Elucidating the Key Structural Features of Carbohydrates and Surfactants Necessary for Inhibiting Ice Recrystallization

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B. Sc. Honours Biochemistry – University of Ottawa, 2009

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
for the Doctorate in Philosophy degree in Chemistry

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Dedicated to my soul mate, Radek, and my mother, father, and brother

“It was a harder day's journey than yesterday's, for there were long and weary hills to climb; and in journeys, as in life, it is a great deal easier to go down hill than up. However, they kept on, with unabated perseverance, and the hill has not yet lifted its face to heaven that perseverance will not gain the summit of at last.”

-Charles Dickens, Nicholas Nickleby
Acknowledgements

I would firstly like to thank Dr. Robert Ben for giving me the opportunity to work in his laboratory five years ago. I was not only encouraged to be a better researcher, critical thinker, and problem solver, but as a better person as a whole. I wholeheartedly want to thank him for his mentorship and guidance and for being an amazing supervisor.

I sincerely want to thank Dr. Mathieu Leclère for teaching me all I know about laboratory techniques. I cannot begin to express how much I appreciate all his help and friendly conversations about food and everything else. Dr. John Trant gave me a tremendous helping hand when I first started in the laboratory and I would like to thank him for the ability to approach him so easily and his enthusiasm to share tips. Chantelle Capicciotti has grown to be a wonderful friend in the laboratory and I especially thank her for her suggestions in preparing seminars, presentations, and conference talks. Malay Doshi has been an invaluable friend and colleague and I most heartily want to thank him for his support and daily discussions about chemistry and life. I especially want to thank Malay for turning on the IRI machine in the mornings for me. I would like to thank Jennie Briard for our discussions about health and fitness and for volunteering to read my thesis. To Thomas Charlton for making me laugh and for printing and delivering my thesis. To Stephanie Abraham for having the same taste in music, which was very fortunate since we shared the bay. To Kyle McClymont and Ibrahim Aboud for making the lab a great place to work. All of you have made me look forward to coming into the lab in the morning. I was very fortunate to work with such amazing colleagues.

To past Ben lab members Matt Alteen, Devin Tonelli, Taz Chema, Roger Tam and Jackie Tokarew. I would especially like to thank the excellent undergraduate students
that I had the pleasure to work with: Amanda Commons, Jessica Poisson, Kyle McClymont, and Michela Febrarro. Thank you for your contributions. To past undergraduate students, Kathryn Davis for becoming my dearest friend, Emily Gardiner, Vik Raina, Ross Mancini, Evan Perley-Robertson, and Adam Ray. To my friends Igor and Jasmine for always being there for me.

Thank you Dr. André Beauchemin, Dr. Jeff Keillor, Dr. Maria DeRosa, and Dr. James Gleason for reading my thesis and providing invaluable suggestions. Their contributions have made this work more complete.

I would like to thank my family, especially my mother for her unconditional support and love, for encouraging me to peruse my dreams, and for knowing how to transform a stressful situation into a manageable one. To my ‘baby’ brother, Michael, for always being there to laugh with.

Finally, I would like to thank Radek. He is the only one who can make me smile and laugh at any time. This work means so much more to me because of his love and support.
Abstract

Ice recrystallization during thawing after cryopreservation results in extensive cellular damage that ultimately leads to cell death and decreased cell viabilities. This is a significant problem particularly with cryopreserved cells utilized in various regenerative medicine therapies. Given the success of these therapies to treat spinal cord injury, cartilage lesions, and cardiac disease, the development of new and improved cryoprotectants that minimize cell damage during freeze-thawing and improve cell viability post-cryopreservation are urgently required. The current cryopreservative dimethyl sulfoxide, DMSO, is associated with cytotoxicity in clinical settings and is not an optimal cryopreservative.

Our laboratory is interested in synthesizing small molecules that possess the property of ice recrystallization inhibition (IRI) activity that can be utilized as cryopreservatives without the cytotoxic effects associated with DMSO. This thesis focuses on the development of small molecule ice recrystallization inhibitors and elucidating the structural features of disaccharides and surfactants that are responsible for potent IRI activity.

The first part of this study examines simple disaccharide derivatives mimicking those found in the native AFGP to determine whether disaccharide structure influences IRI activity. Towards this end, the (1,6)-linked AFGP disaccharide analogue was synthesized, assessed for IRI activity using a splat-cooling assay, and compared to the native (1,3)- and (1,4)-linked AFGP disaccharide analogues. The change in linkage was found to have a profound affect on IRI activity.

The second part of the study focuses on surfactants and gelators as ice recrystallization inhibitors. Our laboratory has demonstrated that carbohydrate-based hydrogelators can be potent inhibitors of ice recrystallization. While our studies have indicated that a delicate balance between hydrophobic and hydrophilic interactions is crucial for ice recrystallization inhibition (IRI) activity, the essential structural features necessary for potent IRI activity remain unknown. To address this issue, structurally diverse amino acid-based surfactants/gelators, anti-ice nucleating agents, and glycoconjugates were synthesized and assessed for IRI activity. The results indicate that long alkyl chains and increased hydrophobicity are important for potent IRI activity and
that the position of these alkyl chains is essential. Also, the counterion of these compounds affects the IRI activity and is related to the counterion degree of hydration. These compounds were assessed for their ability to cryopreserve human liver cells (Hep G2) and human bone marrow cells (Tf-1α) in cell-based assays. Additionally, the best IRI assay solution was determined, which involved studying how the salts of the phosphate buffered saline (PBS) solution modulated IRI activity.

Finally, small molecule ice recrystallization inhibitors were assessed for their ability to protect the viral vectors vaccinia virus, vesicular stomatitis virus, and herpes simplex-1 virus at various storage conditions. This will aid in developing improved preservation protocols for vaccines and viruses utilized in cancer therapy (oncolytic viruses).
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**List of Abbreviations**

<p>| α    | Alpha  |
| β    | Beta   |
| γ    | Gamma  |
| δ    | Delta  |
| ε    | Epsilon|
| $^1$H | Proton |
| $^{13}$C | Carbon |
| 7-AAD | 7-Aminoacetinomycin D |
| Ac   | Acetyl |
| Ac$_2$O | Acetic anhydride |
| AFGP(s) | Antifreeze glycoprotein(s) |
| AFP (s) | Antifreeze protein(s) |
| Anti-INA | Anti-ice nucleation agent |
| Ala  | Alanine |
| Atm  | Atmospheres |
| BF$_3$·OEt$_2$ | Boron trifluoride diethyl etherate |
| Bn   | Benzyl |
| br   | Broad  |
| C-AFGP | C-linked antifreeze glycoprotein |
| C-linked | Carbon-linked |
| CAN  | Ceric ammonium nitrate |
| CBZ  | Carboxybenzyl |
| CDI  | 1,1’-Carbonyldiimidazole |</p>
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TMSOTf</td>
<td>Trimethylsilyl trifluoromethane sulfonate</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
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Chapter 1.  Chapter 1: Introduction to cryopreservation

1.1 Applications of cryopreservation

The use of low temperatures to store cells and tissues is routinely used because cooling to sub-zero temperatures effectively stops any biochemical or enzymatic reactions allowing the biological material to be preserved for prolonged periods of time. Cryopreservation extends into many cell types and tissues. The earliest successful cryopreservation of cells was documented with mammalian spermatozoa by Polge.\textsuperscript{1,2} Subsequent to this, pregnancy resulted from the use of cryopreserved human oocytes.\textsuperscript{3} Then the successful cryopreservation of red blood cells was demonstrated.\textsuperscript{4,5} More recently, the cryopreservation of hematopoietic stem cells\textsuperscript{6} has become important as these cells can be used in regenerative therapies.\textsuperscript{7} The “process of replacing or regenerating human cells, tissues or organs to restore or establish normal function”\textsuperscript{8} can be used to treat spinal cord injury,\textsuperscript{9} coronary artery disease,\textsuperscript{9} heart attacks,\textsuperscript{10} stroke,\textsuperscript{11} other cancers, genetic diseases, immune deficiencies, and blood disorders.\textsuperscript{12} However, the success of these regenerative therapies has been directly linked to the quantity, quality, and functionality of the cells post-thaw.\textsuperscript{13} As a result, new and improved cryopreservation procedures are urgently required.

Cells and tissues are not the only biological materials that require prolonged storage. Over the past ten years, oncolytic viruses are becoming more important for the treatment of cancer and are moving past the stage of laboratory experiments into clinical trials.\textsuperscript{14} Oncolytic viruses are being engineered to infect tumor cells and common oncolytic viral vectors include herpes simplex virus 1, vaccinia virus, and vesicular stomatitis virus.\textsuperscript{14}
Currently, the most common storage methods of viruses are cryopreservation in liquid nitrogen, lyophilization (freeze-drying), and storage at low temperatures (-70°C) in mechanical freezers.\textsuperscript{15,16} Viruses must overcome the same difficulties that cells are confronted with: ice formation, dehydration, and rehydration.\textsuperscript{15} As with cells, current preservation protocols for viruses are not always optimal.\textsuperscript{15} Since viral vectors will soon be used in clinical settings, it is now more important to develop protocols for improved cryopreservation of these viruses. Additionally, global vaccination programs have to overcome problems of thermo-instability of vaccines.\textsuperscript{17} The Global Alliance for Vaccines and Immunizations estimates that approximately 3 million people die each year from vaccine preventable diseases.\textsuperscript{18} While cryopreservation is the preferred method of storage, there are still problems such as agglomeration at low temperatures that need to be addressed.

1.2 Properties of ice and water

To better understand cryopreservation, the fundamentals of water and ice must first be explained.

There are at least 15 forms of ice but ‘ordinary’ ice, which is found at ambient temperatures and pressures, has a hexagonal shape and is defined as I\textsubscript{h} (ice hexagonal).\textsuperscript{19} The other forms of ice arise as a result of high pressures.\textsuperscript{20} However, it was found that cubic ice (I\textsubscript{c}) can form when pure water is frozen at -38°C at normal pressures which may be relevant to cryopreservation.\textsuperscript{21,22} This ice also forms in small amounts in the tropopause region of earth’s atmosphere.\textsuperscript{22}

The structure of I\textsubscript{h} is depicted in Figure 1-1 A. The basic structure is a chair-form when viewed from the z-axis consisting of six water molecules (Figure 1-1 B). When
viewed from the vertical axis, the structure is a boat-form consisting of six water molecules (Figure 1-1 C). In this structure, each water molecule forms hydrogen bonds with four other surrounding water molecules. These structures form hexagonal plates (Figure 1-2) where the top and bottom faces are the basal planes, consisting of the chair-form configuration. The six equivalent side faces are called the prism faces and consist of the boat-form configuration. Due to the differences in hydrogen-bonding on the basal and prism faces, water molecules will add to these faces at different rates depending on conditions. For example, under specific temperatures and water vapor supersaturations,
ice will grow along the prism faces to eventually form the flat, six-pointed shape of a snowflake (Figure 1-3). Under different conditions, ice will grow along the basal face to form long, solid, six-sided columns.

Although ice may look like a solid material, there is in fact liquid water between ice crystals and between ice and air that is in constant equilibrium with the ice crystals. This liquid layer is referred to as the quasi-liquid layer (QLL). Figure 1-4 depicts the

![Diagram showing snow crystal morphology and quasi-liquid layer](image)

**Figure 1-3** The snow crystal morphology diagram showing different types of snow crystals that grow at specified temperatures and water vapor supersaturation. Reproduced from Libbrecht, 2005.

**Figure 1-4** Schematic diagram of the quasi-liquid layer (QLL). The QLL is in between bulk water and ice.
QLL between ice and aqueous water showing the semi-organization of the QLL compared to the random configuration of aqueous water. Its thickness increases as the temperature approaches the melting point. For example, the thickness of the QLL at -0.03°C was measured to be 15 nm, or 40 monolayers of liquid. However, at -10°C less than a monolayer remains. The exact nature of the QLL is still not understood indicating that the equilibrium between ice and water leading to ice crystal growth is complicated.

1.3 Mechanism of cryoinjury

The main cause of damage to cells during cryopreservation is not the low temperatures but rather the transition of water to ice during freezing and the transition from ice to water during thawing. The two different ways cells are damaged during freezing are by osmotic stress or mechanical stress and these physical events are related to cooling and thawing rates.

1.3.1 Slow versus fast cooling

The survival of many cell types in cryopreservation is increased if a slow freezing rate is employed. These observations can be explained with the following. When cells are cooled at a slow rate, ice crystals form outside the cells. As ice crystals grow, they exclude all solutes as the
solutes do not fit into the ice lattice network. The solutes are concentrated in the small spaces between the ice crystals. This results in a large extracellular salt concentration and super-cooled water exits the cell, by osmosis, to balance the highly concentrated salt environment.\textsuperscript{29} As a result, the cells dehydrate. The damaging effects of dehydration are specifically associated with the rise of electrolyte concentration, increasing the ionic strength in the cell and changing of the pH, especially if buffering salts crystalize out. As a result, the conformation of lipid-protein complexes, especially phospholipids found in cell membrane, are altered.\textsuperscript{31} Due to these “solution effects”,\textsuperscript{32} upon thawing, the cells would immediately lyse.\textsuperscript{31} Alternatively, if cells are cooled at a high rate, water does not exit the cells fast enough and the super-cooled water in the cells starts to crystalize and form intracellular ice crystals.\textsuperscript{30} The intracellular ice crystals cause extensive damage to the cell membrane and internal organelles by mechanical stress leading to cell death.\textsuperscript{33} The damage to the cell membrane caused by intracellular ice formation was seen in electron micrographs of frozen liver cells and erythrocytes.\textsuperscript{34,35} The implications for fast cooling and slow cooling are summarized in Figure 1-5 and the general rule exists that a cooling rate that is too high or too low is not beneficial for cell survival.\textsuperscript{32}

Mazur et. al. suggested the two-factor hypothesis of freezing injury:\textsuperscript{32} (1) At high cooling rates, cells are injured by the formation of intracellular ice formation and subsequently injured by ice recrystallization during thawing; and (2) at low cooling rates, cells are injured by the long exposure to the major alteration in intracellular solution caused by dehydration. The optimal cooling rate would inhibit intracellular ice formation and prevent severe cell dehydration.\textsuperscript{36} This has been experimentally confirmed and results are presented in characteristic upside-down $U$-shaped curves shown in Figure 1-
In this scheme, cell survival is plotted as a function of cooling rate and for the three cell types, mouse ova, human lymphocytes, and human red blood cells, there is an optimal cooling rate. Cell viability drops drastically if the cooling rates are too high or too low. At low cooling rates (left side of $U$-curve), cells suffer from dehydration and subsequent ice recrystallization while at high cooling rates (right side of $U$-curve), cells are damaged from intracellular ice formation.

1.3.2 Slow versus fast thawing

The warming rate also has a profound effect on cell survival. Generally, faster thawing rates produce higher cell viabilities compared to slow thawing rates although this is still dependent on the cell type. Even under slow cooling conditions, intracellular ice crystals will be present in the cell in small amounts. If the rate of thawing is slow, the intracellular ice crystals begin to recrystallize into larger ice crystals during the increase in temperature. This phenomenon is known as ice recrystallization and is explained in the next section. The presence of large ice crystals within the cell is very detrimental as these ice crystals can damage delicate cellular structures. However, when cells are thawed at a faster rate, there is insufficient time for the recrystallization of ice and the
small ice crystals simply melt.\textsuperscript{38} The profound effect of ice recrystallization during thawing on cell survival has been demonstrated in yeast, higher plant cells,\textsuperscript{39} ascites tumor cells,\textsuperscript{40} and hamster tissue culture cells.\textsuperscript{41} This correlation is clearly presented in a study by MacKenzie depicted in Figure 1-7.\textsuperscript{42} Yeast cells cooled to -196°C were slowly thawed to various subzero temperatures and then thawed quickly to room temperature. Survival began to drop when the slow warming progressed above -40°C. This is consistent with another study showing that intracellular ice recrystallization can be visualized at temperatures above -40°C.\textsuperscript{43} Although the study by MacKenzie may indicate that fast thawing can increase the survival rate for yeast cells, the optimal thawing rate for other cells may be different.

1.4 Ice recrystallization

Ice recrystallization can occur when a sample is frozen, but ice recrystallization rates are fastest during thawing. There are three types of ice recrystallization: migratory, isomass, and accretion.\textsuperscript{44} Isomass recrystallization refers to the smoothing of surfaces of
rough ice crystals and accretion is the process of two crystals growing together to form one larger crystal.\textsuperscript{44} Migratory ice recrystallization is defined as the growth of large ice crystals at the expense of smaller ice crystals and this is the type that is implicated in decreased cell survival during cryopreservation.\textsuperscript{32} The process of migratory ice recrystallization is mediated through Ostwald ripening.\textsuperscript{44,45} In an ice crystal water molecules at the boundary or interface with water are higher in free energy than water molecules inside the ice crystal since they are not able to form the optimal amount of hydrogen-bonds. Therefore, smaller ice crystals that have a high surface area to volume ratio are thermodynamically less stable than large ice crystals, having a low surface area to volume ratio. As the system attempts to lower its free energy, water molecules from smaller ice crystals migrate to larger ice crystals resulting in a lower surface area to volume ratio.\textsuperscript{44} In Ostwald ripening, the total ice volume of this process remains constant while the average size of the ice crystals increases.\textsuperscript{46} The process of Ostwald ripening can be also simply depicted in the real-life scenario where small oil droplets in water join to form larger oil droplets (Figure 1-8A). As with ice crystals, the system containing many small oil droplets is higher in energy because the surface area to volume ratio is high. As the system attempts to lower its free energy, the oil droplets merge to decrease the surface area to volume ratio.

As the recrystallization of ice is a detrimental factor in cell survival,\textsuperscript{37} there is great interest in identifying compounds which can inhibit ice recrystallization. Figure 1-8 depicts the activity of a lysine-based ice recrystallization inhibitor. Image B shows the large ice crystals of a phosphate buffered saline (PBS) solution compared to very small
ice crystals of the ice recrystallization inhibitor in PBS (Image C). Compounds, which possess ice recrystallization inhibition (IRI) activity, can increase cell viability post-thaw.

1.5 Protection of cells by cryoprotectants

The ability to recover cells that are viable from a temperature of almost -200°C can only be accomplished if the cells were cooled in the presence of a cryoprotective agent (CPA). A CPA is described as ‘any additive which can be provided to cells before freezing and yields a higher post-thaw survival than can be obtained in its absence’.47

The first recorded example of deliberately adding a CPA to protect against freezing damage was by Polge and colleagues in 1949 when glycerol was added to spermatozoa before cryopreservation.2 This small molecule has high solubility in water.
and a relatively low toxicity during short-term exposure to living cells at concentrations from 1 M to 5 M. Glycerol was found to protect many cell types such as human erythrocytes, human bone marrow, human renal cells, and many more. After this, many other CPAs have been discovered and used including methanol, propylene glycol, dimethylacetamide, and dimethyl sulfoxide (DMSO). DMSO, discovered ten years after the first glycerol CPA publication by Polge, was shown to be the most versatile of all CPAs by Lovelock and Bishop as it was also effective at preserving cells that glycerol could not. These CPAs are characterized as permeating CPAs as they are able to traverse the cell membrane and decrease the electrolyte concentration within the cell and reduce the extent of osmotic shrinkage. During freezing without CPAs, cells become dehydrated as water exits the cells to equilibrate the high extracellular salt concentration. The dehydrated cell becomes damaged due to the subsequent intracellular ionic changes. Lovelock proposed that the CPA enters the cell and replaces the water that has exited during freezing resulting in an intracellular salt concentration that would not reach a biologically damaging level. Eventually, when the temperature is sufficiently low (below -80°C), the water left in the cell will cool to a glassy matrix, also know as vitrification. Vitrification excludes intracellular ice crystals that will cause damage during thawing. Additionally, the increasing viscosity of CPAs in the cells may inhibit or slow down ice growth. Therefore, the protection afforded by CPAs is not due to their chemical properties but their colligative properties resulting in the reduction of intracellular ice crystals. An opposing view exists that credits the protection from CPAs to interactions with the phospholipid bilayers resulting in stabilization of the plasma membrane. In one experiment, it was shown that dipole-ion interactions between the
oxygen of DMSO and the phospholipid bilayer increases membrane stability during freeze-thaw.\textsuperscript{51}

The second category of CPAs is non-permeating CPAs, which cannot penetrate the cell membrane. These include polymers such as polyvinyl pyrrolidone, hydroxyethyl starch, polyethylene glycols, dextrans, and other sugars that are not cell permeating such as sucrose. These CPAs are sometimes used alongside permeating CPAs resulting in the use of a lower concentration of permeating CPAs.\textsuperscript{36} The protection offered by non-permeating CPAs is linked with their effects on freezing point depression at high concentrations. As the concentration of non-permeating CPAs increases between ice crystal interfaces during freezing, the hydrogen bonding capacity between the CPA and water increases and the viscosity of these solutions also increases. These effects combine to restrict ice crystal growth which leaves a larger water mass unfrozen\textsuperscript{52} and may suppress or inactivate ice nuclei.\textsuperscript{53}

1.6 Toxicity of cryoprotectants

Since the landmark discovery of glycerol as a CPA by Polge \textit{et al.},\textsuperscript{2} CPAs have been used in every cryopreservation protocol. However, the revival of 100% of the cell population is not possible and the survival rate of some cell types is still very low. A major implication is that if the CPA concentration is increased to suppress freezing injury, the CPA becomes cytotoxic to the cell resulting in cell death.\textsuperscript{54} CPAs are still chemicals that are not normally encountered by living organisms, sometimes at high concentrations, and as such, the toxicity of CPAs is very relevant to cryobiology.

The fact that increasing concentrations of CPAs leads to cytotoxicity is clearly presented by work from Mazur (Figure 1-9).\textsuperscript{55} At a very low DMSO concentration the
cell survival of fetal pancreatic cells was low since DMSO did not offer protection. However, as the concentration of DMSO was increased to 2 M, the survival rate rose above 90%. Unfortunately, the cytotoxicity of DMSO is apparent as cell survival decreased when the DMSO concentration approached 3 M. Similarly, rat hearts which were frozen to -10°C with low concentrations of DMSO recovered fully compared to rat hearts which were frozen to much lower temperatures with higher concentrations of DMSO despite the fact that the hearts at -10°C contained less ice (due to the milder temperature). It was obvious that the hearts which were exposed to higher concentrations of DMSO were more damaged. Another study by Fahy clearly demonstrates that freezing injury is strongly correlated with high DMSO concentrations rather than the amount of ice formed, the amount of unfrozen solution remaining, or the salt concentration during freezing. Similar toxicity results were reported with ethylene glycol, methanol and ethanol, and glycerol. There are many explanations for the cytotoxicity of DMSO and other CPAs. The CPA may inhibit catalases and peroxidases, enzymes which are responsible for the destruction of damaging free radicals which are
implicated in freezing injury.\textsuperscript{58} DMSO may also inhibit fructose diphosphatase which will inhibit glycolysis after freeze-thaw.\textsuperscript{59,60} DMSO tends to precipitate a variety of solutes including calcium salts, magnesium phosphates, inorganic phosphates, and glycerophosphates which increases cell death during thawing and hydration.\textsuperscript{60} Finally, DMSO is able to denature proteins by binding to hydrophobic and aromatic side chains.\textsuperscript{61} Numerous other studies have been conducted on the freezing cryotoxicity of DMSO\textsuperscript{60} and a combination of these effects is most likely the cause of DMSO cytotoxicity.

Perhaps the most prevalent problem with DMSO cytotoxicity is in cryopreserved samples that have been transfused into patients. The use of peripheral blood stem cells (PBSC) is a method used to replace blood-forming stem cells which were destroyed, for example in cancer treatment, and is much less invasive than bone marrow transplantation.\textsuperscript{62} All studies indicate that the amount of DMSO present in the sample is related to the level of toxicity.\textsuperscript{63,64} The most common side effects include nausea and vomiting which is experienced by approximately 50\% of patients.\textsuperscript{63,64,65,66} However in some cases, more severe cytotoxicity has been reported with DMSO. Approximately 2\% of patients suffered adverse effects related to the cardiovascular system (bradyarrhythmia and hypotension), respiratory system, central nervous system, and renal system.\textsuperscript{63,67,68} Another example indicates that DMSO from infusion can cause short-term amnesia and disorientation.\textsuperscript{69,70} Most importantly, almost 90\% of patients who receive higher doses of DMSO suffer from side effects compared to 50\% of patients who receive lower doses of DMSO confirming that DMSO is the cause of the side effects.\textsuperscript{63} There are many reasons as to why DMSO is cytotoxic in humans. Studies have indicated that DMSO depresses the response of diaphragm muscles\textsuperscript{71} and decreases conduction velocity in nerves.\textsuperscript{72} With
cell culture studies, DMSO has shown to impact cellular function by interfering with metabolism, enzymatic activity, cell cycle and apoptosis, calcium concentration, cell differentiation and proliferation.\textsuperscript{70} Although the precise mechanism responsible for DMSO cytotoxicity is unknown, the general consensus is that DMSO concentrations must be reduced, completely washed out before transplantation, or a safer cryoprotectant must be used.\textsuperscript{73} As there are currently no CPAs more effective than DMSO, a large focus in literature has been to develop automated, effective, and low cost methods for the removal of DMSO prior to transplantation.\textsuperscript{70} However, the ultimate goal would be to use CPAs that are effective at cryopreserving cells but do not manifest cytotoxic effects.

### 1.7 Natural cryoprotectants: biological antifreezes

Nature has developed many natural antifreeze proteins that protect plants, insects, fish, frogs, and other organisms from freezing damage. Antifreeze proteins were first discovered by Scholander who reported an abnormally low freezing temperature of blood serum in Arctic fish that was not explained by colligative effects of dissolved solutes in the blood.\textsuperscript{74-76} The sea ocean temperatures that are inhabited by Arctic fish can decrease to -1.9°C yet the fish remain alive although though their blood plasma is at the same temperature. Analysis of blood plasma indicated that the salts found in the plasma only account for water supercooling to -0.8°C.\textsuperscript{77} It was later discovered that antifreeze proteins in blood plasma were responsible for the remainder of the protection.\textsuperscript{78,79} There are two classes of biological antifreezes that have the ability to depress the freezing temperature: antifreeze proteins (AFP) and antifreeze glycoproteins (AFGP).

AFPs are segregated into four categories and their properties are shown in Table 1-1 reproduced from Harding et al.\textsuperscript{80} Additionally, many other AFPs have been found in
organisms other than fish. AFPs are present in insects such as the spruce budworm moth,\textsuperscript{81,82} the yellow mealworm beetle,\textsuperscript{83,84} the fire-coloured beetle,\textsuperscript{85} and the snow flea.\textsuperscript{86} AFPs have also been found in plants,\textsuperscript{87-94} fungi, and bacteria.\textsuperscript{95-100}

Table 1-1 Mass, structural features and natural sources of antifreeze proteins\textsuperscript{80}

<table>
<thead>
<tr>
<th>Mass (kDa)</th>
<th>Structural Features</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3-4.5</td>
<td>Single α-helix</td>
<td>Winter flounder, Scupins</td>
</tr>
<tr>
<td>11-24</td>
<td>Globular with disulfide bonds</td>
<td>Rainbow smelt, Atlantic herring</td>
</tr>
<tr>
<td>6.5</td>
<td>Globular with β-sandwich</td>
<td>Ocean pout, Eel Pout</td>
</tr>
<tr>
<td>12</td>
<td>Helical bundle, alanine rich</td>
<td>Longhorn sculpin</td>
</tr>
</tbody>
</table>

AFGPs refer to a group of eight proteins that make up 3–4% of the blood serum of Antarctic notothenoids and Arctic cod. These proteins are synthesized by the liver and are secreted into the circulatory system where they are dispersed in other fluid compartments of the fish. The eight groups arise from their decreasing molecular weights where AFGP-1 has a molecular weight of 33.7 kDa and AFGP-8 has a molecular weight of 2.6 kDa.\textsuperscript{125} The typical structure of an AFGP is comprised of a threonine-alanine-alanine tripeptide repeat in which the secondary oxygen of the threonine is glycosylated with a β-D-galactosyl-(1,3)-α-D-N-acetyl galactosamine disaccharide (Figure 1-10). There are minor amino acid variations in some AFGPs where occasionally the alanine is replaced with a proline or a threonine is replaced with an arginine. Overall they have similar structures but significant size-variation.\textsuperscript{80}

![Figure 1-10 General structure of AFGP showing the disaccharide linked to the Ala-Ala-Thr peptide repeat.](image)
1.7.1 Antifreeze activity of biological antifreezes

AFPs and AFGPs exhibit two distinct types of antifreeze activity: thermal hysteresis (TH) and ice recrystallization inhibition (IRI) activity. IRI activity was described previously in Section 1.4. Thermal hysteresis activity was the first property of AF(G)Ps quantified in the literature. Thermal hysteresis is defined as the depression of the freezing point relative to the melting point. The TH activity of an Arctic fish AF(G)P is depicted in Figure 1-11. In this diagram, the melting point ($T_m$) of pure water is the same as the freezing point ($T_f$), both corresponding to 0°C. In the presence of salt, a water solution will possess a decreased freezing point and an equally decreased melting point. A water solution in the presence of AFGPs has a lower freezing point relative to the melting point, the TH gap. The ice crystals in this gap have a characteristic hexagonal bipyrimidal shape and do not change size within this gap. The fish are protected as the ice crystals cannot grow any larger and the fish will manage the removal of any ice that forms. Below this

![Figure 1-11](image)

**Figure 1-11** Diagram showing thermal hysteresis (TH). $T_m$ represents the melting point and $T_f$ represents the freezing point.
gap, the ice crystals begin to grow uncontrollably, often with extreme speed, into long spicules and the blood plasma freezes. However, the fish are not at risk of freezing because the ocean waters do not decrease below the freezing point temperature. In fish, TH gaps range from 1 to 1.5°C.

TH has been extensively studied and is attributed to an irreversible adsorption of AF(G)Ps onto the prism faces of a growing ice crystal resulting in the disfavourable addition of water molecules. This phenomenon is referred to as the Kelvin Effect (Figure 1-12A). In this effect, the AF(G)P adsorbs to an ice prism face and water molecules can only adsorb onto the surface between the AF(G)P adsorption sites. As water molecules add to the surface, the radius of curvature increases. The increase of surface tension at the curved surface hinders the addition of subsequent water molecules and ice crystal growth is halted. However, if the temperature is decreased below the TH gap, the

![Figure 1-12](image-url)

**Figure 1-12** The effects of AF(G)Ps binding to ice crystals. A) Schematic representation of the Kelvin Effect. B) Diagram of the adsorption of AF(G)Ps onto the prism plane of an ice crystal causing the hexagonal bipyramidal shape.
curvature increases to a point where the bound AF(G)Ps are frozen over, resulting in rapid expansion of the ice crystal, often forming spicules.\textsuperscript{128} This radius threshold before the ice over-growth occurs is known as the Kelvin radius. The irreversible binding of AF(G)Ps to the ice surfaces therefore inhibits water molecule addition to the prism face of the crystal. Therefore, water molecules add to the more accessible basal plane of the ice crystal resulting in a hexagonal bipyrimidal shape of the ice crystal (\textbf{Figure 1-12B}).

Once a new layer, or step, of a basal plane has formed, AFGPs will add to this layer so that each new layer becomes gradually smaller forming an apex. The conformation of AF(G)Ps allows one face to bind to ice and the other side to interact with water. The face that binds to ice usually contains the threonine residues and it has been shown that in AFP I, the $\beta$-methyl of the threonine is crucial for the function of the antifreeze.\textsuperscript{129-131}

1.7.2 Cryopreservation of biological material with biological antifreezes

Although AF(G)Ps protect Arctic fish from freezing injury, they fail to protect cells and tissues from cryoinjury. One study using AFGPs in the freezing preservation of rat hearts concluded that fish AFGPs ‘not only fail to enhance storage of the isolated rat heart preparation at hypothermic temperature, but cause increased damage under freezing conditions regardless of AFGP concentrations’.\textsuperscript{132} Overall, in literature the use of AF(G)Ps as cryopreservatives is considered ineffective.\textsuperscript{133} One reason that explains why AF(G)Ps are not successful is the formation of speculated ice crystals below the TH gap that severely damages cells as temperatures used in cryopreservation are well bellow the TH gap.\textsuperscript{134} Currently, AF(G)Ps are not utilized for cryopreservation in medicine and industry. However, the property of ice recrystallization inhibition (IRI) that AF(G)Ps
possess is very useful for cryopreservation as the ability to halt the growth of large ice crystals during the thawing phase decreases cell damage.\(^{135}\)

### 1.8 Antifreeze glycoprotein analogues

The Ben laboratory has rationally designed and synthesized C-linked AFGP analogues 101 and 102 (Figure 1-13) that possess the favourable property of potent IRI activity without the damaging effects of TH.\(^{136,137}\) As discussed in Section 1.3.2, ice recrystallization inside the cell is highly damaging and preventing this process would improve cell viabilities.\(^{138,139}\) These C-linked AFGPs are known to exhibit “custom-tailored” antifreeze activity.\(^{140}\) In comparison to the native AFGP that contains a disaccharide glycosylated to a threonine residue, these analogues contained a C-linked α-D-galactosyl unit conjugated to the ε-amine of ornithine in a repeating ornithine-glycine-glycine unit. The C-linkage, in contrast to the typical O-glycosidic linkage, is more resilient to hydrolysis under acidic or basic conditions. 101 exhibits ‘custom tailored antifreeze activity’ as it exhibits potent IRI activity with no TH activity. Although analogue 102 is highly IRI active, it exhibits a very small TH gap of 0.06°C. This TH gap is almost negligible in cryopreservation applications. In a cell viability assay after cryopreservation with human embryonic liver cells, analogues 101 and 102 protect the

![Figure 1-13 Structures of C-linked AFGP analogues 101 and 102.](image)
cells as well as a 2.5% DMSO solution at concentrations of 1 mg/mL. This work confirms the hypothesis that inhibiting ice recrystallization is beneficial for improving cell viability post-thaw. Further structure-function studies were performed to determine the structural features necessary for potent IRI activity. Analogues of the highly IRI potent 101 analogue were studied in which the galactose moiety was replaced with D-glucose (103), D-mannose (104), or D-talose (105) monosaccharide moieties (Figure 1-14). The IRI activity of and 103 – 105 was examined using the splat-cooling assay and domain recognition software to quantify ice crystal size. The y-axis in Figure 1-14 represents the ice crystal mean grain size (MGS) relative to a phosphate buffered saline (PBS) (negative control for inhibition of ice recrystallization). It was found that galactose analogue 101 was the most potent derivative, while glucose analogue 103 exhibited moderate IRI activity. The mannose and talose derivatives, 104 and 105, did not have IRI activity. When the monosaccharides were examined individually, as reducing sugars,
similarity, galactose was the most active while mannose and talose exhibited poor IRI activity (Figure 1-15). As only the configuration of the carbon stereocentres on the pyranose rings changed on these derivatives, it was concluded that the orientation of the hydroxyl groups moderated IRI activity. It has been demonstrated that the stereochemistry of a pyranose affects the hydration of a carbohydrate\textsuperscript{144-146} and it was deduced that hydration is closely correlated to IRI activity.\textsuperscript{140,147} Hydration is described as the number of water molecules that interact closely with a solute. Since these water molecules bind tightly to the solute, they do not fit into the three-dimensional network of the surrounding bulk water. Therefore, a highly hydrated solute does not fit well into bulk water. In the case of carbohydrates, the orientation of the hydroxyl groups (axial or equatorial) on the pyranose ring affects the H-bonding network with bulk water. The hydroxyl groups of galactose, a highly hydrated carbohydrate, are oriented in such a way as to fit poorly into the three-dimensional water network and in this way disorder the
It is hypothesized that the addition of a water molecule from the more disordered quasi-liquid layer to the ice crystal lattice is energetically unfavourable. Inversely, if the quasi-liquid layer was more ordered as in the case of talose, which has low hydration and fits well into bulk water, then water molecule addition to the ice crystal would be more favourable. This is presented in Figure 1-16. A non-hydrated solute will not affect the equilibrium of water molecules adding to ice crystal 1 or ice crystal 2 from the bulk water (represented by solid arrow). With a hydrated solute, the disruption in the bulk water hinders the addition to water molecules to the ice crystals from the bulk water (represented by dashed arrow).

1.8.1 The IRI gauge

In referring to IRI activity, compounds that produce a mean grain size (MGS) of ice crystals at 22 mM of 10% or lower compared to a PBS control, will be referred to as possessing ‘potent’ IRI activity (Figure 1-17). Compounds that have ‘moderate’ IRI
activity produce a MGS of greater than 10 to 80%, while compounds that do not fall into this range will be considered to have ‘weak to no’ IRI activity.

1.9 Small molecule ice recrystallization inhibitors

Nature has provided antifreeze compounds but unfortunately, they cannot be used in the medical field. Furthermore, these are large glycoproteins; the synthesis of which is not amenable to production in large quantities. As such, there has been an effort to develop small molecule ice recrystallization inhibitors.

