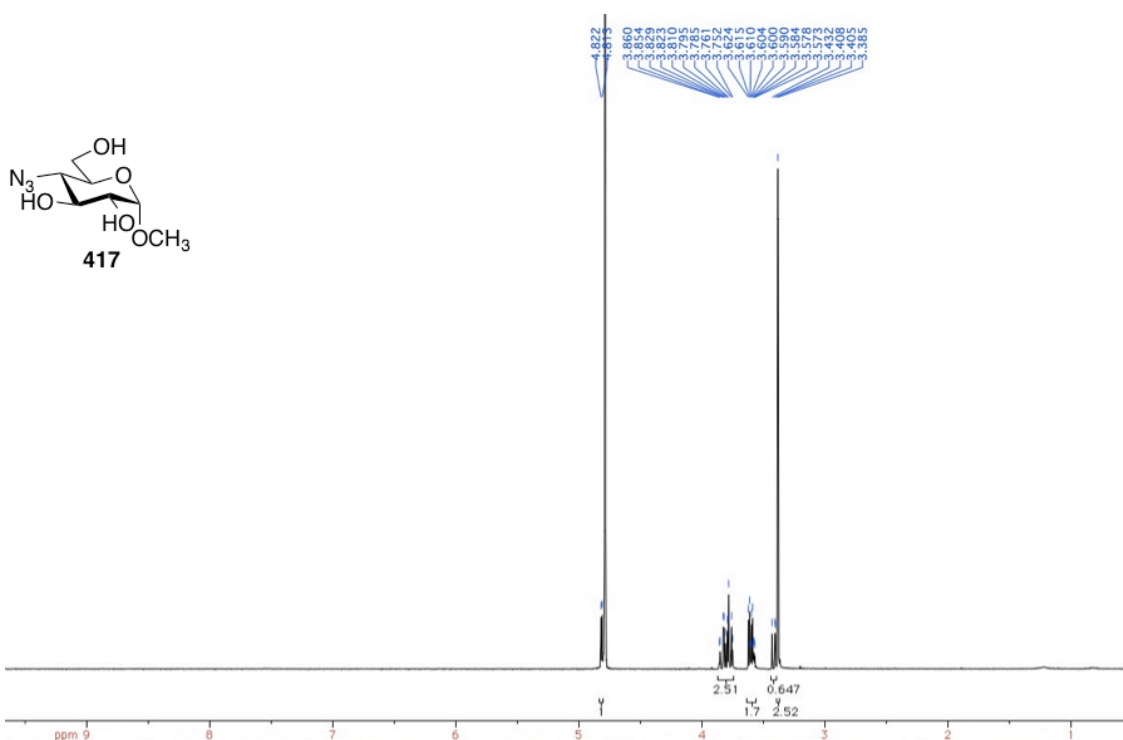


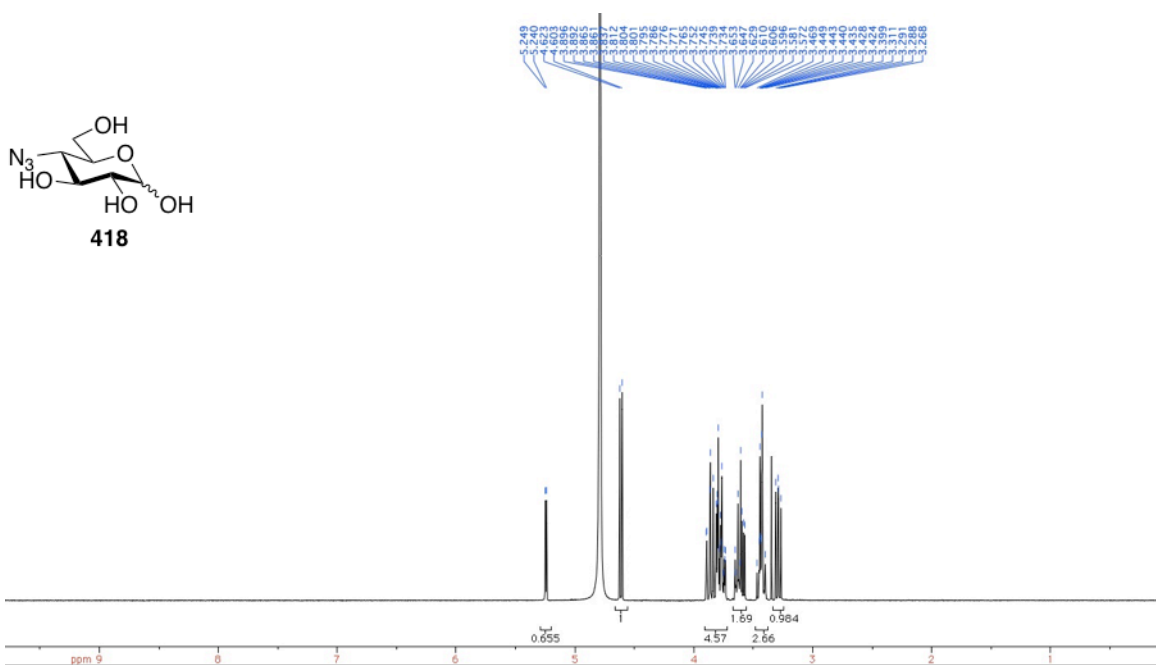


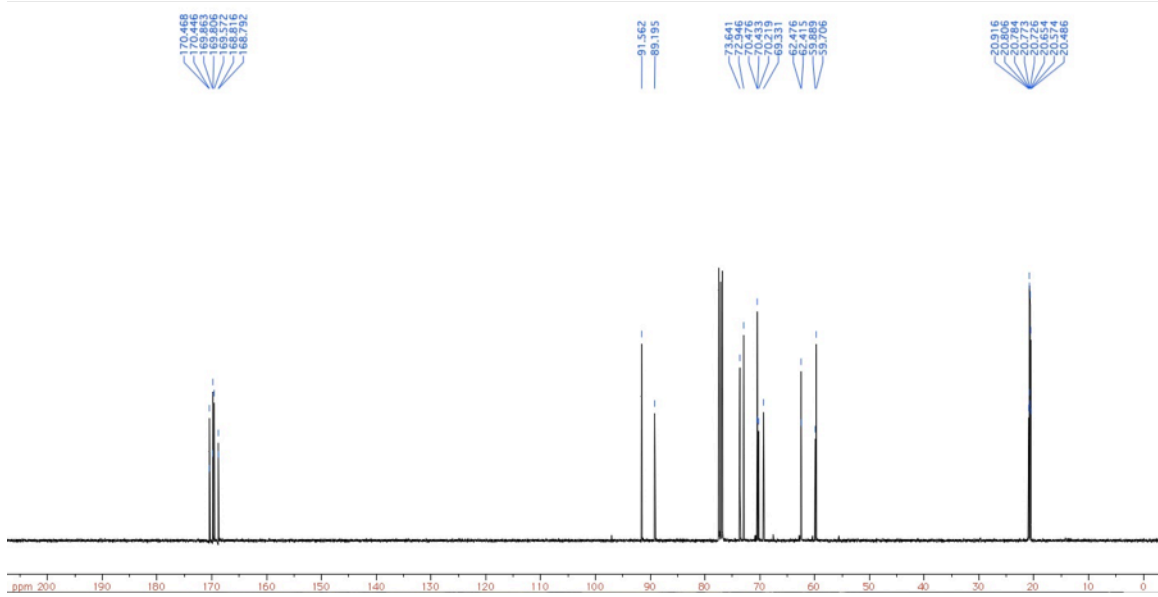
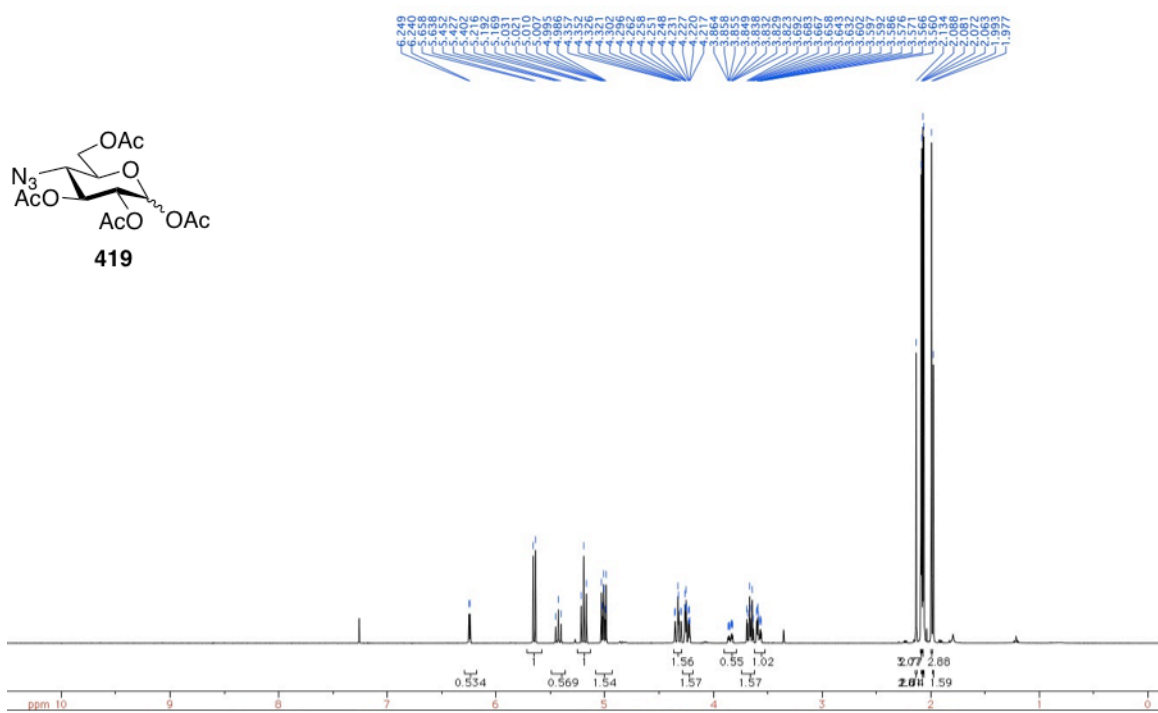


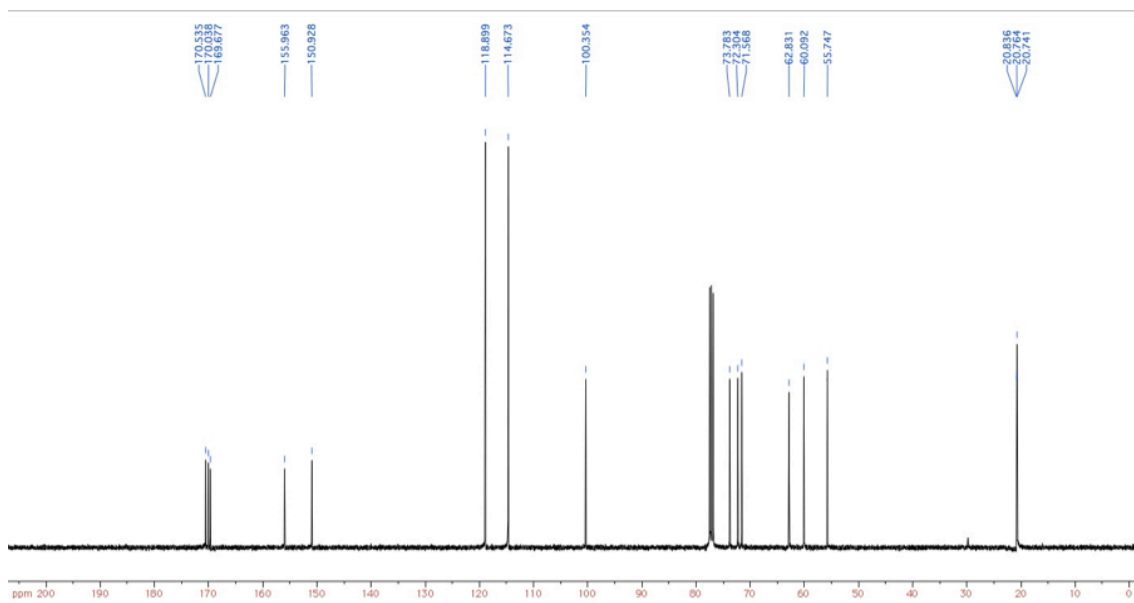
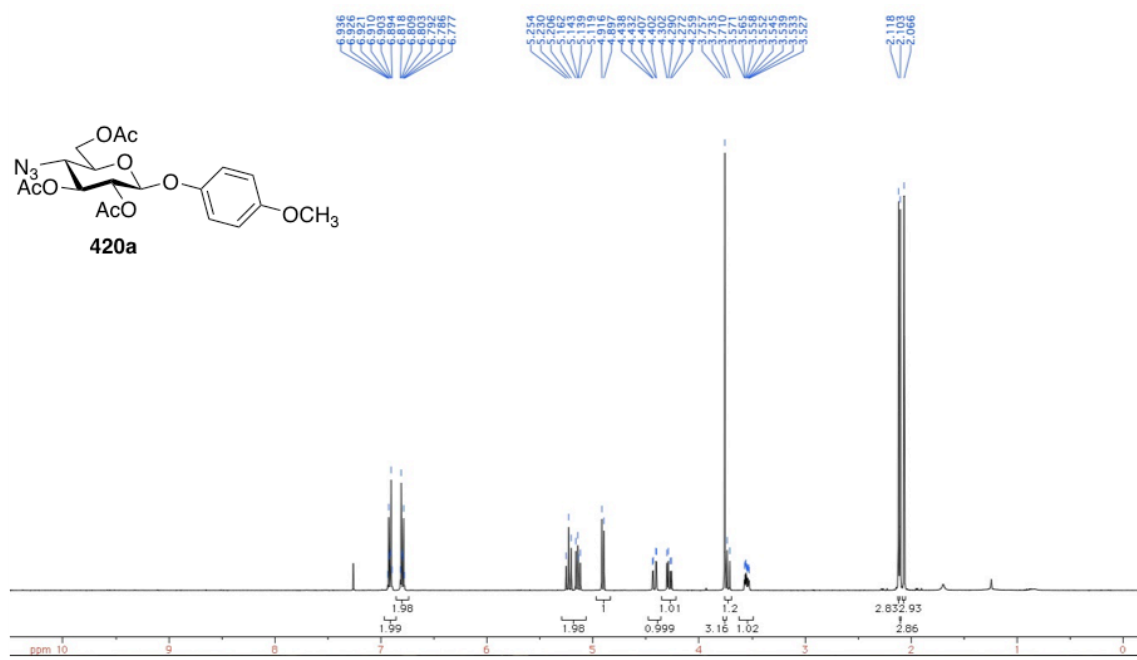


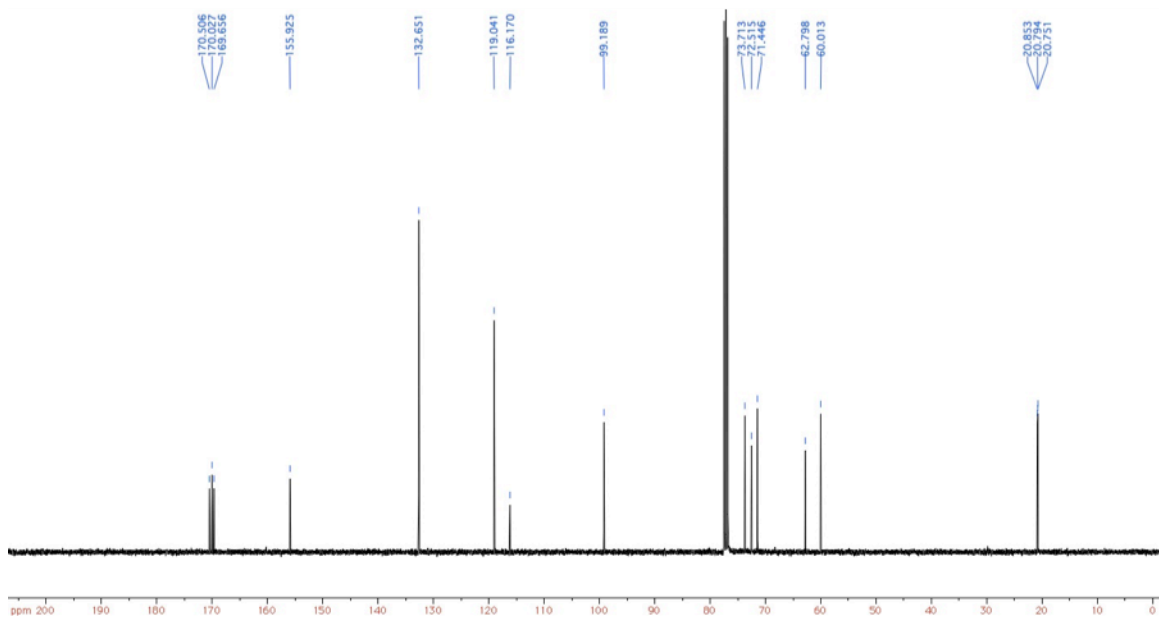
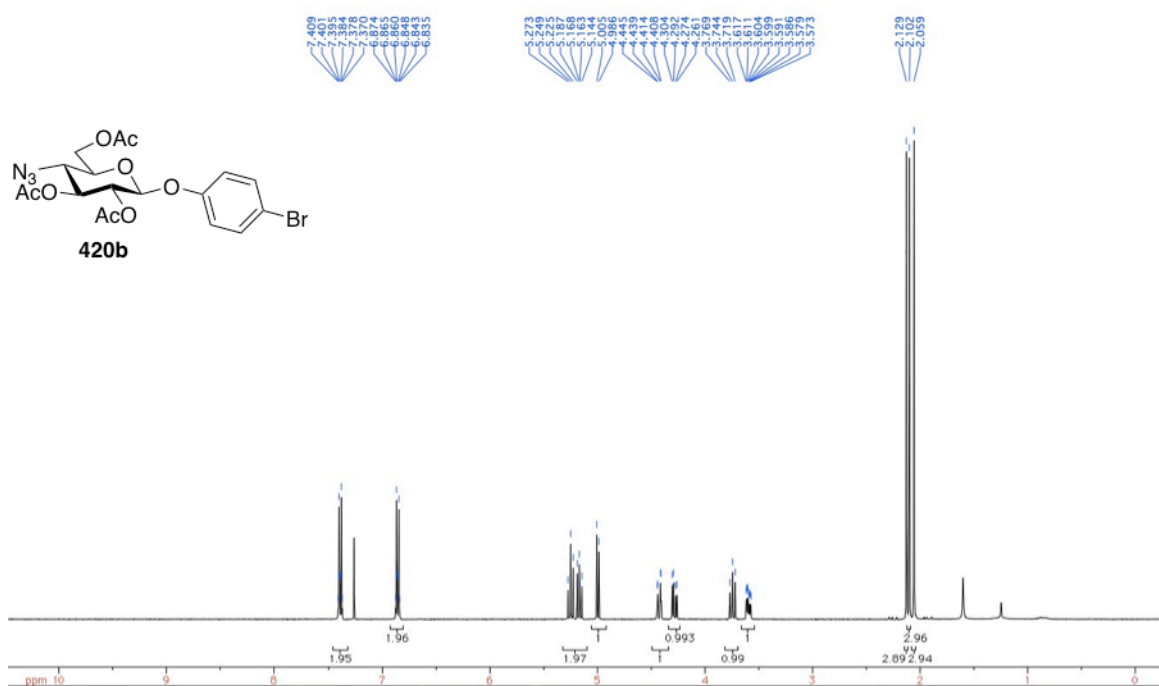


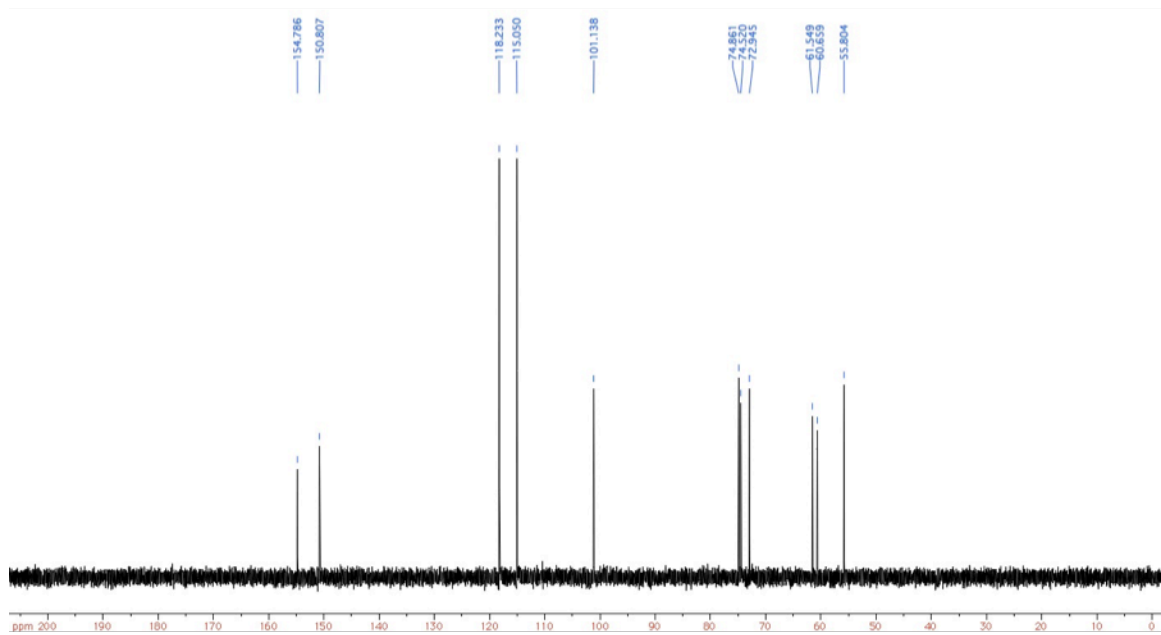
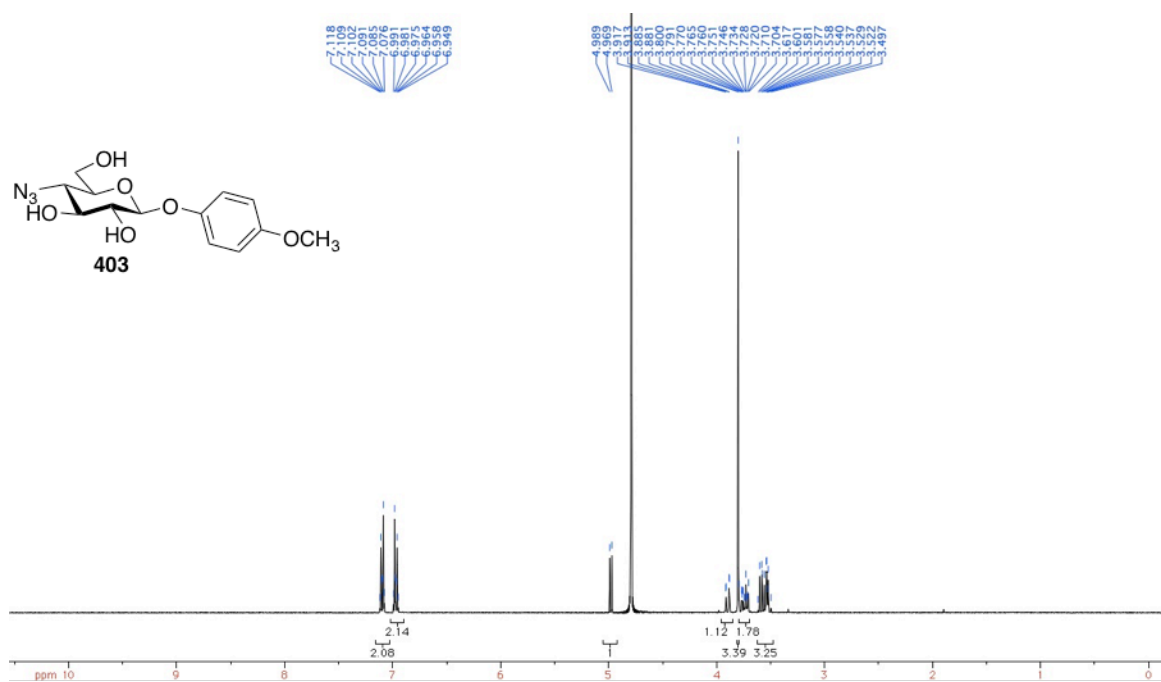


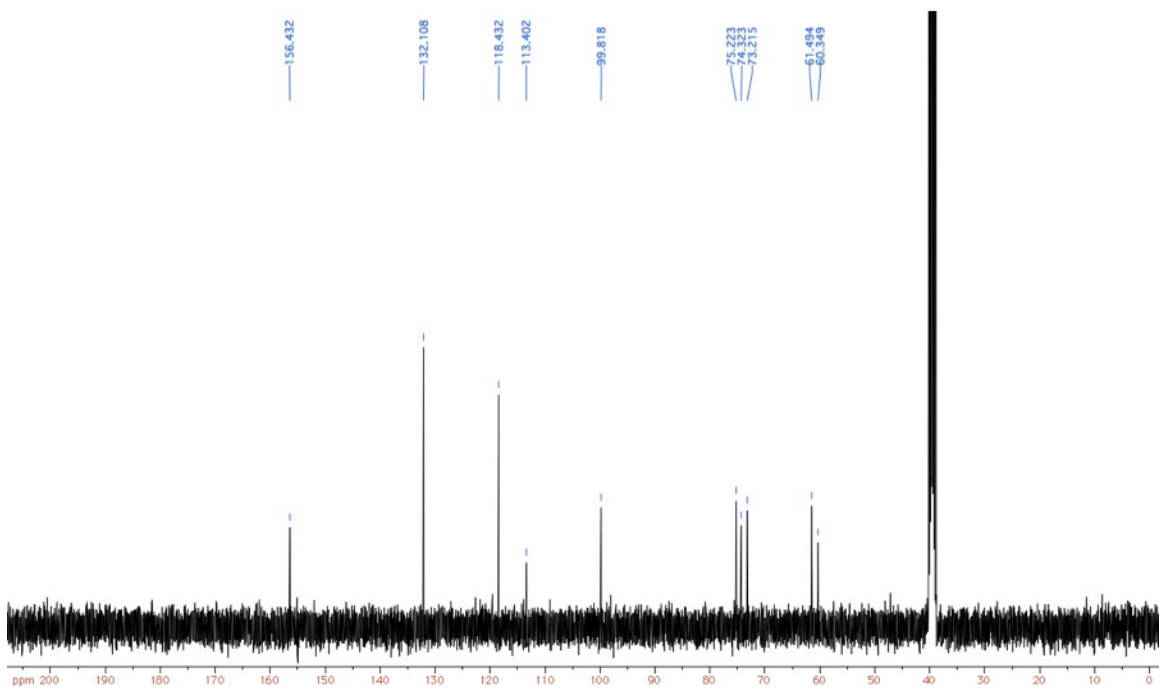
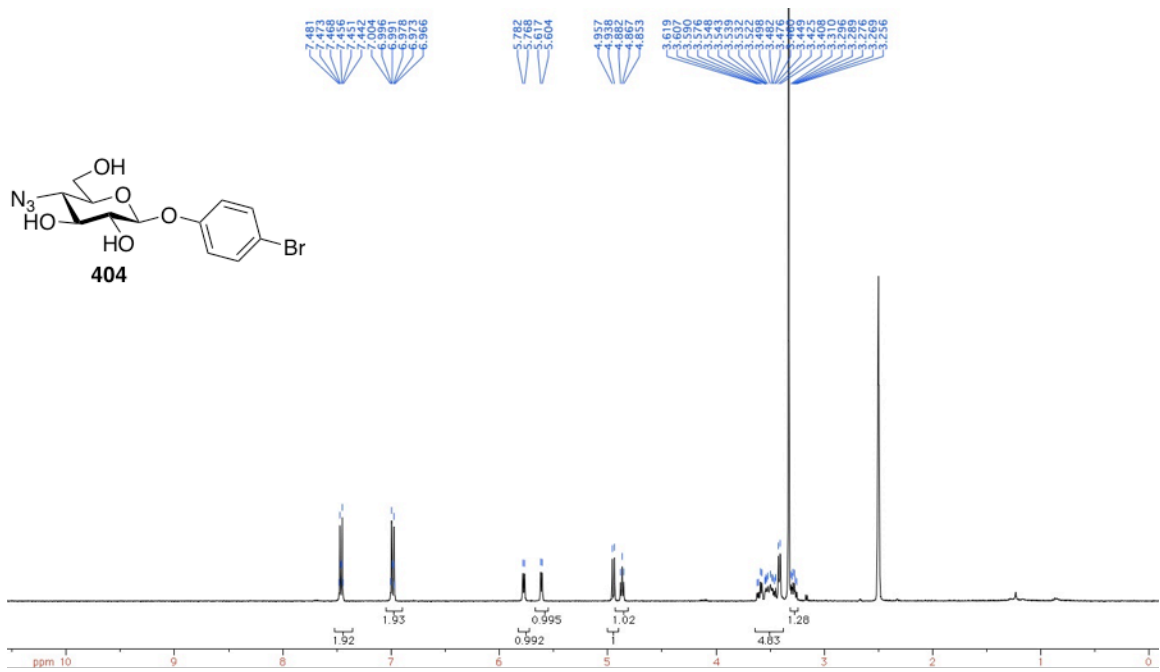


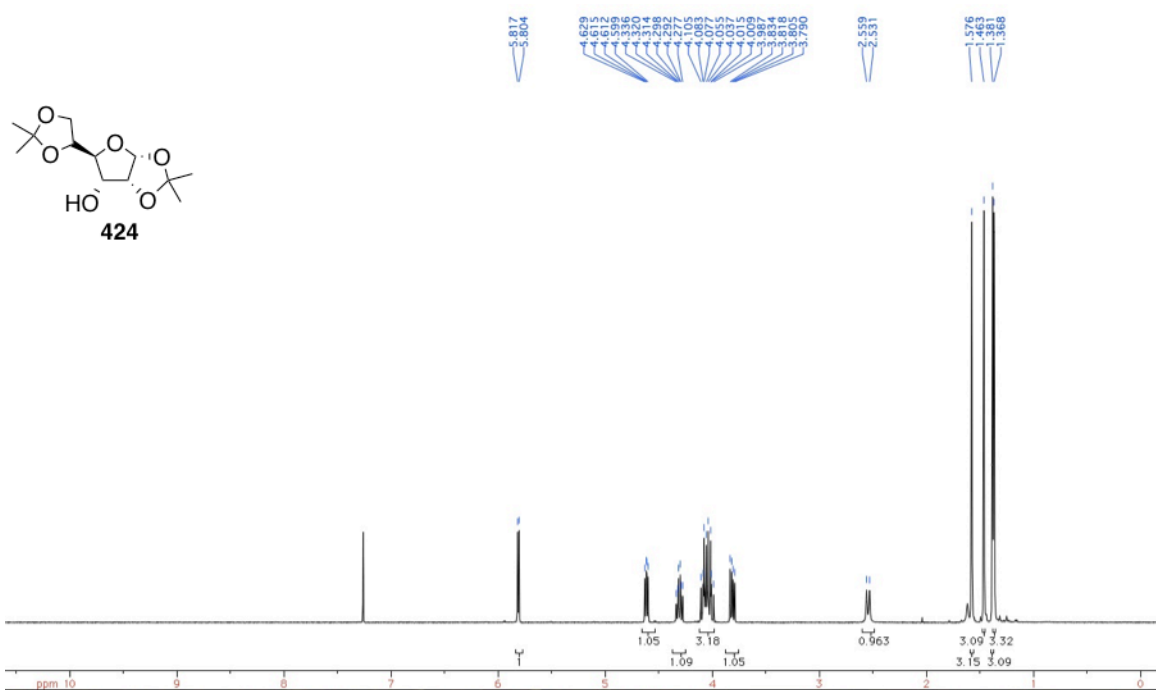


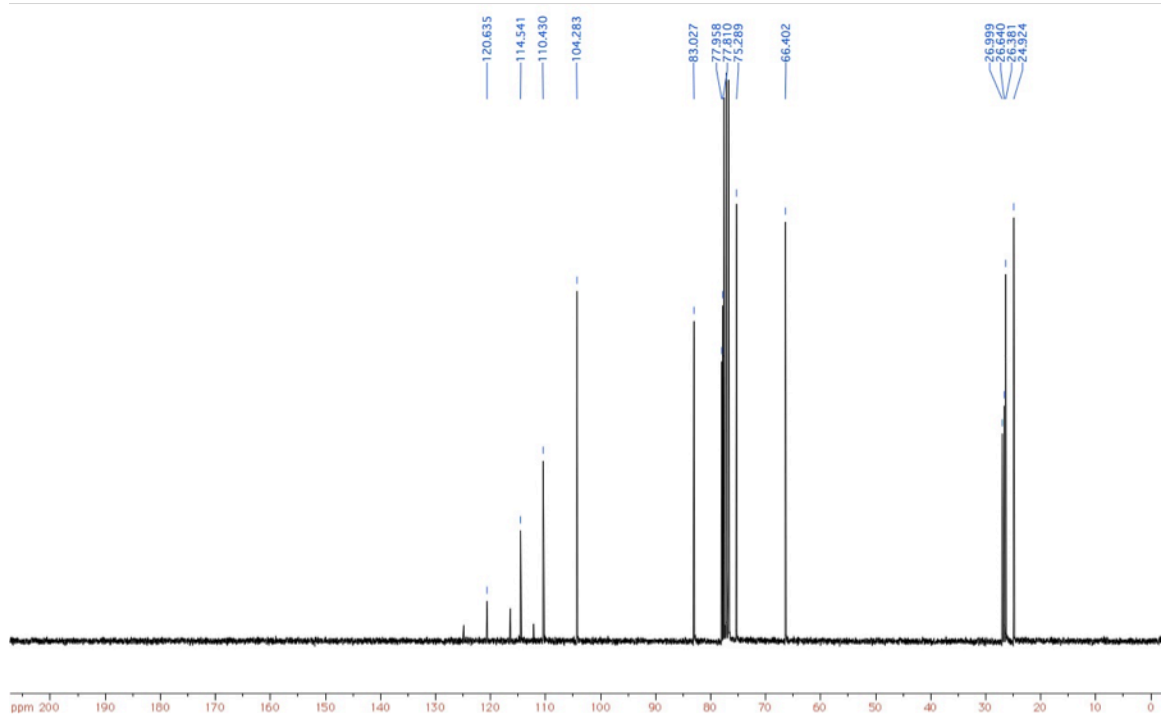
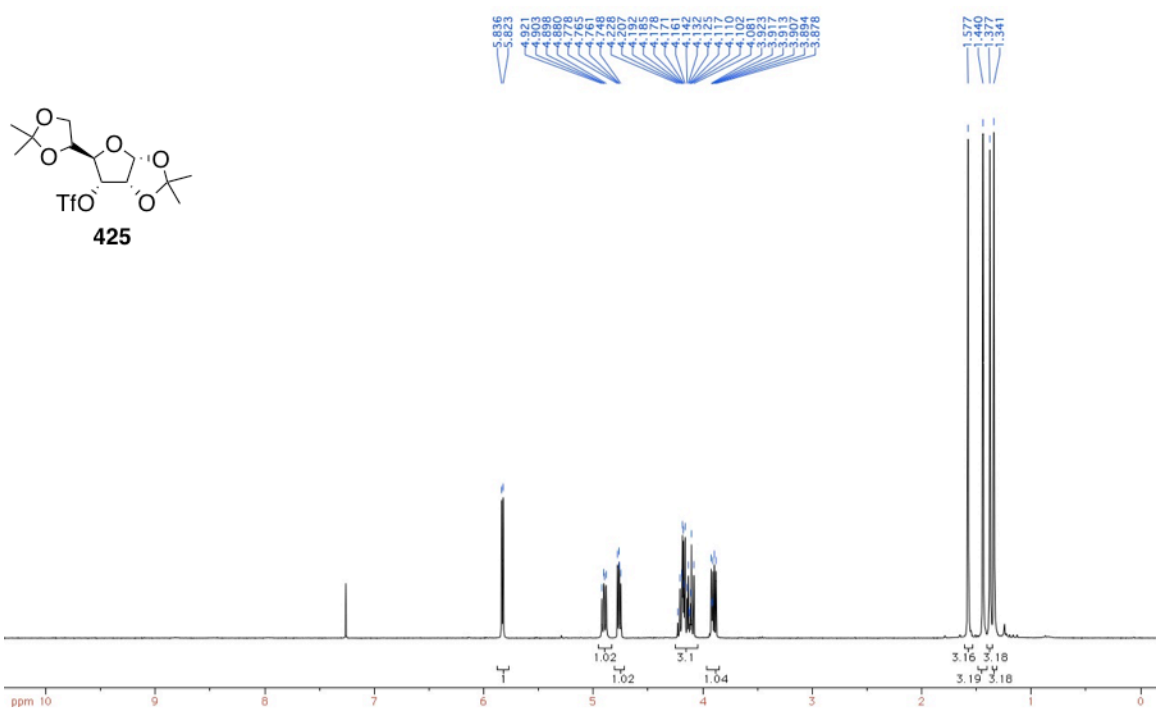


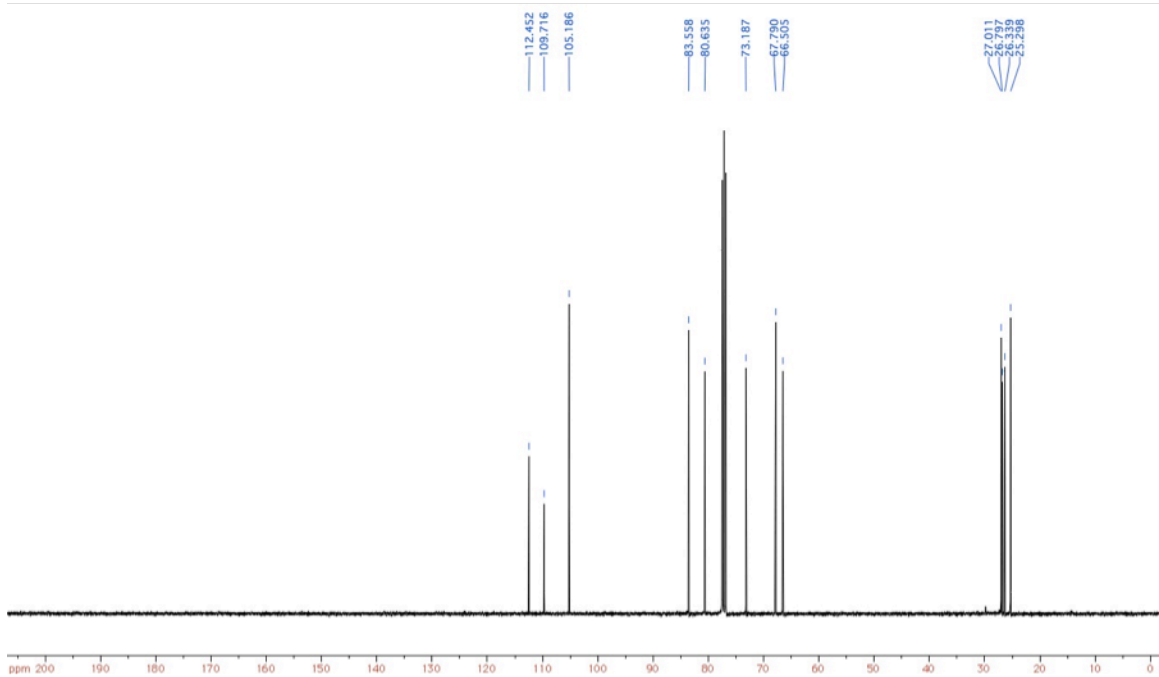
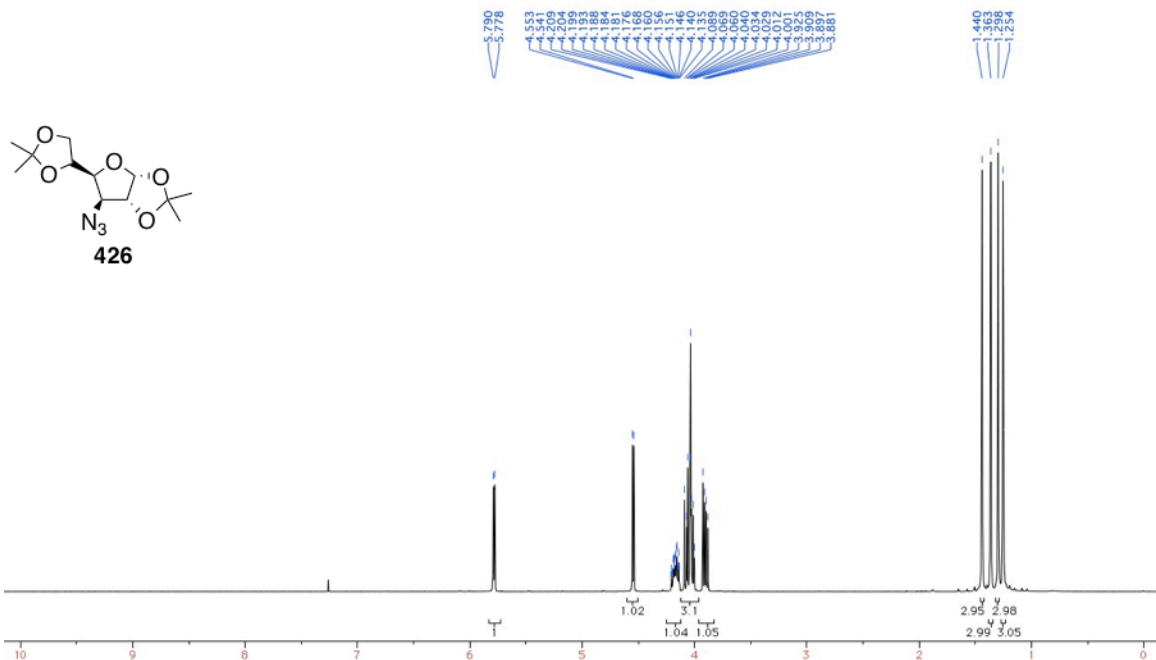


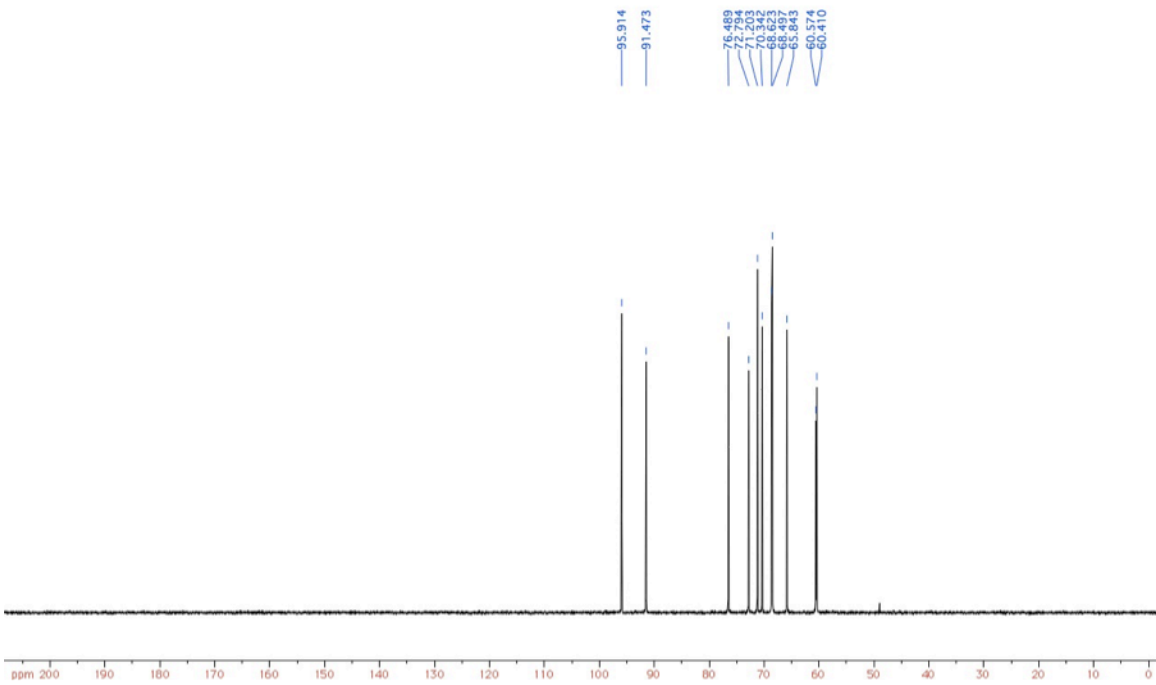
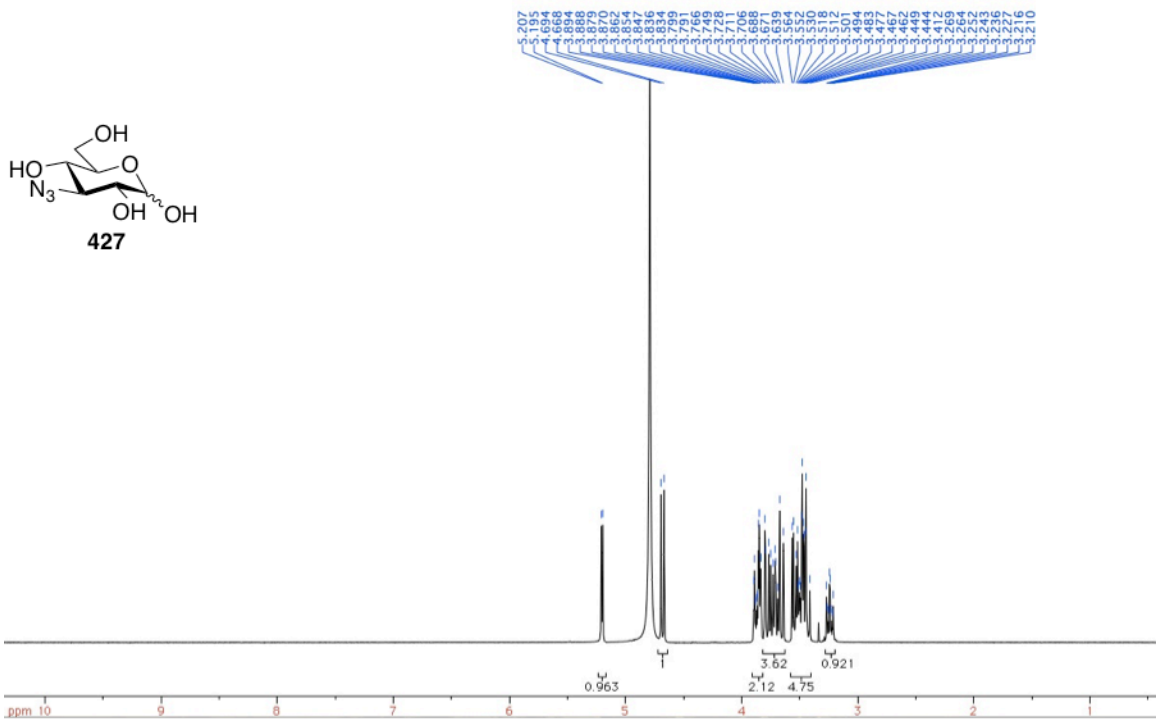


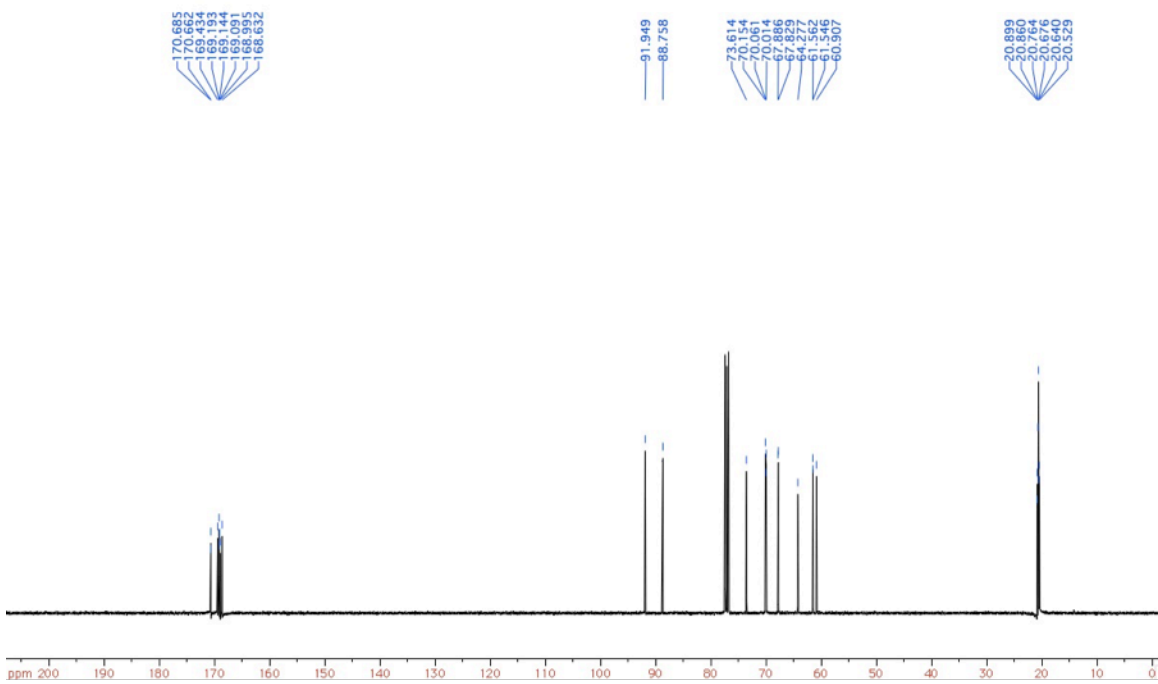
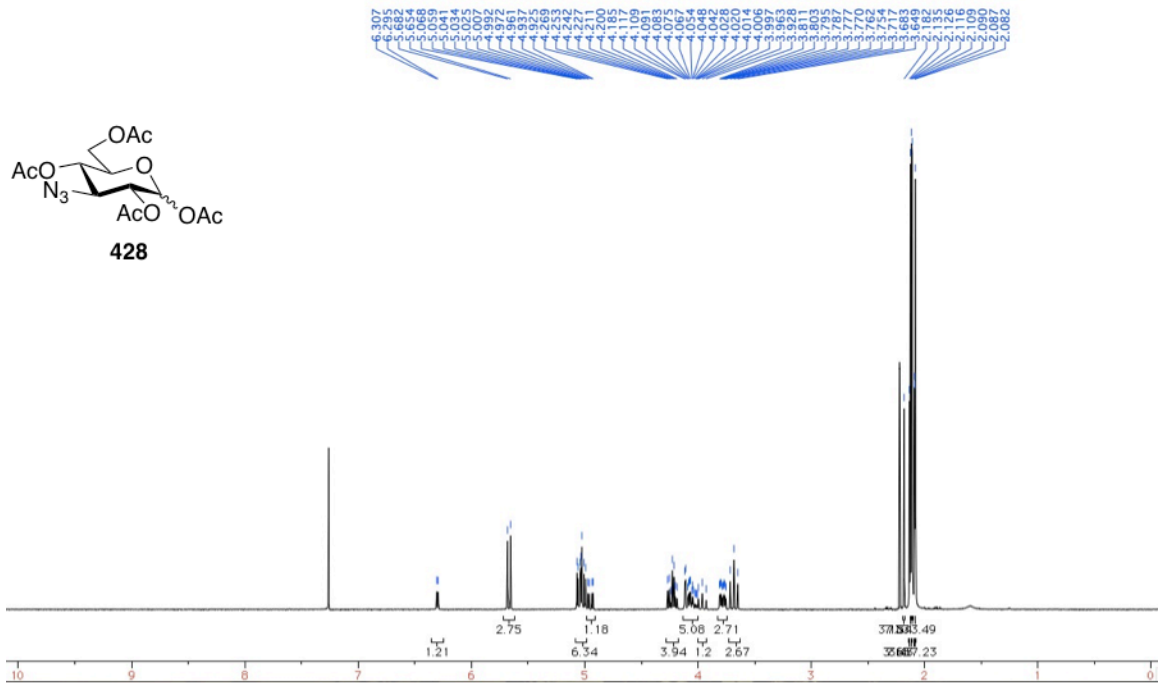


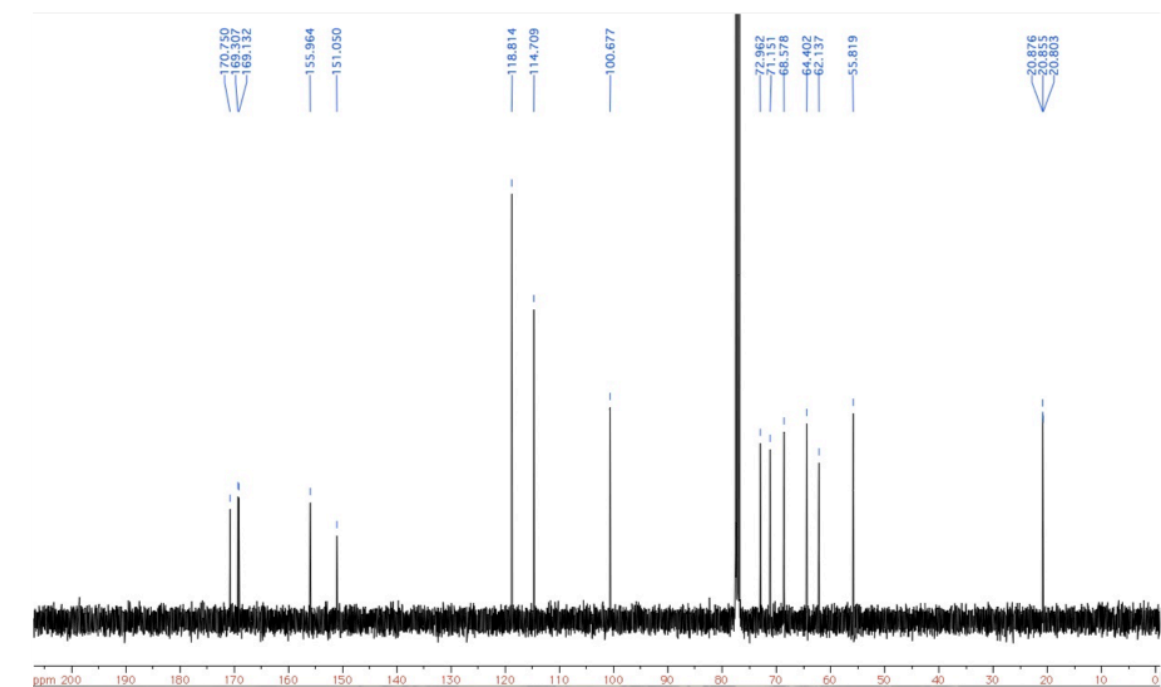


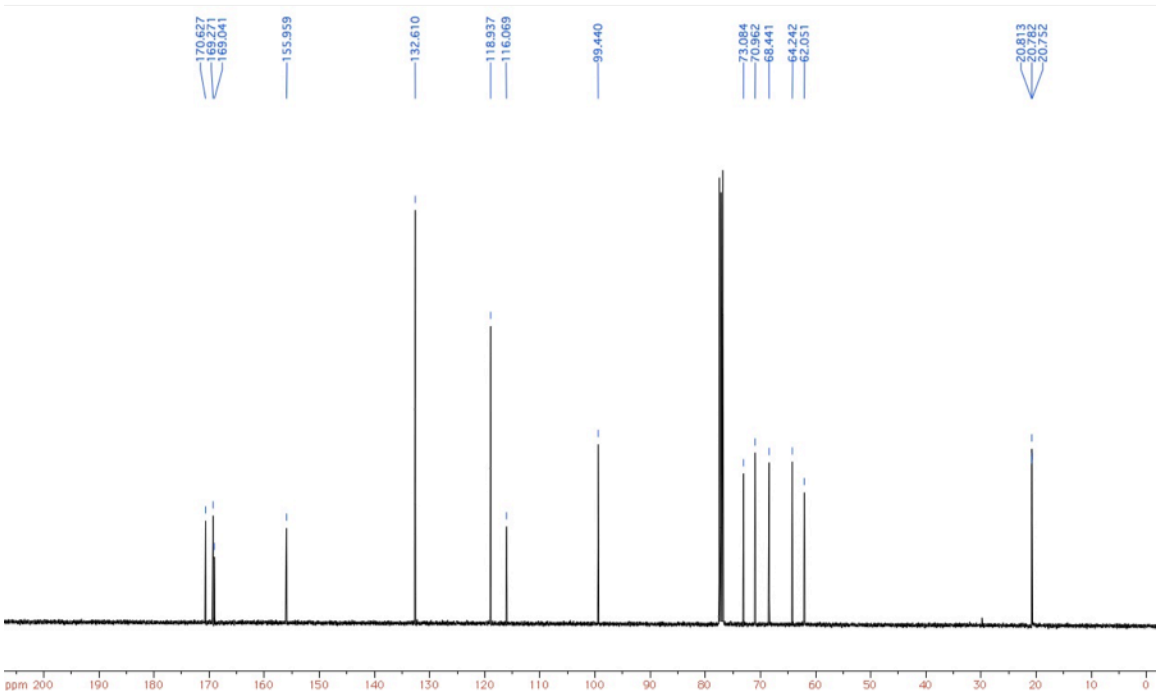


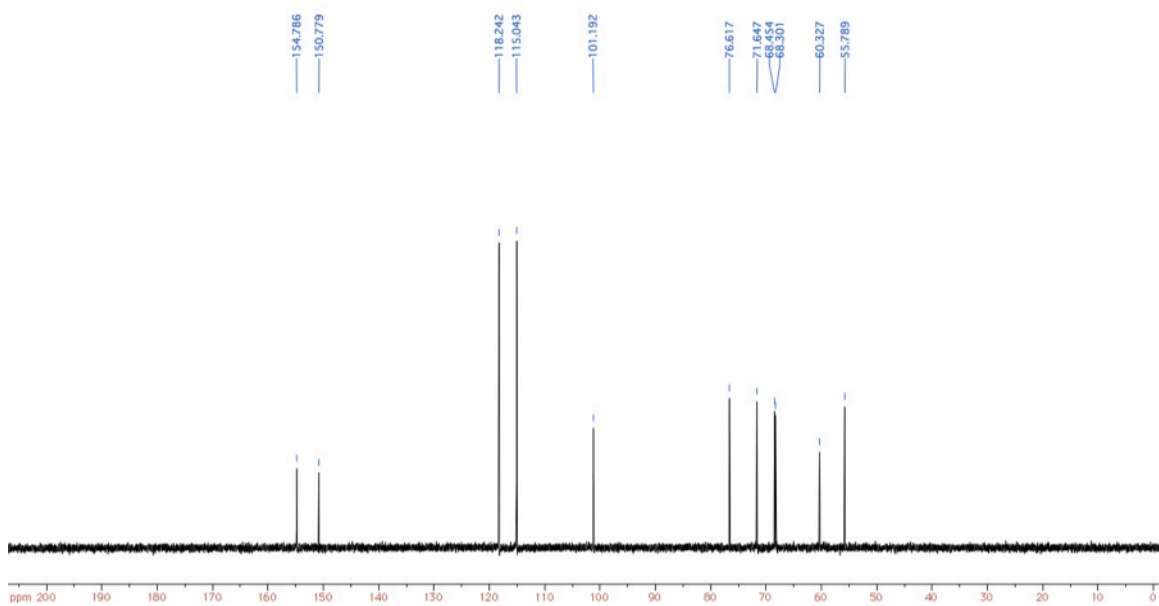
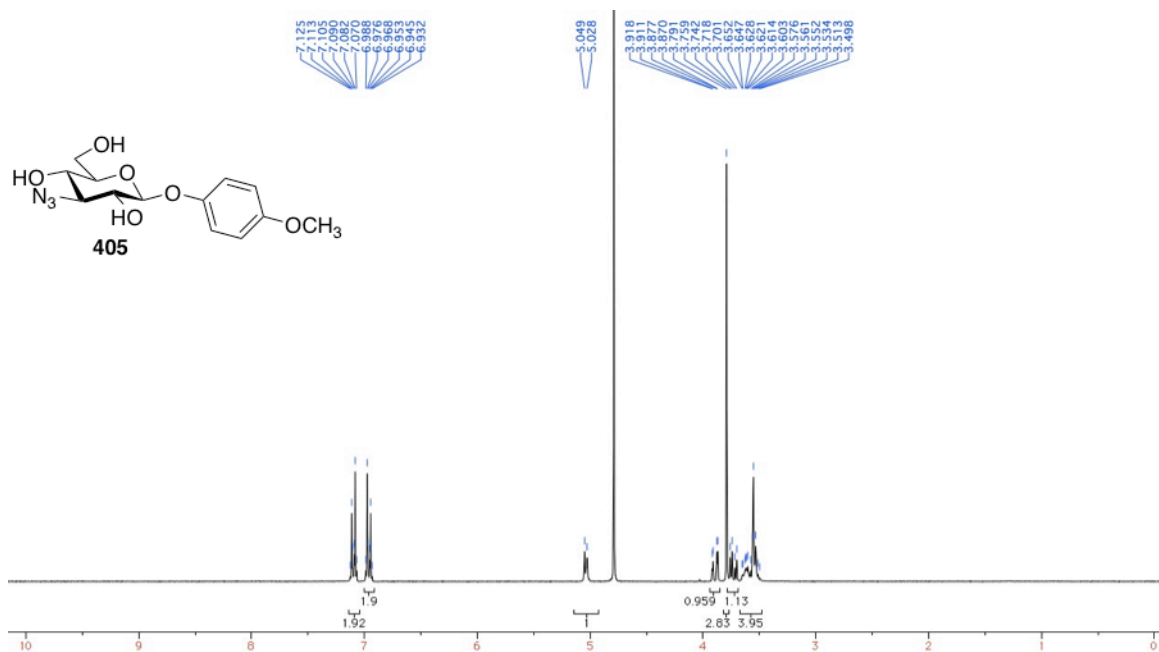


