

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

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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

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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-1 α) 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

α	Alpha
β	Beta
γ	Gamma
δ	Delta
ϵ	Epsilon
^1H	Proton
^{13}C	Carbon
7-AAD	7-Aminoaceticinomyacin D
Ac	Acetyl
Ac_2O	Acetic anhydride
AFGP(s)	Antifreeze glycoprotein(s)
AFP (s)	Antifreeze protein(s)
Anti-INA	Anti-ice nucleation agent
Ala	Alanine
Atm	Atmospheres
$\text{BF}_3\cdot\text{OEt}_2$	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

CPA	Cryoprotective agent
d	Doublet
DBU	Diazabicycloundec-7-ene
dd	Doublet of doublets
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DMDO	dimethyldioxirane
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
dt	Doublet of triplets
Et	Ethyl
Et ₂ O	Diethyl ether
EtOAc	Ethyl acetate
EtOH	Ethanol
Gal	Galactose
Glc	Glucose
GFP	Green fluorescent protein
Hep G2	Human liver hepatocellular carcinoma cells
HCTU	O-(6-Chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate, a coupling agent
IRI	Ice recrystallization inhibition
Lys	Lysine
m	Multiplet
M	Molar
M ⁺	Parent molecular ion
Me	Methyl
MeOH	Methanol
MGS	Mean grain size
MHz	Megahertz
mM	Millimolar
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OGG	Ornithine-glycine-glycine
OMe	<i>O</i> -Methoxy
PBS	Phosphate-buffered saline
Pd/C	Palladium on carbon
PFU	plaque forming unit
q	Quartet
QLL	Quasi-liquid layer
RFP	red fluorescent protein
RT	Room Temperature
s	Singlet
t	Triplet
TEA	Triethylamine
TFA	Trifluoroacetic acid
TH	Thermal hysteresis

Thr	Threonine
TMSOTf	Trimethylsilyl trifluoromethane sulfonate
YFP	Yellow fluorescent protein

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.^{1,2} Subsequent to this, pregnancy resulted from the use of cryopreserved human oocytes.³ Then the successful cryopreservation of red blood cells was demonstrated.^{4,5} More recently, the cryopreservation of hematopoietic stem cells⁶ has become important as these cells can be used in regenerative therapies.⁷ The “process of replacing or regenerating human cells, tissues or organs to restore or establish normal function”⁸ can be used to treat spinal cord injury,⁹ coronary artery disease,⁹ heart attacks,¹⁰ stroke,¹¹ other cancers, genetic diseases, immune deficiencies, and blood disorders.¹² However, the success of these regenerative therapies has been directly linked to the quantity, quality, and functionality of the cells post-thaw.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.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.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.^{15,16} Viruses must overcome the same difficulties that cells are confronted with: ice formation, dehydration, and rehydration.¹⁵ As with cells, current preservation protocols for viruses are not always optimal.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.¹⁷ The Global Alliance for Vaccines and Immunizations estimates that approximately 3 million people die each year from vaccine preventable diseases.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_h (ice hexagonal).¹⁹ The other forms of ice arise as a result of high pressures.²⁰ However, it was found that cubic ice (I_c) can form when pure water is frozen at -38°C at normal pressures which may be relevant to cryopreservation.^{21,22} This ice also forms in small amounts in the tropopause region of earth’s atmosphere.²²