Since it was demonstrated that simple carbohydrates exhibit moderate IRI activity,147 various other monosaccharides were synthesized and assessed for their IRI activity. The effect of an $\alpha$-C-linked allyl group was investigated in analogues 106-109 while 110 and 111 contained a $\beta$-linked C-allyl group (Figure 1-18).147 The effect of fluorine was explored in 112 and its derivatives 113-115.155 Unfortunately, at 22 mM in PBS, these analogues exhibited only moderate IRI activity, not exceeding 60% mean grain size compared to a PBS control. It was also concluded that the presence of simple groups at the C1 position did not affect IRI activity.147,156
As it appears that the most IRI active compounds discussed up to this point were glycopeptide 101 and 102, the C-linked glycopeptides 101 was subsequently truncated to determine the smallest sub-unit that can exhibit potent IRI activity. These C-linked substrates are presented in Figure 1-19. Compounds 116-129 contain increasing alkyl lengths from one to sixteen carbons. The ionizable amine and carboxylic acid groups, and alcohol groups were explored in compounds 130-132, respectively. Additionally, structurally diverse branched alky chains were investigated (133-136), as well as rings (137 and 138), and various alkenes and alkynes (139-143). When the alkyl chain analogues were assessed for IRI activity at 22 mM in PBS, compounds 123 and 124, containing seven and eight carbon atoms, respectively, exhibited very high IRI activity. The analogues with nine and ten carbon atoms (125 and 126) were tested at a lower concentration (5.5 mM) due to solubility problems and these were even more active than 123 and 124 at the same concentration. Even at 5.5 µM, the analogues with the longest alkyl chains, 128 and 129, were as active as galactose at 22 mM while 123-127 had substantially decreased IRI activity at the 5.5 µM concentration. The ionizable groups did
not improve IRI activity as 130 and 131 were only moderately active. However, the presence of an alcohol in 132 did increase IRI activity, but not as much as in 123 and 124 at 22 mM. From the unsaturated alkyl chains (139-143), the analogues with the terminal alkene and alkyne (139 and 140) had improved activity while the remainder where moderately IRI active. Interestingly, the most potent analogues are very similar to non-ionic surfactants. The conclusions from this study were that hydrophobic moieties are beneficial for IRI activity and that small molecule ice recrystallization inhibitors can be developed.\(^\text{157}\)

The Ben group reported the finding of carbohydrate-based small molecule ice recrystallization inhibitors that have potent IRI activity at very low concentrations.\(^\text{158}\) This was the first precedent of small molecules possessing potent IRI activity. From the previous study with the truncated analogues\(^\text{157}\) it was clear that surfactant-like molecules are important for IRI activity, and other compounds that alter bulk water include hydrogelators. Figure 1-20 represents some of the small-molecule surfactants and hydrogelators that were investigated.\(^\text{158}\) The glucose-derived non-ionic surfactant $\beta$-octyl-
d-glucopyranoside 144 did not exhibit high IRI activity at 22 mM and had activity comparable to glucose. This was not surprising since it was previously shown that the substituent at the anomeric position does not modulate IRI activity. However, the galactose-based non-ionic surfactant β-octyl-D-galactopyranoside 145 had much higher IRI activity when compared to galactose. The drastic change in IRI activity effected by a modification of the anomeric position was previously unknown. However, these observations agree with previous studies indicating that galactose derivatives are more IRI active than glucose derivatives because they are more hydrated. Since 144 and 145 are surfactants they form micelles in solution, but it was determined that the ability to form micelles does not affect IRI activity. The open-ring forms of 144 and 145 are the N-octyl-D-aldonamides, 146 and 147, which are known low-molecular weight hydrogelators. The glucose derivative, N-octyl gluconamide 146, had unprecedented potent IRI activity at a low concentration of 0.5 mM (for comparison, all other compounds were tested at 22 mM). Interestingly, the galactose derivative, N-octyl galactonamide 147, was not IRI active at 0.5 mM. This was the first instance where a
glucose derivative was more active than a galactose derivative. Compound 146 can form hydrogels at a concentrations above 33 mM. Therefore, the potent IRI activity at 0.5 mM was not related to the ability to form gels. The N-methylated analogues 148 and 149, where the amide orientation in 149 was reversed, had weak IRI activity indicating that the proton of the amide bond is important for IRI activity. Furthermore, none of these compounds exhibited TH activity or dynamic ice shaping and solid-state NMR studies failed to indicate interaction with ice. This study concluded that potent IRI activity of these compounds is not dependent upon micelle formation, gelation, or interaction with the ice lattice.158

Exploring other surfactants/gelators may result in the discovery of novel small molecule ice recrystallization inhibitors since the relationship between hydrophobic and hydrophilic groups is important for IRI activity.

1.10 References

150. Fletcher, N. Phil. Mag. 1962, 7, 255.
Chapter 2. Goals and Objectives

One of the ultimate goals of the Ben laboratory is to develop cryoprotectants that are non-cytotoxic small molecules capable of increasing cell viabilities or virus infectivity after cryopreservation. This will be accomplished because these small molecules will be effective inhibitors of ice recrystallization and thus protect cells against the injury associated with cryopreservation. Ideally we would like to develop a cryoprotectant that is better than dimethyl sulfoxide. The design of small molecule ice recrystallization inhibitors is a new field and as such work is required to determine the structural features that are responsible for potent ice recrystallization inhibition in a small molecules. The following goals and objectives will explore which structural features of disaccharides, amino acid-based surfactants and gelators, and gemini surfactants affect ice recrystallization inhibition activity. The IRI assay solution has also been implicated in affecting IRI activity. Therefore, the IRI assay solutions will also be examined to investigate how counterions of salts affect ice recrystallization inhibition results.

2.1. Objective 1: Determining the effect of different glycosidic linkages in disaccharide analogues of AFGP affect on IRI activity.

Structure-function studies have demonstrated that the \( \beta\)-d-galactosyl-(1,3)-\( \alpha\)-d-N-acetyl galactosamine disaccharide in native AFGPs (Figure 2-1) is an essential structural feature for thermal hysteresis activity and that changing the glycosidic linkage to \( \beta\)-(1,4) decreases thermal hysteresis activity.\(^1,2\) However, it is unknown how changes in this disaccharide linkage affect IRI. It
is important to investigate the impact of the disaccharide structural features on IRI activity, as it will facilitate the rational design of potent small molecule ice recrystallization inhibitors. The disaccharides with the $\beta$-(1,3) and $\beta$-(1,4) glycosidic linkages were previously synthesized by the Ben group (Figure 2-2a) and the $\beta$-(1,6)-linked disaccharide remained to be prepared. Thus, the goal was to synthesize this derivative and compare the IRI activities of the three disaccharides. The IRI activities of the $\beta$-(1,3)- and $\beta$-(1,4)-linked disaccharides is depicted in Figure 2-2b. As illustrated in this figure there exists a marked difference in IRI activity between these two compounds. It would be interesting to discover what effect the $\beta$-(1,6) glycosidic linkage would have on IRI activity.

**Figure 2-2** a) Structures of target disaccharides and b) IRI activity of AFGP analogues previously synthesized by the Ben group.
2.2. **Objective 2: Determining the effect of hydrophobic groups on IRI activity and cryopreservation ability.**

Glycopeptides previously prepared by the Ben group that possess potent IRI activity are not amenable to large-scale synthesis due to their large size and complex structure. The identification of small molecule carbohydrate-based ice recrystallization inhibitors with high IRI activity at low concentrations (section 1.9) has been a major advance in the field. Since some of these ice recrystallization inhibitors are surfactants and gelators, it follows that other types of surfactants and gelators should be examined. An interesting property of this class of molecules is their ability to sequester bulk water. However, the role (if any) that this property has in the ability of these molecules to inhibit ice recrystallization is not known. Therefore, the second objective is to determine how hydrophobic moieties in surfactants/gelators affect IRI activity.

Many classes of gelators are comprised of amino acid analogues in which the side chain, amine, and carboxylic acid are functionalized. The Hanabusa group has synthesized a variety of lysine-based gelators with diverse modifications (Figure 2-3)

![Figure 2-3 Structures of lysine-based gelators.](image-url)
and demonstrated that modifying the alkyl chain length affects the concentration at which these compounds gel.4-10 However, the role of critical gelation concentration and IRI activity has not been examined. Consequently, di-alkyl anionic lysine derivatives will be synthesized with various alkyl chains lengths (Figure 2-4) and assessed for IRI activity. The carboxylic acid will also be converted to a lithium, sodium, or potassium carboxylate salt to determine the effect of counterion on IRI activity. A more in-depth study of how counterions affect IRI activity is described in section 2.3. Other typed of surfactants include anti-ice nucleating agents (anti-INAs) that consist of quaternary ammonium salts (Figure 2-5a) and lysine alkyl esters (Figure 2-5b).11 These were examined for their potential to protect crops against frost injury caused by bacteria that nucleate ice at higher temperatures. The anti-ice nucleation activity of these compounds was dependent upon the alkyl chain length.11 Since these anti-ice nucleating agents (anti-INAs) inhibit ice nucleation, they may also possess ice recrystallization inhibition properties. Analogues of these anti-INAs with varying alkyl chain lengths will be synthesized and assessed for IRI activity. These experiments will also identify if there is a correlation between ice recrystallization inhibition activity and anti-ice nucleation activity. As the ultimate goal is to identify small molecules that can be used as cryoprotectants with biological materials,
the surfactants/gelators must also be assessed in cell-based cryopreservation assays. Thus, the most IRI active surfactants/gelators will be tested for any cryopreservation ability.

Since simple monosaccharides have moderate to weak IRI activity, it would be interesting to determine whether attaching a monosaccharide moiety to a lysine surfactant would increase the IRI activity. The resulting glycoconjugates would contain a hydrophilic moiety (the monosaccharide) and a hydrophobic moiety (long alkyl chain). Galactose and glucose glycoconjugates (Figure 2-6) would be synthesized containing various alkyl chain lengths and analyzed for IRI activity.

The role of hydrophobic groups will also be studied in ‘gemini surfactants’ that contain long alkyl chains, ionic head groups, spacers, a second ionic group, and another hydrocarbon tail. The significance of these structures is that the two hydrocarbon chains cannot interact with each other due to the rigid linker and association into micelles is disfavoured (Figure 2-7). The gemini surfactants are good model compounds for the study of structural features important for IRI activity since they contain various ionic...
head groups and alkyl chains of different lengths. The hydrophilic moieties in the previously mentioned surfactants/gelators could not be modified, except for the carboxylate counterions. The gemini surfactants allow the study of both the hydrophilic and hydrophobic groups.

2.3. **Objective 3: Investigating whether counterions associated with amines and carboxylic acids influence IRI activity.**

The third objective is to perform a more in-depth study on how amine and carboxylate counterions affect IRI activity. As IRI activity is dependent upon hydration, changing counterions should also modify IRI activity as anions and cations have different hydration shells. Di-alkyl lysine surfactants were synthesized with lithium, sodium, and potassium counterions and studied in Objective 2 (Figure 2-8a). However, a simpler model system would be useful as well as having the ability to study both cationic and anionic counterions. Therefore, para-amino benzoic acid derivatives were synthesized (Figure 2-8b) that have simple structures and possess both cationic (lithium, sodium, and potassium) and anionic (chloride, bromide, and iodide) counterions. The results from these experiments will enable the identification of counterions that may improve IRI activity.

![Figure 2-8 Counterions of a) di-alkyl lysine surfactants and b) para-amino benzoic acid derivatives.](image)
2.4. **Objective 4: Investigating the effect of PBS salts on ice crystal size in the IRI assay**

In the literature, IRI assays are performed by dissolving the compound of interest in a solution containing different solutes (salts or sucrose). These solutes are necessary to inhibit non-specific recrystallization effects when assessing the IRI activity of unknown analytes. It was found that some compounds, especially surfactants, have dramatically different IRI activities in different assay solutions. Furthermore, groups that perform the IRI assay utilize different solutions so results cannot be compared. The solutions include various concentrations of sucrose,\textsuperscript{15-18} Tris-buffered saline,\textsuperscript{19} various concentrations of NaCl,\textsuperscript{20-22} and CaCl\textsubscript{2}.\textsuperscript{23} Therefore, the fourth objective was to investigate what effect the PBS salts have on IRI activity by testing the four PBS salts, NaCl, KCl, Na\textsubscript{2}HPO\textsubscript{4}, and KH\textsubscript{2}PO\textsubscript{4} individually. As well, the sucrose solutions will be tested and evaluated for their effectiveness. The overall aim of these experiments is to understand how external salts affect IRI activity and to encourage the use of one standard solvent for the IRI assay.

2.5. **Objective 5: Assessing the preservation potential of C-Linked AFGPs and carbohydrate-based surfactants/gelators on viruses**

Preliminary data regarding cryopreservation of liver cells indicate that OGG-Gal is not very effective at protecting cells from freezing damage. However, other materials that are being currently cryopreserved include viruses. The differences between eukaryotic cells and viruses are vast and some compounds may preserve viruses more effectively than live cells. The preservation of viruses is becoming increasingly important as oncolytic viruses (viruses that attack tumor cells) are beginning to be used as cancer treatment.\textsuperscript{24} Also, vaccine distribution around the world is still not optimal and better
stabilizers must be developed to prevent vaccine inactivation though improper storage or transportation. Since freezing and thawing damage viruses, using ice recrystallization inhibitors to prevent cryoinjury is a logical step. The last objective is to identify ice recrystallization inhibitors that can preserve viruses, which can be used in oncolytic therapies or in vaccines.

The ice recrystallization inhibitors that will be tested with vaccinia virus, vesicular somatitis virus, and herpes simplex 1 virus, are shown in Figure 2-9, including OGG-Gal. The IRI activities of these compounds vary but they possess the highest IRI activity in their class. Also, they were chosen for their diverse structures to maximize the success of identifying an effective protectant.

2.6. References

Chapter 3. The effect of the glycosidic linkage of AFGP disaccharide analogues on ice recrystallization inhibition activity

One of the differences between native AFPs and AFGPs is that the AFGPs contain a disaccharide moiety. Structure-function studies have demonstrated that the β-d-galactosyl-(1,3)-α-d-N-acetyl galactosamine disaccharide in native AFGPs (Figure 3-1) is an essential structural feature for thermal hysteresis activity. However the structural importance of this moiety and its relationship to the ability of these glycoconjugates to inhibit ice recrystallization has not been investigated. In fact, previously synthesized C-linked AFGPs that are potent inhibitors of ice recrystallization, do not possess this disaccharide. As previous work by the Ben group showed that simple mono- and disaccharides exhibit weak to moderate IRI activity, we sought to investigate how structural changes on the native disaccharide moiety on AFGP8 affects IRI activity. The data from this study will facilitate the rational design of potent small molecule ice recrystallization inhibitors. Disaccharides 301, 302, and 303 were previously synthesized by the Ben group (Figure 3-2). Disaccharide 301 is a close analogue of β-d-galactosyl-(1,3)-α-d-N-acetyl galactosamine found in the native AFGP but the C1 anomeric oxygen is protected as an allyl ether. The presence of the allyl ether simplifies characterization as only the β form is present, rather than the α and β mixture of reducing carbohydrates. Most importantly, earlier work by our laboratory has
demonstrated that simple alkyl groups at C1 do not greatly affect the ability of these structures to inhibit ice recrystallization.\textsuperscript{4} In this study, the $\beta$-C-allyl galactose derivate had less than a 10% decrease in IRI activity relative to the native $O$-linked galactose. However, $\beta$-C-allyl glucose was statistically indistinguishable to the $O$-linked glucose. Following these conclusions, it is expected that the $\beta$-$O$-allyl group may have only a small affect on the IRI activity. Disaccharide 302 is a direct analogue of 301 without the $N$-acetyl group. This was to investigate the effect of the $N$-acetyl group on IRI activity since previous studies have implicated this group as being important for TH activity.\textsuperscript{2} Finally, 303 contains the $\beta$-(1,3)-linkage. The IRI data (Figure 3-2) shows that these disaccharides have moderate IRI activity. Out of these three, 303, containing the $\beta$-(1,4)-linkage is the most active. This is particularly interesting as this is not the glycosidic linkage found in the native AFGP. However, the IRI activity of the $\beta$-(1,6)-linked disaccharide was still unknown.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3-2.png}
\caption{Structures and IRI activity of AFGP analogues 301-303 previously synthesized by the Ben group.}
\end{figure}
To further elucidate how the glycosidic linkage affects IRI activity, the β-(1,6)-linked disaccharide, 304, was synthesized and assayed for IRI activity (Figure 3-3). This completed set will enable the better understanding of the structural features responsible for IRI activity.

3.1. Retrosynthetic analysis of β-D-galactosyl-(1,6)-α-D-N-acetyl galactosamine 304.

The original synthetic approach that is envisioned for the preparation of disaccharide 304 is presented in Scheme 3-1. The key step involves a regioselective ring opening of the glycosyl donor epoxide intermediate 305, by the C-6 unprotected glycosyl acceptor, 308. Consistent with this approach, intermediate 305 would be prepared via epoxidation of the glycal intermediate 306. Glycal 306 must be protected with benzyl groups as acetate protecting groups are destabilizing for the
epoxidation (discussed below). However, benzylated glycal 306 can be easily prepared from acetylated glycal 312. The free C-6 hydroxyl group of glycosyl acceptor 308 can be obtained by the selective deprotection of the tert-butyl silyl (TBS) group at C-6 of 309. Selective protection of C-6 with one equivalent of tert-butyl silyl ether and acetylation of the remaining C-3 and C-4 hydroxyl groups of 310 gives access to 309, the precursor to glycosyl acceptor 308. Finally, the C-2 amino group would be installed though the azido-nitration of 312 followed by alkylation at C-1 to incorporate the O-allyl group. In this synthesis, both the glycosyl donor and acceptor are formed from glycal 308. This is a type of divergent synthesis in which the number of total steps is decreased because the glycosyl donor and acceptor are synthesized from the same initial pathway.

3.1.1. **Synthesis of glycosyl donor 305**

To begin the synthesis of glycosyl donor 305 (Scheme 3-2), galactose is acetylated in 85% yield to give β-D-galactose pentacetate 313. Bromination with HBr resulted in the formation of only the α form of acetobromo-D-galactose due to stabilization provided by the anomeric effect. Glycal 312 was prepared by reductive elimination the C-2 acetate by heating in the presence of Zn dust in an overall yield of 77%. Deprotection of the acetate groups, protection with benzyl groups, and oxidation

Scheme 3-2 Synthesis of glycosyl donor 305.
with in-situ formed dimethyldioxirane (DMDO) from Oxone® yielded glycosyl donor 305 in 80% yield. It was important to exchange the protecting groups from acetates to benzyls for the epoxidation step. Acetate groups reduce the nucleophilicity of the glycal towards DMDO. The epoxidation with an acetylated glycal is reported to require at least six hours resulting in a 7:1 mixture of the desired epoxide 315 and undesired epoxide 316 (Scheme 3-3).\(^8\) Furthermore, since these epoxides cannot be separated into the individual diastereomers by silica column chromatography due to their high reactivity, the whole diastereomeric mixture must be reacted with the glycosyl acceptor. This will yield a mixture of galactose 317 and mannose 318, lowering the overall yield of the desired product 317. In contrast, benzyl groups are more electron donating and the reaction with the more activated benzylated glycal 306 is complete in 2.5 hours with the favoured epoxide as the only product (Scheme 3-3).\(^8\)

**Scheme 3-3** The stereoelectronic effects of acetate vs. benzyl groups on a glycal to be epoxidized.
3.1.2. Synthesis of glycosyl acceptor allyl 2-acetamido-2-deoxy-3,4-O-isopropylidene-β-D-galactopyranoside (325)

The synthesis of the glycosyl donor began with the preparation of glycal 307 in three steps from galactose. Azidonitration\(^9\) of 307 with sodium azide and ceric ammonium nitrate (CAN) furnished 319 in 40% yield (Scheme 3-4a). The proposed mechanism of the azidonitration is depicted in Scheme 3-4b. Ceric ammonium nitrate has the formula Ce\(^{IV}\)(NH\(_4\))\(_2\)(NO\(_3\))\(_6\) and cerium\(^{IV}\) is reduced to cerium\(^{III}\) by a one-electron transfer to the added azide (from NaN\(_3\)). According to the anti-Markovnikov route, the
resulting azide radical will add to C-2 of galactose resulting in a radical at the more stabilized anomeric position. One possible mechanism for the nitration at the anomeric position proceeds by the radical addition of cerium-oxidized NO$_3^−$. Due to the lack of stereospecificity in this reaction, three products are formed (Scheme 3-4b): $α$-2-azido-2-deoxyglycosyl nitrate, $β$-2-azido-2-deoxyglycosyl nitrate, and $α$-2-azido-2-deoxytalosyl nitrate. The talose analogue was formed in trace amounts (approximately 8%) and could not be separated from the galactose derivatives with silica column chromatography or trituration. However, this talose derivative was removed in the next step during purification. The anomeric nitrate was reduced with thiophenol to the reducing sugar 320. Further, direct $O$-alkylation was attempted to install the $O$-allyl group using allyl bromide and sodium hydride as a base. In this reaction, the only product should be the $β$-$O$-allyl product as the $β$ deprotonated C1 oxygen (Scheme 3-4c) is more reactive towards allyl bromide. The increased reactivity is due to the instability brought about by electron cloud repulsion of the anomeric and pyranose oxygens. Unfortunately, the alkylation of 320 was not successful.

Instead, $α$-trichloroacetimidate 322 was synthesized instead (Scheme 3-5a). Trichloroacetimidate 322 would act as a glycosyl donor in a glycosylation with allyl alcohol. The use of trichloroacetimidates is useful as the stereochemistry of the resulting glycoside can be carefully controlled. In this example, under reversible conditions with the base 1,8-diazabicycloundec-7-ene (DBU), the most stable trichloroacetimidate, the $α$-form, is formed preferentially (Scheme 3-5b). Quenching the reaction mixture furnishes the thermodynamic $α$-trichloroacetimidate 322. Conversely, under irreversible conditions using K$_2$CO$_3$ as base, the kinetic $β$-trichloroacetimidate would be formed.
Scheme 3-5  a) Synthetic scheme for synthesis of glycosyl acceptor 325 and b) mechanism for the formation of trichloroacetimidate 322 and allylated 321.

Either α or β glycosides can be obtained from an α-trichloroacetimidate depending on the conditions. A strong promoter in the glycosylation reaction such as TMS-OTf results in the formation of the oxocarbenium ion. Therefore, a mixture of α and β glycosides is possible, especially if the C-2 protecting group is not involved in neighboring group participation (Scheme 3-5a). However, a weaker promoter such as BF₃•OEt₂ results in the selective formation of the β-glycoside via SN₂ inversion of configuration. Displacement of the α-trichloroacetimidate moiety of 322 by allyl alcohol in the presence of BF₃•OEt₂ furnished β-O-allyl galactoside 321 in 95% yield. When BF₃•OEt₂ was replaced with TMS-OTf, a 1:1 mixture of the α and β O-allyl galactoside anomers
resulted. Next, the azide of 321 was reduced with tin chloride and the resulting amine was acetylated furnishing 323 in 48% yield over two steps. Removal of the acetate protecting groups and reaction with 2,2-dimethoxypropane and p-toluene sulfonic acid furnished glycosyl acceptor 325 in which the C-3 and C-4 hydroxyl groups are protected by the isopropylidene and the C-6 hydroxyl group is uncovered. Initially, the kinetic C-4/C-6 isopropylidene is formed. However, after prolonged stirring, the majority of the product is converted to the more thermodynamically stable C-3/C-4 isopropylidene, composing of 63% of the mixture ratio.

3.1.3. Synthesis of Allyl β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-galactopyranoside (304)

The initial glycosylation was attempted with glycosyl acceptor 325 and glycosyl donor 305 with ZnCl₂ as an epoxide activator. However, this resulted in a 1:1 ratio of α and β disaccharides 326 and 327 in 16% overall yield (Scheme 3-6). The major product of the attempted glycosylation was the hydrolyzed epoxide. Due to the stereochemistry of

Scheme 3-6 Attempted synthesis of disaccharide 328.

51
the epoxide, the only product should have been 326 and the 1:1 statistical mixture indicates that the epoxide was opened before the attack of the glycosyl acceptor. One explanation for this result may be that glycosyl acceptor 325 is not reactive enough towards the activated epoxide and after a prolonged period of time, the epoxide is hydrolyzed instead. Despite the lack of stereochemical control, 326 was purified and the selective removal of the benzyl groups in the presence of the allyl ether was attempted. This procedure was especially difficult as most reagents that remove benzyl groups also reduce allyl groups. However, BCl₃ has been shown to de-benzylate carbohydrates in the presence of a C-allyl groups. However, after many attempts this procedure did not furnish the final product 328 and due to the low yield of the glycosylation, this route was abandoned.

3.2. Revised synthesis of allyl β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-galactopyranoside (304)

Due to the difficulty associated with selective removal of benzyl groups in the presence of an allyl group, an acetylated glycosyl donor was chosen for the revised synthesis of disaccharide 304. Acetobromo-α-D-galactose 314 is one of the oldest and simplest glycosyl donors and is used in Koenig-Knorr glycosylation reactions. The Koenig-Knorr reaction is normally performed in the presence of silver carbonate but can also be performed with heavy metal salt promoters such as mercury cyanide. These mercuric salts are weaker promoters than silver carbonate.
The glycosylation reaction with glycosyl acceptor 325 and glycosyl donor 314 in the presence of mercury cyanide afforded the desired disaccharide 329 in 47% yield (Figure 4-7). The only product formed was the β anomer as expected, due to the C-2 neighbouring group participation of glycosyl donor 314. Removal of the isopropylidene group with trifluoroacetic acid and acetate groups with sodium methoxide yielded final product 304 in 85% yield over two steps.

3.3. IRI Activity of AFGP Disaccharide Analogues

Disaccharides 301-304 (synthesized previously5,6) were analyzed for IRI activity using the splat cooling assay. Additionally, monosaccharides 330-332, containing an O-allyl, C-2 N-acetyl, and both O-allyl and C-2 N-acetyl groups were compared to the disaccharides (Figure 3-4). These were tested to identify how the individual structural features affect IRI activity on simple monosaccharides.

Disaccharides 301 to 304 exhibited similar levels of IRI activity (Figure 3-4) comparable to galactose (Gal) itself. The activities of 301 and 304 are statistically indistinguishable to galactose. This indicates that the β-(1,3) and β-(1,6) linkage does not greatly affect the IRI activity of these compounds relative to galactose. However, disaccharide 303 is more active than 301, 304, and Gal. This is interesting as the native disaccharide
in AFGPs possesses a $\beta$-(1,3) linkage, corresponding to 301 but the most active disaccharide 303, possesses the $\beta$-(1,4) linkage. Most importantly, these disaccharides are not conjugated to the native threonine-alanine-alanine peptide and it is unknown whether a glycoconjugate containing disaccharide 303 would be more active than the native AFGP. These glycoconjugate analogues must first be synthesized and analyzed.

The Ben laboratory previously reported that antifreeze activity can be uncoupled, that is, compounds may possess potent IRI activity but not TH activity (custom-tailored antifreeze activity).\textsuperscript{3,17} Similarly, the structural features of compounds may be important for IRI activity but not TH activity. It was reported that the C-2 acetamide is crucial for

\textbf{Figure 3-4} IRI activity of disaccharides 301-304 and monosaccharides Gal, and 330-332. Error bars indicate standard error of the mean (SEM). Asterisks indicate significant difference defined by unpaired Student’s $t$-test ($p < 0.05$) within each set.
TH activity. However, removal of the C-2 acetamide group from disaccharide 301 does not have an effect on IRI activity, as the activity of disaccharide 302 is indistinguishable from 301. It is interesting that this functional group is crucial for TH activity but not for IRI activity. Overall, the C-2 acetamide group of disaccharides is not necessary in ice recrystallization inhibitors.

To determine how the individual functional groups of the disaccharides affect IRI activity, monosaccharides 330 to 332 were tested previously by the Ben group. These results show that allylated galactose derivative 330 is less active than Gal. This is not completely surprising as we have previously demonstrated that β-C-linked allyl-galactose analogs are slightly less active than galactose. This also indicates that, most likely, the absence of the O-allyl group on the disaccharides would increase IRI activity slightly. Similarly, N-acetyl galactosamine 331 is also less active than Gal. This is different than what was seen with the disaccharides where the presence of the acetamide group did not affect IRI activity. But similarly to the disaccharides, the C-2 acetamide group does not improve IRI activity. These data indicate that the role of this group in IRI activity is different than with TH activity where this group was necessary for TH activity. Finally, the presence of both the C1 O-allyl group and C2 acetamide on galactose 332 resulted in very weak IRI activity, similar to that observed with monosaccharides 332 and 333. Therefore, the combined effects of the O-allyl and acetamide groups do not improve IRI activity. Also, the lack of difference in IRI activity between 330 (lacking C2 acetamide) to 332 (containing C2 acetamide) is similar to what was seen in the disaccharide analogues 301 and 302, where the IRI activity was the same. Overall, any modifications made to galactose resulted in a loss of IRI activity.
3.4. Chapter Summary

In summary, the work described in this chapter explores how modifying the glycosidic bond of β-D-galactosyl-(1,3)-α-D-N-acetyl, the disaccharide found in AFGPs, affects IRI activity. Specifically, the synthesis of the (1,6)-linked disaccharide is described and compared to the (1,3)- and (1,4) linked disaccharides. The results indicate that the most active analogue is not the native disaccharide containing a (1,3)-glycosidic bond but the disaccharide with the (1,4)-glycosidic bond. However, it is unknown how this (1,4)-linked disaccharide would perform if conjugated to the actual AFGP protein. As well, the absence of the acetamide group on the (1,3)-linked disaccharide did not affect IRI activity indicating that in contrast to TH, this group is not important for IRI activity. Finally, the results indicated that modifying galactose with a C2 acetamide or with a β-O-allyl would result in lower IRI activity.

3.5. References

Chapter 4: Amino acid-based surfactants/gelators as ice recrystallization inhibitors

The discovery in our laboratory that small molecule carbohydrate-based molecules that function as effective inhibitors of ice recrystallization at low substrate concentrations has been a major advance in this field. In contrast to naturally occurring antifreeze molecules such as AFP and AFGPs the preparation of these small molecules is inexpensive and amenable to large-scale preparation. However, the exact structural features responsible for potent IRI activity are still unknown. Since the parent compounds of these potent ice recrystallizations are gelators and surfactants, it follows that other types of surfactants and gelators should be examined in the hopes of identifying new small molecule ice recrystallization inhibitors. Therefore, the synthesis and analysis of new gelator/surfactant ice recrystallization inhibitors can help in elucidating which attributes confer potent IRI activity.

4.1. IRI activity of cationic anti-ice nucleation agents (anti-INAs)

Low-molecular weight gelators are capable of immobilizing organic or aqueous solvents into three-dimensional networks having morphologies of fibres, rods, or ribbons. Such compounds have a variety of applications including oil spill cleanup, tissue engineering, controlled-release drug formulations and thickeners in cosmetics, paints, and foods. Some types of cationic surfactants are classified as anti-ice nucleation agents (anti-INAs). These are a broad class of compounds that have been used to protect plants from frost injury caused by common strains of ice nucleation active bacteria (Pseudomonas syringae, Pseudomonas fluorescens, Erwinia herbicola, Erwinia ananas,
and *Xanthomonas campestris*. These bacteria have specialized membrane proteins that nucleate ice crystals. Small volumes of water can be supercooled (cooled without the water freezing) to almost -40°C if the water is ultra pure and does not contain any ice nucleators (ions, dust). However, these nucleation active bacteria nucleate ice at higher temperature than water would normally freeze at, causing frost damage in frost-sensitive plants. Due to large crop losses from frost damage, there has been research into finding anti-ice nucleation agents (anti-INAs) that can inhibit the activity of these bacteria. It was concluded that the ice-nucleation active site of plant cells is comprised of an anionic environment because cationic surfactants gave the best results. Two types of surfactants that were tested for their anti-ice nucleation activity were quaternary ammonium surfactants and cationic lysine surfactants. Since some of these compounds inhibited ice nucleation, they may also possess ice recrystallization inhibition properties.

Table 4-1 Cationic quaternary ammonium surfactants

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>401</td>
<td>Bn</td>
<td>C₁₄H₃₃</td>
</tr>
<tr>
<td>402</td>
<td>CH₃</td>
<td>C₁₀H₂₁</td>
</tr>
<tr>
<td>403</td>
<td>CH₃</td>
<td>C₁₄H₃₃</td>
</tr>
</tbody>
</table>

Figure 4-1 IRI activity of cationic quaternary ammonium chloride surfactants 401-403. Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student’s t-test (p<0.05) within each set (separated by vertical lines).
Cationic quaternary ammonium salts, 401 - 403 are commercially available surfactants that are used as disinfectants, surfactants, emulsifiers, fabric softeners, and antistatic agents. In one study, 401 and 402 containing hexadecyl and decyl alkyl chains, respectively, were determined to be very effective inhibitors of ice nucleation and would allow water to supercool more than 5°C in the presence of ice-nucleating active bacteria. Therefore, ice would form below -5°C with the inhibitors and approximately at 0°C without inhibitors. Surfactant 403 was not tested in the indicated study however, 403 was included here to compare with 401, which has the same alkyl chain length but with a benzyl group. These compounds were examined for their ability to inhibit ice recrystallization (Figure 4-1). A 0.5 mg mL\(^{-1}\) NaCl solution was used rather than a PBS solution (which is typically used in this assay) as the compounds were more soluble in the NaCl solution. The use of 0.5 mg mL\(^{-1}\) NaCl has been previously utilized in this assay and is effective at excluding recrystallization effects that are false positive. This solution is also similar to physiological conditions (saline solution) like PBS. Quaternary ammonium salt 401 was a potent inhibitor of ice recrystallization while 402 did not inhibit ice recrystallization. Ammonium salt 403, containing a hexadecyl chain (similar to 401) and a methyl instead of a benzyl substituent, also exhibited potent activity suggesting that a long alkyl chain is essential for IRI activity. The benzyl substituent in 401 only increased the IRI activity slightly when compared to 403, indicating that this functional group is not necessary for high IRI activity. It is noteworthy that while both 401 and 402 were active anti-INAs, only 401 was a potent inhibitor of ice recrystallization. This suggests that there is no correlation between IRI activity and anti-ice nucleating activity and the mechanism for anti-ice nucleation activity is different from
the mechanism responsible for IRI activity. Quaternary ammonium salts 401 and 402 were also assessed for TH activity using a Clifton nanolitre osmometer\textsuperscript{15} but did not have TH gaps or dynamic ice shaping typical of biological antifreezes. Figure 4-2 illustrates the round or disk-like ice crystals indicative of the absence of interactions with the ice lattice.

![Figure 4-2](image)

**Figure 4-2** Round ice crystals of a) 401 and b) 402 showing no dynamic ice shaping.

Due to the high IRI activity of the quaternary ammonium chloride salts, compounds 401-403 were assessed for their ability to cryopreserve Tf-1α cells (human bone marrow erythroblast) and Hep G2 (human liver carcinoma cells). Tf-1α cells are models for studying myeloid progenitor cells that are derived from hematopoietic stem cells used in regenerative medicine. In order to determine whether these compounds exhibited any cryoprotective ability, a cryopreservation assay needed to be developed. In this assay two different concentrations of analyte (22 mM or 220 mM) were added to the cells in the presence of 0% or 2% DMSO. A rate controlled freezing protocol of -1°C/min to -80°C was utilized and then the cells were placed in the vapour of liquid nitrogen (-196°C). After storage for 1-4 days, the cells were removed and quickly thawed in a 37°C water bath. The number of cells post-thaw as determined using a hemocytometer followed by analysis with flow cytometry to determine the number of viable cells and apoptotic cells. In flow cytometry, cells are passed through a laser one at a time to determine if they are alive, dead, or apoptotic.\textsuperscript{16} Dyes are utilized to distinguish
between these cell types. The 7-AAD (7-aminoactinomycin) dye intercalates into double-stranded DNA and does not enter live cells, thereby indicating dead cells. Annexin V is a cellular protein that binds to phosphatidylserines that are only present on the outer membrane of cells undergoing apoptosis. The remaining live cells will not be marked by these dyes. The percentage of recovered cells reflects on the number of cells intact, either alive or dead, while flow cytometry indicates the percentage of live versus dead or apoptotic cells.

Unfortunately, the most IRI active benzylated derivative 401 had very poor solubility in the cell media and could not be assessed. Therefore only 403, which was highly IRI active, was assessed and the non-IRI active 402 was assessed for comparison. Tf-1α cell recovery, viability and apoptosis with 402 and 403 are presented in Figure 4-3. The recovery of cells (Figure 4-3a) with 402 at 22 mM was similar to the recovery with the 2% DMSO control, however, the recovery at 220 mM could not be calculated due to the solution becoming substantially turbid after thawing, preventing counting of cells via hemocytometer. Cell recovery with 220 mM of 403 was excellent while no cells were recovered at 22 mM. Unfortunately, after flow cytometry analysis, no live cells were present upon treatment with either 402 or 403. The fact that cell recovery was high for these compounds indicates that the mechanism of toxicity of 402 and 403 is not related to their surfactant properties of disrupting cell membranes since the cell membranes were intact in the recovered cells. Further investigation revealed that the initial incubation of the cells with 402 and 403 before cryopreservation did not cause cell death. Cells only died after cryopreservation and thawing. However, to determine the toxicity of these compounds, a MTT assay was performed.
In an MTT assay the compounds are incubated with Hep G2 cells for 24 hours at different concentrations. The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is added to the solutions and only living cells will reduce MTT to a purple-coloured formazan dye. After measuring the absorbance, the toxic concentration of the compounds can be deduced. The results in Figure 4-4 indicate that even at very low concentrations of 0.5 mM, quaternary ammonium chloride salts 401 - 403 are highly cytotoxic. This is interesting as the cell recoveries of 402 and 403 (Figure 4-3a) were high.
**Figure 4-4** Cell viability of Hep-G2 cells incubated with quaternary ammonium chloride salts 401-403 in a MTT Assay. Concentrations are in mM.

**Figure 4-5** Cryopreservation of Hep G2 cells with quaternary ammonium chloride salts 402 and 403 showing a) percent recovery and b) percent viability and apoptosis. Error bars represent the standard error of the mean (SEM).
Additionally, Hep G2 cells were cryopreserved using compounds 402 and 403 to determine if better protection can be offered in a different cell type. Hep G2 cells are derived from a human liver carcinoma line. From Figure 4-5, depicting the percent cell recovery, viability, and apoptosis, it is clear that 402 and 403 do not protect cells from cryoinjury as the cell viabilities at both 22 mM and 220 mM were very low. Despite the high cell recovery for 403 at 220 mM in Figure 4-5a, the viability at the same concentration is very low, where almost all the cells were apoptotic (Figure 4-5b). Collectively, the results indicate that even though a compound is highly IRI active, the surfactant-like properties of these compounds are detrimental in vitro.