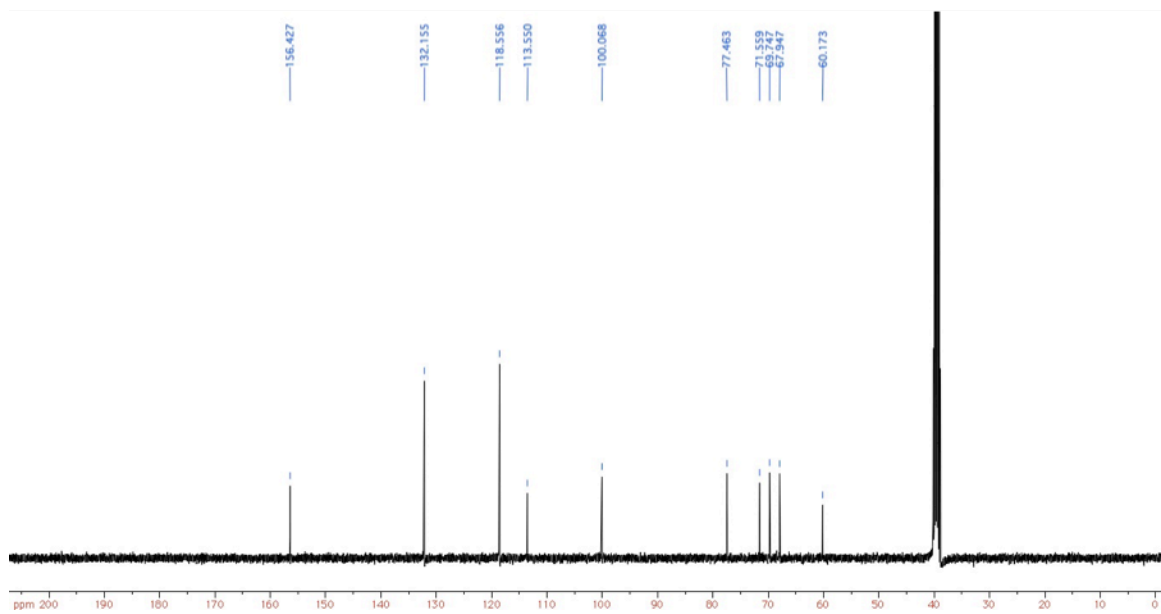
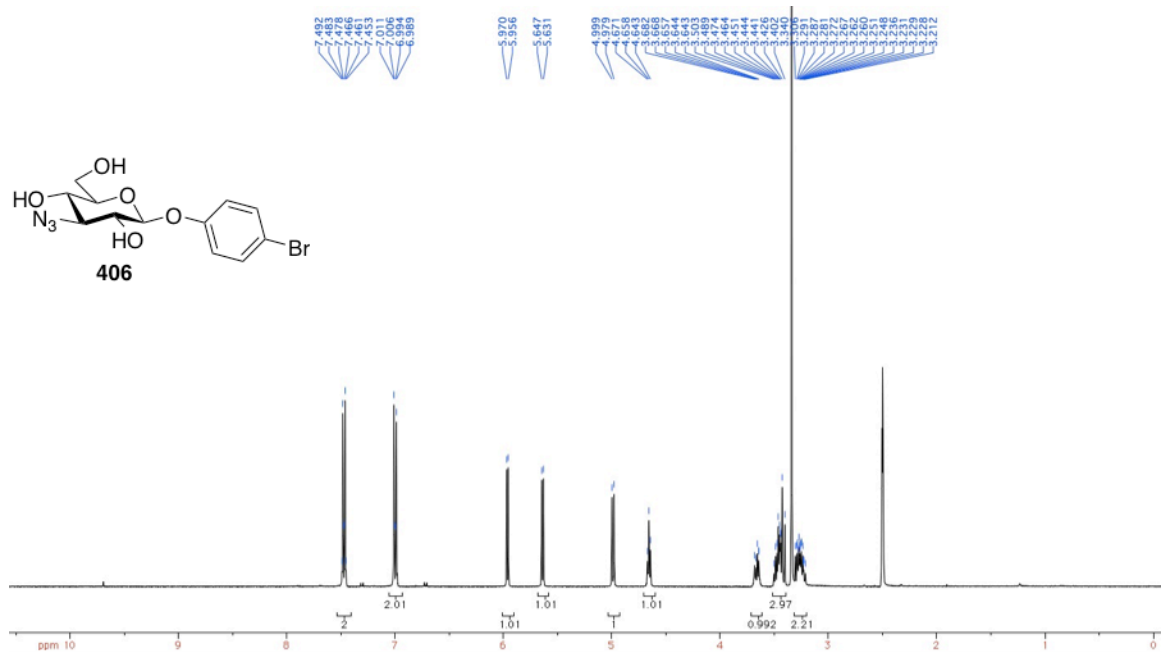


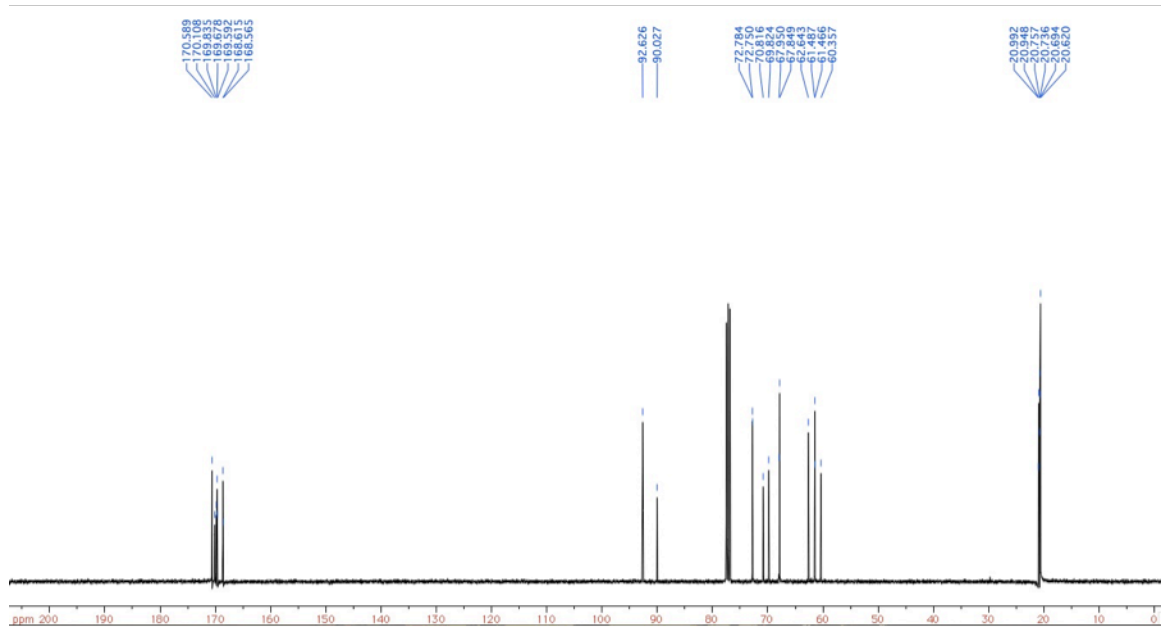


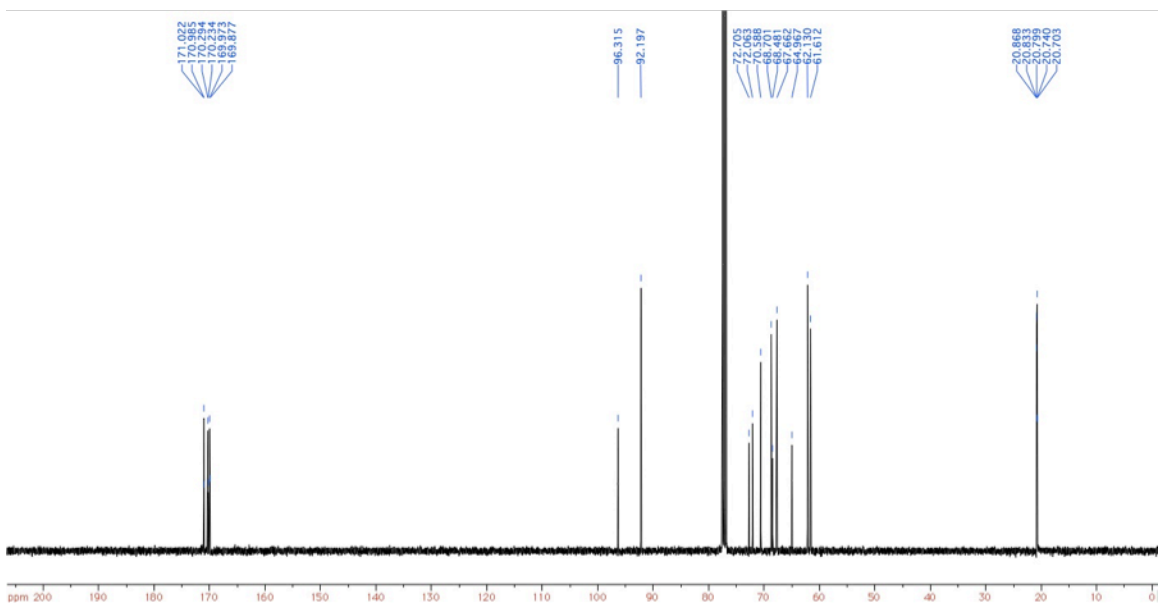
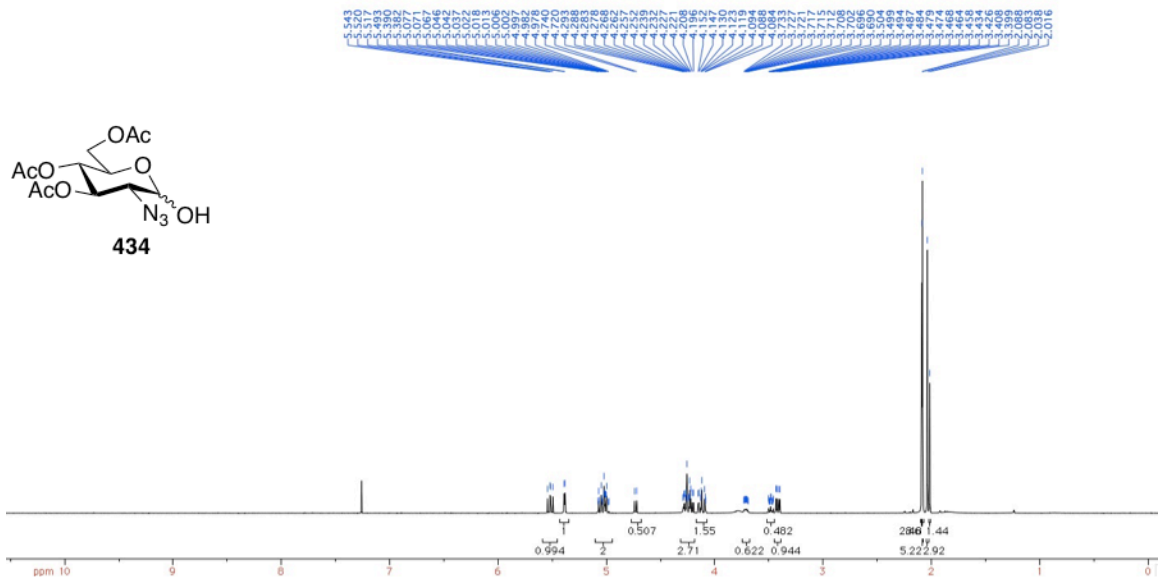


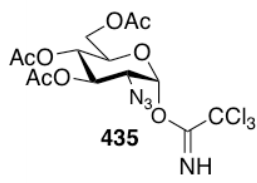


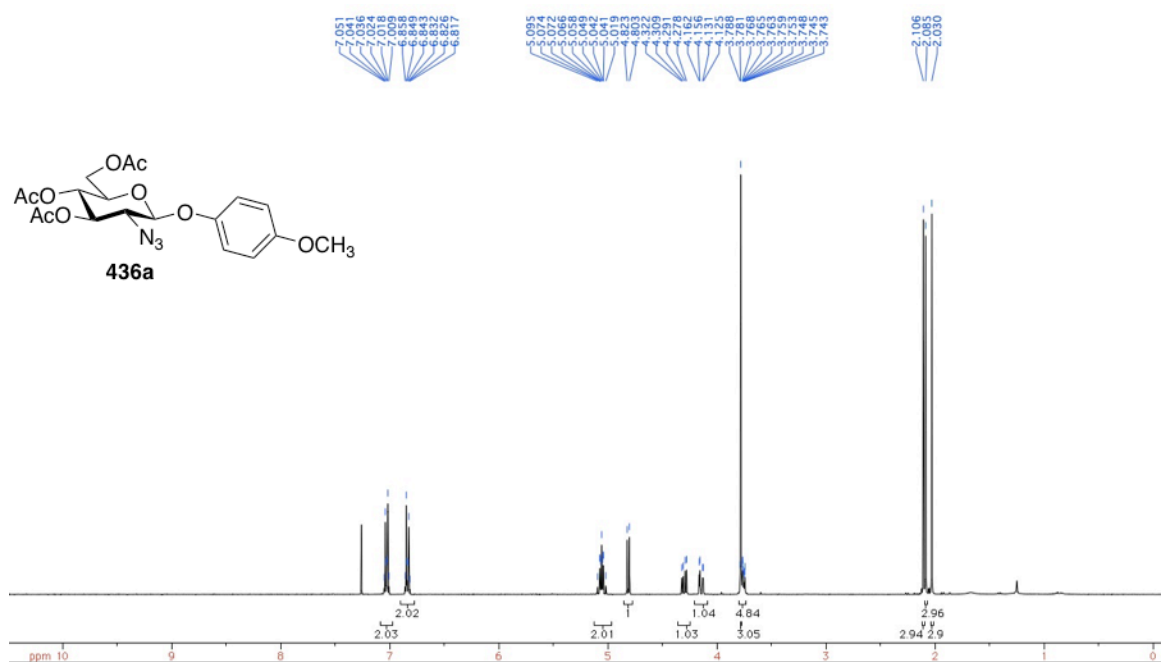


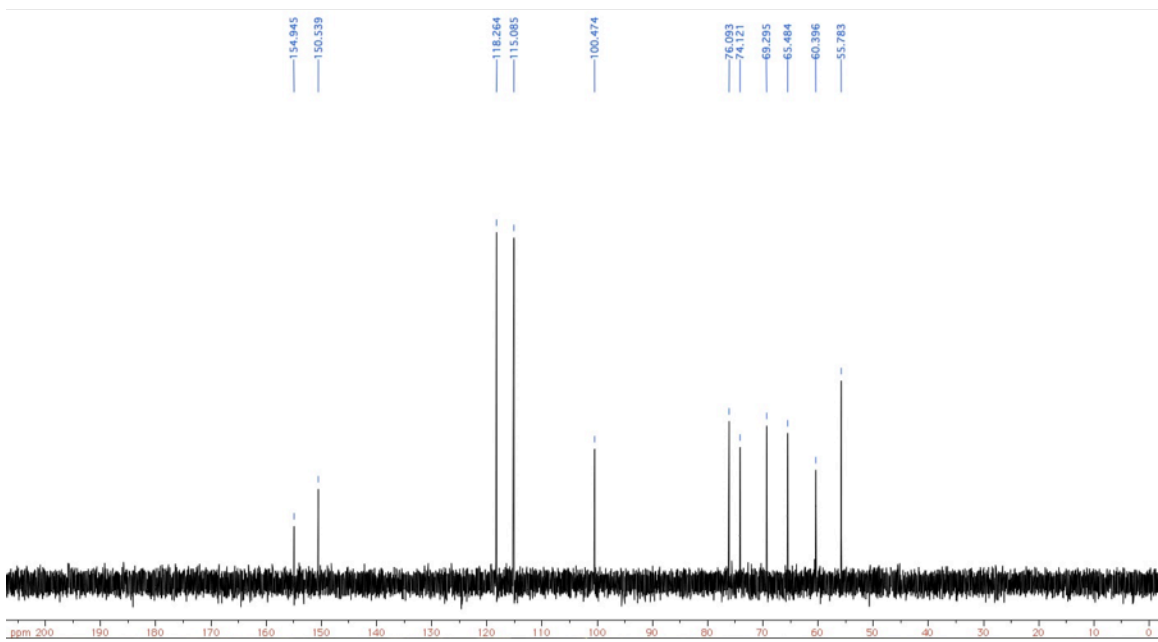
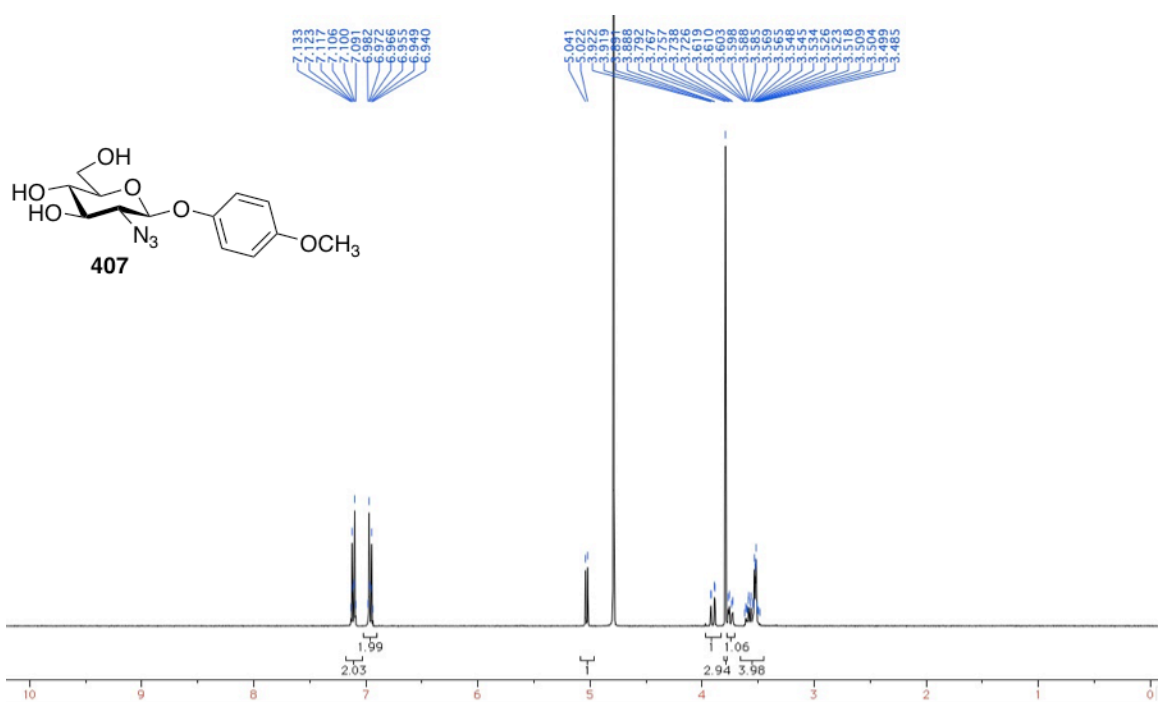


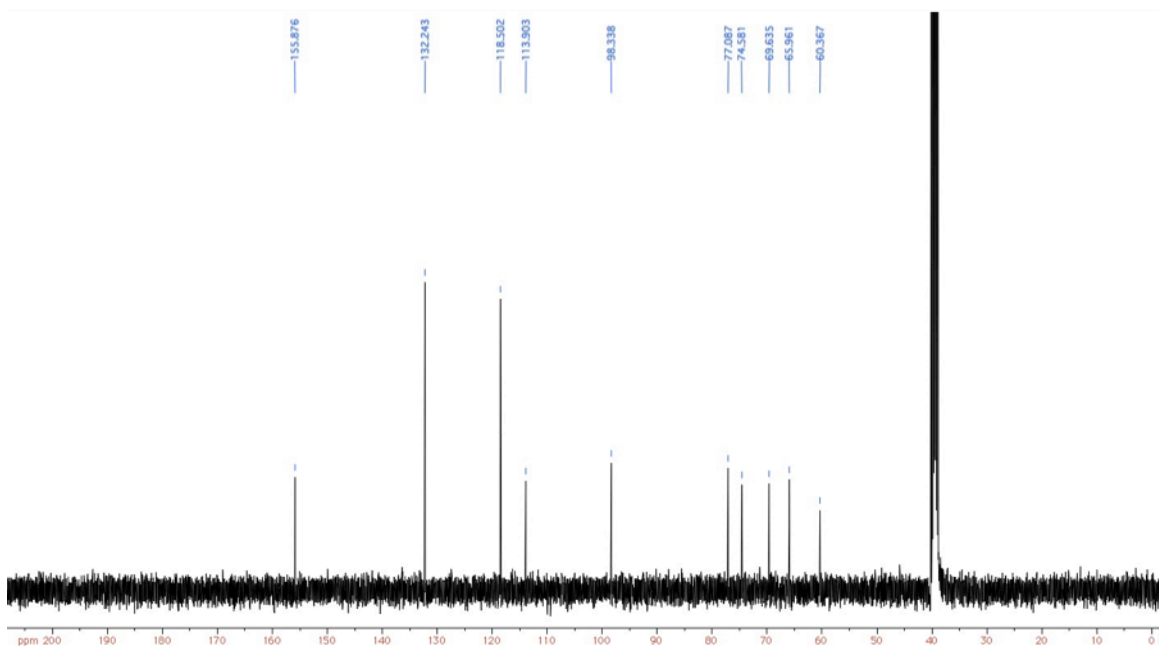


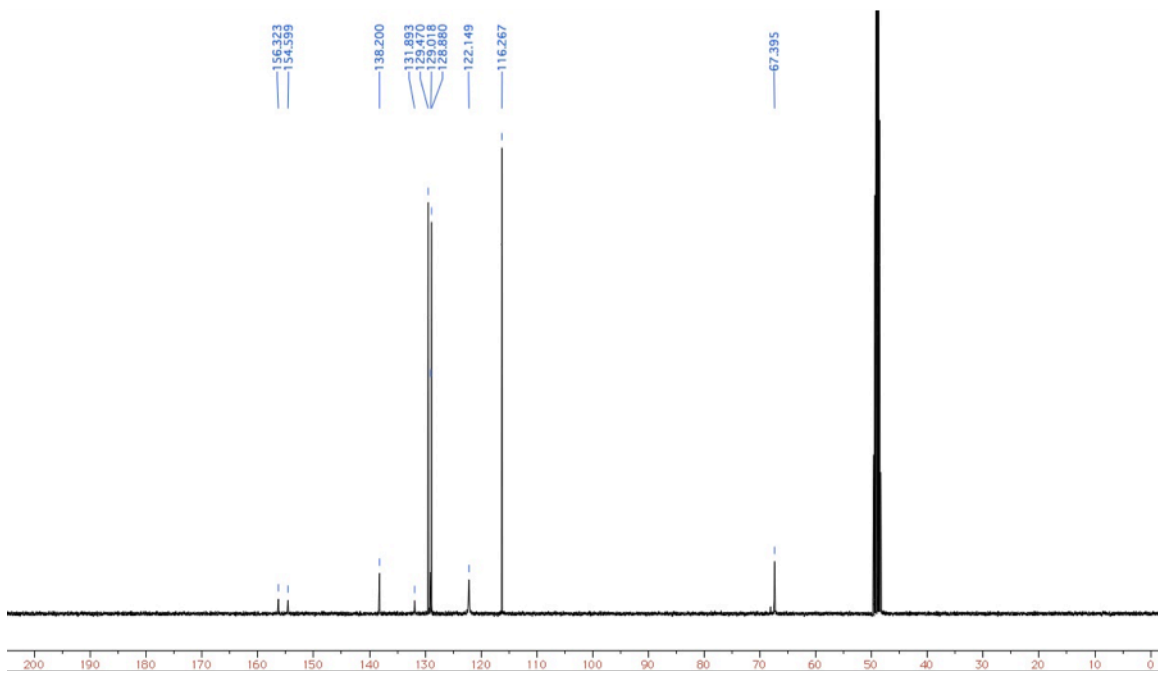
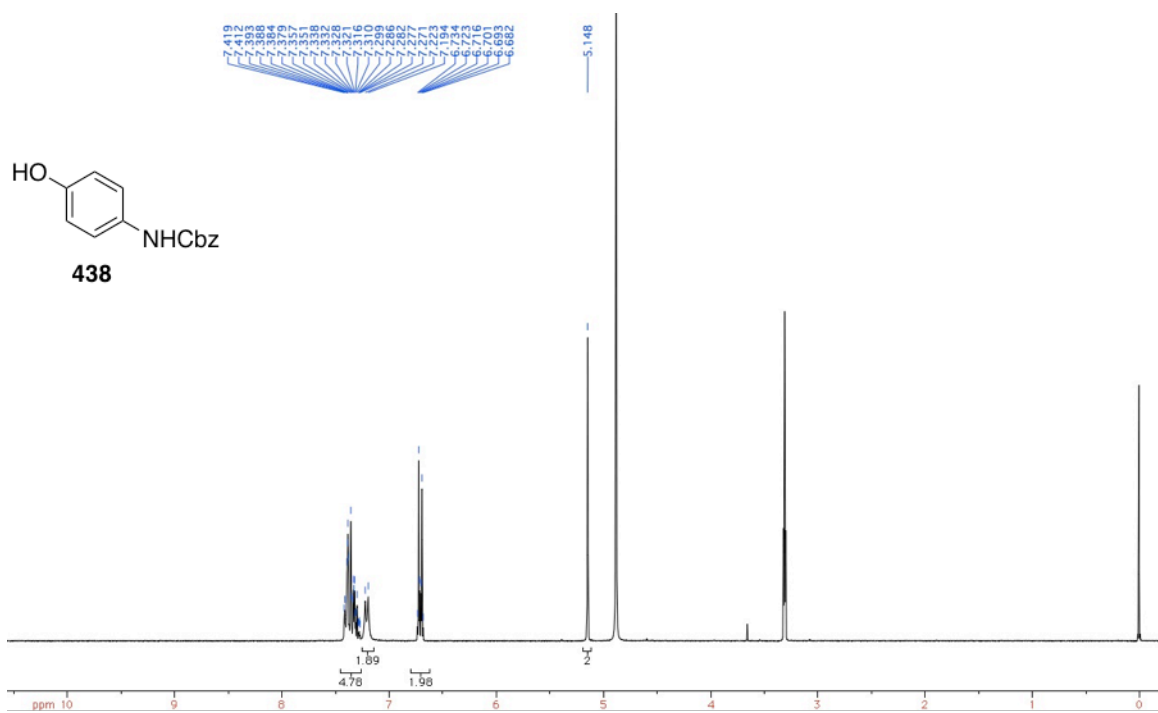


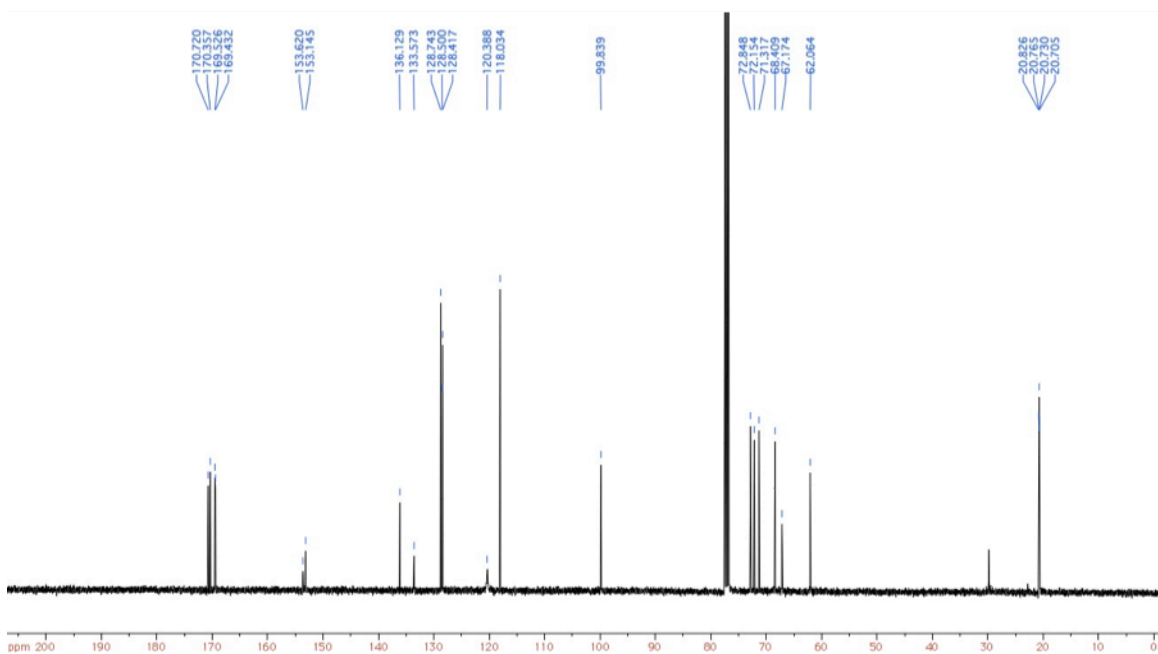
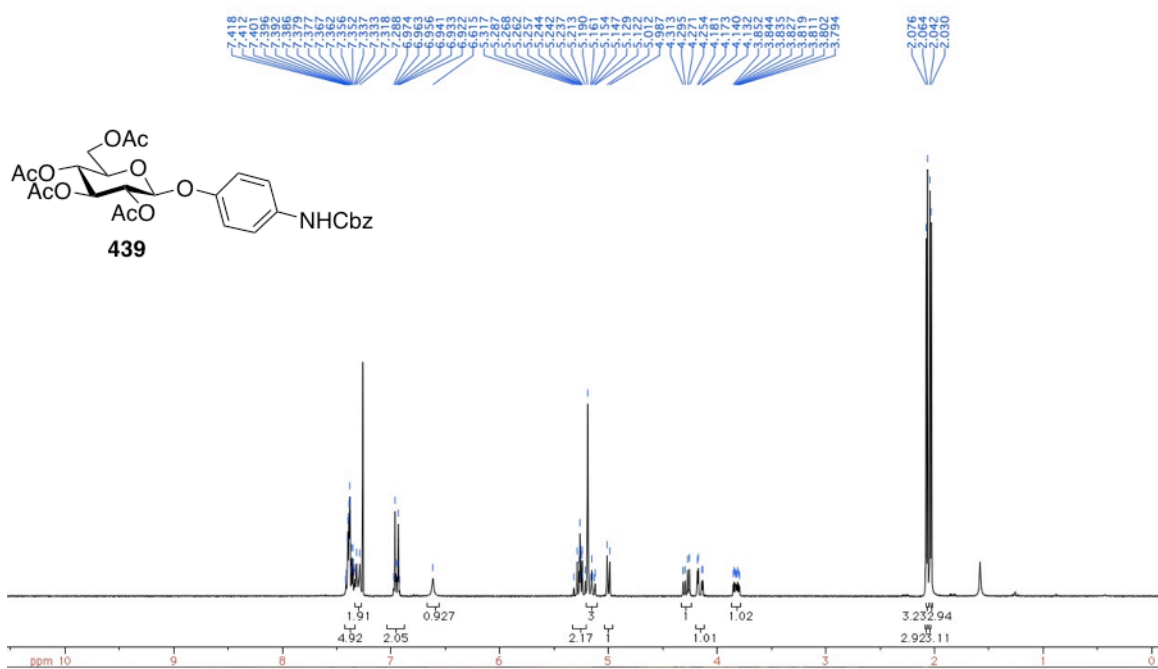


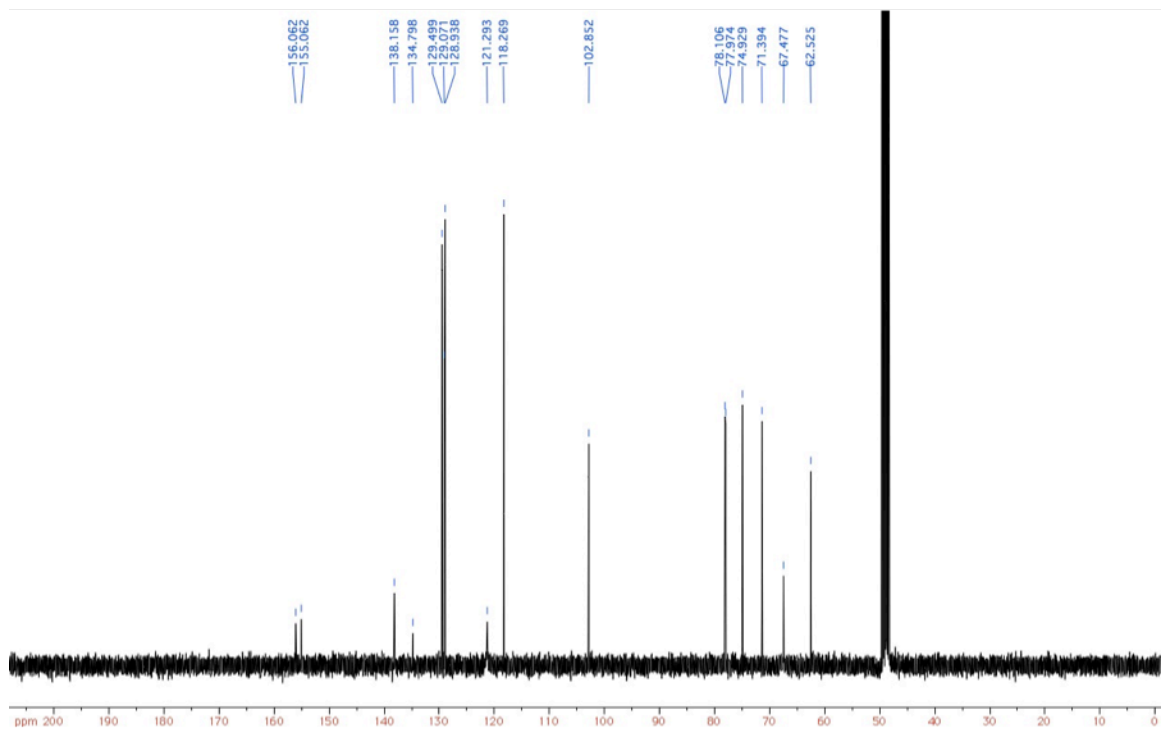
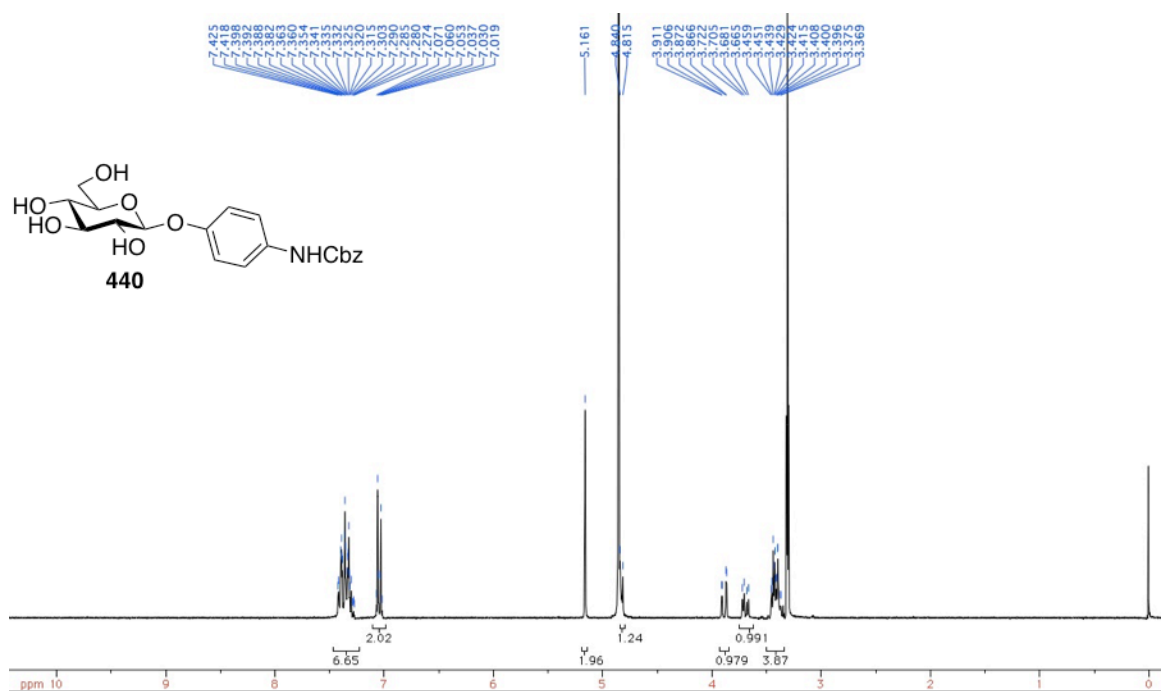


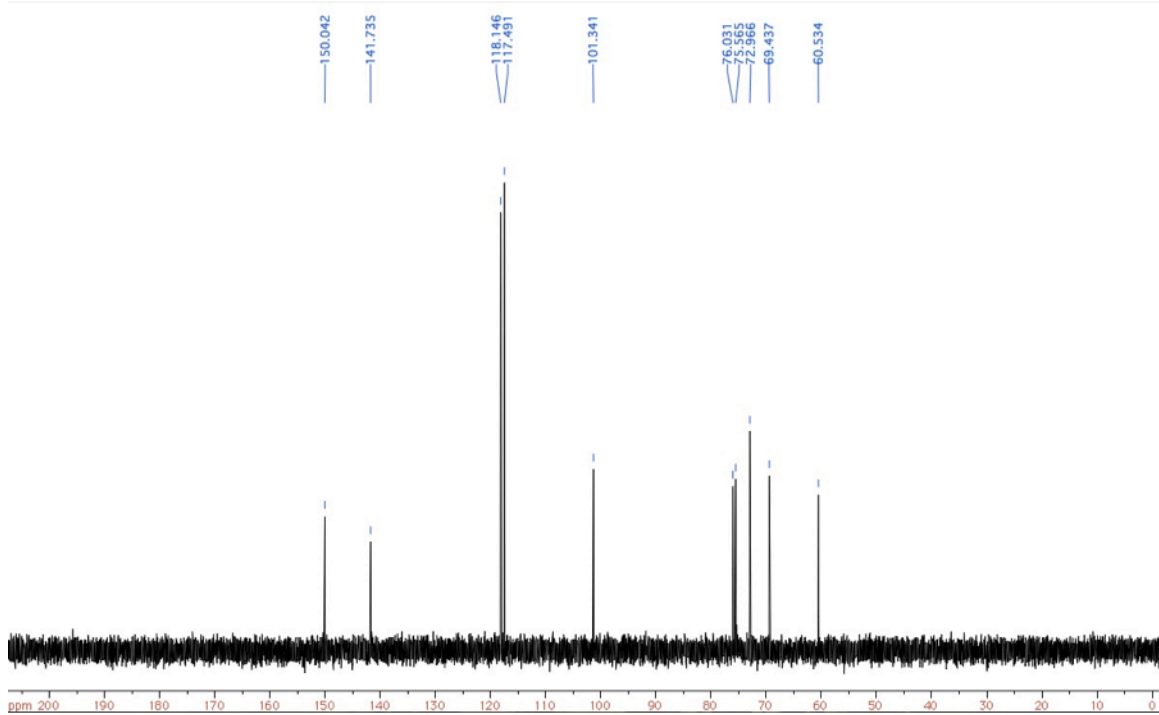
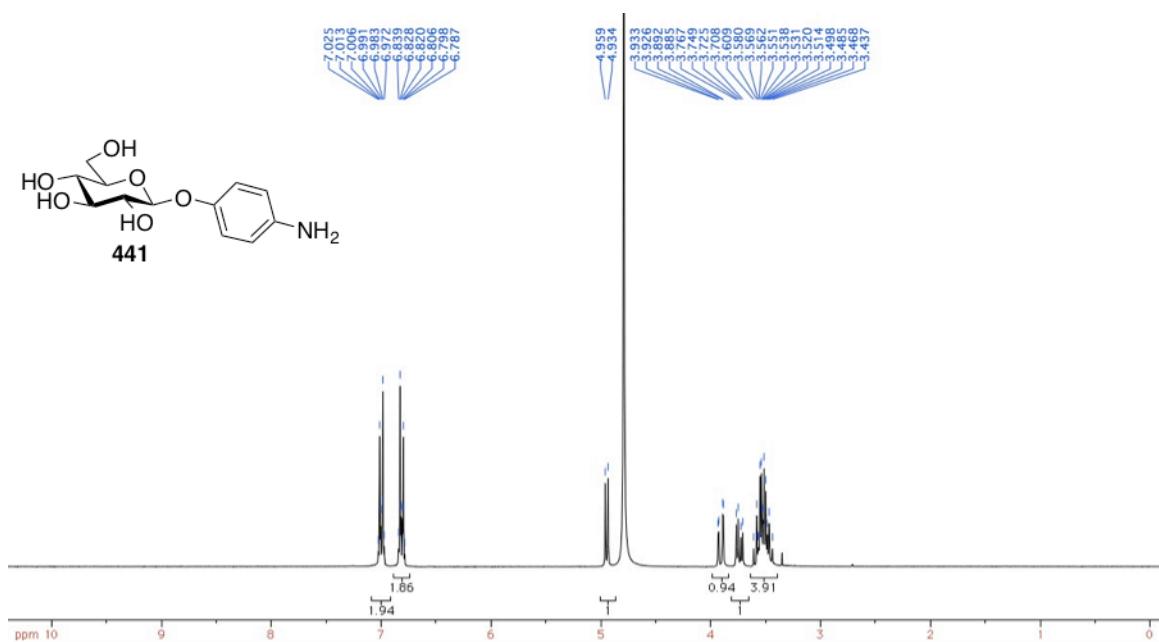


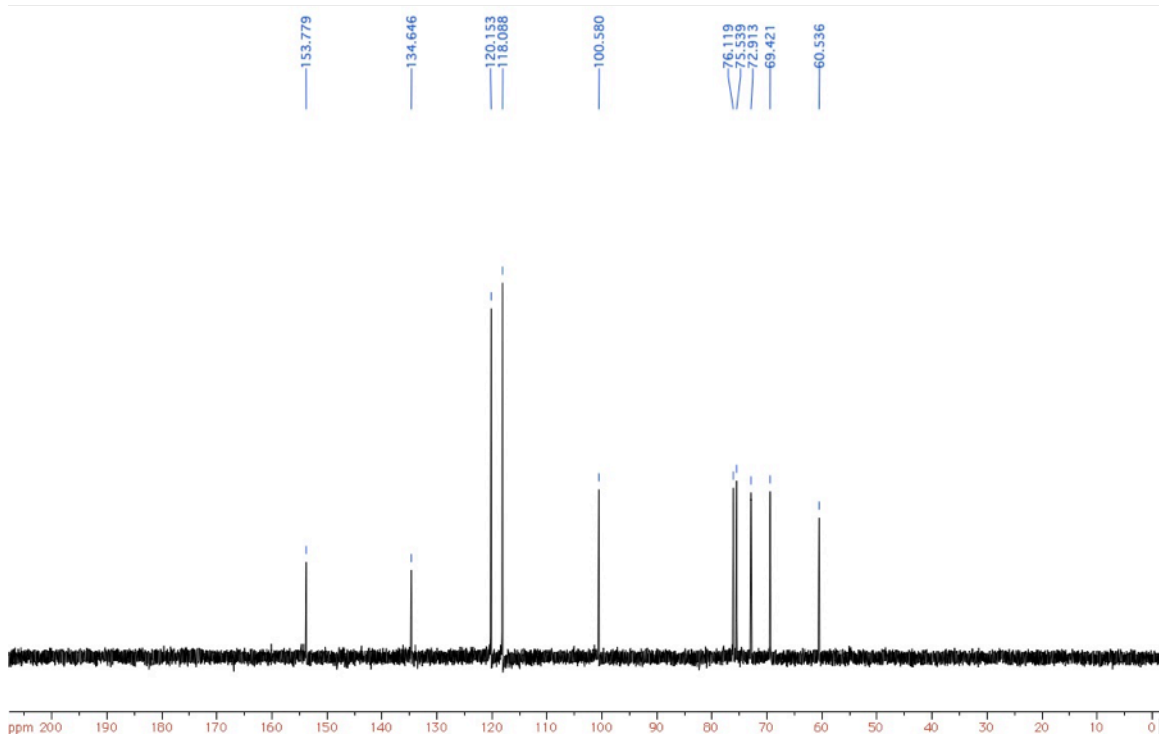
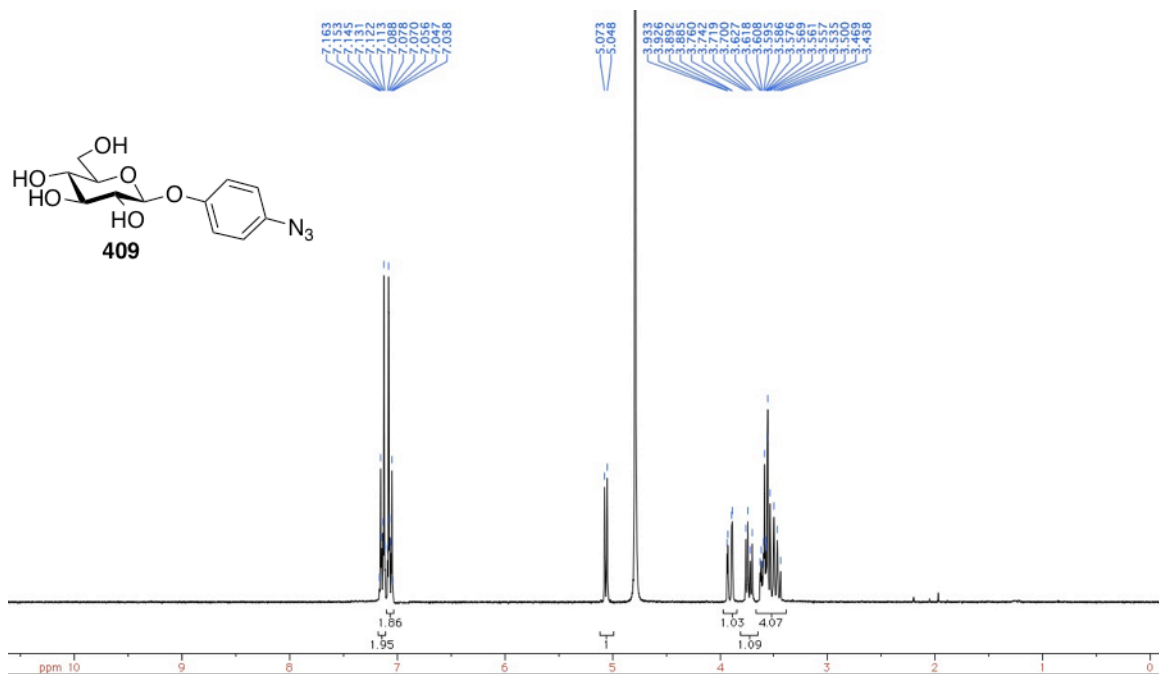


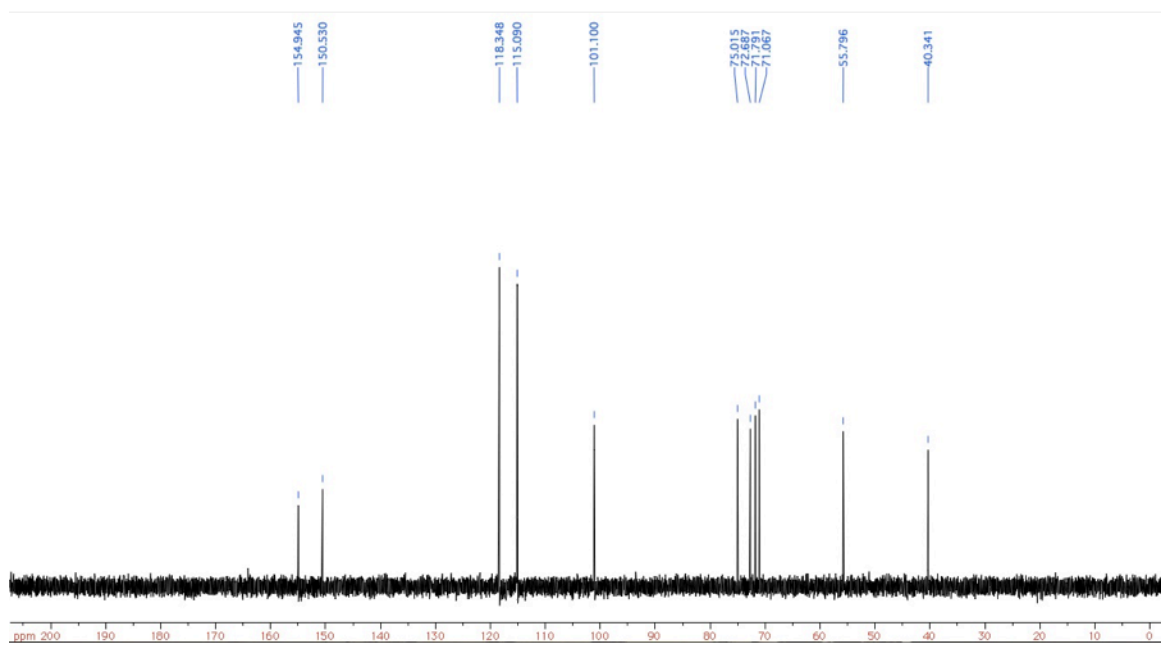
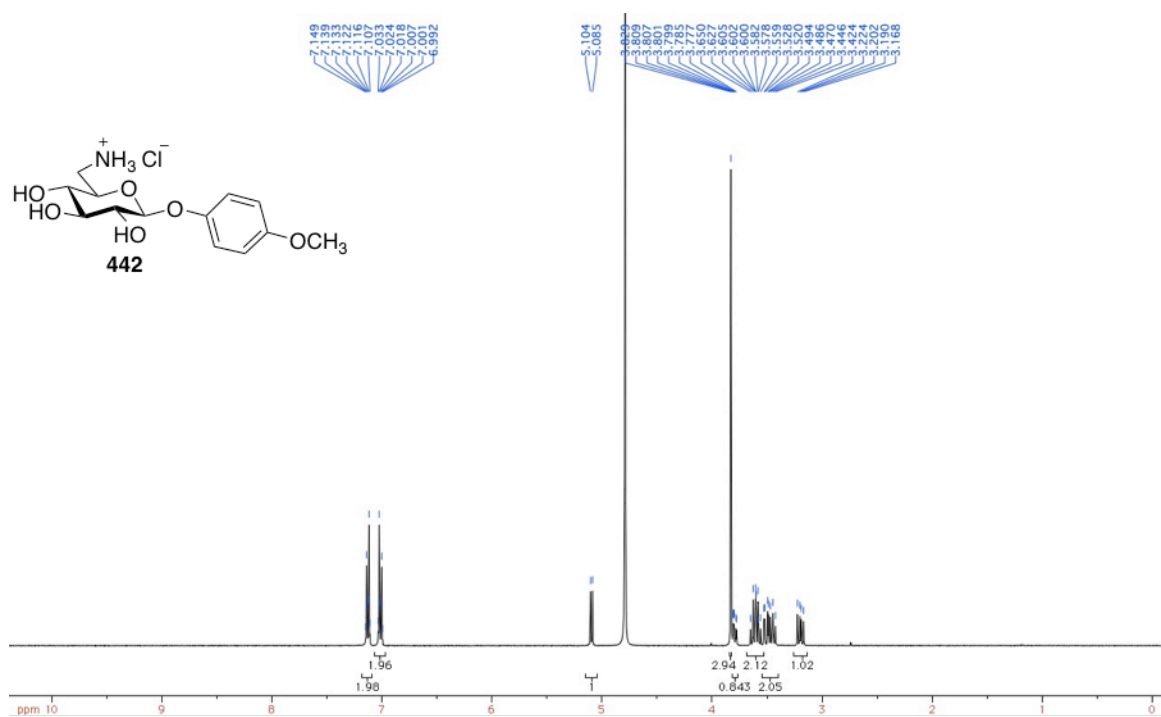


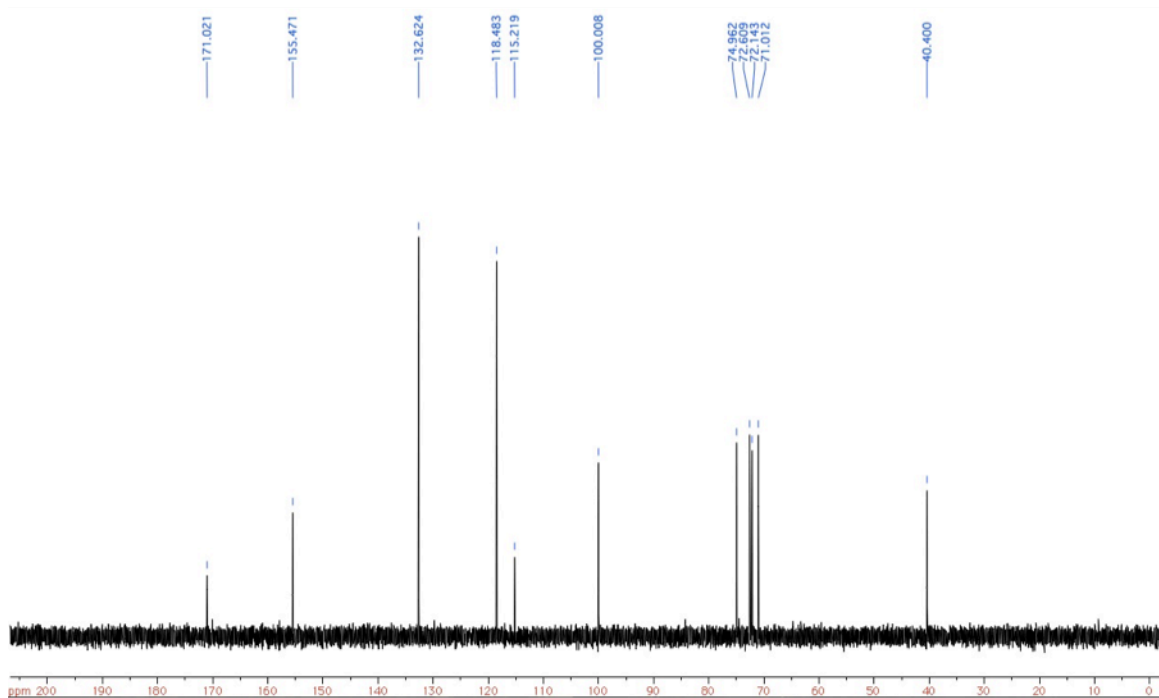
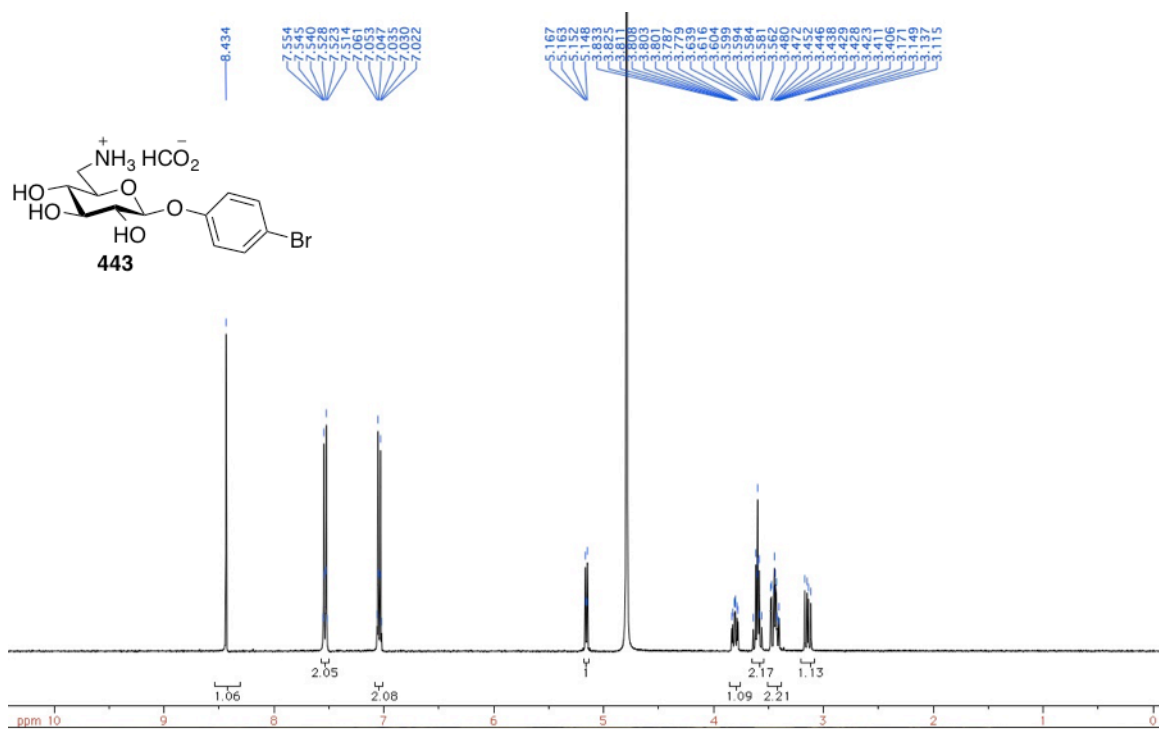


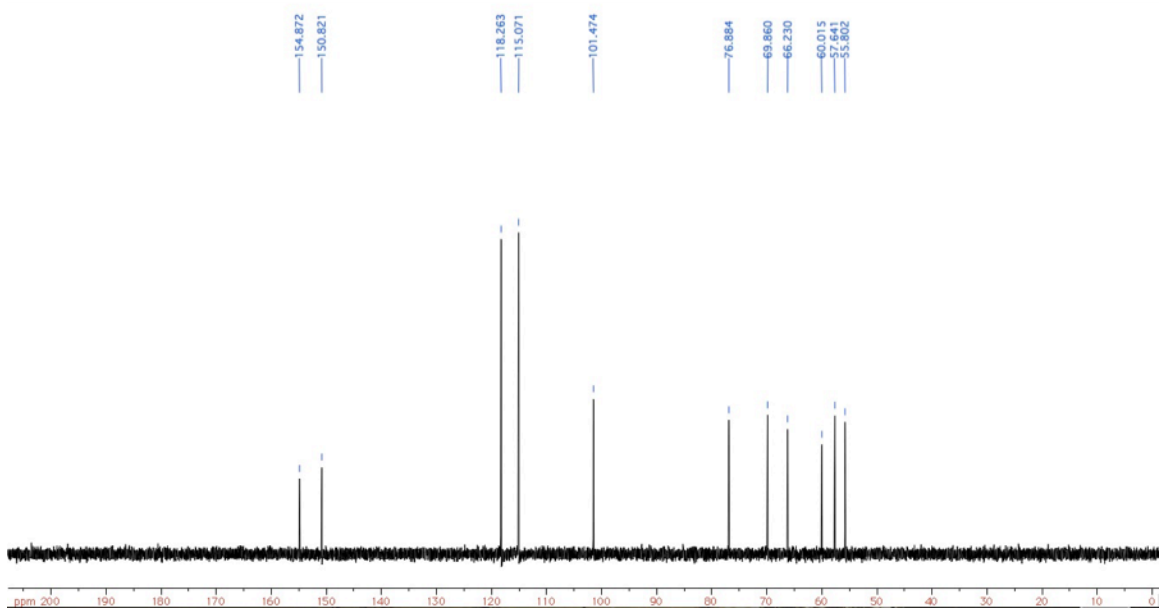
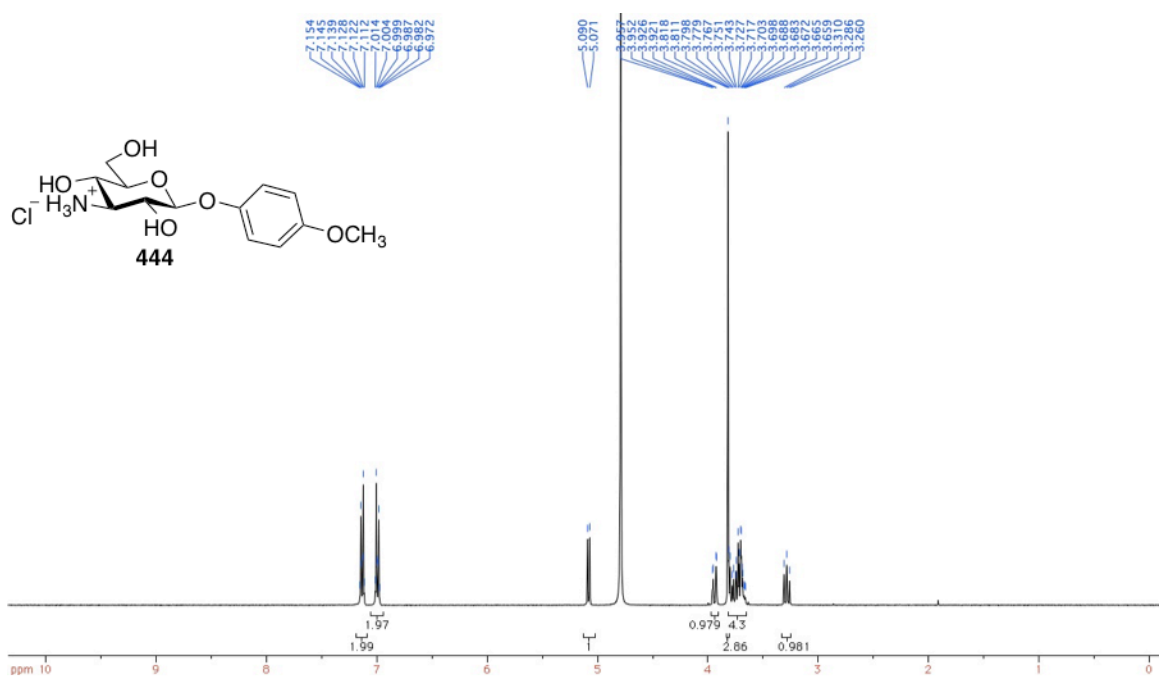