The structure of I_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

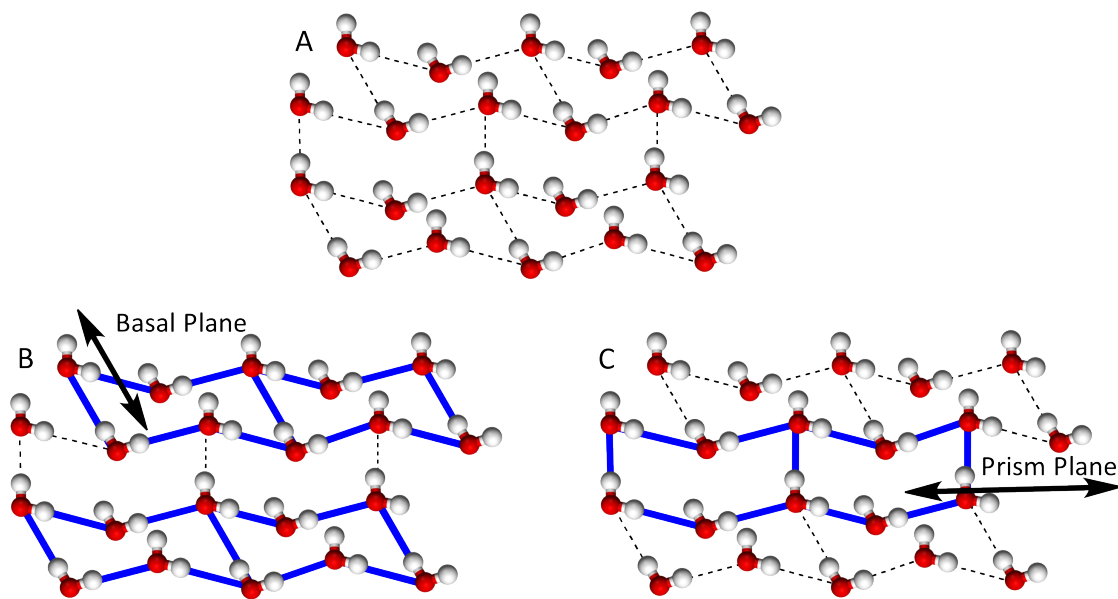


Figure 1-1 Structure of ice. (A) Hexagonal structure of ice where (B) the basal plane is made of chair-form hexagonals and (C) the prism plane is made of boat-form hexagonals.

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,

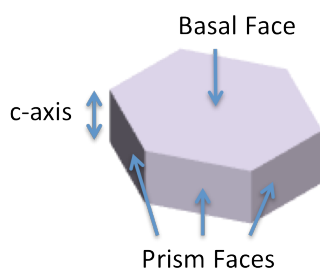


Figure 1-2 Hexagonal structure of ice

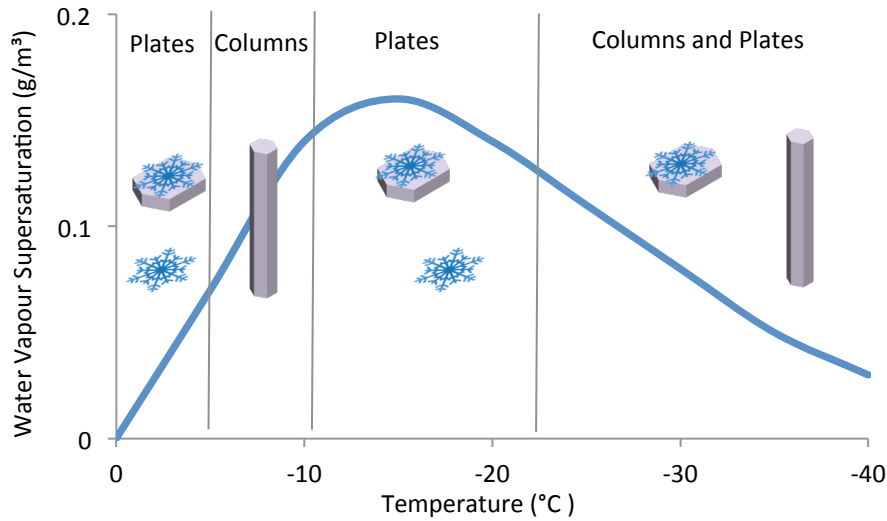


Figure 1-3 The snow crystal morphology diagram showing different types of snow crystals that grow at specified temperatures and water vapor supersaturation. Reproduced from Libbrecht, 2005.²³

ice will grow along the prism faces to eventually form the flat, six-pointed shape of a snowflake (**Figure 1-3**).²³ Under different conditions, ice will grow along the basal face to form long, solid, six-sided columns.²³