The second class of cationic surfactants that were examined as potential anti-INAs to protect crops from frost damage is lysine derivatives 404-409 (Table 4-2). The synthesis involved the esterification of lysine where both amines were protected with a Boc (tert-butyl carbamate) group. The Boc groups were then deprotected to furnish salts 404-409 (Scheme 4-1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Compound</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>404</td>
<td>C₄H₉</td>
<td>407</td>
<td>C₁₀H₂₁</td>
</tr>
<tr>
<td>405</td>
<td>C₆H₁₃</td>
<td>408</td>
<td>C₁₂H₂₅</td>
</tr>
<tr>
<td>406</td>
<td>C₄H₁₇</td>
<td>409</td>
<td>C₁₄H₂₉</td>
</tr>
</tbody>
</table>

These were synthesized and analyzed for IRI activity. In structures 404-409, the lysine C-terminus was esterified with alkyl chains ranging from four to fourteen carbon atoms long and the amino groups were protonated as hydrochloride salts.
The IRI activity of cationic surfactants 404-409 is presented in Figure 4-6. L-Lysine hydrochloride was utilized as a control as it did not exhibit any IRI activity. As the length of the alkyl chain on the C-terminus was increased, the IRI activity increased. A ten-carbon alkyl chain (407) was found to be the most effective inhibitor of ice recrystallization. Analogues possessing alkyl chains longer than ten carbons (408 and 409) exhibited markedly decreased activity although they were fully soluble in the IRI assay solution.

The pH of the solutions with compounds 404–409 were slightly acidic (pH = 3) and attempts to adjust to pH = 7 caused the compounds to precipitate out of solution. To be certain that low pH was not responsible for the IRI activity of 404–409, the IRI activity of a 0.5 mg mL⁻¹ NaCl solution at pH = 3 was examined by adding conc. HCl to the solution. To compensate for any increase in volume and subsequent change in NaCl concentration, a second solution was prepared by adding the same volume of H₂O (pH =

![Scheme 4-1 Synthesis of cationic lysine surfactants.](image)

The IRI activity of cationic surfactants 404-409 is presented in Figure 4-6. L-Lysine hydrochloride was utilized as a control as it did not exhibit any IRI activity. As the length of the alkyl chain on the C-terminus was increased, the IRI activity increased. A ten-carbon alkyl chain (407) was found to be the most effective inhibitor of ice recrystallization. Analogues possessing alkyl chains longer than ten carbons (408 and 409) exhibited markedly decreased activity although they were fully soluble in the IRI assay solution.

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![Figure 4-6 IRI activity of cationic lysine surfactants 404-409.](image)
7). Statistical analysis\(^1\)\(^7\) of these results indicated that the IRI activity of the standard 0.5 mg mL\(^{-1}\) NaCl solution was indistinguishable from both the 0.5 NaCl mg mL\(^{-1}\) solution at pH = 3 and at pH = 7, verifying that pH does not influence activity. The potent IRI activity of 406 and 407 is unprecedented and demonstrates that amino-acid based cationic surfactants can be potent inhibitors of ice recrystallization. It is also interesting to note that while these compounds were weak inhibitors of ice nucleation,\(^8\) they were very effective inhibitors of ice recrystallization indicating that that IRI activity is independent of anti-ice nucleating activity with these cationic lysine derivatives.

4.2. Anionic lysine-based surfactants

There is a large class of gelators based on amino acids. Amino acids naturally have three functional groups that can easily be manipulated and a wide range of gelators have been developed from most amino acids. The Hanabusa group has synthesized a variety of lysine-based gelators with diverse modifications.\(^2\)\(^0\)-\(^2\)\(^3\) The physical and material properties

<table>
<thead>
<tr>
<th>Compound</th>
<th>R(_1)</th>
<th>R(_2)</th>
<th>Compound</th>
<th>R(_1)</th>
<th>R(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>410</td>
<td>C(<em>5)H(</em>{11})</td>
<td>C(_3)H(_7)</td>
<td>415</td>
<td>CH(_3)</td>
<td>C(<em>{11})H(</em>{23})</td>
</tr>
<tr>
<td>411</td>
<td>C(<em>5)H(</em>{11})</td>
<td>C(<em>4)H(</em>{11})</td>
<td>416</td>
<td>C(<em>7)H(</em>{15})</td>
<td>C(<em>{11})H(</em>{23})</td>
</tr>
<tr>
<td>412</td>
<td>C(<em>5)H(</em>{11})</td>
<td>C(<em>3)H(</em>{15})</td>
<td>417</td>
<td>C(<em>{11})H(</em>{23})</td>
<td>C(<em>{11})H(</em>{23})</td>
</tr>
<tr>
<td>413</td>
<td>C(<em>5)H(</em>{11})</td>
<td>C(<em>6)H(</em>{19})</td>
<td>418</td>
<td>C(<em>{11})H(</em>{23})</td>
<td>C(_3)H(_7)</td>
</tr>
<tr>
<td>414</td>
<td>C(<em>5)H(</em>{11})</td>
<td>C(<em>{11})H(</em>{23})</td>
<td>419</td>
<td>C(<em>{11})H(</em>{23})</td>
<td>C(<em>3)H(</em>{15})</td>
</tr>
</tbody>
</table>
of the lysine-based gelators have been extensively studied.\textsuperscript{20-23} The ability of these molecules to sequester water is an interesting property. It is thought that IRI activity may be correlated to the ability to immobilize water. Therefore, compounds 410-421 (Table 4-3) were synthesized and analyzed for IRI activity. These anionic derivatives are acylated at the \(\alpha\)- and \(\varepsilon\)-amino termini with alkyl chains ranging from two to twelve carbons.

It has been previously reported that some of these compounds gel organic solvents as carboxylic acids or carboxylates.\textsuperscript{20} However, the gelling of most aqueous solvents occurred when a water-insoluble carboxylic acid was mixed with a water-soluble carboxylate salt. For example, in Figure 4-7, the water insoluble and water soluble surfactants are not hydrogelators on their own, only when mixed together.\textsuperscript{20} Lysine derivatives 410–419 and 421 (Table 4-2) were synthesized as salts to allow solubilization in the NaCl solution used to dissolve the analyte for the IRI assay. As anticipated, these compounds did not produce gels even at concentrations of 22 mM. Compound 420 was

\begin{figure}
\centering
\includegraphics[width=\textwidth]{hydrogel.png}
\caption{The water insoluble and water soluble surfactants will only form hydrogels if mixed together.}
\end{figure}
tested as a carboxylic acid since it was reported that it was soluble in aqueous NaCl at lower concentrations.\(^{30}\)

### 4.2.1. Synthesis of anionic lysine surfactants

The synthesis of most of the anionic lysine surfactants is shown in **Scheme 4-2.** \(\varepsilon-\)

Carboxybenzyl-protected lysine 422 was treated with the appropriate acyl chloride in a biphasic mixture containing ether and 10% NaOH to install the R\(_1\) alkyl chain. Next, the carboxybenzyl group was removed by hydrogenolysis to furnish free amines 425 or 426, which were acylated a second time to yield carboxylic acids 427-433. The final step converted the carboxylic acids into the corresponding Na, K, or Li carboxylates.

The synthesis of 415, containing an acetamide, was not successful (**Scheme 4-2**) since acetyl chloride is more water-soluble compared to the other alkyl chlorides, and thus more easily hydrolyzed. The biphasic reactions with acetyl chloride resulted in yields lower than 20%. Therefore, carboxylic acid 434 was first protected as a methyl ester under anhydrous acidic conditions and the acetate group was installed at the \(\alpha-\)
amino terminus (435) (Scheme 4-3a).\textsuperscript{20,23} Hydrolysis of the methyl ester under basic conditions and treatment with either NaOH, KOH, or LiOH furnished the respective carboxylates of 415. Since 416 and 417 contain a dodecyl amide, the commercially available 434 was acylated and treated with NaOH to furnish 416 and 417 (Scheme 4-3b). Finally, a derivative of 411 was synthesized that does not contain a carbonyl at the ε-amino terminus (421, Scheme 4-3c). 421 was prepared to determine the effect of the carbonyl in the amide bond on IRI activity. This was accomplished by removing the carboxybenzyl group and alkylating the ε-amino terminus by reductive animation under high-pressure hydrogenation to produce 439. Conversion of the carboxylic acid to a sodium carboxylate furnished 421.
Table 4-3 Structures of anionic lysine surfactants

\[
\begin{align*}
\text{Compound} & \quad \text{R}_1 & \quad \text{R}_2 \\
410 & \text{C}_5\text{H}_{11} & \text{C}_3\text{H}_7 \\
411 & \text{C}_5\text{H}_{11} & \text{C}_3\text{H}_{11} \\
412 & \text{C}_5\text{H}_{11} & \text{C}_7\text{H}_{15} \\
413 & \text{C}_3\text{H}_{11} & \text{C}_3\text{H}_{19} \\
414 & \text{C}_3\text{H}_{11} & \text{C}_{11}\text{H}_{23} \\
415 & \text{CH}_3 & \text{C}_{11}\text{H}_{23} \\
416 & \text{C}_7\text{H}_{15} & \text{C}_{11}\text{H}_{23} \\
417 & \text{C}_{11}\text{H}_{23} & \text{C}_{11}\text{H}_{23} \\
418 & \text{C}_{11}\text{H}_{23} & \text{C}_3\text{H}_7 \\
419 & \text{C}_{11}\text{H}_{23} & \text{C}_3\text{H}_{15} \\
420 & \text{H}_2\text{C}_6\text{H}_4 & \text{N} & \text{O} & \text{H} & \text{O} & \text{H} & \text{N} \\
421 & \text{H}_3\text{C}_6\text{O} & \text{Na} & \text{O} & \text{H} & \text{N} & \text{O} & \text{H} & \text{N} & \text{M} = \text{Li, Na, or K} \\
\end{align*}
\]

Figure 4-8 IRI activity of anionic lysine surfactants as sodium salts (410-421). Error bars indicate that standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student's \( t \)-test \( (p<0.05) \) within each set (separated by vertical lines).

4.2.2. IRI activity of anionic lysine-based surfactants

The IRI activity of 410-421 is presented in Figure 4-8. In compounds 410–414 (Table 4-3), the length of \( \text{R}_1 \) was kept constant at five carbons while \( \text{R}_2 \) was varied between three and eleven carbon atoms. The most IRI active analogue of this series (414) had the longest \( \text{R}_2 \) chain of eleven carbons. \( \text{R}_2 \) chain lengths of three to seven carbons (410–412) resulted in only moderate IRI activity whereas 413, having an \( \text{R}_2 \) length of
nine carbons was much more active. Collectively, these data suggest that from these surfactants, an alkyl chain of eleven carbons at R$_2$ is optimal for IRI activity. Analogues 415-417 demonstrate whether the length of the R$_1$ alkyl chain influenced the IRI activity of 414. In these analogues, R$_2$ contained eleven carbons and R$_1$ was varied with one (415), seven (416), or eleven (417) carbon units. The results in Figure 4-8 indicate that the length of R$_1$ had less of an affect on IRI activity than R$_2$. Although analogue 417, possessing an eleven-carbon alkyl chain at R$_1$, was statistically more active than the rest, analogues 414–417 were all very potent inhibitors of ice recrystallization indicating that a long alkyl chain at R$_1$ is not an essential structural feature. The importance of a long alkyl chain at R$_2$ was further demonstrated in analogues 418 and 419 which both have eleven carbons at R$_1$. Analogue 418 with a three-carbon alkyl chain at R$_2$ was not active, however, as R$_2$ was lengthened to seven carbons (419), the activity increased. However, this analogue was still not as active as 417 where R$_2$ contained eleven carbons. These results further support the fact that a long alkyl chain at the R$_2$ position is critical for high IRI activity. The IRI activity of these compounds appears to be linked to the amphiphilic properties of these molecules given that l-lysine failed to exhibit any IRI activity (Figure 4-6). Attempts to test additional analogues with alkyl chains longer than eleven carbon atoms were unsuccessful as they were not soluble in the NaCl solution at 22 mM. However, to obtain a more accurate account of the IRI activity of these surfactants, a concentration scan for each surfactant needs to be performed. This concentration scan can identify the concentration that results in the highest IRI activity (highest potency).

In summary, the most potent IRI active analogues (414–417) all possessed the eleven-carbon alkyl chain at R$_2$ attached via an amide bond. However, the structural
importance of the amide bond for IRI activity is not known. Previous work by our laboratory with N-octyl gluconamide (NOG) demonstrated that the amide bond linking the hydrophobic alkyl substituent to the hydrophilic carbohydrate substituent was essential for IRI activity in these compounds. Based upon this precedent, a derivative of 411, 421, in which the carbonyl group at the ε-amino terminus was not present (Table 4-3) was tested for IRI activity. 421 exhibited greater IRI activity than the amide analogue, 411 (Figure 4-8). In the 0.5 mg/mL NaCl IRI assay solution, this amine would be protonated. This suggests that a positively charged amine is more IRI active than an amide and provides evidence that the amide bond was not an essential structural feature. In summary, the delicate balance between the hydrophobic and hydrophilic groups is important for potent IRI activity. Analogues of 413 and 414 lacking the carbonyl, containing ten and twelve carbons at the ε-amino terminus were also synthesized, however, they were not soluble in the NaCl solution and could not be tested.

4.2.3. Exploring the relationship between micelle formation and IRI activity

Anti-INAs 401–403 are the only compounds in this study with known critical micelle concentration (CMC) values and they form micelles in solution above the CMC of 0.042 mM, 24 68 mM, 25 and 1.3 mM, 25 respectively. The IRI activity at concentrations below, at, and above the CMC is presented in Figure 4-9. Although the CMC values were reported in water and not in a 0.5 mg mL⁻¹ NaCl solution, it has been reported that CMCs in salt solutions versus pure water vary only slightly. 26,27 The results indicate that there does not seem to be a correlation between micelle formation and IRI activity. For instance, the IRI activity of 402 only changes slightly as the concentration is increased to the CMC (68 mM) from 22 mM and IRI activity remains the same at concentrations
above the CMC (100 mM). Although there is a small difference in the IRI activity of 402 between 22 mM and the CMC, if the formation of micelles had an effect on the activity, we would expect a larger difference in activity between the two concentrations. The IRI activity of 401 increases at and above the CMC values (0.042 and 22 mM, respectively). However, the IRI activity of 403 at its CMC (1.3 mM) is indistinguishable to the IRI activity below its CMC (0.13 mM). Also, when the concentration is increased in 403, the IRI activity also increases. For IRI active compounds, the IRI activity increases simply because of the increase in concentration rather than due to the formation of micelles given that the IRI activity of 403 is the same below and at the CMC. These results are consistent with previous studies on the correlation of CMC with IRI activity.\(^1\)

The increased IRI activity of 401 and 403 with increasing concentrations relative to 402 is likely due to the presence of the longer hydrophobic alkyl chains (sixteen carbons for 401 and 403, while 402 only has a ten-carbon chain). Furthermore, the activity of 401 and 403 is concentration dependent and independent of their ability to form micelles as a 5 mM solution of 403 is well above the CMC but the IRI activity is still not as high as a 22 mM

---

**Figure 4-9** IRI activity of cationic anti-INAs (401–403) below, at, and above the CMC (CMC is indicated with ◆). Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student’s \(t\)-test (\(p<0.05\)) within each set (separated by vertical lines).
solution of 403. The IRI data of 403 can be viewed as a concentration profile. That is, as the concentration of 403 is increased, the IRI also increases.

4.2.4. The effect of functional groups on IRI activity

As anionic lysine derivatives (410–419) were tested as carboxylate salts, it is unknown what effect the carboxylate has on IRI activity compared to a carboxylic acid. Consequently, lysine derivative 415 (a potent inhibitor of ice recrystallization) was prepared as the carboxylic acid (420) and tested for IRI activity. In contrast to carboxylate 415, 420 is reported to gel aqueous solutions and has a minimum gel concentration (MGC) in water and saline at 5.4 mM and 10.8 mM, respectively. Thus, 420 could not be tested at 22 mM in the 0.5 mg mL\(^{-1}\) NaCl solution as it gelled the solution immediately at a concentration of 5 mM and higher. Consequently, it was tested at 1 mM instead (Figure 4-8) where the formation of hydrogels was not observed. Compound 420 was only weakly active when compared to carboxylate derivative 415 at the same concentration suggesting that the carboxylate moiety is beneficial for IRI activity.

4.2.5. Exploring the relationship between carboxylate counterions and gelation on IRI activity.

The effect of counterions on IRI activity was never previously studied. It has been shown that the counterions in lysine gelators affect the minimum gelation concentration.\(^{22}\) Consequently, it was investigated whether changing the counterion would also influence IRI activity. Compounds 412–415 (Table 4-3) were synthesized as lithium and potassium salts and tested for IRI activity (Figure 4-10). The compounds that formed gels are represented by “◆”. Surprisingly, the changes in the counterions affected
each surfactant differently. The IRI activity of the lithium and potassium salts of 412 and 415 was the same as the IRI activity of the corresponding sodium salts. However, the lithium and potassium salts of 413 were less active than the corresponding sodium salt.

An interesting observation was that only the lithium and potassium salts gelled at 22 mM in the NaCl solution after 10 min and the solutions required prolonged heating to dissolve the gel prior to the splat-cooling assay. The sodium salt of 413 did not form hydrogels and remained dissolved. The lithium salt of 414 was less active than the sodium salt, but the potassium salt exhibited similar IRI activity as the sodium salt of 414. This is unlike 413 where the potassium salt had lowered activity. Interestingly, the lithium and potassium salts of 414 still gelled the NaCl standard solution at 22 mM whereas the sodium salt did not. This is the first indication that gelation may not be associated with IRI activity.

When observing the alkyl chain lengths, 412-414 had \( R_2 \) alkyl lengths of seven, nine, and eleven carbons, respectively, while the \( R_1 \) length remained at five carbons. When the averages of all the salts of one compound were taken, 414 still had the highest IRI activity of anionic lysine surfactants/gelators (412–415) as lithium, sodium, and potassium salts. Compounds which gelled at 22 mM in a NaCl standard are indicated with ◆. Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student’s t-test (p<0.05) within each set (separated by vertical lines).
IRI activity since it has the longest R₂ alkyl chain. Surfactant 415, containing eleven carbons at R₂ and one carbon at R₁, in which the sodium salt has the highest IRI activity out of this set, also had the highest IRI activity when including the average of its salts. From these observations, it appears that the counterions have more of a positive effect on compounds that have longer alkyl chains. For example only one counterion in 413 was more potent than the rest while 414, containing a longer R₂ alkyl chain, possessed two counterions, lithium and potassium, that have more potent IRI activity.

Thus far, there does not appear to be a correlation between IRI activity and gelation. Following this observation, 415 (sodium salt of 420) did not gel aqueous solutions and was much more active than 420 (Figure 4-8). As well, the lithium and potassium salts of 413 were gelators, however, they were less active than the sodium salt of 413, which was not a gelator (Figure 4-10). Similarly, the sodium salt of 414 was significantly more IRI active than the lithium salt that formed gels. It is important to note that the lithium and potassium salts of 413 and 414 were tested at the concentration that they gelled at, confirming that gelation ability is not a prerequisite for potent IRI activity. As previously stated, 420 could not be tested closer to its minimum gelation concentration due to the immediate gelation of the solution.

4.2.6. Investigating the relationship between enthalpy of hydration and IRI activity

The enthalpy of hydration refers to the amount of heat (energy) released when one mole of gaseous ions are hydrated by water molecules. Thus, the greater the value, the more hydrated the ion. The enthalpy of hydration for the ions Li, Na, and K are shown in Figure 4-11 and this may aid in explaining the effects of the counterions. From these
three cations, the lithium is the most hydrated while potassium is the least hydrated. Since lithium is the most hydrated, and high hydration is associated with potent IRI activity, the lysine with the lithium cation should be the most active. However, in Figure 4-10, the sodium salt of 413 was the most active while the lithium salt, along with the potassium salt, was significantly less active. Also, the lithium salt of 414 was the only salt with low IRI activity. This poor correlation suggests that enthalpies of hydration are not a good predictor of how the counterions of carboxylates will affect IRI activity with these anionic lysine surfactants. However, the mechanism may be more complicated with these compounds as this involves the delicate balance of hydrophobic and hydrophilic groups. This balance was already shown by the importance of alkyl chain lengths in the anionic lysine derivatives. Consequently, the use of simpler structures as a model may provide more insight, and this will be discussed in chapter 5. In summary, it is clear that changing the counterion affects IRI activity as well as gelation behavior, but a clear correlation is absent. The change in IRI activity appears to be dependent upon substrate structure.

4.2.7. Cryopreservation of cells with anionic lysine derivatives

Anionic lysine surfactants 410, 411, and 413, possessing low, moderate, and high IRI activity, respectively, were assessed for their ability to protect TF-1α cells from cryoinjury. The results in Figure 4-12a indicated that the recovery of cells (intact cells,
either live, dead, or apoptotic, after cryopreservation and thawing) with 410 and 411 was poor to moderate while with 413, no cells were recovered. This is interesting as 413 contained the longest alkyl chain length at R2 of nine carbons. Unfortunately, none of these compounds afforded protection to cells better than a 2% DMSO control as seen in

**Figure 4-12** Cryopreservation of TF-1α cells cells with anionic lysine surfactants 410, 411, and 413 showing a) percent recovery and b) percent viability and apoptosis. Error bars represent the standard error of the mean (SEM).
**Figure 4-12b.** This is most likely due to the surfactant nature of these derivatives. Although these specific derivatives cannot be used with cells in cryopreservation, they may be used in other applications, which will be discussed in the following chapters. A MTT assay was performed to determine if the low viability was caused by the crytotoxicity of compounds 410, 411, and 413. The results of the MTT assay are presented in **Figure 4-13.** Compound 410 begins to become cytotoxic above 22 mM while 411 increases in cytotoxicity above 10 mM. Since a 22 mM concentration was used in the cryopreservation assay, 410 should not be cytotoxic to the cells, and the negligible live cell population at that concentration indicates that the cells die during freeze-thaw, rather than by the short incubation with the compound. It is important to note that the MTT assay was performed with Hep G2 cells while the cryopreservation assay was performed with Tf-1α cells and there may be differences in cytotoxicity in these two cell lines. Finally, 413 was shown to be cytotoxic at concentrations higher than 0.5 mM. Collectively, these results indicate that the least cytotoxic compound contains the shortest alkyl chains (410 possessed three carbons at R₂) and the most cytotoxic compound...
contains the longest alkyl chain (413 possesses nine carbons at R₂). This is unfortunate as the compounds with the longest alkyl chains are the most IRI active.

4.3. Glycoconjugate lysine surfactants

With the anionic lysine surfactants, it was found that a delicate balance between hydrophobicity and hydrophilicity was important for IRI activity. Since monosaccharides possess moderate IRI activity, we wanted to investigate whether attaching a lysine surfactant would improve the IRI activity. This way, the resulting glycoconjugate would contain a hydrophobic and hydrophilic region (Figure 4-14a). It is hypothesized that creating an amphiphilic molecule, as the IRI-active anionic derivatives, would improve IRI activity. Glycoconjugates 440–443 (Figure 4-14b) were envisioned with either a galactose or glucose hydrophilic moiety and a hexyl or dodecyl hydrophobic moiety. Galactose and glucose were chosen since they are the most hydrated monosaccharides and possess the highest IRI activity of the monosaccharides.28,30 It is important to note that the lysine surfactant is glycosylated to the carbohydrate by the α-amino terminus, not the ε-amino terminus, as it was previously demonstrated (section 4.2) that a long alkyl chain on the ε-amino terminus is necessary for potent IRI activity.

Figure 4-14 a) Carbohydrate glycoconjugate with hydrophilic and hydrophobic moieties. b) Structures of glycoconjugates 440–443.
4.3.1. **Synthesis of lysine glycoconjugates**

The retrosynthesis of the lysine glycoconjugates is depicted in Scheme 4-4a. It is envisioned that the glycoconjugates would be formed from coupling a C-linked carboxylic acid of a carbohydrate with the free amine of the lysine surfactant. Furthermore, the carbohydrate would be prepared from bromo-galactose or glucose, 314 or 452. It is imagined that the lysine component would be synthesized by various protecting group manipulations and alkylation of the ε-amino terminus beginning from 422.

The synthesis of the lysine component is depicted in Scheme 4-4b and begins with the installation of a t-buty carbamate group (Boc) at the α-amino terminus of 422 followed by the removal of the carboxybenzyl group at the ε-amino terminus by hydrogenation to furnish 445. Acylation of the resulting free amine under basic conditions with either a hexyl or dodecyl alkyl chain and benzyl protection of the carboxylic acid yielded 448 or 449. Finally, the t-buty carbamate group was removed under acidic conditions to give 450 or 451 as trifluoroacetic acid salts. The remainder of the synthesis involved the preparation of the monosaccharide and the glycosylation (Scheme 4-4c). In the synthesis of the carbohydrate moiety, aceto-bromo sugars 314 and 452 were treated with allyl tributyl tin and triethyl borane under a slow stream of oxygen to furnish C-allyl 543 and 544 through a radical process. Oxidation of the resulting alkene with sodium periodate and ruthenium tetroxide gave carboxylic acids 455 and 456. The carboxylic acid and lysine amine were coupled with the either the coupling agents CDI (1,1’-carbonyldiimidazole) or HCTU under basic conditions to yield glycoconjugates 457-460. Treatment with sodium methoxide removed the acetate groups.
Interestingly, this reaction resulted in a mixture of products that included analogues...
without the benzyl groups and some analogues containing methyl esters. The remaining benzyl groups were removed by hydrogenation that resulted in the formation of methyl esters 465-468. The carboxylate salts, 440-443, were formed by treating the methyl esters with 4 M NaOH or KOH, or 2 M LiOH.

4.3.2. **IRI activity of lysine glycoconjugates**

![Graph showing IRI activity of lysine glycoconjugates](image)

**Figure 4-15** IRI activity of lysine glycoconjugates 440-443 in PBS and NaCl Standard solutions, and lysine surfactants 411 and 414. In 440 – 443 the notation (for example Gal-Hex) represents the carbohydrate (Gal) and the R chain length (Hex) of the glycoconjugate. Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student’s t-test (p<0.05) within each set (separated by vertical lines). Note: in PBS, 441 and 443 are statistically different from 442 and Gal. 422 and Gal are statistically different from 411 and Glc.

It was hypothesized that attaching a lysine surfactant to a monosaccharide would improve the IRI activity of the monosaccharide and **Figure 4-15** indicates that this hypothesis is correct. The derivatives are tested in the 0.5 mg/mL NaCl solution and in a PBS solution, which is the standard testing solution for monosaccharides and disaccharides. In PBS, galactosylated hexanoyl-lysine 440, and galactosylated
dodecanoyl-lysine 441, had higher IRI activity than galactose (Gal). This was similarly seen with the glucosylated glycoconjugates, where 442 and 443 had higher IRI activity than glucose (Glc). It was also expected that the carbohydrates glycosidated with dodecanoyl-lysine, 441 and 443, would have higher IRI activity than the carbohydrates glycosidated with hexanoyl-lysine, 440 and 442, and this was also seen to be true. The long alkyl chain is still important for IRI activity in these glycoconjugates. The comparison of the glycoconjugates to the lysine surfactants themselves, 411 and 414, yields interesting results. Both glycoconjugates containing the hexanoyl-lysine surfactant, 440 and 442, have higher IRI activity than hexanoyl-lysine (411) itself. However, the inverse is seen with glycoconjugates containing dodecanoyl-lysine, 441 and 443. These two glycoconjugates have lower IRI activity than dodecanoyl-lysine, 414. Therefore, it appears that adding a carbohydrate to a short alkyl chain-containing lysine will improve IRI activity; however, IRI activity will decrease if the carbohydrate is added to an already IRI active long alkyl chain-containing lysine. Finally, galactose is more IRI active than glucose and this is expected to follow with the glycoconjugates. The hexanoyl-galactose glycoconjugate, 440, has higher IRI activity than the hexanoyl-glucose glycoconjugates, 442. Interestingly, this trend is not seen with the dodecanoyl glycoconjugates where dodecanoyl-galactose, 441, and dodecanoyl-glucose, 443, have the same IRI activity. This may be due to the fact that the dodecanoyl group has a stronger effect on IRI activity and ‘surpasses’ the IRI activity effects brought about by the carbohydrates.

In the NaCl standard, all compounds follow the same trends as seen in PBS except for the dodecanoyl-galactose glycoconjugate 441. This glycoconjugate is less active than its hexanoyl counterpart, 440. This is surprising as this is the first incidence
that a lysine containing a dodecanoyl alkyl chain at the ε-amine has lower IRI activity than one with a shorter alkyl chain. However similarly as in PBS, both of the galactose glycoconjugates, 440 and 441, possess higher IRI activity than galactose and the glucose glycoconjugates, 442 and 443, are more active than glucose. An interesting observation is that the glycoconjugates have higher IRI activity in the NaCl standard solution but the monosaccharides remain the same in both PBS and NaCl. This observation will be examined more closely in the following chapter.

Overall, the synthesis and testing of the lysine glycoconjugates was important to gain a better understanding of how hydrophobic and hydrophilic moieties affect IRI activity. These data indicated that long hydrophobic alkyl chains are still necessary for IRI activity in glycoconjugates. The results can also be summarized in two ways. Firstly, the addition of any lysine surfactant to galactose or glucose will improve the IRI activity relative to the carbohydrate. Secondly, the addition of a carbohydrate to a lysine will only improve the IRI activity of the lysine surfactant if the alkyl chain is fairly short.

4.4. Glutamine-based surfactants

As the anionic lysine surfactants demonstrated potent IRI activity, we wanted to determine if other amino acid-based surfactants would also yield the same results, and therefore glutamine surfactants were synthesized and analyzed for IRI activity.

![Figure 4-16](image-url) Structural features of glutamine compared to lysine.
activity. It is important to note that the starting material for the glutamine surfactants is glutamic acid, containing a δ-carboxilic acid, not a δ-amide. In comparison to lysine, glutamic acid contains a carboxylic acid at the δ-position, while lysine contains an amine at the ε-position (Figure 4-16). Therefore the linker in glutamic acid is one carbon longer than in lysine. Most importantly, after amidation of δ-carboxylic acid in glutamic acid, the orientation of the resulting amide bond is reversed in comparison to lysine (Figure 4-16). The resulting glutamate can be compared to lysine to determine what effect the orientation of the amide bond has on IRI activity. Secondly, with the lysine anionic surfactants, it was shown that a long alkyl chain at the ε-amino terminus was important, while the length of the alkyl chain at the α-amino terminus was not as important for potent IRI activity. It can be investigated whether this same trend applies to the glutamic acid surfactants. Therefore, glutamic acid surfactants 469-474 (Table 4-4) were synthesized and analyzed for IRI activity.

**Table 4-4 Structures of glutamine surfactants**

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_1 )</th>
<th>( R_2 )</th>
<th>Compound</th>
<th>( R_1 )</th>
<th>( R_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>469</td>
<td>( \text{C}_3\text{H}_7 )</td>
<td>( \text{C}<em>{12}\text{H}</em>{25} )</td>
<td>472</td>
<td>( \text{C}<em>{11}\text{H}</em>{23} )</td>
<td>( \text{C}<em>{12}\text{H}</em>{25} )</td>
</tr>
<tr>
<td>470</td>
<td>( \text{C}<em>2\text{H}</em>{11} )</td>
<td>( \text{C}<em>{12}\text{H}</em>{25} )</td>
<td>473</td>
<td>( \text{C}<em>7\text{H}</em>{15} )</td>
<td>( \text{C}<em>8\text{H}</em>{17} )</td>
</tr>
<tr>
<td>471</td>
<td>( \text{C}<em>7\text{H}</em>{15} )</td>
<td>( \text{C}<em>{12}\text{H}</em>{25} )</td>
<td>474</td>
<td>( \text{C}<em>{11}\text{H}</em>{23} )</td>
<td>( \text{C}<em>8\text{H}</em>{17} )</td>
</tr>
</tbody>
</table>
4.4.1. Synthesis of glutamine surfactants

Scheme 4-4 Glutamine surfactants. a) Synthesis of glutamine surfactants 469 - 474. b) Proposed mechanism of cyclization of glutamine under neutral conditions. Upon the addition of acid, the cyclization is inhibited.

The synthesis of the glutamic acid surfactants is depicted in Scheme 4-4a. Carboxybenzyl and benzyl protected glutamic acid 475 was amidated with an alkyl amine in the presence of HCTU and diisopropylethylamine. The benzyl and carboxybenzyl protecting groups of 476 or 477 were then removed by hydrogenation under acidic conditions. It was crucial to perform the hydrogenation under acidic conditions as the lack of acid lead to a cyclization. The proposed cyclization is presented in Scheme 4-4b in which the free amine attacks the carbonyl to form a five-membered lactam. Under acidic conditions, the nucleophilicity of the amine is decreased due to its conversion into a hydrochloride salt. Derivatives 478 and 479 were acylated a second time under basic conditions to furnish di-alkyl glutamines 480-485. It is important to note that some of the
di-alkyl glutamines degrade on silica and they cannot be purified by silica column chromatography. To overcome this problem, only 0.9 or less equivalents of the acyl alkyl chloride was used (normally 2 equivalents of the acyl chloride is used to increase yield). This way, any remaining unreacted glutamine would be removed by the acidic wash during work-up. Although the yields were poorer for this reaction, the products were pure and were carried onto the next step without further purification. Lastly, 480-485 were treated with NaOH to furnish sodium carboxylates 469-474.

4.4.2. IRI activity of glutamine surfactants

Since lysine itself has a longer linker between the α-carbon and the amine at the ε-carbon, the total length of the alkyl region will be longer by 1 carbon compared to the glutamate surfactants (Figure 4-17). However, it has been previously shown with the lysine surfactants that the IRI activity gradually changes as the length of the alkyl chain is modified. Therefore, even with the one carbon discrepancy, the two surfactants should still be comparable. The IRI activity of the glutamine surfactants is presented in Figure 4-18. The glutamine surfactants are represented by solid colour bars and white bars represent the lysine surfactants, which match most closely to the glutamine surfactants in terms of R₁ and R₂ alkyl chain lengths. The dotted line indicates the similar glutamine and lysine surfactants.
Table 4-4 Structures of glutamine surfactants

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>469</td>
<td>C₃H₇</td>
<td>C₁₂H₂₅</td>
<td>472</td>
<td>C₁₁H₂₃</td>
<td>C₁₂H₂₅</td>
</tr>
<tr>
<td>470</td>
<td>C₃H₁₁</td>
<td>C₁₂H₂₅</td>
<td>473</td>
<td>C₇H₁₅</td>
<td>C₈H₁₇</td>
</tr>
<tr>
<td>471</td>
<td>C₇H₁₅</td>
<td>C₁₂H₂₅</td>
<td>474</td>
<td>C₁₁H₂₃</td>
<td>C₈H₁₇</td>
</tr>
</tbody>
</table>

Figure 4-18 IRI activity of glutamic acid surfactants (469-474). Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student’s t-test (p<0.05) within each set.

Analogues 469-472 possess a dodecyl group at the δ-position since the most IRI potent lysine surfactants contained a dodecanoyl chain at the ε-amino terminus. The R₁ chain length for these analogues increase from three to eleven carbons. Surprisingly, the least potent analogue of this set, 472, had the longest R₁ alkyl chain of 11 carbons. This contrasts greatly with the lysine analogues in which the most potent analogue, 417, had 11 carbons at R₁ and R₂. The most active glutamine analogue of this set was 470 possessing 5 carbons at R₁, however, shortening this chain (469) or lengthening this chain (471 and 472) decreased IRI activity. When comparing 470 and 471 to their lysine counterparts, the IRI activities did not vary substantially. Since a long alkyl chain at R₂ is not necessarily beneficial for IRI activity in glutamine surfactants, 473 and 474 were
synthesized where $R_2$ contained eight carbons and $R_1$ was varied from seven to eleven carbons. The analogue with the shorter $R_1$ alkyl chain, 473, was slightly more active than 474. This corresponds to the previous observations where a shorter alkyl chain at $R_1$ resulted in higher IRI activity. Analogue 419, the comparable lysine surfactant to glutamine analogue 474, is significantly less active indicating that there is no correlation between lysine and glutamic acid surfactants.