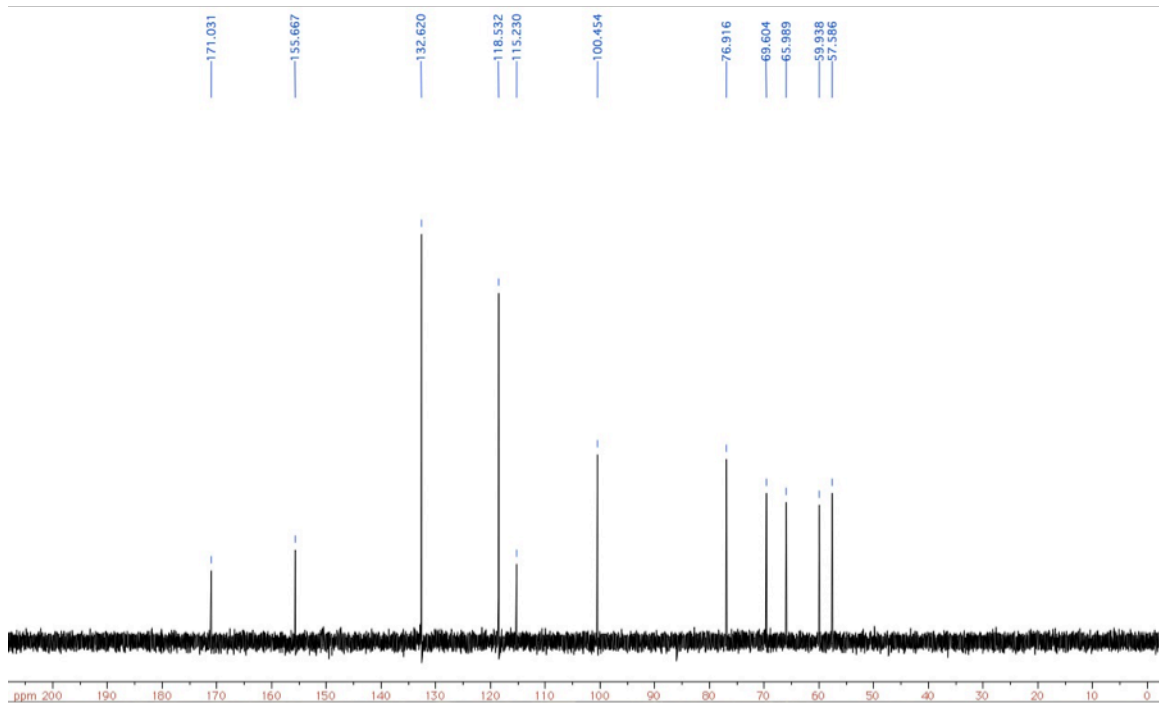
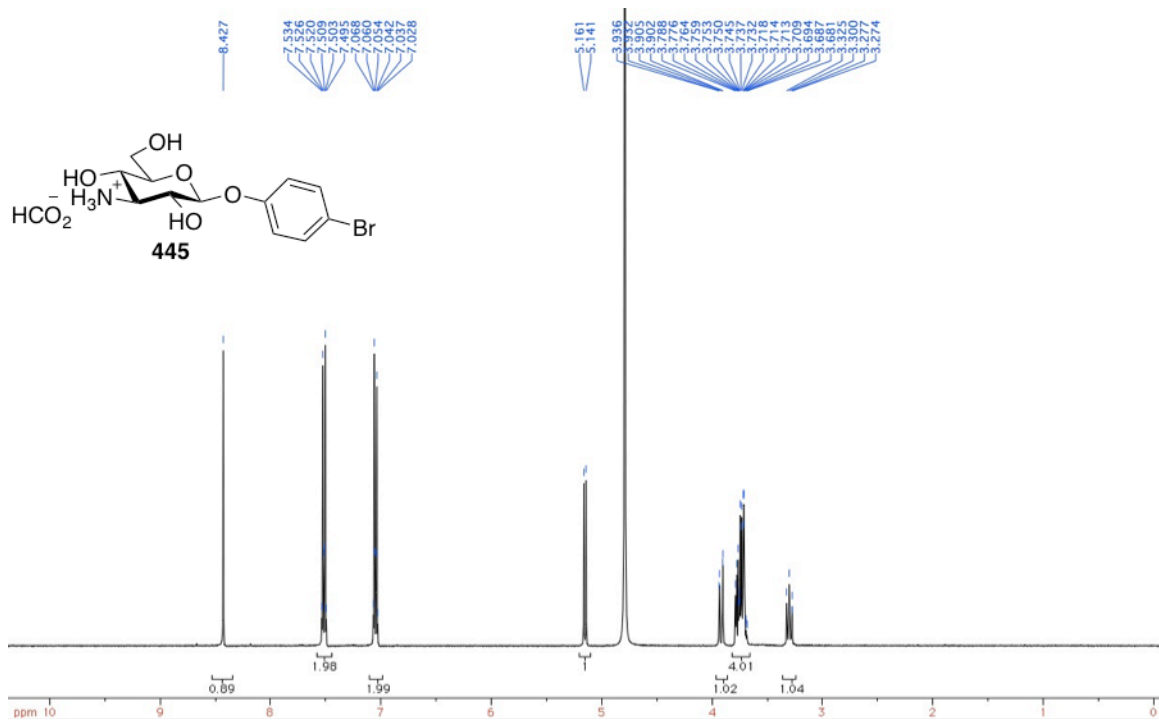












4.6 References

- (1) Tam, R. Y.; Ferreira, S. S.; Czechura, P.; Chaytor, J. L.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 17494.
- (2) Capicciotti, C. J.; Leclère, M.; Perras, F. A.; Bryce, D. L.; Paulin, H.; Harden, J.; Liu, Y.; Ben, R. N. *Chem. Sci.* **2012**, *3*, 1408.
- (3) Balcerzak, A. K.; Febbraro, M.; Ben, R. N. *RSC Adv.* **2013**, *3* (10), 3232.
- (4) Lorber, B.; Bishop, J. B.; DeLucas, L. J. *Biochim. Biophys. Acta* **1990**, *1023*, 254.
- (5) Michel, H.; Oesterhelt, D. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77* (3), 1283.
- (6) Capicciotti, C. J. The Rational Design of Potent Ice Recrystallization Inhibitors for Use as Novel Cryoprotectants, University of Ottawa, 2014.
- (7) Capicciotti, C. J.; Mancini, R. S.; Turner, T. R.; Koyama, T.; Alteen, M. G.; Doshi, M.; Inada, T.; Acker, J. P.; Ben, R. N. *ACS Omega* **2016**, *1* (4), 656.
- (8) Abraham, S.; Keillor, K.; Capicciotti, C. J.; Perley-Robertson, G. E.; Keillor, J. W.; Ben, R. N. *Cryst. Growth Des.* **2015**, *15* (10), 5034.
- (9) Capicciotti, C. J.; Kurach, J. D. R.; Turner, T. R.; Mancini, R. S.; Acker, J. P.; Ben, R. N. *Sci. Rep.* **2015**, *5*, 9692.
- (10) Bräse, S.; Gil, C.; Knepper, K.; Zimmermann, V. *Angew. Chem. Int. Ed.* **2005**, *44* (33), 5188.
- (11) Debets, M. F.; van der Doelen, C. W. J.; Rutjes, F. P. J. T.; van Delft, F. L. *ChemBioChem* **2010**, *11*, 1168.
- (12) Scriven, E. F. V.; Turnbull, K. *Chem. Rev.* **1988**, *88* (2), 297.
- (13) Beckmann, H. S. G.; Wittmann, V. Azides in Carbohydrate Chemistry. In: Bräse, S.; Banert, K., eds. *Organic Azides: Syntheses and Applications*. Chichester, UK: Wiley; 2010. P 469–490.
- (14) Nyffeler, P. T.; Liang, C.-H.; Koeller, K. M.; Wong, C.-H. *J. Am. Chem. Soc.* **2002**, *124* (36), 10773.
- (15) Potter, G. T.; Jayson, G. C.; Miller, G. J.; Gardiner, J. M. *J. Org. Chem.* **2016**, *81* (8), 3443.
- (16) Ma, Y.; Zhang, H.; Gruzdy, V.; Sun, X.-L. *Langmuir* **2011**, *27* (21), 13097.
- (17) Kim, E. J. *Molecules* **2018**, *23* (10), E2411.
- (18) Chakraborty, P. *J. Mol. Genet. Med.* **2018**, *12* (1), 332.
- (19) Huisgen, R. *Angew. Chem. Int. Ed.* **1963**, *2* (10), 565.
- (20) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287* (5460), 2007.
- (21) Kiick, K. L.; Saxon, E.; Tirrell, D. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99* (1), 19.
- (22) Dube, D. H.; Bertozzi, C. R. *Curr. Opin. Chem. Biol.* **2003**, *7* (5), 616.
- (23) Weller, R. L.; Rajski, S. R. *Org. Lett.* **2005**, *7* (11), 2141.
- (24) Lang, K.; Chin, J. W. *ACS Chem. Biol.* **2014**, *9* (1), 16.
- (25) Chakraborty, A.; Wang, D.; Ebright, Y. W.; Ebright, R. H. *Methods Enzym.* **2010**, *472*, 19.
- (26) Prescher, J. A.; Bertozzi, C. R. *Nat. Chem. Biol.* **2005**, *1*, 13.
- (27) Hang, H. C.; Yu, C.; Kato, D. L.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (25), 14846.
- (28) Chang, P. V.; Prescher, J. A.; Hangauer, M. J.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2007**, *129* (27), 8400.
- (29) Reist, E. J.; Spencer, R. R.; Calkins, D. F.; Baker, B. R.; Goodman, L. *J. Org.*

- Chem.* **1965**, 30 (7), 2312.
- (30) Williams, J. M.; Richardson, A. C. *Tetrahedron* **1967**, 23 (3), 1369.
 - (31) Dick, A. J.; Jones, J. K. N. *Can. J. Chem.* **1965**, 43, 977.
 - (32) Lei, Z.; Wang, J.; Mao, G.; Wen, Y.; Tian, Y.; Wu, H.; Li, Y.; Xu, H. *J. Agric. Food Chem.* **2014**, 62, 6065.
 - (33) Pandiakumar, A. K.; Sarma, S. P.; Samuelson, A. G. *Tetrahedron Lett.* **2014**, 55 (18), 2917.
 - (34) Goddard-Borger, E. D.; Stick, R. V. *Org. Lett.* **2007**, 9 (19), 3797.
 - (35) Schmidt, R. R. *Adv. Carbohydr. Chem. Biochem.* **1994**, 50, 21.
 - (36) Schmidt, R. R.; Toepfer, A. *Tetrahedron Lett.* **1991**, 32 (28), 3353.
 - (37) Schmidt, R. R. *Angew. Chem. Int. Ed.* **1986**, 25 (3), 212.
 - (38) Schmidt, R. R.; Michel, J. *Angew. Chem. Int. Ed.* **1980**, 19, 731.
 - (39) Barral, K.; Moorhouse, A. D.; Moses, J. E. *Org. Lett.* **2007**, 9 (9), 1809.
 - (40) Chaytor, J. L.; Tokarew, J. M.; Wu, K.; Leclère, M.; Tam, R. Y.; Capicciotti, J.; Guolla, L.; Moos, E. Von; Findlay, C. S.; Allan, D. S.; Ben, R. N. *Glycobiology* **2012**, 22 (1), 123.
 - (41) Mosmann, T. *J. Immunol. Methods* **1983**, 65 (1–2), 55.
 - (42) Liu, S.; Wang, W.; von Moos, E.; Jackman, J.; Mealing, G.; Monette, R.; Ben, R. N. *Biomacromolecules* **2007**, 8 (5), 1456.
 - (43) Berridge, M. V.; Herst, P. M.; Tan, A. S. *Biotechnol. Annu. Rev.* **2005**, 11, 127.
 - (44) Fung, K. L.; Tepede, A. K.; Pluchino, K. M.; Pouliot, L. M.; Pixley, J. N.; Hall, M. D.; Gottesman, M. M. *Mol. Pharm.* **2014**, 11, 2692.
 - (45) Zahedifard, M.; Faraj, F. L.; Paydar, M.; Looi, C. Y.; Hajrezaei, M.; Hasanpourghadi, M.; Kamalidehghan, B.; Majid, N. A.; Ali, H. M.; Abdulla, M. A. *Nat. Sci. Reports* **2015**, 5, 11544.
 - (46) Yellepeddi, V. K.; Vangara, K. K.; Kumar, A.; Palakurthi, S. *Anticancer Res.* **2012**, 32, 3651.
 - (47) Kwan, Y. P.; Saito, T.; Ibrahim, D.; Al-Hassan, F. M. S.; Oon, C. E.; Chen, Y.; Jothy, S. L.; Kanwar, J. R.; Sasidharan, S. *Pharm. Biol.* **2016**, 54 (7), 1223.
 - (48) Senthilraja, P.; Kathiresan, K. *J. Appl. Pharm. Sci.* **2015**, 5 (3), 80.
 - (49) Galluzzi, L.; Vitale, I.; Abrams, J. M.; Alnemri, E. S.; Baehrecke, E. H.; Blagosklonny, M. V.; Dawson, T. M.; Dawson, V. L.; El-Deiry, W. S. et al. *Cell Death Differ.* **2012**, 19, 107.
 - (50) Elmore, S. *Toxicol. Pathol.* **2007**, 35 (4), 495.
 - (51) Vucic, D.; Dixit, V. M.; Wertz, I. E. *Nat. Rev. Mol. Cell Biol.* **2011**, 12 (7), 439.
 - (52) Zhang, X. D.; Gillespie, S. K.; Hersey, P. *Mol. Cancer Ther.* **2004**, 3 (2), 187.
 - (53) Leffler, J. E.; Temple, R. D. *J. Am. Chem. Soc.* **1967**, 89 (20), 5235.
 - (54) Foye, W. O.; Stoye, L. E. *J. Am. Pharm. Assoc.* **1959**, 48 (4), 201.
 - (55) Baltzly, R.; Phillips, A. P. *J. Am. Chem. Soc.* **1946**, 68 (2), 261.
 - (56) Srinivasa, G. R.; Nalina, L.; Abiraj, K.; Gowda, D. C. *J. Chem. Res.* **2003**, 2003 (10), 630.
 - (57) Balcerzak, A. K. Elucidating the Key Structural Features of Carbohydrates and Surfactants Necessary for Inhibiting Ice Recrystallization, University of Ottawa, 2014.
 - (58) Sinha, S. K.; Brew, K. *Carbohydr. Res.* **1980**, 81, 239.
 - (59) Pedersen, C. M.; Olsen, J.; Brka, A. B.; Bols, M. *Chem. Eur. J.* **2011**, 17 (25),

7080.

- (60) Kawsar, S. M. A.; Islam, M. M.; Chowdhury, S. A.; Hasan, T.; Hossain, M. K.; Manchur, M. A.; Ozeki, Y. *Hacettepe J. Biol. Chem.* **2013**, *41* (3), 195.
- (61) Kakitsubata, Y.; Aramaki, R.; Nishioka, K.; Wakao, M.; Suda, Y. *Tetrahedron Lett.* **2016**, *57*, 1154.
- (62) Fleet, G. W. J.; Peach, J. M.; Smith, P. W.; Austin, G. N.; Baird, P. D.; Watkin, D. J. *Tetrahedron* **1987**, *43* (13), 3095.
- (63) Witzig, C.; Vasella, A.; Chiara, J.; Martin-lomas, M. *Helv. Chim. Acta* **1991**, *74*, 2073.

Chapter 5: Assessing the Cryopreservation Potential of Small Molecule Ice Recrystallization Inhibitors

5.1 Introduction

Cryopreservation is the process whereby biological samples such as cells and tissues are preserved by cooling to subzero temperatures (-78 °C or -196 °C).¹ It enables the storage of cells for 10-20 years depending on the cell type, and thus it is an attractive method for the long-term preservation of cellular products. However, cryopreserving cells while maintaining their viability is a very complex process that requires adequate sample volumes, optimal cooling and warming rates, and cryoprotective agents (CPAs) to maximize post-thaw viability and recovery.²⁻⁵ Many theories and mechanisms of cryoinjury have been proposed; however, the formation and recrystallization of intra- and extracellular ice is widely recognized as a significant contributor to cellular injury and death during cryopreservation.^{6,7} This has been extensively described by Peter Mazur in his two-factor hypothesis which states that each cell type has an optimal cooling and warming rate depending on the water permeability of the cell membrane.⁸ Under optimal cooling rates, the cell has time to sufficiently dehydrate, preventing injury from excessive loss of volume and extended exposure to high concentrations of electrolytes as well as intracellular ice formation.

Cryoprotective agents are employed to mitigate cellular damage and ensure the survival of cells during cryopreservation. There are two classes of CPAs that are typically utilized, permeating or non-permeating agents, depending on the cell type and cooling rate.^{9,10} Permeating CPAs such as dimethyl sulfoxide (DMSO) and glycerol are the most widely used agents for the cryopreservation of clinical products. Although these CPAs ensure high post-thaw cell viability and recovery, the concentration of DMSO used to preserve cells is highly toxic to both cells and patients,¹¹⁻¹³ thus complicating the direct clinical use of frozen-thawed cells. While DMSO can be removed from thawed cells prior to use, the process is complex and time-consuming and it has been shown to be associated with damaging osmotic shock, resulting in cell loss.^{14,15} In the case of glycerol, it is required for successful cryopreservation of red blood cells (RBCs); however, it must be removed post-thaw to avoid intravascular hemolysis.^{5,16} The high concentration of glycerol employed is a significant impediment for routine clinical use owing to the costly

and time-consuming deglycerolization process. Consequently, there is a need to improve current CPAs and cryopreservation protocols to reduce or eliminate the use of DMSO or glycerol.

Given that a significant amount of cells experience cellular injury during cryopreservation due to ice formation and recrystallization,^{6,17} interest has arisen in designing inhibitors of ice recrystallization for use as novel CPAs. Unlike DMSO and glycerol that do not have the ability to control or inhibit ice recrystallization, it is hypothesized that ice recrystallization inhibitors (IRIs) will be beneficial additives for cryopreservation that can increase post-thaw cell viability and recovery, and thus the quantities of DMSO or glycerol may be reduced.

Our laboratory has previously reported that *O*-aryl glucosides, β -PMP-Glc (**103**) and β -pBrPh-Glc (**104**), are effective inhibitors of ice recrystallization and can function as beneficial additives for the freezing of human RBCs.^{18,19} Furthermore, Chapter 4 identified a novel class of small molecule IRIs that are azide derivatives. The structures are provided in Figure 5.1. Since these molecules were shown to be effective inhibitors of ice recrystallization (i.e. $IC_{50} < 50$ mM), they have great potential for use as novel CPAs to improve upon current cryopreservation protocols. In addition to assessing IRI activity, some of the IRI active azido aryl glucosides were also examined for cytotoxic effects in HepG2 cells and it was demonstrated that there was a significant decrease in cell viability following long-term exposure to **405**, **406** and **409** at 37 °C. However, it is important to emphasize that the toxic response varies depending on cell type. In other words, the cytotoxic effects exhibited by **405**, **406** and **409** in HepG2 cells may not necessarily translate to other types of cells such as RBCs. Thus, prior to performing cryopreservation studies utilizing RBCs, the toxicity of **405**, **406** and **409** will be evaluated to ensure that these compounds do not cause a significant amount of cellular damage in the absence of freezing. The cryopreservation potential of the following molecules (**405**, **406** and **409**, Figure 5.1) was explored in RBCs obtained from human peripheral blood to determine if they can be effective cryo-additives.

In addition to RBCs, the cryopreservation of stem cells such as mesenchymal stem cells (MSCs) utilizing small molecule IRIs as additives was also explored. In particular, given that β -pBrPh-Glc (**104**) is a very effective additive for the freezing of human

RBCs,¹⁹ its cryopreservation potential was assessed in MSCs. Conversely, IRI active azide derivatives (**405**, **406** and **409**) were not explored in MSCs due to their lengthy chemical syntheses that are not amenable to large-scale preparations necessary for cryopreservation applications. Additionally, it has been previously demonstrated by our laboratory that *N*-(2-fluorophenyl)-D-gluconamide (2FA (**501**), Figure 5.1) is an effective inhibitor of ice recrystallization with the ability to function as a beneficial additive for the freezing of hematopoietic stem and progenitor cells (HSPCs) from umbilical cord blood (UCB).²⁰ Consequently, we sought to examine the ability of **501** (2FA) to protect other cellular products such as MSCs obtained from human bone marrow.

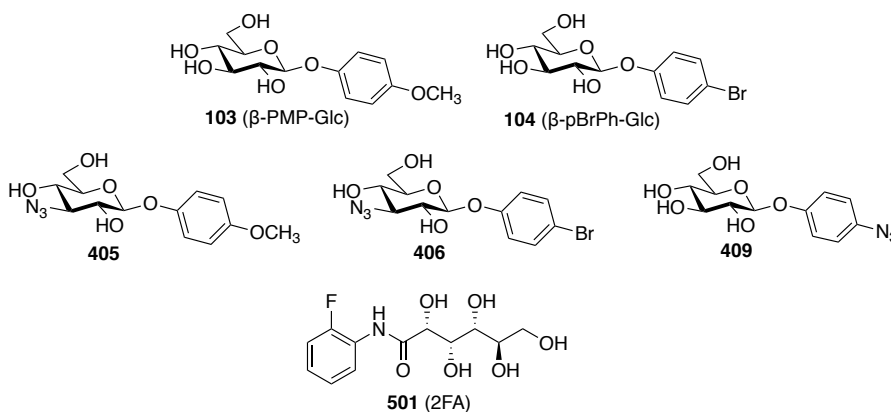


Figure 5.1 Structures of IRI active aryl glucosides **103** (β-PMP-Glc) and **104** (β-pBrPh-Glc), azido aryl glucosides (**405**, **406** and **409**) and *N*-aryl-D-aldoamide **501** (2FA).

5.2 Cryopreservation Ability of Small Molecule IRIs with Human RBCs

5.2.1 Introduction – Cryopreservation of Human RBCs

Red blood cells (RBCs) are the most frequently transfused blood product with approximately 85 million RBC units transfused annually worldwide.²¹ Blood transfusions are a common form of cellular therapy used for surgeries or cancer treatments, for patients with symptomatic anemia and for acute to severe blood loss.^{22,23} Thus, the need for a readily accessible supply of RBCs is very high. RBC units are typically stored at 1 to 6 °C in a refrigerator for up to 42 days.^{5,24,25} The limited storage time can lead to episodic shortages in blood supply and the disposal of outdated blood. In addition, the quality of stored RBCs significantly deteriorates beyond this period as a result of storage lesions that affect the function and *in vivo* survival of transfused RBCs.^{26–28}

Cryopreservation is the only current technology that can provide long-term storage of blood products. This preservation method can extend storage time to at least 10 years^{29,30} and is primarily used to store RBCs for military combat casualty care and to maintain an inventory of autologous RBCs and RBCs with rare phenotypes.^{16,30,31} One of the main reasons this preservation method is not routinely utilized in the clinical setting as a method to manage blood supply is that current cryopreservation protocols employ glycerol as a permeating CPA, which does not allow the direct transfusion of RBCs post-thaw. After thawing, deglycerolization is required to ensure an intracellular glycerol concentration of less than 1% prior to transfusion to avoid intravascular hemolysis.^{5,16,32} The deglycerolization process is time-consuming and less-than-ideal in emergency transfusions where large quantities of RBC units are required in a short period of time. As a result, improvements to the current protocol, such as reducing post-thaw processing times, are necessary in order to provide access to more readily available RBC units.

There are currently two methods that are clinically used for the cryopreservation of RBCs. In Europe, blood banks typically use low concentrations of glycerol (15-20% w/v) with rapid cooling rates (> 100 °C/min) prior to storage in liquid nitrogen.^{33,34} In Canada and the United States, clinical cryopreservation protocols utilize high concentrations of glycerol (40% w/v) with slowing cooling rates (1 °C/min) followed by storage at ≤ -65 °C in a mechanical freezer.^{32,35} While these methods ensure high RBC recoveries, post-thaw processing is required in each case to adequately remove the glycerol. However, the deglycerolization process for the low glycerol method is simpler and takes half the amount of time to deglycerolize a unit compared to the high glycerol method.²⁹ Consequently, in Canada and the United States, great interest has arisen in developing methods that allow for a reduction in the concentration of glycerol to decrease post-thaw processing times without drastically altering the current clinical cryopreservation protocols.

Improvements to the cryopreservation protocol for RBCs have focused on several different aspects including improving the current deglycerolization procedures to allow frozen RBCs to be rapidly prepared for emergency transfusions. Current deglycerolization methods are time-consuming due to limitations on how quickly the glycerol can be removed without causing significant cellular damage. Recently, it was

demonstrated that glycerol could be removed from frozen-thawed RBCs in as little as three minutes with minimal hemolysis using a mathematically optimized three-step removal procedure.³⁶ However, this rapid deglycerolization process required careful control over the composition of the extracellular solution at a time scale of seconds, which is not practical for large-scale use.³⁶ Thus, recent research has focused on designing microfluidic devices for rapid glycerol removal from full RBC units.³⁷

Alternative CPAs have also been explored including non-permeating agents such as hydroxyethyl starch (HES), polyvinylpyrrolidone (PVP), dextran and trehalose.^{5,38–41} Recently, it was reported that RBCs could be cryopreserved using a combination of HES and poly (vinyl alcohol) (PVA) to obtain high cell recoveries, while eliminating the need for glycerol.⁴² Furthermore, it was also demonstrated that highly hydroxylated block copolymer worms were a suitable replacement for HES, and in combination with PVA could result in high recovery.⁴³ However, these methods require rapid cooling rates to achieve high RBC recovery, which are less economical. The application of trehalose as a CPA for RBC cryopreservation has also been recently explored and these studies focus on methods for the intracellular delivery of trehalose into RBCs.^{44–47}

The use of small molecule IRIs as novel CPAs for various cell types has been an active area of research in our laboratory. It has been previously demonstrated that human RBCs could be cryopreserved utilizing reduced concentrations of glycerol (15% versus 40%) in the presence of small molecule IRIs while using current clinical cryopreservation protocols.¹⁹ This result was significant as reducing the quantity of glycerol can decrease post-thaw processing times, enabling frozen RBCs units to be more readily accessible. The details of this work are further described in Section 5.2.2. All of the RBC cryopreservation work was done in collaboration with Dr. Jason Acker's laboratory at Canadian Blood Services (CBS) in Edmonton, Alberta.

5.2.2 Cryopreservation of RBCs using β -PMP-Glc and β -pBrPh-Glc in the Presence of 15% Glycerol

Previous work by our laboratory has shown that two *O*-aryl glucosides, β -PMP-Glc (**103**) and β -pBrPh-Glc (**104**), are beneficial additives for the freezing of human RBCs with the ability to increase the percentage of intact RBCs post-thaw in the presence of reduced concentrations of glycerol (15%) and slow cooling rates.¹⁹ In these initial studies,

RBCs were incubated for 10 minutes at room temperature with 15% glycerol or 15% glycerol containing **103** (110 mM) or **104** (30 mM). Samples (300 μ L volume) were cooled to -5 $^{\circ}$ C before nucleation was induced. Controlled nucleation was performed to ensure that ice nucleation occurs at the same subzero temperature in each vial. The samples were then cooled to -40 $^{\circ}$ C (rate of 1 $^{\circ}$ C/min) and upon stabilization at -40 $^{\circ}$ C, samples were rapidly thawed in a 37 $^{\circ}$ C water bath. RBC hemolysis was measured and the results are reported as the percentage of intact RBCs post-thaw (100 - % hemolysis). In the absence of **103** or **104**, the percentage of intact RBCs post-thaw with the 15% glycerol solution was approximately 40%. Remarkably, the addition of **103** (110 mM) or **104** (30 mM) to a solution of 15% glycerol significantly increased the percentage of intact RBCs post-thaw to approximately 70% and 80%, respectively (Figure 5.2). A concentration scan was previously performed and the optimal concentrations of **103** and **104** for the freezing of RBCs were found to be 110 mM and 30 mM, respectively.⁴⁸

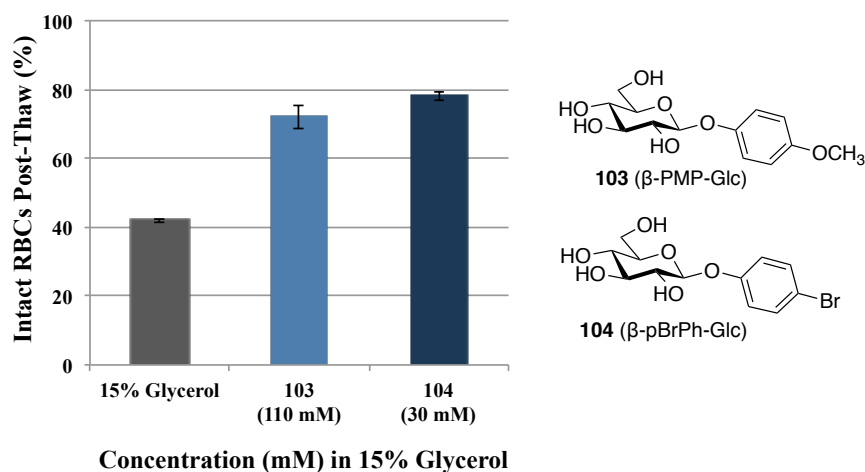


Figure 5.2 Percentage of intact RBCs post-thaw using 15% glycerol in combination with **103** (110 mM) or **104** (30 mM). Samples were frozen at a rate 1 $^{\circ}$ C/min to -40 $^{\circ}$ C and thawed at 37 $^{\circ}$ C in a water bath. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

Given that **103** and **104** could function as beneficial additives for the freezing of RBCs with significantly reduced concentrations of glycerol, deglycerolization conditions were explored. The removal of glycerol is a time-consuming process due to limitations on how quickly glycerol can be removed from frozen-thawed RBCs without causing significant cellular damage.³⁶ The addition and removal of glycerol results in large

osmotic gradients that can lead to cell volume changes, which can be damaging.^{36,49–51} RBCs experience hemolysis if they swell beyond a maximal volume or if they are returned to isotonic conditions after shrinking below a minimal volume.^{36,52,53} The high concentrations of glycerol (40%) utilized for RBC cryopreservation in Canada and the United States requires multistep deglycerolization protocols to minimize the amount of hemolysis caused by osmotic stress.^{32,36,54} The most commonly used method for the removal of glycerol employs an initial wash with 12% saline, an intermediate wash with 1.6% saline and a final wash with 0.9% saline (isotonic).^{36,55}

Previous attempts to deglycerolize RBCs in the presence of **103** or **104** employed washing conditions typically utilized for the low glycerol method.³⁴ However, a significant amount of hemolysis was observed during deglycerolization.⁴⁸ Since it was evident that the deglycerolization protocol for the low glycerol method did not translate to the cryosolutions containing **103** or **104** in 15% glycerol, the osmotic tolerance of RBCs suspended in a cryosolution containing **103** or **104** in 15% glycerol was previously examined to aid in determining the gradient steps of washing solutions that are tolerated and can be used to deglycerolize the RBCs (described in Section 5.2.4).⁴⁸ The RBC recoveries for samples (1 mL volume) that were frozen by placing into dry ice (-78 °C) and deglycerolized using the washing conditions determined from the osmotic tolerance experiments (wash 1 = 5% saline, wash 2 = 1.5% saline and wash 3 = 0.9% saline) are presented in Figure 5.3. The RBC recoveries were quite low in the presence of **103** or **104**, resulting in less than 20% using this method. Ultimately, the deglycerolization of RBCs containing **103** or **104** in 15% glycerol is problematic, resulting in a significant amount of hemolysis.

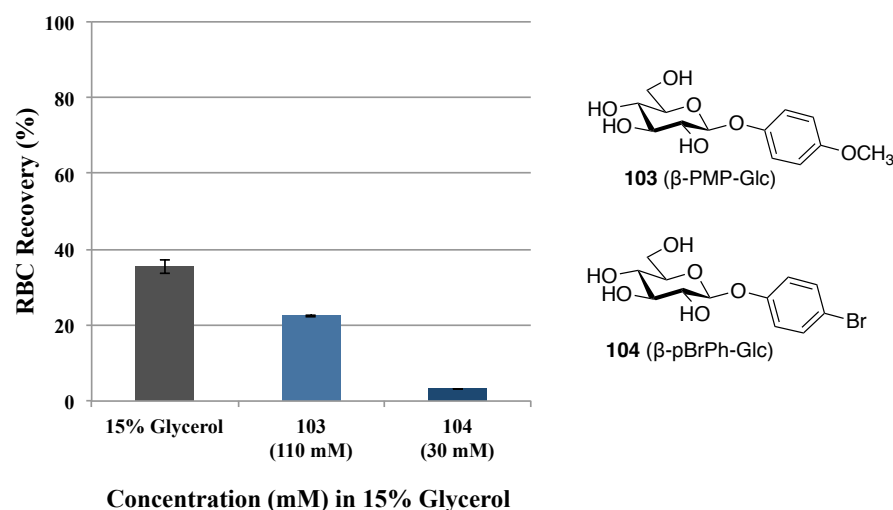


Figure 5.3 Percentage RBC recovery using 15% glycerol in combination with **103** (110 mM) or **104** (30 mM). Samples were frozen by placing into dry ice ($-78\text{ }^{\circ}\text{C}$) and thawed at $37\text{ }^{\circ}\text{C}$ in a water bath. The samples were deglycerolized using wash solutions determined from the osmotic tolerance experiments. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

5.2.2.1 Removal of Excess Cryosolution Pre-Freeze

In an attempt to improve the deglycerolization process, the excess cryosolution was removed prior to freezing to determine whether this could facilitate CPA removal. It was hypothesized that removal of the excess cryosolution containing **103** or **104** may aid in mitigating osmotic imbalances during freezing and may result in less cellular damage during deglycerolization.

In this study, RBCs were suspended in a cryosolution containing 15% glycerol or 15% glycerol + **103** (110 mM) or **104** (30 mM). The RBC samples (1 mL volume) were then centrifuged and the excess cryosolution was removed prior to freezing (“Cryosolution Removal Pre-Freeze”). The samples were frozen rapidly by placing into dry ice ($-78\text{ }^{\circ}\text{C}$) or liquid nitrogen ($-196\text{ }^{\circ}\text{C}$) to ensure that immediate post-thaw hemolysis was low, affording the greatest quantity of RBCs proceeding through the deglycerolization process. The samples were subsequently thawed in a $37\text{ }^{\circ}\text{C}$ water bath. A control experiment was performed in parallel in which the excess cryosolution was removed after thawing (“Cryosolution Removal Post-Thaw”). RBCs were deglycerolized

using the washing conditions previously determined from the osmotic tolerance experiments (wash 1 = 5% saline, wash 2 = 1.5% saline and wash 3 = 0.9% saline).⁴⁸ Following deglycerolization, RBC hemolysis was measured and the results are reported as the percentage of intact RBCs (Figure 5.4).

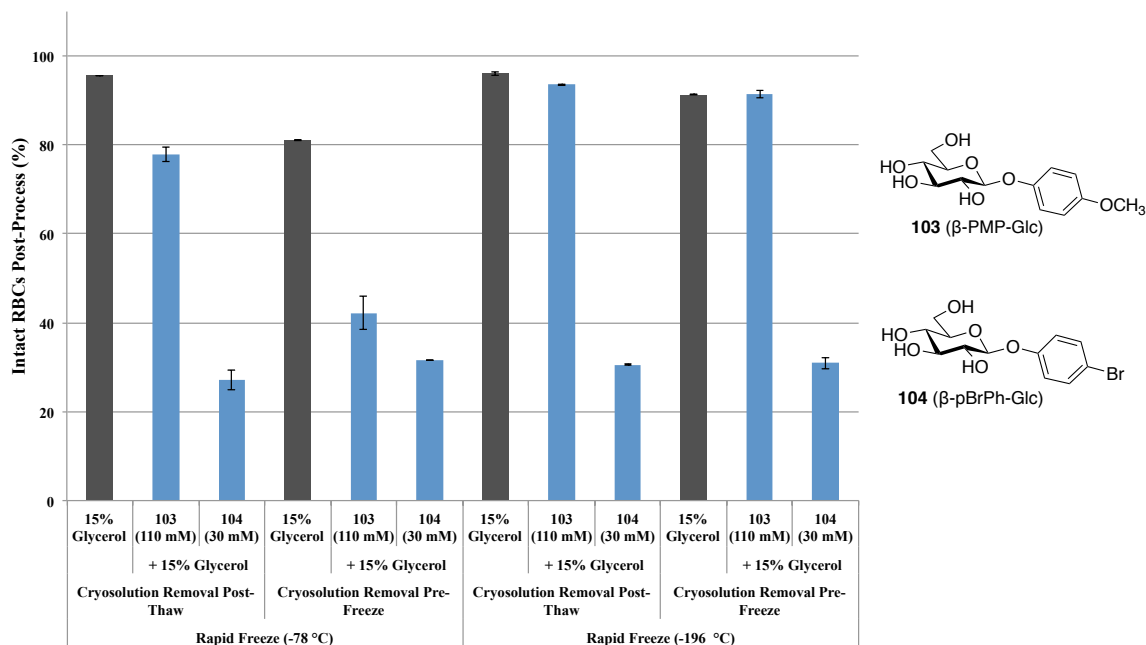


Figure 5.4 Percentage of intact RBCs post-process using 15% glycerol in combination with **103** (110 mM) or **104** (30 mM). Excess cryosolution was removed prior to freezing.

Samples were frozen by placing into dry ice (-78 °C) or liquid nitrogen (-196 °C) and thawed at 37 °C in a water bath. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

Since rapid freezing rates are required for the low glycerol cryopreservation method,³⁴ a greater number of intact RBCs would be expected for RBCs frozen in liquid nitrogen. The freezing rate for RBC samples that are frozen by placing into dry ice is likely slower than liquid nitrogen and this is reflected by the different percentage of intact RBCs. Under rapid freezing conditions to -196 °C, the percentage of intact RBCs remains unchanged in the presence of **103** or **104** when the excess cryosolution is removed prior to freezing. However, under slower freezing conditions to -78 °C, the percentage of intact RBCs decreases in the presence of **103** when the excess cryosolution is removed prior to freezing. This may suggest that excess amounts of **103** in the extracellular solution confer additional protection during freezing. During deglycerolization, after each wash the

supernatant was removed and the amount of hemoglobin (Hgb) in the supernatant was quantified spectrophotometrically using Drabkin's assay.^{56,57} The amount of Hgb in the supernatants was used to evaluate the extent of cellular damage that occurred post-thaw and through each of the washes. The results are reported as the percentage of Hgb lost in each wash step relative to the total amount of Hgb in each sample. The results are presented in Figure 5.5. It is evident that a large amount of Hgb (> 40%) was lost in the wash 3 supernatant for **104** in 15% glycerol for all conditions, indicating that a great amount of RBCs were hemolyzed in the third wash step.

Overall, the results indicate that removal of the excess cryosolution prior to freezing does not facilitate CPA removal. A decrease in the percentage of intact RBCs post-process was observed in the presence of **103** when the excess cryosolution was removed prior to freezing. Furthermore, the highest percentage of hemoglobin was lost in the wash 3 supernatant for both **103** and **104** for all conditions, suggesting that the step-down in osmolality between wash 2 and wash 3 cannot be tolerated, causing hemolysis. Although an additional wash step could be employed to provide a more gradual gradient in osmolality changes, this would be undesirable as it would lengthen the deglycerolization process.

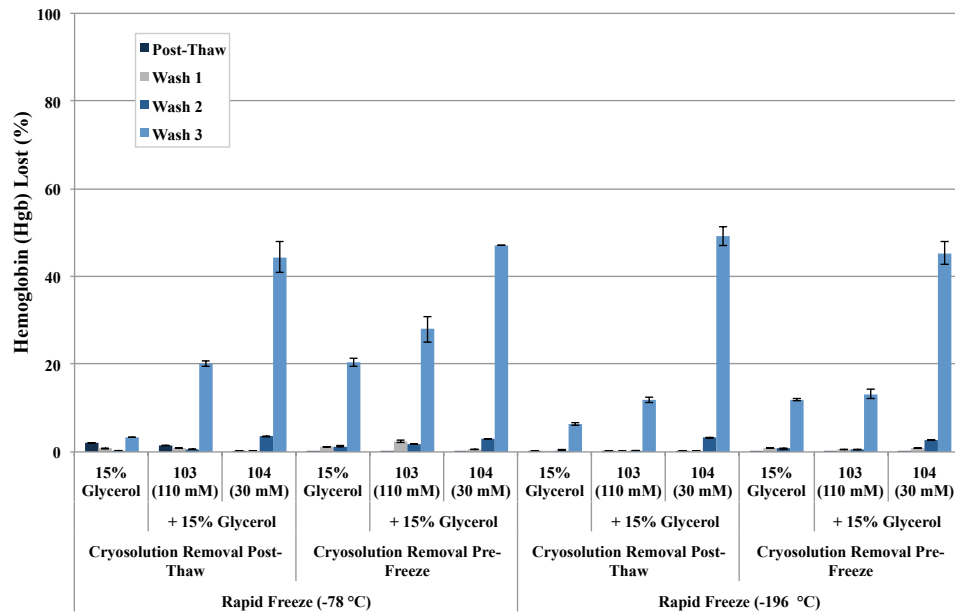


Figure 5.5 Percentage of hemoglobin lost post-thaw and in each wash step during the deglycerolization process when the excess cryosolution was removed prior to freezing.

These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

5.2.3 Cryopreservation of RBCs using β -PMP-Glc and β -pBrPh-Glc in the Presence of Hydroxyethyl Starch

Since the deglycerolization of RBCs containing **103** or **104** in 15% glycerol remained problematic, we sought to explore an alternative approach using hydroxyethyl starch (HES) (a non-permeating CPA). HES is an effective CPA for the cryopreservation of RBCs with the added benefit that its removal from thawed RBCs is not required prior to transfusion.^{5,29,38,58,59} Since HES is degraded in the bloodstream by α -amylase,⁶⁰ it can be directly administered with RBCs,⁶¹ thus eliminating the need for post-thaw processing. Ultimately, this method would eliminate the problems associated with glycerol-preserved RBCs such as osmotic stress that is incurred during the addition and removal of glycerol. In addition to its use as a CPA, HES is the main component in plasma expanders used for the treatment of hypovolemia (low blood volume).⁶² However, since HES is a non-permeating CPA, ultra-fast cooling rates and extremely low storage temperatures (-165 °C or -195 °C) are required to ensure high RBC recovery.^{63–65} Therefore, it was hypothesized that the addition of **103** or **104** to the HES solution could permit the use of slower cooling rates while maintaining high RBC recoveries.

To investigate this, the original protocol for the cryopreservation of RBCs with HES described by Sputtek *et al.* was utilized.⁶⁵ RBCs were suspended in the cryosolution containing HES (11.5% w/w [200/0.5]) supplemented with either **103** (110 mM) or **104** (30 mM). The concentrations selected for each compound were based on the optimal concentrations for the freezing of RBCs using 15% glycerol. The samples (1 mL volume) were cooled rapidly to -196 °C (rate of 115 °C/min) or -78 °C (rate of 5 °C/min) by placing the samples directly into liquid nitrogen or dry ice, or cooled slowly by placement in a -80 °C mechanical freezer (rate of ~2 °C/min). Samples were rapidly thawed at 48 °C in a water bath, diluted with 0.9% saline, and RBC hemolysis was measured using the Drabkin's assay.^{56,57} The results are reported as the percentage of intact RBCs post-thaw (100 - % hemolysis) and shown in Figure 5.6.⁶⁶

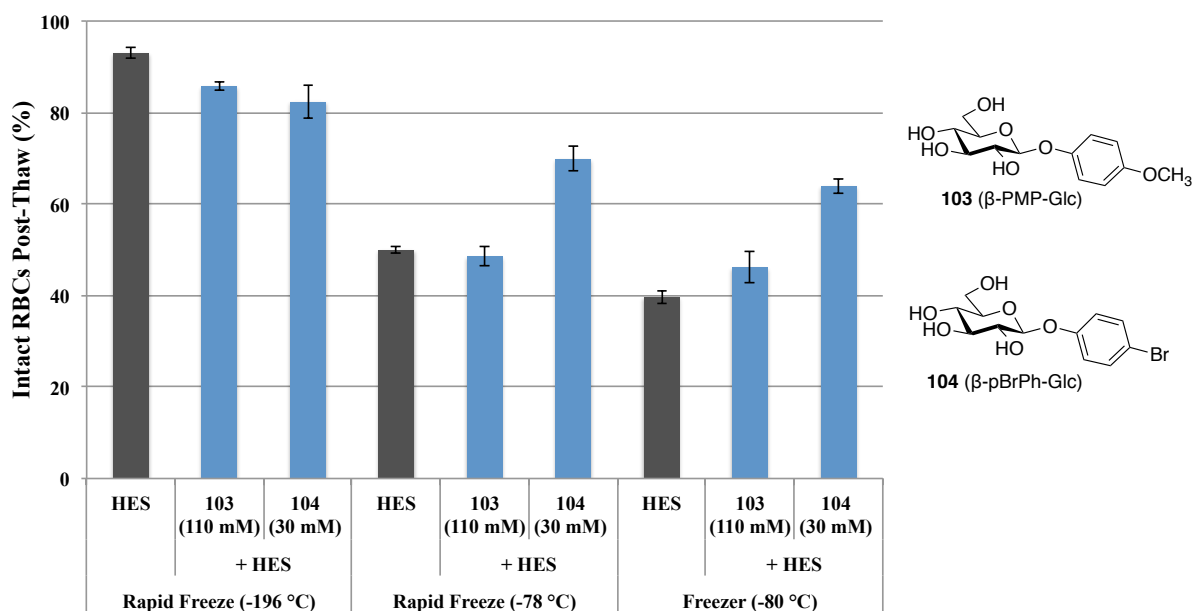


Figure 5.6 Percentage of intact RBCs post-thaw using HES (11.5% w/w in 30 mM NaCl) in combination with **103** (110 mM) or **104** (30 mM). Samples were frozen by placing in liquid nitrogen (-196 °C), dry ice (-78 °C) or a -80 °C freezer and thawed at 48 °C in a water bath. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

Rapid cooling to -196 °C resulted in a high percentage of intact RBCs post-thaw for all cryosolutions (Figure 5.6). This was expected because the cryopreservation of RBCs using HES as a CPA requires fast cooling rates.⁶⁷ In contrast, when the samples were cooled to -78 °C in dry ice, the percentage of intact RBCs with 11.5% HES alone decreased dramatically (from 93% to 50% intact RBCs). This was attributed to the slower cooling rate. The addition of **103** to the HES cryosolution did not improve the percentage of intact RBCs compared to the control. Remarkably, a 30 mM solution of **104** in HES resulted in a 20% increase in the percentage of intact RBCs compared to the HES control. When utilizing even slower cooling rates, which were achieved by placing the samples directly into a -80 °C mechanical freezer, a higher percentage of intact RBCs was observed when **104** was present in solution compared to the HES control. This indicated that **104** was effective at increasing the percentage of intact RBCs post-thaw, compared to the HES control, when slower cooling rates were utilized.⁶⁶

Since a 30 mM solution of **104** in HES could significantly increase the percentage of intact RBCs post-thaw, we then sought to examine the cell recovery post-thaw. RBC

recovery was calculated based on changes in RBC volume, hematocrit, and specific gravity^{68,69} and quantifies the number of RBCs that are recovered after thawing. As depicted in Figure 5.7, the addition of **103** did not improve RBC recovery compared to the HES control when frozen by placing into dry ice (-78 °C) or into a -80 °C freezer. Remarkably, a 30% increase in cell recovery was seen with the addition of **104** under the same freezing conditions.

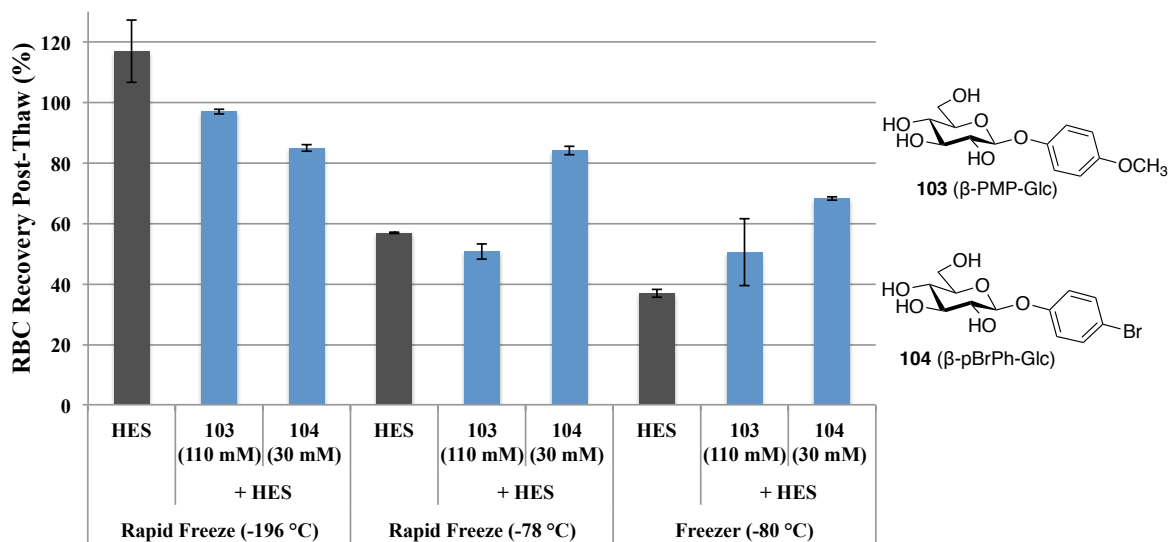


Figure 5.7 Percentage RBC recovery post-thaw using HES (11.5% w/w in 30 mM NaCl) in combination with **103** (110 mM) or **104** (30 mM). Samples were frozen by placing in liquid nitrogen (-196 °C), dry ice (-78 °C) or a -80 °C freezer and thawed at 48 °C in a water bath. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

This result was encouraging as the addition of **104** in HES resulted in significantly higher RBC recovery than HES alone using slower cooling rates (5 °C/min). Thus, interest arose in exploring post-thaw storage of previously frozen RBCs. For these experiments, RBCs were frozen in a cryosolution containing HES (11.5% w/w) supplemented with either **103** (110 mM) or **104** (30 mM) by placement into dry ice (-78 °C). Samples (1 mL volume) were rapidly thawed at 48 °C in a water bath and diluted with 0.9% saline. Thawed samples were stored at 4 °C in a refrigerator and hemolysis was measured at different time points (immediate, 24 h, 48 h). The results are reported as the percentage of intact RBCs (Figure 5.8). It can be seen that the percentage of intact RBCs for the HES control or HES supplemented with **103** does not drastically change over the course of 48 hours.

However, for RBCs frozen with **104**, there is a reduction in the percentage of intact RBCs after 48 hours of storage (62% versus 33%). These results suggest that RBCs cryopreserved with **104** in HES can only be stored up to 24 hours post-thaw prior to transfusion, after which significant hemolysis occurs and RBC quality decreases.

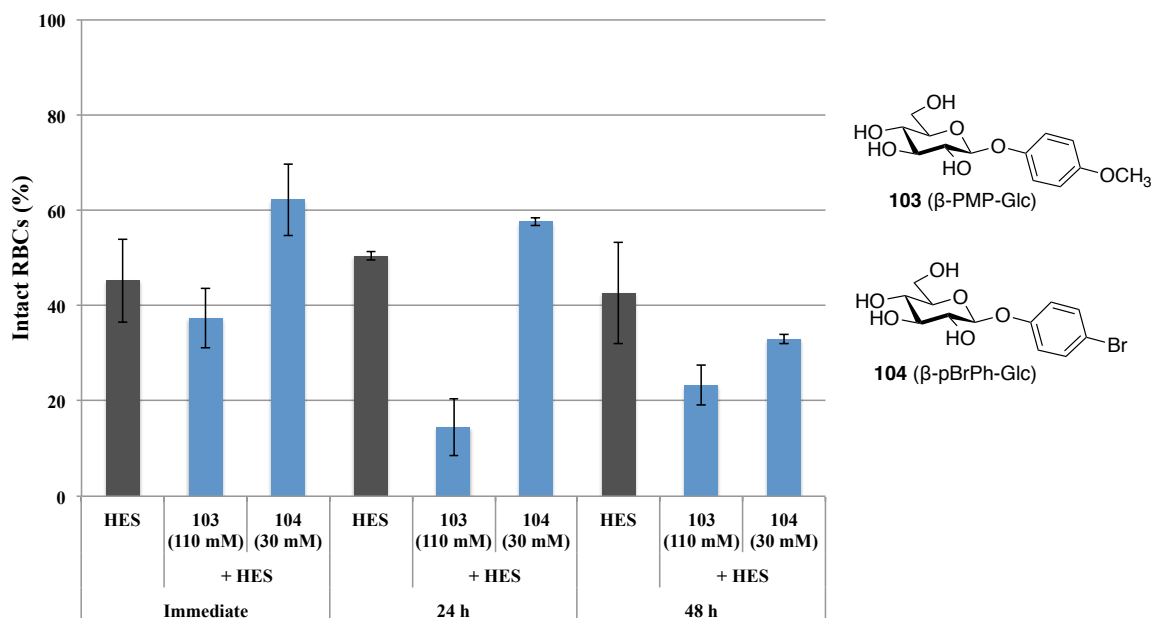


Figure 5.8 Percentage of intact RBCs post-thaw after 48 hours of storage at 4 °C in a refrigerator. After thawing, hemolysis was measured immediately, 24 and 48 hours after storage. These conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

In some cases, it is desirable to remove the HES from thawed RBCs. The main reason for this is that the high concentrations of HES required for cryopreservation lead to highly viscous mixtures,⁷⁰ which are difficult to transfuse into patients (i.e. relatively slow).^{67,71} Therefore, lowering the viscosity of the RBC-HES mixture by partially or fully removing the HES after thawing can permit more rapid administration to patients.^{67,71} This can be accomplished by dilution or a simple wash procedure after thawing to obtain the required viscosity for rapid transfusion.^{67,71} As such, we assessed how the removal of the CPA solution post-thaw would impact the recovery of RBCs.

RBCs were suspended in the cryosolution containing HES (11.5% w/w) supplemented with either **103** (110 mM) or **104** (30 mM). The samples (1 mL volume) were cooled rapidly to -196 °C (rate of 115 °C/min) or -78 °C (rate of 5 °C/min) by placing the samples directly into liquid nitrogen or dry ice. Samples were rapidly thawed

at 48 °C in a water bath and diluted with 0.9% saline, centrifuged, the supernatants were removed and RBCs were resuspended in 0.9% saline (“wash”). Samples treated as “no wash” were diluted with 0.9% saline after thawing and performed in parallel as a control experiment. RBC recovery was calculated to quantify the number of RBCs that are recovered after washing. The results are depicted in Figure 5.9. Recoveries are the highest when the samples were frozen more rapidly (-196 °C). Interestingly, when RBCs are cryopreserved with HES in combination with **103** or **104**, a significant reduction in RBC recovery was observed after washing. Under slower cooling conditions (-78 °C), an even greater reduction in RBC recovery was observed after washing. These results indicate that a significant amount of cells are damaged and lost throughout the washing process. Overall, the removal of the CPA solution post-thaw is detrimental to cell recovery as it causes additional damage.

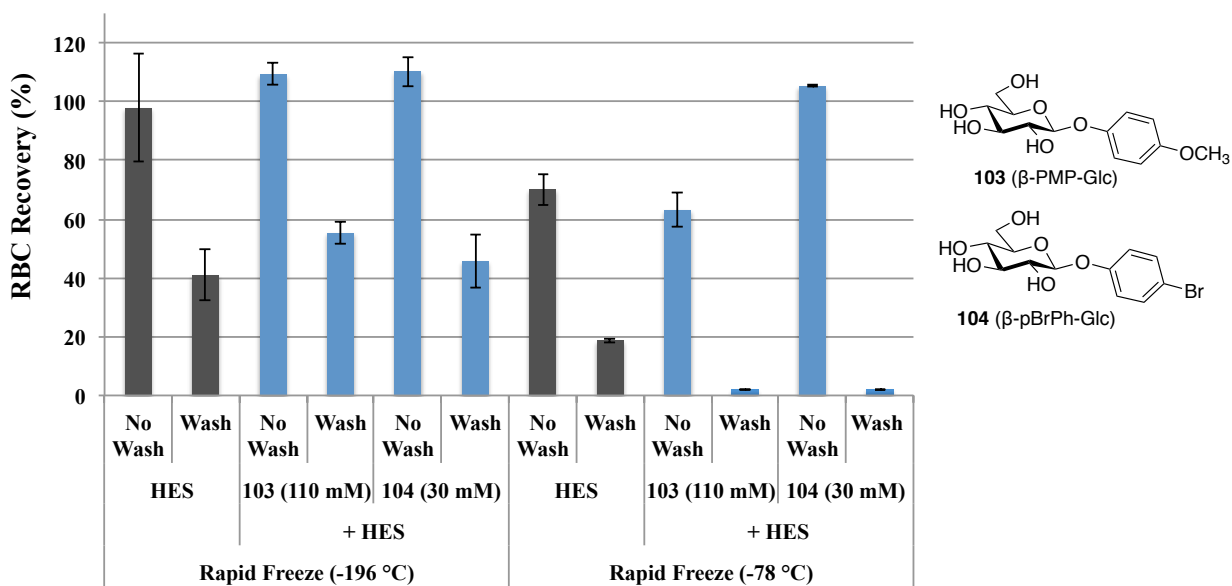


Figure 5.9 Percentage RBC recovery post-thaw after a simple wash procedure. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

Overall, the results from this study demonstrate that RBCs treated with **104** in HES result in significantly higher recovery post-thaw utilizing slower cooling rates (5 °C/min). Additionally, no washing procedure is required, and in principle, frozen RBC units can be thawed and immediately transfused. However, despite these promising results, it has been

reported that the transfusion of HES cryopreserved RBCs results in significantly increased concentrations of plasma hemoglobin.⁷² As a result, HES is not clinically used for the cryopreservation of RBCs due to the inadequate data regarding the safety of HES in patients receiving repeated transfusions.^{5,16} While the use of HES as a CPA for RBCs has no practical clinical use, this study was important because it indicated that **104** could be successfully utilized with different cryosolution formulations. Nevertheless, further studies utilizing IRIs in the presence of HES for the cryopreservation of RBCs were not performed.

5.2.4 Azido Aryl Glucosides as Cryoprotectants for RBCs

Thus far, the deglycerolization of RBCs cryopreserved with **103** (β -PMP-Glc) or **104** (β -pBrPh-Glc) in 15% glycerol has been problematic, resulting in a significant amount of cell damage. Since efforts to optimize the deglycerolization conditions for these novel RBC cryosolutions remained unsuccessful, we sought to investigate different small molecule IRIs, particularly azido aryl glucosides identified in Chapter 4.