In summary, compared to lysine where a long alkyl chain at $R_2$ was important and the alkyl chain at $R_1$ did not affect IRI activity, in the glutamic acid surfactants, the optimal $R_1$ chain length was five carbons. It is clear from the data that placement of two long alkyl chains on a glutamic acid analogue is detrimental to IRI activity (472). As well, contrary to what was seen with the lysine surfactants, a shorter $R_2$ alkyl chain was beneficial for IRI activity. This is depicted in analogues 472 and 474, where the $R_1$ chains remained constant at eleven carbons as the $R_2$ alkyl chain was modified from twelve (472) to eight (474) carbons. The analogue with the shorter (eight) $R_2$ carbons had higher IRI activity. A possible explanation for this observation is the reversed amide bond in glutamic acid. As a delicate balance between hydrophobic and hydrophilic groups is required, the reversed amide can change this balance, requiring $R_2$ to be shorter. The other difference between the lysine and glutamic acid analogues is that the $R_2$ alkyl group in glutamic acid is attached to the $\delta$-carbon rather than the $\varepsilon$-carbon (in lysine). The distance between the amide bond and the carboxylate and $\alpha$-amine is shorter and this may affect the balance between hydrophobicity and hydrophilicity. These data indicate that structural features found in lysine surfactants may not be necessarily generalized to other amino acid surfactants.
4.5. **Gemini surfactants**

Other types of surfactants include ‘gemini surfactants’ which contain, in order, a long alkyl chain, an ionic group, a spacer, a second ionic group, and another hydrocarbon tail whereby the two hydrocarbon chains cannot interact with each other due to the rigid linker and association into micelles is decreased (Figure 4-19). These would be interesting to analyze for IRI activity as the head groups can be modified, unlike the amino acid surfactants where the alkyl chain length can only be modified. The gemini surfactants were provided by GreenCentre Canada, Kingston, Canada and are presented in Figure 4-20. Gemini surfactants 486-489 and 491 posses a quaternary amine hydrophilic head group and 490 possesses a sulfate head group. The quaternary amines are functionalized with methyl (486-491), propanol (487), benzyl (489), and carboxylate (491) groups and the amine counterions are either iodide or chloride. The linker which connects the hydrophilic groups to the hydrophobic alkyl groups is pentaerythritol (2,2-bis(hydroxymethyl)propane-1,3-diol). 486 and 487 contain octyl alkyl chains while 488-490 possess dodecyl alkyl chains. This series of surfactants give the ability to study the effect of alkyl chain length and type of counterion on IRI activity. Finally, 486-489 are cationic surfactants as they possess a positively-charged amine, while 490 is an anionic surfactant due to the negatively-charged sulfate. 491 is zwitterionic as its head group contains both a negatively- and positively-charged species.
4.5.1. IRI activity of Gemini surfactants

The IRI activity of gemini surfactants 486-490 is presented in Figure 4-21 where the cationic, anionic, and zwitterionic head groups are represented by solid, white, and textured bars. The most striking observation is that 486 and 487 have the lowest IRI activity and they are the only gemini surfactants that have the shorter octyl alkyl chain.
Surfactant 488, the exact analogue of 486 but containing dodecyl chains is significantly more IRI active. This follows the conclusions from the lysine surfactants where longer alkyl chains were beneficial for IRI activity. Of the surfactants with dodecyl chains, 488 was the most potent surfactant. 488 possessed trimethylalkyl ammonium as head groups while 489, the other dodecyl-containing cationic surfactant comprised of benzyl head groups, is much less active. A possible explanation is that maintaining the hydrophilicity of the head groups is important for IRI activity as the benzyl groups impart a more hydrophobic character and lower the IRI activity. The other difference between 488 and 490 is the counterion; 488 has an iodide counterion while 489 contains a chloride counterion. While the counterion effects cannot be investigated in this study due to an insufficient number of comparable surfactants, it may be possible that the counterion may also affect the difference in IRI activity between 488 and 489. The anionic gemini surfactant 490, possessed moderate IRI activity indicating that a gemini surfactant comprising of either a positively- or negatively-charged head group can be IRI active. The zwitterionic surfactant 491 was the most weakly IRI active dodecyl-containing surfactant. This is interesting and expected as the net charge on the head groups is zero, rendering the head group less polar, and less hydrophilic. In summary, a long alkyl chain is important for IRI activity in gemini surfactants and the cationic or anionic head groups are tolerated for IRI activity, however, if the head group has a net charge of zero, the IRI activity decreases.

As gemini surfactant 488 was the most IRI active, a concentration scan was performed to identify the concentration that would give the most potent IRI activity and to determine if the IRI activity remains constant at lower concentrations. Surfactant 488
was tested at concentrations ranging from 30 mM to 2 mM and the data is presented in Figure 4-22. The results indicate a positive correlation between IRI activity and concentration where increasing the concentration increases the IRI activity. Although 488 at 30 mM was statistically the most active, the activity at this concentration was not significantly higher than 488 at 22 mM. Furthermore, the 30 mM solution was viscous and testing at higher concentrations would not be possible. At 2 mM, 488 lost its IRI activity and is indistinguishable from the PBS control. Overall, the optimal concentration for 488 is from approximately 17.5 mM to 22 mM, as viscosity will not be a concern. This data may be important if the surfactant is used in application other than cryopreservation.

4.6. Chapter Summary

This chapter explored the IRI activity of amino acid-based anti-INAs, surfactants/gelators and glycoconjugates, and gemini surfactants. For the anti-INAs, (cationic lysine
surfactants and quaternary ammonium salts) the data indicated that IRI activity is not related to anti-ice nucleation activity. As well, these compounds began to indicate that long alkyl chains are important for IRI activity. With the lysine surfactants/gelators, it was clearly shown that potent IRI activity is dependent upon the presence of long alkyl chains and increased hydrophobicity. For di-acylated anionic lysine, the most active compound had the long alkyl chain attached to the ε-amino terminus, while alkyl chains attached to the α-amino terminus did not affect IRI activity significantly. These data also indicated that IRI activity is not correlated to the ability to form hydrogels or micelles and the best ice recrystallization inhibitors will be amphiphilic molecules with a finely tuned balance between hydrophobic and hydrophilic components. The importance of long alkyl chains was further supported by the lysine glycoconjugates and gemini surfactants. Interestingly, the glutamine surfactants did not follow this trend. One of the unresolved

![Graphical summary of the influences on IRI activity.](image)

**Figure 4-23** Graphical summary of the influences on IRI activity.
observations in this chapter involves how the counterions of the lysine surfactants modulate IRI activity. In Chapter 5, further experiments are performed to try to resolve this problem.

4.7. References

16. Biosciences, B. San Jose, California, 2000; Vol. 11-11032-01.

Chapter 5. Investigating the effect of counterions and salts on IRI activity

As seen in previous chapters, structural modifications made to various disaccharides and surfactants/gelators may modulate IRI activity. Chapter 3 described how modifying the glycosidic linkage in disaccharides modulates IRI activity. The lysine-based surfactants and gelators in Chapter 4 indicated that alkyl chain lengths play a crucial role in IRI activity. However, the structural features of these compounds are not the only factors that affect IRI activity. These other factors include carboxylate counterions that are associated with small molecules, as seen in Chapter 4 with the di-alkyl anionic lysine derivatives, and the salts that are present in standard solutions used in the IRI assay. This chapter will describe how carboxylate, amine, and IRI assay salt counterions modulate IRI activity.

5.1. The effect of PABA counterions on IRI activity

Chapter 4 indicated that modifying the carboxylate counterion of di-alkyl anionic lysine surfactants from lithium, sodium, to potassium modulates IRI activity. However, a conclusion for how the counterions affected the IRI activity could not be made since the trends did not correlate to the counterion enthalpy of hydration. Therefore, this counterion study was performed on simpler compounds, with the expectation that this could help elucidate how counterions affect IRI activity. The simpler compounds are based on para-amino benzoic acid (PABA). The presence of an amine and carboxylic group on PABA allows the study of both cationic and anionic counterions by protonating
the amine with a hydrogen halide or by deprotonating the carboxylic acid with an alkali base (Scheme 5-1a). A single six-carbon alkyl chain can be easily installed on each of these functional groups resulting in a simple aryl core, which contains the hydrophilic salt at one end and the hydrophobic alkyl chain at the other end. In comparison to the di-alkyl lysine surfactants, the PABA analogues only contain one short alkyl chain, a simple aryl core, and one functional group that is a salt.

The synthesis of the PABA salts is detailed in Scheme 5-1b,c. To produce the carboxylate with a cationic counterion, PABA was treated with hexanoyl chloride under basic conditions to install the hexanoyl alkyl chain in 82% yield and the carboxylic acid was then converted into the carboxylate salts 501, 502, and 503 with 2 M LiOH or 4M NaOH or KOH (Scheme 5-1b). In the synthesis of the amine salts, the amine of PABA was first protected as a Boc-carbamate and the carboxylic acid was amidated with hexyl amine with HCTU and DIPEA in DCM in 86% yield (Scheme 5-1c).
amine in the presence of HCTU under basic conditions to furnish 509 in 86% yield (Scheme 5-1c). The final step involved treating Boc-protected 509 with anhydrous HCl, HBr, or HI to simultaneously remove the Boc group and protonate the resulting amine.

5.2. Effect of PABA counterions on IRI activity

<table>
<thead>
<tr>
<th>Cations</th>
<th>Li⁺</th>
<th>Na⁺</th>
<th>K⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydration</td>
<td>-545</td>
<td>-418</td>
<td>-351</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anions</th>
<th>Cl⁻</th>
<th>Br⁻</th>
<th>I⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydration</td>
<td>-338</td>
<td>-304</td>
<td>-261</td>
</tr>
</tbody>
</table>

Values are in kJ mol⁻¹

*Figure 5-1* Enthalpy of hydration of cations and anions

According to the enthalpy of hydration (*Figure 5-1*), the order of cations from least to most hydrated is K, Na, and Li.¹ Since IRI activity is correlated to the hydration of monosaccharides and disaccharides,² the most potent PABA analogue should contain the Li counterion and the least IRI active analogue should possess the K counterion. Similarly, the order of anions from least to most hydrated is I, Br, and Cl and it is hypothesized that the iodide PABA analogue would be the least IRI active while the chloride PABA analogue would be the most IRI active. *Figure 5-2* shows the IRI activity of all PABA analogues and indicates that IRI activity is indeed correlated to the hydration of the counterions. In the NaCl standard solution, the analogue with the most hydrated cation (Li) exhibited the best IRI activity and the analogue with least hydrated counterion (K) exhibited the weakest IRI activity. Similar results were observed when anions were systematically changed such that the analogue with Cl was the most active. When PBS
was used as the standard, all PABA compounds were inactive, and statistically indistinguishable except for 501, containing the Li counterion. Due to the inactivity of these compounds in PBS, the effect of the counterions could not be determined. These results differ from the counterion data obtained from the di-alkyl lysine surfactants/gelators in Chapter 4. From that data, the IRI activity of two di-alkyl lysine surfactants was not affected with the change in counterion (Li, Na, or K), while with 413, only the Na counterion was more potent and in 414, the Na and K counterions had potent IRI activity, but not the Li counterion. But with the PABA compounds that are structurally less complex than the di-alkyl lysine surfactants, the presence of only one alkyl chain may enable the counterion hydration to have more of an effect on IRI activity. Another interesting study that can be performed is to dissolve the PABA carboxylic acid 507 in the IRI assay solution and add one equivalent of LiOH, NaOH, or KOH. Since the counterions will be dispersed in solution, the results from this study should be comparable to the current data. The difference in IRI activity between the NaCl and PBS solutions is also an interesting observation and will be discussed in the following section.

**Figure 5-2** IRI activity of PABA-based compounds 501 – 505 with different counterions. Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student’s t-test (p<0.05) within each set (separated by vertical lines). Note: In NaCl, 502 and 504 are statistically indistinguishable, and in PBS, 501 and 502 are statistically indistinguishable.
5.3. Determining the “best” IRI assay solutions for standardization of the assay

Di-alkyl anionic lysine surfactants in Chapter 4 were tested in a 0.5 mg mL\(^{-1}\) NaCl solution for the IRI assay since some compounds had improved solubility in this solution. This NaCl solution ensures that only compounds that are IRI active will produce small crystals. This is further depicted in Figure 5-3.\(^3\) This figure shows the ice crystals from ice wafers after the splat-cooling assay containing 0.02 mg mL\(^{-1}\) of an active antifreeze peptide and a peptide that does not possess any antifreeze activity at different NaCl concentrations. The two lowest NaCl concentrations (0 and 0.01 mg/mL) result in small ice crystals with both the antifreeze active peptide and inactive peptide giving the appearance that the inactive peptide is actually active. However, an increase in the NaCl concentration to 0.1 and 0.5 mg/mL results in large ice crystals for the inactive peptide, which shows that the inactive peptide is indeed inactive. This data is significant and reveals that salts are required in the IRI assay solution.

![Image of Figure 5-3](image-url)

**Figure 5-3** Effect of NaCl on recrystallization inhibition by an active and inactive antifreeze protein. Protein concentration is 0.02 mg/mL. Only NaCl 0.1 and 0.5 mg/mL destroy false-positives. Picture from Knight, 1995.\(^3\)
Another common solution used in the IRI assay is PBS (phosphate buffered saline). This solution is buffered with phosphate salts, imitating physiological conditions. Surprisingly, lysine surfactants 412, 413, and 415 had very different IRI activity in the NaCl solution compared to the PBS solution as presented in Figure 5-4. The IRI activities in the PBS solution were much lower and even the highly active 415 had only moderate IRI activity in PBS. Furthermore, galactose and glucose had the same IRI activity in NaCl and PBS. This striking difference between the lysine surfactants and the monosaccharides indicates that the increase in IRI activity caused by NaCl is dependent on the surfactant nature of the lysine analogues. This raises the question of whether different salts dissolved in these solutions influence the IRI activity of a compound.

Furthermore, with the IRI assay, there are many solutions that are used by various research groups. Some of these solutions include 30% sucrose, 4-6 20% sucrose, 7 Tris-buffered saline, 8 8.6 mM NaCl (0.5 mg/mL), 3,9 15 mM NaCl, 10 200 mM NaCl, 10 and 0.5 mg/mL CaCl₂. 11 As different solutions are used, it is difficult to compare IRI activities between different research groups that use this assay. Therefore, it would be beneficial to standardize the IRI assay solution so that results can be compared across all research groups. In the following sections, the effect of PBS on IRI activity will be explored.

**Figure 5-4** IRI activity of anionic lysine surfactants and monosaccharides in NaCl or PBS solution. Error bars indicate the standard error of the mean (SEM).
In an attempt to investigate which salts in PBS cause the low IRI activity of lysine analogues, the salts present in PBS were tested individually at the concentration found in PBS with an anionic lysine surfactant as analyte, that was IRI active in the 0.5 mg mL\(^{-1}\) NaCl standard solution (Figure 5-5). The solid bars represent the PBS standard or 415 in the PBS and NaCl standard solutions. All salt mixtures were compared to the PBS control. The IRI activity of 415 in the individual salts, 8 mg/mL NaCl, 0.2 mg/mL KCl, 1.4 mg/mL Na\(_2\)HPO\(_4\), and 0.25 mg/mL KH\(_2\)PO\(_4\), was still higher than in PBS. However, 415 in the NaCl solution had the highest IRI activity. Interestingly, this solution had the largest amount of salt by weight. Collectively, these results indicate that the weak IRI activity of 415 in PBS is not due to a single salt, but rather to a combination of salts. Thus, 415 was tested in mixtures of salts which included NaCl + Na\(_2\)HPO\(_4\), NaCl + KCl,
KCl + KH$_2$PO$_4$, and Na$_2$HPO$_4$ + KH$_2$PO$_4$. The salts were grouped together by either similar cations (Na and K) or anions (Cl and PO$_4$) and are at concentrations that exist in PBS. 415 in the NaCl + Na$_2$HPO$_4$ had statistically indistinguishable IRI activity to 415 in PBS while the other salt combinations yielded higher IRI activity. It appears that this particular salt combination allows for more ice recrystallization to occur resulting in larger ice crystals and is responsible for the lower IRI activity of lysine analogues in the PBS solution. In comparison to the 0.5 mg/mL NaCl standard, the NaCl + Na$_2$HPO$_4$ combination contains a total of 9.4 mg/mL of salt (8 mg/mL of NaCl and 1.4 mg/mL Na$_2$HPO$_4$) which may simply indicate that a solution with a larger concentration of salts decreases the IRI activity of a compound. Despite the fact that KCl + KH$_2$PO$_4$ is less IRI active than Na$_2$HPO$_4$ + KH$_2$PO$_4$ which contains a higher concentration of salts, the difference between these two activities is very small. However, this discrepancy may also be caused by the different hydration values of the salts.

Perhaps a more accurate analysis of these observations can involve the ionic strength, instead of the total salt concentration of these solutions. The ionic strength of a solution is a measure of the concentration of ionic charge and is measured in molarity. This is defined by the formula:

$$I = \frac{1}{2} \cdot \sum c_i \cdot Z_i^2$$

where

- $I$ = ionic strength (M)
- $c_i$ = concentration of the $i$th species (M)
- $Z_i^2$ = charge of the $i$th species
Table 5-1 Ionic strengths of solutions

<table>
<thead>
<tr>
<th>0.25 mg/mL KH₂PO₄</th>
<th>0.2 mg/mL KCl</th>
<th>0.5 mg/mL NaCl</th>
<th>1.4 mg/mL Na₂HPO₄</th>
<th>8 mg/mL NaCl</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.84</td>
<td>2.68</td>
<td>4.52</td>
<td>8.56</td>
<td>29.6</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>31.4</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>137</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>171</td>
<td></td>
</tr>
</tbody>
</table>

Values are in mM

The ionic strengths of the solutions found in the previous figure are presented in Table 5-1 and the correlation between ionic strength and IRI activity of sodium $N^\alpha$-ethanoyl-$N^\varepsilon$-dodecanoyl-L-lysinate (415) at 22 mM is shown in Figure 5-6. Generally, it appears that as the ionic strength of the solution is increased, the IRI activity decreases. The correlation is not apparent when the solutions that have ionic strengths of 0 – 30 mM as the IRI ranges from 1% to 15% in no order. However, the solutions with the higher ionic strengths (> 130 mM) are much less IRI active. An experiment with a more continuous range of ionic strengths can be more helpful.

To more closely determine what effect the ionic strength has on IRI activity, NaCl was tested at different concentrations in the absence of a lysine analogue and compared to the PBS solution. The concentration of NaCl was increased from 0 to 200 mg/mL.

![Figure 5-6 Correlation between ionic strength and IRI activity of 22 mM sodium $N^\alpha$-ethanoyl-$N^\varepsilon$-dodecanoyl-L-lysinate (415) compared to a PBS control.](image-url)
It is clear from this graph that the IRI activity decreases as the salt concentration increases, similar to the ionic strength and IRI activity figure. The IRI activity of NaCl from 200 mM to 10 mM is statistically indistinguishable and only begins to increase at 5 mM. As a reference, the concentration of NaCl in PBS is 137 mM.

It is expected that if increasing concentrations of NaCl leads to lower IRI activity, then other salts in PBS may have the same effect. Therefore, the other PBS salts were tested at the lower concentrations than they appear in PBS and at the same concentration that NaCl is present in PBS, 137 mM. The results are presented in Figure 5-8 where a) represents the concentrations of the salts as in PBS and b) represents the salts at 137 mM. It is interesting to see that even at the same concentration (Figure 5-8b), the salts do not have the same IRI activity. Na$_2$HPO$_4$ and KH$_2$PO$_4$ at 137 mM are still much more IRI potent than NaCl and KCl. It is also interesting to see that the trend of decreasing IRI activity with increasing concentration is not true with Na$_2$HPO$_4$ as this salt at 137 mM has
much higher IRI activity than at 9.9 mM. However, with KCl and KH$_2$PO$_4$, the higher concentrations are less IRI active, as expected. Most importantly the solutions in Figure 8b have the same ionic strength (137 mM) with the exception of the Na$_2$HPO$_4$, which has an ionic strength of 411 mM. This contrasts with the previous data where the solutions with the higher ionic strengths had lower IRI activity.

Since the concentrations of the salts in Figure 5-8b are the same, we revisited the enthalpies of hydration for anions and counterions. The individual salts in PBS are highlighted in Table 5-2 and are ordered according to hydration. The enthalpy of hydration for the phosphate is unknown as it involves complexes from H$_2$PO$_4^-$ to PO$_3^{3-}$. The Hofmeister series ranks cations and anions according to how well they precipitate proteins out of solution, the Hofmeister series is directly related to the hydration of ions as their hydration affects how well they can precipitate proteins. In this ranking, phosphate is ranked as being more hydrated than Cl. When the results are compared to this ordering of salts, a trend emerges where the more hydrated salts have higher IRI
activity than the less hydrated salts. For example, when comparing NaCl and KCl, the variable ions are Na (more hydrated) and K (less hydrated). In the graph, NaCl has higher IRI activity than KCl. Similarly, KH₂PO₄ has higher IRI activity than KCl and it also possess the more hydrated phosphate ion compared to Cl. These trends apply to the last two possible combinations (Na₂HPO₄ is more active than NaCl, and Na₂HPO₄ is more active than KH₂PO₄). These observations are summarized in Table 5-2. However, there are some problems with this analysis. There are two ions of Na in each molecule of Na₂HPO₄ so in the comparison between KH₂PO₄, there will be twice as many Na ions than K ions (the ionic strength takes into account the number of ions). However, the difference between the IRI activity of Na₂HPO₄ and KH₂PO₄ is large, so that if the concentration of KH₂PO₄ was doubled, Na₂HPO₄ could still be more active. Collectively, these data indicate that the IRI activity of PBS salts is not necessarily due to their concentration in solution or the ionic strength of the solution, but due to their different hydration values.

Table 5-2 Comparison of PBS salts with the Hofmeister series.

<table>
<thead>
<tr>
<th></th>
<th>Hydration</th>
</tr>
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<tbody>
<tr>
<td>Na⁺</td>
<td>-418</td>
</tr>
<tr>
<td>K⁺</td>
<td>-351</td>
</tr>
<tr>
<td>phosphate</td>
<td>?</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>-338</td>
</tr>
</tbody>
</table>

Values are in kJ mol⁻¹⁻¹

<table>
<thead>
<tr>
<th>More IRI Active</th>
<th>vs.</th>
<th>Less IRI Active</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>KCl</td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>KH₂PO₄</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>KCl</td>
<td></td>
</tr>
<tr>
<td>Na₂PO₄</td>
<td>NaCl</td>
<td></td>
</tr>
</tbody>
</table>

The variable ions are underlined.
One of the very common IRI assay solvents is a sucrose solution, commonly 30% w/v. To investigate how this solution compares to PBS, a 30% sucrose solution was tested with anionic lysine surfactant 415. The results in Figure 5-9a,b demonstrate that 415 has the same IRI activity in the 30% sucrose solution as in PBS indicating that this solution is comparable to PBS. However, Figure 5-9c,d shows the relative sizes of the ice crystals in these two solutions. The ice crystals in the PBS solution are on average 5.5 µm while the ice crystals in the 30% sucrose solution are 0.4 µm. The very small ice crystals in the sucrose solution increase the difficulty of accurately defining the ice

*Figure 5-9* Comparison between PBS and 30% sucrose IRI assay solutions. a) IRI activity of 415 in PBS. b) IRI activity of 415 in 30% sucrose. c) Comparison of the mean grain sizes of PBS and 30% sucrose. d) Picture of PBS and 30% sucrose ice crystals.
crystal boundaries. Furthermore, the ice crystals overlap in the sucrose solution and have a rounder appearance than the ice crystals in PBS, again increasing the difficulty of distinguishing the boundaries (Figure 5-9d). The very sharp boundary edges and larger size of ice crystals in PBS make this solution ideal for discerning ice crystal boundaries. Overall, PBS is a more physiological relevant solution than either 0.5 mg mL\(^{-1}\) NaCl or 30% sucrose. Saline solution, which is the most physiologically-balanced solution since it is used in intravenous infusions, has 9 mg/mL of NaCl. PBS has 8 mg/mL of NaCl which is closer to saline than the 0.5 mg/mL NaCl solution. Since the goal is to discover small molecule ice recrystallization inhibitors which can increase cell viability after cryopreservation, PBS should be the common IRI assay solution since ice recrystallization inhibitors will be dissolved in physiological solutions for cryopreservation.

5.4. Chapter summary

This chapter describes the effects of ions acting as counterions or are present in the IRI assay solutions on IRI activity. In simple para-amino benzoic acid compounds, where either the amine was protonated with a salt, or a salt carboxylate was present, the analogues that possess the highest IRI activity are those that contained the most hydrated counterions. Of the anionic counterions, the best to worst IRI activity was obtained with the following: Cl, Br, and I, which also corresponds to their hydration. Similarly, the highest to lowest hydrated cationic counterions are Li, Na, K, and IRI activity also followed this trend. This indicated that the hydration of counterions affects the overall IRI activity of the compound and this should be taken into account if ice recrystallization inhibitors are salts. Secondly, the IRI activity of anionic lysine surfactants is lower in
PBS than in the 0.5 mg/mL NaCl assay solution. This is due to two reasons. Firstly, larger amounts of salts (as in PBS) decrease the IRI activity and secondly, the hydration of the salts modulates IRI activity. For simple monosaccharides, however, the IRI activity is the same in both PBS and NaCl and the conclusions ascertained above only apply to the lysine surfactants. Finally, it was determined that PBS, due to its clear ice crystal shapes sizes and closeness to physiological conditions, is the best choice for the IRI assay solution.

5.5. References

Chapter 6. Ice recrystallization inhibitors as stabilizers for viral vectors.

6.1. Introduction

Oncolytic viruses are agents that have been engineered to infect cancer cells and destroy them. The strategies that these viruses use to target cancer cells involve the same machineries that cause their unregulated cell growth.\textsuperscript{1,2} The advantages of using oncotytic viruses as cancer therapeutics include the ability to rapidly modify the viruses with recombinant DNA technology to create ‘designer viruses’\textsuperscript{3} and the natural effect that the viruses have to stimulate tumor-specific inflammation.\textsuperscript{1} These treatments are in the stages of clinical trials presently.\textsuperscript{2} As these therapies will become more common, the preservation and stabilization of these oncolytic viruses will become more important as they will be used in clinical settings. The methods by which viruses are stored long-term are similar to those employed with cells, and therefore, there are still obstacles since the infectivity of the virus must be maintained if it is to be used as cancer therapy.\textsuperscript{4} This chapter will describe compounds, which can maintain virus infectivity after storage. In addition to the field of oncolytic viruses, world vaccination programs are still suffering due to relative thermo-instability of vaccines that contain live attenuated viruses.\textsuperscript{5} Over three million deaths are prevented in developing countries due to vaccination programs.\textsuperscript{6} However, there are still a number of vaccines which are not being stored or transported properly and this can result in the loss of vaccine efficacy. The most important factor in storing or transporting vaccines is maintaining the cold chain (vaccines which are
refrigerated at every point in transport); however, this is sometimes difficult to achieve in developing countries. Breaking the cold chain typically involves exposure to high temperatures or freezing temperatures. The World Health Organization has mandated that in order for a vaccine to be considered effective, less than a 10-fold decrease in original titer can be tolerated. Therefore, the development of stabilizers that can maintain vaccine activity at higher temperatures or prevent cryoinjury during freezing would be beneficial to further improve world vaccination programs.

Oncolytic viral vectors that are commonly used in the laboratory include vaccinia virus (VV), vesicular stomatitis virus (VSV), and herpes simplex virus type 1 (HSV-1) (Figure 6-1). VV is a large double-stranded DNA virus that was used in the vaccine to eradicate smallpox, which is caused by the variola virus. VV is naturally attracted to tumor cells but it is being engineered to be even more specific by the following routes. Normal dividing cells require thymidine kinase for the synthesis of deoxyribonucleotides; however, cancer cells have high concentrations of deoxyribonucleotides and thymidine kinase is not required. Thus, developing a thymidine kinase knockout VV will enable it to only flourish in cancer cells where it will have high supplies of deoxyribonucleotides. Also, deleting the vaccinia growth factor (VGF) gene in VV, which is important for stimulating cell growth in normal cells for the proliferation of the virus, will allow VV to survive only in cancer cells that are already proliferating. VSV is a small bullet-
shaped negative single-stranded RNA virus that mainly infects livestock, causing ulcers in the mouth, and is in the same family as the rabies virus.\textsuperscript{17} VSV is very sensitive and is destroyed by interferons that are released by normal cells but will proliferate in tumor cells that have defective interferon pathways.\textsuperscript{18} As well, VSV is being developed as a vector for vaccines against HIV-1,\textsuperscript{19} Ebola virus,\textsuperscript{20} hepatitis B\textsuperscript{21} and C.\textsuperscript{22} Finally, HSV-1 is a double-stranded DNA virus and causes cold sores in humans.\textsuperscript{17} A virus based on HSV-1, talimogene laherparepvec by Amgen, is the first oncolytic virus that has been proven effective in Phase III clinical trials in March 2013.\textsuperscript{23}

The use of ice recrystallization inhibitors at stabilizers of vaccines and viruses at sub-zero temperatures has never been reported. As these types of compounds can protect cells from cryoinjury,\textsuperscript{24} they may also have the same effect on viruses.

6.2. **Ice recrystallization inhibitors**

Thirteen compounds, that have varying levels of IRI activity were initially assessed for their ability to protect VV and VSV after 10 freezing and thawing cycles. These compounds are presented in Figure 6-2. Only three compounds proved to be
successful at protecting the viral vectors: the $C$-linked AFGP analogue, OGG-Gal (101), the most potent small molecule ice recrystallization inhibitor $N$-octyl gluconamide, (NOGlc, 146), and $N$-octyl galactonamide (NOGal, 157). These were further assessed with HSV-1 in addition to VV and VSV in various conditions: room temperature storage, freezing and thawing cycles, and lyophilization. These assays were performed in collaboration with Dr. Maxim Berezovski’s laboratory. The IRI activity of these compounds is shown in Figure 6-3. The most active compound is OGG-Gal, which has high IRI activity at a low concentration of 5.5 µM. NOGlc is the second most potent compound. Replacing the glucose alditol portion of NOGlc with a galactose alditol (NOGal) results in a decrease of IRI activity, indicating that the configuration of the carbon bearing the C4 hydroxyl group modulates IRI activity.

6.3. **Stabilization of viral vectors in cryopreservation**

Freezing is one of the most common methods for the preservation of viruses and it is typical to conduct multiple freeze-thaw cycles to determine the robustness of viruses to freezing. Therefore, the effect of the ice recrystallization inhibitors on the infectivity of viral vectors after 10 freeze-thaw cycles at -20°C was analyzed. In these assays, the virus was incubated with the ice recrystallization inhibitors at the given conditions and the infectivity of the virus was determined using plaque forming assay. In this assay, virus titers in varying dilutions are added to monolayers of Vero cells (kidney epithelial cells)
in multi-well plates and then covered in media containing agarose to prevent the virus particles from moving over large areas. A plaque is formed when the virus infects a cell and the cell lyses and spreads the infection to adjacent cells. Uninfected cells surround this circular plaque as the agarose prevents indiscriminant spreading. The plaque forming unit (PFU) is determined by quantifying the plaques, taking into account the dilution factor. **Figure 6-4** depicts the plaque forming assay where the white spots are individual plaques. The viral vectors VV, VSV, and HSV-1 used in these experiments are recombinant forms containing green, yellow, or red fluorescent protein (GFP, YFP, and RFP, respectively), enabling the quantification of plaques by fluorescence. In **Figure 6-5a**, VV with OGG-Gal, NOGal, and NOGlc lost only 0.3, 1.3, and 0.5 Log$_{10}$ PFU mL$^{-1}$, respectively, compared to a 1.7 Log$_{10}$ PFU mL$^{-1}$ infectivity loss with the PBS control. The best ice recrystallization inhibitor with VV was therefore OGG-Gal as the infectivity was statistically indistinguishable from the infectivity prior to freezing. VSV treated with OGG-Gal and NOGlc lost more than 1/3 of its original infectivity but with NOGal, VSV
lost more than half of its infectivity (Figure 6-5b). This is the same loss that was seen with PBS. Overall, higher protection is offered to VV than VSV and OGG-Gal and NOGlc were better at protecting the viruses than NOGal. This is not surprising as NOGal had the weakest IRI activity from the three ice recrystallization inhibitors. The ability to prevent ice recrystallization decreases cryoinjury since smaller ice crystals are less prone to damage the virus. The fact that OGG-Gal and NOGlc are more effective with VV than with VSV may correlate to the size and characteristics of the virus. One speculation may be that VV is much larger than VSV and ice crystals are hypothesized to be more damaging to particles with larger volumes. Thus, the ice recrystallization inhibitors may have a more beneficial effect on the larger VV. In terms of virus characteristics, VV is an enveloped virus, possessing a lipid membrane that is derived from the host upon budding. Enveloped viruses are sensitive to chemical or physical conditions that disrupt the membrane including desiccation, heat, detergents, and extreme pH’s. Despite the fact that desiccation and large temperature changes are present in cryopreservation, VV is still more robust than VSV and the ice recrystallization inhibitors
are still very effective protectants, maybe stabilizing the lipid envelope. The ice recrystallization inhibitors with HSV-1 did not show any significant improvements.

6.4. Stabilization of viral vectors during lyophilization

Lyophilization is a well-established technique for stabilizing vaccines. Vaccines are more stable when desiccated and refrigerated until the time of use, however, they still lose some of their infectivity during storage time. Since lyophilization is the sublimation of water from a frozen sample at low pressure, ice recrystallization would occur during the time the ice sublimes. It is hypothesized that the ice recrystallization inhibitors could protect the viral vectors from this type of cryoinjury. Figure 6-6a illustrates the infectivity of VV after lyophilization with the ice recrystallization inhibitors. The most promising compound was OGG-Gal, which reduced the infectivity by \( 0.7 \log_{10} \text{PFU mL}^{-1} \) while VV in the PBS control lost its infectivity by \( 2 \log_{10} \text{PFU mL}^{-1} \). NOGal and NOGlc only protected VV slightly compared to PBS. The ice recrystallization inhibitors had no protecting effect on VSV as the infectivity of the virus with the compounds was similar to the infectivity in the PBS control, which was reduced

![Figure 6-6](image.png)

*Figure 6-6* Effect of ice recrystallization inhibitors on a) VV and b) VSV after lyophilization.
by almost 5 Log\textsubscript{10} units (Figure 6-6b). Even in lyophilization, VV is the more robust virus and the ice recrystallization inhibitors are more effective with VV than VSV. The lyophilization procedure used for these experiments was the same but it is known that similar to cell types requiring different cryopreservation procedures, different viruses require different lyophilization procedures.\textsuperscript{30} The procedure may have been more effective for VSV but not as effective for VV. The ice recrystallization inhibitors with HSV-1 did not show any significant improvements.

6.5. Stabilization of viral vectors at room temperature

Perhaps the most important condition assessed was storage at room temperature. This is important especially for vaccines, as one of the main causes of vaccine inactivation is the lack of refrigeration and storage at ambient temperatures. As illustrated in Figure 6-7, NOGlc dramatically increases the storage time of both VV and HSV-1 at room temperature. With VV (Figure 6-7a), even after 40 days, the infectivity does not decrease below 1 Log\textsubscript{10} PFU mL\textsuperscript{-1} of the original infectivity while VV in PBS loses more than 1 Log\textsubscript{10} infectivity within eight days. This is also seen with HSV-1 (Figure 6-7b)

![Figure 6-7 Effect of NOGlc on viral vectors at 22°C. a) VV infectivity during 40 days. b) HSV-1 infectivity during 9 days. Error bars indicate the standard error of the mean.](image)
when at nine days, the virus with NOGlc loses only 0.4 \( \log_{10} \) PFU mL\(^{-1}\) infectivity, while the control loses more than 1 \( \log_{10} \) PFU mL\(^{-1}\) infectivity only after one day. These results are very relevant to vaccine stabilization as the addition of these ice recrystallization inhibitors can dramatically prevent vaccine damage caused by a break in the cold chain. These experiments were not performed with VSV as this virus had extremely low stability at ambient temperatures. Also, OGG-Gal and NOGal did not protect VV or HSV-1 at room temperature. These results are interesting as there was no freezing involved in this assay and NOGlc still had a protecting effect on VV and HSV-1 indicating that NOGlc must be stabilizing the viruses in ways other than inhibiting ice recrystallization. Both VV and HSV-1, but not VSV, possess a lipid bilayer envelope, thus, NOGlc may stabilize this membrane. However, more experiments must be performed to identify how exactly NOGlc stabilizes these viruses.

6.6. **Assessment of viral aggregation on infectivity**

Many virus particles have the capacity to form aggregates which result in loss of infectivity.\(^{31}\) In addition to damage sustained from ice crystals during cryopreservation, the infectivity of the viral vectors can also be decreased due to aggregation. As ice recrystallization inhibitors protect viral particles from cryoinjury, they were also analyzed for their effect on the aggregation of viral particles. In this aggregation assay, two VSV mutants are used containing either yellow fluorescent protein (YFP) or red fluorescent protein (RFP). Only one virus particle possessing either RFP or YFP will infect a single host cell. When viral particles aggregate, statistically, the aggregate will contain both YFP and RFP. Therefore, when the aggregate enters the cell, the plaque will contain YFP and RFP. The overlap of YFP and RFP fluoresce indicates that the plaque was formed by
infection of an aggregate. Figure 6-8a shows the plaques containing YFP (first panel), RFP (second panel), and the merge of both YFP and RFP representing aggregates (third panel). The results for this assay with VSV and OGG-Gal after one freeze-thaw cycle are shown in Figure 6-8b. With untreated VSV, 34% of cells were infected by viral aggregates. However, only 16% of cells were infected by aggregates when VSV was treated with OGG-Gal. This demonstrates that OGG-Gal must stabilize the viral particles in a way that they are prevented from aggregating at room temperature and during freezing.

6.7. Chapter Summary

This chapter describes the stabilizing effects of the ice recrystallization inhibitors, OGG-Gal, N-octyl gluconamide, and N-octyl galactonamide on vaccinia virus, vesicular stomatitis virus, and herpes simplex virus type 1. Three storage conditions were evaluated: freezing and thawing cycles, lyophilization, and room temperature storage and
all results are summarized in Table 6-1. With 10 freeze-thaw cycles, OGG-Gal and NOGlc, but not NOGal, proved to be the most effective at maintaining viral infectivity with VV and VSV. This was expected as OGG-Gal and NOGlc were the most potent ice recrystallization inhibitors and NOGal was the weakest ice recrystallization inhibitor in this set. These results indicate that there is a correlation between ice recrystallization inhibition activity^32 and protection of viruses though freeze-thaw cycles. OGG-Gal also inhibits the aggregation of virus particles, which adds to the increased infectivity of VSV with OGG-Gal. The protective effects of the ice recrystallization inhibitors were less pronounced with lyophilization of the viruses. The most effective ice recrystallization inhibitor with both lyophilized VV and VSV was OGG-Gal. In room temperature storage, NOGlc stabilized VV to a significant extent where almost the full infectivity of the virus was observed after 40 days. Without NOGlc, VV lost all of its infectivity after 40 days.
This is perhaps the most significant result and it raises the question of the mechanism of stabilization since freezing was not involved in this experiment. According to the World Heath Organizations, vaccines can be utilized if they lose less than 1 Log$_{10}$ of the original titer amount.\textsuperscript{9,10} Thus, according to these data, NOGlc would be effective stabilizers of vaccines since NOGlc can stabilize VSV and HSV-1 for prolonged periods of time at ambient temperature. For the storage of oncolytic viruses for cancer therapy, OGG-Gal would be the most effective as it protected VV and VSV in freezing and thawing cycles. The different activities of the ice recrystallization inhibitors in the three different viruses indicate that each type of virus requires different storage protocols.

6.8. References

Chapter 7. Experimental Procedures and Characterization

Data

7.1. General Experimental

All anhydrous reactions were performed in flame-dried or oven-dried glassware under a positive pressure of dry argon or nitrogen. Air or moisture-sensitive reagents and anhydrous solvents were transferred with oven-dried syringes or cannula. All flash chromatography was performed with EMD 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) ultraviolet light and/or staining (ceric ammonium molybdate, potassium permanganate, or phosphomolybdate stain solution).