The deglycerolization studies and analysis of RBC recoveries have revealed important insights into the possible cause of failure in regards to RBCs cryopreserved with **103** or **104** in 15% glycerol. The low RBC recoveries observed in the presence of **103** or **104** were hypothesized to be the result of membrane interactions during freezing, causing the RBCs to be more susceptible to osmotic stress during deglycerolization. This hypothesis was based on observations made from the RBC osmotic tolerance studies as explained below. Previous studies examined the osmotic tolerance of RBCs after treatment with **103** or **104** in 15% glycerol to aid in determining the gradient steps of washing solutions that are tolerated and can be used to deglycerolize the RBCs.⁴⁸ The osmolalities of the NaCl solutions are provided in Table 5.1. In these studies, RBCs were incubated at room temperature with either 15% glycerol, **103** (110 mM) + 15% glycerol or **104** (30 mM) + 15% glycerol. A SAGM control (saline-adenine-glucose-mannitol) was included since RBCs are typically stored in this isotonic solution. Following incubation, the samples were centrifuged, the supernatants were removed and the packed RBCs were resuspended in various NaCl solutions and hemolysis was measured spectrophotometrically to determine the appropriate NaCl concentration for wash 1. The

percentage hemolysis after resuspension in the NaCl solutions following incubation with each cryosolution is shown in Figure 5.10. The osmotic tolerance profiles indicated that after incubation with the cryosolutions, RBCs could be resuspended in NaCl solutions ranging from 4.0-6.0% NaCl, where minimal hemolysis was observed. These NaCl concentrations correspond to osmolalities of 1235-1760 mOsm/kg (Table 5.1). Therefore, after thawing, RBC samples should be washed with NaCl solutions between 4.0-6.0% without causing significant hemolysis.⁴⁸

Subsequently, NaCl solutions that could be tolerated after wash 1 were investigated. Following incubation with the cryosolutions and removal of the supernatants, the packed RBCs were resuspended in the selected wash 1 solution (5% NaCl, 1498 mOsm/kg). The samples were centrifuged, the supernatants were removed and the packed RBCs were resuspended in various NaCl solutions and hemolysis was measured spectrophotometrically to determine the appropriate NaCl concentration for wash 2. The osmotic tolerance profiles following wash 1 are shown in Figure 5.11. It was observed that both 15% glycerol and **103** (110 mM) + 15% glycerol could be resuspended and washed in an isotonic solution (0.9% NaCl, 300 mOsm/kg) following wash 1. In contrast, **104** (30 mM) + 15% glycerol could only be resuspended in NaCl solutions greater than 3% (> 929 mOsm/kg). NaCl solutions at concentrations less than 3% resulted in significant hemolysis.⁴⁸ Interestingly, these results indicated that when **104** is present in the cryosolution, the RBCs have a significantly lower osmotic tolerance than RBCs with 15% glycerol alone.

Table 5.1 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).

% NaCl (w/v)	Osmolality (mOsm/kg)
0.1	33
0.2	64
0.3	99
0.35	114
0.4	131
0.5	161
0.55	176
0.6	193
0.65	208
0.75	240
0.9	285
1	317
1.5	475
2	623
2.5	774
3	929
3.5	1074
4	1235
4.5	1374
5	1498
5.5	1628
6	1760
6.5	1903
7	2064
7.5	2216
8	2355
8.5	NR (> 3000)
9	NR (> 3000)
9.5	NR (> 3000)
10	NR (> 3000)

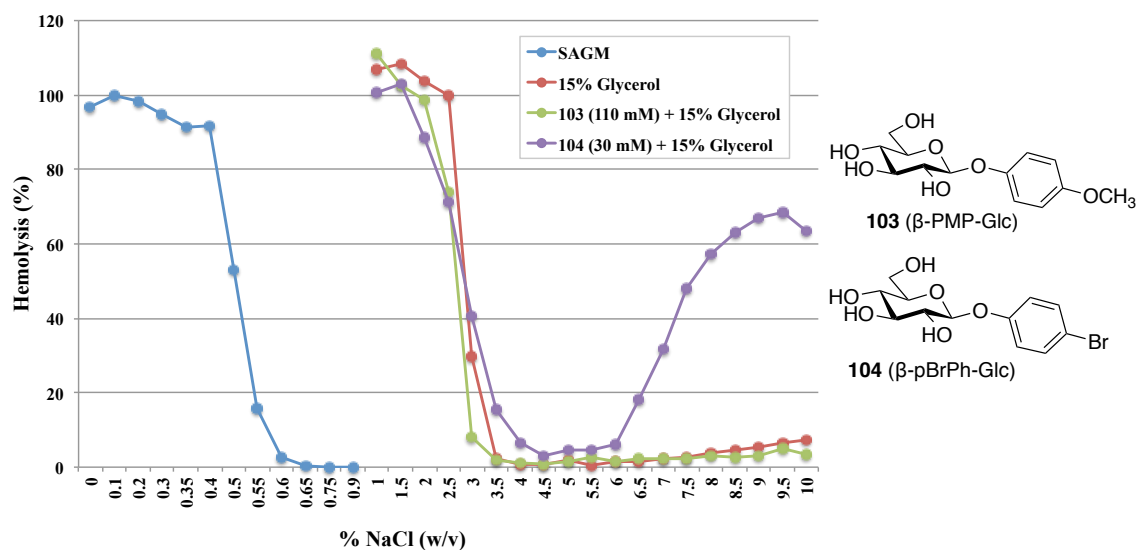


Figure 5.10 Osmotic tolerance of RBCs incubated with SAGM or cryosolutions. After incubation with the indicated solution, packed RBCs were resuspended in various NaCl solutions and assessed for hemolysis.⁴⁸

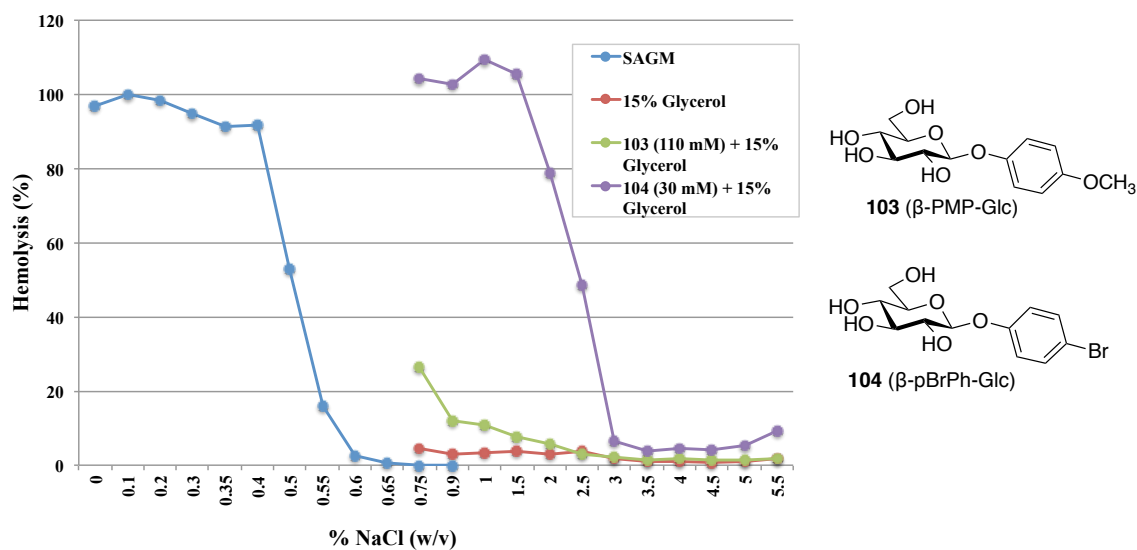


Figure 5.11 Osmotic tolerance of RBCs after wash 1. Following removal of cryosolution supernatant, packed RBCs were resuspended in wash 1 solution consisting of 5% NaCl. After incubation with wash 1, packed RBCs were resuspended in various NaCl solutions and assessed for hemolysis.⁴⁸

It was hypothesized that this was the result of a potential interaction with the RBC membrane as RBCs that were suspended in a solution of **104** (30 mM) + 15% glycerol were very susceptible to hemolysis by large differences in solution osmolalities.

Accordingly, it was postulated that the low RBC recoveries observed in the presence of **104** were due to membrane interactions during freezing, causing the RBCs to be more susceptible to osmotic stress during deglycerolization, leading to an increase in cell damage. Thus, improvements to the deglycerolization process of RBCs frozen with *O*-aryl glycosides were explored by investigating different small molecule IRIs. In particular, it was hypothesized that IRI active azido aryl glucosides (Chapter 4) could improve the deglycerolization process because these molecules may be less hydrophobic than **104** (β -pBrPh-Glc), and thus they may be less likely to interact with the RBC membrane during freezing. This could facilitate their removal during deglycerolization and avoid extensive osmotic stress. The following azido aryl glucosides (Figure 5.12) were assessed to determine their ability to function as beneficial cryo-additives that reduce cellular damage post-thaw and improve RBC recovery.

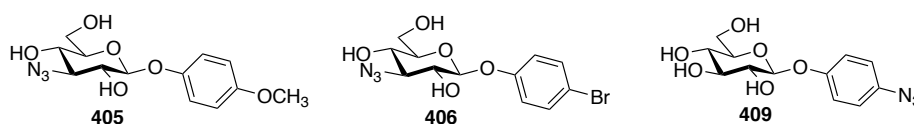


Figure 5.12 Structures of azido aryl glucosides (**405**, **406** and **409**).

5.2.4.1 *In Vitro* Toxicity of Azido Aryl Glucosides in RBCs

In Chapter 4 (Section 4.2.3), the cytotoxic effects of **405**, **406** and **409** were assessed in HepG2 cells using the conventional MTT assay and dose-response curves were generated to quantify cytotoxicity (IC_{50}). It was demonstrated that the azido aryl glucosides **405**, **406** and **409** caused significant loss of HepG2 cell viability after incubation for 18 h at 37 °C, displaying IC_{50} values of 9 mM, 3 mM and 12 mM, respectively. Since these compounds were shown to be quite toxic to HepG2 cells, the toxicity of **405**, **406** and **409** was examined in RBCs prior to performing cryopreservation studies. To determine whether these compounds cause hemolysis, a hypothermic storage experiment was performed where RBCs were suspended in 0.9% saline/0.2% dextrose containing **405** (30 mM), **406** (10 mM) or **409** (20 mM) and stored at 4 °C in a refrigerator for 7 days. The concentrations selected for each IRI were based on their maximum solubility in 0.9% saline/0.2% dextrose. The percentage of hemolysis was measured at different time points (immediate, 24 h, 48 h and 7 days). Thus, the amount of hemolysis after 7 days of hypothermic storage was used to evaluate the degree of toxicity

in RBCs. The percentage of RBC hemolysis after hypothermic storage with **405** (30 mM), **406** (10 mM) or **409** (20 mM) is shown in Figure 5.13. It is evident that even after 7 days of storage at 4 °C, less than 0.6% hemolysis was observed for all solutions, indicating that **405**, **406** and **409** do not exhibit any toxicity in RBCs at their maximal concentrations. This result was encouraging and demonstrated that compounds **405**, **406** and **409** are suitable candidates for RBC cryopreservation studies.

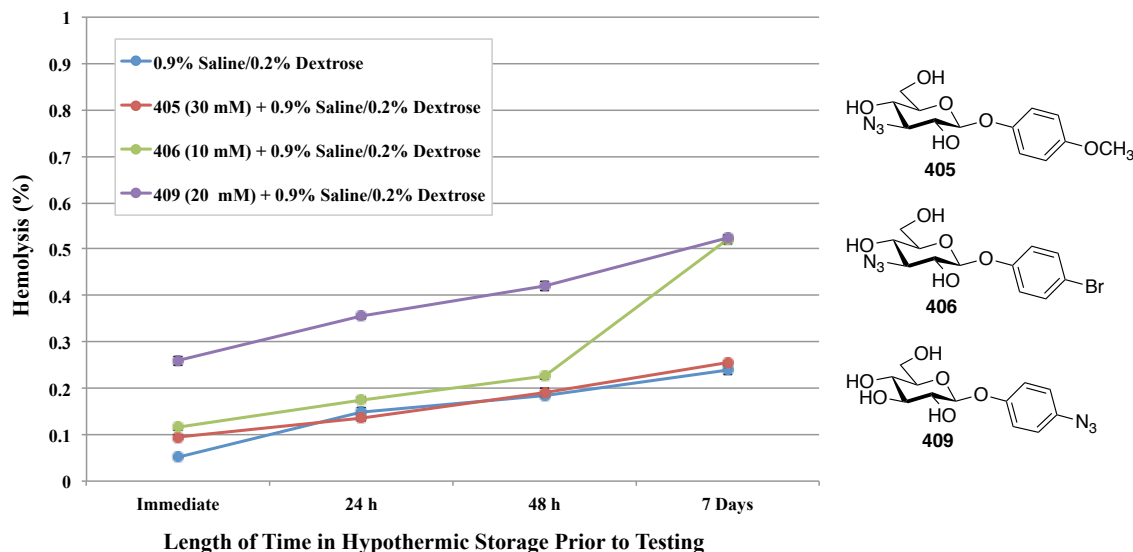


Figure 5.13 Percentage of RBC hemolysis after hypothermic storage (4 °C) with the indicated solution for up to 7 days. These conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

In addition to assessing hemolysis, other RBC parameters such as mean cell volume (MCV), the percentage of methemoglobin (MetHb) and membrane deformability were assessed at each time point (immediate, 24 h, 48 h and 7 days). Mean cell volumes were measured using an automated hematology analyzer. A normal range for MCV is between 80 and 96 fL.⁷³ A low MCV (i.e. < 80 fL) or a high MCV (i.e. > 96 fL) indicate either RBC shrinkage or swelling as a result of osmotic imbalances or abnormal RBC morphologies.⁷⁴ Mean cell volumes of RBCs after hypothermic storage at 4 °C in a solution of **405**, **406** or **409** in 0.9% saline/0.2% dextrose are shown in Figure 5.14. Similar to the 0.9% saline/0.2% dextrose control, RBCs stored in 0.9% saline/0.2% dextrose containing **405** or **406** have normal MCVs over the course of 7 days (~92-96 fL). In contrast, RBCs stored in 0.9% saline/0.2% dextrose containing **409** have

considerably larger than normal MCVs at each time measurement (~114 fL). It is important to note that variations in RBC deformability and morphology can lead to artifacts in the electronic determination of MCV.⁷⁵ Electronic cell counters have a limited range for accurate MCV determination and changes in morphology can result in false elevations of the MCV value.^{76–78} Thus, the increased MCV value may suggest that **409** is causing morphological changes or that **409** is being internalized, leading to an increase in the intracellular solute concentration which may cause RBC swelling. However, it is not possible to determine whether **409** is internalized based on MCV alone and thus internalization was further assessed in Chapter 7.

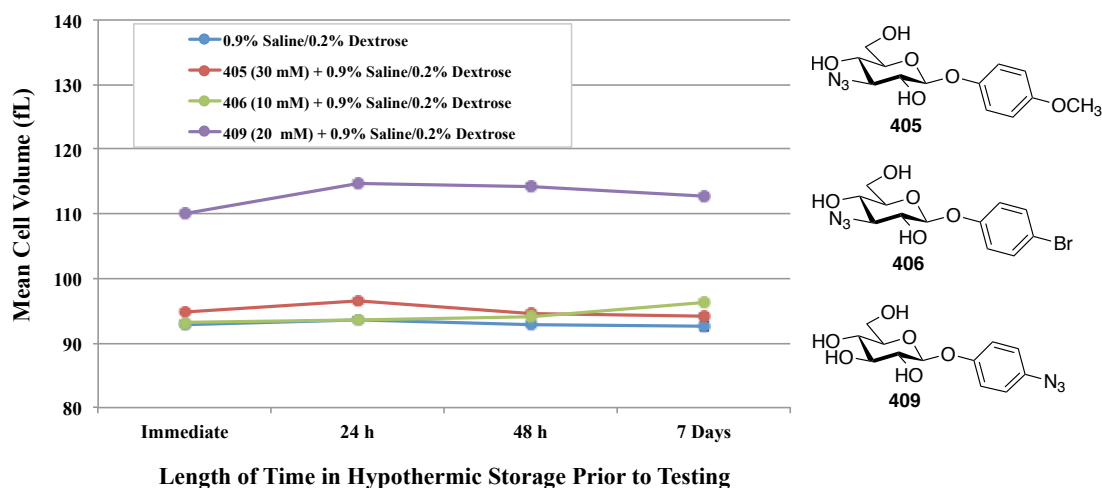


Figure 5.14 Mean cell volume after hypothermic storage (4 °C) with the indicated solution for up to 7 days. These conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

The percentage of methemoglobin (metHb) was also measured. RBCs are exposed to several oxidizing agents during their life span, resulting in the oxidation of hemoglobin to methemoglobin.⁷⁹ However, the metHb that is formed in normal RBCs is reduced to hemoglobin enzymatically in order to maintain metHb levels less than 3%.^{80,81} While hemoglobin is capable of carrying oxygen, facilitating delivery from the lungs to tissues, its oxidized form (metHb) cannot bind oxygen under physiological conditions, therefore leading to a decrease in the ability of RBCs to transport oxygen.^{79,81} Thus, evaluating the extent of hemoglobin oxidation during storage of RBCs is an important biological marker of oxidative stress. The percentage of methemoglobin after hypothermic storage of RBCs at 4 °C in a solution of **405**, **406** or **409** in 0.9% saline/0.2% dextrose is depicted in

Figure 5.15. Compared to the 0.9% saline/0.2% dextrose control, it can be seen that methHb levels for RBCs stored in 0.9% saline/0.2% dextrose containing **405** or **409** were higher after 24 hours. After 7 days of hypothermic storage, the percentage of methHb for RBCs stored with **405** or **409** increased substantially (30% and 14%, respectively). In contrast, the percentage of methHb for RBCs stored in 0.9% saline/0.2% dextrose containing **406** did not change throughout storage and remained similar to the control. After 7 days of hypothermic storage, RBC methHb levels for the 0.9% saline/0.2% dextrose control and **406** were within the physiological range (< 3%).⁸⁰ These results suggest that **405** and **409** behave as oxidizing agents in RBCs, accelerating the oxidation of hemoglobin and eventually overwhelming the capacity of reducing endogenous systems.⁸² It has been reported in the literature that 4-methoxyphenol is capable of initiating oxidation of hemoglobin to methemoglobin.⁸³ Given the structure of **405**, which contains a methoxy substituent (-OCH₃) at the *para*-position of the aryl ring, it seems plausible that **405** may be oxidizing hemoglobin via a mechanism similar to that of 4-methoxyphenol. Thus, it is not surprising that RBCs stored with **405** result in elevated levels of methHb.

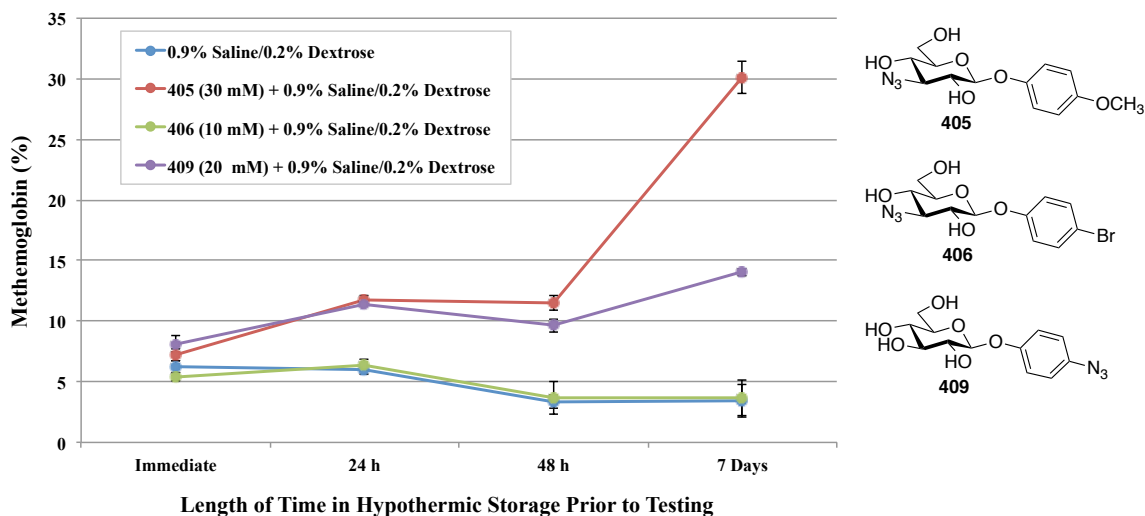


Figure 5.15 Percentage of methemoglobin after hypothermic storage (4 °C) with the indicated solution for up to 7 days. These conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

RBC membrane deformability was measured by ektacytometry using the laser assisted optical rotational cell analyzer (LORCA).⁸⁴ The ability of RBCs to deform is essential for their navigation through the microvasculature and subsequent delivery of

oxygen to tissues.⁸⁵ In recent years, studies have demonstrated that hypothermically stored RBCs have decreased deformability, which can affect their ability to enter the microvasculature, resulting in impaired perfusion and oxygen delivery to tissues.⁸⁵⁻⁸⁸ RBC deformability is measured by subjecting a suspension of RBCs in a high viscous medium to flow stress and applying varying shear stresses, causing RBC deformation.⁸⁴ By passing a laser through the suspension, the deformability of RBCs can be measured from the diffraction patterns of the laser.⁸⁴ This results in a characteristic deformability curve which is then linearized via the Eadie-Hofstee technique to obtain two parameters describing RBC deformation; EI_{max} and K_{EI} .⁸⁴ EI_{max} is defined as the maximum elongation index predicted at an infinite shear stress and therefore it is an indicator of overall RBC deformability.⁸⁴ K_{EI} is defined as the shear stress required to elongate to half of the EI_{max} and thus it is an indicator of RBC rigidity.⁸⁴ Therefore, a high EI_{max} indicates very deformable RBCs whereas a high K_{EI} indicates very rigid RBCs as more shear stress is required to reach half of the EI_{max} . EI_{max} and K_{EI} values after hypothermic storage of RBCs at 4 °C in a solution of **405**, **406** or **409** in 0.9% saline/0.2% dextrose are shown in Figure 5.16. Normal ranges for EI_{max} and K_{EI} values are 0.56-0.58 and 2.40-2.94, respectively.⁸⁴ Compared to the 0.9% saline/0.2% dextrose control, the EI_{max} values for RBCs stored in 0.9% saline/0.2% dextrose containing **405** or **406** were of similar magnitude throughout 7 days of hypothermic storage. In contrast, the EI_{max} value for RBCs stored with **409** was immediately considerably lower than the control and did not change throughout storage. The markedly decreased EI_{max} value of ~0.24 indicates that the RBCs are less deformable in the presence of **409**. In other words, the RBCs do not retain the ability to elongate. At the immediate time measurement, RBCs stored in 0.9% saline/0.2% dextrose containing **405** or **406** had K_{EI} values that were similar to the control, whereas RBCs stored in **409** had a noticeably decreased K_{EI} value. The decreased K_{EI} value (~0.78) is indicative of a decrease in cellular rigidity. However, after 7 days of hypothermic storage, K_{EI} values for RBCs stored in **405**, **406** or **409** were similar to the 0.9% saline/0.2% dextrose solution. Collectively, the observed decrease in EI_{max} and K_{EI} values suggests potential alterations to the structure of the RBC membrane, resulting in changes in membrane rigidity (increase in membrane fluidity).^{84,89} Overall, it is evident that RBCs stored with **409** lead to impairment of RBC deformability.

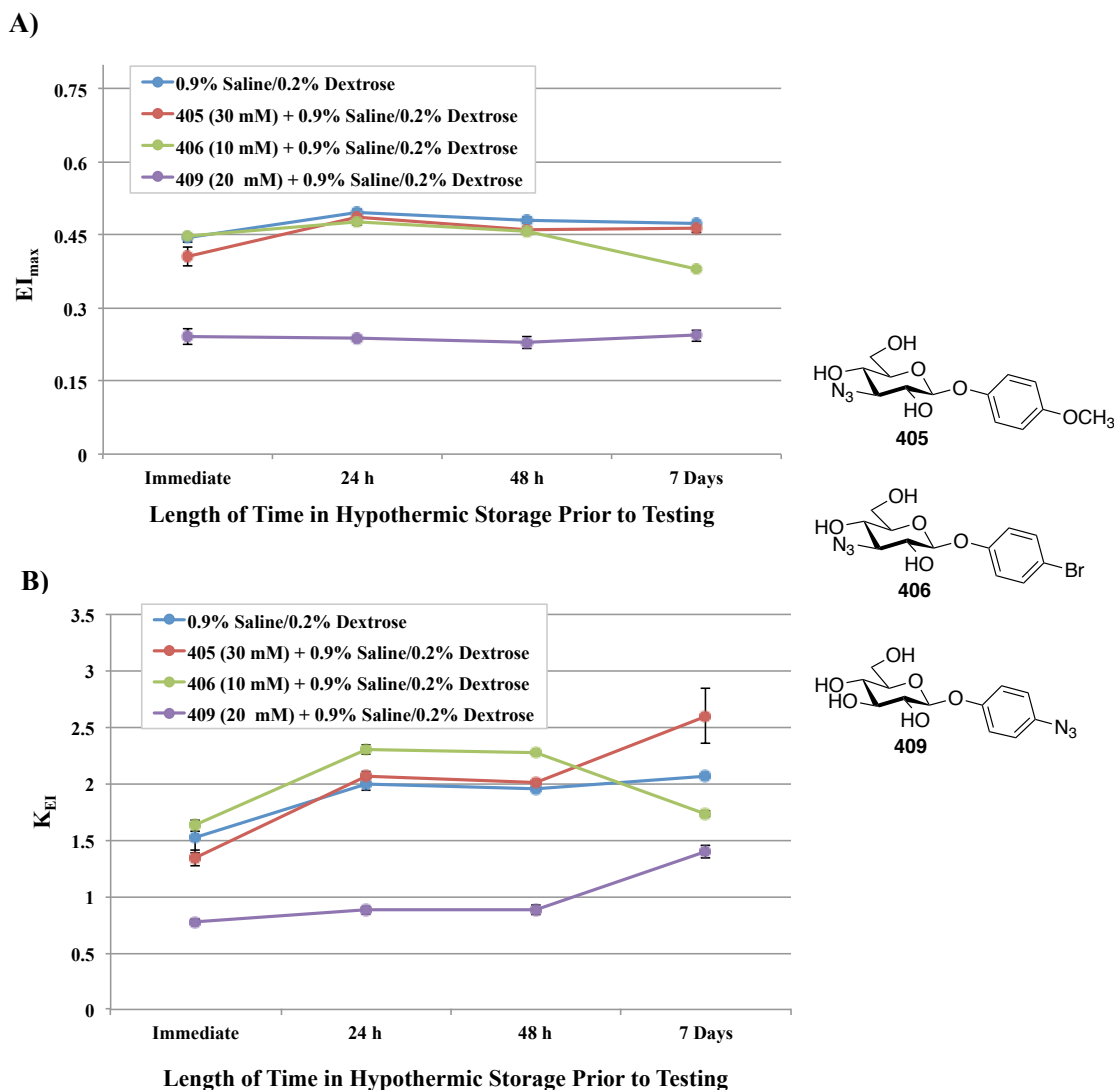


Figure 5.16 RBC deformability parameters (A) EI_{max} and (B) K_{EI} after hypothermic storage (4 °C) with the indicated solution for up to 7 days. These conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

In summary, the hypothermic storage study indicated that RBCs stored in 0.9% saline/0.2% dextrose containing **405**, **406** or **409** do not exhibit any toxicity as less than 0.6% hemolysis was observed after 7 days of storage. Thus, given the non-toxic nature of compounds **405**, **406** and **409** in RBCs, they are suitable candidates for RBC cryopreservation studies. Furthermore, the hypothermic storage study provided some insight into how these compounds affect RBC quality in the absence of freezing by measuring mean cell volumes, methemoglobin levels and membrane deformability.

5.2.4.2 Freezing of RBCs using Azido Aryl Glucosides in the Presence of 15% Glycerol

To assess the ability of azido aryl glucosides **405**, **406** and **409** to effectively cryopreserve RBCs in the presence of 15% glycerol, an initial study was performed where RBCs were suspended in a cryosolution containing 15% glycerol or 15% glycerol + **405** (30 mM), **406** (10 mM) or **409** (20 mM). The concentrations selected for each compound were based on their maximum solubility. RBCs were incubated for 10 minutes at room temperature prior to cooling to -5 °C. The samples (300 µL volume) were then nucleated at -5 °C to ensure that ice nucleation occurred at the same subzero temperature in each vial and subsequently cooled to -40 °C (rate of 1 °C/min) or -78 °C by placement into dry ice. The samples were rapidly thawed in a 37 °C water bath and RBC hemolysis was measured utilizing the Drabkin's assay.^{56,57} The results are reported as the percentage of intact RBCs post-thaw (100 - % hemolysis) shown in Figure 5.17.

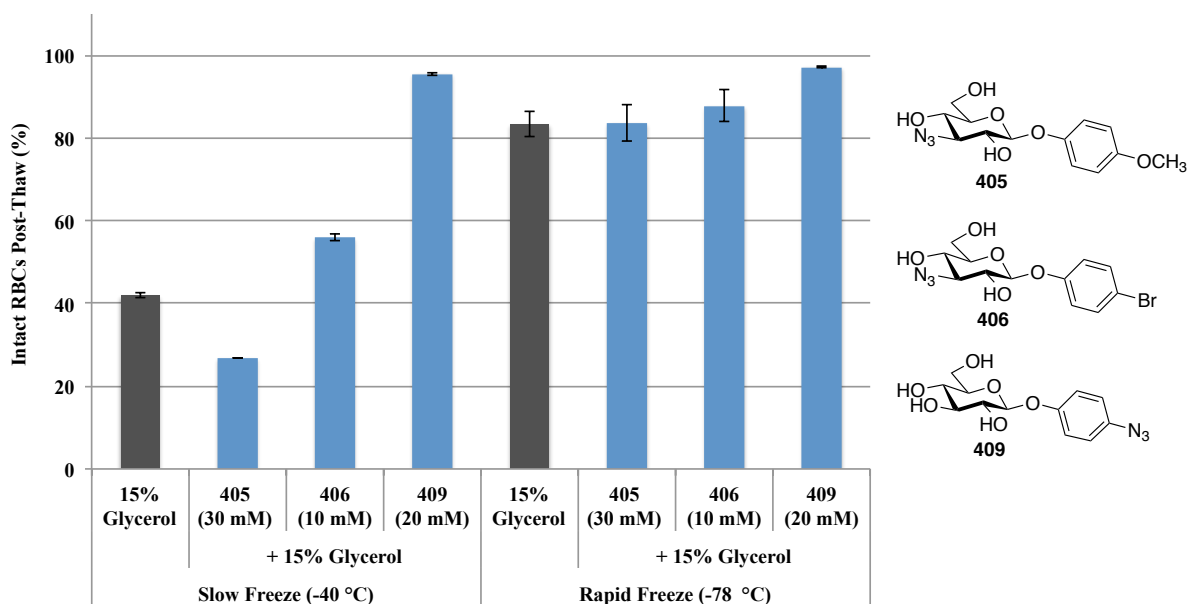


Figure 5.17 Percentage of intact RBCs post-thaw using 15% glycerol in combination with **405** (30 mM), **406** (10 mM) or **409** (20 mM). Samples were frozen to -40 °C (1 °C/min) or -78 °C by placement into dry ice and thawed at 37 °C in a water bath. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

Under rapid freezing conditions (i.e. -78 °C), the percentage of intact RBCs post-thaw were above 80% for all conditions. This was expected because the cryopreservation of RBCs using 15% glycerol requires fast cooling rates.^{33,34} Under slow cooling conditions

(i.e. -40 °C at a rate of 1 °C/min), the 15% glycerol control resulted in approximately 40% intact RBCs post-thaw. The addition of **409** (20 mM) to a solution of 15% glycerol significantly increased the percentage of intact RBCs post-thaw to approximately 95%. Unfortunately, the addition of **406** (10 mM) to a solution of 15% glycerol resulted in only a slight increase in the percentage of intact RBCs post-thaw (56% intact RBCs), whereas **405** (30 mM) was ineffective in increasing the percentage of intact RBCs post-thaw relative to the 15% glycerol control. Since **409** was capable of significantly increasing the percentage of intact RBCs post-thaw using slow cooling rates (1 °C/min), we sought to explore whether concentrations lower than 20 mM could be utilized in combination with 15% glycerol. As such, **409** was prepared at a final concentration of 5 mM, 10 mM and 15 mM in 15% glycerol and RBC samples were frozen at a controlled rate of 1 °C/min to -40 °C, rapidly thawed in a 37 °C water bath and hemolysis was measured to determine the lowest concentration of **409** that could be used to effectively cryopreserve RBCs. A 20 mM solution of **409** in 15% glycerol was included for comparison. The percentage of intact RBCs post-thaw in the presence of different concentrations of **409** in 15% glycerol is depicted in Figure 5.18. It is evident that the optimal concentration of **409** for the freezing of RBCs is 20 mM as this concentration results in the highest percentage of intact RBCs post-thaw. At concentrations lower than 20 mM, a reduction in the percentage of intact RBCs post-thaw was observed. It is important to note that a concentration scan was not performed on **405** and **406** since lowering their concentrations would unlikely improve their effectiveness.

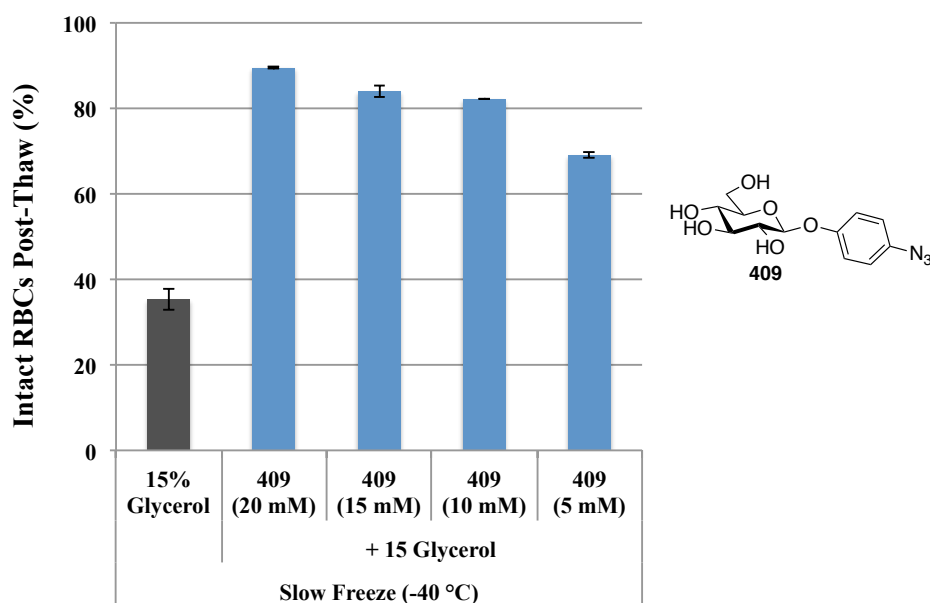


Figure 5.18 Percentage of intact RBCs post-thaw using 15% glycerol in combination with **409** at the indicated concentrations. Samples were frozen to -40 °C at a rate of 1 °C/min) and thawed at 37 °C in a water bath. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

Since **409** was capable of significantly increasing the percentage of intact RBCs post-thaw in the presence of 15% glycerol and slow cooling rates, we also sought to explore whether the concentration of glycerol could be reduced further. For this study, **409** was prepared at a final concentration of 20 mM in either 10%, 5% or 0% glycerol and RBC samples were cooled to -40 °C (rate of 1 °C/min) or -78 °C by placement into dry ice and subsequently thawed in a 37 °C water bath. RBC hemolysis was measured and the results are reported as the percentage of intact RBCs post-thaw (100 - % hemolysis) shown in Figure 5.19. A 20 mM solution of **409** in 15% glycerol was included for comparison. Under rapid freezing conditions (i.e. -78 °C), the percentage of intact RBCs post-thaw were well below 80% for conditions that employed glycerol concentrations less than 15%. The addition of **409** (20 mM) to a solution of 15% glycerol was as effective as 15% glycerol alone under rapid freezing conditions. Under slow cooling conditions (i.e. -40 °C at a rate of 1 °C/min), a solution of **409** (20 mM) in 15% glycerol remained the most effective for freezing RBCs as glycerol concentrations lower than 15% resulted in a significant reduction in the percentage of intact RBCs post-thaw. Overall, these studies

indicated that the optimal cryosolution resulting in a high percentage of intact RBCs post-thaw is 15% glycerol supplemented with **409** (20 mM).

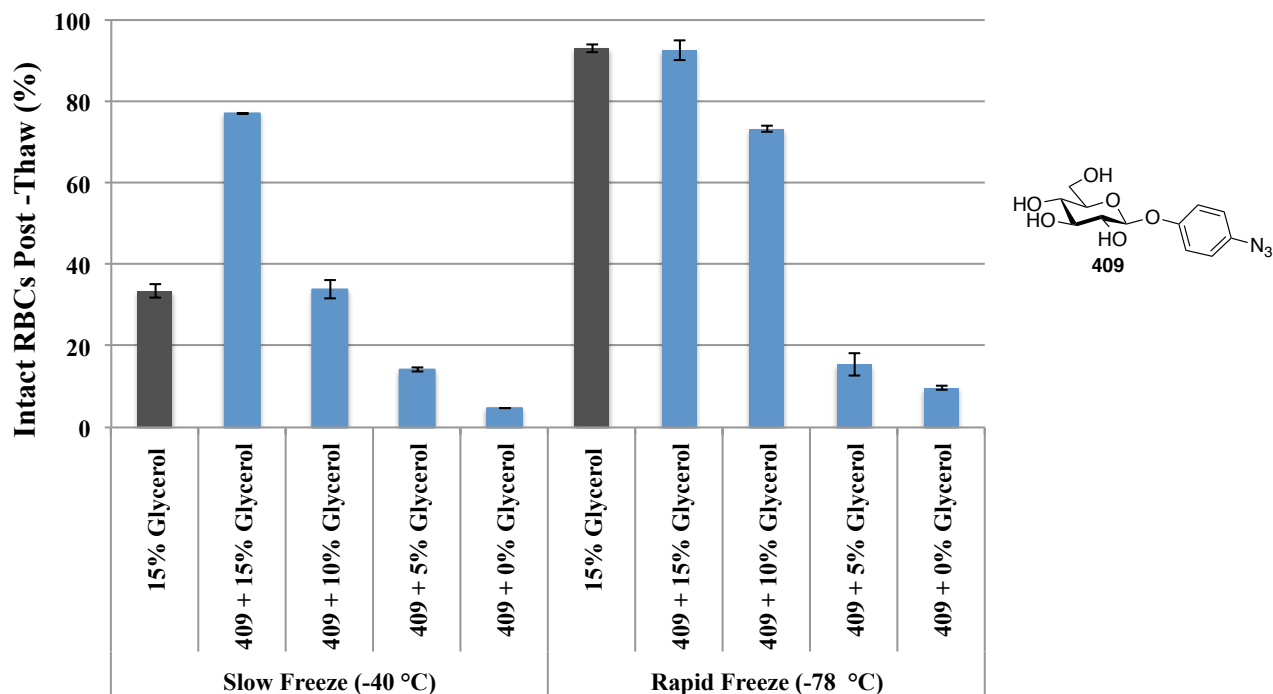


Figure 5.19 Percentage of intact RBCs post-thaw using various concentrations of glycerol in combination with **409** (20 mM). Samples were frozen to -40 °C (1 °C/min) or -78 °C by placement into dry ice and thawed at 37 °C in a water bath. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

5.2.4.3 Osmotic Tolerance of RBCs after Treatment with 15% Glycerol and Azido Aryl Glucosides

With the ability of **409** to significantly increase the percentage of intact RBCs post-thaw relative to 15% glycerol alone, the ability to deglycerolize RBCs was then investigated. However, prior to deglycerolizing RBCs frozen with **409** (20 mM) in 15% glycerol, the osmotic tolerance of RBCs was examined to aid in determining the gradient steps of washing solutions that are tolerated and can be used to deglycerolize the RBCs.^{2,52} The osmolalities of the NaCl solutions are provided in Table 5.1 (Section 5.2.4). Although the addition of **405** (30 mM) or **406** (10 mM) to a solution of 15% glycerol was ineffective in increasing the percentage of intact RBCs post-thaw, the osmotic tolerance of RBCs suspended in these cryosolutions was also examined as proof

of concept that azido aryl glucosides may interact with the RBC membrane less and may be less susceptible to osmotic stress.

In these studies, RBCs were incubated at room temperature with either 15% glycerol, **405** (30 mM) + 15% glycerol, **406** (10 mM) + 15% glycerol or **409** (20 mM) + 15% glycerol. A SAGM control (saline-adenine-glucose-mannitol) was included since RBCs are typically stored in this isotonic solution. Following incubation, the samples were centrifuged, the supernatants were removed and the packed RBCs were resuspended in various NaCl solutions and hemolysis was measured spectrophotometrically to determine the appropriate NaCl concentration for wash 1. The percentage hemolysis after resuspension in the NaCl solutions following incubation with each cryosolution is shown in Figure 5.20. The osmotic tolerance profiles indicate that after incubation with the cryosolutions, RBCs could be resuspended in NaCl solutions ranging from 4.5-6.0% NaCl, where minimal hemolysis was observed. These NaCl concentrations correspond to osmolalities of 1374-1760 mOsm/kg (Table 5.1). Therefore, after thawing, RBC samples should be washed with NaCl solutions between 4.5-6.0% without causing significant hemolysis.

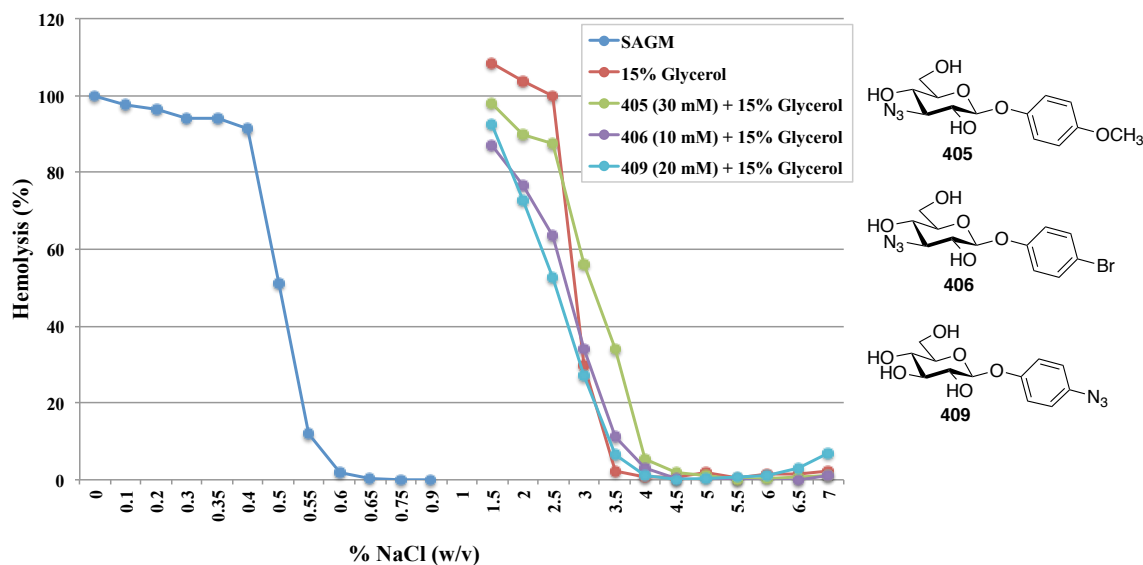


Figure 5.20 Osmotic tolerance of RBCs incubated with SAGM or cryosolutions. After incubation with the indicated solution, packed RBCs were resuspended in various NaCl solutions and assessed for hemolysis.

Subsequently, NaCl solutions that could be tolerated after wash 1 were investigated. Following incubation with the cryosolutions and removal of the supernatants, the packed

RBCs were resuspended in the wash 1 solution (4.5% NaCl, 1374 mOsm/kg). This solution was selected because it was the lowest saline concentration that the RBCs could tolerate without causing significant hemolysis (Figure 5.20). The samples were centrifuged, the supernatants were removed and the packed RBCs were resuspended in various NaCl solutions and hemolysis was measured spectrophotometrically to determine the appropriate NaCl concentration for wash 2. The osmotic tolerance profiles following wash 1 are shown in Figure 5.21. It was observed that both 15% glycerol and **409** (20 mM) + 15% glycerol could be resuspended and washed in an isotonic solution (0.9% NaCl, 300 mOsm/kg) with less than 5% hemolysis. In addition, **405** (30 mM) and **406** (10 mM) could also be subjected to a final wash with isotonic saline (0.9% NaCl, ~20% hemolysis). These studies indicated that the ideal wash solutions for **405** (30 mM), **406** (10 mM) and **409** (20 mM) in 15% glycerol are as follows: wash 1 = 4.5% NaCl (~1400 mOsm/kg); wash 2 = 0.9% NaCl (isotonic, ~300 mOsm/kg). Thus, a two-step wash procedure can be used to deglycerolize RBCs cryopreserved with **405**, **406**, or **409** in 15% glycerol and the RBCs can be returned to isotonic conditions.

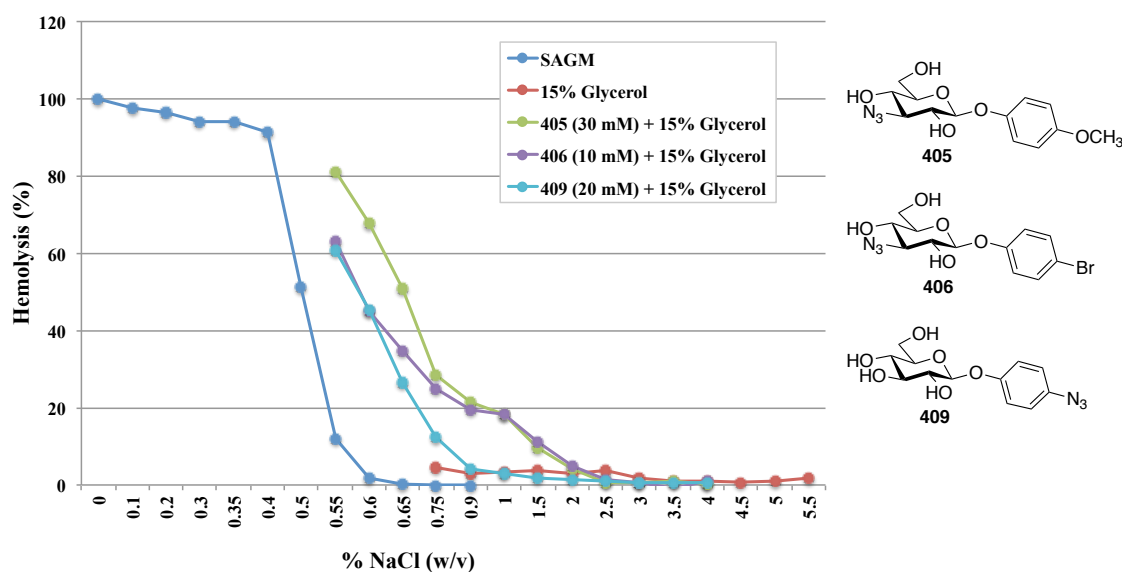


Figure 5.21 Osmotic tolerance of RBCs after wash 1. Following removal of cryosolution supernatant, packed RBCs were resuspended in wash 1 solution consisting of 4.5% NaCl. After incubation with wash 1, packed RBCs were resuspended in various NaCl solutions and assessed for hemolysis.

These results were encouraging as RBCs treated with **405**, **406**, or **409** + 15% glycerol could tolerate large differences in solution osmolalities without causing extensive cell

damage resulting in hemolysis. It was previously shown that the second wash of the deglycerolization process was problematic, resulting in a significant amount of hemolysis, especially in the presence of **104**.⁴⁸ Specifically, it was demonstrated that RBCs suspended in a cryosolution containing **104** (30 mM) + 15% glycerol could only be washed with NaCl solutions greater than 3% (> 929 mOsm/kg) (previously discussed in Section 5.2.4). Compared to the osmotic tolerance profile for **104** following wash 1, the profiles for **405**, **406**, or **409** shift drastically to lower osmolalities and the RBCs can tolerate resuspension in an isotonic (0.9%) saline solution for wash 2 (Figure 5.22). These results clearly indicate that RBCs suspended in **405**, **406**, or **409** + 15% glycerol have higher osmotic tolerances than **104** in 15% glycerol. It was hypothesized that compounds **405**, **406**, and **409** may be less hydrophobic than **104** and thus they may be less strongly affiliated with the RBC membrane. As a result, RBCs treated with **405**, **406**, or **409** are less susceptible to osmotic stress. Thus, RBCs cryopreserved with **405**, **406**, or **409** in 15% glycerol may suffer less osmotic damage during deglycerolization, resulting in less hemolysis and higher cell recoveries.

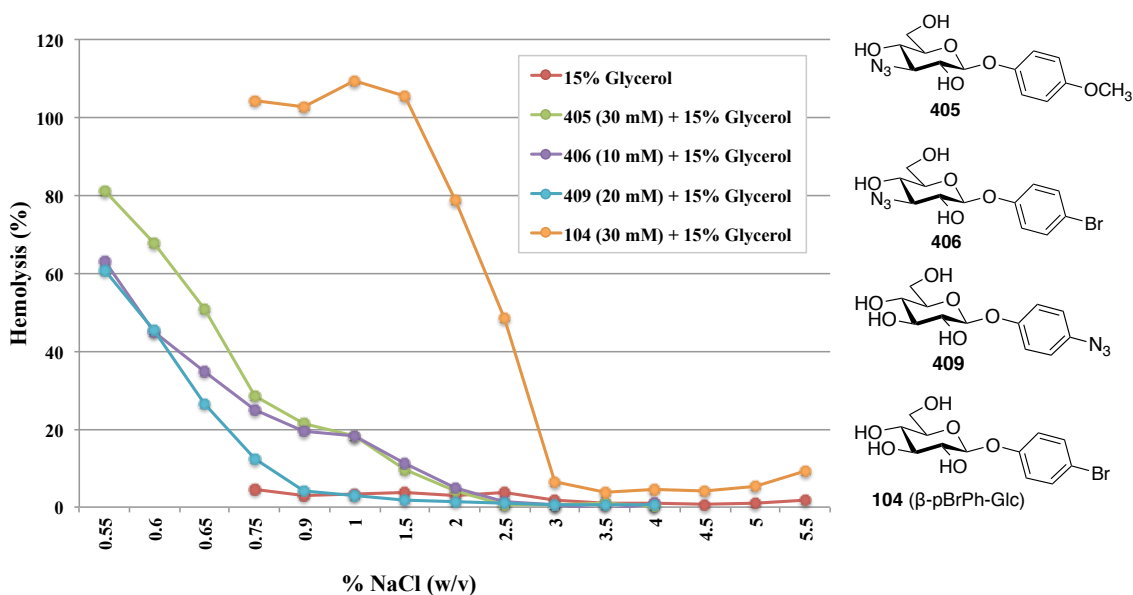


Figure 5.22 Comparison of osmotic tolerance profiles for RBCs suspended in the indicated solution after wash 1.

5.2.4.4 Post-Thaw Deglycerolization of RBCs in the Presence of 15% Glycerol and Azido Aryl Glucosides

The ideal washing conditions, established from the osmotic tolerance studies, were then applied for the deglycerolization of RBCs frozen with either 15% glycerol, **405** (30 mM) + 15% glycerol, **406** (10 mM) + 15% glycerol or **409** (20 mM) + 15% glycerol. In this experiment, two different cooling rates were utilized. The reason for this was; (1) the faster cooling rate (~ 5 °C/min) would ensure that immediate post-thaw hemolysis was lower to afford a greater quantity of RBCs proceeding through the deglycerolization process and (2) the slower cooling rate (~ 1 °C/min) would more closely mimic the freezing conditions that are employed clinically in Canada for RBC cryopreservation.

RBCs suspended in the appropriate cryosolution were cooled rapidly to -78 °C (rate of ~ 5 °C/min) by plunging the samples directly into dry ice or cooled slowly by placing the samples in a -80 °C mechanical freezer (rate of ~ 1 °C/min). The samples (1 mL volume) were thawed quickly at 37 °C in a water bath and subjected to deglycerolization using the following wash conditions: wash 1 = 4.5% NaCl (~ 1400 mOsm/kg); wash 2 = 0.9% NaCl (~ 300 mOsm/kg); resuspension in 0.9% saline. After each wash, the supernatant was removed and the amount of hemoglobin (Hgb) in the supernatant was quantified spectrophotometrically using Drabkin's assay.^{56,57} The amount of Hgb in the supernatants was used to evaluate the extent of cellular damage that occurred post-thaw and through each of the washes. The results are reported as the percentage of Hgb lost in each wash step relative to the total amount of Hgb in each sample. The results are presented in Figure 5.23. It is evident that a large amount of Hgb ($> 40\%$) was lost in the post-thaw supernatant for 15% glycerol, **405** (30 mM) + 15% glycerol and **406** (10 mM) + 15% glycerol for both freezing conditions, indicating extensive RBC damage during the freeze-thaw process. This was not surprising since **405** and **406** were initially shown to be ineffective in increasing the percentage of intact RBCs post-thaw (Section 5.2.4.2, Figure 5.17) without deglycerolization. Conversely, RBCs cryopreserved with **409** (20 mM) + 15% glycerol resulted in less than 30% Hgb lost in the post-thaw supernatant for both freezing conditions. The % Hgb lost in the wash 1 supernatant for all cryosolutions and freezing conditions ranged from 8-18%, indicating that this first wash solution does not cause a substantial amount of hemolysis. Remarkably, despite the large difference in

osmolality between wash 1 (~1400 mOsm/kg) and wash 2 (~300 mOsm/kg), less than 10% Hgb was lost in the wash 2 supernatant for all cryosolutions and freezing conditions. These results demonstrate that RBCs cryopreserved with **405**, **406** or **409** in 15% glycerol experience less cellular damage during deglycerolization, resulting in less hemolysis through each of the washes.

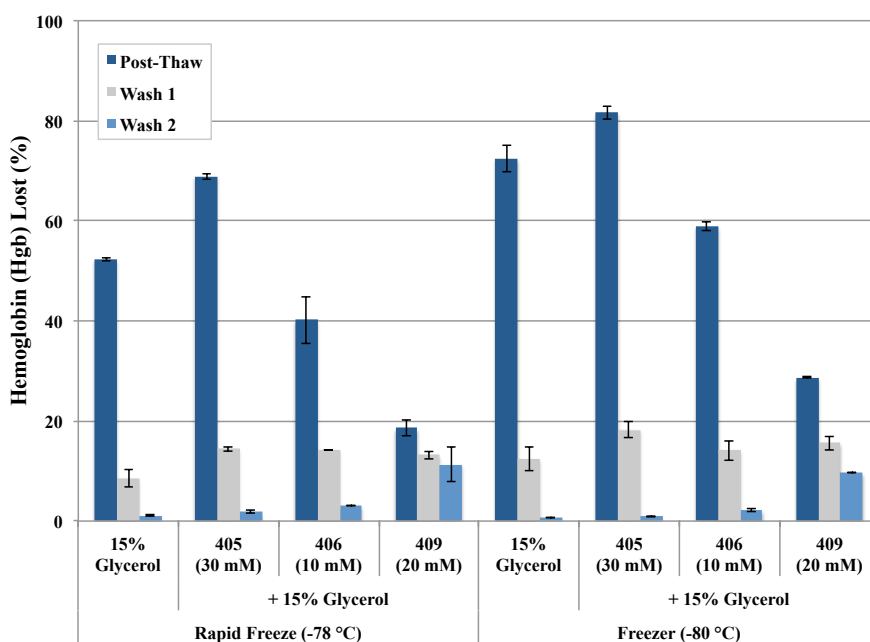


Figure 5.23 Percentage of hemoglobin lost post-thaw and in each wash step during the deglycerolization process. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

The overall RBC recoveries after the deglycerolization process are shown in Figure 5.24. Under rapid freezing conditions (i.e. -78 °C at a rate of 5 °C/min), RBCs that were cryopreserved with **405** (30 mM) or **406** (20 mM) + 15% glycerol afforded a recovery of 16% and 47%, respectively, compared to the 15% glycerol control which yielded a recovery of 35%. A significant increase in recovery was observed in the presence of **409** (20 mM) + 15% glycerol (recovery of 57%). Conversely, under slower cooling conditions (i.e. -80 °C at a rate of 1 °C/min), a reduction in RBC recovery was observed for **406**. Interestingly, RBCs that were cryopreserved with **409** (20 mM) + 15% glycerol resulted in no substantial decrease in recovery, despite utilizing even slower cooling rates. These results were encouraging for two reasons; (1) significantly higher recoveries were obtained with **409** compared to **104** (β -pBrPh-Glc) (recovery of 57% versus 3%) and (2)

slow cooling rates can be effectively utilized with **409** in the presence of 15% glycerol. These deglycerolization experiments were performed using a total volume of 1 mL. Consequently, given the promising results on a 1 mL scale, we sought to examine the ability of **409** + 15% glycerol to effectively cryopreserve RBCs utilizing a larger volume.

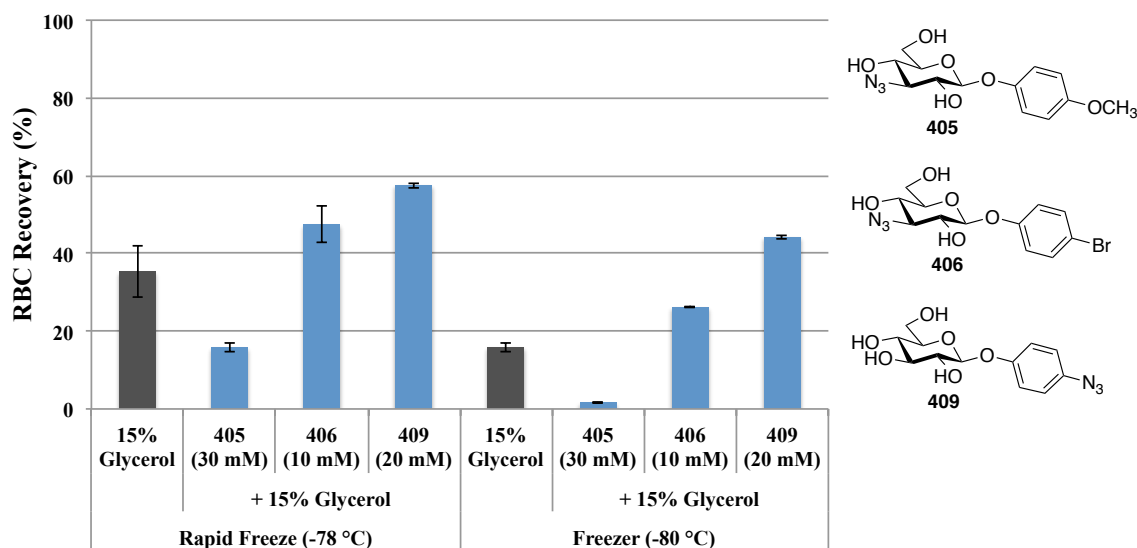


Figure 5.24 Percentage RBC recovery after deglycerolization using 15% glycerol in combination with **405** (30 mM), **406** (10 mM) or **409** (20 mM). Samples were frozen to -78 °C (~5 °C/min) by placement into dry ice or -80 °C (~1 °C/min) by placement in a freezer and thawed at 37 °C in a water bath. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

5.2.4.5 Large-Scale Cryopreservation of RBCs in the Presence of **409** and 15% Glycerol

To evaluate the potential of **409** + 15% glycerol in clinical applications, a scale-up method was explored. The volume of a typical RBC unit is approximately 300 mL.⁵⁴ The previous studies focused on the cryopreservation of relatively small volumes, typically in cryovials (1 mL). In such sample volumes, heat transfer is relatively rapid and uniform cooling rates are achieved.⁹⁰ However, in larger sample volumes, heat transfer occurs more slowly as a result of the low surface area to volume ratio and thus the cooling rate in large volumes is significantly slower.⁹¹ In addition, with larger volumes, the thawing rate is considerably slower,⁹⁰ increasing the extent of ice recrystallization resulting in increased cellular damage. As a result, the variation in the cooling and warming rates can negatively impact successful cryopreservation. Towards this end, the slower freezing and

warming rates for a larger volume are beneficial since it has been demonstrated that **409** can protect RBCs from ice recrystallization injury associated with slow cooling rates.