All solvents used for anhydrous reactions were distilled. Tetrahydrofuran (THF) and diethyl ether were distilled from sodium/benzophenone under nitrogen. Dichloromethane, acetonitrile, triethylamine, benzene and diisopropylethylamine (DIPEA) were distilled from calcium hydride. N,N-dimethylformamide (DMF) was stored over activated 4Å molecular sieves under argon.

$^1$H (300, 400, or 500 MHz) and $^{13}$C NMR (75, 100 or 125 MHz) spectra were recorded at ambient temperature on a Bruker Avance 300, Bruker AM 360, Bruker Avance 400 or Bruker Avance 500 spectrometer. Deuterated chloroform (CDCl$_3$), methanol (CD$_3$OD),
or deuterium oxide (D$_2$O) were used as NMR solvents, unless otherwise stated. Chemical
shifts are reported in ppm downfield from TMS and corrected using the solvent residual
peak or TMS as an internal standard. Splitting patterns are designated as follows: s,
singlet; d, doublet; t, triplet; q, quartet; 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 µL/min using electrospray ionization (ESI) or a
Voyager DE-Pro matrix-assisted desorption ionization-time of flight (MALDI-TOF),
(Applied Biosystem, Foster City, CA) mass spectrometer operated in the
reflectron/positive-ion mode with DHB in 20% EtOH/H$_2$O as the MALDI matrix. High
resolution mass spectrometry (HRMS) data was acquired on Applied Biosystems/Sciex
QStar (Concord, ON). Samples in MeOH or H$_2$O were mixed with Agilent ES tuning mix
for internal calibration, and infused into the mass spectrometer at 5 µL/min.

7.2. Assessing Ice Recrystallization Inhibition (IRI) Activity

Sample analysis for IRI activity was performed using the “splat cooling” method as
previously described.$^1$ In this method, the analyte was dissolved in phosphate buffered
saline (PBS) solution and a 10 µL droplet of this solution was dropped from a
micropipette through a two meter high plastic tube (10 cm in diameter) onto a block of
polished aluminum precooled to approximately -80 °C. The droplet froze instantly on the
polished aluminum block and was approximately 1 cm in diameter and 20 µm thick. This
wafer was then carefully removed from the surface of the block and transferred to a
cryostage held at -6.4 °C for annealing. After a period of 30 min, the wafer was
photographed between crossed polarizing filters using a digital camera (Nikon CoolPix 5000) fitted to the microscope. A total of three images were taken from each wafer. During flash freezing, ice crystals spontaneously nucleated from the supercooled solution. These initial crystals were relatively homogeneous in size and quite small. During the annealing cycle, recrystallization occurred, resulting in a dramatic increase in ice crystal size. A quantitative measure of the difference in recrystallization inhibition of two compounds X and Y is the difference in the dynamics of the ice crystal size distribution. Image analysis of the ice wafers was performed using a novel domain recognition software (DRS) program.\textsuperscript{2} This processing employed the Microsoft Windows Graphical User Interface to allow a user to visually demarcate and store the vertices of ice domains in a digital micrograph. The data was then used to calculate the domain areas. All data was plotted and analyzed using Microsoft Excel. The mean grain (or ice crystal) size (MGS) of the sample was compared to the MGS of the control PBS solution for that same day of testing. IRI activity is reported as the percentage of the MGS (% MGS) relative to the PBS control, and the % MGS for each sample was plotted along with its standard error of the mean. Large percentages represent a large MGS, which is indicative of poor IRI activity. Unpaired Student’s \textit{t}-tests were performed to a 95% confidence level.

7.3. \textbf{Assessing Thermal Hysteresis (TH)}

Nanoliter osmometry was performed using a Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, NY), as described by Chakrabartty and Hew.\textsuperscript{3} All of the
measurements were performed in doubly distilled water. Ice crystal morphology was observed through a Leitz compound microscope equipped with an Olympus 20× (infinity-corrected) objective, a Leitz Periplan 32X photo eyepiece, and a Hitachi KPM2U CCD camera connected to a Toshiba MV13K1 TV/VCR system. Still images were captured directly using a Nikon CoolPix digital camera.

7.4. Biological Assay Procedures

7.4.1. TF-1α cell culture

TF1-α (human bone marrow erythroblasts, ATCC CRL-2451) were cultured in RPM1 media supplemented with 10% FBS (fetal bovine serum) and 1% penicillin–streptomycin in 150 mm Corning® petri dishes. Cells were incubated in a 37°C incubator supplied with 5% CO₂. The media was changed every two days. This involved transferring the cell solution from plates to 50 mL falcon tubes and centrifuging at 1000 rpm for 5 min. The media was discarded from falcon tubes and the pellet was re-suspend in 5 mL of RPM1 and agitated to disperse cells. A 1/8 dilution of the cell solution was prepared in an eppendorf tube with trypan blue as a dye and the cells were counted with a hemocytometer. The cell solution was transferred to a new cell culture dish and 20 mL of RPM1 was added. The cells were split every week or if more than 20 million cells were per plate.

7.4.2. TF-1α cryopreservation
The compound cryo solutions were prepared in falcon tubes with RPM1. Cells were transferred from cell culture plates to 50 mL falcon tubes and centrifuging at 1000 rpm for 5 min. The media was discarded from falcon tubes and the pellet was re-suspend in 5 mL of RPM1 and agitated to disperse cells. A 1/8 dilution of the cell solution was prepared in an eppendorf tube with trypan blue as a dye and the cells were counted with a hemocytometer. 3 million cells were added to each cryo tube and centrifuged at 1000 rpm for 5 min. The media was removed from tubes and the cryo solution was added with and either media or DMSO so that the total volume was 100μL per tube. The cryo tubes were agitated to disperse cells, transferred to a Mr. Frosty and placed in -80°C freezer for 16 hours. The cryo tubes were stored in the vapor phase of liquid nitrogen for a minimum of 24 hours.

7.4.3. **TF-1α post cryopreservation flow cytometry**

Samples were quickly thawed in a 37°C water bath. 900 μL of 1X Annexin 5 binding buffer was added to each cryo tube the cells were counted with a hemocytometer with trypan blue as a dye. 400 uL of the solution in cryo tube was transferred to flow tubes. 7 μL of 7-AAD and 7 μL of Annexin V-FITC was added to flow tubes and the tubes were agitated and incubated in the dark for 15 min. The solution in flow tubes was diluted with 1 x Annexin 5 binding buffer to 1 mL (572 μL). Flow cytometry analysis on 100,000 cells was carried out on a Beckman Coulter FC500 flow cytometer. Annexin V-FICT was measured with 525 nm optical filter (FL-1), while 7-AAD was measured with 675 nm optical filter (FL-4). Viability was determined by the total number of 7-AAD− cells detected. Apoptosis was determined by the number of 7-AAD− cells that were Annexin
Cell recoveries were calculated from total cells counted and the sample volume analyzed, determined from the hemocytometer count. Time between thawing and analysis by flow cytometry was less than 1 h. All samples were tested in triplicate.

7.4.4. **Hep G2 cell culture**

HepG2 cells (human liver hepatocellular carcinoma cells, ATCC, HB-8065) were cultured in Eagle’s minimum essential media (MEM) supplemented with 10% FBS (fetal bovine serum), 1% non-essential amino acids, 1 mM sodium pyruvate and 1% penicillin–streptomycin in 75cm² Corning® flasks. Cells were incubated in a 37°C incubator supplied with 5% CO₂. Passages 5–18 were used in this study. No evidence of overgrowth or morphological changes consistent with apoptosis was observed. The media was changed every two days and the cells were split every seven days. The splitting involved removing media solution from flasks, adding 3 mL of Accutase solution and incubating at 37°C for 5 min. The detached cells were transferred to a 50 mL falcon tube and pelleted at 1000 rpm for 5 min. The media was discarded from falcon tubes and the pellet was resuspended in 5 mL of MEM and agitated to disperse cells. A 1/8 dilution of the cell solution was counted with a hemocytometer with trypan blue as a dye. The cell solution was transferred to a new flask and 20 mL of RPM1 was added.

7.4.5. **Hep G2 cryopreservation**

Hep G2 cells were cultured and detached as described above. Cells were transferred from flasks to 50 mL falcon tubes and centrifuging at 1000 rpm for 5 min. The media was
discarded from falcon tubes and the pellet was re-suspend in 5 mL of RPM1 and agitated to disperse cells. A 1/8 dilution of the cell solution was counted on a hemocytometer with trypan blue as a dye. 3 million cells were added to each cryo tube and centrifuged at 1000 rpm for 5 min. The media was removed from tubes and the cryo solution was added with and either media or DMSO so that the total volume was 100µL per tube. The cryo tubes were agitated to disperse cells, transferred to a Mr. Frosty and placed in -80°C freezer for 16 hours. The cryo tubes were stored in the vapour phase of liquid nitrogen for a minimum of 24 hours.

7.4.6. **Hep G2 post-cryopreservation flow cytometry**

Samples were quickly thawed in a 37°C water bath. 400 µL of 1X dilution of the Annexin 5 binding buffer was added to each cryo tube and 100 µL of this solution was transferred to flow tubes. 5 µL of 7-AAD and 4 µL of PE Annexin 5 was added to flow tubes and the tubes were agitated and incubated in the dark for 15 min. 10 µL of Countbright® beads was added and the solution in flow tubes was diluted with 1 x Annexin 5 binding buffer to 1 mL (381 µL). The samples were analyzed on the flow cytometer. Flow cytometry analysis on 100,000 cells was carried out on a Beckman Coulter FC500 flow cytometer. Annexin V-PE was measured with 575nm optical filter (FL-2), while 7-AAD was measured with 675 nm optical filter (FL-4). Viability was determined by the total number of 7-AAD- cells detected. Apoptosis was determined by the number of 7-AAD- cells that were Annexin V+. Cell recoveries were calculated from total cells counted and the sample volume analyzed, determined from the number of counting beads detected and the concentration of beads in the analyzed solution.
Counting beads were measured with 610 nm optical filter (FL3). Time between thawing and analysis by flow cytometry was less than 1 h. All samples were tested in triplicate.

7.4.7. MTT assay with HepG2 Cells

The MTT assay was performed as described previously. HepG2 cells were plated in 96-well plates and treated with 100 µL of compound MEM solution and incubated at 37°C for 16 h with 5% CO₂. Cells incubated with MEM without compound were used as a negative control, and cells supplemented with 1% Triton-X were used as a positive control. Following incubation, the media was removed by aspiration and 200 µL of fresh media and 50 µL of MTT solution (5 mg/mL) in HBSS (Hank’s balanced salt solution) were added and the plates were incubated at 37°C with 5% CO₂ for 3 h. The plates were then centrifuged, the media aspirated and 200 µL of MTT solubilization solution (10% Triton X-100, 0.1 N HCl in isopropanol) was added to each well. The plates were incubated at room temperature in the dark for 2-4 h and the absorbance of each well was then read at a wavelength of 570 nm with a multiwell plate reader (AD 34°C Absorbance Detector, Beckman Coulter, Inc., Mississauga, ON). Viability was reported as a percentage of the control. All experiments were repeated at least three times in 10 consecutive wells for each condition.

7.5. Experimental Data

Allyl β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-galactopyranoside (304)
(25 mg, 0.03 mmol) was dissolved in CH$_2$Cl$_2$ (0.5 mL) and TFA was added until pH reached approx. 2. The solution was stirred at room temperature for 30 min after which triethyl amine was added until pH 7 and the solvent was removed. The residue was diluted in CH$_2$Cl$_2$ and washed with H$_2$O. The aqueous phase was washed twice with CH$_2$Cl$_2$ and the combined organic phases were dried with MgSO$_4$, filtered, and concentrated under reduced pressure. The residue was purified on preparatory thin layer chromatography (9:1 EtOAc:MeOH) to yield white solid. The solid was dissolved in MeOH (0.5 mL) and 1M NaOMe in MeOH was added until pH reached approx. 11. The reaction mixture was stirred at room temperature for 14 hrs after which the reaction was neutralized with Dowex 50 (H$^+$) resin, filtered, and concentrated. The product was purified by preparatory thin layer chromatography (4:2:2:2 EtOAc:ACN:MeOH:H$_2$O) to yield white product 204 (14 mg, 85%).

$^1$H NMR (500 MHz, D$_2$O) $\delta$ ppm 5.89 (dddd, J = 16.9, 10.5, 6.2, 5.2, 1H), 5.30 (dddd, J = 17.3, 1.5, 1.3 Hz, 1H), 5.24 (dddd, J = 10.5, 1.5, 1.3 Hz, 1H), 4.50 (d, J = 8.50 Hz, 1H), 4.45 (d, J = 7.86 Hz, 1H), 4.33 (dddd, J = 13.2, 5.1, 1.4 Hz, 1H), 4.15 (ddd, J = 13.1, 6.3, 1.2 Hz, 1H), 4.06 (dd, J = 10.8, 4.0 Hz, 1H), 3.96 (dd, J = 3.08, <1.0 Hz, 1H), 3.88 (m, 3H), 3.73 (m, 5H), 3.64 (dd, J = 10.0, 3.4 Hz, 1H), 3.52 (dd, J = 9.9, 7.8 Hz, 1H), 2.02 (s, 3H).

$^{13}$C NMR (500 MHz, D$_2$O) $\delta$ ppm 174.74, 133.36, 118.06, 103.20, 100.57, 75.09, 73.75, 72.62, 70.81, 70.67, 70.51, 68.91, 68.53, 67.75, 60.91, 52.25, 22.11.

ESI-MS m/z calcd for C$_{17}$H$_{29}$NO$_{11}$ [M + Na]$^+$: 446.16. Found: 446.37.

2,3,4,6-tetra-O-acetyl-$\alpha$-D-galactopyranosyl bromide (314)

Compound was prepared from 313 as previously described.$^5$ All spectral data was consistent with that reported in the literature.$^5$

3,4,6-tri-O-acetyl-$\beta$-galactal (307)

Compound was prepared as previously described.$^6$ All spectral data was consistent with that reported in the literature.$^6$

2-azido-2-deoxy-3,4,6-tri-O-acetyl-$\beta$-galactopyranosyl nitrate (319)
Compound was prepared from 307 as previously described.\(^7\)

307 (3.5 g, 13 mmol) was dissolved in acetonitrile (120 mL) and cooled to -15°C. Ceric ammonium nitrate (25.5 g, 46.5 mmol, 3.6 eq) and sodium nitrate (1.3 g, 19.4 mmol, 1.5 eq) were added and the mixture was stirred at -15°C for 1 hour and then at ambient temperature for 1 hour. The organic phase was diluted with EtOAc and washed with H\(_2\)O two times and a third time with brine. The organic phase was dried with Mg SO\(_4\), filtered, and concentrated under reduced pressure. The oil was purified with silica column chromatography (8:2 hexanes:EtOAc) to yield 319 (1.9 g, 5.2 mmol, 40% yield) as a clear colorless oil. Note that a small proportion of the talose derivative is present which is removed in the next step. All spectral data was consistent with that reported in the literature.\(^7\)

\(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) ppm 6.34 (d, J = 4.2 Hz, 1H), 5.50 (d, J = 2.2 Hz, 1H), 5.25 (dd, J = 11.3, 3.2 Hz, 1H), 4.36 (bt, J = 6.7 Hz, 1H), 4.14-4.10 (m, 3H), 2.17 (s, 4H), 2.07 (s, 3H), 2.04 (s, 3H).

\(^13\)C NMR (500 MHz, CDCl\(_3\)) \(\delta\) ppm 170.4, 169.9, 169.6, 97.0, 69.6, 68.7, 66.8, 61.0, 56.1, 20.74, 20.71, 20.69. ESI-MS m/z calcd for C\(_{12}\)H\(_{17}\)N\(_4\)O\(_{10}\)\(^+\) [M+K]\(^+\): 415.05. Found: 415.32.

3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-\(\beta\)-D-galactopyranose (320)

319 (1.1 g, 2.8 mmol) was dissolved in acetonitrile (50 mL) and cooled to 0°C. Thiophenol (0.86 mL, 8.5 mmol, 3 eq) and diisopropylethylamine (0.48 mL, 2.8 mmol, 1 eq) was added and the mixture was stirred for 2 hours at 0°C or until reaction is complete via TLC. The mixture was extracted with 10% HCl, saturated NaHCO\(_3\), and brine and the organic phase was dried with MgSO\(_4\) and concentrated under reduced pressure. The resulting yellow oil was purified by silica column chromatography (6:4 hexanes:EtOAc) to yield 320 as a colourless oil (0.70 g, 2.1 mmol, 75 %). All spectral data was consistent with that reported in the literature.\(^8\)

\(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) ppm 5.45 (d, J = 2.7, 2H), 5.42 (s, 2H), 5.39 (dd, J = 11.0, 3.2, 2H), 5.33 (d, J = 3.1, 1H), 4.81 (dd, J = 10.9, 3.3, 1H), 4.70 (dd, J = 7.7, 3.7, 1H), 4.46 (t, J = 6.6, 2H), 4.33 (s, 1H), 4.13-4.07 (m, 6H), 3.91 (t, J = 6.5, 1H), 3.74 (dd, J = 11.0, 3.3, 2H), 3.68-3.66 (m, 3H), 2.16 (s, 3H), 2.15 (d, J = 5.5, 8H), 2.06 (s, 6H), 2.05 (s, 12H). \(^13\)C NMR (400 MHz, CDCl\(_3\)) \(\delta\) ppm 170.79, 170.77, 170.29, 170.11, 170.10, 96.5, 92.5, 71.3, 71.0, 68.5, 67.8, 66.70, 66.61, 62.11, 61.93, 61.7, 58.2, 20.83, 20.80, 20.76, 20.74. ESI-MS m/z calcd for C\(_{12}\)H\(_{17}\)N\(_3\)O\(_8\) [M + K]\(^+\): 370.06. Found: 370.16.

Allyl 3,4,6-tri-O-acetyl-2-azido-2-deoxy-\(\beta\)-D-galactopyranoside (321)

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To a solution of α-D-trichloroacetimidate 322 (0.155 g, 0.326 mmol) in anhydrous CH₂Cl₂ (6 mL) cooled to -20°C, allyl alcohol (67 µL, 0.98 mmol, 3 eq) and BF₃·OEt₂ (8.3 µL, 0.065 mmol, 0.2 eq) were added dropwise and the reaction mixture was stirred for 2 hrs. The solvent was removed under reduced pressure and the product was purified by silica column chromatography (8:2 hexanes:EtOAc) to afford 321 (0.11 g, 88%). All spectral data was consistent with that reported in the literature.

1H NMR (400 MHz, CDCl₃) δ ppm 5.95 (ddddd, J = 17.2, 10.5, 6.2, 5.3, 1H), 5.38-5.33 (m, 2H), 5.26 (dd, J = 10.9, 3.4, 1H), 4.78 (dd, J = 10.9, 3.4, 1H), 4.43-4.40 (m, 2H), 4.21-4.09 (m, 3H), 3.84 (td, J = 6.7, 1.1, 1H), 3.71 (dd, J = 11.0, 8.0, 1H), 2.15 (s, 3H), 2.05 (s, 3H), 2.05 (s, 3H).

13C NMR (400 MHz, CDCl₃) δ ppm 170.39, 170.10, 169.85, 133.02, 118.35, 101.11, 71.08, 70.68, 70.65, 66.35, 61.24, 60.82, 20.65, 20.62, 20.62.


3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl trichloroacetimidate (322)

Compound was prepared from 320 as previously described.⁸

320 (1.9 g, 5.7 mmol) was dissolved in CH₂Cl₂ (50 mL), cooled to 0°C, and then trichloroacetonitrile (5.7 mL, 57 mmol, 10 eq) and 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (0.4 mL, 2.9 mmol, 0.5 eq) was added. The mixture was stirred form 2 hrs at 0°C after which the solvent was removed under reduced pressure and the dark brown oil was purified by silica column chromatography (8:2 hexanes:EtOAc + 1% Et₂N) to provide 322 as a colourless oil (2.0 g, 4.1 mmol, 72% yield). All spectral data was consistent with that reported in the literature.⁸

1H NMR (400 MHz, CDCl₃) δ ppm 8.79 (s, 1H), 6.51 (d, J = 3.6 Hz, 1H), 5.54 (dd, J = 1.1, 3.1 Hz, 1H), 5.38 (dd, J = 11.1, 3.2 Hz, 1H), 4.41 (dt, J = 6.6, 0.7 Hz, 1H), 4.14 (dd, J = 11.3, 6.6 Hz, 1H), 4.09-4.00 (m, 2H), 2.17 (s, 3H), 2.07 (s, 3H), 2.00 (s, 3H).

13C NMR (400 MHz, CDCl₃) δ 170.4, 170.0, 169.8, 160.8, 94.6, 90.7, 69.2, 68.8, 67.0, 61.3, 57.2, 20.7, 20.7, 20.7. ESI-MS m/z calcd for C₁₃H₁₇Cl₃N₄O₈ [M + K]⁺: 512.97. Found: 513.11.

Allyl 3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-β-D-galactopyranoside (323)

To a stirred solution of SnCl₂ (0.86 g, 3.8 mmol) in CH₃CN (8 mL), the following were added consecutively: thiophenol (1.68 g, 15.2 mmol, 1.6 mL), Et₂N (1.6 mL, 11.4
mmol), and 321 (0.94 g, 2.53 mmol) in CH$_3$CN (30 mL). The reaction mixture was stirred at room temperature for 30 min and then diluted with CH$_2$Cl$_2$ and washed with 10% NaOH. The aqueous phase was extracted with CH$_2$Cl$_2$ and the combined organic extracts were dried with MgSO$_4$ and concentrated under reduced pressure. The residue was dissolved in pyridine (23 mL) and Ac$_2$O (9.6 mL) and stirred at room temperature for 14 hrs. The solution was evaporated to dryness and purified by silica column chromatography (0.5:9.5 hexanes:EtOAc) to yield 323 (0.49 g, 48%). Spectral data is consistent with the partial listing in the literature.\textsuperscript{10,11}

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ ppm 5.87 (dddd, $J$ = 16.9, 10.5, 6.2, 5.1 Hz, 1H), 5.55 (d, $J$ = 8.6 Hz, 1H, NH), 5.38-5.23 (m, 3H), 5.19 (br dd, $J$ = 10.4, 1.3 Hz, 1H), 4.74 (d, $J$ = 8.4 Hz, 1H), 4.35 (ddd, $J$ = 13.0, 5.1, 1.3 Hz, 1H), 4.20-4.05 (m, 3H), 3.97 (ddd, $J$ = 11.2, 8.5 Hz, 1H), 3.81 (dd, $J$ = 10.8, 9.0 Hz, 1H), 3.76 (dd, $J$ = 11.8, 4.5, 1H), 3.66 (dd, $J$ = 7.8, 11.8, 1H), 2.03 (s, 3H). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ ppm 170.44, 170.41, 170.33, 170.25, 133.53, 117.81, 99.84, 70.59, 69.99, 69.80, 66.76, 61.46, 51.65, 23.44, 20.67, 20.65, 20.65. ESI-MS m/z calcd for C$_{17}$H$_{25}$NO$_9$ [M + K]$^+$: 426.12. Found: 426.10.

Allyl 2-acetamido-2-deoxy-β-D-galactopyranoside (324)

323 (0.143 g, 0.36 mmol) was dissolved in MeOH (1 mL) and a solution of 1M NaOMe in MeOH was added until pH reached approx. 11. The reaction mixture was stirred at room temperature for 14 hrs after which the solution was neutralized with Dowex 50 (H$^+$) resin, filtered, and concentrated. The product was purified by silica column chromatography (9:1 CH$_2$Cl$_2$:MeOH) to yield white product 324 (0.05 g, 57%). All spectral data was consistent with that reported in the literature.\textsuperscript{12}

$^1$H NMR (300 MHz, D$_2$O) $\delta$ ppm 5.90 (dddd, $J$ = 17.1, 10.4, 6.3, 5.2 Hz, 1H), 5.30 (ddd, $J$ = 17.3, 1.7, 1.6, 1H), 5.25 (ddd, $J$ = 10.4, 1.7, 1.2, 1H), 4.49 (d, $J$ = 8.5 Hz, 1H), 4.34 (ddd, $J$ = 13.2, 5.1, 1.4 Hz, 1H), 4.16 (ddd, $J$ = 13.2, 6.3, 1.3 Hz, 1H), 3.9 (dd, $J$ = 10.8, 8.4 Hz, 1H), 3.71 (dd, $J$ = 10.8, 3.4 Hz, 1H), 3.93 (d, $J$ = 3.30, <1.0 Hz, 1H), 3.66 (m, 1H), 3.76 (dd, $J$ = 11.8, 4.5, 1H), 3.81 (dd, $J$ = 7.8, 11.8, 1H), 2.03 (s, 3H). $^{13}$C NMR (400 MHz, D$_2$O) $\delta$ ppm 174.81, 133.43, 118.13, 100.56, 75.12, 71.07, 70.38, 67.81, 60.95, 52.40, 22.18. ESI-MS m/z calcd for C$_{11}$H$_{19}$NNaO$_6$ [M + Na]$^+$: 284.11. Found: 284.20.

Allyl 2-acetamido-2-deoxy-3,4-O-isopropylidene-β-D-galactopyranoside (325)

To a solution of 324 (0.16 g, 0.62 mmol) in DMF (3 mL), 2,2-dimethoxy propane (0.30 mL, 2.5 mmol) and p-toluenesulfonylic acid (12 mg, 0.062 mmol) were added and the mixture was stirred at room temperature for 15 hrs. The solution was neutralized with triethyl amine, concentrated, and purified by silica column chromatography (100% EtOAc, 1% triethyl amine) to yield two major fractions, the first fraction being 325 (0.11
\( ^1H \text{ NMR} \) (500 MHz, D\textsubscript{2}O) \( \delta \) ppm 5.86 (dddd, \( J = 16.9, 11.0, 6.0, 6.0 \) Hz, 1H), 5.26 (br d, \( J = 17.3 \) Hz, 1H), 5.21 (br d, \( J = 10.4 \) Hz, 1H), 4.51 (d, \( J = 8.95 \) Hz, 1H), 4.32-4.27 (m, 2H), 4.22 (dd, \( J = 8.7, 5.0 \) Hz, 1H), 4.12 (dd, \( J = 13.4, 6.3 \) Hz, 1H), 4.01 (br dd, \( J = 5.9 \) Hz, 1H), 3.83-3.78 (m, 3H), 1.99 (s, 1H), 1.51 (s, 1H), 1.33 (s, 1H).  

\( ^13C \text{ NMR} \) (500 MHz, D\textsubscript{2}O) \( \delta \) ppm 174.46, 133.34, 118.03, 111.04, 99.49, 76.62, 73.10, 73.05, 70.09, 60.85, 53.80, 27.06, 25.36, 22.11.  

ESI-MS m/z calcd for C\textsubscript{14}H\textsubscript{23}NO\textsubscript{6}[M + H]\textsuperscript{+}: 302.16, C\textsubscript{14}H\textsubscript{23}NNaO\textsubscript{6}[M + Na]\textsuperscript{+}: 324.14. Found: 302.25, 324.23.

**Allyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-4,6-O-isopropylidene-β-D-galactopyranoside (329)**

![Structure](image)

To a suspension of 325 (74 mg, 0.25 mmol) in a 1:1 chloroform: nitromethane mixture (5 mL) with 3 Å molecular sieves (0.23 g), galactopyranosyl bromide 314 (0.15 g, 0.37 mmol) was added and the mixture was stirred for 30 min at room temperature. Hg(CN)\textsubscript{2} (0.11 g, 0.42 mmol) was added and refluxed for 1 hr. The solution was brought to room temperature and stirred for 15 hrs after which the solution was diluted with CH\textsubscript{2}Cl\textsubscript{2}, washed with saturated NaHCO\textsubscript{3}, brine, dried with MgSO\textsubscript{4}, filtered, and concentrated. The residue was purified with preparatory thin layer chromatography (100% EtOAC) to yield 329 (0.073 g, 47%). Complete purification was performed in the next step.

**L-Lysine alkyl ester dihydrochloride derivatives (404-409)**

**General Procedure**

\( N^\alpha,N^\varepsilon-\text{Bis}(t\text{-butyloxy carbonyl})\text{-}L\text{-}\text{lysine and carbonyldiimidazole (1.1 eq)} \) were dissolved in anhydrous DCM (final concentration of 0.1 M) and the mixture was stirred at room temperature for 45 min. Alcohol (1.1 eq) was added and the mixture was stirred overnight. The solvent was evaporated under reduced pressure and the residue was purified via silica column chromatography (8:2 petroleum ether: EtOAc) to yield \( N^\alpha,N^\varepsilon\)-bis(t-butyloxy carbonyl)-L-lysine alkyl ester as a yellowish oil. The oil was suspended in 2 M HCl in anhydrous Et\textsubscript{2}O (final concentration of 0.1 M) and stirred at room temperature for 2 hrs after which the residue was concentrated under reduced pressure. The oil was washed with Et\textsubscript{2}O x 4 and residual Et\textsubscript{2}O was removed under reduced pressure to yield L-lysine alkyl ester dihydrochloride as a yellow oil.

**L-Lysine butyl ester dihydrochloride (404)**
The procedure above was used to give compound 404 in an isolated yield of 62%.

$^1$H NMR (400 MHz, $D_2$O) δ 4.26 (td, $J = 6.5, 1.8$ Hz, 2H), 4.13 (t, $J = 6.4$ Hz, 1H), 2.98 (t, $J = 7.7$ Hz, 2H), 1.97 (m, 2H), 1.67 (m, 4H), 1.49 (m, 2H), 1.35 (m, 2H), 0.89 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (400 MHz, $D_2$O) δ 170.11, 67.12, 52.60, 38.92, 29.62, 29.22, 26.21, 21.42, 18.36, 12.76. ESI-MS m/z calcd for C$_{10}$H$_{22}$N$_2$O$_2$ [M+H]$^+$: 203.18. Found: 203.26.

1-L-Lysine hexyl ester dihydrochloride (405)

The procedure above was used to give compound 405 in an isolated yield of 58%.

$^1$H NMR (500 MHz, $D_2$O) δ 4.23 (td, $J = 6.3, 4.0$ Hz, 2H), 4.10 (t, $J = 6.3$ Hz, 1H), 2.96 (t, $J = 7.7$ Hz, 2H), 1.95 (m, 2H), 1.66 (m, 4H), 1.49 (m, 1H), 1.43 (m, 1H), 1.32 (m, 2H), 1.25 (m, 4H), 0.82 (t, $J = 6.4$ Hz, 3H). $^{13}$C NMR (400 MHz, $D_2$O) δ 170.10, 67.39, 52.57, 38.89, 30.45, 29.21, 27.46, 26.18, 24.58, 21.77, 21.38, 13.17. ESI-MS m/z calcd for C$_{12}$H$_{26}$N$_2$O$_2$ [M+H]$^+$: 231.21. Found: 231.24.

1-L-Lysine octyl ester dihydrochloride (406)

The procedure above was used to give compound 6 in an isolated yield of 70%.

$^1$H NMR (400 MHz, $D_2$O) δ 4.27 (td, $J = 6.3, 3.2$ Hz, 2H), 4.14 (t, $J = 6.4$ Hz, 1H), 3.00 (t, $J = 7.9$ Hz, 2H), 1.99 (m, 2H), 1.70 (m, 4H), 1.50 (m, 2H), 1.14-1.21 (m, 10H), 0.85 (t, $J = 6.1$ Hz, 3H). $^{13}$C NMR (400 MHz, $D_2$O) δ 170.10, 67.38, 52.57, 38.90, 30.96, 29.21, 28.23, 28.12, 27.49, 26.19, 24.89, 21.91, 21.39, 13.31. ESI-MS m/z calcd for C$_{14}$H$_{30}$N$_2$O$_2$ [M+H]$^+$: 259.41. Found: 259.34.

1-L-Lysine decyl ester dihydrochloride (407)

The procedure above was used to give compound 407 in an isolated yield of 72%.

$^1$H NMR (400 MHz, $D_2$O) δ 4.26 (td, $J = 6.2, 2.2$ Hz, 2H), 4.14 (s, 1H), 3.00 (t, $J = 7.6$ Hz, 2H), 1.98 (m, 2H), 1.70 (m, 4H), 1.50 (m, 2H), 1.38-1.20 (m, 14H), 0.84 (t, $J = 6.0$ Hz, 3H). $^{13}$C NMR (400 MHz, $D_2$O) δ 169.92, 67.01, 52.56, 48.81, 31.47, 29.26, 29.07, 29.03, 28.84, 28.65, 27.73, 26.20, 25.16, 22.24, 21.47, 13.57. ESI-MS m/z calcd for C$_{16}$H$_{34}$N$_2$O$_2$ [M+H]$^+$: 287.27. Found: 287.35.

1-L-Lysine dodecyl ester dihydrochloride (408)
Nα,Nε-Bis(t-butyloxycarbonyl)-L-lysine (0.25 g, 0.71 mmol) and carbonyldiimidazole (0.13 g, 0.78 mmol) were dissolved in anhydrous DCM (7.1 mL) and the mixture was stirred at room temperature for 45 min. Dodecanol (0.19 mL, 0.85 mmol) was added and the mixture was stirred overnight. The solvent was evaporated under reduced pressure and the residue was purified via silica thin layer chromatography (toluene x 3 elution) to yield Nα,Nε-bis(t-butyloxycarbonyl)-L-lysine dodecyl ester (0.22 g, 0.41 mmol, 58%) as a yellowish oil. The oil (0.023 g, 0.047 mmol) was suspended in 2 M HCl in anhydrous Et2O (0.5 mL) and stirred at room temperature for 2 hrs after which the residue was concentrated under reduced pressure. The oil was washed with Et2O x 4 and residual Et2O was removed under reduced pressure to yield L-lysine dodecyl ester dihydrochloride (408) as a yellow oil (0.017 g, 0.043 mmol, 96%).

1H NMR (500 MHz, D2O) δ 4.23 (m, 2H), 4.12 (t, J = 6.4 Hz, 1H), 2.97 (t, J = 7.7, 2H), 1.95 (m, 2H), 1.67 (m, 4H), 1.47 (m, 2H), 1.36-1.18 (m, 18H), 0.82 (t, J = 6.9 Hz, 3H).

13C NMR (400 MHz, D2O) δ 170.08, 67.32, 52.61, 38.94, 31.28, 29.54, 29.28, 28.89, 28.80, 28.72, 28.62, 28.35, 27.62, 26.24, 25.02, 21.47, 13.46.


L-lysine tetradecyl ester dihydrochloride (409)

Nα,Nε-Bis(t-butyloxycarbonyl)-L-lysine (0.21 g, 0.60 mmol) and carbonyldiimidazole (0.11 g, 0.66 mmol) were dissolved in anhydrous DCM (6.0 mL) and the mixture was stirred at room temperature for 45 min. Tetradecanol (0.16, 0.72 mmol) was added and the mixture was stirred overnight. The solvent was evaporated under reduced pressure and the residue was purified via silica thin layer chromatography (toluene x 3 elution) to yield Nα,Nε-bis(t-butyloxycarbonyl)-L-lysine tetradecyl ester (0.20 g, 0.37 mmol, 61%) as a yellowish oil. The oil (0.20 g, 0.37 mmol) was suspended in 2 M HCl in anhydrous Et2O (0.5 mL) and stirred at room temperature for 2 hrs after which the residue was concentrated under reduced pressure. The oil was washed with Et2O x 4 and residual Et2O was removed under reduced pressure to yield L-lysine tetradecyl ester dihydrochloride (409) as a yellow oil (0.15 g, 0.36 mmol, 98%).

1H NMR (500 MHz, D2O) δ 4.23 (m, 2H), 4.11 (t, J = 6.4 Hz, 1H), 2.97 (t, J = 7.7 Hz, 2H), 1.96 (m, 2H), 1.67 (m, 4H), 1.47 (m, 2H), 1.36-1.18 (m, 22H), 0.82 (t, J = 6.4 Hz, 3H).


(c) General Procedure for synthesis of di-alkyl lysine derivatives (420–434)

General Procedure for synthesis of salts (410–419, 421)
Sodium Salts:
The di-alkyl L-lysine was dissolved in MeOH (final concentration of 0.1 M) and 4 M NaOH (1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure. The residue was dissolved in H₂O and filtered through a Celite pad and dried by lyophilisation to yield a white powder or a yellowish oil. All yields were > 95%.

Lithium Salts:
The above procedure was used except a 2 M LiOH solution was used.

Potassium Salts:
The above procedure was used except a 4 M KOH solution was used.