The previous small-scale cryopreservation studies demonstrated the following: (1) the optimal cryosolution resulting in a high percentage of intact RBCs post-thaw is 15% glycerol supplemented with **409** (20 mM), (2) the ideal wash solutions to deglycerolize the RBCs are 4.5% NaCl (wash 1) and 0.9% NaCl (wash 2) and (3) slow cooling rates can be effectively utilized with **409** in the presence of 15% glycerol. At this point, prior to performing large-scale cryopreservation, we sought to explore additional factors of the freezing process such as cell concentration. The influence of cell concentration during freezing on post-thaw viability and recovery has been well studied.⁹²⁻⁹⁸ In particular, it has been shown that increasing the hematocrit (i.e. packed cell volume) during the cryopreservation of RBCs increases hemolysis upon thawing.⁹²⁻⁹⁴ Considering the effect of hematocrit on the recovery of RBCs after cryopreservation, it was hypothesized that decreasing the hematocrit may improve the percentage of intact RBCs post-thaw. To test this hypothesis, different cell:cryoprotectant volume ratios were investigated to determine the optimal ratio that could result in the highest percentage of intact RBCs post-thaw. It is important to note that in the initial deglycerolization studies utilizing 1 mL volumes (Section 5.2.4.4), the final cell:cryoprotectant volume ratio was 1:1 representing a hematocrit of approximately 30%. In this experiment, RBCs were suspended in the cryosolution containing **409** (20 mM) + 15% glycerol at RBC:CPA ratios of either 1:1, 1:1.5, 1:2, 1:2.5 or 1:3 while maintaining the same total volume per sample (3 mL). This experiment was performed using a 3 mL volume since the cooling and warming rates would be slower than those observed with the 1 mL volume, providing a better representation of a large sample volume. The samples were incubated for 10 minutes at room temperature prior to cooling to either -78 °C (rate of 2.4 °C/min) by placement into dry ice or -196 °C (rate of 77 °C/min) by immersion in liquid nitrogen. The samples were rapidly thawed in a 37 °C water bath and RBC hemolysis was measured utilizing the Drabkin's assay.^{56,57} The results are reported as the percentage of intact RBCs post-thaw (100 - % hemolysis) shown in Figure 5.25.

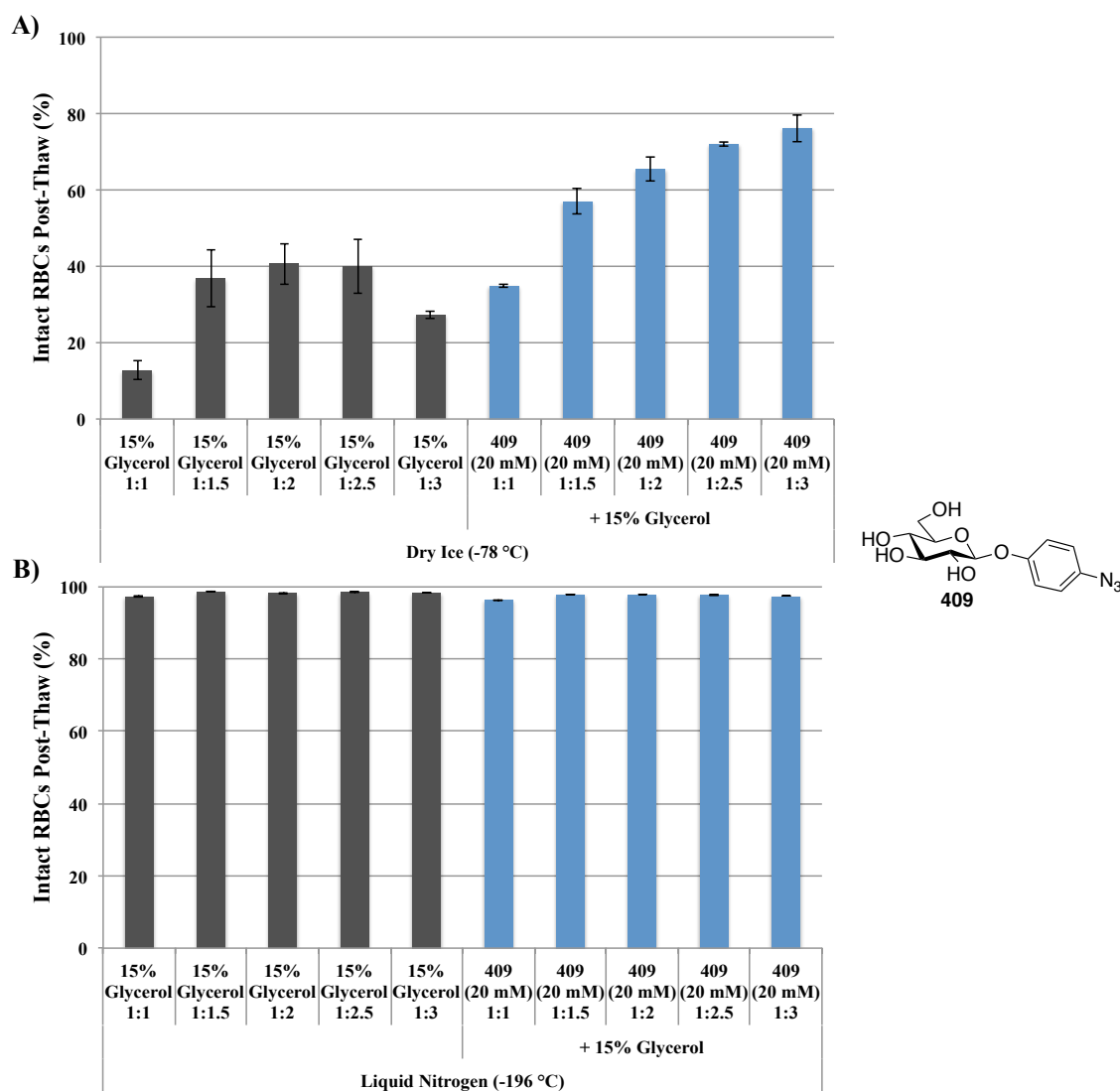


Figure 5.25 Percentage of intact RBCs post-thaw following cryopreservation at different RBC:CPA ratios. Samples were frozen to -78 °C (rate of 2.4 °C/min) by placement into dry ice (**A**) or -196 °C (rate of 77 °C/min) by immersion in liquid nitrogen (**B**) and thawed at 37 °C in a water bath. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

Under rapid freezing conditions (i.e. -196 °C at a rate of 77 °C/min), the percentage of intact RBCs post-thaw was above 90% for all conditions with no observed difference in the cell:cryoprotectant volume ratios. This was expected because the cryopreservation of RBCs using 15% glycerol requires fast cooling rates.^{33,34} Under slower cooling conditions (i.e. -78 °C at a rate of 2.4 °C/min), RBCs cryopreserved in 15% glycerol had less than 40% intact RBCs post-thaw, irrespective of the cell:cryoprotectant ratio.

However, a RBC cryosolution containing **409** (20 mM) + 15% glycerol in a 1:3 cell:cryoprotectant ratio was considerably more effective than a 1:1 ratio, resulting in the highest percentage of intact RBCs post-thaw. This study validated the hypothesis and indicated that a 1:3 ratio of RBCs to cryoprotectant solution (**409** (20 mM) + 15% glycerol) should be utilized in conjunction with slow cooling rates to maximize the percentage of intact RBCs post-thaw. Thus, for the preliminary large-scale cryopreservation experiment, a 1:3 ratio of RBCs to cryoprotectant solution was used in combination with the ideal wash solutions determined from the osmotic tolerance experiments for deglycerolization. Although these conditions differed from the previous small-scale protocol, they were a logical starting point to evaluate the scale-up method.

For the large-scale experiment, RBCs were prepared in a 1L Origen freezing bag in a 1:3 ratio of RBCs to cryoprotectant solution (15% glycerol or **409** (20 mM) + 15% glycerol) to obtain a total sample volume of 250 mL. The samples were frozen directly in dry ice (-78 °C) or liquid nitrogen (-196 °C) prior to storage in a -80 °C freezer. The samples were thawed in a 37 °C water bath, a 2 mL aliquot was taken and tested immediately for hemolysis (Figure 5.26). The results are reported as the percentage of intact RBCs post-thaw and signify the amount of cellular damage that occurred during the freeze-thaw process. Under both freezing conditions, the percentage of intact RBCs post-thaw was greater than 80%, indicating minimal RBC damage during the freeze-thaw process. However, the percentage of intact RBCs post-thaw was slightly higher using liquid nitrogen freezing conditions. This was expected since the cooling rate utilizing liquid nitrogen would be faster than dry ice and the cryopreservation of RBCs using 15% glycerol requires fast cooling rates.^{33,34} Nevertheless, these results demonstrated that immediate post-thaw hemolysis was low and a large quantity of RBCs was proceeding through the deglycerolization process.

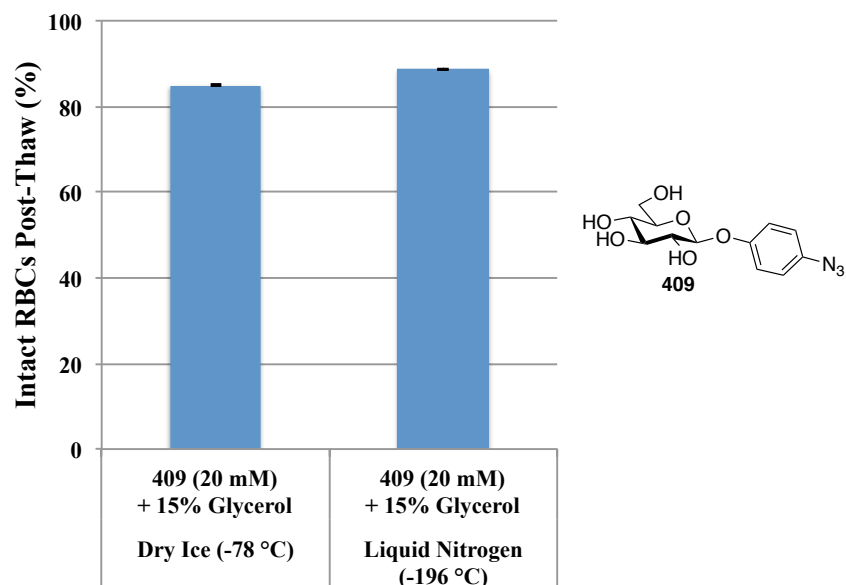


Figure 5.26 Percentage of intact RBCs post-thaw using 15% glycerol in combination with **409** (20 mM). Samples were frozen to -78 °C by placement into dry ice or -196 °C by immersion in liquid nitrogen, thawed at 37 °C in a water bath and aliquots were taken (n = 2). Error bars represent the standard error of the mean (SEM).

The RBC samples were deglycerolized utilizing the washing conditions determined from the osmotic tolerance experiments: wash 1 = 4.5% NaCl (~1400 mOsm/kg) and wash 2 = 0.9% NaCl (~300 mOsm/kg) followed by resuspension in AS-3 to obtain a hematocrit of 50%. However, a pre-dilution step was employed prior to wash 1 to provide a more gradual step-down from the high osmolality of the cryosolution (> 3000 mOsm/kg) to the significantly lower osmolality of wash 1 (~1400 mOsm/kg). This pre-dilution step involved the addition of the wash 1 solution (~750 mL of 4.5% NaCl) to the RBCs suspended in cryosolution to gradually decrease the osmolality of the cryosolution closer to the osmolality of the wash 1 solution. It was hypothesized that using a pre-dilution step prior to wash 1 would result in the RBCs being more tolerable to resuspension in lower osmolality solutions. After each wash, the supernatant was removed and the amount of hemoglobin (Hgb) in the supernatant was quantified spectrophotometrically using Drabkin's assay.^{56,57} The amount of Hgb in the supernatants was used to evaluate the extent of cellular damage that occurred through each of the

washes. The results are reported as the percentage of Hgb lost in each wash step relative to the total amount of Hgb in each sample. The results are presented in Figure 5.27.

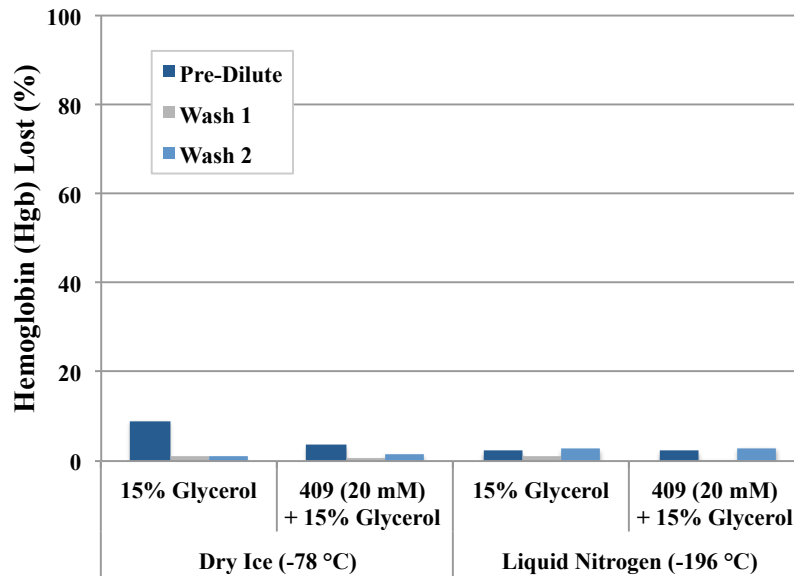


Figure 5.27 Percentage of hemoglobin lost in each wash step during the deglycerolization process (n = 1).

Remarkably, very little hemoglobin (less than 10%) was lost in each step during deglycerolization, indicating that the RBCs experience minimal cellular damage during the washing procedure, resulting in less hemolysis through each of the washes.

The overall RBC recovery after the deglycerolization process is depicted in Figure 5.28. Under liquid nitrogen freezing conditions, RBCs cryopreserved with 15% glycerol alone or **409** (20 mM) + 15% glycerol afforded recoveries of 62% and 65%, respectively, suggesting that the addition of **409** (20 mM) to a solution of 15% glycerol provided no additional protection under these conditions. However, under slower cooling conditions (i.e. dry ice), RBCs cryopreserved with **409** (20 mM) + 15% glycerol afforded a considerably higher recovery than those cryopreserved with 15% glycerol alone (recovery of 70% versus 30%). For the small volume cryopreservation experiments (1 mL), placement of the samples into dry ice produced a cooling rate of approximately 5 °C/min. Although the exact cooling rate was not determined for the large sample volume (250 mL), it is likely much slower because heat transfer will be significantly compromised compared with those achieved in cryovials. This preliminary large-scale

study further emphasizes that RBCs can be effectively cryopreserved with **409** in the presence of 15% glycerol using slow cooling rates. Furthermore, these RBCs can be deglycerolized without causing extensive cell damage. These preliminary results highlight the need for optimization studies.

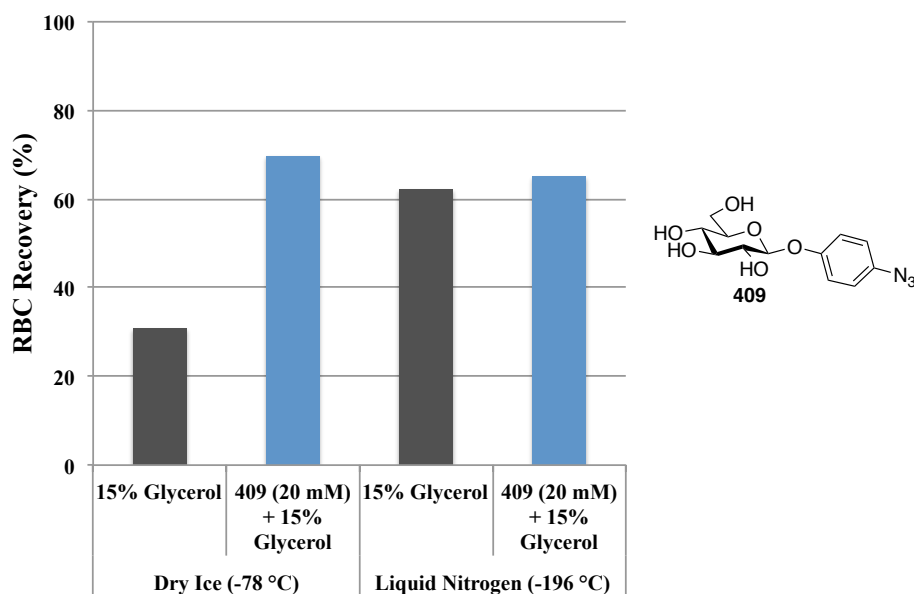


Figure 5.28 Percentage RBC recovery after deglycerolization using 15% glycerol in combination with **409** (20 mM). Samples were frozen to -78 °C by placement into dry ice or -196 °C by immersion in liquid nitrogen and thawed at 37 °C in a water bath (n = 1).

5.2.4.6 RBC Functional Analysis After Cryopreservation with **409** + 15% Glycerol

The *in vitro* quality of RBCs frozen with **409** (20 mM) + 15% glycerol in dry ice (-78 °C), stored in a -80 °C freezer, deglycerolized and stored at 4 °C in AS-3 for 7 days was assessed and compared to RBC units cryopreserved with 40% glycerol. *In vitro* assays included an assessment of hemolysis; measurements of mean cell volume (MCV), adenosine triphosphate (ATP), 2,3-diphosphoglycerate (DPG), and methemoglobin (MetHb) concentration; oxygen affinity; membrane deformability; and extracellular potassium and sodium levels. These assays were performed 24 hours and 7 days after deglycerolization and the measurements are listed in Table 5.2. In RBCs frozen with 15% glycerol or **409** (20 mM) + 15% glycerol, the percentage of hemolysis was less than 5% after storage at 4 °C for 7 days, indicating that minimal cell damage was occurring throughout storage. Mean cell volumes were measured using an automated hematology

analyzer. A typical range for MCV values for RBCs cryopreserved in 40% glycerol, deglycerolized and stored at 4 °C for 24 hours is 84.2-97.2 fL.⁸⁰ RBCs cryopreserved with 15% glycerol or **409** (20 mM) + 15% glycerol had larger than normal MCVs after 24 hours and 7 days of post-thaw storage. A high MCV (i.e. > 97.2 fL) indicates either RBC swelling as a result of osmotic imbalances or abnormal RBC morphologies.⁷⁴ Since 15% glycerol has a noticeably higher than normal MCV (~120 fL), this may suggest that either the residual glycerol concentration is above 1%, causing RBC swelling or that cryopreservation and subsequent deglycerolization affect RBC morphology.

The percentage of methemoglobin (metHb) was also measured. RBCs are exposed to several oxidizing agents during their life span, resulting in the oxidation of hemoglobin to methemoglobin.⁷⁹ However, the metHb that is formed in normal RBCs is reduced to hemoglobin enzymatically in order to maintain metHb levels less than 3%.^{80,81} While hemoglobin is capable of carrying oxygen, facilitating delivery from the lungs to tissues, its oxidized form (metHb) cannot bind oxygen under physiological conditions, therefore leading to a decrease in the ability of RBCs to transport oxygen.^{79,81} Thus, evaluating the percentage of methemoglobin after deglycerolization is an important biological marker of oxidative stress. The percentage of methemoglobin after 24 hours and 7 days of post-thaw storage is shown in Table 5.2. A typical range for metHb (%) for RBCs cryopreserved in 40% glycerol, deglycerolized and stored at 4 °C for 24 hours is 0.32-1.32%.⁸⁰ The metHb levels for RBCs cryopreserved with 15% glycerol or **409** (20 mM) + 15% glycerol were elevated 24 hours after deglycerolization (~6%). It is likely that cryopreservation and subsequent deglycerolization affect the metHb reduction pathway in RBCs. While the metHb levels for RBCs frozen with 15% glycerol decreased to 3.2% after storage for 7 days, the metHb levels for RBCs frozen with **409** (20 mM) + 15% glycerol increased to 9.0%. This is consistent with previous results that revealed that RBCs stored at 4 °C with **409** for greater than 24 hours possess elevated levels of metHb (Section 5.2.4.1, Figure 5.15).

Table 5.2 RBC quality measurements after 24 hours and 7 days of post-thaw storage.

	15% Glycerol (n = 1)	409 (20 mM) + 15% Glycerol (n = 1)
<i>RBC hemolysis, %</i>	3.4 (4.2)	3.1 (4.1)
<i>Mean cell volume, fL</i>	120.8 (119.2)	108 (107.7)
<i>Methemoglobin, %</i>	6.3 (3.2)	6.4 (9.0)
<i>ATP, $\mu\text{mol/g Hb}$</i>	3.3 (0.4)	3.6 (1.1)
<i>2,3-DPG, $\mu\text{mol/g Hb}$</i>	0.2	1.7
<i>p50, mmHg</i>	16.5 (15.8)	16.6 (15.3)
<i>El_{max}</i>	0.41 (0.41)	0.45 (0.43)
<i>K_{El}</i>	1.77 (2.13)	1.93 (1.90)
<i>Extracellular potassium, mmol/L</i>	< 1.0 (3.4)	1.1 (4.5)
<i>Extracellular sodium, mmol/L</i>	193 (196)	227 (218)

^aValue in brackets represents day 7 testing

The storage of RBCs at 4 °C is associated with several biochemical changes that result in the loss of *in vivo* properties.⁹⁹ For instance, these changes include a decrease in intracellular ATP and 2,3-DPG levels.^{100–102} Reduced levels of ATP impair RBC metabolic activities including glycolysis and maintenance of membrane integrity, thereby reducing RBC deformability and promoting alterations in their shape, which results in decreased RBC viability.^{103–105} The ease with which hemoglobin releases oxygen to the tissues is controlled by 2,3-DPG.¹⁰⁶ 2,3-DPG alters the oxygen affinity of hemoglobin such that an increase in the concentration of 2,3-DPG reduces the affinity of hemoglobin for oxygen.¹⁰⁶ Therefore, increased levels of 2,3-DPG improve tissue oxygenation whereas decreased levels can lead to tissue hypoxia.¹⁰⁶ Due to the interaction of 2,3-DPG with hemoglobin and its subsequent effect on oxygen affinity, changes in the concentration of 2,3-DPG will shift the oxygen-hemoglobin dissociation curve which is characterized by the p50 value (the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen).¹⁰⁶ Thus, low levels of 2,3-DPG shift the oxygen-hemoglobin dissociation curve to the left and decrease the p50 value. In other words, the

affinity of hemoglobin for oxygen increases and the ability of RBCs to deliver oxygen to tissues becomes limited.⁸⁰ Conversely, high levels of 2,3-DPG shift the oxygen-hemoglobin dissociation curve to the right and increase the p50 value.¹⁰⁶ The levels of ATP were measured after 24 hours and 7 days of post-thaw storage (Table 5.2). A normal range for ATP levels for RBCs cryopreserved in 40% glycerol, deglycerolized and stored at 4 °C for 24 hours is 2.98-3.78 $\mu\text{mol/g Hb}$.⁸⁰ After storage for 24 hours, RBCs cryopreserved with 15% glycerol or **409** (20 mM) + 15% glycerol possessed ATP levels within the normal range. However, after storage for 7 days, ATP levels were depleted for RBCs cryopreserved with 15% glycerol (0.4 $\mu\text{mol/g Hb}$) or **409** (20 mM) + 15% glycerol (1.1 $\mu\text{mol/g Hb}$), indicating decreased RBC viability.¹⁰⁷ This is expected as ATP levels decrease over time with hypothermic storage.¹⁰⁰⁻¹⁰² Conversely, re-suspension of deglycerolized RBCs in AS-3 solution for 24 hours likely provided additional nutrients to maintain ATP levels post-cryopreservation.

The levels of 2,3-DPG were also measured 24 hours after deglycerolization (Table 5.2). A typical range for 2,3-DGP levels for RBCs cryopreserved in 40% glycerol, deglycerolized and stored at 4 °C for 24 hours is 0.72-9.60 $\mu\text{mol/g Hb}$.⁸⁰ RBCs cryopreserved with 15% glycerol or **409** (20 mM) + 15% glycerol possessed 2,3-DPG levels of 0.2 $\mu\text{mol/g Hb}$ and 1.7 $\mu\text{mol/g Hb}$, respectively. It appears that 2,3-DPG levels for RBCs cryopreserved with 15% glycerol alone are reduced compared to RBC units cryopreserved with 40% glycerol, which may indicate an increased affinity of hemoglobin for oxygen and a decreased ability of RBCs to deliver oxygen to tissues. However, due to the small number of samples examined ($n = 1$), it is not possible to conclude whether this value is statistically different. Nevertheless, RBCs cryopreserved with **409** (20 mM) + 15% glycerol possessed 2,3-DPG levels within the normal range, suggesting that oxygen transport function is not impaired.

The partial pressure of oxygen at which hemoglobin is 50% saturated, known as the p50, was measured after 24 hours and 7 days of post-thaw storage (Table 5.2). The p50 is a conventional measure of hemoglobin affinity for oxygen such that an increased p50 value indicates decreased affinity whereas a lower p50 value indicates higher affinity.¹⁰⁶ Typical p50 levels for RBCs cryopreserved in 40% glycerol, deglycerolized and stored at 4 °C for 24 hours are approximately 21.4 mmHg.¹⁰⁸ RBCs cryopreserved with 15%

glycerol or **409** (20 mM) + 15% glycerol possessed similar p50 levels (~17 mmHg) after storage for 24 hours. After 7 days of storage at 4 °C, the p50 levels were consistent with those previously reported for RBC units cryopreserved with 40% glycerol.¹⁰⁸ These results further suggest that oxygen transport function is not reduced.

RBC membrane deformability was measured by ektacytometry using the laser assisted optical rotational cell analyzer (LORCA).⁸⁴ The ability of RBCs to deform is essential for their navigation through the microvasculature and subsequent delivery of oxygen to tissues.⁸⁵ As such, a decrease in RBC deformability can affect their ability to enter the microvasculature, resulting in impaired perfusion and oxygen delivery to tissues.^{85–88} RBC deformability is measured by subjecting a suspension of RBCs in a high viscous medium to flow stress and applying varying shear stresses, causing RBC deformation.⁸⁴ By passing a laser through the suspension, the deformability of RBCs can be measured from the diffraction patterns of the laser.⁸⁴ This results in a characteristic deformability curve which is then linearized via the Eadie-Hofstee technique to obtain two parameters describing RBC deformation; EI_{max} and K_{EI} .⁸⁴ EI_{max} is defined as the maximum elongation index predicted at an infinite shear stress and therefore it is an indicator of overall RBC deformability.⁸⁴ K_{EI} is defined as the shear stress required to elongate to half of the EI_{max} and thus it is an indicator of RBC rigidity.⁸⁴ Thus, a high EI_{max} indicates very deformable RBCs whereas a high K_{EI} indicates very rigid RBCs as more shear stress is required to reach half of the EI_{max} . EI_{max} and K_{EI} values after RBC cryopreservation with 15% glycerol or **409** (20 mM) + 15% glycerol are shown in Table 5.2. RBC deformability was measured after 24 hours and 7 days of post-thaw storage. Typical EI_{max} and K_{EI} values for RBCs cryopreserved in 40% glycerol, deglycerolized and stored at 4 °C for 24 hours are approximately 0.55 and 2.36, respectively.^{109,110} The EI_{max} values for RBCs cryopreserved with 15% glycerol or **409** (20 mM) + 15% glycerol were similar (~0.4) to those previously reported and remained unchanged after 7 days of storage. Similarly, RBCs cryopreserved with 15% glycerol or **409** (20 mM) + 15% glycerol possessed comparable K_{EI} values to those reported for 40% glycerol.¹⁰⁹ These results suggest that cryopreservation utilizing **409** (20 mM) + 15% glycerol and subsequent deglycerolization does not impact RBC deformability.

Extracellular potassium (K^+) and sodium (Na^+) were measured after 24 hours and 7 days of post-thaw storage. Under normal conditions, the RBC membrane functions as a permeability barrier, allowing the cell to maintain different concentrations of ions intracellularly.¹¹¹ The sodium/potassium adenosine triphosphate (Na^+/K^+ ATPase) pump is responsible for correcting minor ion leakages such that potassium is pumped into the cell in exchange for sodium, maintaining a gradient where potassium is the principal intracellular cation and sodium is the principal extracellular cation.^{111,112} However, at temperatures of 4 °C, typically employed for the storage of RBCs, the Na^+/K^+ ATPase pump has limited functionality.^{111,113,114} This results in changes in cation gradients such that potassium progressively leaks from the RBCs into the surrounding medium, gradually increasing the extracellular potassium concentration.¹¹² A typical range for extracellular potassium and extracellular sodium for RBCs cryopreserved in 40% glycerol and deglycerolized is 0.6-1.0 mmol/L and 137.9-140.7 mmol/L, respectively.¹¹⁵ After 24 hours of storage, RBCs cryopreserved with 15% glycerol or **409** (20 mM) + 15% glycerol possessed extracellular potassium concentrations of approximately 1.0 mmol/L. This is consistent with previously reported values as thawed RBC units usually have low potassium content due to the removal of these extracellular substances during washing.¹¹⁶ During storage, the extracellular potassium concentration increased from 1.0 mmol/L 24 hours after deglycerolization to approximately 4.0 mmol/L after 7 days of storage. This is expected as extracellular potassium levels can increase by 0.5-1.0 mmol/L per day of refrigerated storage.¹¹⁷ Extracellular sodium concentrations for RBCs cryopreserved with 15% glycerol or **409** (20 mM) + 15% glycerol appeared to be slightly elevated compared to those reported for 40% glycerol, which may be due to altered permeability of the RBC membrane resulting from cell damage during cryopreservation.

Overall, the *in vitro* assays suggest that RBCs frozen with **409** (20 mM) + 15% glycerol in dry ice (-78 °C), stored in a -80 °C freezer, deglycerolized and stored at 4 °C in AS-3 possess comparable quality and function to those cryopreserved with 40% glycerol. However, a major limitation of this study was the small number of samples studied (n = 1), which did not permit the adequate examination of RBC *in vitro* quality and function. Nevertheless, the findings obtained from this preliminary large-scale study underscore the need for further investigation.

5.2.5 Summary for RBC Cryopreservation

Previous work by our laboratory demonstrated that small molecule IRIs **103** (β -PMP-Glc) and **104** (β -pBrPh-Glc) are effective additives for the freezing of RBCs with the ability to increase the percentage of intact RBCs post-thaw in the presence of 15% glycerol and slow cooling rates. This initial discovery was significant since post-thaw processing times would be greatly reduced with low glycerol concentrations and would provide access to more readily available RBC units. The next steps involved exploring deglycerolization conditions to determine whether the glycerol could be adequately removed from RBCs cryopreserved with **103** or **104** in 15% glycerol; however, it was shown that a significant amount of cellular damage occurred, resulting in very low cell recoveries. An attempt to improve the deglycerolization process focused on removing the excess cryosolution prior to freezing; however, this did not facilitate CPA removal as a decrease in the percentage of intact RBCs post-process was observed. Furthermore, an alternative approach using HES was explored since this method would eliminate the problems associated with glycerol-preserved RBCs. It was demonstrated that the addition of **104** to a solution of HES was capable of increasing the percentage of intact RBCs post-thaw and cell recovery when slow cooling rates were utilized. However, HES is not clinically used for the cryopreservation of RBCs due to the inadequate data regarding the safety of HES in patients receiving repeated transfusions and therefore, this study served to demonstrate that **104** can be successfully utilized with different cryosolution formulations.

While efforts to optimize the deglycerolization conditions for these novel RBC cryosolutions remained unsuccessful, these studies were crucial because they provided insight into the possible cause of failure. In particular, osmotic tolerance studies that were previously performed demonstrated that RBCs suspended in **104** + 15% glycerol had significantly lower osmotic tolerances following wash 1. It was hypothesized that this was the result of a potential interaction with the RBC membrane, and ultimately low cell recoveries were observed because of membrane interactions during freezing, causing the RBCs to be more susceptible to osmotic stress during deglycerolization. Thus, it was postulated that IRI active azido aryl glucosides may improve the deglycerolization process since these molecules may be less likely to interact with the RBC membrane

during freezing, facilitating their removal during deglycerolization. Given the non-toxic nature of **405**, **406** and **409** in RBCs, their cryopreservation potential was explored and it was initially demonstrated that **409** was a more effective cryoprotectant than **405** or **406** in the presence of 15% glycerol and slow cooling rates. Osmotic tolerance studies were performed to help determine the gradient steps of washing solutions that are tolerated and can be used to deglycerolize the RBCs. It was immediately evident that RBCs suspended in **405**, **406** or **409** + 15% glycerol had a much higher osmotic tolerance after wash 1 and could be resuspended in isotonic saline (0.9%). Therefore, a two-step wash method was employed and initial small-scale (1 mL volume) deglycerolization studies demonstrated that RBCs cryopreserved with azido aryl glucosides experience less RBC damage during deglycerolization. The most remarkable observation was that RBCs cryopreserved with **409** + 15% glycerol resulted in significantly higher recoveries than those obtained with **104** (β -pBrPh-Glc) utilizing slow cooling conditions. The potential of this IRI in clinical applications was evaluated by exploring a scale-up method in which the total sample volume was increased to 250 mL. An increase in cell recovery was observed when RBCs were frozen with **409** (20 mM) + 15% glycerol by placement into dry ice (recovery of 70%). RBC quality parameters were measured to evaluate the function of RBC metabolism and membrane post-cryopreservation and the *in vitro* assays suggested that RBCs frozen with **409** (20 mM) + 15% glycerol possess comparable quality and function to those cryopreserved with 40% glycerol.

Overall, these results demonstrate that azido aryl glucosides are beneficial additives for the freezing of RBCs in the presence of reduced concentrations of glycerol. More importantly, while a number of modifications to the large-scale cryopreservation protocol were made, this preliminary work demonstrates that it is possible to successfully scale up to larger volumes (~250 mL) using **409** (20 mM) in 15% glycerol. Further optimization is required to utilize automated deglycerolization methods, which could yield greater RBC recovery with increased removal of free hemoglobin, and would reduce operator time and increase the overall efficiency of the deglycerolization process.

5.3 Cryopreservation Ability of Small Molecule IRIs with MSCs

5.3.1 Introduction – Cryopreservation of MSCs

Mesenchymal stem cells (MSCs) are multipotent stem cells with the capacity to differentiate into osteocytes, adipocytes, and chondrocytes as well as neurocytes and hepatocytes.¹¹⁸ MSCs were initially isolated from bone marrow; however, various tissues such as adipose tissue, amniotic fluid, endometrium, dental tissue and umbilical cord tissue have been reported to harbor potential MSCs.¹¹⁸ Due to the tissue repair properties of MSCs,¹¹⁹ they are ideal candidates for several biomedical applications, in particular cell-based therapies for the treatment of various inflammatory diseases,¹²⁰ cardiac repair,¹²¹ musculoskeletal diseases,¹²² and osteoarthritis.¹²³ Furthermore, the immunosuppressive properties of MSCs are advantageous compared to other cell types as it permits allogeneic transplantation without the need for immunosuppressive therapy.¹⁴ Despite the great advantages that MSCs have over other stem cells, their clinical applications are impeded by several barriers of which include obtaining a sufficient amount of cells. Typically, large numbers of MSCs are obtained by culturing or pooling from multiple donors;¹²⁴ however, not all MSCs can be cultured for long periods of time as it has been shown that they lose their potency during *in vitro* expansion.¹¹⁸ When MSCs are continuously subcultured, it increases the possibility of contamination, genotypic drift, chromosomal abnormalities and phenotypic instability.^{125–127} In addition, early passage MSCs were shown to display increased differentiation ability to osteocytes, adipocytes and chondrocytes compared to those at higher passage numbers and on long-term culture.¹²⁸ It has also been reported that the engraftment efficiency of MSCs decreases with higher passage number.¹²⁹ Consequently, the only feasible method that can provide a large number of MSCs in an “off-the-shelf” format for clinical applications is cryopreservation and cell banking.

Several cryopreservation techniques have been employed to preserve MSCs; however, the challenges lie in optimizing the freezing protocols to ensure that post-thaw MSCs maintain their viability and functional properties, and retain the characteristics of freshly isolated MSCs. Conventional methods of MSC cryopreservation commonly use 10% DMSO in an electrolyte solution (ex. PlasmaLyte A, PLA) containing an added protein (ex. Human Serum Albumin, HSA). The cell solutions are typically frozen in a

controlled rate freezer¹³⁰ or at a rate of 1 to 5 °C/min to approximately -100 °C at which point the cells are stored in liquid nitrogen.¹³¹ Although these protocols yield high post-thaw viability and recovery,¹³⁰ DMSO is cytotoxic at the concentrations used to preserve cells, therefore complicating the direct clinical use of frozen-thawed cells. Upon the infusion of MSCs frozen in DMSO, it has been shown that patients develop mild complications such as nausea and vomiting.¹³² Furthermore, DMSO has been reported to possess a wide spectrum of toxicities and adverse effects on the central nervous system,^{133,134} respiratory,^{135,136} hemolytic,¹³⁷ gastrointestinal,¹³ hepatic,¹³⁷ cardiovascular^{135,138} and renal systems.¹³ Due to these toxic effects, it can necessitate the removal of DMSO from thawed cells prior to use; however, this process is both complex and time-consuming¹⁴ and has been shown to be associated with damaging osmotic shock,¹⁵ resulting in cell loss. Nevertheless, despite its toxicity, DMSO remains the only effective CPA.

Considering the therapeutic potential of MSCs for a variety of diseases, there is a need to ensure that cryopreserved MSCs maintain their functional capacity in order to be effective therapeutics. In recent years, several research groups have examined the impact of cryopreservation on MSC functionality, particularly on its immunomodulatory functions. It has been suggested that freshly thawed MSCs display impaired immunomodulatory and blood regulatory properties.^{127,139–141} These results highlight a possible cause for the failure of clinical trials on MSC-based immunotherapy using freshly thawed cell products as compromised MSC function may result in diminished efficacy.^{127,141,142} Consequently, since the treatment efficacy of MSC transplantation depends on the quality of transplanted MSCs, the impact of cryopreservation on MSC function must be well understood in order to advance MSC-based therapies.

Improvements to the cryopreservation protocol for MSCs have primarily focused on developing procedures that utilize reduced concentrations of DMSO to minimize toxicity or that replace DMSO with non-toxic alternatives. In particular, several studies have demonstrated that the concentration of DMSO could be reduced to 5% without significantly comprising the post-thaw viability of MSCs.^{143–147} Furthermore, a DMSO-free cryopreservation protocol for MSCs has recently been reported which involves the addition of different sugars (sucrose, trehalose or raffinose) into the cell culture medium

for 24 h prior to cryopreservation and subsequent freezing in the presence of sugars.^{148,149} The application of sugars on both the pre-treatment and cryopreservation stages was shown to result in a significant increase in MSC viability.¹⁴⁸ The majority of these studies limited their assessment of cellular function post-thaw to membrane integrity, failing to adequately provide insight on the functional capacity of MSCs. A recent study examined the effects of optimized DMSO-free cryopreservation solutions on the molecular, cellular and biological properties of MSCs.¹⁵⁰ In this study, MSCs were cryopreserved in solutions containing different combinations of sugars, sugar alcohols and small molecule additives and post-thaw MSC samples underwent extensive functional characterization including immunomodulatory functions, gene expression analysis of growth factors, adhesion molecules, transcription factors, chemokines, and stress genes, which are all associated with the functionality and potency of MSCs.¹⁵⁰ It was demonstrated that MSCs frozen using these osmolyte-based cryopreservation solutions exhibited comparable post-thaw recovery and improved post-thaw attachment compared to MSCs cryopreserved with DMSO.¹⁵⁰ In addition, these novel cryosolutions retained the differentiation ability of MSCs and were shown to improve post-thaw function of MSCs via effects on the epigenome and expression of cytoprotective genes.¹⁵⁰

Given the recent evidence that suggests that freshly thawed MSCs may have compromised function, we sought to examine the ability of small molecule IRIs to function as beneficial additives to improve the post-thaw function of MSCs. Furthermore, since MSCs are nucleated cells that possess the ability to differentiate into a variety of cell types,¹⁵¹ they are an appropriate *in vitro* model for studying the effects of IRIs on cell proliferation and differentiation.

5.3.2 Cryopreservation of MSCs using β -pBrPh-Glc and 2FA in the Presence of DMSO

It has been previously demonstrated that **104** (β -pBrPh-Glc) is an effective additive for the freezing of human RBCs with the ability to increase the percentage of intact RBCs post-thaw in the presence of reduced concentrations of glycerol (15%) and slow cooling rates. However, this relied on post-thaw integrity alone, which is not indicative of functional capacity. Thus, the cryopreservation potential of **104** (β -pBrPh-Glc) was explored in MSCs to assess its functional capacity and determine whether post-thaw

MSC function could be improved in the presence of **104**. Conversely, although IRI active azido aryl glucosides were shown to be effective additives for the freezing of RBCs (Section 5.2.4), they were not explored in MSCs due to their lengthy chemical syntheses that are not amenable to large-scale preparations necessary for cryopreservation applications. In addition to **104** (β -pBrPh-Glc), *N*-(2-fluorophenyl)-D-gluconamide (**501** (2FA), Figure 5.29) was also explored for the cryopreservation of MSCs. It has been previously demonstrated that this class of novel small molecules, *N*-aryl-D-aldonamides, possess the ability to inhibit ice recrystallization and function as beneficial additives to the standard 10% DMSO solution to improve the post-thaw function of hematopoietic stem and progenitor cells (HSPCs) from umbilical cord blood (UCB).²⁰ Specifically, it was demonstrated that **501** (2FA) was the most effective inhibitor of ice recrystallization and resulted in a significant increase in the number of post-thaw committed progenitors and CD34+ cell numbers.²⁰ This highlighted the ability of **501** (2FA) supplemented with 10% DMSO to increase the post-thaw function and potency of HSPCs from UCB. Consequently, interest arose in examining the ability of **501** (2FA) to protect other cellular products. Thus, small molecule IRIs, **104** (β -pBrPh-Glc) and **501** (2FA), were assessed for their ability to function as beneficial additives for the cryopreservation of MSCs to improve post-thaw function.

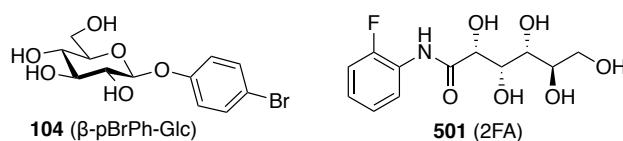


Figure 5.29 Structures of **104** (β -pBrPh-Glc) and **501** (2FA).

This preliminary study involved the use of bone marrow-derived MSCs, which were prepared at 2.0×10^6 cells/mL in standard freezing media (5% Human Albumin (HA) and 10% DMSO in PlasmaLyte A (PLA)) supplemented with either **104** (30 mM) or **501** (15 mM). Human albumin is a protein that provides cellular stabilization and PlasmaLyte A is an electrolyte solution that provides physiological osmolality and pH.¹⁵² Thus, the standard media formulation for MSC freezing is suitable for clinical use.¹⁵² The concentration selected for **104** was based on the previous optimal concentration for the freezing of human RBCs, whereas the concentration selected for **501** was based on its maximum solubility in standard freezing media (5% HA-PLA and 10% DMSO) at 37 °C.

The samples were frozen in a controlled rate freezer using an optimized step-down program in which the starting temperature was set at 18 °C, and the temperatures were subsequently modulated as indicated: 5 °C/min until the chamber reached 4 °C, 1 °C/min until the sample reached -6 °C, 30 °C/min until the chamber reached -40 °C, 10 °C/min until the chamber reached -18 °C, 1 °C/min until the chamber reached -40 °C, 5 °C/min until the chamber reached -80 °C, 10 °C/min until the chamber reached -180 °C, followed by transfer to liquid nitrogen storage. This step-down program was initially optimized for a solution of 5% HA-PLA and 10% DMSO to ensure high post-thaw viability and recovery. Thus, to ensure that this step-down program could be utilized for solutions containing **104** or **501** in 5% HA-PLA and 10% DMSO, freezing curves were recorded. The cooling profiles are depicted in Figure 5.30.

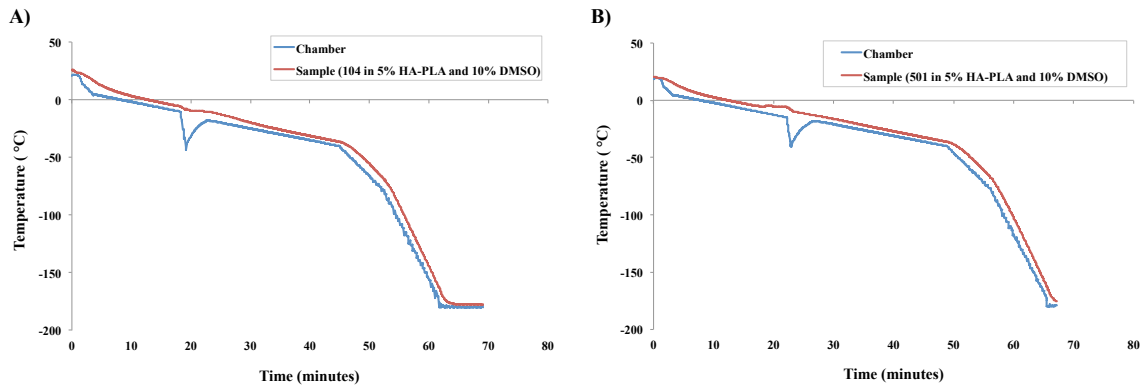


Figure 5.30 Cooling profiles for solutions of **104** (A) or **501** (B) in 5% HA-PLA and 10% DMSO using the optimized step-down program.

The cooling and warming steps of the freezing profile (30 °C/min to -40 °C, 10 °C/min to -18 °C) correspond to a downward spike in the temperature of the chamber included to “flatten” the temperature rebound of the sample resulting from the latent heat of fusion of ice formation (nucleation).³ This method of rapid temperature reduction is often used in controlled rate freezers to control ice nucleation and provide temperature compensation for release of the latent heat of fusion to result in improved post-thaw viability.¹⁵³ It is evident that the step-down program previously optimized can be utilized for solutions containing **104** or **501** as uniform cooling profiles were achieved with minimal latent heat evolution upon freezing (Figure 5.30).

Samples were removed from liquid nitrogen storage and thawed at room temperature until no ice was observed. Cell viability after thawing was measured by Trypan Blue

exclusion and Annexin V and propidium iodide (PI) staining, and cell recovery was calculated. Viable cells were identified as both Annexin V and PI negative. The results are shown in Table 5.3.

Table 5.3 Cell viability, recovery and proliferation post-thaw.

	5% HA-PLA and 10% DMSO (n = 1)	104 (30 mM) in 5% HA-PLA and 10% DMSO (n = 3)	501 (15 mM) in 5% HA-PLA and 10% DMSO (n = 3)
<i>Cell viability, % (Trypan Blue)</i>	92.5	87.6 ± 0.8	90.9 ± 0.9
<i>Cell viability, % (AV-/PI-)</i>	82.3	82.0 ± 4.2	91.7 ± 0.6
<i>Cell recovery, %</i>	80.0	73.3 ± 6.7	91.3 ± 0.7
<i>Proliferation, % in S-phase</i>	6.9	5.6 ± 0.5	23.6 ± 1.9

^aResults shown as mean ± SEM.

MSCs cryopreserved in 5% HA-PLA and 10% DMSO possessed a recovery of 80% and cell viabilities of 92.5% and 82.3% as measured by Trypan Blue exclusion and Annexin V and PI staining, respectively. In comparison, MSCs cryopreserved in 5% HA-PLA and 10% DMSO containing **104** (30 mM) or **501** (15 mM) had similar recoveries of 73.3 ± 6.7% or 91.3 ± 0.7%, respectively. In the presence of **104** (30 mM) or **501** (15 mM), cell viability post-thaw was also comparable to the control (5% HA-PLA and 10% DMSO). In addition to assessing cell viability and recovery, cell proliferation was measured 48 hours post-thaw as a surrogate marker of recovery. The S-phase of the cell cycle represents the period in which cells replicate their DNA,¹⁵⁴ and thus cell proliferation is characterized by *de novo* DNA synthesis during this phase.¹⁵⁴ Cell proliferation was analyzed in post-thaw samples cryopreserved with **104** or **501** using the EdU (5-ethynyl-2'-deoxyuridine) assay.¹⁵⁵ EdU is a thymidine nucleoside analog containing an alkyne that is incorporated into newly synthesized DNA strands of proliferating cells. The alkyne group of the incorporated EdU is then detected through its reaction with a fluorescent-labeled azide in a Cu(I)-catalyzed [3+2] cycloaddition reaction ("click" chemistry).¹⁵⁵ Using flow cytometry, the percentage of S-phase cells in the population can be determined. The results are shown in Table 5.3. In the control sample (5% HA-PLA and 10% DMSO), approximately 7% of cells were in the S-phase of the cell cycle.

However, an increase in the fraction of cells in the S-phase of the cell cycle was observed for those cryopreserved with **501** ($23.6 \pm 1.9\%$), indicating that *de novo* DNA synthesis is occurring at a much higher rate. This effect was specific to **501** because cells cryopreserved with **104** possessed a similar fraction of cells in the S-phase to that of the control.

In addition, post-thaw samples were cultured for three days to evaluate cell attachment and growth. After three days in culture, MSCs were harvested, counted and cell viability was measured by Trypan Blue exclusion and Annexin V and PI staining. In addition, the cells were imaged and there were no apparent morphological changes in those cryopreserved with **104** or **501** compared to the control (Figure 5.31).

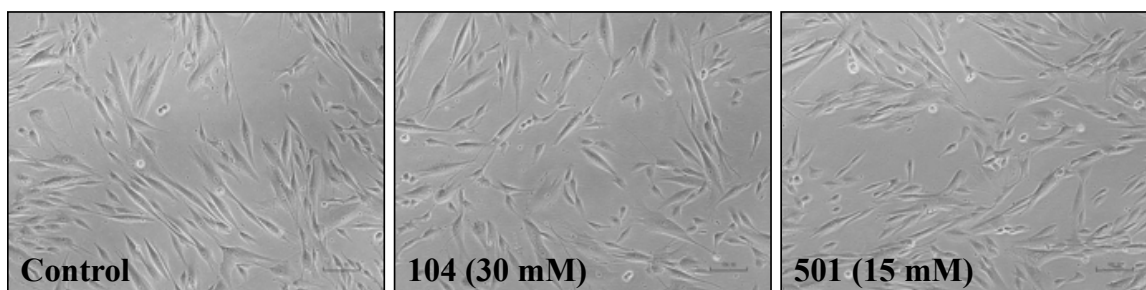


Figure 5.31 Morphology of post-thaw MSCs after three days in culture.

After three days in culture, MSCs cryopreserved in 5% HA-PLA and 10% DMSO supplemented with **104** (30 mM) or **501** (15 mM) possessed cell viabilities comparable to the control as measured by Trypan Blue exclusion and Annexin V and PI staining (Table 5.4). The characteristics of cultured MSCs were measured in terms of population doubling and population doubling time (Table 5.4). Population doubling refers to the total number of times the cells in culture have increased two-fold (doubled).¹⁵⁶ The population doublings for MSCs cryopreserved with **501** (15 mM) in 5% HA-PLA and 10% DMSO and cultured post-thaw for three days were greater (2.7 ± 0.3) than those cryopreserved in 5% HA-PLA and 10% DMSO, signifying that these cells possess augmented replicative capacity. There were no differences observed between those cryopreserved with **104** (30 mM) in 5% HA-PLA and 10% DMSO and the control in terms of population doublings. The ability of MSCs to proliferate *in vitro* was also evaluated by measuring the population doubling time, which refers to the time required for a population to double the number of cells.¹⁵⁷ MSCs cryopreserved with **501** (15 mM) in 5% HA-PLA and 10%

DMSO and cultured post-thaw for three days had lower doubling times (27.4 ± 3.6 h) compared to those cryopreserved with **104** (30 mM) or 5% HA-PLA and 10% DMSO alone, which did not show differences in their doubling times (59.7 ± 5.3 h versus 52.2 h). These results are significant because greater proliferation of MSCs post-thaw when cryopreserved with **501** would allow for shorter culture time or large expansion to generate a sufficient quantity of MSCs for clinical use.

Table 5.4 Cell viability, population doubling and doubling time after 3 days in culture post-cryopreservation.

	5% HA-PLA and 10% DMSO (n = 1)	104 (30 mM) in 5% HA-PLA and 10% DMSO (n = 3)	501 (15 mM) in 5% HA-PLA and 10% DMSO (n = 3)
Cell viability, % (Trypan Blue)	92.8	89.1 ± 1.6	95.3 ± 0.6
Cell viability, % (AV-/PI-)	91.5	94.5 ± 0.07	93.2 ± 1.2
Population doubling	1.4	1.2 ± 0.1	2.7 ± 0.3
Doubling time, h	52.2	59.7 ± 5.3	27.4 ± 3.6

^aResults shown as mean $\pm$ SEM.

5.3.3 Summary for MSC Cryopreservation

It has been previously demonstrated that **104** (β -pBrPh-Glc) and **501** (2FA) are effective additives for the freezing of human RBCs and the freezing of HSPCs from UCB, respectively. As a result, we sought to explore the cryopreservation potential of **104** and **501** in bone marrow-derived MSCs to examine their ability to function as beneficial additives to improve MSC function post-thaw. In this preliminary study, MSCs were cryopreserved in 5% HA-PLA and 10% DMSO supplemented with either **104** (30 mM) or **501** (15 mM) using an optimized step-down program. Upon thawing, cells were assessed for viability, recovery and proliferation. Although there were no differences in cell viability and recovery post-thaw for all conditions, analysis of proliferation showed that proliferative capacity was substantially increased when MSCs were cryopreserved in 5% HA-PLA and 10% DMSO containing **501** (15 mM). Furthermore, MSCs that were cultured after cryopreservation with **501** had lower doubling times and underwent more population doublings. These results are encouraging given that MSCs cryopreserved with

501 (15 mM) in 5% HA-PLA and 10% DMSO demonstrate marked improvement in post-thaw MSC function. Additional testing is currently on-going to increase the number of technical and biological replicates and ultimately, further functional analysis is required.

5.4 Experimental Procedures

Small molecule IRIs **103** (β -PMP-Glc), **104** (β -pBrPh-Glc) and **501** (2FA) used in this chapter for cryopreservation studies were prepared as previously described.^{20,158} IRI active azido aryl glucosides **405**, **406** and **409** used in this chapter for cryopreservation studies were prepared as described in the experimental section of Chapter 4. Cryopreservation protocols for RBCs and MSCs are described below. Culture of MSCs, cryopreservation, and EdU cell proliferation assays were done in collaboration with Liliana Davila, a research technician under the supervision of Dr. David Courtman at the Ottawa Hospital Research Institute (OHRI).

5.4.1 Blood Collection and Preparation

All RBCs were obtained from NetCAD (Canadian Blood Services' Network Centre for Applied Development). Whole blood was collected from healthy volunteers using standardized phlebotomy guidelines approved by Canadian Blood Services (CBS). Informed consent was obtained from all donors. All experimental protocols were approved by NetCAD and CBS. Ethics approvals were obtained from Research Ethics Board (REB) at CBS and the University of Alberta. For cryovial experiments, whole blood units were collected and processed by NetCAD (Vancouver, BC). The whole blood was processed using the buffy coat (BC) method to produce leukocyte reduced SAGM RBC units, which has been previously described.⁵⁶ ABO matched RCCs were pooled and split to reduce donor to donor variability.

5.4.2 RBC Freezing and Thawing Experiments using 15% Glycerol

The freezing solution consisted of a 30% glycerol solution prepared from a commercially available glycerol solution (57 Glycerolyte, Baxter) by diluting it with 0.9% saline/0.2% dextrose. An equal volume of freezing solution was added to 150 μ L of RBCs for a final volume of 300 μ L. The final concentrations of all freezing solutions are

as indicated in the text. RBC suspensions were transferred to cryotubes and incubated at room temperature for 10 minutes prior to immersion in a methanol bath cooled to -5 °C. A thermocouple was inserted into a RBC/15% glycerol sample (temperature probe) to monitor temperature at 1 s intervals. Once the internal solution from the temperature probe reached -5 °C, ice nucleation was induced by touching the cryotubes with pre-cooled (in liquid nitrogen) forceps. RBC samples were then held at -5 °C for 5 minutes. Samples were then cooled at a rate of 1 °C/min to -40 °C or placed into dry ice (-78 °C) and thawed in a 37 °C water bath.

5.4.3 Post-Thaw Hemolysis

Post-thaw hematocrits (hct) and percentage hemolysis were determined for all freezing experiments by comparing the supernatant hemoglobin concentration to total hemoglobin concentration using the cyanmethemoglobin Drabkin's method.^{56,57} The percentage of intact RBCs post-thaw was calculated using the measured percentage hemolysis values according to the following equation: % intact RBCs post-thaw = 100 - % hemolysis.

5.4.4 RBC Deglycerolization Experiments

The freezing solution consisted of a 30% glycerol solution prepared from a commercially available glycerol solution (57 Glycerolyte, Baxter) by diluting it with 0.9% saline/0.2% dextrose. An equal volume of freezing solution was added to 500 µL of RBCs for a final volume of 1.0 mL. The final concentrations of all freezing solutions are as indicated in the text. RBC suspensions were transferred to cryotubes and incubated at room temperature for 10 minutes prior to placement into dry ice (-78 °C) or a -80 °C freezer. A thermocouple was inserted into a RBC/15% glycerol sample (temperature probe) to monitor temperature at 1 s intervals. Following thawing at 37 °C in a water bath, RBC samples were centrifuged at 2,200 g for 5 minutes at 4 °C and the supernatant was removed. Packed RBCs were then resuspended in wash 1 solution by adding an equal volume of the removed supernatant. Samples were centrifuged at 2,200 g for 5 minutes at 4 °C and the supernatant was removed. This process was repeated for wash 2 and wash 3 (if necessary). Following removal of the supernatant, the remaining packed RBCs were resuspended in 0.9% saline or AS-3 solution.

5.4.5 Calculation of RBC Recovery

All empty vials, pre-processed vials and post-deglycerolized vials were weighed and recorded. Percentage cell recovery was calculated based on changes in RBC volume and hematocrit (hct) according to the following equation: % cell recovery = (post-weight (g) x post-specific gravity (g/mL) x post-hct)/ (pre-weight (g) x pre-specific gravity (g/mL) x pre-hct) x 100.

5.4.6 RBC Deglycerolization Experiment with Removal of Excess Cryosolution Pre-Freeze

The freezing solution consisted of a 30% glycerol solution prepared from a commercially available glycerol solution (57 Glycerolyte, Baxter) by diluting it with 0.9% saline/0.2% dextrose. An equal volume of freezing solution was added to 500 µL of RBCs for a final volume of 1.0 mL. The final concentrations of all freezing solutions are as indicated in the text. RBC suspensions were transferred to cryotubes and incubated at room temperature for 10 minutes. RBC samples were centrifuged at 2,200 g for 5 minutes at 4 °C and the supernatant was removed prior to placement into dry ice (-78 °C) or immersion in liquid nitrogen (-196 °C). A thermocouple was inserted into a RBC/15% glycerol sample (temperature probe) to monitor temperature at 1 s intervals. Following thawing at 37 °C in a water bath, RBCs were suspended in wash 1 solution (500 µL) and centrifuged at 2,200 g for 5 minutes at 4 °C and the supernatant was removed. This process was repeated for wash 2 and wash 3. Following removal of wash 3 supernatant, the remaining packed RBCs were resuspended in 0.9% saline.

5.4.7 RBC Freezing and Thawing Experiments using 11.5% HES

The freezing solution consisted of an HES solution (23% w/w in 60 mM NaCl) prepared from commercially available 200/0.5 HES (Fresenius Kabi) by diluting it with 60 mM NaCl. An equal volume of freezing solution was added to 500 µL of RBCs for a final volume of 1.0 mL. The final concentrations of all freezing solutions are as indicated in the text. Samples were cooled by plunging into liquid nitrogen (-196 °C) or dry ice (-78 °C), or by placing in a -80 °C freezer, and thawed immediately in a 48 °C water bath. After thawing, RBCs were diluted with 0.9% saline (500 µL). Samples treated with a simple wash procedure were centrifuged at 16,000 g for 5 minutes at 4 °C, the

supernatant was removed and RBCs were resuspended in 0.9% saline (500 μ L).

5.4.8 RBC In Vitro Toxicity (Hypothermic Storage)

An equal volume of 0.9% saline/0.2% dextrose was added to 5.5 mL of RBCs for a final volume of 11 mL. The final concentrations of all solutions are as indicated in the text. RBC properties were measured immediately or after 24 h, 48 h or 7 days of storage at 4 °C in a refrigerator.

5.4.9 RBC Indices

Mean cell volume (MCV) was measured using a hematology analyzer (Coulter AcT 8, Beckman Coulter, Inc., Fullerton, CA).

5.4.10 Hemoglobin Oxidation

Hemoglobin oxidation was measured according to methods previously described.⁵⁶ Briefly, RBC samples were diluted (1:500) in distilled water and absorbance was read in a microplate at 560, 576, 630, and 700 nm. The percentage of methemoglobin (metHb) was determined using extinction coefficients of oxyhemoglobin (oxyHb) and metHb.¹⁵⁹

5.4.11 Ektacytometric Analysis

RBC deformability was measured according to methods previously described.¹⁶⁰ Briefly, RBCs were suspended in high molecular weight polymer polyvinylpyrrolidone (PVP) and analyzed using a laser-assisted optical rotational cell analyzer (LORCA) (Lorrcar® Maxxis osmoscan, Mechatronics). The data were linearized using an Eadie-Hofstee analysis and two parameters were extracted: (1) EI_{max} (the maximum RBC elongation under shear stress); and (2) K_{EI} (the shear stress required for RBCs to reach half of their EI_{max}).

5.4.12 RBC Osmotic Tolerance

RBCs (500 μ L) were suspended with cryosolutions (500 μ L) and incubated at room temperature for 10 minutes. Following incubation, 10 μ L of the RBC/cryosolution mixture was added to 1 mL of different concentrations of saline (concentrations indicated in the text). RBCs were allowed to incubate in the saline solutions for 30 minutes at room

temperature, centrifuged at 2,200 g for 5 minutes at room temperature and the absorbance of the supernatant was measured at 540 nm. For the osmotic tolerances after washes, following incubation with cryosolution or wash solution, samples were centrifuged at 2,200 g for 5 minutes 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). Following this, 10 µL of the RBC/wash solution mixture was added to 1 mL of different concentrations of saline (concentrations indicated in the text). RBCs were allowed to incubate in the saline solutions for 30 minutes at room temperature, centrifuged at 2,200 g for 5 minutes at room temperature and the absorbance of the supernatant was measured at 540 nm. Hemolysis was calculated as described above.

5.4.13 RBC Freezing and Thawing Experiment using Different RBC:CPA Ratios

RBC freezing solutions were prepared at 1:1, 1:1.5, 1:2, 1:2.5 and 1:3 (RBC:CPA) in such a way to maintain a final concentration of 15% glycerol. The total sample volumes were 3.0 mL and the final concentrations of all freezing solutions are as indicated in the text. RBC suspensions were transferred to cryotubes and incubated at room temperature for 10 minutes prior to placement into dry ice (-78 °C) or immersion in liquid nitrogen (-196 °C). A thermocouple was inserted into a RBC/15% glycerol sample (temperature probe) to monitor temperature at 1 s intervals. Samples were then thawed in a 37 °C water bath.

5.4.14 Large-Scale RBC Deglycerolization Experiment

RBCs were prepared in 1L Origen freezing bags using a 1:3 ratio of RBCs:cryosolution for a final volume of 250 mL. The final concentrations of all freezing solutions are as indicated in the text. The samples were placed into metal freezing cassettes and frozen by placement into dry ice (-78 °C) or immersion in liquid nitrogen (-196 °C) prior to storage in a -80 °C freezer. The samples were thawed in a 37 °C water bath for approximately 5-10 minutes. The 1L bags were deglycerolized manually using centrifugation with supernatant extraction between washes. After thawing, wash 1 solution (~750 mL) was added, centrifuged at 2,200 g for 10 minutes at 4 °C and the supernatant was removed using a plasma extractor. Packed RBCs were then resuspended

in wash 1 solution (~750 mL), centrifuged at 2,200 g for 10 minutes at 4 °C and the supernatant was removed using a plasma extractor. Lastly, wash 2 solution (~750 mL) was added, centrifuged at 2,200 g for 10 minutes at 4 °C and the supernatant was removed using a plasma extractor. The remaining packed RBCs were resuspended in AS-3 solution to obtain a hematocrit of 50%. The pre-processed bag and post-deglycerolized bag were weighed and recorded to determine cell recovery.

5.4.15 Adenosine Triphosphate Concentration

Adenosine triphosphate (ATP) concentrations were measured according to methods previously described.⁵⁶ RBC samples were deproteinized on ice in 10 or 12% trichloroacetic acid at 5:6 or 1:1 dilution, respectively, centrifuged at 3,000 g for 10 minutes at 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.

5.4.16 2,3-Diphosphoglycerate Concentration

2,3-Diphosphoglycerate (DPG) concentrations were measured according to methods previously described.⁵⁶ RBC samples were deproteinized in 0.6 mol/L perchloric acid at a dilution of 1:5 on ice, centrifuged at 3,000 g for 10 minutes at 4 °C to obtain a clear supernatant, which was diluted (8:1) with 2.5 mol/L potassium carbonate and incubated on ice for 60 minutes. Extracts were centrifuged at 3,000 g for 5 minutes at 4 °C and measured using a commercial kit (2,3-DPG, Roche, Mannheim, Germany) adapted from the manufacturer's protocol to a microplate method. Absorbance (340 nm) measured the reduction of NADH, which was directly related to the concentration of 2,3-DPG. An in-house 2.5 mmol/L standard was used as an assay control (2,3-diphospho-d-glyceric acid pentasodium salt). The 2,3-DPG concentration was converted to $\mu\text{mol/g}$ Hb.

5.4.17 Oxygen Affinity (p50)

Oxygen affinity was measured according to methods previously described.⁵⁶ RBCs (50 μ L) were added to 5 mL of buffer solution (Hemox, TCS Scientific Corp., New Hope, PA), 10 μ L of antifoaming agent (TCS Scientific), and 20 μ L 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 Hb 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 quality control.

5.4.18 Extracellular K^+ and Na^+

Extracellular potassium (K^+) and sodium (Na^+) concentrations were measured according to methods previously described.⁵⁶ RBC samples were centrifuged at 2,200 g for 10 minutes at 4 °C, and extracellular K^+ and Na^+ were measured in the supernatant using an automated chemistry analyzer (DXC800, Beckman Coulter, Inc.).

5.4.19 MSC Culture

Donor MSCs previously cryopreserved in passage 1 in Master cell banks were thawed and cultured in NutriStem XF media supplemented with 0.6% NutriStem XF supplement mix and 10 μ g/mL gentamicin in 175 cm² Corning® flasks. Cells were incubated in a 37 °C incubator supplied with 5% CO₂ and cultures were maintained by changing media every three days. After four days in culture, the cells were removed from the flasks using 10 mL TrypLE Select for use in experiments.

5.4.20 MSC Freezing and Thawing Experiments

MSCs were cultured and detached as described above. Cells were diluted with culture media and counted using a hemocytometer. Aliquots containing 2×10^6 cells were added to 15 mL conical tubes and cells were pelleted by centrifugation at 400 g for 5-15 minutes at room temperature. The supernatant was removed and the cells were resuspended in 1 mL of 5% HA-PLA and 10% DMSO supplemented with the desired

IRI. The final concentrations of all freezing solutions are as indicated in the text. Cell suspensions were transferred to 2 mL cryogenic vials and frozen in a controlled rate freezer using an optimized step-down program (described in the text). Samples were then stored in the vapor phase of liquid nitrogen (-196 °C). Following storage, samples were thawed slowly at room temperature by adding 1 mL of pre-warmed media (37 °C) to the vial.

5.4.21 MSC Viability

Cell viability was evaluated using Trypan Blue exclusion method.¹⁶¹ An aliquot of the cell suspension was diluted 1:4 in Trypan Blue and the cells were loaded immediately onto a hemocytometer. The number of viable and non-viable cells was counted and the percentage of viable cells was calculated. A standard apoptosis assay was also performed by staining cells with Annexin V-Alexa Fluor 488 and propidium iodide (PI), and quantifying fluorescence on a flow cytometer.¹⁶² Viable cells were identified as both Annexin V and PI negative. Time between thawing and analysis by flow cytometry was less than 1 h.

5.4.22 MSC Recovery

Cell counts were obtained with a hemocytometer and percentage cell recovery was calculated by dividing the total cell count by the initial cell count and multiplying by 100.

5.4.23 EdU Cell Proliferation Assay

Thawed cell contents were seeded in 75 cm² Corning® flasks at 200,000 cells/flask and incubated in a 37 °C incubator supplied with 5% CO₂ for two days. Cultures were then incubated with 10 µM EdU for 4 hours. After 4 hours, the cells were removed from the flasks using TrypLE Select and the cell suspension was centrifuged at 400 g for 5 minutes at room temperature. The supernatant was removed and the cells were washed once with 1% bovine serum albumin (BSA) in PBS. The cells were resuspended in 1% BSA in PBS, fixed for 15 minutes in 4% paraformaldehyde in PBS and washed with 1% BSA in PBS. Following membrane permeabilization, the cells were directly incubated for 30 minutes at room temperature in the EdU detection cocktail (Invitrogen, Click-iT EdU Alexa Fluor 488 Flow Cytometry assay). For 1 sample reaction, the following amounts of

the reaction components were mixed in 438 µl PBS: 10 µl copper (II) sulfate solution (Component F, 100 mM CuSO₄), 2.5 µl Alexa Fluor 488 azide (Component B in 130 µl DMSO) and 50 µl buffer additive (Component G). The cells were washed and resuspended in 1X Click-iT saponin-based permeabilization and wash reagent. The samples were analyzed using a flow cytometer.

5.4.24 MSC Population Doublings and Doubling Time

Population doubling time was calculated using the following equation:

$$N(t) = N(0) \times 2^{(t/d)}$$

Where:

$N(t)$ = Number of cells harvested after “t” days

$N(0)$ = Number of cells seeded initially

t = Number of days in culture

d = Doubling Time

Based on the equation, (t/d) = population doublings.

5.5 References

- (1) Stacey, G. N.; Masters, J. R. *Nat. Protoc.* **2008**, 3 (12), 1981.
- (2) Meryman, H. T. *Transfusion* **2007**, 47, 935.
- (3) Baust, J. G.; Gao, D.; Baust, J. M. *Organogenesis* **2009**, 5 (3), 90.
- (4) Bakhach, J. *Organogenesis* **2009**, 5 (3), 119.
- (5) Scott, K. L.; Lecak, J.; Acker, J. P. *Transfus. Med. Rev.* **2005**, 19 (2), 127.
- (6) Mazur, P. Principles of Cryobiology. In: Fuller, B.; Lane, N.; Benson, E., eds. Life in the Frozen State. Boca Raton, FL: CRC Press; 2004.
- (7) Acker, J. P.; McGann, L. E. *Cell Transplant.* **2002**, 11 (6), 563.
- (8) Mazur, P.; Leibo, S. P.; Chu, E. H. *Exp. Cell Res.* **1972**, 71 (2), 345.
- (9) McGann, L. E. *Cryobiology* **1978**, 15 (4), 382.
- (10) Meryman, H. T. *Cryobiology* **1971**, 8 (2), 173.
- (11) Zenhausern, R.; Tobler, A.; Leoncini, L.; Hess, O. M.; Ferrari, P. *Ann. Hematol.* **2000**, 79 (9), 523.
- (12) Brockbank, K. G. M. *MOJ Cell Sci. Rep.* **2016**, 3 (4), 3.
- (13) Berz, D.; Colvin, G. Cryopreservation of Hematopoietic and Non-Hematopoietic Stem Cells – A Review for the Clinician. In: T. Demirer, editor. New Advances in Stem Cell Transplantation. New York: InTech; 2012. p 231–266.
- (14) Thirumala, S.; Goebel, W. S.; Woods, E. J. *Organogenesis* **2009**, 5 (3), 143.
- (15) Wood, E. J.; Pollok, K. E.; Byers, M. A.; Perry, B. C.; Purttman, J.; Heimfeld, S.; Gao, D. *Transfus. Med. Hemotherapy* **2007**, 34, 276.
- (16) Hess, J. R. *Transfus. Med.* **2004**, 14 (1), 1.
- (17) Farrant, J. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **1977**, 278 (959), 191.
- (18) Abraham, S.; Keillor, K.; Capicciotti, C. J.; Perley-Robertson, G. E.; Keillor, J. W.; Ben, R. N. *Cryst. Growth Des.* **2015**, 15 (10), 5034.

- (19) Capicciotti, C. J.; Kurach, J. D. R.; Turner, T. R.; Mancini, R. S.; Acker, J. P.; Ben, R. N. *Sci. Rep.* **2015**, *5*, 9692.
- (20) Briard, J. G.; Jahan, S.; Chandran, P.; Allan, D.; Pineault, N.; Ben, R. N. *ACS Omega* **2016**, *1*, 1010.
- (21) Takei, T.; Amin, N. A.; Schmid, G.; Dhingra-Kumar, N.; Rugg, D. J. *Acquir. Immune Defic. Syndr.* **2009**, *52* (Suppl 2), S127.
- (22) Klein, H. G.; Spahn, D. R.; Carson, J. L. *Lancet* **2007**, *370* (9585), 415.
- (23) Sharma, S.; Sharma, P.; Tyler, L. N. *Am. Fam. Physician* **2011**, *83* (6), 719.
- (24) Yap, C.; Lau, L.; Krishnaswamy, M.; Gaskell, M.; Yui, M. *Ann. Thorac. Surg.* **2008**, *86*, 554.
- (25) Hebert, P. C.; Chin-Yee, I.; Fergusson, D.; Blajchman, M.; Martineau, R.; Clinch, J.; Olberg, B. *Anesth. Analg.* **2005**, *100*, 1433.
- (26) Högman, C. F.; Meryman, H. T. *Transfus. Med. Rev.* **1999**, *13* (4), 275.
- (27) Tinmouth, A.; Fergusson, D.; Yee, I. C.; Hebert, P. C.; Investigators, A.; Group, the C. C. C. T. *Transfusion* **2006**, *46* (11), 2014.
- (28) Zimrin, A. B.; Hess, J. R. *Vox Sang.* **2009**, *96* (2), 93.
- (29) Bohoněk, M. Cryopreservation of Blood. In: P. Kochhar, editor. *Blood Transfusion in Clinical Practice*. Rijeka, HR: InTech; 2012. P 233–242.
- (30) Henkelman, S.; Noorman, F.; Badloe, J. F.; Lagerberg, J. W. M. *Vox Sang.* **2015**, *108*, 103.
- (31) Asghar, W.; El Assal, R.; Shafiee, H.; Anchan, R. M.; Demirci, U. *Biotechnol. J.* **2014**, *9* (7), 895.
- (32) Meryman, H. T.; Hornblower, M. *Transfusion* **1972**, *12* (3), 145.
- (33) Krijnen, H. W.; De Wit, J. J.; Kuivenhoven, A. C.; Loos, J. A.; Prins, H. K. *Vox Sang.* **1964**, *9*, 559.
- (34) Rowe, A. W.; Eyster, E.; Kellner, A. *Cryobiology* **1968**, *5* (2), 119.
- (35) Tullis, J. L.; Ketchel, M. M.; Pyle, H. M.; B, P. R.; Gibson, J. G.; Tinch, R. J.; Driscoll, S. G. *JAMA* **1958**, *168* (4), 399.
- (36) Lusianti, R. E.; Benson, J. D.; Acker, J. P.; Higgins, A. Z. *Biotechnol. Prog.* **2013**, *29* (3), 609.
- (37) Lusianti, R. E.; Higgins, A. Z. *Biomicrofluidics* **2014**, *8* (5), 054124.
- (38) Knorpp, C. T.; Merchant, W. R.; Gikas, P. W.; Spencer, H. H.; Thompson, N. W. *Science* **1967**, *157* (3794), 1312.
- (39) Williams, R. J. *Cryobiology* **1983**, *20* (5), 521.
- (40) Stolzing, A.; Naaldijk, Y.; Fedorova, V.; Sethe, S. *Transfus. Apher. Sci.* **2012**, *46* (2), 137.
- (41) Pellerin-Mendes, C.; Million, L.; Marchand-Arvier, M.; Labrude, P.; Vigneron, C. *Cryobiology* **1997**, *35* (2), 173.
- (42) Deller, R. C.; Vatish, M.; Mitchell, D. A.; Gibson, M. I. *ACS Biomater. Sci. Eng.* **2015**, *1*, 789.
- (43) Mitchell, D. E.; Lovett, J. R.; Armes, S. P.; Gibson, M. I. *Angew. Chem. Int. Ed.* **2016**, *55*, 2801.
- (44) Holovati, J. L.; Gyongyossy-Issa, M. I. C.; Acker, J. P. *Cryobiology* **2009**, *58*, 75.
- (45) Stoll, C.; Holovati, J. L.; Acker, J. P.; Wolkers, W. F. *Biotechnol. Prog.* **2012**, *28* (2), 364.
- (46) Brumfiel, G. *Nature* **2004**, *428*, 14.

- (47) Quan, G. B.; Han, Y.; Liu, M. X.; Fang, L.; Du, W.; Ren, S. P.; Wang, J. X.; Wang, Y. *Cryobiology* **2011**, 62 (2), 135.
- (48) Capicciotti, C. J. The Rational Design of Potent Ice Recrystallization Inhibitors for Use as Novel Cryoprotectants, University of Ottawa, 2014.
- (49) Gilmore, J. A.; Liu, J.; Gao, D. Y.; Critser, J. K. *Hum. Reprod.* **1997**, 12 (1), 112.
- (50) Gao, D. Y.; Liu, J.; Liu, C.; McGann, L. E.; Watson, P. F.; Kleinhans, F. W.; Mazur, P.; Critser, E. S.; Critser, J. K. *Hum. Reprod.* **1995**, 10 (5), 1109.
- (51) Wusteman, M. C.; Pegg, D. E.; Robinson, M. P.; Wang, L.-H.; Fitch, P. *Cryobiology* **2002**, 44 (1), 24.
- (52) Parpart, A. K.; Lorenz, P. B.; Parpart, E. R.; Gregg, J. R.; Chase, A. M. *J. Clin. Invest.* **1947**, 26 (4), 636.
- (53) Meryman, H. T. *Nature* **1968**, 218, 333.
- (54) Turner, T.; Hansen, A.; Kurach, J.; Acker, J. P. *Transfus. Med. Hemotherapy* **2017**, 44, 30.
- (55) Brecher, M. E. *Technical Manual*; Bethesda, MD: AABB, 2005.
- (56) Acker, J. P.; Hansen, A. L.; Kurach, J. D. R.; Turner, T. R.; Croteau, I.; Jenkins, C. *Transfusion* **2014**, 54 (10), 2534.
- (57) Drabkin, D. L. *Am. J. Med. Sci.* **1948**, 215, 110.
- (58) Lionetti, F. J.; Hunt, S. M. *Cryobiology* **1975**, 12 (2), 110.
- (59) Sputtek, A.; Horn, E. P.; Schulte Am Esch, J.; Kuhn, P. *Anesthesiol Intensivmed Notfallmed Schmerzther* **2001**, 36 (Suppl), S162.
- (60) Mitra, S.; Khandelwal, P. *Indian J. Anaesth.* **2009**, 53 (5), 592.
- (61) Thomas, M. J. G.; Parry, E. S.; Nash, S. G.; Bell, S. H. *Transfus. Sci.* **1996**, 17 (3), 385.
- (62) Hightower, C. M.; Salazar Vazquez, B. Y.; Cabrales, P.; Tsai, A. G.; Al., E. *Transfusion* **2012**, 53 (1), 49.
- (63) Sputtek, A. Cryopreservation of Red Blood Cells and Platelets. In: Day, J. G.; Stacey, G. N., eds. *Cryopreservation and Freeze-Drying Protocols. Methods in Molecular Biology*. Totowa, NJ: Humana Press; 2007. p 283–301.
- (64) Sputtek, A.; Rau, G. *Infusionsther Transfusionsmed.* **1992**, 19 (6), 269.
- (65) Sputtek, A.; Singbartl, G.; Langer, R.; Schleinzner, W.; Henrich, H. A.; Kuhn, P. *CryoLetters* **1995**, 16, 283.
- (66) Poisson, J. S.; Briard, J. G.; Turner, T. R.; Acker, J. P., and Ben, R. N. *Bioprocess. J.* **2017**, 15 (4), 16.
- (67) Allen, E. D.; Weatherbee, L.; Spencer, H. H.; Lindenauer, S. M.; Permod, P. A. *Cryobiology* **1976**, 13 (5), 500.
- (68) Umlas, J.; O'Neill, T. P. *Transfusion* **1980**, 20 (6), 720.
- (69) Silver, H.; Umlas, J.; Kenney, P.; Anderson, J.; Pina, B.; Cross, D. *Transfusion* **1982**, 22 (3), 254.
- (70) Neff, T. A.; Fischler, L.; Mark, M.; Stocker, R.; Reinhart, W. H. *Anesth. Analg.* **2005**, 100 (6), 1773.
- (71) Knorpp, C. T.; Starkweather, W. H.; Spencer, H. H.; Weatherbee, L. *Cryobiology* **1971**, 8, 511.
- (72) Horn, E.-P.; Sputtek, A.; Standl, T.; Rudolf, B.; Kuhn, P.; Schulte am Esch, J. *Anesth. Analg.* **1997**, 85 (4), 739.
- (73) Moll, R.; Davis, B. *Medicine* **2017**, 45 (4), 198.

- (74) Reinhart, W. H.; Piety, N. Z.; Goede, J. S.; Shevkoplyas, S. S. *Microvasc. Res.* **2015**, *98*, 102.
- (75) Marvel, J. S.; Suter, S. P.; Krogstad, D. J.; Zarkowsky, H. S.; Williamson, J. R. *Blood Cells* **1991**, *17* (3), 497.
- (76) Mohandas, N.; Kim, Y. R.; Tycko, D. H.; Orlik, J.; Wyatt, J.; Groner, W. *Blood* **1986**, *68* (2), 506.
- (77) Gray, M. L.; Hoffman, R. A.; Hansen, W. P. *Cytometry* **1983**, *3* (6), 428.
- (78) Mohandas, N.; Clark, M. R.; Kissinger, S.; Bayer, C.; Shohet, S. B. *Blood* **1980**, *56* (1), 125.
- (79) Kanas, T.; Acker, J. P. *FEBS J.* **2010**, *277*, 343.
- (80) Lecak, J.; Scott, K.; Young, C.; Hannon, J.; Acker, J. P. *Blood Components* **2004**, *44*, 1306.
- (81) Wallace, W. J.; Caughey, W. S. *Biochem. Biophys. Res. Commun.* **1975**, *62* (3), 561.
- (82) Alanazi, M. Q. *J. Hematol. Thromboembolic Dis.* **2017**, *5* (3), 270.
- (83) Stolze, K.; Nohl, H. *Free Radic. Res. Commun.* **1991**, *11* (6), 321.
- (84) Stadnick, H.; Onell, R.; Acker, J. P.; Holovati, J. L. *Clin. Hemorheol. Microcirc.* **2011**, *47*, 229.
- (85) Relevo, H.; Koshkaryev, A.; Manny, N.; Yedgar, S.; Barshtein, G. *Blood Components* **2008**, *48*, 136.
- (86) Barshtein, G.; Manny, N.; Yedgar, S. *Transfus. Med. Rev.* **2011**, *25* (1), 24.
- (87) Berezina, T. L.; Zaets, S. B.; Morgan, C.; Spillert, C. R.; Kamiyama, M.; Spolarics, Z.; Deitch, E. A.; Machiedo, G. W. *J. Surg. Res.* **2002**, *102*, 6.
- (88) Izzo, P.; Manicone, A.; Spagnuolo, A.; Lauta, V. M.; Di Pasquale, A.; Di Monte, D. *Clin. Hemorheol. Microcirc.* **1999**, *21* (3–4), 335.
- (89) Cooper, R. A. *J. Supramol. Struct.* **1978**, *8* (4), 413.
- (90) Kilbride, P.; Lamb, S.; Milne, S.; Gibbons, S.; Erro, E.; Bundy, J.; Selden, C.; Fuller, B.; Morris, J. *Cryobiology* **2016**, *73* (1), 47.
- (91) Massie, I.; Selden, C.; Hodgson, H.; Fuller, B.; Gibbons, S.; Morris, G. J. *Tissue Eng.* **2014**, *20* (9), 693.
- (92) De Loecker, W.; Koptelov, V. A.; Grischenko, V. I.; De Loecker, P. *Cryobiology* **1998**, *37*, 103.
- (93) Mazur, P.; Cole, W. *Cryobiology* **1985**, *22*, 509.
- (94) Pegg, D. E. *Cryobiology* **1981**, *18*, 221.
- (95) Wagner, C. T.; Burnett, M. B.; Livesey, S. A.; Connor, J. *Cryobiology* **2000**, *41*, 178.
- (96) Rowley, B. S. D.; Bensinger, W. I.; Gooley, T. A.; Buckner, C. D. *Blood* **1994**, *83* (9), 2731.
- (97) Matsumura, Y.; Ujihira, M.; Nogawa, S.; Kimura, K.; Ichikawa, H.; Mabuchi, K. *CryoLetters* **2007**, *28* (5), 337.
- (98) Beaujean, F.; Bourhis, J.-H.; Bayle, C.; Jouault, H.; Divine, M.; Rieux, C.; Janvier, M.; Forestier, C. Le; Pico, J. L. *Bone Marrow Transplant.* **1998**, *22*, 1091.
- (99) Brecher, M. E.; Zylstra-Halling, V. W.; Pineda, A. A. *Am. J. Clin. Pathol.* **1991**, *96* (6), 767.
- (100) Högman, C. F.; de Verdier, C.-H.; Ericson, A.; Hedlund, K.; Sandhagen, B. *Vox Sang.* **1985**, *48* (5), 257.

- (101) Greenwalt, T. J.; Bryan, D. J.; Dumaswala, U. J. *Vox Sang.* **1984**, 47 (4), 261.
- (102) Gabrio, B. W.; Finch, C. A.; Huennekens, F. M. *Blood* **1956**, 11, 103.
- (103) Dern, R. J.; Brewer, G. J.; Wiorkowski, J. J. *J. Lab. Clin. Med.* **1967**, 69, 968.
- (104) Wolfe, L. C. *Transfusion* **1985**, 25, 185.
- (105) Almac, E.; Ince, C. *Best Pr. Clin. Anaesthesiol.* **2007**, 21 (2), 195.
- (106) Macdonald, R. *Anaesthesia* **1977**, 32, 544.
- (107) Karger, R.; Lukow, C.; Kretschmer, V. *Transfus. Med. Hemotherapy* **2012**, 39 (4), 277.
- (108) Valeri, C. R.; Ragno, G. *Artif. Cells Blood Substit. Immobil. Biotechnol.* **2008**, 36 (1), 3.
- (109) Nunes, J. The Adhesion of Stored Red Blood Cells to Human Umbilical Vein Endothelial Cells, University of Alberta, 2013.
- (110) Henkelman, S.; Lagerberg, J. W. M.; Graaff, R.; Rakhorst, G.; van Oeveren, W. *Blood Components* **2010**, 50, 2393.
- (111) Flatt, J. F.; Bawazir, W. M.; Bruce, L. J. *Front. Physiol.* **2014**, 5, 214.
- (112) Opoku-Okrah, C.; Acquah, B. K.; Dogbe, E. E. *Pan. Afr. Med. J.* **2015**, 20, 236.
- (113) Marjanovic, M.; Willis, J. S. *J. Physiol.* **1992**, 456, 575.
- (114) Minetti, G.; Ciana, A.; Profumo, A.; Zappa, M.; Vercellati, C.; Zanella, A.; Arduini, A.; Brovelli, A. *Biochim. Biophys. Acta.* **2001**, 1527 (3), 149.
- (115) Bandarenko, N.; Hay, S. N.; Holmberg, J.; Whitley, P.; Taylor, H. L.; Moroff, G.; Rose, L.; Kowalsky, R.; Brumit, M.; Rose, M.; Sawyer, S.; Johnson, A.; McNeil, D.; Popovsky, M. A. *Transfusion* **2004**, 44 (11), 1656.
- (116) Lagerberg, J. W. M.; Truijens-de Lange, R.; de Korte, D.; Verhoeven, A. J. *Transfusion* **2007**, 47, 2242.
- (117) Bailey, D. N.; Bove, J. R. *Transfusion* **1975**, 15 (3), 244.
- (118) Ullah, I.; Subbarao, R. B.; Rho, G. J. *Biosci. Rep.* **2015**, 35 (2), e00191.
- (119) Kalinina, N. I.; Sysoeva, V. Y.; Rubina, K. A.; Parfenova, Y. V.; Tkachuk, V. A. *Acta Naturae* **2011**, 3 (4), 30.
- (120) Zhang, J.; Huang, X.; Wang, H.; Liu, X.; Zhang, T.; Wang, Y.; Hu, D. *Stem Cell Res. Ther.* **2015**, 6 (234), 1.
- (121) Thakker, R.; Yang, P. *Curr. Treat. Options Cardiovasc. Med.* **2014**, 16 (7), 1.
- (122) Bashir, J.; Sherman, A.; Lee, H.; Kaplan, L.; Hare, J. M. *PM&R* **2014**, 6 (1), 61.
- (123) Freitag, J.; Bates, D.; Boyd, R.; Shah, K.; Barnard, A.; Huguenin, L.; Tenen, A. *BMC Musculoskelet. Disord.* **2016**, 17 (230), 1.
- (124) Yong, K. W.; Safwani, W.; Xu, F.; Abas, W.; Choi, J.; Pingguan-Murphy, B. *Biopreserv. Biobank.* **2015**, 13 (4), 231.
- (125) Vacanti, V.; Kong, E.; Suzuki, G.; Sato, K.; Canty, J. M.; Lee, T. *J. Cell. Physiol.* **2005**, 205 (2), 194.
- (126) Ben-David, U.; Mayshar, Y.; Benvenisty, N. *Cell Stem Cell* **2011**, 9 (2), 97.
- (127) Marquez-Curtis, L. A.; Janowska-Wieczorek, A.; McGann, L. E.; Elliott, J. A. W. *Cryobiology* **2015**, 71 (2), 181.
- (128) Chen, G.; Yue, A.; Ruan, Z.; Yin, Y.; Wang, R.; Ren, Y.; Zhu, L. *Cell Tissue Bank* **2014**, 15 (4), 513.
- (129) Sohni, A.; Verfaillie, C. M. *Stem Cells Int.* **2013**, 2013, 130763.
- (130) Pollock, K.; Sumstad, D.; Kadidlo, D.; McKenna, D. H.; Hubel, A. *Cytherapy* **2015**, 17 (1), 38.

- (131) Haack-Sørensen, M.; Kastrup, J. *Methods Mol. Biol.* **2011**, 698, 161.
- (132) Zambelli, A.; Poggi, G.; Da Prada, G.; Pedrazzoli, P.; Cuomo, A.; Miotti, D.; Periotti, C.; Preti, P.; Robustelli della Cuna, G. *Anticancer Res.* **1998**, 18 (6B), 4705.
- (133) Rowley, S.; MacLeod, B.; Heimfeld, S.; Holmberg, L.; Bensinger, W. *Cytotherapy* **1999**, 1 (4), 311.
- (134) Windrum, P.; Morris, T. C. *Bone Marrow Transplant.* **2003**, 31 (4), 315.
- (135) Windrum, P.; Morris, T. C.; Drake, M. B.; Niederwieser, D.; Ruutu, T. *Bone Marrow Transplant.* **2005**, 36 (7), 601.
- (136) Benekli, M.; Anderson, B.; Wentling, D.; Bernstein, S.; Czuczman, M.; McCarthy, P. *Bone Marrow Transplant.* **2000**, 25 (12), 1299.
- (137) Yi, X.; Liu, M.; Luo, Q.; Zhuo, H.; Cao, H.; Wang, J.; Han, Y. *FEBS Open Bio* **2017**, 7, 485.
- (138) Davis, J. M.; Rowley, S. D.; Braine, H. G.; Piantadosi, S.; Santos, G. W. *Blood* **1990**, 75 (3), 781.
- (139) Moll, G.; Alm, J. J.; Davies, L. C.; von Bahr, L.; Heldring, H.; Stenbeck-Funke, L.; Hamad, O. A.; Hinsch, R.; Ignatowicz, L.; Locke, M.; Lonnie, H.; Lambiris, J. D.; Termura, Y.; Nilsson-Ekdahl, K.; Nilsson, B.; Le Blanc, K. *Stem Cells* **2015**, 32 (9), 2430.
- (140) Francois, M.; Copland, I. B.; Yuan, S.; Romieu-Mourez, R.; Waller, E. K.; Galipeau, J. *Cytotherapy* **2012**, 14, 147.
- (141) Galipeau, J. *Cytotherapy* **2013**, 15, 2.
- (142) Galipeau, J. *ISBT Sci. Ser.* **2013**, 8, 100.
- (143) Yuan, Z.; Lourenco, S. D. S.; Sage, E. K.; Kolluri, K. K.; Lowdell, M. W.; Janes, S. M. *Cytotherapy* **2016**, 18, 860.
- (144) Haack-Sørensen, M.; Bindslev, L.; Mortensen, S.; Friis, T.; Kastrup, J. *Cytotherapy* **2007**, 9 (4), 328.
- (145) Janz, F. de L.; Debes, A. de A.; Cavaglieri, R. de C.; Duarte, S. A.; Romao, C. M.; Moron, A. F.; Zugaib, M.; Bydlowski, S. P.; Bydlowski, P. *J. Biomed. Biotechnol.* **2012**, 2012, 649353.
- (146) Liu, Y.; Xu, X.; Ma, X.; Martin-Rendon, E.; Watt, S.; Cui, Z. *Biotechnol. Prog.* **2010**, 26 (6), 1635.
- (147) Briquet, A.; Halleux, A.; Lechanteur, C.; Beguin, Y. *Cytotherapy* **2013**, 15 (11), 1385.
- (148) Petrenko, Y. A.; Roguiska, O. Y.; Mutsenko, V. V.; Petrenko, A. Y. *CryoLetters* **2014**, 35 (3), 239.
- (149) Roguiska, O. Y.; Petrenko, Y. A.; Petrenko, A. Y. *Cytotechnology* **2017**, 69 (2), 265.
- (150) Pollock, K.; Samsonraj, R. M.; Dudakovic, A.; Thaler, R.; Stumbras, A.; McKenna, D. H.; Dosa, P. I.; Wijnen, A. J. Van; Hubel, A. *Stem Cells Dev.* **2017**, 26 (11), 828.
- (151) Han, I.; Kwon, B.-S.; Park, H.-K.; Kim, K. S. *Int. Neurol. J.* **2017**, 21 (Suppl 1), S24.
- (152) Pal, R.; Hanwate, M.; Totey, S. M. *J. Tissue Eng. Regen. Med.* **2008**, 2, 436.
- (153) Brockbank, K. G. M.; Covault, J. C.; Taylor, M. J. *Cryopreservation Guide, T. Scientific, Ed.*; 2007.

- (154) Laskey, R. A.; Fairman, M. P.; Blow, J. J. *Science* **1989**, *246* (4930), 609.
- (155) Salic, A.; Mitchison, T. J. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (7), 2415.
- (156) Greenwood, S. K.; Hill, R. B.; Sun, J. T.; Armstrong, M. J.; Johnson, T. E.; Gara, J. P.; Galloway, S. M. *Environ. Mol. Mutagen.* **2004**, *43* (1), 36.
- (157) Hashimoto, M.; Nozoe, T.; Nakaoka, H.; Okura, R.; Akiyoshi, S.; Kaneko, K.; Kussell, E.; Wakamoto, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113* (12), 3251.
- (158) Capicciotti, C. J.; Mancini, R. S.; Turner, T. R.; Koyama, T.; Alteen, M. G.; Doshi, M.; Inada, T.; Acker, J. P.; Ben, R. N. *ACS Omega* **2016**, *1* (4), 656.
- (159) Benesch, R. E.; Benesch, R.; Yung, S. *Anal. Biochem.* **1973**, *55* (1), 245.
- (160) Almizraq, R.; Tchir, J. D. R.; Holovati, J. L.; Acker, J. P. *Transfusion* **2013**, *53* (10), 2258.
- (161) Strober, W. *Curr. Protoc. Immunol.* **1997**, *21* (1), A.3B.1.
- (162) Wlodkowic, D.; Skommer, J.; Darzynkiewicz, Z. *Methods Mol. Biol.* **2009**, *559*, 19.

Chapter 6: Investigating the Ability of Small Molecule IRIs to Mitigate Cell Lysis During Transient Warming

6.1 Introduction

The formation and recrystallization of intra- and extracellular ice is widely recognized as a significant contributor to cellular injury and death during cryopreservation.^{1,2} The phenomenon of ice recrystallization occurs when intra- or extracellular ice formed during freezing melts and coalesces to form larger, more damaging ice crystals during warming.³ While the amount of ice stays constant, changes in ice crystal shape and size can cause injury to cells.³ Conventional cryoprotective agents (CPAs) such as dimethyl sulfoxide (DMSO) and glycerol ensure high post-thaw cell viability and recovery by replacing the cell water content, therefore, they prevent cell dehydration and reduce the concentration of damaging solutes.^{1,4,5} Consequently, DMSO and glycerol function by a number of different mechanisms but do not mitigate or control ice recrystallization.

Over the past few years, our laboratory has reported several different classes of small molecules capable of inhibiting ice recrystallization such as lysine-based surfactants, non-ionic carbohydrate-based amphiphiles (alkyl and aryl aldonamides) and *O*-linked alkyl and aryl glycosides.^{6–10} The use of these small molecule ice recrystallization inhibitors (IRIs) as novel CPAs has become an important strategy to improve cell viability and function post-thaw. In particular, it has been previously reported that *O*-aryl glucosides, β -PMP-Glc (**103**) and β -pBrPh-Glc (**104**), are effective inhibitors of ice recrystallization and can function as beneficial additives for the freezing of human red blood cells (RBCs) in the presence of reduced concentrations of glycerol (15% w/v).^{11,12} This discovery was significant as reducing the concentration of glycerol can decrease deglycerolization times, enabling frozen RBC units to be more readily accessible. Furthermore, Chapter 4 identified a novel class of small molecule IRIs that are azide derivatives. These molecules were shown to be effective inhibitors of ice recrystallization with the ability to protect RBCs from cryoinjury during freezing and thawing (Chapter 5). The structures are provided in Figure 6.1. Given the ability of these small molecule IRIs to function as effective additives for the freezing of RBCs, we sought to explore whether

their ability to mitigate ice growth *in vitro* is correlated to increased post-thaw viability using RBCs as an appropriate model.

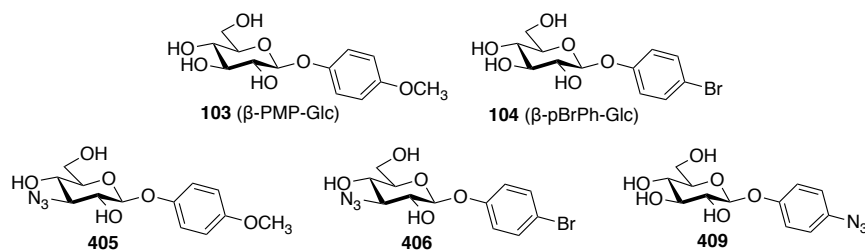


Figure 6.1 Structures of IRI active aryl glucosides **103** (β -PMP-Glc) and **104** (β -pBrPh-Glc) and azido aryl glucosides (**405**, **406** and **409**).

In recent years, there has been an increase in the number of studies examining the impact of transient warming events (TWEs) on cryopreserved cellular therapy products.^{13–18} TWEs are described as brief exposures of a cryopreserved product to temperatures above the critical storage temperature.¹³ These uncontrolled temperature fluctuations on the frozen product can occur during shipping and transfer between cryogenic containers or even during storage where routine sample access of cell banks stored in liquid nitrogen can expose adjacent samples to ambient temperatures.¹⁷ It has been established that TWEs on frozen cells are a major contributor to reduced post-thaw viability and functionality in sperm,¹⁹ placental cord blood,^{13,14} peripheral blood mononuclear cells,^{15,16} and mesenchymal stem cells.^{17,18} Thus, this chapter also explores whether small molecule IRIs can protect RBCs against injury resulting from ice recrystallization during transient warming events.

6.2 Controlling Ice Growth *In Vitro*

It was previously demonstrated that small molecule IRIs **103** (β -PMP-Glc, 110 mM) and **104** (β -pBrPh-Glc, 30 mM) are effective cryoprotectants for RBCs with the ability to increase the percentage of intact RBCs post-thaw to 70-80% using only 15% glycerol and slow cooling rates (Chapter 5, Section 5.2.2). It was hypothesized that the increase in the percentage of intact RBCs post-thaw was associated with a reduction in ice crystal size in the presence of an IRI. Although **103** and **104** were previously shown to effectively reduce ice crystal size, this assay was performed in a PBS solution in the absence of cells.¹¹ Thus, we sought to explore whether these molecules were capable of reducing the

ice crystal size in the presence of RBCs. For these studies, compound **104** (β -pBrPh-Glc) was initially selected because it was shown to be a more effective cryoprotectant for RBCs than **103** (β -PMP-Glc). An experiment was performed in which RBCs were frozen using a Linkam Cryostage and the ice was imaged.²⁰ Using this approach, RBCs were suspended in 15% glycerol or **104** (30 mM) + 15% glycerol and cooled to -40 °C at a rate of 25 °C/min. The sample was warmed to -10 °C at a rate of 10 °C/min and held at this temperature for 10 minutes prior to taking an image. During this time, ice recrystallization occurred. This experiment was also repeated in the absence of RBCs. The images of the ice crystals are shown in Figure 6.2. It is evident that the percentage of ice in the presence of 15% glycerol or **104** (30 mM) + 15% glycerol stays constant even in the presence of RBCs.²⁰ This indicates that the presence of RBCs does not appear to have an influence on the amount of ice in the sample. However, in the presence of **104** (30 mM) + 15% glycerol (with and without RBCs), less ice was observed compared to the 15% glycerol control. It is important to note that in these images, the percentage of the frozen fraction is small because the holding temperature of -10 °C is close to the colligative freezing point depression of the 15% glycerol solution (-4 °C) and thus a large portion of the sample is unfrozen.²¹

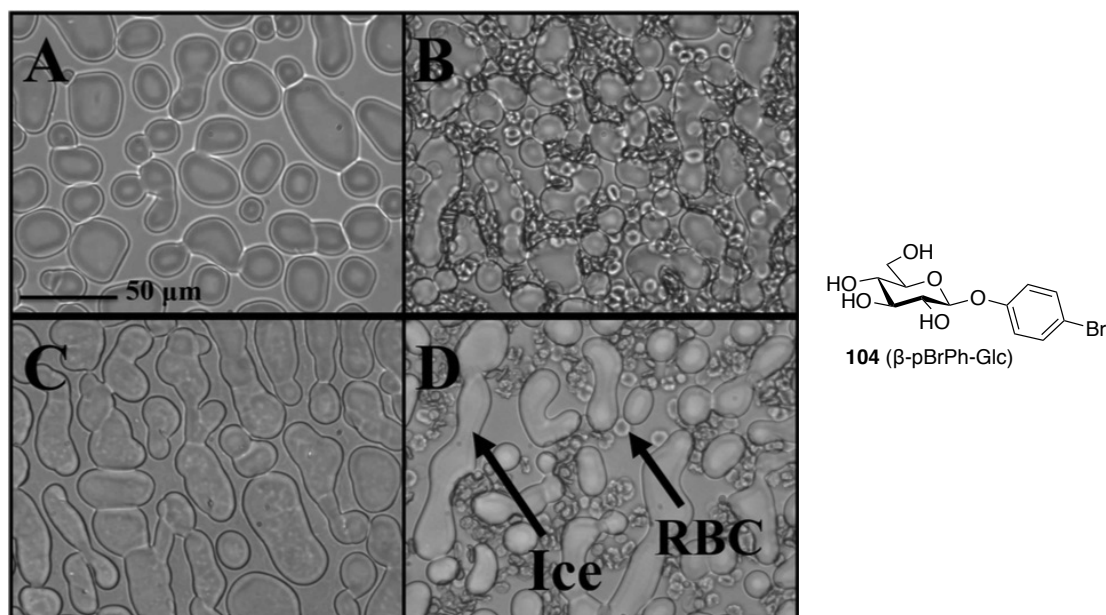


Figure 6.2 Images of ice in the presence of (A) 15% glycerol; (B) 15% glycerol + RBCs; (C) **104** (30 mM) + 15% glycerol; (D) **104** (30 mM) + 15% glycerol + RBCs. Samples

were cooled to -40 °C (rate of 25 °C/min), warmed to -10 °C (rate of 10 °C/min) and held at this temperature for 10 minutes prior to taking an image.

However, the typical assay utilized to evaluate the IRI activity of **104** was performed in conditions with high amounts of ice present. Thus, the experiment described above was repeated at a lower holding temperature of -20 °C to ensure higher ice volume.²⁰ Briefly, RBCs were suspended in 15% glycerol or **104** (30 mM) + 15% glycerol and cooled to -40 °C at a rate of 25 °C/min. The sample was warmed to -20 °C at a rate of 10 °C/min and held at this temperature for 10 minutes prior to taking an image. The images of the ice crystals are shown in Figure 6.3. It is evident that a larger fraction of the sample is frozen which more closely mimics the situation in a frozen sample. The ice crystal size is noticeably reduced in the presence of **104** (30 mM) + 15% glycerol compared to the 15% glycerol control.²⁰ Using a domain recognition software,²² the percentage of frozen fraction in each image was calculated. In the presence of **104** (30 mM) + 15% glycerol, the percentage of frozen fraction was 86% relative to 92% for 15% glycerol alone. The average ice crystal size was also found to be smaller (4x) when **104** was present in the 15% glycerol solution compared to 15% glycerol alone. This study demonstrated that **104** (30 mM) + 15% glycerol is capable of reducing the average ice crystal size of extracellular ice compared to 15% glycerol alone, supporting the positive correlation between inhibition of ice recrystallization and increased percentage of intact RBCs post-thaw.

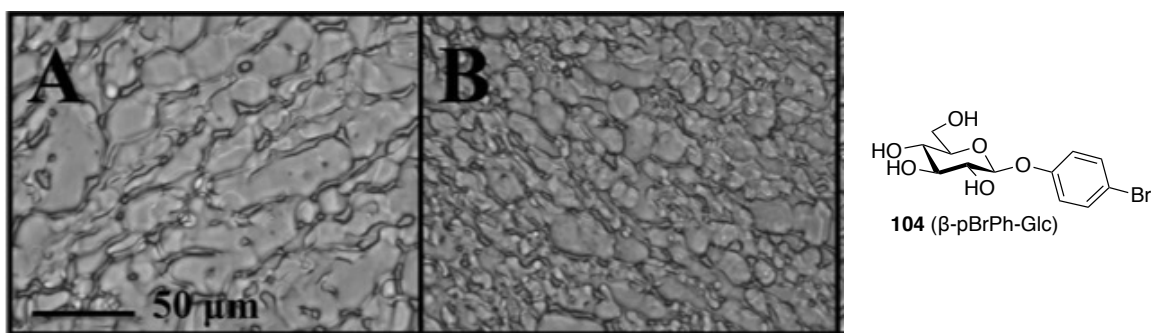


Figure 6.3 Images of ice in the presence of RBCs with (A) 15% glycerol; (B) **104** (30 mM) + 15% glycerol. Samples were cooled to -40 °C (rate of 25 °C/min), warmed to -20 °C (rate of 10 °C/min) and held at this temperature for 10 minutes prior to taking an image.

6.3 Exacerbating Cellular Injury from Ice Recrystallization

Given the fact that the addition of **104** to a solution of 15% glycerol resulted in smaller ice crystal size *in vitro*, we sought to explore whether this molecule could maintain smaller ice crystal size when the extent of ice recrystallization in the frozen sample was increased. An experiment was performed using a cryomicroscope in which RBCs were suspended in 15% glycerol or **104** (30 mM) + 15% glycerol and rapidly cooled to -80 °C at a rate of 90 °C/min, held at this temperature for 15 minutes and then rapidly warmed to -20 °C at a rate of 90 °C/min.²⁰ The sample was held at this temperature for 15 minutes prior to being cooled to -80 °C again and warmed to -20 °C before being thawed. Images of the ice crystals were acquired throughout the experiment and are presented in Figure 6.4.

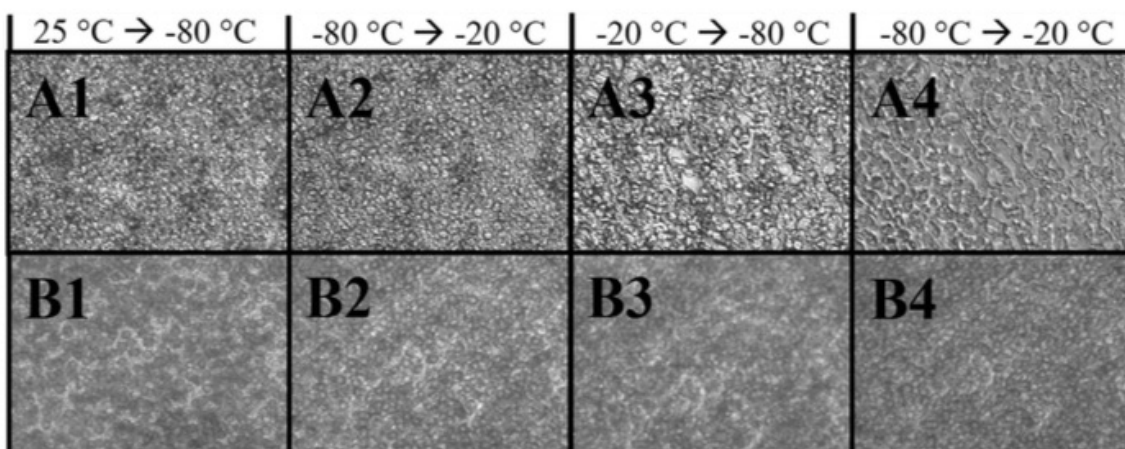


Figure 6.4 Images of ice in the presence of RBCs with (A1-A4) 15% glycerol; (B1-B4) **104** (30 mM) + 15% glycerol. Freezing and warming rates were 90 °C/min and each phase of the cycle (RT to -80 °C, -80 °C to -20 °C etc.) was held for 15 minutes.

Remarkably, in the presence of **104** (30 mM) + 15% glycerol, the ice crystal sizes remained constant throughout the experiment even after warming to -20 °C and repeated cycling. Moreover, the average ice crystal size did not change from the initial ice crystal size observed upon cooling to -80 °C.²⁰ In contrast, in the presence of 15% glycerol alone, the ice crystal sizes began to increase after several warming and cooling cycles. This was expected since the amount of ice recrystallization will increase with each successive cycle and glycerol is not capable of mitigating or controlling ice recrystallization. This experiment demonstrated that **104** was capable of controlling and preventing subsequent ice crystal growth in a frozen sample upon repeated warming.²⁰

The 15% glycerol control is representative of transient warming events that occur when frozen samples are transferred to and from freezers and between blood banks. Importantly, TWEs on frozen cells have been recognized as a major contributor to reduced post-thaw viability and functionality in sperm,¹⁹ placental cord blood,^{13,14} peripheral blood mononuclear cells,^{15,16} and mesenchymal stem cells.^{17,18}

Based on the observation that **104** could maintain smaller ice crystal size even after several warming and cooling cycles, it was hypothesized that IRIs could prevent transient warming injury by inhibiting ice recrystallization during freezing and thawing/warming, resulting in increased post-thaw viability. An experiment was performed using small molecule IRIs **103** and **104** in which RBCs were suspended in 15% glycerol, **103** (110 mM) + 15% glycerol or **104** (30 mM) + 15% glycerol and frozen by placement into dry ice at -78 °C and then warmed to -20 °C in a methanol bath.²⁰ Upon stabilization at -20 °C, the sample was cooled to -78 °C and this process was repeated for several cycles (1, 3, and 5 cycles) before the sample was thawed in a 37 °C water bath (depicted in Figure 6.5). This experiment was designed in such a way to mimic transient warming. Fast cooling rates were chosen to maximize the quantity of RBCs that would survive using only 15% glycerol, making it easier to observe a decrease in the percentage of intact RBCs after several warming and cooling cycles.

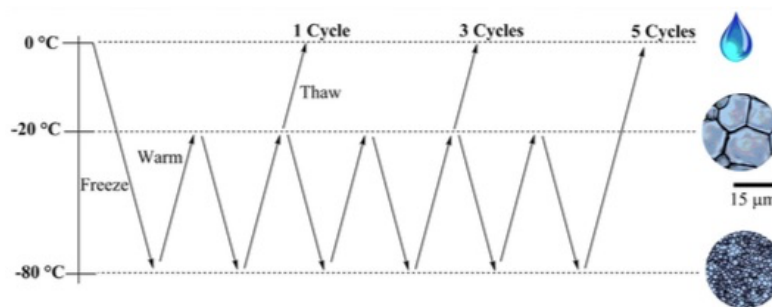


Figure 6.5 A schematic representation of the transient warming experiment. Each -78 °C to -20 °C to -78 °C transition represents one cycle.

After thawing, hemolysis was measured and the results are reported as the percentage of intact RBCs post-thaw (Figure 6.6). The percentage of intact RBCs post-thaw was compared to a 15% glycerol control. It is evident that after one cycle, 15% glycerol has a high percentage of intact RBCs post-thaw, signifying very little hemolysis. However, **103** (110 mM) + 15% glycerol appeared to be less effective, resulting in approximately 50%

intact RBCs post-thaw. In contrast, **104** (30 mM) + 15% glycerol was as effective as 15% glycerol. Increasing the amount of ice recrystallization by subjecting the samples to three cycles decreased the percentage of intact RBCs post-thaw for both the 15% glycerol control (55%) and **103** (110 mM) + 15% glycerol (40%). Interestingly, the percentage of intact RBCs post-thaw for **104** (30 mM) + 15% glycerol remained constant (92%).²⁰ After five cooling and warming cycles, RBCs frozen with 15% glycerol and **103** (110 mM) + 15% glycerol had only 36% and 39% intact RBCs post-thaw, respectively. However, RBCs frozen with **104** (30 mM) + 15% glycerol were capable of maintaining a high percentage of intact RBCs post-thaw (90%). Each successive cooling and warming cycle increases the amount of ice recrystallization and thus, increases the extent of cellular damage. Consequently, it is not surprising that a decrease in the number of intact RBCs post-thaw was observed with 15% glycerol since it is not capable of mitigating or controlling ice recrystallization. The most remarkable observation is that **104** (30 mM) + 15% glycerol is very effective at preventing cellular injury resulting from ice recrystallization during TWEs.²⁰

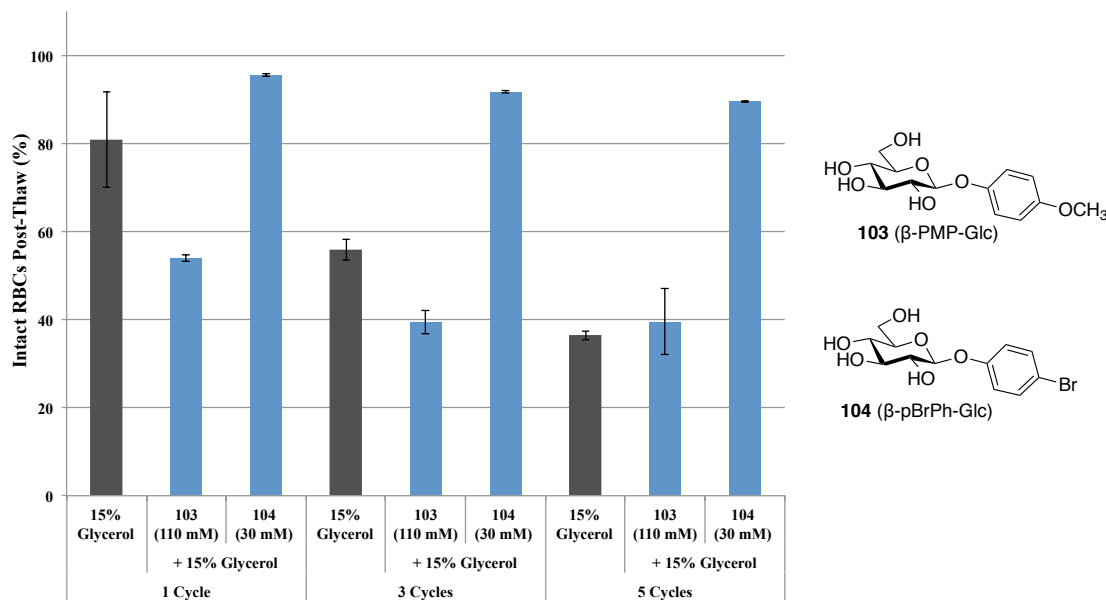


Figure 6.6 Percentage of intact RBCs post-thaw after transient warming injury using 15% glycerol in combination with **103** (110 mM) or **104** (30 mM). Samples were frozen by placement into dry ice (-78 °C), warmed to -20 °C and allowed to stabilize prior to cooling to -78 °C (represents one cycle). After 1, 3 and 5 cycles, the samples were

thawed in a 37 °C water bath. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

Given the significant results obtained with **104** (30 mM) + 15% glycerol, we sought to explore whether azido aryl glucosides **405**, **406** and **409** could prevent transient warming injury. The experiment was performed as described above. Briefly, RBCs were suspended in 15% glycerol, **405** (30 mM) + 15% glycerol, **406** (10 mM) + 15% glycerol or **409** (20 mM) + 15% glycerol and frozen by placement into dry ice at -78 °C and then warmed to -20 °C in a methanol bath. Upon stabilization at -20 °C, the sample was cooled to -78 °C and this process was repeated for several cycles (1, 3, and 5 cycles) before the sample was thawed in a 37 °C water bath. The concentrations selected for each compound were based on their maximum solubility. The results are reported as the percentage of intact RBCs post-thaw (Figure 6.7) and are compared to the 15% glycerol control.

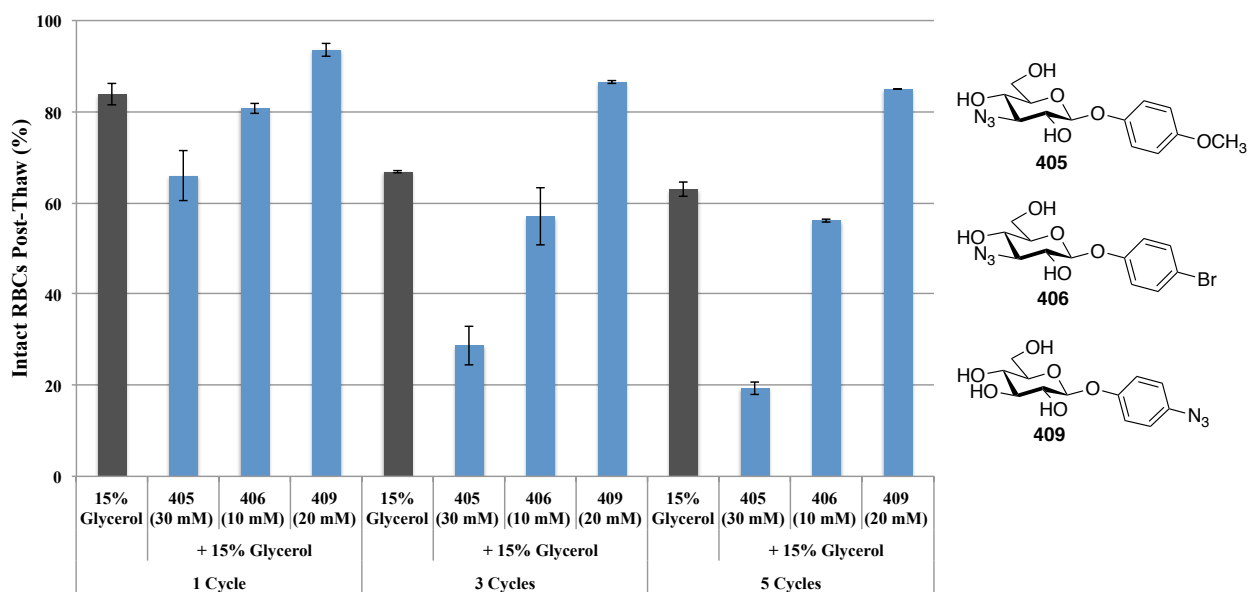


Figure 6.7 Percentage of intact RBCs post-thaw after transient warming injury using 15% glycerol in combination with **405** (30 mM), **406** (10 mM) or **409** (20 mM). Samples were frozen by placement into dry ice (-78 °C), warmed to -20 °C and allowed to stabilize prior to cooling to -78 °C (represents one cycle). After 1, 3 and 5 cycles, the samples were thawed in a 37 °C water bath. These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

After one cycle, 15% glycerol had a high percentage of intact RBCs post-thaw, signifying very little hemolysis. Similarly, **405** (30 mM), **406** (10 mM) and **409** (20 mM) in 15%

glycerol appeared to be as effective, displaying no substantial difference relative to 15% glycerol. As the amount of ice recrystallization in the samples was increased after three cycles, the percentage of intact RBCs post-thaw decreased significantly for **405** (30 mM) + 15% glycerol (29%) while RBCs frozen with **406** (10 mM) + 15% glycerol possessed a similar number of intact RBCs post-thaw to that of 15% glycerol. However, the percentage of intact RBCs post-thaw for **409** (20 mM) + 15% glycerol remained constant (87%). After five cooling and warming cycles, RBCs frozen with **405** (30 mM) + 15% glycerol had only 19% intact RBCs post-thaw. RBCs frozen with 15% glycerol or **406** (10 mM) + 15% glycerol were similar, possessing 63% and 56% intact RBCs post-thaw, respectively. Interestingly, RBCs frozen with **409** (20 mM) + 15% glycerol were capable of maintaining a high percentage of intact RBCs post-thaw (85%). This result was encouraging as **409** (20 mM) + 15% glycerol was previously shown to be an effective cryoprotectant for RBCs using slow cooling rates and also enabling successful deglycerolization without causing extensive cell damage (Chapter 5, Section 5.2.4.4). The fact that **409** (20 mM) + 15% glycerol was also very effective at preventing transient warming injury further demonstrates the utility of this ice recrystallization inhibitor as a cryoprotectant.