Sodium N⁺-hexanoyl-N⁺-butanoyl-L-lysinate (410)

The procedure above was used from the di-alkyl L-lysine 427.

\[ ^1H \text{ NMR} (300 \text{ MHz, D}_2O) \delta 4.00 (dd, J = 9.0, 4.2 \text{ Hz, 1 H}), 3.04 (t, J = 6.7 \text{ Hz, 2 H}), 2.14 (td, J = 4.1, 7.4 \text{ Hz, 2 H}), 2.06 (t, J = 7.3 \text{ Hz, 2H}), 1.66 (m, 1H), 1.53-1.24 (m, 7H), 1.24-1.02 (m, 6H), 0.76 (t, J = 7.10 \text{ Hz, 3H}), 0.85 (t, J = 6.5 \text{ Hz, 3H}). \]

\[ ^13C \text{ NMR} (300 \text{ MHz, D}_2O) \delta 179.39, 176.80, 176.61, 54.80, 38.97, 37.66, 35.64, 31.16, 30.39, 27.80, 25.00, 22.61, 21.56, 18.99, 13.18, 12.66. \text{ ESI-MS m/z calcd for C}_{16}H_{29}N_2O_4 - [M]: 313.21. \text{ Found: 313.17.} \]

Sodium N⁺,N⁺-bis(hexanoyl)-L-lysinate (411)

The procedure above was used from the di-alkyl L-lysine 428.

\[ ^1H \text{ NMR} (500 \text{ MHz, D}_2O) \delta 4.10 (dd, J = 9.2, 4.4 \text{ Hz, 1H}), 3.13 (t, J = 6.8 \text{ Hz, 2H}), 2.24 (m, 2H), 2.17 (t, J = 7.4 \text{ Hz, 2H}), 1.76 (m, 1H), 1.68-1.41 (m, 7H), 1.38-1.17 (m, 11H), 0.83 (t, J = 7.0 \text{ Hz, 3H}), 0.82 (t, J = 7.0 \text{ Hz, 3H}). \]

\[ ^13C \text{ NMR} (300 \text{ MHz, D}_2O) \delta 179.33, 177.03, 176.56, 54.85, 39.03, 35.77, 35.73, 31.30, 30.48, 30.41, 27.87, 25.10, 25.06, 22.65, 21.63, 21.60, 13.20, 13.17. \text{ ESI-MS m/z calcd for C}_{20}H_{37}N_2O_4 - [M]: 341.24. \text{ Found: 341.25.} \]

Lithium, Sodium, or Potassium N⁺-hexanoyl-N⁺-octanoyl-L-lysinate (412)

The procedures above were used from the di-alkyl L-lysine 429.
$^1$H NMR (500 MHz, D$_2$O) $\delta$ 4.11 (dd, $J = 9.3$, 3.9 Hz, 1H), 3.14 (bt, $J = 6.5$ Hz, 2H), 2.24 (m, 2H), 2.17 (t, $J = 7.4$ Hz, 2H), 1.78 (m, 1H), 1.71-1.40 (m, 7H), 1.40-1.12 (m, 14H), 0.83 (t, $J = 6.7$ Hz, 3H), 0.81 (t, $J = 7.0$ Hz, 3H). $^{13}$C NMR (400 MHz, D$_2$O) $\delta$ 179.33, 175.96, 175.93, 54.81, 38.95, 36.02, 35.93, 31.45, 31.42, 30.85, 28.74, 28.65, 28.14, 25.74, 25.34, 22.76, 22.29, 21.96, 13.62, 13.52. ESI-MS m/z calcd for C$_{20}$H$_{37}$N$_2$O$_4$ [M]: 369.28. Found: 369.25.

Lithium, Sodium, or Potassium $N^\alpha$-hexanoyl-$N^\varepsilon$-decanoyl-$\ell$-lysinate (413)

The procedures above were used from the di-alkyl L-lysine 430.

$^1$H NMR (500 MHz, D$_2$O) $\delta$ 4.13 (bdd, $J = 9.2$, 4.1 Hz, 1H), 3.15 (m, 1H), 2.26 (m, 2H), 2.17 (bt, 2H), 1.79 (m, 1H), 1.57-1.25 (m, 7H), 1.42-0.12 (m, 22H), 0.87 (t, $J = 6.9$ Hz, 3H), 0.83 (t, $J = 6.9$ Hz, 3H). $^{13}$C NMR (500 MHz, D$_2$O) $\delta$ 179.21, 175.54, 175.32, 54.68, 38.83, 36.07, 35.91, 31.77, 31.36, 30.97, 29.50, 29.35, 29.22, 29.13, 29.19, 25.88, 25.39, 22.75, 22.46, 22.06, 13.69, 13.63. ESI-MS m/z calcd for C$_{22}$H$_{41}$N$_2$O$_4$ [M]: 397.31. Found: 397.24.

Lithium, Sodium, or Potassium $N^\alpha$-hexanoyl-$N^\varepsilon$-dodecanoyl-$\ell$-lysinate (414)

The procedures above were used from the di-alkyl L-lysine 431.

$^1$H NMR (400 MHz, D$_2$O) $\delta$ 4.15 (dd, $J = 9.4$, 3.8 Hz, 1H), 3.16 (m, 2H), 2.28 (m, 2H), 2.19 (t, $J = 7.4$ Hz, 2H), 1.80 (m, 1H), 1.73-1.42 (m, 7H), 1.42-0.12 (m, 22H), 0.87 (t, $J = 6.9$ Hz, 3H), 0.83 (t, $J = 6.9$ Hz, 3H). $^{13}$C NMR (500 MHz, D$_2$O) $\delta$ 179.27, 175.26, 174.96, 174.96, 54.64, 38.82, 36.17, 36.07, 31.94, 31.77, 31.42, 30.00, 29.90, 29.79, 29.50, 29.03, 29.01, 28.26, 28.08, 25.93, 22.80, 22.57, 22.55, 13.92, 13.67. ESI-MS m/z calcd for C$_{24}$H$_{45}$N$_2$O$_4$ [M]: 425.34. Found: 425.33.

Lithium, Sodium, or Potassium $N^\alpha$-ethanoyl-$N^\varepsilon$-dodecanoyl-$\ell$-lysinate (415)

Compounds were prepared as previously described from 20. $^{13}$ All spectral data was consistent with that reported in the literature.

Sodium $N^\alpha$-octanoyl-$N^\varepsilon$-dodecanoyl-$\ell$-lysinate (416)

The procedure above was used from the di-alkyl L-lysine 437.

$^1$H NMR (500 MHz, D$_2$O) $\delta$ 4.14 (bd, $J = 8.5$ Hz, 1H), 3.15 (m, 2H), 2.27 (bt, 2H), 2.18 (m, 2H), 1.80 (m, 1H), 1.79 (s, 1H), 1.57-1.26 (m, 7H), 1.26-0.92 (m, 26H), 0.85 (t, $J =
6.8 Hz, 3H), 0.80 (t, J = 6.7 Hz, 3H). $^{13}$C NMR (126 MHz, $D_2$O) δ 179.27, 175.26, 174.96, 54.64, 38.82, 36.17, 36.07, 31.94, 31.77, 31.42, 30.00, 30.00, 29.90, 29.79, 29.50, 29.50, 29.03, 29.01, 28.26, 26.08, 25.93, 22.80, 22.57, 22.55, 13.92, 13.92. ESI-MS m/z calcd for C$_{26}$H$_{46}$N$_2$O$_4$ [M] : 453.37. Found: 453.37.

Sodium $N^\alpha$,$N^\varepsilon$-bis(dodecanoyl)-l-lysinate (417)

The procedure above was used from the di-alkyl l-lysine 438.

$^1$H NMR (300 MHz, $D_2$O + 10 % CD$_3$OD) δ 4.18 (dd, J = 7.7, 4.7 Hz, 1H), 3.06 (t, J = 6.8 Hz, 2H), 2.14 (t, J = 7.5 Hz, 2H), 2.07 (t, J = 7.5 Hz, 2H), 1.74 (m, 1H), 1.68-1.36 (m, 7H), 1.36-1.10 (m, 34H), 0.85 (t, J = 6.7 Hz, 3H), 0.81 (t, J = 6.7 Hz, 3H). $^{13}$C NMR (300 MHz, $D_2$O + 10 % CD$_3$OD) δ 179.19, 176.23, 175.40, 55.99, 40.35, 37.48, 37.25, 33.87, 33.14, 30.84, 30.82, 30.81, 30.80, 30.75, 30.71, 30.58, 30.55, 30.55, 30.54, 30.52, 30.47, 30.43, 30.12, 27.19, 27.12, 24.24, 23.80, 14.50, 14.50. ESI-MS m/z calcd for C$_{30}$H$_{57}$N$_2$O$_4$ - [M] : 509.43. Found: 509.42.

Sodium $N^\alpha$-dodecanoyl-$N^\varepsilon$-butanoyl-l-lysinate (418)

The procedure above was used from the di-alkyl l-lysine 432.

$^1$H NMR (500 MHz, $D_2$O) δ ppm 4.14 (dd, J = 8.8, 3.8 Hz, 1H), 3.14 (m, 2H), 2.26 (t, J = 7.4 Hz, 2H), 1.79 (m, 1H), 1.70-1.40 (m, 7H), 1.40-1.10 (m, 26H), 0.85 (t, J = 0.5 Hz, 3H). $^{13}$C NMR (500 MHz, $D_2$O) δ ppm 179.15, 175.59, 175.22, 54.66, 38.87, 37.83, 36.06, 31.81, 31.47, 29.67, 29.61, 29.38, 29.28, 29.06, 28.22, 25.86, 22.74, 22.45, 19.14, 13.77, 13.11. ESI-MS m/z calcd for C$_{22}$H$_{41}$N$_2$O$_4$- [M] : 397.31. Found: 397.34.

Sodium $N^\alpha$-dodecanoyl-$N^\varepsilon$-octanoyl-l-lysinate (419)

The procedure above was used from the di-alkyl l-lysine 433.

$^1$H NMR (500 MHz, $D_2$O) δ 4.13 (dd, J = 8.7, 3.3 Hz, 1H), 3.15 (m, 2H), 2.26 (m, 2H), 2.18 (t, J = 7.4 Hz, 2H), 1.79 (m, 1H), 1.70-1.40 (m, 7H), 1.40-1.10 (m, 26H), 0.85 (t, J = 7.0 Hz, 3H), 0.82 (t, J = 6.6 Hz, 3H). $^{13}$C NMR (500 MHz, $D_2$O) δ 179.25, 175.30, 175.10, 54.62, 38.75, 36.16, 36.07, 31.93, 31.77, 31.40, 29.97, 29.91, 29.85, 29.72, 29.50, 29.33, 29.05, 29.03, 28.22, 26.05 25.90, 22.70, 22.58, 22.55, 13.88, 13.77. ESI-MS m/z calcd for C$_{26}$H$_{48}$N$_2$O$_4$ [M] : 453.37. Found: 453.43.

$N^\alpha$-ethanoyl-$N^\varepsilon$-dodecanoyl-l-lysine (420)
Compound was prepared as previously described. All spectral data was consistent with that reported in the literature.

**Sodium N\textsuperscript{\alpha}-hexanoyl-N\textsuperscript{\epsilon}-hexyl-L-lysinate (421)**

The procedure above was used from the di-alkyl L-lysine 439.

\textsuperscript{1}H NMR (500 MHz, D\textsubscript{2}O) δ 4.13 (dd, J = 8.5, 4.3 Hz, 1H), 2.71 (m, 3H), 2.25 (m, 2H), 2.18 (t, J = 7.4 Hz, 2H), 1.79 (m, 1H), 1.70-1.40 (m, 7H), 1.42-1.14 (m, 13H), 0.84 (t, J = 6.9 Hz, 3H), 0.83 (t, J = 7.0 Hz, 3H). \textsuperscript{13}C NMR (500 MHz, D\textsubscript{2}O) δ 179.21, 176.59, 54.66, 48.00, 47.72, 35.65, 31.23, 30.66, 30.42, 27.02, 26.61, 25.76, 25.02, 22.61, 21.76, 21.61, 13.20, 13.17. ESI-MS m/z calcd for C\textsubscript{18}H\textsubscript{35}N\textsubscript{2}O\textsubscript{3} [M] : 327.27. Found: 327.21.

**N\textsuperscript{\alpha}-Hexanoyl-N\textsuperscript{\epsilon}-carboxybenzyl-L-lysine (423)**

N\textsuperscript{\alpha}-Carboxybenzyl-L-lysine 422 (0.61 g, 2.18 mmol) was dissolved in 10 % NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0 °C and hexanoyl chloride (0.46 mL, 3.26 mmol, 1.5 eq) was added slowly to the ether layer with no mixing. The biphasic solution was vigorously stirred at 0 °C for 4 hrs and then at room temperature for 18 hrs. The ether was evaporated under a stream of air and the solution was carefully acidified with concentrated HCl to ca. pH = 1. The mixture was extracted with DCM five times. The combined organic phases were washed with brine, dried with MgSO\textsubscript{4}, and concentrated under reduced pressure. The crude product was purified by silica column chromatography (3:7 Pet Ether: EtOAc + 1 % AcOH) to yield white product (0.73 g, 1.94 mmol, 89%).

\textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) δ 10.22 (bs, 1H), 7.26 (m, 5H), 6.58 (d, J = 7.09 Hz, 1H), 5.01 (bs, 2H), 4.51 (m, 1H), 3.11 (dt, J = 6.4, 6.2 Hz, 2H), 2.15 (t, J = 7.6 Hz, 2H), 1.80 (m, 1H), 1.68 (m, 1H), 1.61-1.11 (m, 11H), 0.80 (t, J = 6.84 Hz, 3H). \textsuperscript{13}C NMR (400 MHz, CDCl\textsubscript{3}) δ 174.90, 174.47, 156.91, 136.35, 128.51, 128.13, 127.97, 67.28, 66.73, 52.98, 40.30, 36.26, 31.32, 29.39, 25.27, 22.30, 22.12, 13.90. ESI-MS m/z calcd for C\textsubscript{20}H\textsubscript{30}N\textsubscript{2}O\textsubscript{5} [M+H] : 379.23. Found: 379.33.

**N\textsuperscript{\alpha}-Dodecanoyl-N\textsuperscript{\epsilon}-carboxybenzyl-L-lysine (424)**

N\textsuperscript{\epsilon}-Carboxybenzyl-L-lysine 422 (0.31 g, 1.09 mmol) was dissolved in 10 % NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0 °C and dodecanoyl chloride (0.38 mL, 1.64 mmol, 1.5 eq) was added slowly to the ether layer with no mixing. The biphasic solution was vigorously stirred at 0 °C for
4 hrs and then at room temperature for 18 hrs. The ether was evaporated under a stream of air and the solution was carefully acidified with concentrated HCl to ca. pH = 1. The mixture was extracted with DCM five times. The combined organic phases were washed with brine, dried with MgSO₄, and concentrated under reduced pressure. The crude product was purified by silica column chromatography (3:7 Pet Ether: EtOAc + 1 % AcOH) to yield white product (0.73 g, 1.94 mmol, 89%).

**1H NMR** (300 MHz, (CD₂)₂SO) δ 7.78 (d, J = 7.8 Hz, 1H), 7.73 (m, 5H), 7.04 (bs, 1H), 5.02 (s, 2H), 4.16 (td, J = 8.4, 5.1 Hz, 1H), 2.99 (dd, J = 13.0, 6.7 Hz, 2H), 2.11 (dt, J = 7.2, 6.9 Hz, 2H), 1.68 (m, 1H), 1.49 (m, 2H), 1.42 (m, 2H), 1.35-1.20 (m, 18H), 0.86 (t, J = 6.89 Hz, 3H).


Nα-Hexanoyl-L-lysine (425)

Nα-Hexanoyl-Nε-carboxybenzyl-L-lysine (423) (0.053 g, 0.14 mmol) was dissolved in MeOH (final concentration of 0.1 M) and Pd(OH)₂ on carbon (5 mg) was added. H₂ atmosphere was introduced into the flask and the mixture was stirred for 16 hours at room temperature after which the solution was filtered through a Celite pad and concentrated under reduced pressure to yield a white powder (425) (0.034 g, 0.09 mmol, 98%).

**1H NMR** (300 MHz, D₂O) δ 7.71 (d, J = 7.7 Hz, 1H), 4.03 (m, 1H), 2.84 (t, J = 7.6 Hz, 2H), 2.13 (t, J = 7.5 Hz, 2H), 1.74-1.34 (m, 6H), 1.36-1.04 (m, 6H), 0.72 (t, J = 6.8 Hz, 3H). **13C NMR** (500 MHz, D₂O) δ 178.97, 176.65, 54.60, 39.13, 35.61, 30.99, 30.41, 26.22, 24.96, 22.04, 21.57, 13.12. **ESI-MS m/z** calcd for C₁₂H₂₄N₂O₃ [M+H]⁺: 245.19. Found: 245.24.

**Nα-Dodecanoyl-L-lysine (426)**

Nα-Dodecanoyl-Nε-carboxybenzyl-L-lysine (424) (0.12 g, 0.25 mmol) was dissolved in MeOH (final concentration of 0.1 M) and Pd(OH)₂ on carbon (5 mg) was added. H₂ atmosphere was introduced into the flask and the mixture was stirred for 16 hours at room temperature after which the solution was filtered through a Celite pad and concentrated under reduced pressure to yield a white powder (426) (0.079 g, 0.21 nmol, 96%).

**1H NMR** (300 MHz, D₂O) δ 4.19 (bt, J = 6.3 Hz, 1H), 2.84 (t, J = 7.5 Hz, 2H), 2.15 (t, J = 7.5 Hz, 2H), 1.74 (m, 1H), 1.77-1.51 (m, 5H), 1.51-1.17 (m, 19H), 0.81 (t, J = 6.6 Hz, 3H). **13C NMR** (500 MHz, (CD₂)₂SO) δ 175.31, 172.57, 52.27, 37.52, 34.33, 30.24, 30.10, 27.79, 27.76, 27.68, 27.52, 27.49, 27.41, 25.02, 24.02, 20.75, 20.47, 11.45. **ESI-MS m/z** calcd for C₁₈H₃₆N₂O₃ [M+H]⁺: 329.28. Found: 329.38.
General procedure for di-alkyl lysine derivatives (427-433)

$N^\alpha$-Alkyl-$\varepsilon$-lysine (425 or 426) was dissolved in 10 % NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0 °C and alkyl acyl chloride (1.5 eq) was added slowly to the ether layer with no mixing. The biphasic solution was vigorously stirred at 0 °C for 4 hrs and then at room temperature for 18 hrs. The ether was evaporated under a stream of air and the solution was carefully acidified with concentrated HCl to ca. pH = 1. The white precipitate was filtered, washed with water then dried. If the product did not precipitate, the mixture was extracted with DCM five times. The combined organic phases were washed with brine, dried with MgSO$_4$, filtered, and concentrated under reduced pressure. The crude product was purified by silica column chromatography (2:8 Pet Ether:EtOAc + 1 % AcOH to 9:1 EtOAc:MeOH + 1 % AcOH) to yield white product.

$N^\alpha$-Hexanoyl-$\varepsilon$-butanoyl-$\varepsilon$-lysine (427)

The procedure above was used with $N^\alpha$-hexanoyl-$\varepsilon$-lysine (425) (0.033 g, 0.13 mmol) and butyryl chloride (20 µL, 0.20 mmol) to yield 427 (0.014 g, 0.045 mmol, 34%).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 6.84 (d, $J = 7.2$ Hz, 1H), 6.06 (t, $J = 4.1$, 1H), 4.55 (td, $J = 7.2$, 5.0, 5.0 Hz, 1H), 3.33 (m, 1H), 3.20 (m, 1H), 2.25 (t, $J = 7.3$, 2H), 2.18 (t, $J = 7.5$, 2H), 1.83 (m, 2H), 1.64 (m, 4H), 1.53 (m, 2H), 1.44-1.19 (m, 6H), 0.93 (t, $J = 7.4$, 7.4 Hz, 3H), 0.88 (t, $J = 7.4$, 7.4 Hz, 3H).

$^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 174.92, 174.37, 174.30, 52.21, 38.89, 38.39, 36.26, 31.46, 31.34, 28.76, 25.33, 22.29, 22.27, 19.19, 13.88, 13.66. ESI-MS m/z calcd for C$_{16}$H$_{30}$N$_2$O$_4$ [M +H]$^+$: 315.23. Found: 315.25.

$N^\alpha$-$N^\varepsilon$-Bis(hexanoyl)-$\varepsilon$-lysine (428)

The procedure above was used with $N^\alpha$-hexanoyl-$\varepsilon$-lysine (425) (0.033 g, 0.14 mmol) and hexanoyl chloride (28 µL, 0.20 mmol) to yield 428 (0.034 g, 0.10 mmol, 74%).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 6.81 (d, $J = 6.2$ Hz, 1H), 6.03 (t, $J = 4.8$, 1H), 4.54 (m, 1H), 3.31 (s, 1H), 3.20 (s, 1H), 2.25 (t, $J = 7.5$, 7.5 Hz, 4H), 2.19 (t, $J = 7.5$, 7.5 Hz, 2H), 1.83 (m, 2H), 1.57 (m, 3H), 1.30 (m, 11H), 0.89 (t, $J = 6.2$, 6.2 Hz, 3H), 0.89 (t, $J = 6.2$, 6.2 Hz, 3H).

$^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 174.63, 174.59, 174.33, 53.53, 50.69, 38.93, 36.63, 36.37, 31.48, 31.44, 31.41, 28.88, 25.52, 25.39, 22.37, 22.21, 13.94, 13.94. ESI-MS m/z calcd for C$_{18}$H$_{34}$N$_2$O$_4$ [M +H]$^+$: 343.26. Found: 343.3.

$N^\alpha$-Hexanoyl-$N^\varepsilon$-octanoyl-$\varepsilon$-lysine (429)
The procedure above was used with Nα-hexanoyl-l-lysine (425) (0.026 g, 0.11 mmol) and octanoyl chloride (28 µL, 0.62 mmol) to yield 429 (0.032 g, 0.086 mmol, 80%).

\[ ^1H \text{NMR} \quad \delta \quad 6.80 \ (d, \ J = 6.5 \text{ Hz}, \ 1H), \ 5.82 \ (m, \ 1H), \ 4.52 \ (t, \ J = 7.3, \ 4.8, \ 4.8 \text{ Hz}, \ 1H), \ 3.36 \ (m, \ 1H), \ 3.18 \ (m, \ 1H), \ 2.35 \ (t, \ J = 7.5 \text{ Hz}, \ 1H), \ 2.27 \ (t, \ J = 7.1, \ 1.1 \text{ Hz}, \ 2H), \ 2.19 \ (t, \ J = 7.6 \text{ Hz}, \ 2H), \ 1.90 \ (m, \ 1H), \ 1.81 \ (m, \ 1H), \ 1.63 \ (m, \ 4H), \ 1.53 \ (m, \ 2H), \ 1.45-1.23 \ (m, \ 13H), \ 0.89 \ (t, \ J = 6.7, \ 3H), \ 0.87 \ (t, \ J = 6.1, \ 3H). \]

\[ ^{13}C \text{NMR} \quad \delta \quad 174.59, \ 174.59, \ 174.32, \ 52.13, \ 38.93, \ 36.68, \ 36.37, \ 31.70, \ 31.50, \ 35.50, \ 31.41, \ 29.28, \ 29.10, \ 28.93, \ 25.86, \ 25.39, \ 22.61, \ 22.37, \ 22.23, \ 14.07, \ 13.95. \]

ESI-MS m/z calcd for C20H38N2O4 [M+H]+: 371.29. Found: 371.32.

**Nα-Hexanoyl-Nε-decanoyl-l-lysine (430)**

The procedure above was used with Nα-hexanoyl-l-lysine (425) (0.022 g, 0.090 mmol) decanoyl chloride (28 µL, 0.13 mmol) to yield 430 (0.027 g, 0.67 mmol, 75%).

\[ ^1H \text{NMR} \quad \delta \quad 6.83 \ (bd, \ J = 5.2 \text{ Hz}, \ 1H), \ 6.18 \ (bs, \ 1H), \ 4.45 \ (bd, \ J = 4.8 \text{ Hz}, \ 1H), \ 3.57 \ (q, \ J = 7.2, \ 7.2, \ 7.2 \text{ Hz}, \ 2H), \ 3.21 \ (m, \ 2H), \ 2.22 \ (t, \ J = 7.7, \ 7.7 \text{ Hz}, \ 2H), \ 2.16 \ (t, \ J = 7.7, \ 7.7, \ 2H), \ 1.86 \ (m, \ 1H), \ 1.71 \ (m, \ 1H), \ 1.67-1.44 \ (m, \ 6H), \ 1.44-1.18 \ (m, \ 21H), \ 0.87 \ (t, \ J = 7.0 \text{ Hz}, \ 3H), \ 0.87 \ (t, \ J = 6.7 \text{ Hz}, \ 3H). \]

\[ ^{13}C \text{NMR} \quad \delta \quad 173.69, \ 173.41, \ 173.37, \ 57.94, \ 39.18, \ 36.78, \ 36.73, \ 31.88, \ 31.51, \ 29.51, \ 29.43, \ 29.31, \ 28.01, \ 25.93, \ 25.48, \ 22.68, \ 22.54, \ 22.42, \ 14.12, \ 13.99, \ 8.10, \ 8.10. \]

ESI-MS m/z calcd for C22H42N2O4 [M+H]+: 399.32. Found: 399.36.

**Nα-Hexanoyl-Nε-dodecanoyl-l-lysine (431)**

The procedure above was used with Nα-hexanoyl-l-lysine (425) (0.032 g, 0.13 mmol) and dodecanoyl chloride (45 µL, 0.20 mmol) to yield 431 (0.049 g, 0.11 mmol, 87%).

\[ ^1H \text{NMR} \quad \delta \quad 7.11 \ (bs, \ 1H), \ 6.63 \ (bs, \ 1H), \ 4.37 \ (bs, \ 1H), \ 3.16 \ (bs, \ 2H), \ 2.18 \ (m, \ 4H), \ 1.79 \ (m, \ 1H), \ 1.71-1.40 \ (m, \ 7H), \ 1.40-1.15 \ (m, \ 22H), \ 0.86 \ (bt, \ J = 5.88, \ 5.88 \text{ Hz}, \ 6H). \]

\[ ^{13}C \text{NMR} \quad \delta \quad 174.29, \ 174.13, \ 174.13, \ 38.93, \ 36.64, \ 36.38, \ 31.87, \ 31.47, \ 29.65, \ 29.61, \ 29.61, \ 29.57, \ 29.43, \ 29.43, \ 29.43, \ 29.32, \ 29.32, \ 25.91, \ 25.44, \ 22.64, \ 22.38, \ 14.06, \ 13.94. \]

ESI-MS m/z calcd for C24H46N2O4 [M +H]+: 427.36. Found: 427.54.

**Nα-Dodecanoyl-Nε-butanoyl-l-lysine (432)**

The procedure above was used with Nα-dodecanoyl-l-lysine (426) (0.010 g, 0.030 mmol) and butryl chloride (5 µL, 0.046 mmol) to yield 432 (0.011 g, 0.027 mmol, 87%).
$^1$H NMR (500 MHz, CDCl$_3$) δ 6.96 (bs, 1H), 6.37 (bs, 1H), 4.41 (bs, 1H), 3.20 (bs, 2H), 2.22 (m, 2H), 2.17 (t, $J = 7.3$ Hz, 2H), 1.83 (m, 1H), 1.75-1.42 (m, 6H), 1.42-1.16 (m, 19H), 0.92 (t, $J = 7.4$ Hz, 3H), 0.88 (t, $J = 6.7$ Hz, 3H). $^{13}$C NMR (400 MHz, CDCl$_3$) δ 174.11, 174.02, 174.02, 57.93, 38.83, 38.51, 36.43, 31.90, 29.68, 29.64, 29.60, 29.59, 29.45, 29.43, 29.35, 29.31, 28.94, 25.80, 22.67, 19.22, 14.09, 13.75. ESI-MS m/z calcd for C$_{22}$H$_{42}$N$_2$O$_4$ [M +H]$^+$: 399.32. Found: 399.42.

$^{N^\alpha}$-Dodecanoyl-$^{N^\varepsilon}$-octanoyl-$\ell$-lysine (433)

The procedure above was used with $^{N^\alpha}$-dodecanoyl-$\ell$-lysine (426) (0.011 g, 0.033 mmol) and octanoyl chloride (9 µL, 0.049 mmol) to yield 433 (0.009 g, 0.019 mmol, 57%).

$^1$H NMR (500 MHz, CDCl$_3$) δ 6.85 (d, $J = 6.9$ Hz, 1H), 5.79 (t, $J = 5.4$ Hz, 1H), 4.49 (m, 1H), 3.38 (m, 1H), 3.19 (m, 1H), 2.27 (td, $J = 7.3$, 3.3, 2H), 2.19 (t, $J = 7.7$ Hz, 2H), 1.90 (m, 1H), 1.82 (s, 1H), 1.68-1.57 (m, 4H), 1.57-1.48 (m, 2H), 1.45-1.15 (m, 26H), 0.87 (t, $J = 6.8$ Hz, 6H). $^{13}$C NMR (400 MHz, CDCl$_3$) δ 174.74, 174.58, 174.29, 52.26, 38.54, 36.76, 36.35, 31.90, 31.67, 30.73, 29.64, 29.62, 29.53, 29.37, 29.34, 29.31, 29.23, 29.11, 28.98, 25.78, 25.68, 22.68, 22.59, 21.82, 14.11, 14.05. ESI-MS m/z calcd for C$_{26}$H$_{50}$N$_2$O$_4$ [M +H]$^+$: 455.39. Found: 455.52.

$^{N^\alpha}$-Octanoyl-$^{N^\varepsilon}$-dodecanoyl-$\ell$-lysine (437)

The procedure above was used with $^{N^\varepsilon}$-dodecanoyl-$\ell$-lysine (434) (0.034 g, 0.10 mmol) and octanoyl chloride (26 µL, 0.15 mmol) to yield 437 (0.029 g, 0.065 mmol, 63%).

$^1$H NMR (500 MHz, CDCl$_3$) δ 6.79 (d, $J = 6.7$ Hz, 1H), 5.89 (m, 1H), 4.53 (s, 1H), 3.35 (m, 1H), 3.20 (m, 1H), 2.26 (m, 2H), 2.19 (m, 2H), 1.90 (m, 1H), 1.81 (m, 1H), 1.71-1.47 (m, 6H), 1.46-1.20 (m, 26H), 0.87 (t, $J = 6.6$ Hz, 6H), 0.87 (t, $J = 6.6$ Hz, 6H). $^{13}$C NMR (400 MHz, CDCl$_3$) δ 174.60, 174.51, 174.29, 52.10, 38.54, 36.76, 36.35, 31.90, 31.67, 30.73, 29.64, 29.62, 29.53, 29.37, 29.34, 29.31, 29.23, 29.11, 28.98, 25.78, 25.68, 22.68, 22.59, 21.82, 14.11, 14.05. ESI-MS m/z calcd for C$_{26}$H$_{50}$N$_2$O$_4$ [M +H]$^+$: 455.39. Found: 455.52.

$^{N^\alpha}$,$^{N^\varepsilon}$-Bis(dodecanoyl)-$\ell$-lysine (438)

The procedure above was used with $^{N^\varepsilon}$-dodecanoyl-$\ell$-lysine (434) (33 mg, 0.10 mmol) and dodecanoyl chloride (35 µL, 0.15 mmol) to yield 438 (0.045 g, 0.088 mmol, 87%).

$^1$H NMR (500 MHz, CDCl$_3$) δ 6.83 (d, $J = 7.1$ Hz, 1H), 5.84 (t, $J = 5.9$ Hz, 1H), 4.51 (m, 1H), 3.36 (m, 1H), 3.18 (m, 1H), 2.26 (dt, $J = 7.3$, 2.19 Hz, 2H), 2.18 (m, 2H), 1.89 (m, 1H), 1.81 (m, 1H), 1.62 (m, 4H), 1.53 (m, 2H), 1.45-1.19 (m, 34H), 0.87 (t, $J = 7.0$ Hz, 6H).
Hz, 6H). $^{13}$C NMR (300 MHz, CDCl$_3$) $\delta$ 174.81, 174.47, 174.35, 52.31, 38.41, 36.83, 36.33, 31.90, 30.59, 29.66, 29.63, 29.61, 29.53, 29.51, 29.38, 29.35, 29.34, 29.34, 29.31, 29.16, 25.78, 25.67, 22.68, 21.77, 14.11, 14.11. ESI-MS m/z calcd for C$_{30}$H$_{58}$N$_2$O$_4$ [M +H]$^+$: 511.45. Found: 511.58.

$^{N^\alpha}$-Hexanoyl-$^{N^\varepsilon}$-hexyl-$^{L}$-lysine (439)

423 (0.03 g, 0.079 mmol) was dissolved in MeOH and the following were added: hexanal (14 µL, 0.12 mmol), acetic acid (9 µL, 0.16 mmol), Pd(OH)$_2$ on carbon (3 mg). The mixture was subjected to 40 bar of H$_2$ in a Parr Bomb while stirring at 50 °C for 18 hrs. The mixture was cooled to room temperature for 1 hr before opening Parr Bomb to atmosphere. The Pd(OH)$_2$ on carbon was filtered and the filtrate was concentrated under reduced pressure. The residue was purified via silica column chromatography (5:1:1:1 EtOAc:MeOH:H$_2$O:CH$_3$CN, Rf = 0.3) to yield 439 as a yellowish oil (8.6 mg, 0.026 mmol, 33%).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 6.77 (bs, 1H), 4.20 (td, $J = 5.2, 5.8$ Hz, 1H), 2.87 (m, 3H), 2.18 (bt, $J = 7.6$ Hz, 2H), 1.87 (m, 1H), 1.80-1.53 (m, 7H), 1.52-1.12 (m, 13H), 0.89 (t, $J = 6.8$ Hz, 3H), 0.86 (t, $J = 6.9$ Hz, 3H). $^{13}$C NMR (500 MHz, CDCl$_3$) $\delta$ 176.34, 173.25, 53.62, 47.90, 47.48, 36.56, 31.95, 31.47, 31.22, 29.68, 26.40, 25.93, 25.75, 25.45, 22.44, 22.41, 13.97, 13.91. ESI-MS m/z calcd for C$_{18}$H$_{36}$N$_2$O$_3$ [M +H]$^+$: 329.28. Found: 329.29.

Sodium $^{N^\varepsilon}$-hexanoyl-$^{L}$-lysinate-1-(α-D-galactopyranoside) (440)

Benzyl $^{N^\varepsilon}$-hexanoyl-$^{L}$-lysine-1-(2,3,4,6-tetra-O-acetyl-α-D-galactopyranoside) (457) (0.19 g, 0.26 mmol) was dissolved in MeOH (2 mL) and 0.1 M NaOMe was added dropwise until the solution was ca. pH = 12. The reaction mixture was stirred at room temperature for 14 hrs after which the reaction was neutralized with Dowex 50 (H$^+$) resin, filtered, and concentrated under reduced pressure to yield benzyl $^{N^\varepsilon}$-dodecanoyl-$^{L}$-lysine-1-(α-D-galactopyranoside). The residue was dissolved in MeOH (5 mL) and Pd(OH)$_2$ on carbon (0.019 g) was added. The air in the flask was evacuated and H$_2$ was introduced and the mixture was stirred for 16 hours at room temperature after which the solution was filtered through a Celite pad and concentrated under reduced pressure to yield $^{N^\varepsilon}$-hexanoyl-$^{L}$-lysine-1-(α-D-galactopyranoside). This product was dissolved in MeOH (final concentration of 0.1 M) and 4 M NaOH (1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure to give 440 as a white powder (0.12 g, 0.25 mmol, 92% yield).

$^1$H NMR (500 MHz, D$_2$O) $\delta$ 4.51 (dt, $J = 10.0, 4.9, 1$H), 4.18 (dd, $J = 7.8, 4.9, 1$H), 4.01
(m, 2H), 3.88 (t, J = 5.6, 1H), 3.79-3.63 (m, 3H), 3.17 (t, J = 6.6, 2H), 2.74 (dd, J = 15.1, 10.5, 1H), 2.64 (dd, J = 15.1, 3.9, 1H), 2.22 (t, J = 7.3, 2H), 1.79 (m, 1H), 1.68 (m, 1H), 1.64-1.46 (m, 4H), 1.39-1.20 (m, 6H), 0.87 (t, J = 6.8, 3H). \(^{13}\)C NMR (500 MHz, D\(_2\)O) \(\delta\) 179.1, 177.1, 172.8, 72.7, 72.5, 69.6, 68.6, 67.6, 60.7, 55.1, 39.0, 35.7, 32.3, 31.3, 30.3, 27.9, 25.1, 22.4, 21.6, 13.1. \textbf{ESI-MS} \(m/z\) calcd for C\(_{20}\)H\(_{35}\)N\(_2\)O\(_9\)- [M]: 447.23. Found: 447.34.

\textbf{Sodium N\textsuperscript{ε}-dodecanoyl-\textit{L}-lysinate-1-(\textalpha-\textd-galactopyranoside) (441)}

Benzyl N\textsuperscript{ε}-dodecanoyl-\textit{L}-lysine-1-(2,3,4,6-tetra-O-acetyl-\textalpha-\textd-galactopyranoside) (458) (0.031 g, 0.039 mmol) was dissolved in MeOH (2 mL) and 0.1 M NaOMe was added dropwise until the solution was ca. pH = 12. The reaction mixture was stirred at room temperature for 14 hrs after which the reaction was neutralized with Dowex 50 (H\(^+\)) resin, filtered, and concentrated under reduced pressure to yield benzyl N\textsuperscript{ε}-dodecanoyl-\textit{L}-lysine-1-(\textalpha-\textd-galactopyranoside). The residue was dissolved in MeOH (3 mL) and Pd(OH)$_2$ on carbon (0.003 g) was added. The air in the flask was evacuated and H\(_2\) was introduced and the mixture was stirred for 16 hours at room temperature after which the solution was filtered through a Celite pad and concentrated under reduced pressure to yield N\textsuperscript{ε}-dodecanoyl-\textit{L}-lysine-1-(\textalpha-\textd-galactopyranoside). This product was dissolved in MeOH (final concentration of 0.1 M) and 4 M NaOH (1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure to give 441 as a white powder (0.19 g, 0.034 mmol, 87% yield).

\(^1\)H NMR (300 MHz, D\(_2\)O) \(\delta\) 4.34 (m, 2H), 3.82 (m, 3H), 3.64 (m, 1H), 3.49 (m, 1H), 3.08 (m, 2H), 2.62 (m, 1H), 2.47 (m, 1H), 2.09 (m, 2H), 1.84-0.93 (m, 25H), 0.83 (m, 3H). \(^{13}\)C NMR (500 MHz, DMSO) \(\delta\) 173.0, 172.6, 171.5, 73.4, 70.6, 68.5, 67.7, 59.7, 52.12, 52.00, 48.8, 38.3, 35.7, 33.0, 31.5, 30.8, 29.22, 29.21, 29.15, 28.97, 28.93, 28.84, 25.6, 22.9, 22.3, 14.2. \textbf{ESI-MS} \(m/z\) calcd for C\(_{26}\)H\(_{48}\)N\(_2\)O\(_9\)- [M + H]: 532.34. Found 532.17.