6.4 Chapter Summary

Previous work by our laboratory demonstrated that small molecule IRIs **103** (β -PMP-Glc) and **104** (β -pBrPh-Glc) are effective additives for the freezing of RBCs with the ability to increase the percentage of intact RBCs post-thaw in the presence of 15% glycerol and slow cooling rates. It was hypothesized that this increase in the percentage of intact RBCs post-thaw was associated with a reduction in ice crystal size in the presence of an IRI. To determine whether these molecules were capable of mitigating ice growth *in vitro*, cryomicroscopy studies were conducted in the presence of RBCs. It was found that **104** was capable of reducing the average ice crystal size of extracellular ice in the presence of RBCs, supporting the positive correlation between inhibition of ice recrystallization and increased percentage of intact RBCs post-thaw. It was also demonstrated that **104** and **409** in 15% glycerol are very effective at preventing cellular injury resulting from ice recrystallization during transient warming events. These

attributes make small molecule IRIs very valuable as novel CPAs with a unique mode of action.

6.5 Experimental Procedures

Small molecule IRIs **103** (β -PMP-Glc) and **104** (β -pBrPh-Glc) used in this chapter for cryopreservation studies were prepared as previously described.^{10,23} IRI active azido aryl glucosides **405**, **406** and **409** used in this chapter for cryopreservation studies were prepared as described in the experimental section of Chapter 4.

6.5.1 Blood Collection and Preparation

All RBCs were obtained from NetCAD (Canadian Blood Services' Network Centre for Applied Development). Whole blood was collected from healthy volunteers using standardized phlebotomy guidelines approved by Canadian Blood Services (CBS). Informed consent was obtained from all donors. All experimental protocols were approved by NetCAD and CBS. Ethics approvals were obtained from Research Ethics Board (REB) at CBS and the University of Alberta. For cryovial experiments, whole blood units were collected and processed by NetCAD (Vancouver, BC). The whole blood was processed using the buffy coat (BC) method to produce leukocyte reduced SAGM RBC units, which has been previously described.²⁴ ABO matched RCCs were pooled and split to reduce donor to donor variability.

6.5.2 RBC Cryomicroscopy

Cryomicroscopy was performed according to methods previously described.²⁰ The nucleation and growth of extracellular ice in solutions containing IRIs were documented using a cryomicroscope that consists of a Nikon 80i fluorescent microscope with a long working distance condenser and objectives, CCD cameras (Hamamatsu ORCA) interfaced to a personal computer and a convection cryomicroscope stage (Linkam FDCS196). The freezing and thawing of samples was imaged in real time and the ice crystal areas were assessed using Image J software.

6.5.3 RBC Transient Warming Experiments

The freezing solution consisted of a 30% glycerol solution prepared from a

commercially available glycerol solution (57 Glycerolyte, Baxter) by diluting it with 0.9% saline/0.2% dextrose. An equal volume of freezing solution was added to 150 μ L of RBCs for a final volume of 300 μ L. The final concentrations of all freezing solutions are as indicated in the text. RBC suspensions were transferred to cryotubes and incubated at room temperature for 10 minutes prior to placement into dry ice (-78 °C). A temperature probe was used for temperature measurements at 1 s intervals. Once the internal solution reached -78 ± 2 °C, the samples were immersed in a methanol bath cooled to -20 °C. Once the internal solution reached -20 °C, the samples were placed into dry ice again. RBC samples were held in dry ice until the internal solution from the temperature probe reached -78 ± 2 °C, after which the samples were either thawed (representing one cycle of transient warming) or immersed in a methanol bath cooled to -20 °C. One, three and five cycles of immersion in a -20 °C methanol bath and dry ice were performed. Samples were thawed in a 37 °C water bath.

6.5.4 Post-Thaw Hemolysis

Post-thaw hematocrits (hct) and percentage hemolysis were determined for all freezing experiments by comparing the supernatant hemoglobin concentration to total hemoglobin concentration using the cyanmethemoglobin Drabkin's method.^{24,25} The percentage of intact RBCs post-thaw was calculated using the measured percentage hemolysis values according to the following equation: % intact RBCs post-thaw = 100 - % hemolysis.

6.6 References

- (1) Mazur, P. Principles of Cryobiology. In: Fuller, B.; Lane, N.; Benson, E., eds. Life in the Frozen State. Boca Raton, FL: CRC Press; 2004.
- (2) Acker, J. P.; McGann, L. E. *Cell Transplant.* **2002**, *11* (6), 563.
- (3) Woods, E. J.; Thirumala, S.; Badhe-Buchanan, S. S.; Clarke, D.; Mathew, A. J. *Cytotherapy* **2016**, *18* (6), 697.
- (4) Elliott, G. D.; Wang, S.; Fuller, B. J. *Cryobiology* **2017**, *76*, 74.
- (5) Fuller, B. J. *CryoLetters* **2004**, *25* (6), 375.
- (6) Capicciotti, C. J.; Leclère, M.; Perras, F. A.; Bryce, D. L.; Paulin, H.; Harden, J.; Liu, Y.; Ben, R. N. *Chem. Sci.* **2012**, *3*, 1408.
- (7) Balcerzak, A. K.; Febbraro, M.; Ben, R. N. *RSC Adv.* **2013**, *3* (10), 3232.
- (8) Balcerzak, A.; Capicciotti, C. J.; Briard, J. G.; Ben, R. N. *RSC Adv.* **2014**, *4* (80), 42682.
- (9) Briard, J. G.; Fernandez, M.; De Luna, P.; Woo, T. K.; Ben, R. N. *Sci. Rep.* **2016**,

- 6, 26403.
- (10) Capicciotti, C. J.; Mancini, R. S.; Turner, T. R.; Koyama, T.; Alteen, M. G.; Doshi, M.; Inada, T.; Acker, J. P.; Ben, R. N. *ACS Omega* **2016**, *1* (4), 656.
 - (11) Abraham, S.; Keillor, K.; Capicciotti, C. J.; Perley-Robertson, G. E.; Keillor, J. W.; Ben, R. N. *Cryst. Growth Des.* **2015**, *15* (10), 5034.
 - (12) Capicciotti, C. J.; Kurach, J. D. R.; Turner, T. R.; Mancini, R. S.; Acker, J. P.; Ben, R. N. *Sci. Rep.* **2015**, *5*, 9692.
 - (13) Elmoazzen, H.; Howell, A.; Pasha, R.; Turner, T.; Pineault, N.; Acker, J. P.; Halpenny, M. *Stem Cells Transl. Med.* **2018**, *7* (Suppl 1), S27.
 - (14) Mrowiec, Z. R.; Angelina, A.; Laluf, J.; Yildirim, C.; Verter, F.; Todd, D. J. *Transfus. Med.* **2012**, *5* (4), 153.
 - (15) Germann, A.; Oh, Y. J.; Schmidt, T.; Schon, U.; Zimmermann, H.; von Briesen, H. *Cryobiology* **2013**, *67* (2), 193.
 - (16) Smith, J. G.; Joseph, H. R.; Green, T.; Field, J. A.; Wooters, M.; Kaufhold, R. M.; Antonello, J.; Caulfield, M. J. *Clin. Vaccine Immunol.* **2007**, *14* (5), 527.
 - (17) Chabot, D.; Tremblay, T.; Pare, I.; Bazin, R.; Loubaki, L. *Cytotherapy* **2017**, *19* (8), 978.
 - (18) Fink, J.; Karnieli, O. *J. Cell Ther.* **2017**, *19* (5), S112.
 - (19) Carrell, D. T.; Wilcox, A. L.; Urry, R. L. *Andrologia* **1996**, *28* (6), 315.
 - (20) Briard, J. G.; Poisson, J. S.; Turner, T. R.; Capicciotti, C. J.; Acker, J. P.; Ben, R. N. *Sci. Rep.* **2016**, *6*, 23619.
 - (21) Lane, L. B. *Ind. Eng. Chem.* **1925**, *17* (9), 924.
 - (22) Jackman, J.; Noestheden, M.; Moffat, D.; Pezacki, J. P.; Findlay, S.; Ben, R. N. *Biochem. Biophys. Res. Commun.* **2007**, *354*, 340.
 - (23) Briard, J. G.; Jahan, S.; Chandran, P.; Allan, D.; Pineault, N.; Ben, R. N. *ACS Omega* **2016**, *1*, 1010.
 - (24) Acker, J. P.; Hansen, A. L.; Kurach, J. D. R.; Turner, T. R.; Croteau, I.; Jenkins, C. *Transfusion* **2014**, *54* (10), 2534.
 - (25) Drabkin, D. L. *Am. J. Med. Sci.* **1948**, *215*, 110.

Chapter 7: Investigating the Uptake and Internalization of Small Molecule Ice Recrystallization Inhibitors

7.1 Introduction

The most widely utilized cryoprotectants are permeating agents such as dimethyl sulfoxide (DMSO) and glycerol. Permeating cryoprotective agents (CPAs) have the ability to move freely across the cell membrane of several different cell types¹ and offer protection against slow freezing injury by replacing the intracellular water content, thereby preventing cell dehydration and reducing the concentration of damaging solutes.¹⁻³ In general, permeating CPAs provide a better cryoprotective effect than non-permeating CPAs and are required for optimal cryopreservation. However, the major problem with cell permeating CPAs such as DMSO and glycerol is cytotoxicity.⁴⁻⁶ Thus, there is a need to develop novel CPAs that are as effective as permeating agents but exhibit significantly less toxicity.

Small molecule ice recrystallization inhibitors (IRIs) represent a new class of CPAs that function by a different mechanism than conventional CPAs (i.e. DMSO and glycerol) and protect cells against cryoinjury by controlling or inhibiting ice recrystallization. The use of small molecule IRIs as an alternative to DMSO and glycerol for the cryopreservation of various cell types is attractive as they exhibit less toxicity. However, it is not known whether small molecule IRIs are functioning as cell permeating CPAs.

The *O*-aryl glycosides represent a novel class of small molecule IRIs with the ability to protect red blood cells (RBCs) against cryoinjury during freezing in the presence of reduced concentrations of glycerol (15%) and slow cooling rates.⁷ Previous studies have speculated that the increase in the percentage of intact RBCs post-thaw observed with **103** (β -PMP-Glc) may be due to its ability to interact with the RBC membrane and/or permeate the cell.⁷ These postulations were based on studies that were carried out using an interrupted graded freezing protocol to determine the mechanism of cryoinjury in the presence of **103**. The interrupted graded freezing technique has been previously demonstrated to be an excellent analytical technique for simulating and identifying the progressive cell injury that occurs during freezing and thawing of biological systems and to evaluate differential protective effects of intra- and extracellular cryoprotectants.⁸⁻¹¹ In this experiment, samples were cooled at a low linear cooling rate to defined subzero

temperatures before being either thawed directly to assess the impact of cryoinjury during the initial cooling (direct thaw) or plunged into a low storage temperature (-78 °C) before thawing to assess the impact of damage from rapid cooling (plunge thaw). It was found that the percentage of intact RBCs post-thaw significantly increased following slow cooling when **103** was added to a 15% glycerol solution and that the cryobiological response was consistent with that of a cell permeating cryoprotectant (Figure 7.1).⁷ This suggested that **103** was being internalized into RBCs.

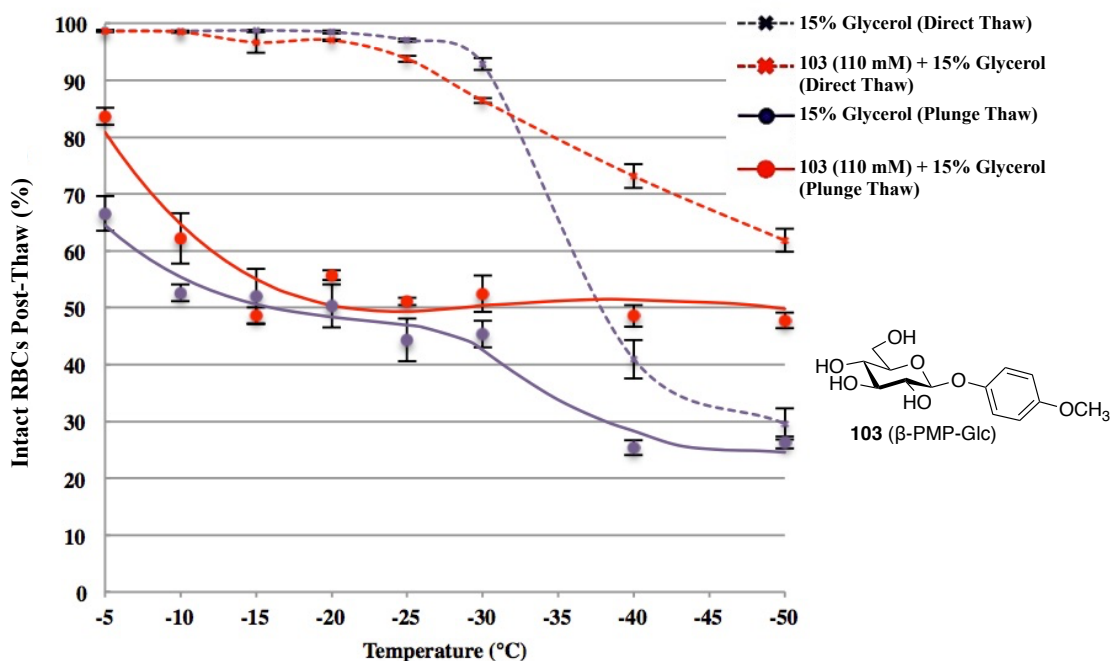


Figure 7.1 Interrupted graded freezing of RBCs with **103** (β-PMP-Glc). Percentage of intact RBCs post-thaw (100 – % hemolysis) with and without 110 mM **103** in 15% glycerol. Samples were cooled at a rate of 1 °C/min to predefined subzero temperatures then either directly thawed (dashed lines) or plunged into dry ice (-78 °C) prior to thawing in a 37 °C water bath (solid lines). These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).⁷

Since the “cooling” profile for **103** suggested that this compound might be conferring protection against cryoinjury through a mechanism consistent with that of a cell permeating CPA, subsequent studies were carried out to investigate whether the observed increase in the percentage of intact RBCs post-thaw was the result of internalization of **103**. It has been previously demonstrated that the rate of sugar transport across the cell membrane can be considerably slowed at 0 °C.¹² Thus, an experiment was performed where RBCs were incubated with **103** in 15% glycerol for 0, 5, 10 and 30 minutes at

room temperature or 0 °C prior to freezing to determine whether the internalization of **103** was important for its cryoprotective ability.⁷ The pre-incubation study was shown to yield a higher percentage of intact RBCs post-thaw with a longer incubation (10 minutes) compared to no incubation (immediate freezing) and slowing the rate of sugar transport at 0 °C was shown to result in a decrease in the percentage of intact RBCs post-thaw.⁷ This data suggested that this molecule may be internalized into RBCs and internalization may be important for its protective effect during freezing. Given these preliminary results, we sought to further investigate the internalization of small molecule IRIs to determine if these molecules are crossing the cell membrane and behaving as permeating CPAs. IRI permeation was evaluated in two different cell models, RBCs and human umbilical vein endothelial cells (HUVECs).

7.2 Internalization Studies for the Freezing of RBCs in the Presence of IRIs

7.2.1 Interrupted Graded Freezing of RBCs

Previous work by our laboratory has shown that **104** (β -pBrPh-Glc) is a beneficial additive for the freezing of human RBCs with the ability to increase the percentage of intact RBCs post-thaw in the presence of 15% glycerol and slow cooling rates.⁷ However, an interrupted graded freezing protocol has not yet been performed with **104**. Thus, the freezing of human RBCs was carried out using an interrupted graded freezing protocol to assess the cryoprotective effectiveness of this IRI and determine the mechanism of cryoinjury in the presence of **104**.¹³ This would determine whether the “cooling” profile for **104** is consistent with that of a permeating CPA.

An interrupted graded freezing procedure allows for the separation of cell damage, which occurs during an initial cooling phase, from that which occurs during subsequent cooling to a storage temperature. In this experiment, samples were cooled at a low linear cooling rate to defined subzero temperatures before being either thawed directly to assess the impact of cryoinjury during the initial cooling (direct thaw) or plunged into a low storage temperature (-78 °C) before thawing to assess the impact of damage from rapid cooling (plunge thaw).¹³ Thawing was performed in a 37 °C water bath and RBC hemolysis was measured utilizing the Drabkin’s assay.^{14,15} The results are reported as the percentage of intact RBCs post-thaw (100 - % hemolysis) shown in Figure 7.2. In the

presence of 15% glycerol, RBCs that were thawed immediately after slow cooling (1 °C/min) to a defined subzero temperature showed a significant decrease in the percentage of intact RBCs post-thaw with lower subzero temperatures.¹³ This decrease in the number of intact RBCs post-thaw is attributed to the deleterious effect of exposure to increasing concentrations of extracellular solutes, which is not completely mitigated by the presence of intra- and extracellular glycerol. At temperatures above −25 °C, slow freezing resulted in a high percentage of intact RBCs post-thaw, as glycerol has the ability to permeate the cell membrane and reduce the amount of ice formed, thereby reducing osmotic and chemical damage from the increasing solute concentrations.

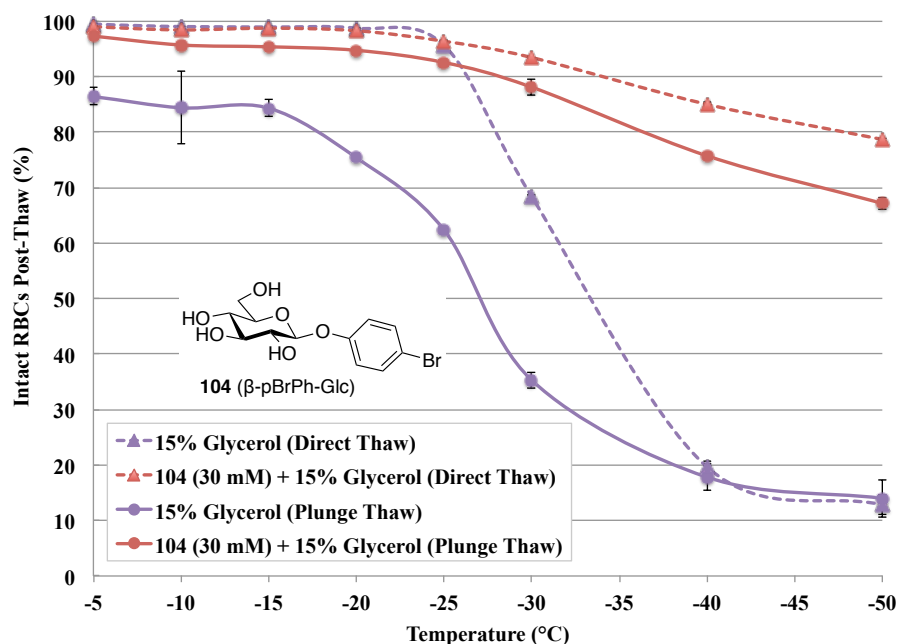


Figure 7.2 Interrupted graded freezing of RBCs with **104** (β -pBrPh-Glc). Percentage of intact RBCs post-thaw (100 – % hemolysis) with and without 30 mM **104** in 15% glycerol. Samples were cooled at a rate of 1 °C/min to predefined subzero temperatures then either directly thawed (dashed lines) or plunged into dry ice (−78 °C) prior to thawing in a 37 °C water bath (solid lines). These freezing conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

When **104** (30 mM) was used in combination with 15% glycerol, the percentage of intact RBCs during slow cooling (direct thaw) was significantly higher than the 15% glycerol alone at the lower subzero temperatures. At −50 °C, the percentage of intact RBCs post-thaw was considerably higher (79%) in the presence of **104** (30 mM) compared with 15%

glycerol alone (13%).¹³ In comparison to samples that were directly thawed from defined subzero temperatures, RBC samples that were rapidly cooled (plunged) to $-78\text{ }^{\circ}\text{C}$ after slow cooling generated additional injury, as shown in Figure 7.2. Damage during the plunge from high subzero temperatures is attributed to injury associated with the formation of intracellular ice.^{8,10} These experiments were performed using small sample volumes (300 μL) to achieve fast cooling rates that are required to induce intracellular ice formation. In the presence of 15% glycerol alone, the percentage of intact RBCs progressively decreased when samples were slowly cooled to temperatures below $-15\text{ }^{\circ}\text{C}$. When **104** (30 mM) was used in combination with 15% glycerol, the percentage of intact RBCs increased as a function of temperature by 10 to 55% compared with the glycerol-only control group.¹³

In the cryopreservation of RBCs, glycerol functions as a permeating cryoprotectant to mitigate damage during freezing and thawing.^{16,17} As shown in Figure 7.2, when used alone, glycerol is able to maintain the integrity of RBCs that are thawed directly from high subzero temperatures (direct thaw) or rapidly cooled to $-78\text{ }^{\circ}\text{C}$ (plunge thaw) from high subzero temperatures. However, if slow cooling occurs to sufficiently low subzero temperatures (-15 to $-25\text{ }^{\circ}\text{C}$) before the direct thaw or plunge thaw, then significant damage can occur. This cryobiological behavior is consistent with the “two-factor hypothesis” of freezing injury,¹⁸ where the permeant cryoprotectant glycerol functions to displace intracellular water and thereby minimize the probability of damage from intracellular ice formation but is unable to fully protect cells from the damaging osmotic and toxic effects of concentrating solutes that occur during slow cooling. The addition of **104** (30 mM) to the 15% glycerol solution significantly increased the percentage of intact RBCs post-thaw under both slow cooling (direct thaw) and rapid cooling (plunge thaw) conditions (Figure 7.2).¹³ The increase in the percentage of intact RBCs after slow cooling to defined subzero temperatures (direct thaw) suggests that **104** has the ability to mitigate damage caused by prolonged exposure to high solute concentrations. The ability to maintain RBC integrity following rapid cooling to $-78\text{ }^{\circ}\text{C}$ over a large range of subzero temperatures (-5 to $-30\text{ }^{\circ}\text{C}$) before damage from solute concentration during slow cooling further suggests that **104** is being internalized into RBCs.¹³

Alternatively, the increased cellular damage in samples that were rapidly cooled to

$-78\text{ }^{\circ}\text{C}$ (plunge thaw) may also be attributed to additional damage that results from increased exposure to damaging solute concentrations during cooling. In this instance, the increase in the percentage of intact RBCs following cooling to $-78\text{ }^{\circ}\text{C}$ in the presence of **104** suggests that this IRI also has the ability to mitigate damage caused by the formation of extracellular ice.¹³

In Chapter 5, it was shown that azido aryl glucoside **409** was also a beneficial additive for the freezing of RBCs with the ability to increase the percentage of intact RBCs post-thaw in the presence of 15% glycerol and slow cooling rates. Thus, similar studies were performed with **409** to determine the mechanism of cryoinjury. As described above, the freezing of RBCs was carried out using an interrupted graded freezing protocol in which samples were cooled at a low linear cooling rate to defined subzero temperatures before being either thawed directly to assess the impact of cryoinjury during the initial cooling (direct thaw) or plunged into a low storage temperature ($-78\text{ }^{\circ}\text{C}$) before thawing to assess the impact of damage from rapid cooling (plunge thaw). Thawing was performed in a $37\text{ }^{\circ}\text{C}$ water bath and RBC hemolysis was measured utilizing the Drabkin's assay.^{14,15} The results are reported as the percentage of intact RBCs post-thaw (100 - % hemolysis) shown in Figure 7.3.

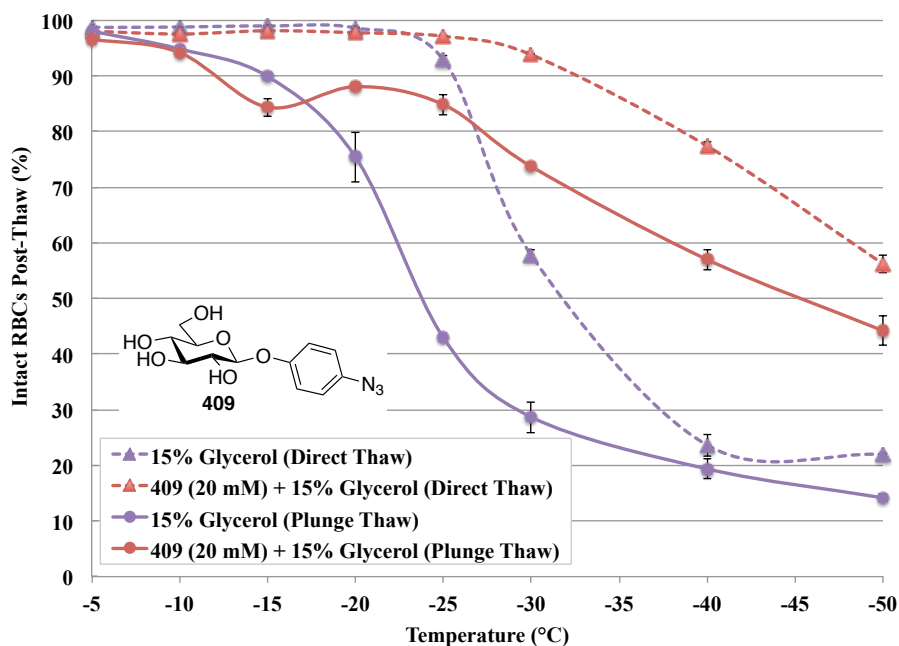


Figure 7.3 Interrupted graded freezing of RBCs with **409**. Percentage of intact RBCs post-thaw (100 - % hemolysis) with and without 20 mM **409** in 15% glycerol. Samples

were cooled at a rate of 1 °C/min to predefined subzero temperatures then either directly thawed (dashed lines) or plunged into dry ice (−78 °C) prior to thawing in a 37 °C water bath (solid lines). These freezing conditions were repeated in duplicate for each solution.

Error bars represent the standard error of the mean (SEM).

The “cooling” profile for 15% glycerol (Figure 7.3, dashed lines) is typical of that observed with a cell permeating cryoprotectant. RBCs that were thawed immediately after slow cooling (1 °C/min) to a defined subzero temperature showed a progressive decrease in the percentage of intact RBCs post-thaw with lower subzero temperatures. At temperatures above −25 °C, slow freezing resulted in a high percentage of intact RBCs post-thaw whereas slow freezing to lower subzero temperatures (i.e. below −30 °C) resulted in a lower number of intact RBCs. When **409** (20 mM) was used in combination with 15% glycerol, the “cooling” profile was similar to that obtained for **104**. During slow cooling (direct thaw), the addition of **409** resulted in a significant increase in the percentage of intact RBCs compared to 15% glycerol alone at the lower subzero temperatures. For instance, at −40 °C, the percentage of intact RBCs with a 15% glycerol solution was 24% whereas this value increased to 77% with the addition of **409** (20 mM). A similar result was also obtained at −50 °C where the percentage of intact RBCs post-thaw was significantly higher (56%) in the presence of **409** (20 mM) relative to 15% glycerol alone (22%). Compared to samples that were directly thawed from defined subzero temperatures, RBC samples that were rapidly cooled (plunged) to −78 °C after slow cooling generated additional injury resulting from the formation of intracellular ice.^{8,10} As previously described, in the presence of 15% glycerol, the percentage of intact RBCs post-thaw progressively decreased when samples were slowly cooled to temperatures below −15 °C. However, the addition of **409** (20 mM) to 15% glycerol resulted in an increase in the percentage of intact RBCs post-thaw at all temperatures below −25 °C (Figure 7.3, solid lines). Analogous to the results obtained with **104**, the addition of **409** to the 15% glycerol solution significantly increased the percentage of intact RBCs post-thaw under both slow cooling (direct thaw) and rapid cooling (plunge thaw) conditions. However, the fact that the addition of **409** to the 15% glycerol solution resulted in a higher percentage of intact RBCs (reduced hemolysis) under slow cooling (direct thaw) conditions is consistent with the profile of a cell permeating cryoprotectant.

Collectively, the results from the interrupted graded freezing protocol suggest that both **104** and **409** are being internalized into RBCs.

7.2.2 The Effect of Pre-Freeze Incubation on Post-Thaw Recovery of RBCs

The “cooling” profiles obtained for both **104** and **409** in 15% glycerol suggest that these IRIs may be conferring protection against cryoinjury through a mechanism consistent with that of a cell permeating cryoprotectant. Thus, we sought to explore whether the observed increase in the percentage of intact RBCs post-thaw was the result of internalization of **104** and **409**. In RBCs, glucose is readily transported across the cell membrane through facilitated diffusion. This process is mediated by glucose transporters (GLUTs) that are highly expressed on the RBC surface and are substrate specific.^{19,20} The transport of glucose and its analogues is highly dependent on the stereochemistry of the hydroxyl groups on the pyranose ring and ultimately the shape of the molecule.^{21,22} As a result, depending on the structure, glucose derivatives may not be transported or the rate of transport across the cell membrane may be significantly decreased.

It has been demonstrated that the rate of sugar transport across the cell membrane can be considerably slowed at reduced temperatures (i.e. 0 °C) and accelerated at elevated temperatures (i.e. 37 °C).^{12,23–28} To elucidate whether the internalization of **104** and **409** is important for their cryoprotective ability, an experiment was performed where RBCs were incubated with **104** (30 mM) or **409** (20 mM) in 15% glycerol for 5, 10, 30 and 60 minutes at either 0 °C, room temperature (RT) or 37 °C prior to freezing. The logic behind this experiment was that if internalization is slow, increased incubation time and temperature will increase the rate of sugar transport across the cell membrane and yield higher post-thaw recovery. It is important to note that the incubation time for RBC samples that were placed in a 37 °C water bath was limited to 30 minutes because if the rate of sugar transport is in fact faster at this temperature, an incubation time of 30 minutes would be sufficient to observe a difference in post-thaw recovery. Following incubation, RBC samples were cooled to -5 °C in a methanol bath and nucleated at this temperature (-5 °C) to ensure that ice nucleation occurred at the same subzero temperature in each vial. The samples were then cooled to -40 °C (rate of 1 °C/min) and rapidly thawed in a 37 °C water bath. RBC hemolysis was measured utilizing the

Drabkin's assay^{14,15} and the results are reported as the percentage of intact RBCs post-thaw (100 - % hemolysis) shown in Figures 7.4 and 7.5.

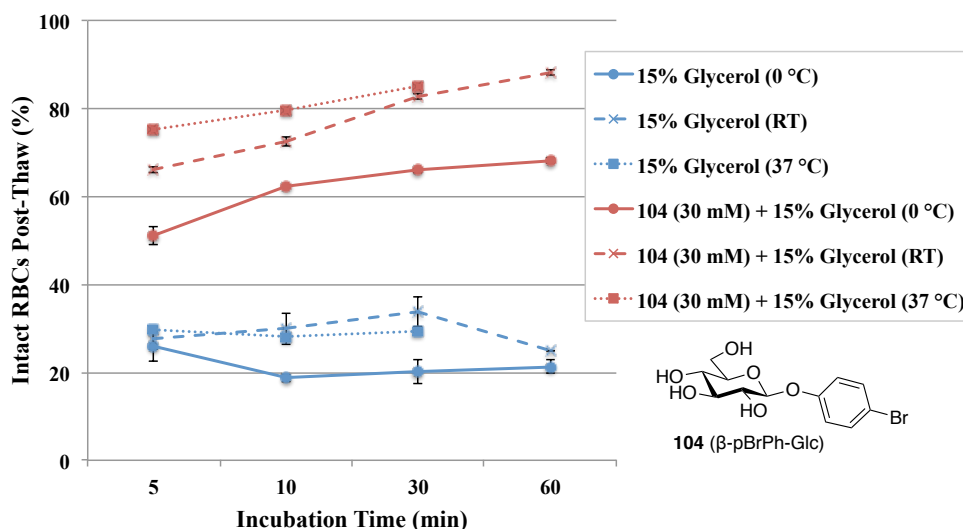


Figure 7.4 The effect of pre-freeze incubation of cryosolutions on the percentage of intact RBCs post-thaw. RBC samples were incubated for the indicated time and at the indicated temperature with a solution of **104** (30 mM) + 15% glycerol prior to freezing to $-40\text{ }^{\circ}\text{C}$ (rate of $1\text{ }^{\circ}\text{C}/\text{min}$). Samples were thawed at $37\text{ }^{\circ}\text{C}$ in a water bath. These conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

The 15% glycerol solution resulted in a similar percentage of intact RBCs post-thaw independent of the pre-freeze incubation time or temperature (approximately 20-35% intact RBCs, Figure 7.4). This is not surprising since it is well established that RBCs are highly permeable to glycerol even at $0\text{ }^{\circ}\text{C}$.^{17,29,30} Consequently, at the lower temperatures and shorter pre-freeze incubation times, the RBCs are likely glycerolized before freezing. Interestingly, in the presence of **104** (30 mM), the percentage of intact RBCs increased as a function of pre-freeze incubation time and temperature. For instance, with a pre-freeze incubation of 5 minutes at $0\text{ }^{\circ}\text{C}$, the percentage of intact RBCs post-thaw with a solution of **104** (30 mM) in 15% glycerol was 51% whereas this value increased to 75% with elevated temperatures (i.e. $37\text{ }^{\circ}\text{C}$). Similar results were also obtained at 10 and 30 minutes. A pre-freeze room temperature incubation appeared to result in an intermediate percentage of intact RBCs post-thaw between $0\text{ }^{\circ}\text{C}$ and $37\text{ }^{\circ}\text{C}$ (Figure 7.4). However, it is evident that utilizing a pre-freeze incubation of 30 minutes at $37\text{ }^{\circ}\text{C}$ is as effective as a pre-freeze incubation of 60 minutes at room temperature as there were no differences in

the percentage of intact RBCs post-thaw. This data suggests that this molecule may be internalized into RBCs and internalization may be important for its protective effect during freezing.

Figure 7.5 depicts the effect of pre-freeze incubation of a solution of **409** (20 mM) in 15% glycerol on the percentage of intact RBCs post-thaw. As described above, the 15% glycerol solution resulted in a similar percentage of intact RBCs post-thaw independent of the pre-freeze incubation time or temperature (approximately 20-35% intact RBCs, Figure 7.5).

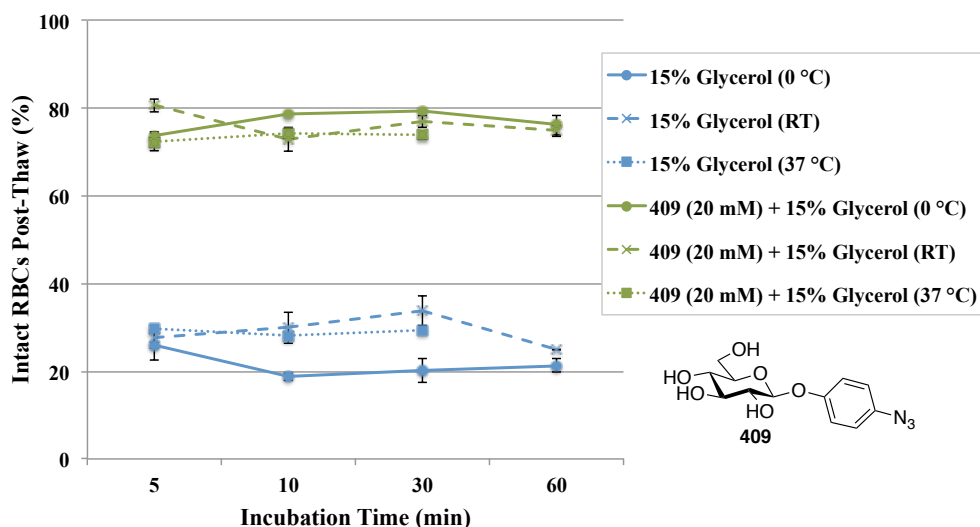


Figure 7.5 The effect of pre-freeze incubation of cryosolutions on the percentage of intact RBCs post-thaw. RBC samples were incubated for the indicated time and at the indicated temperature with a solution of **409** (20 mM) + 15% glycerol prior to freezing to $-40\text{ }^{\circ}\text{C}$ (rate of $1\text{ }^{\circ}\text{C}/\text{min}$). Samples were thawed at $37\text{ }^{\circ}\text{C}$ in a water bath. These conditions were repeated in duplicate for each solution. Error bars represent the standard error of the mean (SEM).

Interestingly, in the presence of **409** (20 mM), the percentage of intact RBCs post-thaw also remained constant independent of the pre-freeze incubation time or temperature (approximately 72-80% intact RBCs). In fact, there were no drastic differences in the percentage of intact RBCs post-thaw for all pre-freeze incubation conditions. This trend is consistent with that observed for a solution of 15% glycerol alone which is known to permeate RBCs very fast. The fact that a solution of **409** (20 mM) in 15% glycerol is capable of increasing the percentage of intact RBCs post-thaw independent of the pre-freeze incubation time or temperature suggests that IRI permeation may occur quickly even at lower temperatures. As a result, a sufficient quantity of **409** may be internalized

prior to freezing. Alternatively, the increased percentage of intact RBCs post-thaw may be attributed to the ability of **409** to function as an extracellular (non-permeating) cryoprotectant. However, this seems less likely since previous work has suggested that this compound might be conferring protection against cryoinjury through a mechanism consistent with that of a cell permeating CPA (Section 7.2.1).

Given the interesting results described above, we sought to examine the morphology of the RBCs in the presence of **104** and **409** in 15% glycerol in the absence of freezing. In this experiment, RBCs were suspended in a solution containing **104** (30 mM) or **409** (20 mM) in 15% glycerol and examined under a microscope. The RBCs were imaged immediately (~1 min), at approximately 5 minutes and after 15-20 minutes total elapsed time from the addition of the cryosolution. The images are depicted in Figure 7.6.

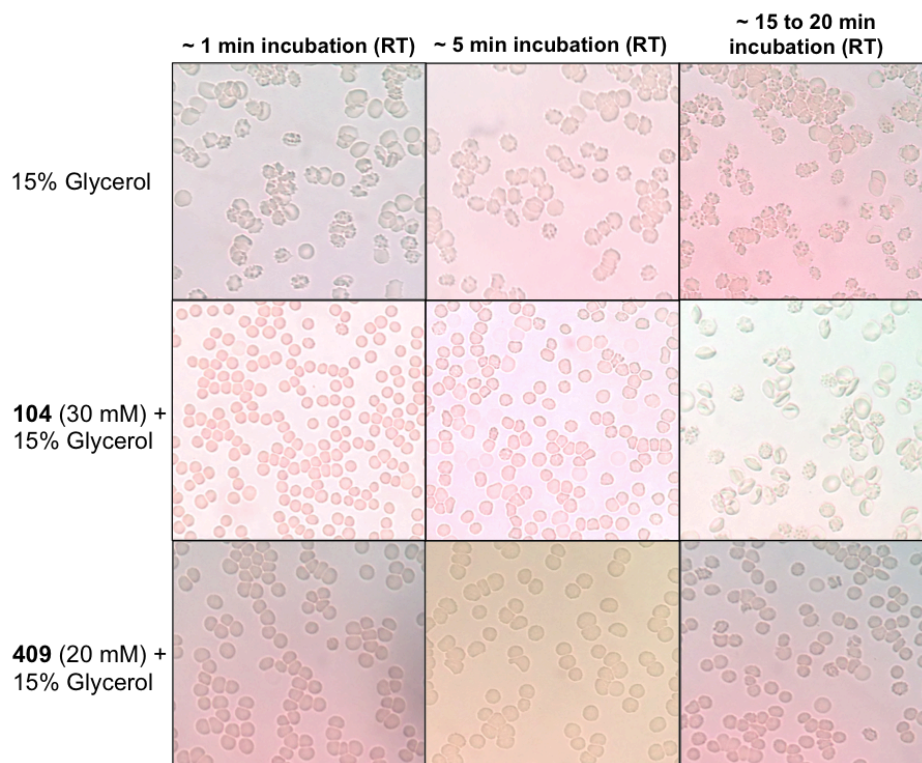


Figure 7.6 The effect of incubation of cryosolutions on RBC morphology. RBC samples were incubated for the indicated time at room temperature (RT) with a solution of 15% glycerol, **104** (30 mM) + 15% glycerol or **409** (20 mM) + 15% glycerol.

Normal, mature RBCs are biconcave, disc-shaped cells; however, it is well established that RBCs undergo shape transformations when subjected to various physical and chemical factors.³¹ In the presence of 15% glycerol, a characteristic change in the shape

of the RBCs was seen such that there was moderate crenation of the RBCs (i.e. spiky appearance) immediately and even after 15-20 minutes of incubation (Figure 7.6). In contrast, RBCs incubated at room temperature with **104** (30 mM) in 15% glycerol appeared swollen and spherocytic immediately (~1 min) and at approximately 5 minutes. However, after 15-20 minutes of incubation, there was a drastic change in RBC morphology from that observed at 5 minutes. It appeared as though the RBCs recovered to their normal biconcave disc shape with few crenated cells. Conversely, RBCs incubated at room temperature in the presence **409** (20 mM) + 15% glycerol were sphere-shaped even after 15-20 minutes, suggesting that cell flexibility may be altered.³² The qualitative morphological analysis showed that both **104** and **409** induced changes in the shape of the RBCs that were different from those observed with 15% glycerol alone. These morphological alterations may suggest changes to the RBC surface or changes in membrane properties in the presence of IRIs.

7.3 Assessing the Uptake of Small Molecule IRIs by RBCs

Thus far, the internalization of small molecule IRIs into RBCs has been assessed under cryopreservation conditions. While the studies described above (Section 7.2) suggest that these molecules behave as cell permeating CPAs, IRI permeation has not yet been investigated in the absence of freezing. Consequently, the uptake of *O*-aryl glycosides (Figure 7.7) by RBCs was assessed to provide additional information to support the hypothesis of internalization.

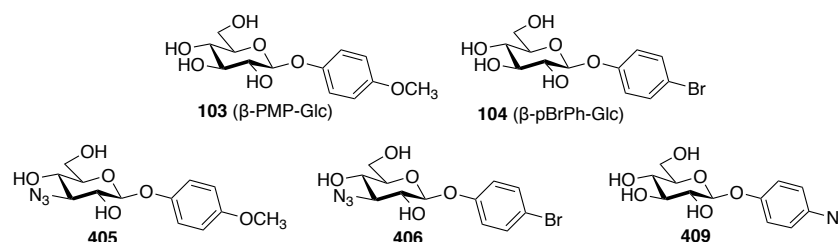


Figure 7.7 Structures of *O*-aryl glucosides assessed in RBC uptake experiments.

As previously mentioned, glucose is readily transported across the cell membrane through facilitated diffusion, a process that does not require energy but involves molecular binding of the sugar to a specific site(s) on the membrane.²¹ This process is mediated by GLUTs that display strong specificity for D-stereoisomers of pentoses and hexoses adopting the chair configuration of the pyranose ring (i.e. D-glucose, D-

galactose).²¹ As such, the transport of glucose and its analogues is highly dependent on the stereochemistry of the hydroxyl groups on the pyranose ring and the shape of the molecule.^{21,22} GLUT1 was the first glucose transporter isoform to be identified and is one of the most extensively studied of all membrane transport proteins.¹⁹ In RBCs, GLUT1 is highly expressed on the RBC surface and is quantitatively the only significant isoform, comprising 10-20% of the integral membrane protein content.^{19,33} As a result, glucose transport in RBCs has been extensively characterized. GLUT1 possesses high substrate specificity for D-glucose and sugar transport is proposed to occur via an asymmetric mechanism with two conformational states (Figure 7.8).²¹ Studies by Barnett *et al.* suggested that the hydroxyl groups at C-1 and C-3 of D-glucose serve as hydrogen bond acceptors when D-glucose is positioned in the GLUT1 sugar uptake site (outward-facing conformation).^{19,21} The binding of D-glucose to the outward-facing binding site triggers a conformational change in the transporter, effectively moving the bound glucose through the protein where it is now bound to the inward-facing binding site. After the release of glucose into the RBC, it is rapidly phosphorylated to glucose-6-phosphate by hexokinase, preventing it from leaving the cell.³⁴ This aids in keeping the intracellular concentration of free glucose low to maintain the concentration gradient.¹⁹ There is also some evidence to suggest that the C-4 hydroxyl group of D-glucose may form a hydrogen bond with GLUT1 as D-galactose (C-4 epimer of D-glucose) has 10-fold lower affinity for the transporter than D-glucose.^{19,21} Alternatively, it is possible that the non-*gluco* configuration of the sugar hydroxyl group may sterically hinder transport.^{19,21} It was also demonstrated that the C-6 hydroxyl group does not appear to form a hydrogen bond with GLUT1 and bulky substituents are tolerated at this position. Conversely, bulky substituents at C-1 are not tolerated.^{19,21} However, for the sugar exit site (inward-facing conformation), the reverse appears to be true such that bulky substituents at C-1 are tolerated but large substituents at C-6 are not.^{19,21} These studies clearly demonstrate the stringent structural requirements for binding to the GLUT1 transport system. Towards this end, it was hypothesized that the transport of *O*-aryl glycosides (Figure 7.7) across the RBC membrane will not be mediated by GLUT1 as these molecules contain bulky substituents at C-1. To test this hypothesis, the uptake of *O*-aryl glycosides by RBCs was evaluated in the absence and presence of a GLUT1 inhibitor.

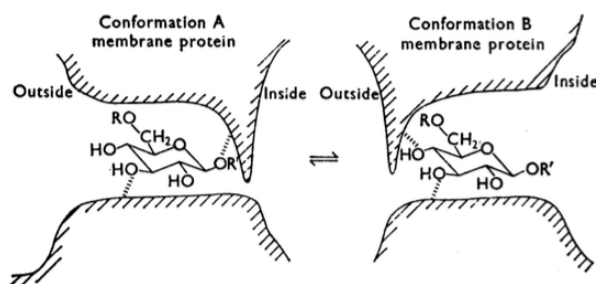


Figure 7.8 Possible model for sugar transport in the human red blood cell.²¹

The uptake of *O*-aryl glycosides by RBCs was studied using capillary electrophoresis-mass spectrometry (CE-MS) and the experimental protocol is depicted in Figure 7.9. Prior to performing the experiment, RBCs were suspended in ten volumes of isotonic phosphate buffered saline solution (PBS) and incubated for 20 minutes at 37 °C to deplete endogenous intracellular sugar.²² The obtained RBCs were then suspended in a PBS solution containing **103**, **104**, **405**, **406**, or **409**. All compounds were assessed at a concentration of 15 mM with the exception of **406**, which was insoluble at 15 mM, thus, it was assessed at 10 mM in PBS.

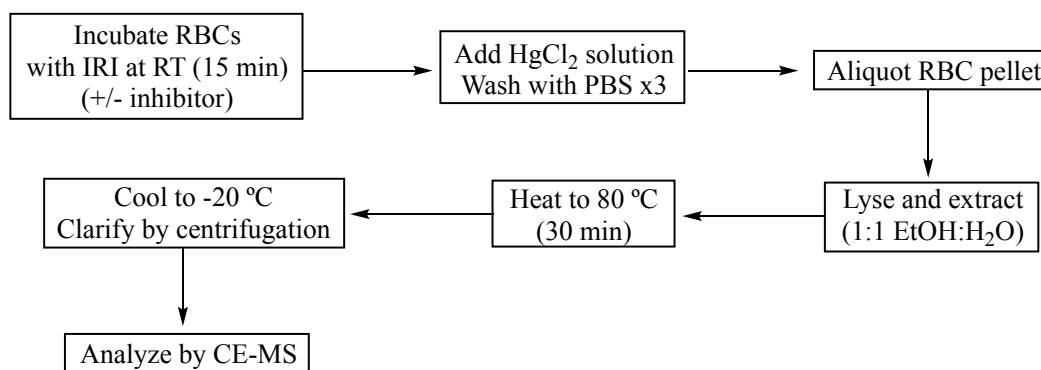


Figure 7.9 Flow chart of the experimental protocol of the RBC uptake study.

RBC samples were incubated for 15 minutes at room temperature prior to adding ice-cold stopping solution consisting of 1% NaCl aqueous solution containing 2 mM mercuric chloride (HgCl_2) and 1.25 mM potassium iodide (KI) to quench sugar flux across the cell membrane.^{22,35} The RBCs were washed with PBS three times to remove excess compound and the contents of the isolated cells were released upon the addition of aqueous ethanol. The RBC samples were heated to 80 °C for 30 minutes to denature proteins, cooled to -20 °C to further precipitate proteins and debris and clarified by

centrifugation. The supernatants were analyzed by CE-MS to qualitatively assess uptake. Capillary electrophoresis (CE) is a powerful analytical technique for the separation of polar molecules.³⁶ In this separation method, analytes migrate within a capillary tube filled with a conducting buffer solution under the influence of an applied electric field.³⁷ The inner surface of the capillary is negatively charged and an electrical double layer is formed on the capillary wall when cations of the buffer solution bind to the negatively charged capillary wall.³⁷ When an electric field is applied, cations move to the negatively charged cathode, resulting in a solution flow, the electroosmotic flow (Figure 7.10).³⁷ The mobility of an individual molecule through the capillary depends on several factors including the charge on the molecule, buffer system (pH and ionic strength), voltage applied, temperature, length and diameter of the capillary, and the nature of the capillary wall.^{37,38} The difference in mobilities between the different analytes in the sample leads to their separation. CE coupled to mass spectrometry (MS) combines the high separation efficiency of CE and identification of molecular masses in MS. For CE-MS, the typical liquid-to-gas ion transformation method used is electrospray ionization (ESI) as analytes can be sprayed directly into the mass spectrometer from the CE.³⁶

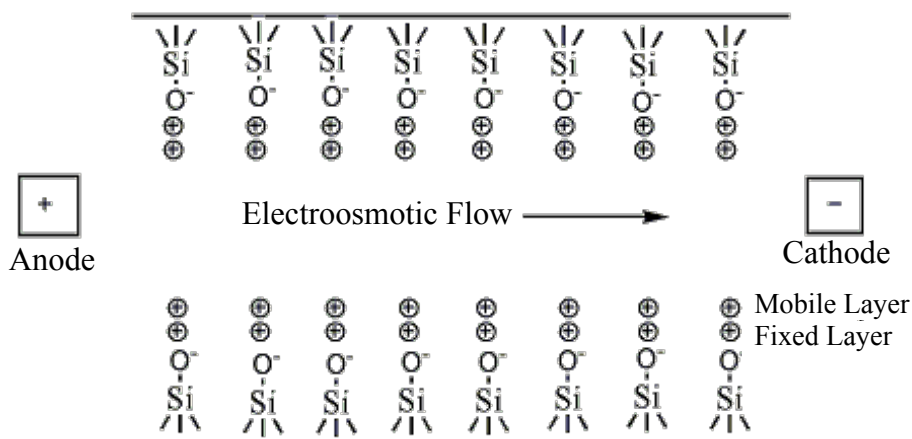


Figure 7.10 Schematic diagram of the interior of a capillary tube in the presence of a buffer solution.

As the analytes appear in the mass spectrometer detector, their presences are signaled by a peak. Thus, a chromatogram displays the detector responses (intensity) against the retention time and provides a spectrum of peaks for a given sample, which represents the analytes present in the sample eluting from the column at different times. From the mass

spectrum that is produced, the component of each peak can be identified from the mass-to-charge ratio (m/z) of the molecular ion. Using this method, it allows us to identify whether the desired compound is present, and the area of the peak reflects the amount of compound that is present. Thus, for this experiment, a qualitative analysis was performed in which the presence of the desired molecular ion peak in the mass spectrum of the cell lysate indicated that the analyte of interest was present in the sample, suggesting internalization into RBCs.

The incubations were also carried out in the presence of the GLUT1 inhibitor cytochalasin B (CCB) to determine whether the uptake of **103**, **104**, **405**, **406**, and **409** was mediated by the GLUT1 transporter. Cytochalasin B, the best-characterized GLUT1 inhibitor, is a membrane-permeable, intracellular sugar-transport inhibitor such that it binds at or close to the GLUT1 sugar efflux site.^{21,39–41} Thus, it acts as a non-competitive inhibitor of sugar uptake.^{39,40} For comparative purposes, the uptake experiment was carried out with a non-metabolizable glucose analogue, 3-*O*-methyl-D-glucose (3-OMG), since it is transported across the RBC membrane by GLUT1 with an affinity that is not much lower than that of D-glucose.⁴² Furthermore, it is well established that the uptake of 3-OMG into RBCs can be inhibited by cytochalasin B.⁴³

The CE-MS results are shown in Figure 7.11 and are reported as the area under the curve (AUC). For all compounds assessed, the desired molecular ion peak was present in the mass spectrum of the cell lysate, suggesting internalization into RBCs. It is important to note that the y-axis scale (i.e. AUC) differs for each compound because different analytes have different affinities for the detector. Since the AUC is based on the number of counts taken by the MS detector at the point of retention, analytes with higher affinity for the detector will give a larger peak area. This in turn can falsely indicate the amount of compound present. As a result, in order to directly compare different compounds, standard curves with known concentrations of analytes should be generated in the future to provide a quantitative analysis. As depicted in Figure 7.11, for each compound, AUC values are shown for RBC samples treated with (“+ inhibitor”) and without (“- inhibitor”) the GLUT1 inhibitor (cytochalasin B). The areas under the curve of the identified molecules were used as a measure of the difference in uptake between samples treated without and with inhibitor. The glucose analogue 3-OMG was used as a positive control

considering that its transport across the RBC membrane is mediated by GLUT1. In the absence of the GLUT1 inhibitor, a peak corresponding to the molecular weight of 3-OMG was detected, indicating that this compound was internalized. The value of the AUC was found to be considerably lower (6x) in the presence of the GLUT1 inhibitor, indicating that the quantity of compound detected was less. Consequently, this confirmed that cytochalasin B was in fact inhibiting the GLUT1 transporter, resulting in decreased uptake of 3-OMG by RBCs. The extent of the difference in uptake of 3-OMG between samples treated without and with inhibitor was thus used as a standard for comparison.

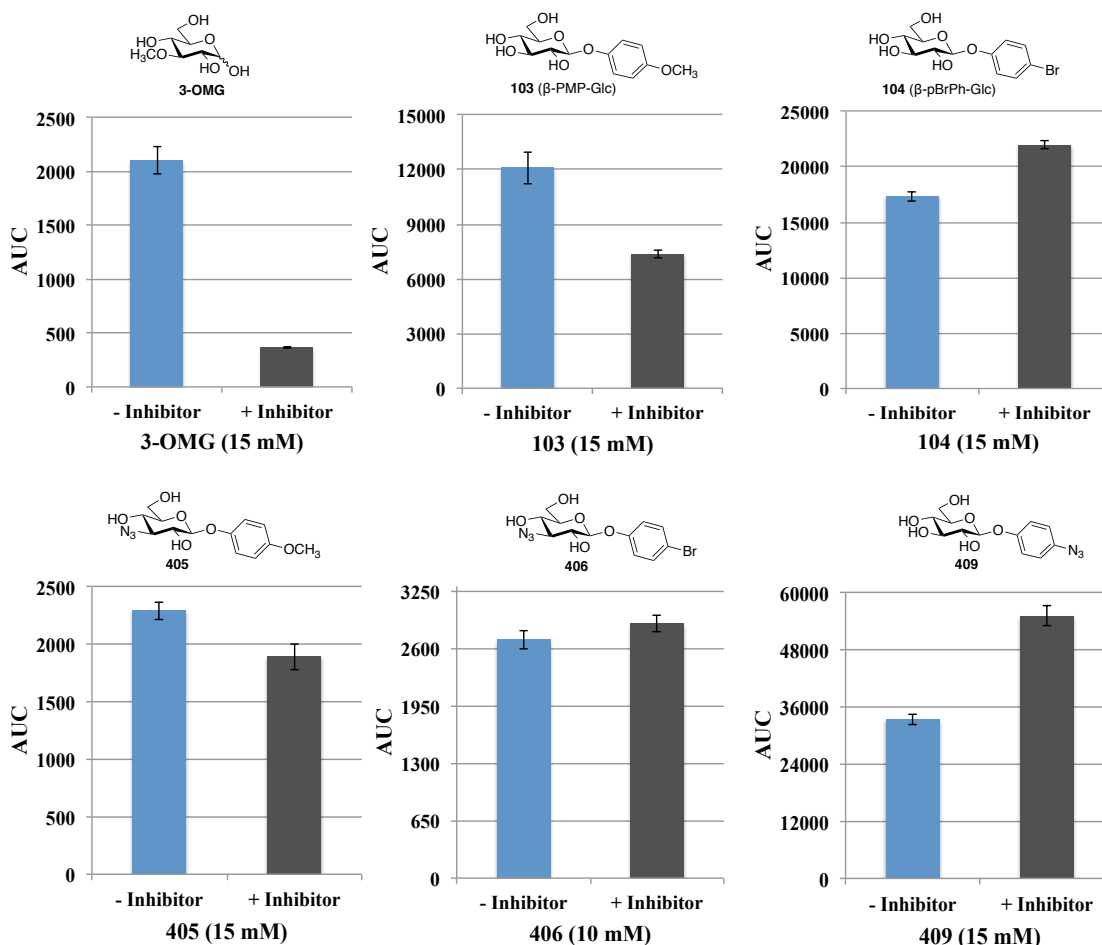


Figure 7.11 Values of area under the curve (AUC) for RBC samples treated with the indicated IRI in the presence (“+ inhibitor”) or absence (“- inhibitor”) of the GLUT1 inhibitor (cytochalasin B). These conditions were repeated in triplicate for each solution. Error bars represent the standard error of the mean (SEM).

Relative to 3-OMG, RBC samples that were incubated with compounds **104**, **405** or **406** in the absence or presence of the GLUT1 inhibitor displayed AUC values that were

similar. In contrast, RBC samples treated with compounds **103** or **409** displayed AUC values that were 1.5 times less and 0.6 times more in the presence of the GLUT1 inhibitor, respectively. In the case of **103**, although the quantity of compound detected in the cell lysate was less in the presence of the GLUT1 inhibitor, the extent of the difference in uptake was not comparable to that obtained with 3-OMG, a known substrate of GLUT1. As a result, it seems likely that **103** is not transported across the RBC membrane by GLUT1. Overall, these results suggest that compounds **103**, **104**, **405**, **406**, and **409** have the ability to cross the RBC membrane, leading to internalization. This is consistent with previously reported results that demonstrated that sugars substituted at C-1 such as phenyl- β -D-glucopyranoside have the ability to permeate the RBC membrane.²¹ The experiments in the presence of the GLUT1 inhibitor validate the hypothesis that the uptake of *O*-aryl glucosides by RBCs is not mediated by GLUT1. These findings are also consistent with previously reported results that demonstrated that the substitution of glucose at the anomeric position (C-1) leads to compounds with low or no binding to GLUT1.^{21,22,44} Therefore, the uptake of compounds **103**, **104**, **405**, **406**, and **409** by RBCs seems to support a mechanism of permeation different from the use of the GLUT1 transporter.

7.4 Controlling Intracellular Ice Recrystallization in HUVECs

In Sections 7.2 and 7.3, the internalization of small molecule IRIs was evaluated in RBCs. Specifically, an interrupted graded freezing technique was used to evaluate IRI permeation into RBCs and mitigate cell damage during freezing and thawing. It was shown that the addition of **103**, **104** or **409** to 15% glycerol significantly increased the percentage of intact RBCs post-thaw during slow cooling compared with the 15% glycerol-only control group (Section 7.2.1).⁷ The characteristics of the cryobiological behavior of the RBCs subjected to the interrupted graded freezing suggested that these aryl glycoside-based IRIs are internalized into the RBCs. To extend our understanding of the internalization of these molecules, IRI permeation was evaluated in a different cell model, in particular human umbilical vein endothelial cells (HUVECs). Since HUVECs have been used to study intracellular ice formation and growth,^{45,46} this provided a novel opportunity to investigate the ability of small molecule IRIs to permeate the cell

membrane of vascular endothelial cells and inhibit intracellular ice recrystallization.¹³ Our approach does not attempt to prevent intracellular ice formation but rather control ice growth once intracellular ice formation has occurred to minimize subsequent cellular damage.

The consequences of rapid cooling and the formation of intracellular ice have been reported and studied for over 30 years. While a current tenet in cryobiology is that intracellular ice formation is lethal to cells, the degree to which intracellular ice is damaging and the mechanism by which this injury occurs has been largely speculative. The most widely held view is that intracellular ice formation damage occurs as a result of mechanical damage due to recrystallization;¹⁸ however, it has been suggested that the amount, size, or location of intracellular ice may be critically important.⁹ Small molecule IRIs provide an ideal tool to study intracellular ice formation in much more detail than has been previously possible.