\textbf{Sodium N\textsuperscript{ε}-hexanoyl-\textit{L}-lysinate-1-(\textalpha-\textd-glucopyranoside) (442)}

Benzyl N\textsuperscript{ε}-hexanoyl-\textit{L}-lysine-1-(2,3,4,6-tetra-O-acetyl-\textalpha-\textd-glucopyranoside) (459) (0.12 g, 0.17 mmol) was dissolved in MeOH (2 mL) and 0.1 M NaOMe was added dropwise until the solution was ca. pH = 12. The reaction mixture was stirred at room temperature for 14 hrs after which the reaction was neutralized with Dowex 50 (H\(^+\)) resin, filtered, and concentrated under reduced pressure to yield benzyl N\textsuperscript{ε}-hexanoyl-\textit{L}-lysine-1-(\textalpha-\textd-glucopyranoside). The residue was dissolved in MeOH (5 mL) and Pd(OH)$_2$ on carbon
(0.012 g) was added. The air in the flask was evacuated and H₂ was introduced and the mixture was stirred for 16 hours at room temperature after which the solution was filtered through a Celite pad and concentrated under reduced pressure to yield N'-dodecanoyl-L-lysine-1-(α-d-galactopyranoside). This product was dissolved in MeOH (final concentration of 0.1 M) and 4 M NaOH (1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure to give 442 as a white powder (0.075 g, 0.16 mmol, 94% yield).

\[^1\text{H NMR} \ (300 \text{ MHz, MeOD}) \ \delta \ 4.33 \ (\text{dq, } J = 11.8, 3.8, 1H), 4.18 \ (\text{dd, } J = 7.5, 4.8, 1H), 3.71 \ (\text{dd, } J = 11.8, 2.3, 1H), 3.63-3.38 \ (m, 5H), 3.07 \ (m, 2H), 2.67-2.47 \ (m, 2H), 2.08 \ (t, J = 7.6, 2H), 1.77 \ (m, 1H), 1.66-1.37 \ (m, 5H), 1.37-1.17 \ (m, 6H), 0.83 \ (t, J = 6.9, 3H). \]^1\text{H NMR} \ (300 \text{ MHz, CDCl}_{3}) \ \delta \ 173.8, 172.1, 170.8, 170.0, 169.48, 169.45, 169.2, 135.3, 128.64, 128.50, 128.32, 70.02, 69.96, 69.7, 69.4, 68.1, 67.2, 61.9, 52.1, 38.6, 36.8, 34.1, 31.46, 31.34, 29.1, 25.5, 22.42, 22.24, 20.78, 20.73, 20.70, 20.69, 14.0. \]^13\text{C NMR} \ (300 \text{ MHz, MeOD}): \ \delta \ 179.2, 176.2, 173.2, 75.7, 74.9, 74.3, 72.5, 72.0, 62.9, 56.3, 40.3, 37.1, 33.8, 33.5, 32.6, 30.2, 26.8, 24.2, 23.4, 14.3. \text{ESI-MS} \ m/z \ \text{calcd for } C_{20}H_{36}N_{2}O_7^+ \ \text{[M]}: 447.23. \text{Found: } 446.99.

\text{Sodium } N'-\text{dodecanoyl-L-lysinate-1(α-d-glucopyranoside) (443)}

Benzy l \ N'-dodecanoyl-L-lysine-1-(2,3,4,6-tetra-O-acetyl-α-d-glucopyranoside) (460) (0.083 g, 0.11 mmol) was dissolved in MeOH (5 mL) and 0.1 M NaOMe was added dropwise until the solution was ca. pH = 12. The reaction mixture was stirred at room temperature for 14 hrs after which the reaction was neutralized with Dowex 50 (H⁺) resin, filtered, and concentrated under reduced pressure to yield benzy l \ N'-dodecanoyl-L-lysine-1-(α-d-glucopyranoside). The residue was dissolved in MeOH (5 mL) and Pd(OH)₂ on carbon (0.008 g) was added. The air in the flask was evacuated and H₂ was introduced and the mixture was stirred for 16 hrs at room temperature after which the solution was filtered through a Celite pad and concentrated under reduced pressure to yield \ N'-dodecanoyl-L-lysine-1-(α-d-glucopyranoside). This product was dissolved in MeOH (final concentration of 0.1 M) and 4 M NaOH (1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure to give 443 as a white powder (0.075 g, 0.16 mmol, 94% yield).

\[^1\text{H NMR} \ (500 \text{ MHz, MeOD}) \ \delta \ 4.37-4.33 \ (m, 2H), 3.71 \ (dd, J = 11.8, 2.5, 1H), 3.63 \ (dd, J = 11.8, 5.4, 1H), 3.56 \ (dd, J = 9.3, 5.9, 1H), 3.50 \ (ddd, J = 8.9, 5.6, 2.9, 1H), 3.43 \ (t, J = 8.9, 1H), 3.10 \ (t, J = 6.9, 2H), 2.63 \ (dd, J = 15.0, 10.6, 1H), 2.53 \ (dd, J = 14.9, 3.9, 1H), 2.10 \ (t, J = 7.5, 2H), 1.77 \ (m, 1H), 1.64 \ (m, 1H), 1.53 \ (m, 2H), 1.45 \ (m, 2H), 1.35 \ (m, 2H), 1.29-1.20 \ (m, 17H), 0.84 \ (t, J = 6.9, 3H). \]^13\text{C NMR} \ (500 \text{ MHz, MeOD}) \ \delta \ 174.9, 172.90, 172.72, 73.88, 73.71, 73.2, 71.0, 70.3, 61.3, 52.3, 38.6, 35.8, 32.2, 31.7, 30.7, 29.33, 29.33, 29.23, 29.07, 29.04, 28.91, 28.5, 25.7, 22.8, 22.3, 13.1. \text{ESI-MS} \ m/z \ \text{calcd for } C_{28}H_{47}N_{2}O_9^- \ \text{[M]}: 531.33. \text{Found: } 531.17
\[ N^\alpha-(\text{tert-Butoxycarbonyl})-N^\varepsilon-\text{carboxybenzyl-L-lysine (444)} \]

\[ N^\varepsilon-\text{Carboxybenzyl-L-lysine 422 (8.2 g, 39.4 mmol) was suspended in 10\% Et}_3\text{N in MeOH (200 mL) and di-}\text{tert-butyl dicarbonate (12.9 g, 58.5 mmol, 2 eq) was added. The mixture was refluxed for 1 hour and stirred at ambient temperature for 1 hour after which the solvent was removed under reduced pressure. The residue was dissolved in EtOAc and washed with cold 10\% HCl to remove residual TFA salts. The organic phase was concentrated under reduced pressure to yield 444 as a colorless oil (11 g, 28.8 mmol, 98 \% yield). 444 was used directly in the next step.} \]

\[ N^\alpha-(\text{tert-Butoxycarbonyl})-L-lysine (445) \]

\[ N^\alpha-(\text{tert-Butoxycarbonyl})-N^\varepsilon-\text{hexanoyl-L-lysine (446)} \]

\[ 1^H\text{ NMR (300 MHz, } C\text{DCl}_3) \delta 4.06 (m, 1H), 2.98 (t, J = 7.6, 2H), 1.84 (m, 1H), 1.68 (m, 3H), 1.47 (m, 2H), 1.40 (s, 9H). \]

\[ 1^C\text{ NMR (300 MHz; } C\text{DCl}_3) \delta 176.6, 157.7, 81.4, 53.6, 39.2, 30.1, 27.7, 27.7, 27.7, 26.2, 22.1. \]

\[ \text{ESI-MS } m/z \text{ calcd for C}_{11}\text{H}_{23}\text{N}_2\text{O}_3^+ [M+H]^+: 247.17. \text{ Found: 247.20.} \]

\[ 1^H\text{ NMR (400 MHz, } C\text{DCl}_3) \delta 6.13 (s, 1H), 5.36 (d, J = 7.9, 1H), 4.25 (q, J = 5.9, 1H), 3.22 (q, J = 6.4, 2H), 2.17 (t, J = 7.7, 2H), 1.83 (mz, 1H), 1.74-1.67 (m, 1H), 1.58 (m, 4H), 1.42 (s, 9H), 1.40-1.24 (m, 6H), 0.87 (t, J = 6.9, 3H). \]

\[ 1^C\text{ NMR (400 MHz, } C\text{DCl}_3) \delta 175.4, 174.5, 156.0, 80.1, 53.3, 39.3, 36.8, 32.3, 31.5, 29.0, 28.5, 28.5, 28.5, 25.6, 22.53, 22.47, 14.1. \text{ ESI-MS } m/z \text{ calcd for C}_{17}\text{H}_{33}\text{N}_2\text{O}_5^+ [M+H]^+: 345.24. \text{ Found: 345.27.} \]
$N^\alpha$-(tert-Butoxycarbonyl)-$N^\varepsilon$-dodecanoyl-$L$-lysine (447)

$N^\alpha$-(tert-Butoxycarbonyl)-L-lysine (445) (0.078 g, 0.32 mmol) was dissolved in 10 % NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0°C and dodecanoyl chloride (0.11 mL, 0.47 mmol, 1.5 eq) was added slowly to the ether layer with no mixing. The biphasic solution was vigorously stirred at 0°C for 4 hrs and then at room temperature for 18 hrs. The ether was evaporated under a stream of air and the solution was carefully acidified with concentrated HCl to ca. pH = 1. The mixture was extracted with CH$_2$Cl$_2$ five times. The combined organic phases were washed with brine, dried with MgSO$_4$, and concentrated under reduced pressure. The crude product was purified by silica column chromatography (5:5 petroleum ether:EtOAc + 1% AcOH to 6:3:1 EtOAc:petroleum ether:MeOH + 1% AcOH) to yield 447 as a white product (0.062 g, 0.15 mmol, 46 % yield).

$^1$H NMR (500 MHz, CDCl$_3$) δ 5.99 (bs, 1H), 5.33 (d, J = 7.8, 1H), 4.27 (q, J = 5.9, 1H), 3.23 (q, J = 6.2, 2H), 2.17 (t, J = 7.7, 2H), 1.85 (m, 1H), 1.71 (m, 1H), 1.60 (m, 2H), 1.53 (m, 2H), 1.43 (s, 9H), 1.38-1.15 (m, 18H), 0.86 (t, J = 6.9, 3H).

$^{13}$C NMR (400 MHz, CDCl$_3$) δ 175.4, 174.4, 156.0, 80.1, 53.2, 39.4, 36.9, 32.3, 32.0, 29.76, 29.74, 29.65, 29.48, 29.48, 29.45, 29.0, 28.5, 28.5, 26.0, 22.8, 22.5, 14.3. ESI-MS m/z calcd for C$_{23}$H$_{44}$N$_2$O$_5$ + NH$_4$-CH$_3$CN$^+$ complex (ammonia-acetonitrile) [M+NH$_4$-CH$_3$CN$^+$]: 487.39. Found: 487.48.

Benzyl $N^\alpha$-(tert-butoxycarbonyl)-$N^\varepsilon$-hexanoyl-$L$-lysine (448)

$N^\alpha$-(tert-Butoxycarbonyl)-$N^\varepsilon$-hexanoyl-$L$-lysine (446) (1.4 g, 4.0 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (10 mL) and 1,1'-Carbonyldiimidazole (CDI) (0.85 g, 5.4 mmol, 1.2 eq) was added. After 30 min of stirring, benzyl alcohol (0.48 g, 4.4 mmol, 1.1 eq) was added and the mixture was stirred for 16 hours. The solvent was removed under reduced pressure and the residue was purified by silica column chromatography (7:3 petroleum ether:EtOAc to 4:6 petroleum ether:EtOAc) to afford 448 as an oil (1.5 g, 3.4 mmol, 86% yield).

$^1$H NMR (300 MHz, CDCl$_3$) δ 7.34 (m, 5H), 5.68 (bs, 1H), 5.15 (q, J = 11.3, 2H), 5.15 (bs, 1H), 4.29 (q, J = 6.4, 1H), 3.17 (q, J = 6.5, 2H), 2.12 (t, J = 7.6, 2H), 1.82-1.74 (m, 1H), 1.69-1.44 (m, 4H), 1.41 (s, 9H), 1.37-1.21 (m, 6H), 0.87 (t, J = 6.9, 3H). $^{13}$C NMR (300 MHz, CDCl$_3$) δ 173.4, 172.7, 155.6, 135.5, 128.68, 128.53, 128.37, 80.0, 67.1, 53.4, 39.1, 36.8, 32.4, 31.6, 29.1, 28.4, 28.4, 25.6, 22.64, 22.48, 14.0. ESI-MS m/z calcd for C$_{24}$H$_{38}$N$_2$NaO$_5$ $^+$ [M+Na$^+$]: 457.27. Found: 457.33.

Benzyl $N^\alpha$-(tert-butoxycarbonyl)-$N^\varepsilon$-dodecanoyl-$L$-lysine (449)
\(N^\alpha-\text{(tert-Butoxycarbonyl)}-N^\varepsilon\text{-dodecanoyl-}\varepsilon\text{-lysine (447)}\) (0.84 g, 1.6 mmol) was dissolved in anhydrous \(\text{CH}_2\text{Cl}_2\) (20 mL) and 1,1'-carbonyldiimidazole (CDI) (0.29 g, 1.8 mmol, 1.1 eq) was added. After 30 min of stirring, benzyl alcohol (0.19 g, 1.8 mmol, 1.1 eq) was added and the mixture was stirred for 16 hours. The solvent was removed under reduced pressure and the residue was purified by silica column chromatography (7:3 petroleum ether:EtOAc to 4:6 petroleum ether:EtOAc) to afford 449 as an oil (0.70 g, 1.7 mmol, 99% yield).

\[1^1\text{H NMR (300 MHz, CDCl}_3) \delta 7.34 (m, 5H), 5.51 (bs, 1H), 5.16 (q, J = 11.1, 2H), 5.10 (bs, 1H), 4.31 (m, 1H), 3.18 (q, J = 6.4, 2H), 2.12 (t, J = 7.6, 2H), 1.79 (m, 1H), 1.70-1.54 (m, 3H), 1.39-1.18 (m, 18H), 0.87 (t, J = 6.6, 3H). \]

\[13^1\text{C NMR (500 MHz, CDCl}_3) \delta 173.4, 172.7, 155.7, 135.5, 128.76, 128.61, 128.47, 80.1, 67.2, 53.3, 39.2, 37.0, 32.6, 32.1, 29.8, 29.7, 29.5, 29.5, 29.9, 29.1, 28.5, 28.5, 26.0, 22.84, 22.67, 14.3 \text{ ESI-MS m/z calcd for C}_{30}\text{H}_{51}\text{N}_2\text{O}_5^+ [M+H]^+: 519.38. Found: 419.23.}\]

**Benzyl N\(^\varepsilon\)-hexanoyl-\(\varepsilon\)-lysine monohydrotrifluoroacetate (450)**

Benzyl \(N^\alpha-\text{(tert-butoxycarbonyl)}-N^\varepsilon\text{-hexanoyl-}\varepsilon\text{-lysine (448)}\) (1.5 g, 3.4 mmol) was dissolved in \(\text{CH}_2\text{Cl}_2\) (10 mL), and trifluoroacetic acid was added dropwise until the solution became ca. pH = 1. The mixture was stirred at ambient temperature for 2 hours or until completion of reaction was seen via TLC. The solvent was removed under reduced pressure to yield 450 as a yellow oil (1.4 g, 3.1 mmol, 98% yield), which solidifies to a white solid upon standing.

\[1^1\text{H NMR (500 MHz, CDCl}_3) \delta 7.37-7.29 (m, 5H), 6.54 (bs, 1H), 5.18 (q, J = 16.8, 2H), 4.05 (t, J = 6.1, 1H), 3.17 (d, J = 5.3, 2H), 2.17 (t, J = 7.7, 2H), 2.02-1.91 (m, 2H), 1.55 (q, J = 7.5, 2H), 1.52 (m, 3H), 1.36-1.21 (m, 5H), 0.86 (t, J = 7.0, 3H). \]

\[13^1\text{C NMR (400 MHz, CDCl}_3) \delta 176.8, 169.5, 161.3, 160.9, 134.3, 129.1, 128.9, 128.6, 68.7, 53.3, 39.2, 36.0, 31.3, 29.6, 28.3, 25.6, 22.2, 21.6, 13.8 \text{ ESI-MS m/z calcd for C}_{10}\text{H}_{31}\text{N}_2\text{O}_3^+ [M+H]^+: 335.26. Found: 335.26.}\]

**Benzyl N\(^\varepsilon\)-dodecanoyl-\(\varepsilon\)-lysine monohydrotrifluoroacetate (451)**

Benzyl \(N^\alpha-\text{(tert-butoxycarbonyl)}-N^\varepsilon\text{-hexanoyl-}\varepsilon\text{-lysine (449)}\) (0.015 g, 0.035 mmol) was dissolved in \(\text{CH}_2\text{Cl}_2\) (5 mL), and trifluoroacetic acid was added dropwise until the solution became ca. pH = 1. The mixture was stirred at ambient temperature for 2 hours or until completion of reaction was seen via TLC. The solvent was removed under reduced pressure to yield 451 as a yellow oil (0.0084 g, 0.034 mmol, 98% yield).

\[1^1\text{H NMR (300 MHz, MeOD} \delta 7.39 (m, 5H), 5.28 (q, J = 9.3, 2H), 4.07 (t, J = 6.3, 1H), 3.12 (t, J = 6.8, 2H), 2.15 (t, J = 7.5, 2H), 1.91 (m, 2H), 1.59 (m, 2H), 1.48 (m, 3H), 1.38-1.35 (m, 17H), 0.90 (t, J = 6.7, 3H) \]

\[13^1\text{C NMR (500 MHz, CDCl}_3) \delta 169.6, 134.4, 128.95, 128.80, 128.5, 68.4, 53.1, 39.0, 36.4, 32.0, 29.74, 29.74, 29.74, 29.74, 29.62, 29.46, 29.46, 29.44, 29.35, 28.5, 26.0, 22.8, 21.8, 14.2 \text{ ESI-MS m/z calcd for C}_{25}\text{H}_{43}\text{N}_2\text{O}_3^+ [M]^+: 519.38. Found: 419.23.}\]
2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (452)

Compound was prepared from 313 as previously described. All spectral data was consistent with that reported in the literature.

Allyl 2,3,4,6-tetra-O-acetyl-α-D-galactopyranoside (453)

Allyl 2,3,4,6-tetra-O-acetyl-α-D-galactopyranoside (314) (14.3 g, 34.8 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ under argon and allyl tributyl tin (27 mL, 86.9 mmol, 2.5 eq) and 1M Et$_2$B (2.5 mL) was added. Air was bubbled through the solution by means of a 10 gauge long needle with a flow rate of 5 mL/hr for a total of 40 mL of air. The solvent was removed by reduced pressure and the residue was dissolved in CH$_3$CN and pentanes. The CH$_3$CN phase was washed 3 times with pentanes to remove the tin reagent. The residue was concentrated and purified by silica column chromatography (5:4:1 toluene:petroleum ether:EtOAc) to provide 453 as a colourless oil (8.2 g, 21.9 mmol, 63% yield).

$^1$H NMR (500 MHz, CDCl$_3$) δ 5.69 (ddt, J = 16.8, 10.0, 6.8, 1H), 5.28 (t, J = 9.1, 1H), 5.15-5.01 (m, 3H), 4.92 (t, J = 9.1, 1H), 4.22 (dt, J = 10.7, 5.3, 1H), 4.15 (dd, J = 12.2, 5.3, 1H), 4.02 (dd, J = 12.1, 2.1, 1H), 3.81 (ddd, J = 8.9, 5.6, 2.7, 1H), 2.53-2.47 (m, 1H), 2.28 (dt, J = 15.3, 5.1, 1H), 2.02 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H). $^{13}$C NMR (400 MHz, CDCl$_3$): δ 170.2, 169.7, 169.22, 169.17, 132.9, 117.4, 71.5, 69.99, 69.94, 68.58, 68.54, 61.9, 30.2, 20.3, 20.3, 20.3, 20.3. ESI-MS m/z calcd for C$_{17}$H$_{24}$NaO$_9$+ [M]$: 395.13. Found: 395.13.

Allyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (454)

Compound was prepared from 313 as previously described. All spectral data was consistent with that reported in the literature.

2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl acid (455)

Allyl 2,3,4,6-tetra-O-acetyl-α-D-galactopyranoside (453) was dissolved in CH$_2$Cl$_2$ and CH$_3$CN and H$_2$O was added in the following proportions: 2:2:3 CH$_2$Cl$_2$:CH$_3$CN:H$_2$O for a total volume of 32 mL. Sodium periodate (7.7 g, 36.1 mmol, 6 eq) and ruthenium trichloride (0.037 g, 0.18 mmol, 0.03 eq) were added and the mixture was stirred at
ambient temperature for 16 hrs. The mixture was extracted with CH₂Cl₂ and then 1% HCl. The organic phase was dried with MgSO₄, concentrated under reduced pressure, and purified by silica column chromatography (8:2 CH₂Cl₂:EtOAc to 5:5 CH₂Cl₂:EtOAc) to produce 455 as a colorless oil (1.0 g, 2.6 mmol, 43% yield).

_1H NMR (500 MHz, CDCl₃) δ 5.39 (dd, J = 3.0, 1H), 5.30 (dd, J = 9.0, 5.1, 1H), 5.15 (dd, J = 9.0, 3.3, 1H), 4.68 (dd, J = 8.8, 5.3, 1H), 4.20 (dd, J = 10.9, 7.1, 1H), 4.13 (td, J = 6.0, 2.7, 1H), 4.08 (dd, J = 10.9, 5.0, 1H), 2.69 (dd, J = 15.6, 8.9, 1H), 2.61 (dd, J = 15.6, 5.6, 1H), 2.10 (s, 3H), 2.04 (s, 3H), 2.01 (s, 3H), 2.01 (s, 3H).

_13C NMR (500 MHz, CDCl₃) δ 175.1, 170.9, 170.23, 170.11, 169.8, 69.4, 69.0, 67.9, 67.6, 67.2, 61.2, 33.1, 20.78, 20.73, 20.73, 20.73.

ESI-MS m/z calcd for C₁₆H₂₁O₁₁ [M]⁻: 389.11. Found: 388.94.

2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl acid (456)

_1H NMR (500 MHz, CDCl₃) δ 5.26 (t, J = 8.8, 1H), 5.17 (m, 1H), 5.00 (t, J = 8.8, 1H), 4.68 (m, 1H), 4.24 (dd, J = 12.2, 4.9, 1H), 4.11 (dd, J = 8.0, 4.7, 1H), 3.94 (m, 1H), 2.80 (m, 1H), 2.69 (dd, J = 15.5, 5.2, 1H), 2.07 (s, 3H), 2.04 (s, 3H). _13C NMR (500 MHz, CDCl₃) δ 175.6, 170.9, 170.2, 169.66, 169.53, 169.53, 70.16, 70.06, 69.1, 68.3, 62.0, 33.0, 20.80, 20.77, 20.72. ESI-MS m/z calcd for C₁₆H₂₁O₁₁ [M]⁻: 389.11. Found: 389.02.

Benzyl Nε-hexanoyl-L-lysine-1-(2,3,4,6-tetra-O-acetyl-α-D-galactopyranoside) (457)

2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl acid (455) (0.73 g, 1.8 mmol) was dissolved in anhydrous CH₂Cl₂ (18 mL) under argon, and 1,1'-Carbonyldiimidazole (CDI) (0.32 g, 2.0 mmol, 1.1 eq) was added, and the mixture was stirred at ambient temperature for 30 min. Diisopropylethylamine (0.66 mL, 3.8 mmol, 2.1 eq) and benzyl Nε-hexanoyl-L-lysine monohydrotrifluoroacetate (450) (1.1 g, 2.1 mmol, 1.1 eq) was added and the mixture was stirred for 16 hours, after which the solvent was removed under reduced pressure and the residue was purified by silica column chromatography (3:7 petroleum ether:EtOAc to 0.5:9.5 petroleum ether:EtOAc) to yield 457 as a white powder (0.87 g, 1.2 mmol, 68% yield).

_1H NMR (500 MHz, CDCl₃) δ 7.38-7.31 (m, 5H), 6.71 (d, J = 7.5, 1H), 5.69 (bt, J = 4.81, 1H), 5.42 (t, J = 3.0, 1H), 5.27 (dd, J = 8.5, 4.7, 1H), 5.17 (dd, J = 8.40, 3.40 Hz, 1H) 5.15 (q, J = 13.8, 2H), 4.68 (dt, J = 9.5, 4.7, 1H), 4.58 (td, J = 7.7, 4.7, 1H), 4.27-4.21 (m, 2H), 4.13 (dd, J = 9.9, 4.0, 1H), 3.18 (q, J = 6.4, 2H), 2.66 (dd, J = 15.3, 9.6, 1H), 2.47 (dd, J = 15.3, 4.6, 1H), 2.14 (t, J = 7.6, 2H), 2.11 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 2.00 (s, 3H), 1.87-1.81 (m, 1H), 1.73-1.68 (m, 1H), 1.61 (quintet, J = 7.5, 2H), 1.50-1.40
(m, 2H), 1.35-1.24 (m, 6H), 0.88 (t, J = 7.0, 3H). $^{13}$C NMR (400 MHz, CDCl$_3$) δ 173.8, 172.2, 170.8, 170.12, 169.93, 169.74, 169.4, 135.4, 128.75, 128.64, 128.44, 69.6, 69.0, 68.12, 68.00, 67.3, 67.1, 61.0, 52.2, 38.8, 36.8, 34.5, 31.69, 31.57, 29.2, 25.6, 22.51, 22.38, 20.90, 20.87, 20.86, 20.79, 14.1. ESI-MS m/z calcd for C$_{35}$H$_{51}$N$_2$O$_{13}$ $^{[M+H]^+}$: 707.34. Found: 707.44.

Benzyl N$^\epsilon$-dodecanoyl-$\tau$-lysine-1-(2,3,4,6-tetra-O-acetyl-$\alpha$-$\delta$-galactopyranoside) (458)

2,3,4,6-tetra-O-acetyl-$\alpha$-$\delta$-galactopyranosyl acid (455) (0.156 g, 0.40 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (5 mL) under argon, and diisopropylethyamine (0.07 mL, 0.44 mmol, 1 eq), and HCTU (0.18 g, 0.44 mmol, 1.1 eq) was added, and the mixture was stirred at ambient temperature for 30 min. Diisopropylethyamine (0.14 mL, 0.88 mmol, 2.1 eq) and benzyl N$^\epsilon$-dodecanoyl-$\tau$-lysine monohydrotrifluoro acetate (451) (1.1 g, 2.1 mmol, 1.1 eq) was added and the mixture was stirred for 16 hours, after which the solvent was removed under reduced pressure and the residue was purified by silica column chromatography (3:7 petroleum ether:EtOAc to 0.5:9.5 petroleum ether:EtOAc) to yield 458 as a white powder (0.23 g, 0.31 mmol, 78% yield).

$^1$H NMR (500 MHz, CDCl$_3$) δ 7.34 (m, 5H), 6.70 (d, J = 7.6, 1H), 5.73 (bs, 1H), 5.41 (t, J = 2.8, 1H), 5.27 (dd, J = 8.3, 4.6, 1H), 5.19-5.11 (m, 3H), 4.67 (dt, J = 9.4, 4.6, 1H), 4.59 (q, J = 5.8, 1H), 4.29-4.21 (m, 2H), 4.13-4.09 (m, 1H), 3.18 (q, J = 6.1, 2H), 2.65 (dd, J = 15.2, 9.6, 1H), 2.46 (dd, J = 15.3, 4.4, 1H), 2.14 (t, J = 7.3, 2H), 2.10 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 2.00 (s, 3H), 1.84 (m, 1H), 1.70 (m, 1H), 1.59 (m, 2H), 1.50-1.40 (m, 2H), 1.35-1.27 (m, 18H), 0.86 (t, J = 6.9, 3H). $^{13}$C NMR (500 MHz, CDCl$_3$) δ 173.2, 172.2, 170.8, 170.12, 169.98, 169.8, 169.4, 135.4, 128.73, 128.62, 128.42, 69.6, 69.2, 69.0, 68.7, 68.0, 67.3, 67.0, 61.0, 52.1, 38.7, 36.8, 34.5, 32.0, 31.7, 29.74, 29.72, 29.62, 29.50, 29.45, 29.45, 29.45, 29.1, 26.0, 22.8, 22.3, 20.87, 20.80, 14.2. ESI-MS m/z calcd for C$_{41}$H$_{63}$N$_2$O$_{13}$ $^{[M+H]^+}$: 791.43. Found: 791.21.

Benzyl N$^\epsilon$-hexanoyl-$\tau$-lysine-1-(2,3,4,6-tetra-O-acetyl-$\alpha$-$\delta$-galactopyranoside) (459)

2,3,4,6-tetra-O-acetyl-$\alpha$-$\delta$-galactopyranosyl acid (456) (0.24 g, 0.61 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (5 mL) under argon, and 1,1'-Carbonyldiimidazole (CDI) (0.11 g, 0.67 mmol, 1.1 eq) was added, and the mixture was stirred at ambient temperature for 30 min. Diisopropylethyamine (0.44 mL, 2.2 mmol, 2.1 eq) and benzyl N$^\epsilon$-hexanoyl-$\tau$-lysine monohydrotrifluoro acetate (450) (0.33 g, 0.67 mmol, 1.1 eq) was added and the mixture was stirred for 16 hours, after which the solvent was removed under reduced
pressure and the residue was purified by silica column chromatography (3:7 petroleum ether:EtOAc to 0.5:9.5 petroleum ether:EtOAc) to yield \(459\) as a white powder (0.23 g, 0.31 mmol, 78\% yield).

\(^1\)H NMR (500 MHz, \(CDCl_3\)) \(\delta\) 7.34-7.29 (m, 5H), 6.90 (m, 1H), 5.87 (bs, 1H), 5.23 (t, J = 8.5, 1H), 5.16-5.08 (m, 3H), 5.00 (t, J = 8.5, 1H), 4.64 (dt, J = 10.1, 5.0, 1H), 4.54 (td, J = 7.8, 4.6, 1H), 4.23 (dd, J = 12.2, 4.5, 1H), 4.10 (dd, J = 12.3, 2.9, 1H), 4.02 (dt, J = 8.4, 4.1, 1H), 3.15 (m, 2H), 2.75 (dd, J = 15.1, 10.2, 1H), 2.51 (dd, J = 15.2, 4.5, 1H), 2.12 (t, J = 7.6, 2H), 2.01 (m, J = 6.8, 12H), 1.80 (dd, J = 9.4, 5.2, 1H), 1.69 (m, 1H), 1.59 (m, 2H), 1.43 (dt, J = 13.6, 6.8, 2H), 1.31-1.22 (m, 6H), 0.86 (t, J = 6.9, 3H). \(^{13}\)C NMR (500 MHz, \(CDCl_3\)) \(\delta\) 173.8, 172.1, 170.8, 170.0, 169.48, 169.45, 169.2, 135.3, 128.64, 128.50, 128.3, 70.02, 69.96, 69.7, 69.4, 68.1, 67.2, 61.9, 52.1, 38.6, 36.8, 34.1, 31.46, 31.34, 29.1, 25.5, 22.42, 22.24, 20.78, 20.73, 20.70, 20.69, 14.0 ESI-MS \(m/z\) calcd for C\(_{35}\)H\(_{50}\)N\(_2\)O\(_{13}\)\(^+\) [M+H\(^+\)]: 707.33. Found: 707.21.

**Benzyl \(N^\alpha\)-dodecanoyl-L-lysine-1-(2,3,4,6-tetra-O-acetyl-\(\alpha\)-d-glucopyranoside) (460)**

2,3,4,6-tetra-O-acetyl-\(\alpha\)-d-glucopyranosyl acid (456) (0.14 g, 0.36 mmol) was dissolved in anhydrous CH\(_2\)Cl\(_2\) (5 mL) under argon, and diisopropylethylamine (0.06 mL, 0.36 mmol, 1 eq) and HCTU (0.16 g, 0.39 mmol, 1.1 eq) was added, and the mixture was stirred at ambient temperature for 30 min. Diisopropylethylamine (0.12 mL, 0.72 mmol, 2.1 eq) and benzyl \(N^\alpha\)-dodecanoyl-L-lysine monohydrotrifluoroacetate (451) (0.21 g, 0.39 mmol, 1.1 eq) was added and the mixture was stirred for 16 hours, after which the solvent was removed under reduced pressure and the residue was purified by silica column chromatography (3:7 petroleum ether:EtOAc to 0.5:9.5 petroleum ether:EtOAc) to yield \(460\) as a white powder (0.17 g, 0.24 mmol, 67\% yield).

\(^1\)H NMR (500 MHz, \(CDCl_3\)) \(\delta\) 7.37-7.30 (m, 5H), 6.74 (d, J = 7.6, 1H), 5.85 (bs, 1H), 5.24 (t, J = 8.4, 1H), 5.15 (q, J = 14.2, 3H), 5.01 (t, J = 8.4, 1H), 4.65 (m, 1H), 4.57 (m, 1H), 4.26 (dd, J = 12.3, 4.6, 1H), 4.14 (dd, J = 12.3, 3.2, 1H), 4.04 (ddd, J = 8.4, 4.5, 3.7, 1H), 3.21 (m, 1H), 3.15 (m, 1H), 2.75 (dd, J = 15.2, 10.2, 1H), 2.53 (dd, J = 15.2, 4.4, 1H), 2.16 (m, 2H), 2.04 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.93 (m, 1H), 1.71 (m, 1H), 1.60 (m, 2H), 1.46 (m, 2H), 1.34-1.20 (m, 18H), 0.86 (t, J = 7.0, 3H). \(^{13}\)C NMR (500 MHz, \(CDCl_3\)) \(\delta\) 174.1, 172.1, 170.9, 170.0, 169.57, 169.57, 169.3, 135.4, 128.74, 128.61, 128.43, 70.20, 70.04, 69.7, 69.4, 68.2, 67.3, 61.9, 52.2, 38.9, 36.8, 34.3, 32.0, 31.5, 29.74, 29.73, 29.64, 29.39, 29.45, 29.15, 29.43, 29.0, 26.0, 22.8, 22.3, 20.88, 20.81, 20.79, 14.2. ESI-MS \(m/z\) calcd for C\(_{41}\)H\(_{63}\)N\(_2\)O\(_{13}\)\(^+\) [M+H\(^+\)]: 791.43. Found: 791.30.

**Sodium \(N^\alpha\)-butanoyl-\(N^\theta\)-dodecyl-L-glutamate (469)**

\(N^\alpha\)-butanoyl-\(N^\theta\)-dodecyl-L-glutamine (480) (0.023 g, 0.06 mmol) was dissolved in MeOH
(final concentration of 0.1 M) and 4 M NaOH (1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure to yield 469 (0.024 g, 0.059 mmol, 99% yield).

\[ ^1H \text{NMR} \ (500 \text{ MHz}, D_2O) \delta 4.08 \ (dd, J = 8.6, 4.0, 1H), \ 3.06 \ (bs, 2H), \ 2.18 \ (m, 4H), \ 2.01 \ (m, 1H), \ 1.85 \ (m, 1H), \ 1.52 \ (dq, J = 14.5, 7.2, 2H), \ 1.39 \ (m, 2H), \ 1.27-1.01 \ (m, 18H), \ 0.82 \ (t, J = 7.3, 3H), \ 0.75 \ (t, J = 5.1, 3H). \ \] 13C NMR (500 MHz, D2O) δ 178.3, 175.4, 174.5, 168.3, 54.7, 39.4, 37.7, 32.7, 31.9, 29.87, 29.82, 29.82, 29.57, 29.44, 29.0, 28.0, 27.1, 22.6, 18.9, 13.7, 13.2. ESI-MS m/z calcd for C21H39N2O4 [M]-: 383.29. Found: 383.23.

Sodium N<sup>a</sup>-hexanoyl-N<sup>δ</sup>-dodecyl-L-glutamate (470)

\[
\text{H}_3\text{C}_{12-}\text{O}^+ \text{Na}^+ \text{H}_2\text{C}_{20-}
\]

N<sup>a</sup>-hexanoyl-N<sup>δ</sup>-dodecyl-L-glutamine (481) (0.025 g, 0.06 mmol) was dissolved in MeOH (final concentration of 0.1 M) and 4 M NaOH (1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure to yield 470 (0.026 g, 0.06 mmol, 99% yield).

\[ ^1H \text{NMR} \ (500 \text{ MHz}, D_2O) \delta 4.14 \ (dd, J = 9.3, 4.0, 1H), \ 3.12 \ (t, J = 6.2, 2H), \ 2.24 \ (m, 4H), \ 2.07 \ (m, 1H), \ 1.92 \ (m, 1H), \ 1.56 \ (dt, J = 13.4, 6.8, 2H), \ 1.44 \ (m, 2H), \ 1.35-1.11 \ (m, 22H), \ 0.84 \ (t, J = 6.9, 3H), \ 0.79 \ (t, J = 6.5, 3H). \ \] 13C NMR (500 MHz, D2O): δ 178.3, 175.3, 174.5, 54.6, 39.5, 36.0, 32.6, 32.0, 31.7, 30.10, 30.04, 29.89, 29.79, 29.65, 29.4, 29.1, 27.9, 27.1, 25.3, 22.6, 22.2, 13.77, 13.77. ESI-MS m/z calcd for C23H43N2O4- [M]-: 411.32. Found: 411.28.

Sodium N<sup>a</sup>-octanoyl-N<sup>δ</sup>-dodecyl-L-glutamate (471)

\[
\text{H}_3\text{C}_{12-}\text{O}^+ \text{Na}^+ \text{H}_2\text{C}_{18-}
\]

N<sup>a</sup>-octanoyl-N<sup>δ</sup>-dodecyl-L-glutamine (482) (0.021 g, 0.048 mmol) was dissolved in MeOH (final concentration of 0.1 M) and 4 M NaOH (1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure to yield 471 as a white powder (0.022 g, 0.047 mmol, 98% yield).

\[ ^1H \text{NMR} \ (500 \text{ MHz}, D_2O) \delta 4.07 \ (dd, J = 9.2, 3.7, 1H), \ 3.05 \ (m, 2H), \ 2.22-2.11 \ (m, 4H), \ 2.00 \ (m, 1H), \ 1.84 \ (m, 1H), \ 1.49 \ (m, 2H), \ 1.37 \ (m, 2H), \ 1.25-1.05 \ (m, 26H), \ 0.77 \ (t, J = 6.6, 3H), \ 0.71 \ (t, J = 6.3, 3H). \ \] 13C NMR (500 MHz; D2O): δ 178.3, 175.3, 174.5, 54.6, 39.5, 36.0, 32.6, 32.0, 31.7, 30.10, 30.04, 29.93, 29.82, 29.5, 29.13, 29.06, 28.97, 27.9, 27.2, 25.7, 22.6, 13.9, 13.7. ESI-MS m/z calcd for C25H47N2O4- [M]-: 439.35. Found: 439.32.

Sodium N<sup>a</sup>-dodecanoyl-N<sup>δ</sup>-dodecyl-L-glutamate (472)

\[
\text{H}_3\text{C}_{12-}\text{O}^+ \text{Na}^+ \text{H}_2\text{C}_{23-}
\]

N<sup>a</sup>-dodecanoyl-N<sup>δ</sup>-dodecyl-L-glutamine (483) (0.032 g, 0.064 mmol) was dissolved in
MeOH (final concentration of 0.1 M) and 4 M NaOH (1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure to yield 472 as a white powder (0.033 g, 0.063 mmol, 98% yield).

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 4.12 (dd, $J = 7.6$, 4.9, 1H), 3.01 (d, $J = 7.2$, 2H), 2.10 (q, $J = 8.0$, 4H), 1.97 (m, 1H), 1.80 (m, 1H), 1.49 (t, $J = 6.7$, 2H), 1.36 (t, $J = 6.6$, 2H), 1.17 (d, $J = 13.5$, 34H), 0.77 (t, $J = 6.9$, 6H). $^{13}$C NMR (500 MHz, MeOD) $\delta$ 178.4, 175.6, 161.5, 55.8, 40.5, 37.4, 33.8, 33.1, 30.80, 30.80, 30.77, 30.77, 30.77, 30.76, 30.68, 30.53, 30.56, 30.50, 30.50, 30.50, 30.43, 30.41, 28.1, 27.0, 23.8, 14.5, 14.5. ESI-MS m/z calcd for C$_{29}$H$_{55}$N$_{2}$O$_{4}$ [M$^-$]: 495.42. Found: 495.44.

Sodium N$^a$-dodecanoyl-N$^\delta$-dodecyl-$\alpha$-glutamate (473)

$^a$-dodecanoyl-N$^\delta$-dodecyl-$\alpha$-glutamine (484) (0.018 g, 0.047 mmol) was dissolved in MeOH (final concentration of 0.1 M) and 4 M NaOH (1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure to yield 473 as a white powder (0.019 g, 0.046 mmol, 98% yield).

$^1$H NMR (500 MHz, D$_2$O) $\delta$ 4.06 (dd, $J = 9.2$, 4.0, 1H), 3.03 (m, 2H), 2.15 (m, 4H), 1.97 (m, 1H), 1.82 (m, 1H), 1.47 (m, 2H), 1.36 (t, $J = 6.3$, 2H), 1.08-1.07 (m, 19H), 0.74 (t, $J = 13.5$, 34H), 0.71 (t, $J = 7.0$ Hz, 6H). $^{13}$C NMR (400 MHz, D$_2$O) $\delta$ 178.3, 175.4, 174.7, 54.7, 39.6, 36.1, 32.7, 31.91, 31.71, 29.50, 29.38, 29.06, 29.01, 28.0, 27.0, 25.7, 22.57, 22.55, 13.82, 13.70. ESI-MS m/z calcd for C$_{21}$H$_{39}$N$_{2}$O$_{4}$ [M$^-$]: 383.29. Found: 383.29.

Sodium N$^a$-dodecanoyl-N$^\delta$-octyl-$\alpha$-glutamate (474)

N$^a$-dodecanoyl-N$^\delta$-octyl-$\alpha$-glutamine (485) (0.007 g, 0.017 mmol) was dissolved in MeOH (final concentration of 0.1 M) and 4 M NaOH (1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure to yield 474 as a white powder (0.019 g, 0.046 mmol, 98% yield).

$^1$H NMR (500 MHz, D$_2$O) $\delta$ 4.20 (dd, $J = 8.7$, 3.1, 1H), 3.19 (m, 2H), 2.30 (m, 4H), 2.12 (m, 1H), 1.96 (m, 1H), 1.62 (m, 2H), 1.52 (m, 2H), 1.24-1.22 (m, 25H), 0.89 (t, $J = 5.5$ Hz, 3H), 0.86 (t, $J = 6.9$ Hz, 3H). $^{13}$C NMR (400 MHz, D$_2$O) $\delta$ 178.4, 175.2, 174.7, 54.6, 39.6, 36.1, 32.7, 32.0, 30.20, 30.20, 30.13, 30.02, 29.93, 29.70, 29.56, 29.49, 29.44, 29.44, 29.1, 28.0, 27.1, 25.9, 22.7, 13.89, 13.74. ESI-MS m/z calcd for C$_{25}$H$_{47}$N$_{2}$O$_{4}$ [M$^-$]: 439.35. Found: 439.36.

Benzyl N$^a$-(carboxybenzyl)-N$^\delta$-octyl-$\alpha$-glutamate (476)

To a solution of anhydrous CH$_2$Cl$_2$ and benzyl N$^a$-(carboxybenzyl)-$\alpha$-glutamic acid (475)
(0.20 g, 0.53 mmol) under argon, diisopropylethylamine (0.11 mL, 0.64 mmol, 1.2 eq) and HCTU (0.24 g, 0.58 mmol, 1.1 eq) was added and the mixture was stirred for 30 min. Octyl amine (0.18 mL, 1.1 mmol, 2 eq) was added and after 16 hrs, the solvent was removed under reduced pressure and the residue was purified by silica column chromatography (7:3 petroleum ether:EtOAc to 6:4 petroleum ether:EtOAc) to yield 476 as a white solid (0.23 g, 0.49 mmol, 92% yield).

\[ ^1H \text{NMR} (300 MHz, CDCl}_3 \delta 7.27 (m, 10H), 5.90 (d, J = 6.7, 2H), 5.13 (d, J = 3.5, 2H), 5.06 (s, 2H), 4.35 (td, J = 8.2, 3.3, 1H), 3.14 (q, J = 6.7, 2H), 2.19-2.11 (m, 3H), 2.03-1.92 (m, 1H), 1.41 (m, J = 6.1, 2H), 1.31 (m, J = 7.0, 10H), 0.86 (t, J = 6.7, 3H). \]

\[ ^13C \text{NMR} (400 MHz, CDCl}_3 \delta 171.91, 171.73, 156.4, 136.3, 135.3, 128.76, 128.64, 128.51, 128.30, 128.19, 128.08, 67.3, 67.0, 53.8, 39.7, 32.5, 31.8, 29.6, 29.47, 29.42, 28.8, 27.1, 23.6, 22.8, 22.5, 14.3. \]

ESI-MS m/z calcd for C\textsubscript{28}H\textsubscript{39}N\textsubscript{2}O\textsubscript{5}\textsuperscript{+}[M+H\textsuperscript{+}]: 483.29. Found: 483.31.

Benzyl N\textsuperscript{\alpha}-(carboxybenzyl)-N\textsuperscript{\delta}-dodecyl-L-glutamine (477)

To a solution of anhydrous CH\textsubscript{2}Cl\textsubscript{2} and benzyl N\textsuperscript{\alpha}-(carboxybenzyl)-L-glutamic acid (475) (0.061 g, 0.16 mmol) under argon and N,N\textsuperscript{\prime}-Diisopropylcarbodiimide (DIC) (0.021 g, 0.16 mmol, 1.1 eq) was added and the mixture was stirred for 30 min. Dodecyl amine (0.025 mL, 0.16 mmol, 1.1 eq) was added and after 16 hrs, the solvent was removed under reduced pressure and the residue was purified by silica column chromatography (7:3 petroleum ether:EtOAc to 6:4 petroleum ether:EtOAc) to yield 477 as a white solid (0.065 g, 0.12 mmol, 74% yield).

\[ ^1H \text{NMR} (500 MHz, CDCl}_3 \delta 7.31-7.29 (m, 10H), 5.73 (d, J = 7.8, 2H), 5.14 (dt, J = 20.5, 9.7, 2H), 5.08 (s, 2H), 4.37 (t, J = 7.3, 1H), 3.16 (m, 2H), 2.17 (m, 3H), 1.97 (m, 1H), 1.42 (m, 2H), 1.30-1.27 (m, 18H), 0.86 (t, J = 7.0, 3H). \]

\[ ^13C \text{NMR} (300 MHz, CDCl}_3 \delta 171.91, 171.73, 156.4, 136.3, 135.3, 128.76, 128.64, 128.51, 128.30, 128.19, 128.08, 67.3, 67.0, 53.8, 42.3, 39.8, 32.6, 32.0, 29.77, 29.73, 29.73, 29.65, 29.47, 29.42, 28.8, 27.1, 23.6, 22.8, 22.5, 14.3. \]

ESI-MS m/z calcd for C\textsubscript{32}H\textsubscript{47}N\textsubscript{2}O\textsubscript{5}\textsuperscript{+}[M+H\textsuperscript{+}]: 539.35. Found: 539.31.

N\textsuperscript{\delta}-octyl-L-glutamine monohydrochloride (478)

Benzyl N\textsuperscript{\alpha}-(carboxybenzyl)-N\textsuperscript{\delta}-octyl-L-glutamine (476) (0.19 g, 0.38 mmol) was dissolved in MeOH (5 mL), 10% HCl (0.05 mL) and Pd/C (0.019 g) was added, and the air in the flask was replaced by H\textsubscript{2}. The mixture was stirred for 16 hrs at ambient temperature and then filtered through a Celite pad and concentrated under reduced pressure to yield 478 as a white solid (0.11 g, 0.39 mmol, 98% yield).

\[ ^1H \text{NMR} (300 MHz, MeOD) \delta 3.89 (t, J = 6.2, 1H), 3.19 (dt, J = 3.3, 1.6, 1H), 3.04 (t, J = 7.1, 2H), 2.36 (t, J = 7.1, 2H), 2.06 (m, 2H), 1.38 (t, J = 6.6, 2H), 1.18-1.11 (m, 10H), 0.77 (t, J = 6.7, 3H). \]

\[ ^13C \text{NMR} (400 MHz, MeOD) \delta 174.0, 171.5, 53.6, 42.3, 39.8, 32.6, 32.0, 29.77, 29.73, 29.65, 29.47, 29.42, 28.8, 27.1, 23.6, 22.8, 22.5, 14.3. \]

ESI-MS m/z calcd for C\textsubscript{13}H\textsubscript{27}N\textsubscript{2}O\textsubscript{5}\textsuperscript{+}[M+H\textsuperscript{+}]: 259.20. Found: 259.22.
**N^δ-dodecyl-L-glutamine monohydrochloride (479)**

![Chemical Structure](attachment:image.png)

Benzyl N^δ- (carboxybenzyl)-N^δ-dodecyl-L-glutamine (477) (0.30 g, 0.56 mmol) was dissolved in MeOH (5 mL), 10% HCl (0.05 mL) and Pd/C (0.019 g) was added, and the air in the flask was replaced by H\textsubscript{2}. The mixture was stirred for 16 hrs at ambient temperature and then filtered through a Celite pad and concentrated under reduced pressure to yield 479 as a white solid (0.19 g, 0.54 mmol, 98% yield).

\(^{1}\text{H} \text{NMR} \) \(300 \text{ MHz, MeOD}\) \(\delta 3.90 \text{ (t, } J = 6.2, 1\text{H}), 3.18 \text{ (dt, } J = 3.2, 1.6, 1\text{H}), 3.04 \text{ (t, } J = 7.0, 2\text{H}), 2.35 \text{ (t, } J = 7.2, 2\text{H}), 2.13-1.96 \text{ (m, 2H), 1.37 \text{ (t, } J = 6.4, 2\text{H}), 1.10-1.08 \text{ (m, 18H), 0.77 \text{ (t, } J = 6.6, 3\text{H})} \)

\(^{13}\text{C} \text{NMR} \) \(300 \text{ MHz, CDCl}_3\) \(\delta 170.72, 170.63, 51.6, 31.3, 29.1, 29.1, 29.0, 29.0, 28.80, 28.75, 26.5, 26.1, 22.1, 14.0 \)

ESI-MS \( m/z \) calcd for C\(_{17}\)H\(_{35}\)N\(_2\)O\(_3\)\(^+\) [M+H]\(^+\): 315.26. Found: 315.15.

**N^α-butanoyl-N^δ-dodecyl-L-glutamine (480)**

\![Chemical Structure](attachment:image.png)

N^δ-dodecyl-L-glutamine monohydrochloride (479) (0.022 g, 0.063 mmol) was dissolved in 10 % NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0 °C and butyric chloride (6 \(\mu\)L, 0.056 mmol, 0.9 eq) was added slowly to the ether layer with no mixing. The biphasic solution was vigorously stirred at 0 °C for 4 hrs and then at room temperature for 18 hrs. The ether was evaporated under a stream of air and the solution was carefully acidified with concentrated HCl to ca. pH = 1. The mixture was extracted with DCM five times. The combined organic phases were washed with brine, dried with MgSO\(_4\), and concentrated under reduced pressure to yield 480 (0.022 g, 0.056 mmol, 90%).

\(^{1}\text{H} \text{NMR} \) \(500 \text{ MHz, CDCl}_3\) \(\delta 7.45 \text{ (d, } J = 5.3, 1\text{H}), 6.22 \text{ (m, 1H)), 4.40 \text{ (q, } J = 5.6, 1\text{H}), 3.26 \text{ (m, 2H), 2.66 \text{ (m, 1H), 2.45 \text{ (m, 1H), 2.24 \text{ (t, } J = 7.5, 2\text{H), 2.13 \text{ (m, 1H), 2.06 \text{ (m, 1H), 1.67 \text{ (sextet, } J = 7.4, 2\text{H), 1.51 \text{ (t, } J = 6.9, 2\text{H), 1.29-1.21 \text{ (m, 18H), 0.95 \text{ (t, } J = 7.4, 3\text{H), 0.88 \text{ (t, } J = 7.0, 3\text{H})} \)

\(^{13}\text{C} \text{NMR} \) \(500 \text{ MHz, CDCl}_3\) \(\delta 174.8, 174.3, 172.9, 52.9, 40.3, 38.4, 33.0, 32.1, 29.80, 29.78, 29.74, 29.68, 29.50, 29.46, 29.41, 28.3, 27.1, 22.8, 19.1, 14.3, 13.8. ESI-MS \( m/z \) calcd for C\(_{21}\)H\(_{40}\)N\(_2\)O\(_4\)\(^+\) + NH\(_4\)-CH\(_3\)CN\(^+\) complex (ammonia-acetonitrile) [M+NH\(_4\)-CH\(_3\)CN]\(^+\): 443.36. Found: 443.46.

**N^α-hexanoyl-N^δ-dodecyl-L-glutamine (481)**

\![Chemical Structure](attachment:image.png)

N^δ-dodecyl-L-glutamine monohydrochloride (479) (0.018 g, 0.057 mmol) was dissolved in 10 % NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0 °C and hexanoyl chloride (12 \(\mu\)L, 0.085 mmol, 1.5 eq) was added slowly to the ether layer with no mixing. The biphasic solution was vigorously stirred at 0 °C for 4 hrs and then at room temperature for 18 hrs. The ether was
evaporated under a stream of air and the solution was carefully acidified with concentrated HCl to ca. pH = 1. The mixture was extracted with DCM five times. The combined organic phases were washed with brine, dried with MgSO₄, and concentrated under reduced pressure to yield. The crude product was purified with silica column chromatography (9.5:0.5 CH₂Cl₂:MeOH) to furnish 481 (0.019 g, 0.047 mmol, 83% yield).

\(^{1}H\) NMR (500 MHz, CDCl₃) δ 7.39 (d, J = 5.4, 1H), 6.31 (bs, 1H), 4.40 (q, J = 5.3, 1H), 3.26 (q, J = 6.3, 2H), 2.64 (m, 1H), 2.45 (m, 1H), 2.25 (t, J = 7.6, 2H), 2.14 (m, 1H), 2.05 (m, 1H), 1.63 (quintet, J = 7.3, 2H), 1.51 (dt, J = 13.3, 6.4, 2H), 1.35-1.21 (m, 22H), 0.89 (t, J = 6.9 Hz, 3H), 0.88 (t, J = 0.87 Hz, 3H). \(^{13}C\) NMR (500 MHz, CDCl₃) δ 174.9, 174.2, 172.9, 52.9, 40.3, 36.5, 32.9, 32.1, 31.5, 29.86, 29.78, 29.74, 29.68, 29.50, 29.44, 29.41, 28.4, 27.1, 25.4, 22.8, 22.5, 14.3, 14.1 ESI-MS m/z calcd for C_{23}H_{44}N_{2}NaO_{4} \([M+Na]^+\): 435.32. Found: 435.37.

N\(^\alpha\)-octanoyl-N\(^\delta\)-dodecyl-L-glutamine (482)

N\(^\delta\)-dodecyl-L-glutamine monohydrochloride (479) (0.022 g, 0.062 mmol) was dissolved in 10 % NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0 °C and octanoyl chloride (10 µL, 0.055 mmol, 0.9 eq) was added slowly to the ether layer with no mixing. The biphasic solution was vigorously stirred at 0 °C for 4 hrs and then at room temperature for 18 hrs. The ether was evaporated under a stream of air and the solution was carefully acidified with concentrated HCl to ca. pH = 1. The mixture was extracted with DCM five times. The combined organic phases were washed with brine, dried with MgSO₄, and concentrated under reduced pressure to yield 482 (0.021 g, 0.048 mmol, 78%).

\(^{1}H\) NMR (500 MHz, CDCl₃) δ 7.38 (d, J = 3.9, 1H), 6.38 (bs, 1H), 4.40 (m, 1H), 3.26 (bd, J = 5.5, 2H), 2.62 (m, 1H), 2.44 (m, 1H), 2.25 (t, J = 7.5, 2H), 2.13 (m, 1H), 2.05 (m, 1H), 1.63 (m, 2H), 1.51 (m, 2H), 1.34-1.21 (m, 26H), 0.88 (t, J = 6.7, 6H). \(^{13}C\) NMR (500 MHz, CDCl₃) δ 174.8, 174.0, 173.4, 52.9, 40.3, 36.6, 32.8, 32.1, 31.8, 29.80, 29.78, 29.76, 29.71, 29.50, 29.44, 29.44, 29.31, 29.13, 28.5, 27.1, 25.7, 22.83, 22.75, 14.27, 14.22. ESI-MS m/z calcd for C_{25}H_{47}N_{2}O_{4} \([M]^+\): 439.35. Found: 439.18.

N\(^\alpha\)-dodecanoyl-N\(^\delta\)-dodecyl-L-glutamine (483)

N\(^\delta\)-dodecyl-L-glutamine monohydrochloride (479) (0.030 g, 0.096 mmol) was dissolved in 10 % NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0 °C and dodecanoyl chloride (35 µL, 0.15 mmol, 1.5 eq) was added slowly to the ether layer with no mixing. The biphasic solution was vigorously stirred at 0 °C for 4 hrs and then at room temperature for 18 hrs. The ether was evaporated under a stream of air and the solution was carefully acidified with concentrated HCl to ca. pH = 1. The mixture was extracted with DCM five times. The
combined organic phases were washed with brine, dried with MgSO₄, and concentrated under reduced pressure. The crude product was purified by silica column chromatography (9:5:0.5 CH₂Cl₂:MeOH) to yield 483 (0.040 g, 0.081 mmol, 84% yield).

1H NMR (500 MHz, CDCl₃) δ 7.60 (bs, 1H), 7.30 (bs, 1H), 4.14 (m, 1H), 3.11 (m, 2H), 2.18 (m, 3H), 2.02 (m, 1H), 1.84 (m, 1H), 1.48 (m, 3H), 1.33-1.07 (m, 36H), 0.86 (t, J = 6.5, 6H).

13C NMR (500 MHz, CDCl₃) δ 177.8, 174.7, 174.0, 54.4, 40.0, 36.6, 32.1, 30.0 - 29.64 (17 carbons), 27.4, 26.1, 22.9, 14.3, 14.3. ESI-MS m/z calcd for C₂₀H₆₅N₂NaO₄⁺ [M+Na⁺]: 519.41. Found: 519.44.

Nα-dodecanoyl-Nδ-dodecyl-L-glutamine (484)

Nα-dodecanoyl-Nδ-octyl-L-glutamine (485)
(400 MHz, CDCl₃) δ 174.9, 174.2, 173.1, 52.8, 40.3, 36.6, 32.9, 32.06, 31.93, 29.77, 29.76, 29.64, 29.49, 29.49, 29.44, 29.37, 29.36, 29.34, 28.4, 27.1, 25.7, 22.84, 22.78, 14.27, 14.23. ESI-MS m/z calcld for C_{25}H_{47}N_{2}O_{4}⁻ [M]⁻: 439.35. Found: 439.16.

**Lithium 4-hexanamidobenzoate (501)**

4-hexanamidobenzoic acid (507) (0.051 g, 0.22 mmol) was dissolved in MeOH (final concentration of 0.1 M) and 2 M LiOH (0.11 mL, 1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure (0.051 g, 0.21 mmol, 99% yield).

**1H NMR** (400 MHz, D₂O) δ 7.85 (d, J = 8.6, 2H), 7.47 (d, J = 8.5, 2H), 2.38 (t, J = 7.4, 2H), 1.65 (quintet, J = 7.2, 2H), 1.31 (m, 4H), 0.86 (t, J = 7.0, 3H). **13C NMR** (400 MHz, DMSO) δ 171.3, 169.5, 140.5, 133.4, 129.8, 117.6, 36.4, 30.9, 24.8, 21.9, 13.9. ESI-MS m/z calcld for C_{13}H_{16}NO_{3}⁻ [M]⁻: 234.11. Found: 234.06.

**Sodium 4-hexanamidobenzoate (502)**

4-hexanamidobenzoic acid (507) (0.045 g, 0.19 mmol) was dissolved in MeOH (final concentration of 0.1 M) and 4 M NaOH (0.046 mL, 1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure (0.048 g, 0.19 mmol, 99% yield).

**1H NMR** (400 MHz, D₂O) δ 7.85 (d, J = 8.5, 2H), 7.46 (d, J = 8.4, 2H), 2.32 (m, 2H), 1.60 (m, 2H), 1.27 (m, 4H), 0.84 (d, J = 3.9, 3H). **13C NMR** (400 MHz, DMSO) δ 171.3, 170.3, 140.1, 134.6, 129.7, 117.6, 36.4, 30.9, 24.9, 21.9, 13.9. ESI-MS m/z calcld for C_{13}H_{16}NO_{3}⁻ [M]⁻: 234.11. Found: 234.05.

**Potassium 4-hexanamidobenzoate (503)**

4-hexanamidobenzoic acid (507) (0.049 g, 0.21 mmol) was dissolved in MeOH (final concentration of 0.1 M) and 4 M KOH (0.052 mL, 1 eq) was added. The mixture was stirred at room temperature for 30 min after which the solvent was evaporated under reduced pressure (0.055 g, 0.20 mmol, 97% yield).

**1H NMR** (400 MHz, D₂O) δ 7.85 (d, J = 8.5, 2H), 7.46 (d, J = 8.4, 2H), 2.32 (t, J = 7.4, 2H), 1.60 (m, 2H), 1.27 (m, 4H), 0.84 (t, J = 6.6, 3H). **13C NMR** (400 MHz, DMSO) δ 171.8, 169.8, 140.4, 135.5, 130.0, 118.0, 36.9, 31.4, 25.4, 22.4, 14.4. ESI-MS m/z calcld for C_{13}H_{16}NO_{3}⁻ [M]⁻: 234.11. Found: 234.06.
4-(Hexylcarbamoyl)benzenaminium chloride (504)

*tert*-Butyl (4-(hexylcarbamoyl)phenyl)carbamate (509) (0.027 g, 0.0833 mmol) was dissolved in anhydrous 2 M HCl in ether (5 mL) and stirred at ambient temperature for 16 hrs. The white precipitate and solvent was transferred to a falcon tube and centrifuged at 1000 rpm for 1 min. The solvent was carefully decanted, fresh ether was added (5 mL), the mixture was mixed until the precipitate was distributed thoroughly, and the falcon tube was centrifuged again. This was repeated until decanted ether is colourless. The white precipitate was dried under air to yield 504 (0.010 g, 0.039 mmol, 47% yield).

$^1$H NMR (500 MHz, $D_2O$) $\delta$ 7.81 (d, $J = 8.7$, 2H), 7.45 (d, $J = 8.7$, 2H), 3.34 (t, $J = 6.9$, 2H), 1.56 (quintet, $J = 7.2$, 2H), 1.32 (m, 2H), 1.26 (m, 4H), 0.81 (t, $J = 7.0$, 3H). $^{13}$C NMR (500 MHz, $D_2O$) $\delta$ 169.5, 134.4, 133.4, 128.7, 123.0, 40.0, 30.6, 28.1, 25.7, 21.8, 13.2. ESI-MS m/z calcd for C$_{13}$H$_{21}$N$_2$O$^+$ [M$^+$]: 221.16. Found: 221.13.

4-(Hexylcarbamoyl)benzenaminium bromide (505)

*tert*-Butyl (4-(hexylcarbamoyl)phenyl)carbamate (507) (0.024 g, 0.074 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (5 mL) and anhydrous HBr was bubbled though the solution until a precipitate formed. The solution was stirred at ambient temperature for an additional hour. The white precipitate and solvent was transferred to a falcon tube and centrifuged at 1000 rpm for 1 min. The solvent was carefully decanted, fresh ether was added (5 mL), the mixture was mixed until the precipitate was distributed thoroughly, and the falcon tube was centrifuged again. This was repeated until decanted ether is colourless. The white precipitate was dried under air to yield 505 (0.016 g, 0.053 mmol, 72% yield).

$^1$H NMR (400 MHz, $D_2O$) $\delta$ 7.84 (d, $J = 8.5$, 2H), 7.50 (d, $J = 8.5$, 2H), 3.36 (t, $J = 6.9$, 2H), 1.58 (quintet, $J = 7.1$, 2H), 1.37-1.22 (m, 6H), 0.83 (t, $J = 7.0$, 3H). $^{13}$C NMR (400 MHz, $D_2O$) $\delta$ 169.5, 134.8, 133.1, 128.9, 123.3, 40.1, 30.7, 28.2, 25.8, 21.9, 13.3. ESI-MS m/z calcd for C$_{13}$H$_{21}$N$_2$O$^+$ [M$^+$]: 221.16. Found: 221.13.

4-(Hexylcarbamoyl)benzenaminium iodide (506)

*tert*-Butyl (4-(hexylcarbamoyl)phenyl)carbamate (510) (0.040 g, 0.12 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (5 mL) and anhydrous HI was bubbled though the solution until a precipitate formed. The solution was stirred at ambient temperature for an additional hour. The white precipitate and solvent was transferred to a falcon tube and centrifuged at 1000 rpm for 1 min. The solvent was carefully decanted, fresh ether was added (5 mL), the mixture was mixed until the precipitate was distributed thoroughly, and the falcon tube was centrifuged again. This was repeated until decanted ether is
colourless. The white precipitate was dried under air to yield \textbf{506} (0.036 g, 0.11 mmol, 85\% yield).

$^1$H NMR (500 MHz, $D_2O$) $\delta$ 7.79 (d, $J = 8.6$, 2H), 7.44 (d, $J = 8.6$, 2H), 3.29 (t, $J = 6.8$, 2H), 1.53 (quintet, $J = 7.2$, 2H), 1.28-1.18 (m, 6H), 0.78 (t, $J = 7.0$, 3H). $^{13}$C NMR (500 MHz, $D_2O$) $\delta$ 169.1, 134.6, 132.9, 128.9, 123.2, 40.0, 30.8, 28.3, 25.9, 21.9, 13.3. ESI-MS $m/z$ calcd for C$_{13}$H$_{17}$N$_2$O$^+$ [M$^+$]: 221.16. Found: 221.13.

\textbf{4-hexanamidobenzoic acid (507)}

4-Aminobenzoic acid (1.0 g, 7.3 mmol) was dissolved in 10\% NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0 °C and hexanoyl chloride (1.5 mL, 10.9 mmol, 1.5 eq) was added slowly to the ether layer with no mixing. The biphasic solution was vigorously stirred at 0 °C for 4 hrs and then at room temperature for 18 hrs. The ether was evaporated under a stream of air and the solution was carefully acidified with concentrated HCl to ca. pH = 1. The white precipitate was filtered and washed with H$_2$O until washings had a neutral pH. The crude product was purified by silica column chromatography (5:5 petroleum ether:EtOAc to 7:3 petroleum ether:MeOH) to yield white product (1.4 g, 6.0 mmol, 82\%).

$^1$H NMR (300 MHz, DMSO) $\delta$ 10.18 (bs, 1H), 7.87 (m, 2H), 7.70 (m, 2H), 2.33 (t, $J = 7.4$, 2H), 1.59 (quintet, $J = 7.3$, 2H), 1.29 (m, 4H), 0.87 (t, $J = 6.9$, 3H). $^{13}$C NMR (300 MHz, DMSO) $\delta$ 171.8, 167.0, 143.4, 131.6, 123.3, 117.4, 81.5, 28.3, 28.3, 28.3. ESI-MS $m/z$ calcd for C$_{13}$H$_{16}$NO$_3^-$ [M$^-$]: 234.11. Found: 234.01.

\textbf{4-((tert-Butoxycarbonyl)amino)benzoic acid (508)}

4-Aminobenzoic acid was dissolved in a mixture of 1:2 H$_2$O:dioxane for a total volume of 2.5 mL and Et$_2$N (0.2 mL, 1.4 mmol, 2 eq) was added. After 5 min of stirring at ambient temperature, di-tert-butyldicarbonate (0.32 g, 1.4 mmol) was added. The mixture was stirred for 16 hrs and the solvent was removed under reduced pressure and the crude product was purified by silica column chromatography (100\% petroleum ether to 5:5 EtOAc:MeOH) to yield \textbf{508} as a white powder (0.12 g, 0.49 mmol, 67\% yield).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.04 (d, $J = 8.8$, 2H), 7.46 (d, $J = 8.8$, 2H), 1.54 (s, 9H). $^{13}$C NMR (300 MHz, CDCl$_3$) $\delta$ 170.7, 152.2, 143.4, 131.6, 123.3, 117.4, 81.5, 28.3, 28.3, 28.3. ESI-MS $m/z$ calcd for C$_{12}$H$_{14}$NO$_4^-$ [M$^-$]: 236.09. Found: 236.02.

\textit{tert-Butyl (4-(hexylcarbamoyl)phenyl)carbamate (509)}

To a solution of anhydrous CH$_2$Cl$_2$ and 4-((tert-Butoxycarbonyl)amino)benzoic acid
(508) (0.048 g, 0.20 mmol) under argon, diisopropylethylamine (0.054 mL, 0.20 mmol, 1.1 eq) and HCTU (0.093 g, 0.22 mmol, 1.1 eq) was added and the mixture was stirred for 30 min. Hexyl amine (0.023 mL, 0.22 mmol, 1.1 eq) was added and after 16 hrs, the solvent was removed under reduced pressure and the residue was purified by silica column chromatography (8:2 petroleum ether:EtOAc to 7:3 petroleum ether:EtOAc) to yield 509 (0.056 g, 0.18 mmol, 86% yield).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.68 (d, J = 8.6, 2H), 7.41 (d, J = 8.6, 2H), 7.03 (m, 1H), 6.30 (m, 1H), 3.39 (q, J = 6.7, 2H), 1.58 (m, 2H), 1.49 (s, 9H), 1.36-1.20 (m, 6H), 0.86 (t, J = 6.4, 3H). $^{13}$C NMR (300 MHz, CDCl$_3$) $\delta$ 167.1, 152.6, 141.5, 128.9, 128.0, 117.9, 81.0, 40.2, 31.6, 28.4, 28.4, 28.4, 26.8, 22.7, 14.1. ESI-MS m/z calcd for C$_{18}$H$_{29}$N$_2$O$_3$ $^+$ [M+H]$^+$: 321.22. Found: 321.17.
7.6. Spectroscopic Data
AcO OAc
AcO
N3 ONO2

319

171
\[
\text{H}_2\text{C}_7\text{N}\text{H}_3\text{N}\text{C}_7\text{H}_11\text{N}\text{C}_7\text{H}_11\text{N}\text{C}_7\text{H}_11\text{N}\text{C}_7\text{H}_11\text{N}\text{C}_7\text{H}_11\text{N}\text{C}_7\text{H}_11
\]
\[
\text{H}_2\text{C}_{11}\overset{\text{H}}{\text{N}}\overset{\text{BnO}}{\text{O}}\overset{\text{NHBOc}}{\text{O}}
\]
$\text{H}_2\text{O}\text{C}_2\text{H}_{12}\text{N}^-\text{H}^+\text{O}\text{C}_{11}\text{H}_{23}$

[Chemical structure and spectroscopy graph]

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References

Conclusions

The overall aims were to synthesize small molecule ice recrystallization inhibitors and elucidate the structural features responsible for potent ice recrystallization inhibition activity for the potential use as cryopreservatives with cells and viruses. In particular, the goals of this study were: (1) to determine how the glycosidic linkage in AFGP disaccharide analogues modulates IRI activity; (2) to synthesize lysine, glutamine, and \textit{para}-amino benzoic acid-based surfactants and gelators, and glycoconjugates, and determine which structural features affect IRI activity; (3) to assess the synthesized surfactants and gelators for their ability to preserve cells in cryopreservation; (4) to determine the best solution that should be utilized in the IRI assay; and (5) to identify ice recrystallization inhibitors that preserve viral vectors in various storage conditions.

The first goal was achieved by synthesizing the $\beta$-(1,6)-linked AFGP disaccharide analogue and assessing it for IRI activity. When compared to the native $\beta$-(1,3)- and $\beta$-(1,4)-linked AFGP disaccharide, the derivative that had the highest IRI activity was the $\beta$-(1,4)-linked disaccharide. This was not the disaccharide that is found in the native AFGP, which contains the $\beta$-(1,3)-linkage. Additionally, it was found that the C2 \textit{N}-acetyl group of the disaccharide is not necessary for IRI activity. This is of particular interest because it indicates that features that are important for thermal hysteresis are not necessarily those necessary for IRI activity. Overall, these data indicate that the glycosidic linkage of disaccharides does influence IRI activity.

Secondly, structurally diverse cationic and anionic lysine surfactants and glycoconjugates as well as glutamine surfactants were synthesized and analyzed for IRI activity. The results indicate that potent IRI activity is dependent upon the presence of
long alkyl chains and increased hydrophobicity. In particular, for bis-acylated anionic lysines, the most active compound had the alkyl chain attached to the e-amino terminus. Other conclusions from this study are that anti-ice nucleation activity, gelation, and the ability to form gels is independent of high IRI activity. The IRI activity of the glycoconjugates containing a lysine surfactant indicated that the addition of a hydrophobic moiety to a monosaccharide will increase the IRI activity with respect to the carbohydrate. The best ice recrystallization inhibitors will be amphiphilic molecules with a finely-tuned balance between hydrophobic and hydrophilic components. One of the most important conclusions from these studies shows that the IRI activity of compounds containing amine and carboxylate counterions correspond to the enthalpy of hydration of the counterions. The para-amino benzoic acid derivatives indicated that the higher the degree of hydration of the counterion, the higher the IRI activity. Future work for these surfactnats should focus on determining the exact role that the long alkyl chains play in the mechanism of ice recrystallization.

The cryopreservation of human liver cells (Hep G2) and human bone marrow cells (Tf-1α) with cationic quaternary ammonium chloride surfactants and anionic lysine surfactants indicated high IRI activity does not necessarily correlate with the ability to protect cells from cryoinjury. Although these compounds are not optimal for cells, they may have applications in the fields of frost prevention on various surfaces.

It was determined that the most optimal solution for the IRI assay is PBS. This solution is very similar to physiological conditions and prevents the appearance of high IRI activity of know IRI-inactive compounds. This study also demonstrated that IRI
activity of compounds is dependent upon the assay solution and that the degree of hydration of the individual ions present in the assay solutions modulate IRI activity.

Lastly, three ice recrystallization inhibitors (OGG-Gal, N-octyl gluconamide, and N-octyl galactonamide) were found to offer protection to the viral vectors vaccinia virus (VV), vesicular stomatitis virus (VSV), and herpes simplex virus 1 (HSV-1) at three different storage conditions. With 10 freeze-thaw cycles, OGG-Gal and NOGlc proved to be the most effective at maintaining viral infectivity with VV and VSV. These results indicate also that there is a correlation between ice recrystallization inhibition activity and protection of viruses though freeze-thaw cycles. In lyophilization, the three ice recrystallization inhibitors the protective effects were less pronounced but still OGG-Gal and NOGlc offered the best protection with VV. The most significant result from this study is that NOGlc was extremely effective at stabilizing VV at room temperature. This facilitates the exploration of ambient temperature vaccine stabilization, a major problem currently.

Combined, these studies promote the rational design of small molecules that have the potential to protect cells and viruses in cryopreservation. This was accomplished by determining the structural features of disaccharides and surfactants that are important for potent IRI activity.
Claims to original research

1. Synthesis and analysis of the ice recrystallization inhibition (IRI) activity of the \(\beta\-(1,6)\)-linked AFGP disaccharide analogue, lysine surfactants, glutamine surfactants, and PABA analogues.

2. Analysis of the ice recrystallization inhibition (IRI) activity of PBS salts.

3. Analysis of cytotoxicity and cryopreservation potential of small molecule ice recrystallization inhibitors with a human liver cell line (HepG2 cells) and human bone marrow cell line (Tf-1\(\alpha\)).

Publications

Balcerzak, A. K.; Capicciotti, C. J.; Briard, J. G.; Ben, R. N. Designing ice recrystallization inhibitors: from antifreeze (glyco)proteins to small molecules *RSC Advances* 2014, 4, 42682.