The effect of IRIs (**103** and **104**) on the intracellular growth of ice crystals was assessed in HUVECs using fluorescence cryomicroscopy.¹³ Unfortunately, **409** could not be assessed because under fluorescence illumination, the aryl azide functionality underwent photolytic decomposition, resulting in the formation of a nitrene and nitrogen gas.⁴⁷ The evolution of nitrogen gas was evident under the microscope, producing large bubbles. This was not surprising since aryl azides are known to be photolabile.^{47–49} For the cryomicroscopy experiments,⁵⁰ HUVECs were treated with SYTO 13 in a 0.9% NaCl solution and frozen using fast cooling rates (90 °C/min). These fast cooling rates were selected to prevent cellular dehydration associated with slow cooling rates. In addition, extracellular ice was not nucleated (or seeded) in the samples, as nucleated samples frozen with high cooling rates greatly affect intracellular ice formation.⁵¹ Rapid cooling (90 °C/min) of HUVECs in 0.9% NaCl to –40 °C resulted in intracellular ice in all of the cells (Figure 7.12).¹³ In these images, the ice crystals appear as large black spots in the cell nuclei, as the nucleic-acid-bound SYTO 13 fluorophore is excluded from the ice lattice. As the sample warmed, ice recrystallization resulted in the formation of larger ice crystals. These ice crystals grew steadily in size, and, ultimately, the number of ice crystals decreased as the average surface area of the total ice in the cell was reduced (Figure 7.12A). For example, at –40 °C, we observed 57 distinct ice crystals, at –20 °C,

we observed 23, and at $-10\text{ }^{\circ}\text{C}$, only 10 ice crystals were observed (Figure 7.12A). Also, the average ice crystal size in the nuclei of the cells was very homogeneous, and the number of ice crystals in each cell appeared to be proportional to the volume of the nucleus.¹³

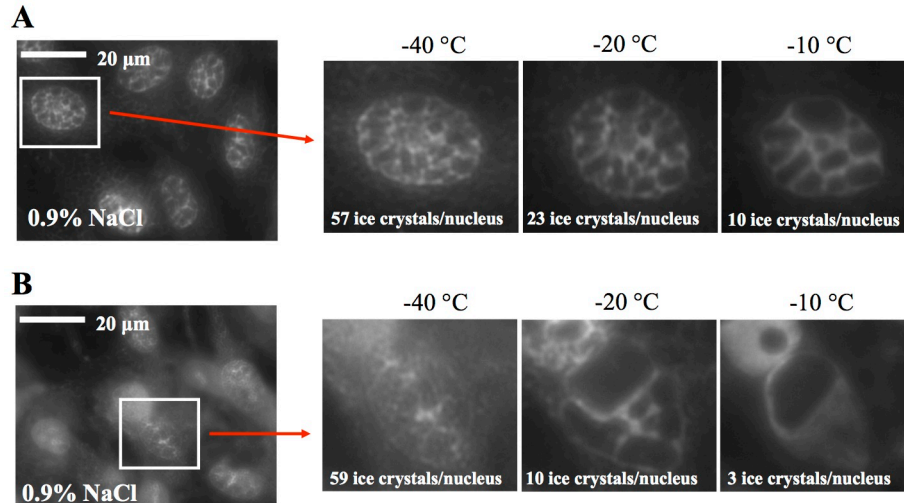


Figure 7.12 Intracellular ice formation at different warming rates. HUVECs were cooled at $90\text{ }^{\circ}\text{C}/\text{min}$ to $-40\text{ }^{\circ}\text{C}$ in 0.9% NaCl with SYTO 13 ($5\text{ }\mu\text{M}$). (A) Warming at $25\text{ }^{\circ}\text{C}/\text{min}$ and (B) warming at $2\text{ }^{\circ}\text{C}/\text{min}$. Under fluorescence illumination, intracellular ice crystals appear as black spots in the nuclei of the cells.

The experiment was repeated in the same manner; however, the frozen cells were warmed at a significantly slower rate of $2\text{ }^{\circ}\text{C}/\text{min}$ (Figure 7.12B). This increased the rate of ice recrystallization, and consequently, larger ice crystals were observed. In addition, significantly more cellular damage would be expected with the slower warming rate.^{52–54} As illustrated in Figure 7.12B, the intracellular ice looks similar to that in Figure 7.12A when HUVECs were frozen at a cooling rate of $90\text{ }^{\circ}\text{C}/\text{min}$ to $-40\text{ }^{\circ}\text{C}$. In fact, the number of ice crystals at $-40\text{ }^{\circ}\text{C}$ was 59, whereas in Figure 7.12A, there were 57.¹³ However, upon slowly warming the sample, the number of ice crystals in each nucleus decreased and the average size of each crystal increased. For instance, at -20 and $-10\text{ }^{\circ}\text{C}$, there were fewer ice crystals, and the average size of each was much larger than those observed with faster warming rates. At $-10\text{ }^{\circ}\text{C}$, slower thawing resulted in three ice crystals, whereas with the faster thawing rate, we observed 10.¹³

The number of ice crystals in each HUVEC nucleus in the expanded field of view was counted, and the mean grain size (MGS) was determined.⁵⁵ In some instances, it was

not possible to examine every cell in the field of view because some cells were not in focus, so only those with clearly visible ice crystals were measured. On average, one to nine different nuclei were measured per field of view. This data were plotted as a function of temperature (Figure 7.13).¹³ Both curves are exponential in nature, and whereas the initial ice crystal size is equal under both fast and slow thaw conditions, ice crystal size is considerably larger under slow thaw conditions.

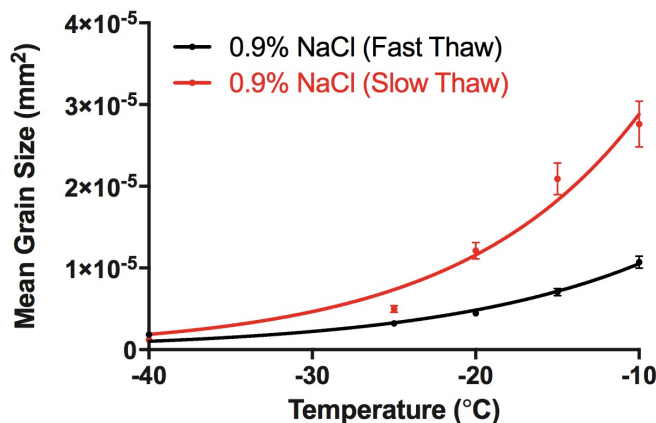


Figure 7.13 Mean grain size (mm^2) of ice crystals as a function of temperature ($^{\circ}\text{C}$) under faster ($25^{\circ}\text{C}/\text{min}$) and slower thawing ($2^{\circ}\text{C}/\text{min}$) conditions. Error bars represent the standard error of the mean (SEM). The solid lines through the data points were fitted using a monoexponential growth equation.

Given that we were able to observe intracellular ice recrystallization in the nuclei of HUVECs and verify the warming rate dependence, these experiments were repeated in the presence of **103** and **104** because they have proven to be very effective at inhibiting ice recrystallization (Figures 7.14 and 7.15). Compound **103** (β -PMP-Glc) was dissolved in 0.9% NaCl to a final concentration of 110 mM. This is the optimal concentration that has been utilized previously in our work with human RBCs.⁷ It is immediately evident from the images in Figure 7.14 that after rapid cooling to -40°C , the intracellular ice crystals are extremely small compared to those observed with HUVECs in 0.9% NaCl (Figure 7.12) with identical cooling rates.¹³ In comparison with the 0.9% NaCl control (Figure 7.12), it is evident that there are very little changes in the intracellular ice between the temperatures of -40 to -20°C when **103** is present in solution. While ice recrystallization does ultimately occur, there is a very rapid change in the ice structure; however, this does not occur until -10°C (Figure 7.14A). At temperatures below -10°C , there are no visible changes in the intracellular ice. It is also worth pointing out that even

at $-10\text{ }^{\circ}\text{C}$ the size of the ice crystals is smaller than those observed with 0.9% NaCl under the same cooling and warming conditions.¹³ This is consistent with less ice recrystallization occurring. However, thawing the cells at a slower rate of $2\text{ }^{\circ}\text{C}/\text{min}$ produces very different results. At $-20\text{ }^{\circ}\text{C}$, individual ice crystals are clearly visible (Figure 7.14B) and ice recrystallization is evident, but the average size of these crystals is much smaller than those observed in the 0.9% NaCl control with fast thawing rates (Figure 7.12A). However, these ice crystals are larger than those observed in Figure 7.14A at $-20\text{ }^{\circ}\text{C}$. As expected, the total number of ice crystals present in each nucleus is greater because the inhibition of ice recrystallization is not as effective at a slower warming rate. The fact that intracellular ice recrystallization is inhibited and we observe smaller ice crystals with **103** suggests this compound is internalized in HUVECs.¹³ This result further supports the previous RBC work with this compound suggesting that internalization is occurring.⁷

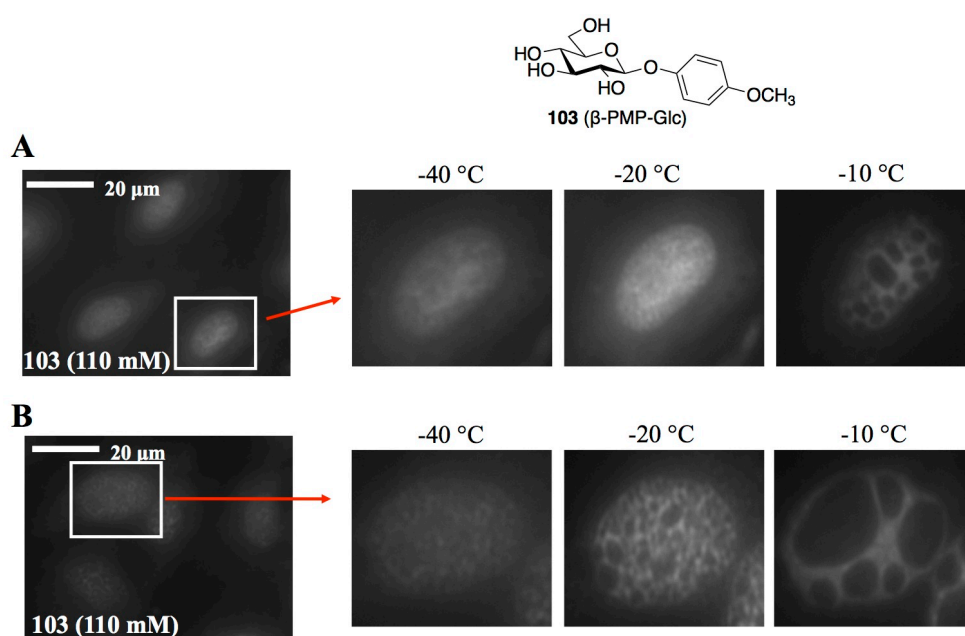


Figure 7.14 Intracellular ice formation at different warming rates in the presence of 110 mM **103** (β -PMP-Glc). HUVECs were cooled at $90\text{ }^{\circ}\text{C}/\text{min}$ to $-40\text{ }^{\circ}\text{C}$ in 0.9% NaCl with SYTO 13 ($5\text{ }\mu\text{M}$). **(A)** Warming at $25\text{ }^{\circ}\text{C}/\text{min}$ and **(B)** warming at $2\text{ }^{\circ}\text{C}/\text{min}$. Under fluorescence illumination, intracellular ice crystals appear as black spots in the nuclei of the cells.

Compound **104** is also very effective at inhibiting intracellular ice recrystallization (Figure 7.15) but at a lower concentration of 30 mM compared with 110 mM. A concentration of 30 mM was chosen because this is the optimal concentration that has been utilized previously in our work with human RBCs. Rapid cooling of HUVECs in the presence of 30 mM **104** (β -pBrPh-Glc) produced very similar results to those observed with **103**. At $-40\text{ }^{\circ}\text{C}$, there is a larger number of ice crystals, and the average size of each ice crystal is much smaller (Figure 7.15) than that observed with the 0.9% NaCl control.¹³ The intracellular ice is initially composed of very small crystals and remains unchanged even when the sample is warmed to approximately $-20\text{ }^{\circ}\text{C}$. However, ice recrystallization occurs rapidly at $-10\text{ }^{\circ}\text{C}$, but the size of the ice crystals remains much smaller than the 0.9% NaCl control.

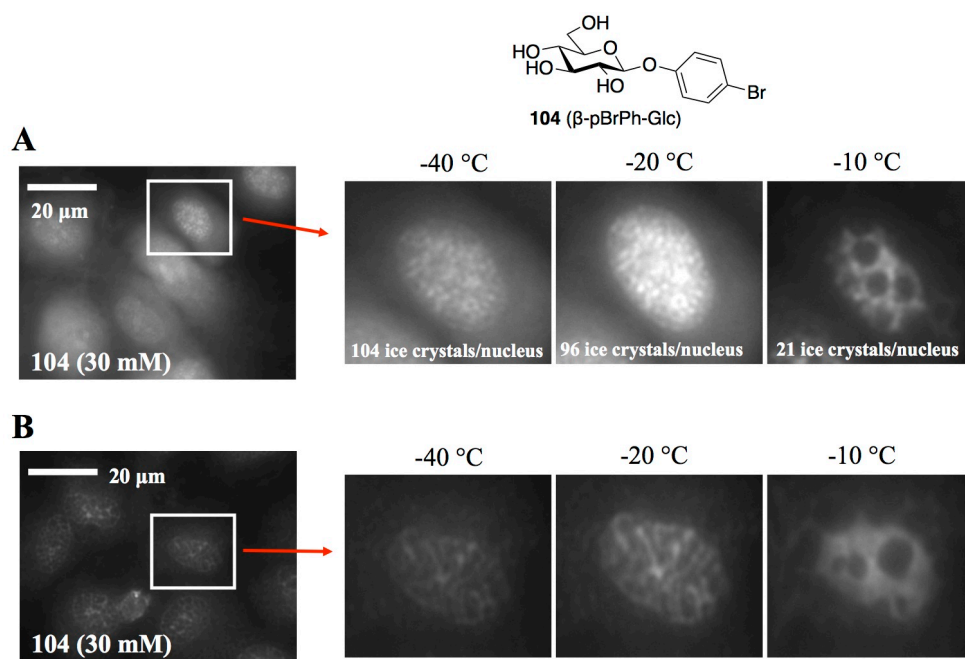


Figure 7.15 Intracellular ice formation at different warming rates in the presence of 30 mM **104** (β -pBrPh-Glc). HUVECs were cooled at $90\text{ }^{\circ}\text{C}/\text{min}$ to $-40\text{ }^{\circ}\text{C}$ in 0.9% NaCl with SYTO 13 ($5\text{ }\mu\text{M}$). **(A)** Warming at $25\text{ }^{\circ}\text{C}/\text{min}$ and **(B)** warming at $2\text{ }^{\circ}\text{C}/\text{min}$. Under fluorescence illumination, intracellular ice crystals appear as black spots in the nuclei of the cells.

As described above, the number of ice crystals in each HUVEC nucleus in the expanded field of view was counted, the mean grain size (MGS) was determined,⁵⁵ and the data was plotted as a function of temperature. Figure 7.16 illustrates the mean grain size of intracellular ice as a function of temperature when samples are thawed at $25\text{ }^{\circ}\text{C}/\text{min}$ (fast

thaw). It is evident that **103** and **104** are effective inhibitors of intracellular ice recrystallization. Each curve is exponential in nature, as observed with 0.9% NaCl.¹³

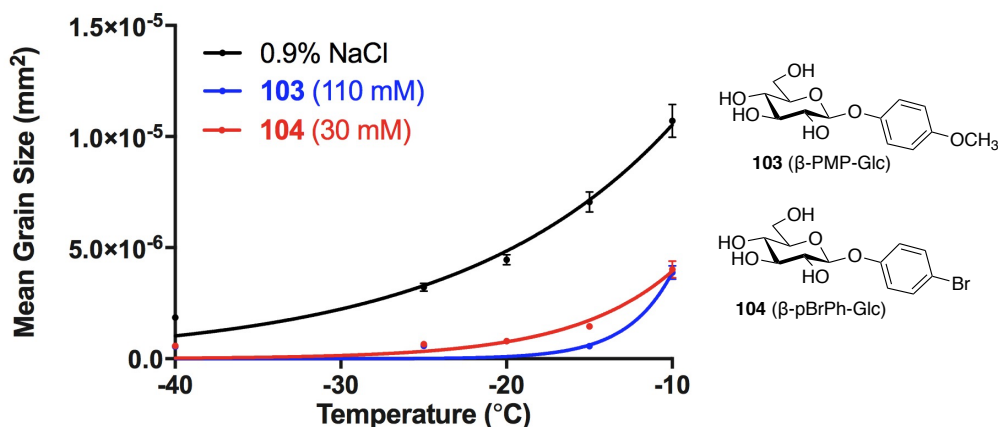


Figure 7.16 Correlation of intracellular ice crystal size (mm²) as a function of temperature (°C) using a warming rate of 25 °C/min. Error bars represent the standard error of the mean (SEM). The solid lines through the data points were fitted using a monoexponential growth equation.

Figure 7.17 shows the data obtained from the slower thawing rate experiments. Under slower thawing conditions, it is apparent that **104** (β-pBrPh-Glc) is a much better inhibitor than **103** (β-PMP-Glc) and is able to effectively inhibit ice recrystallization; however, **103** is only effective up to temperatures of -15 °C, at which point it is no longer effective. Yet under faster thawing conditions, **103** is an effective inhibitor of intracellular ice recrystallization (Figure 7.16).¹³

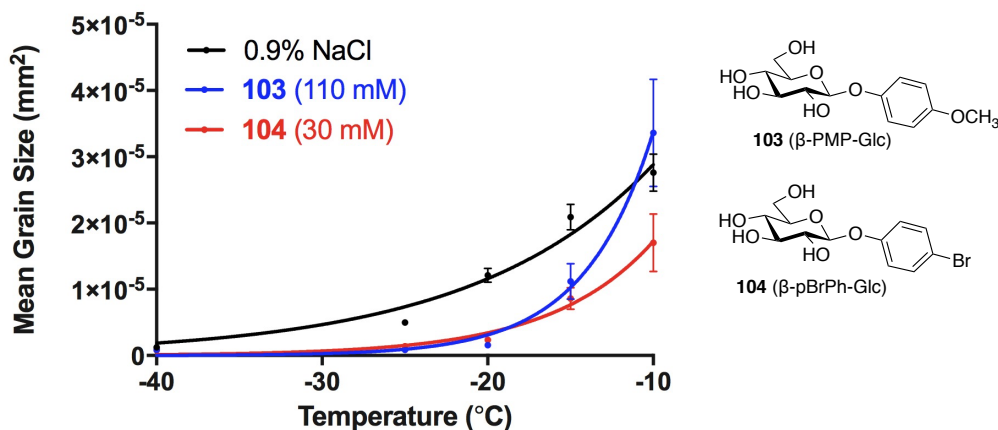


Figure 7.17 Correlation of intracellular ice crystal size (mm²) as a function of temperature (°C) using a warming rate of 2 °C/min. Error bars represent the standard

error of the mean (SEM). The solid lines through the data points were fitted using a monoexponential growth equation.

This work demonstrates that small molecule IRIs **103** and **104** are capable of permeating the cell membrane of vascular endothelial cells and controlling the rate and extent of intracellular ice recrystallization in the nuclei of HUVECs over a broad range of subzero temperatures. These findings are highly significant because controlling intracellular ice growth using small molecule IRIs can be an important approach to improve the recovery and function of cryopreserved cells and tissues. These results emphasize the potential of IRIs as cell permeating cryoprotectants for the preservation of cells and tissues and provide insight into the mechanism of intracellular ice growth.

7.4.1 Assessing Cytotoxicity of β -pBrPh-Glc in HUVECs

HUVECs treated with the IRIs were shown to retain a large number of small ice crystals during warming to high subzero temperatures and demonstrated a significant inhibition of intracellular ice recrystallization. In particular, **104** (β -pBrPh-Glc) was shown to be very effective at inhibiting intracellular ice recrystallization under both fast and slow thawing conditions. The ability of **104** to control intracellular ice growth emphasizes its tremendous potential as a CPA for the preservation of cells and tissues to improve post-thaw recovery and function. Ideally, the optimal CPA should also exhibit little to no cytotoxicity. Thus, we sought to examine the cytotoxicity of **104** in HUVECs in the absence of freezing using the Alamar Blue (resazurin) assay.⁵⁶ This is a colorimetric assay that measures cellular metabolic activity as a means of assessing cell viability. It is based on the ability of mitochondrial and cytosolic dehydrogenase enzymes in metabolically active cells to reduce the water-soluble blue non-fluorescent dye resazurin to the pink, highly fluorescent resorufin.⁵⁶ Fluorescence is measured using a spectrophotometer to quantify cell viability. Thus, an intense pink colour represents viable, metabolically active cells whereas a purple/blue colour signifies reduced cell number and thus cytotoxicity of a given compound.

The cytotoxic effect of **104** was determined by assessing cell viability in HUVECs at various concentrations (Figure 7.18). It is evident that **104** is highly cytotoxic even at low concentrations. A 1 mM solution of **104** resulted in a cell viability of 77% and increasing

this concentration to 5 or 10 mM drastically decreased viability to approximately 10%. A further increase in the concentration of **104** (i.e. 20 mM) increased these cytotoxic effects, resulting in a cell viability of 0%.

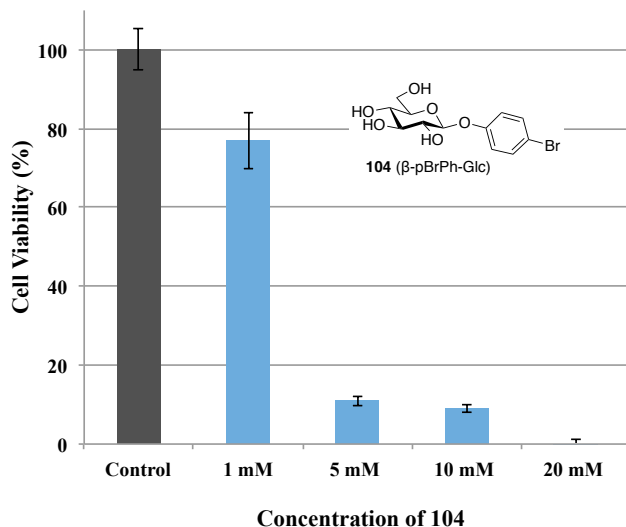


Figure 7.18 Percent cell viability of HUVECs treated with various concentrations of **104** (β -pBrPh-Glc) following incubation for 24 h at 37 °C as determined by Alamar Blue assay. Error bars represent the standard error of the mean (SEM).

Sugar conjugates are ubiquitous and found in virtually all eukaryotic cells. Examples of these conjugates include *O*-glycosides such as oligosaccharides and glycolipids. Given the ubiquitous nature of *O*-glycosides, enzymes that are responsible for the hydrolysis of carbohydrate moieties are also found in all living organisms.^{57,58} Consequently, it is well established that *O*-glycosides are susceptible to cleavage by hydrolytic enzymes such as glycosidases.⁵⁹ Towards this end, it was hypothesized that the glycosidic linkage in **104** may be enzymatically cleaved to produce glucose (**Glc**) and *p*-bromophenol (**pBrPh**) in which the latter component (**pBrPh**) may be responsible for the observed cytotoxicity in HUVECs. To investigate this, HUVECs were treated with the individual components of **104** that would be formed upon cleavage (i.e. **Glc** and/or **pBrPh**) and cytotoxicity was determined by assessing cell viability as described above. HUVECs were also treated with **104** for comparison. The results are presented in Figure 7.19. As shown previously, a 1 mM solution of **104** resulted in a cell viability of 70%. In contrast, it is evident that glucose (**Glc**) is non-toxic at concentrations of 1 mM. However, a 1 mM solution of *p*-bromophenol (**pBrPh**) is highly cytotoxic, resulting in a cell viability of 15%. Similarly,

a solution of equal concentrations of glucose (**Glc**) and *p*-bromophenol (**pBrPh**) was found to be highly cytotoxic (cell viability of 15%). Thus, it is apparent that even at low concentrations of 1 mM, **pBrPh** is highly cytotoxic to HUVECs. Similar results were obtained at higher concentrations in which a 5 mM solution of glucose (**Glc**) exhibited low toxicity (cell viability of 86%) whereas solutions containing **pBrPh** (5 mM) were highly toxic to HUVECs (cell viabilities of 0%). The fact that a 5 mM solution of **104** is considerably more toxic than a 1 mM solution suggests that cytotoxicity may be dependent on the relative concentration of **pBrPh** in solution. Overall, these results indicate that the cytotoxicity exhibited by **104** in HUVECs may be due to cleavage of the glycosidic linkage via the action of glycosidases and subsequent release of the toxic product *p*-bromophenol (**pBrPh**). Interestingly, compound **104** (β -pBrPh-Glc) that is a beneficial, non-toxic additive for the freezing of human RBCs is highly cytotoxic to HUVECs. This demonstrates that the cytotoxicity of **104** is cell type-dependent and that **104** is better suited as a CPA for RBCs.

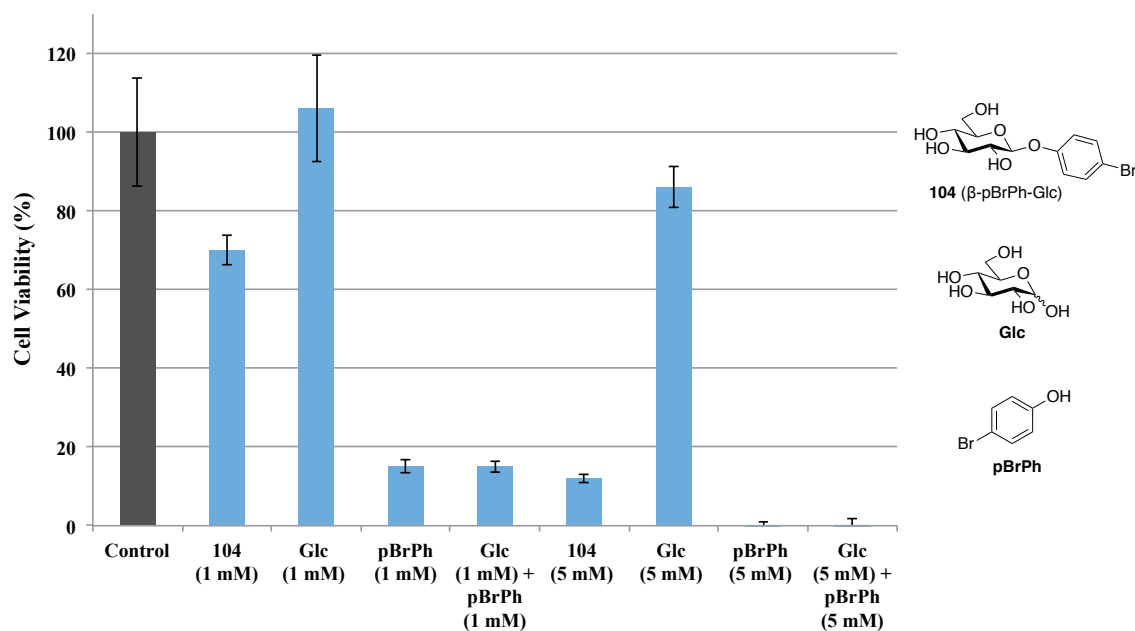


Figure 7.19 Percent cell viability of HUVECs treated with **104** (β -pBrPh-Glc) and its individual components (**Glc** and **pBrPh**) following incubation for 24 h at 37 °C as determined by Alamar Blue assay. Error bars represent the standard error of the mean (SEM).

7.5 Chapter Summary

Previous studies by our laboratory have speculated that the increase in the percentage of intact RBCs post-thaw observed with **103** (β -PMP-Glc) may be due to its ability to interact with the RBC membrane and/or permeate the cell. Consequently, further investigation was performed to determine if small molecule IRIs are crossing the cell membrane and behaving as permeating CPAs. In addition to **103**, it has also been demonstrated that **104** (β -pBrPh-Glc) and azido aryl glucoside **409** are effective additives for the freezing of human RBCs. Thus, an interrupted graded freezing protocol was performed to determine the mechanism of cryoinjury in the presence of **104** and **409**. It was found that the percentage of intact RBCs post-thaw was significantly increased following slow cooling when **104** or **409** was added to a 15% glycerol solution and that the cryobiological response was consistent with that of a cell permeating CPA, suggesting that **104** and **409** are internalized into the RBCs. Since the rate of sugar transport across the RBC membrane is known to be temperature-dependent, additional studies were conducted in which RBCs were incubated with **104** or **409** in 15% glycerol for various time periods at different temperatures prior to freezing to explore whether the observed increase in the percentage of intact RBCs post-thaw was the result of internalization. In the presence of **104**, the percentage of intact RBCs post-thaw increased as a function of pre-freeze incubation time and temperature whereas in the presence of **409**, the percentage of intact RBCs post-thaw remained constant independent on the pre-freeze incubation time or temperature. This suggests that the rates of transport of **104** and **409** across the RBC membrane may be different; however, further studies are required to support this claim.

To extend our understanding of the internalization of these molecules, the uptake of glucose derivatives (*O*-aryl glucosides) by RBCs was studied in the absence of freezing using CE-MS. Additionally, to determine whether the uptake of *O*-aryl glucosides was mediated by the GLUT1 transporter, these experiments were carried out in the presence of a GLUT1 inhibitor (cytochalasin B). It was demonstrated that the *O*-aryl glucosides have the ability to cross the cell membrane utilizing a mechanism different from the use of the GLUT1 transporter.

Lastly, we investigated the ability of small molecule IRIs **103** (β -PMP-Glc) and **104** (β -pBrPh-Glc) to permeate the cell membrane of HUVECs and control intracellular ice recrystallization using fluorescence cryomicroscopy. HUVECs treated with the IRIs were shown to retain a large number of small ice crystals during warming to high subzero temperatures and demonstrated a significant inhibition of intracellular ice recrystallization. Under slow thawing conditions, **104** (β -pBrPh-Glc) was shown to be most effective at inhibiting intracellular ice recrystallization. This work suggests that these IRIs are capable of permeating the cell membrane of vascular endothelial cells and further supports the RBC work with these compounds suggesting that internalization is occurring. Collectively, the results from the internalization studies described in this chapter provide additional evidence supporting the hypothesis of internalization.

7.6 Experimental Procedures

Small molecule IRIs **103** (β -PMP-Glc) and **104** (β -pBrPh-Glc) used in this chapter for internalization studies were prepared as previously described.⁶⁰ IRI active azido aryl glucosides **405**, **406** and **409** used in this chapter for internalization studies were prepared as described in the experimental section of Chapter 4. The capillary electrophoresis-mass spectrometry experiments were done in collaboration with Dr. Gleb Mironov, a former graduate student under the supervision of Dr. Maxim Berezovski at the University of Ottawa.

7.6.1 Blood Collection and Preparation

All RBCs were obtained from NetCAD (Canadian Blood Services' Network Centre for Applied Development). Whole blood was collected from healthy volunteers using standardized phlebotomy guidelines approved by Canadian Blood Services (CBS). Informed consent was obtained from all donors. All experimental protocols were approved by NetCAD and CBS. Ethics approvals were obtained from Research Ethics Board (REB) at CBS and the University of Alberta. For cryovial experiments, whole blood units were collected and processed by NetCAD (Vancouver, BC). The whole blood was processed using the buffy coat (BC) method to produce leukocyte reduced SAGM RBC units, which has been previously described.¹⁴ ABO matched RCCs were pooled and split to reduce donor to donor variability.

7.6.2 Interrupted Graded Freezing of RBCs

The freezing solution consisted of a 30% glycerol solution prepared from a commercially available glycerol solution (Glycerolyte 57, Baxter) by diluting it with 0.9% saline/0.2% dextrose. An equal volume of freezing solution was added to 150 μ L of RBCs for a final volume of 300 μ L. The final concentrations of all freezing solutions are as indicated in the text. RBC suspensions were transferred to glass vials (6 $\times$ 50 mm culture tubes) and incubated at room temperature for 10 min prior to immersion in a methanol bath precooled to -5°C . A thermocouple was inserted into a RBC/15% glycerol sample to monitor temperature at 1 s intervals. Once the internal temperature of the solution reached -5°C , extracellular ice nucleation was induced in each sample by touching the vials with precooled (in liquid nitrogen) forceps. Samples were held at -5°C for 5 min and then cooled at $1^{\circ}\text{C}/\text{min}$ to defined subzero temperatures before being either immediately thawed in a 37°C water bath or rapidly cooled by plunging into dry ice (-78°C) and then thawed in a 37°C water bath.

7.6.3 Post-Thaw Hemolysis

Post-thaw hematocrits (hct) and percentage hemolysis were determined for all freezing experiments by comparing the supernatant hemoglobin concentration to total hemoglobin concentration using the cyanmethemoglobin Drabkin's method.^{14,15} The percentage of intact RBCs post-thaw was calculated using the measured percentage hemolysis values according to the following equation: % intact RBCs post-thaw = $100 - \% \text{ hemolysis}$.

7.6.4 RBC Incubation Experiments

The freezing solution consisted of a 30% glycerol solution prepared from a commercially available glycerol solution (Glycerolyte 57, Baxter) by diluting it with 0.9% saline/0.2% dextrose. An equal volume of freezing solution was added to 150 μ L of RBCs for a final volume of 300 μ L. The final concentrations of all freezing solutions are as indicated in the text. RBC suspensions were transferred to glass vials (6 $\times$ 50 mm culture tubes) and incubated either at 0°C (in an ice bath slurry in the refrigerator), at room temperature or at 37°C in a water bath for various time periods (5, 10, 30 and/or 60

minutes) prior to immersion in a methanol bath cooled to -5 °C. A thermocouple was inserted into a RBC/15% glycerol sample (temperature probe) to monitor temperature at 1 s intervals. Once the internal solution from the temperature probe reached -5 °C, ice nucleation was induced by touching the cryovials with pre-cooled (in liquid nitrogen) forceps. RBC samples were then held at -5 °C for 5 minutes. Samples were cooled at a rate of 1 °C/min to -40 °C and thawed in a 37 °C water bath.

7.6.5 RBC Wet Preparation Microscopy

The freezing solution consisted of a 30% glycerol solution prepared from a commercially available glycerol solution (Glycerolyte 57, Baxter) by diluting it with 0.9% saline/0.2% dextrose. An equal volume of freezing solution was added to 100 µL of RBCs for a final volume of 200 µL. Using a capillary tube, a small drop of the RBC sample was placed on a glass slide and covered with a glass coverslip. The slide was immediately placed on a microscope and the cells were imaged immediately, at approximately 5 minutes and after 15-20 minutes total elapsed time from the addition of the freezing solution.

7.6.6 RBC Internalization Experiments

RBCs were washed in ten volumes of Dulbecco's phosphate buffered saline (DPBS), centrifuged at 3,500 rpm for 15 min at room temperature, resuspended in DPBS for 20 minutes in a 37 °C water bath, centrifuged and washed three more times at room temperature.²² An equal volume of IRI prepared in PBS was added to 150 µL of washed RBCs for a final volume of 300 µL and samples were incubated at room temperature for 15 minutes prior to adding 1 mL of ice-cold stopping solution consisting of 2 mM HgCl₂, 1.25 mM KI and 1% NaCl aqueous solution. The final concentrations of all solutions are as indicated in the text. RBC samples were centrifuged at 1,000 g for 3 min at 4 °C, the supernatant was removed and RBCs were washed twice with DPBS. An 80 µL aliquot of the RBC pellet was added to 0.5 mL of 1:1 EtOH: H₂O and vortexed to lyse cells. RBC samples were placed on a Thermomixer at 80 °C for 30 minutes and cooled to -20 °C in a refrigerator for 10 minutes followed by centrifugation (17,200 RCF, 10 min, 4 °C) to clarify the lysate. The supernatant was mixed with 15 mM Tris-Acetate buffer for analysis of internalization by capillary electrophoresis-mass spectrometry (CE-MS).

7.6.7 RBC Internalization Experiments in the Presence of GLUT-1 Inhibitor

RBCs were washed in ten volumes of Dulbecco's phosphate buffered saline (DPBS), centrifuged at 3,500 rpm for 15 min at room temperature, resuspended in DPBS for 20 minutes in a 37 °C water bath, centrifuged and washed three more times at room temperature.²² Prior to adding an equal volume of IRI prepared in PBS to 150 µL of washed RBCs, the GLUT1 inhibitor (Cytochalasin B, 200 µM) was added and samples were incubated at room temperature for 5 minutes. RBC samples were then treated as described above.

7.6.8 Capillary Electrophoresis-Mass Spectrometry

All solutions were filtered through 0.22-µm pore size nylon membrane filters (Millipore). The bare-silica capillary was purchased from Polymicro (Phoenix, USA). The sample storage and capillary temperature were maintained at 4 ± 0.5 and 15 ± 0.5 °C, respectively. The electric field in CE separation was 330 V/cm with a positive electrode at the injection end. For all experiments, the capillary was 90 cm long with an inner diameter of 50 µm and an outer diameter of 360 µm. The equilibrium mixture was injected into the capillary from the inlet end by a pressure pulse of $6 \text{ s} \times 5 \text{ psi}$. Before each experiment, the capillary was rinsed by 75 psi pressure with: 0.1 M NaOH for 3 min, ddH₂O for 3 min, 15 mM tris-acetate buffer for 5 min. A Synapt G2 high definition mass spectrometer from Waters (UK) was coupled with a PA800plus Pharmaceutical Analysis CE system (Beckman Coulter, USA) through a CE-ESI sprayer from Micromass (UK) and used in all CE-MS experiments. Liquid cooling tubing was home modified to apply the cooling liquid to the whole capillary. Electrospray ionization conditions were as follows: capillary voltage 3.2 kV, positive mode, sampling cone voltage 80 V, extraction cone voltage 5 V, source temperature 120 °C, cone gas 50 L/h, nano flow gas 0.15 Bar, purge gas 3 L/h, mobility cell bias voltage 3 V. Sheath liquid (50:50 methanol:1% acetic acid) was delivered with flow rate of 1.5 µl/min.

7.6.9 HUVEC Culture for Cryomicroscopy Experiments

Human umbilical vein endothelial cells (HUVECs; C2519A, Lonza) were purchased as pooled primary cells frozen at passage 1 in a cryopreservation medium containing

endothelial growth medium (EGM) with 10% fetal bovine serum (FBS) and 10% DMSO. The cells were cultured at 37 °C and 5% CO₂ in EGM consisting of endothelial basal media (EBM) supplemented with a bullet kit (LONZA, CC-3162) containing human recombinant fibroblast growth factor B, epidermal growth factor, vascular endothelial growth factor, insulin growth factor, hydrocortisone, ascorbic acid, heparin, and FBS and in the absence of antibiotics. Cells were harvested when they reached 80% coverage by treatment with trypsin-EDTA (Lonza, CC-5012) for 2 to 3 min at room temperature, followed by the addition of trypsin neutralizing solution (Lonza, CC-5002). Cell suspensions were centrifuged at 1000 rpm for 5 min in an Eppendorf 5810R tabletop centrifuge, resuspended in EGM, and counted using a Coulter Z₂ particle count and size analyzer (Beckman Coulter). Confluent monolayers were obtained by growing cells on sterile, fibronectin-coated, 12 mm circular coverslips placed in 24-well plates.

7.6.10 Freezing of HUVEC Monolayers and Visualization of Intracellular Ice

The nucleation and growth of intracellular ice was documented using a cryomicroscope that consisted of a Nikon 80i fluorescence microscope with a long working distance condenser and objectives, CCD cameras (ORCA, Hamamatsu) interfaced to a personal computer, and a convection cryomicroscope stage (FDCS196, Linkam Scientific). To study intracellular ice formation without seeding of ice at a predefined subzero temperature, samples were cooled (rate of 90 °C/min) from room temperature (23 °C) to -40 °C. The samples were then warmed slowly (rate of 2 °C/min) or quickly (rate of 25 °C/min) to room temperature, and images were taken every 10 °C. The number and area of individual intracellular ice crystals were assessed, as previously described,⁵⁵ by manually circling all of the ice crystals within a field of view using ImageJ with corrections made for the magnification of the image.

To visualize and characterize intracellular ice formation, samples were stained with 5 µM of SYTO 13 in 0.9% NaCl solution prior to freezing on the cryomicroscope for 10 min at room temperature. In cases where the IRIs were present, the compounds were predissolved in 100 µL of 0.9% NaCl, and SYTO 13 was then added to the solution. SYTO 13 is a DNA/RNA binding stain that permeates the cell nucleus and fluoresces upon excitation by UV light. After incubation at room temperature for 10 min, the

solution was removed, the coverslip was washed, and 0.9% NaCl was added prior to the coverslip being placed in the Linkam stage.

7.6.11 HUVEC Culture for Cytotoxicity Experiments

Human umbilical vein endothelial cells (HUVECs; C2519A, Lonza) were purchased as pooled primary cells frozen at passage 1 in a cryopreservation medium containing endothelial growth medium (EGM) with 10% fetal bovine serum (FBS) and 10% DMSO. The cells were cultured at 37 °C and 5% CO₂ in EGM consisting of endothelial basal media (EBM) supplemented with a bullet kit (LONZA, CC-3162) containing human recombinant fibroblast growth factor B, epidermal growth factor, vascular endothelial growth factor, insulin growth factor, hydrocortisone, ascorbic acid, heparin, and FBS and in the absence of antibiotics. Cells were harvested for use in experiments when they reached 80% coverage by treatment with trypsin-EDTA (Lonza, CC-5012) for 2 to 3 min at room temperature, followed by the addition of trypsin neutralizing solution (Lonza, CC-5002).

7.6.12 Alamar Blue Assay with HUVECs

HUVECs were plated in 96-well plates (~10,000 cells/well) and incubated overnight at 37 °C with 5% CO₂ to allow cells to adhere. Cells were then treated with 100 µL of EGM supplemented with the test compound at various concentrations and incubated at 37 °C with 5% CO₂ for 23 h. Cells incubated with EGM without supplement were used as a negative control and cells supplemented with 1% Triton-X were used as a positive control. Following incubation, 10 µL of resazurin solution (125 µg/mL) in DPBS (Dulbecco's phosphate buffered saline) was added to each well and the plates were incubated at 37 °C with 5% CO₂ for 1 h. The fluorescence of each well was measured using a fluorescence plate reader with an excitation at 530 nm (emission at 590 nm). Each experiment was repeated at least two times in 6 consecutive wells for each condition. Cell viability was calculated as a percentage of the control.

7.7 References

- (1) Fuller, B. J. *CryoLetters* **2004**, 25 (6), 375.
- (2) Mazur, P. Principles of Cryobiology. In: Fuller, B.; Lane, N.; Benson, E., eds. *Life in the Frozen State*. Boca Raton, FL: CRC Press; 2004.

- (3) Elliott, G. D.; Wang, S.; Fuller, B. J. *Cryobiology* **2017**, 76, 74.
- (4) Fahy, G. M. *Cryobiology* **1986**, 23, 1.
- (5) Fahy, G. M.; Lilley, J. T. H.; Linsdell, H.; Douglas, M. S. J.; Meryman, H. T. *Cryobiology* **1990**, 27, 247.
- (6) Best, B. P. *Rejuvenation Res.* **2015**, 18 (5), 422.
- (7) Capicciotti, C. J.; Kurach, J. D. R.; Turner, T. R.; Mancini, R. S.; Acker, J. P.; Ben, R. N. *Sci. Rep.* **2015**, 5, 9692.
- (8) McGann, L. E. *Cryobiology* **1978**, 15 (4), 382.
- (9) Acker, J. P.; McGann, L. E. *Cell Transplant.* **2002**, 11 (6), 563.
- (10) McGann, L. E.; Yang, H. Y.; Walteson, M. *Cryobiology* **1988**, 25 (3), 178.
- (11) Ross-Rodriguez, L. U.; Elliott, J. A.; McGann, L. E. *Cryobiology* **2010**, 60 (2), 106.
- (12) Baldwin, S. A. *Biochim. Biophys. Acta.* **1993**, 1154 (1), 17.
- (13) Poisson, J. S.; Acker, J. P.; Briard, J. G.; Meyer, J. E.; Ben, R. N. *Langmuir* **2018**, Article ASAP.
- (14) Acker, J. P.; Hansen, A. L.; Kurach, J. D. R.; Turner, T. R.; Croteau, I.; Jenkins, C. *Transfusion* **2014**, 54 (10), 2534.
- (15) Drabkin, D. L. *Am. J. Med. Sci.* **1948**, 215, 110.
- (16) Meryman, H. T. *Cryobiology* **1971**, 8 (2), 173.
- (17) Lovelock, J. E. *Biochim. Biophys. Acta.* **1953**, 11, 28.
- (18) Mazur, P.; Leibo, S. P.; Chu, E. H. *Exp. Cell Res.* **1972**, 71 (2), 345.
- (19) Carruthers, A.; DeZutter, J.; Ganguly, A.; Devaskar, S. U. *Am. J. Physiol. Endocrinol. Metab.* **2009**, 297 (4), E836.
- (20) Concha, I. I.; Velasquez, F. V.; Martinez, J. M.; Angulo, C.; Droppelmann, A.; Reyes, A. M.; Slebe, J. C.; Vera, J. C.; Golde, D. W. *Blood* **1997**, 89 (11), 4190.
- (21) Barnett, J. E. G.; Holman, G. D.; Chalkley, R. A.; Munday, K. A. *Biochem. J.* **1975**, 145, 417.
- (22) Garcia-Alvarez, I.; Garrido, L.; Fernandez-Mayoralas, A. *ChemMedChem* **2007**, 2 (4), 496.
- (23) Brahm, J. J. *J. Physiol.* **1983**, 339, 339.
- (24) Carruthers, A. *Physiol. Rev.* **1990**, 70 (4), 1135.
- (25) Sen, A. K.; Widdas, W. F. J. *J. Physiol.* **1962**, 160, 392.
- (26) Lacko, L.; Wittke, B.; Geck, P. J. *Cell. Physiol.* **1973**, 82, 213.
- (27) Hankin, B. L.; Stein, W. D. *Biochim. Biophys. Acta.* **1972**, 288, 127.
- (28) Moore, T. J.; Schlowsky, B. J. *Lipid Res.* **1969**, 10, 216.
- (29) Moore, T. J. *J. Lipid Res.* **1968**, 9, 642.
- (30) Mazur, P.; Miller, R. H. *Cryobiology* **1976**, 13 (5), 507.
- (31) Bakaltcheva, I. B.; Odeyale, C. O.; Spargo, B. J. *Biochim. Biophys. Acta.* **1996**, 1280, 73.
- (32) Johnsson, R.; Vuopio, P. *Acta. Haematol.* **1978**, 60 (6), 329.
- (33) Gorga, F. R.; Lienhard, G. E. *Biochemistry* **1982**, 21 (8), 1905.
- (34) van Wijk, R.; van Solinge, W. W. *Blood* **2005**, 106 (13), 4034.
- (35) Bloch, R. J. *Biol. Chem.* **1974**, 249 (11), 3543.
- (36) Cai, J.; Henion, J. J. *Chromatogr. A* **1995**, 703, 667.
- (37) Mantovani, V.; Galeotti, F.; Maccari, F.; Volpi, N. *Electrophoresis* **2018**, 39, 179.
- (38) Linhardt, R. J.; Pervin, A. J. *Chromatogr. A.* **1996**, 720 (1–2), 323.

- (39) Basketter, D. A.; Widdas, W. F. *J. Physiol.* **1978**, 278, 389.
- (40) Devés, R.; Krupka, R. M. *Biochim. Biophys. Acta.* **1978**, 510 (2), 339.
- (41) Zoccoli, M. A.; Baldwin, S. A.; Lienhard, G. E. *J. Biol. Chem.* **1978**, 253 (19), 6923.
- (42) Baker, G. F.; Widdas, W. F. *J. Physiol.* **1988**, 395, 57.
- (43) Sage, J. M.; Carruthers, A. *Am. J. Physiol. Cell Physiol.* **2014**, 306 (10), C910.
- (44) Fernández, C.; Nieto, O.; Fontenla, J. A.; Rivas, E.; de Ceballos, M. L.; Fernández-Mayoralas, A. *Org. Biomol. Chem.* **2003**, 1, 767.
- (45) Yang, G.; Zhang, A.; Xu, L. X. *Cryobiology* **2009**, 58, 96.
- (46) Prickett, R. C.; Marquez-Curtis, L. A.; Elliott, J. A. W.; McGann, L. E. *Cryobiology* **2015**, 70, 156.
- (47) Gritsan, N. P.; Platz, M. S. *Chem. Rev.* **2006**, 106 (9), 3844.
- (48) Reddington, S.; Watson, P.; Rizkallah, P.; Tippmann, E.; Jones, D. D. *Biochem. Soc. Trans.* **2013**, 41, 1177.
- (49) Sumranjit, J.; Chung, S. J. *Molecules* **2013**, 18, 10425.
- (50) Acker, J. P.; Croteau, I. M. *J. Microsc.* **2004**, 215 (2), 131.
- (51) Yang, G.; Zhang, A.; Xu, L. X. *Cryobiology* **2011**, 63 (1), 38.
- (52) Wu, L. K.; Tokarew, J. M.; Chaytor, J. L.; von Moos, E.; Li, Y.; Pali, C.; Ben, R. N.; Allan, D. S. *Carbohydr. Res.* **2011**, 346 (1), 86.
- (53) Gage, A. A.; Guest, K.; Montes, M.; Caruana, J. A.; Whalen, D. A. *Cryobiology* **1985**, 22 (2), 175.
- (54) Gage, A. A.; Baust, J. M.; Baust, J. G. *Cryobiology* **2009**, 59 (3), 229.
- (55) Abraham, S.; Keillor, K.; Capicciotti, C. J.; Perley-Robertson, G. E.; Keillor, J. W.; Ben, R. N. *Cryst. Growth Des.* **2015**, 15 (10), 5034.
- (56) Rampersad, S. N. *Sensors* **2012**, 12, 12347.
- (57) Ati, J.; Lafite, P.; Daniellou, R. *Beilstein J. Org. Chem.* **2017**, 13, 1857.
- (58) Ball, A. L.; Chambers, K. A.; Hewinson, M.; Navaratnarajah, S.; Samrin, L.; Thomas, N.; Tyler, A. E. H.; Wall, A. J.; Lloyd, M. D. *J. Enzyme Inhib. Med. Chem.* **2008**, 23 (1), 131.
- (59) Lee, B. H.; You, H. J.; Park, M. S.; Kwon, B.; Ji, G. E. *J. Microbiol. Biotechnol.* **2006**, 16 (4), 497.
- (60) Capicciotti, C. J.; Mancini, R. S.; Turner, T. R.; Koyama, T.; Alteen, M. G.; Doshi, M.; Inada, T.; Acker, J. P.; Ben, R. N. *ACS Omega* **2016**, 1 (4), 656.

Conclusions

The overall goal of this thesis was to design and synthesize novel small molecules that are effective inhibitors of ice recrystallization. Given that many cryopreservation protocols are suboptimal, small molecule IRIs are attractive for their use as novel cryoprotectants to improve upon current protocols. The objectives of this thesis were: (1) to elucidate additional structural features that are important for the IRI activity of small molecules; (2) to determine the influence of an azido moiety on the IRI activity of aryl glucosides; (3) to explore the cryopreservation potential of small molecule IRIs in RBCs and MSCs; and (4) to investigate whether small molecule IRIs are crossing the cell membrane and functioning as permeating cryoprotectants.

The first part of this thesis examined the IRI activity of three diverse classes of small molecules. Carbohydrate-based surfactants were previously found to be effective inhibitors of ice recrystallization; however, the precise mechanism by which these molecules inhibit ice recrystallization remains unknown. As the isomerization of azobenzene can provide spatiotemporal control over molecular structure and properties, this strategy could potentially be employed to photo-modulate IRI activity and may be used as a tool to gain mechanistic insight. Thus, the first class of small molecules investigated were carbohydrate-based (fluoro)surfactants bearing an azobenzene moiety. While the majority of the carbohydrate-based (fluoro)surfactants were weak to moderate inhibitors of ice recrystallization, the results reaffirmed the importance of the identity of the carbohydrate moiety and regiochemistry for the IRI activity of these molecules. The *trans-cis* photoisomerization studies also highlighted the importance of the isomeric state of the azobenzene tail group for IRI activity as some carbohydrate-based (fluoro)surfactants exhibited reduced activity in their *cis* states. The second class of small molecules investigated were fluorinated aryl glycosides, as aryl glycosides have been previously shown to be effective inhibitors of ice recrystallization and a fluorine atom on the carbohydrate moiety has not yet been investigated for its influence on IRI activity. Generally speaking, the results indicated that fluorinated aryl glycosides were not effective inhibitors of ice recrystallization and replacing the C-6 hydroxyl group with a fluorine atom decreased both IRI activity and solubility. Lastly, a small diverse class of monosaccharides bearing a phosphate group was investigated as IRIs. This study

represented a novel opportunity to develop pyranoses with unique functional groups as IRIs and served to generate preliminary data. This work demonstrated that the majority of the phosphate sugars examined were effective inhibitors of ice recrystallization, highlighting the potential importance of phosphate functional groups for IRI activity.

In addition, aryl glycosides such as β -PMP-Glc and β -pBrPh-Glc were previously found to effectively inhibit ice recrystallization. Furthermore, these small molecule IRIs were shown to be effective cryo-additives for the freezing of human RBCs with the ability to increase post-thaw RBC integrity utilizing reduced concentrations of glycerol (15% w/v) and slow cooling rates. This was significant as decreased glycerol concentrations can result in faster deglycerolization methods. Therefore, structure-function studies were carried out on β -PMP-Glc and β -pBrPh-Glc to elucidate key structural features that increase their IRI activity and may increase their cryoprotective ability. In particular, this work explored the influence of an azido moiety on the IRI activity of β -PMP-Glc and β -pBrPh-Glc. The most effective inhibitors of ice recrystallization were the 3-azido analogues of β -PMP-Glc and β -pBrPh-Glc and the *para*-azido glucoside. In fact, the 3-azido analogues of β -PMP-Glc and β -pBrPh-Glc were found to be significantly more IRI active than their parent compounds.

This thesis also explored the cryopreservation potential of small molecule IRIs in RBCs and MSCs. Previous work demonstrated that β -PMP-Glc and β -pBrPh-Glc can protect RBCs from cellular injury during freezing using 15% glycerol, thus, RBCs cryopreserved with these novel cryosolutions were subjected to deglycerolization. However, it was found that a significant amount of cellular damage occurred, resulting in low cell recoveries. Although efforts to optimize the deglycerolization conditions for these novel RBC cryosolutions remained unsuccessful, these studies were crucial because they provided insight into the possible cause of failure. The low RBC recoveries observed in the presence of β -PMP-Glc or β -pBrPh-Glc were hypothesized to be the result of membrane interactions during freezing, causing the RBCs to be more susceptible to osmotic stress during deglycerolization. Thus, this led to the hypothesis that IRI active azido aryl glucosides may improve the deglycerolization process since these molecules may be less likely to interact with the RBC membrane during freezing, facilitating their removal during deglycerolization. The most promising results were obtained with the

para-azido glucoside, which was discovered to be a beneficial additive for the freezing of RBCs. The addition of this IRI to a solution of 15% glycerol significantly improved cell recovery. This work demonstrated that the structure of an IRI could be rationally modified for a specific *in vitro* application.

The ability of small molecule IRIs to improve the cryopreservation of MSCs was investigated and it was found that the addition of an *N*-aryl-aldonamide (2FA) to the standard 10% DMSO solution improved the post-thaw function of MSCs. Specifically, results from this study demonstrated that significantly higher proliferation rates were observed post-thaw in MSCs cryopreserved with 2FA. This is significant because freshly thawed MSCs were found to display compromised function and this may result in diminished efficacy.

Lastly, this thesis also investigated the ability of small molecule IRIs to cross the cell membrane and function as permeating cryoprotectants utilizing two different cell models, RBCs and HUVECs. These studies suggested that aryl glycoside IRIs are capable of permeating the cell membrane of RBCs and vascular endothelial cells to mitigate the response of these cells to freezing and thawing. This work also demonstrated that β -PMP-Glc and β -pBrPh-Glc can control the rate and extent of intracellular ice recrystallization in the nuclei of HUVECs over a broad range of subzero temperatures. These findings are highly significant because controlling intracellular ice growth using small molecule IRIs can be an important approach to improve the recovery and function of cryopreserved cells and tissues.

Overall, the work presented in this thesis emphasizes that small molecule IRIs have tremendous potential as novel cell-permeating cryoprotectants for the preservation of cells and tissues that can improve upon current protocols.

Future Directions

The aim of this thesis was to design novel small molecules that are effective inhibitors of ice recrystallization for their use as cryoprotective agents. Throughout this thesis, several different classes of small molecule IRIs were identified that can help direct future research in the field.

A consistent theme throughout this thesis was the emphasis on identifying highly effective inhibitors of ice recrystallization for cryopreservation applications. In Chapter 3, carbohydrate-based surfactants bearing an azobenzene moiety proved to be weak to moderate inhibitors of ice recrystallization and their use in cellular applications was limited due to their surfactant-like nature. Within the class of fluorinated aryl glycosides, difluorinated derivative **327** was one of the most effective IRIs reported in this thesis. However, cryopreservation applications are limited due to its poor aqueous solubility. It was apparent that monosaccharides bearing a phosphate group could be effective inhibitors of ice recrystallization and this preliminary work highlighted the potential importance of phosphate functional groups for IRI activity. The relationship between the phosphate group and IRI activity needs to be further explored through the IRI testing of novel synthesized phosphate sugars.

The results from Chapter 4 demonstrated that certain azido derivatives of β -PMP-Glc and β -pBrPh-Glc could be effective inhibitors of ice recrystallization. In particular, the 3-azido aryl glucosides **405** and **406** have proven to be the most effective small molecule IRIs. However, the use of these IRIs as novel cryoprotectants is limited due to their complex and lengthy synthesis that is not amenable to large-scale preparation and their poor aqueous solubility. Contrarily, amino aryl glycosides represent a promising new class of IRIs owing to their ability to effectively inhibit ice recrystallization and their improved aqueous solubility. The amino aryl glycosides prepared in this thesis should be evaluated for cytotoxicity and cryopreservation ability in order to determine if they can be effective cryoprotectants.

In Chapter 5, human RBCs cryopreserved with azido aryl glycoside **409** + 15% glycerol were shown to result in increased cell recovery compared to 15% glycerol alone utilizing slow cooling conditions. These results demonstrate that IRI **409** is a beneficial additive for the freezing of RBCs in the presence of reduced concentrations of glycerol.

While deglycerolization conditions were explored, further optimization is still required to increase cell recovery. The utilization of automated deglycerolization methods could yield greater RBC recovery with increased removal of free hemoglobin, and would reduce operator time and increase the overall efficiency of the deglycerolization process. Future studies should also focus on optimizing the glycerolization stage and freezing stage of the cryopreservation protocol.

Another major theme throughout this thesis was investigating the internalization of small molecule IRIs to determine whether these molecules are crossing the cell membrane and behaving as permeating cryoprotectants. In Chapter 7, the internalization of small molecule IRIs into RBCs and HUVECs provided insight into how these molecules are behaving as cryoprotectants. These studies suggested that small molecule IRIs are capable of permeating the cell membrane and controlling intracellular ice recrystallization. While this work suggests that these molecules are being internalized and behaving as permeating cryoprotectants, substantial work lies ahead to provide conclusive evidence for internalization. Furthermore, it remains unclear as to how these molecules are crossing the cell membrane. Additional studies are needed to further probe the mechanism of internalization of small molecule IRIs. The azido aryl glycosides presented in this thesis can be used as tools for studying internalization in greater detail considering that the azide can be tagged with probes using click chemistry. Therefore, future work should include utilizing the azido aryl glycosides to further study internalization and cellular localization of these molecules in order to gain mechanistic insight.

Contributions to Original Research

1. Analysis of the ice recrystallization inhibition (IRI) activity of carbohydrate-based (fluoro)surfactants **301-322** and monosaccharide phosphate sugars **332-338**.
2. Synthesis and analysis of the IRI activity of fluorinated aryl glycosides **323-328**.
3. Synthesis and analysis of the IRI activity of azido aryl glucosides **401-409**. Cytotoxicity assessment of **405**, **406** and **409** in a human liver cancer cell line (HepG2 cells). Synthesis and analysis of the IRI activity of amino aryl glucosides **441-445**.
4. Analysis of the cryopreservation potential of small molecule ice recrystallization inhibitors in human RBCs and bone marrow-derived MSCs.
5. Investigating the internalization of small molecule ice recrystallization inhibitors into human RBCs and HUVECs.

Publications Resulting from this Thesis

1. **Poisson, J. S.**; Acker, J. P.; Briard, J. G.; Meyer, J. E. and Ben, R. N. Modulating intracellular ice growth with cell permeating small molecule ice recrystallization inhibitors. *Langmuir* **2019**, 35 (23), 7452–7458. Article was selected to be featured in ACS Editors' Choice.
2. **Poisson, J. S.**; Briard, J. G.; Turner, T. R.; Acker, J. P. and Ben, R. N. Hydroxyethyl starch supplemented with ice recrystallization inhibitors greatly improves cryopreservation of human red blood cells. *BioProcessing Journal* **2017**, 15 (4), 16–21.
3. Adam, M.; Hu, Y.; **Poisson, J. S.**; Pottage, M.; Ben, R. N. and Wilkinson, B. Photoswitchable carbohydrate-based fluorosurfactants as tuneable ice recrystallization inhibitors. *Carbohydrate Research* **2017**, 439, 1-8.
4. Adam, M. K.; **Poisson, J. S.**; Hu, Y.; Prasannakumar, G.; Tabor, R. F.; Ben, R. N. and Wilkinson, B. L. Carbohydrate-based surfactants as photocontrollable inhibitors of ice recrystallization. *RSC Advances* **2016**, 6, 39240-39244.
5. Briard, J. G.; **Poisson, J. S.**; Turner, T. R.; Capicciotti, C. J.; Acker, J. P. and Ben, R. N. Small molecule ice recrystallization inhibitors mitigate red blood cell lysis during freezing, transient warming and thawing. *Scientific Reports* **2016**, 6, 23619.