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).²⁴ **Figure 1-4** depicts the

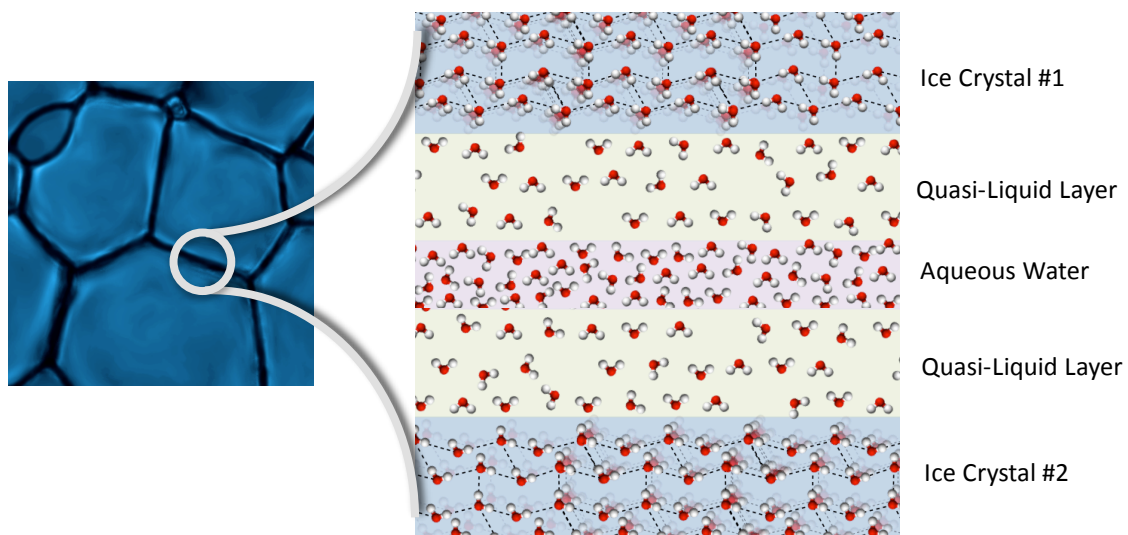


Figure 1-4 Schematic diagram of the quasi-liquid layer (QLL). The QLL is in between bulk water and ice.

QLL between ice and aqueous water showing the semi-organization of the QLL compared to the random configuration of aqueous water.²⁵ Its thickness increases as the temperature approaches the melting point.²⁶ 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.²⁷ 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.²⁸ 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

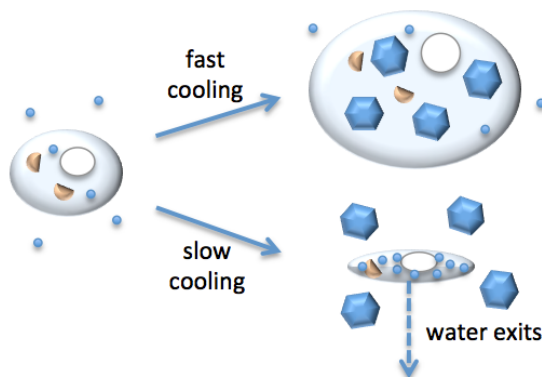


Figure 1-5 Schematic representation of cells that are cooled rapidly with the formation of intracellular ice or cells that are cooled slowly while being dehydrated.

The survival of many cell types in cryopreservation is increased if a slow freezing rate is employed.^{29,30} These observations can be explained with the following. When cells are cooled at a slow rate, ice crystals form outside the cells.²⁸ 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.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 crystallize out. As a result, the conformation of lipid-protein complexes, especially phospholipids found in cell membrane, are altered.³¹ Due to these “solution effects”,³² upon thawing, the cells would immediately lyse.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.³⁰ The intracellular ice crystals cause extensive damage to the cell membrane and internal organelles by mechanical stress leading to cell death.³³ The damage to the cell membrane caused by intracellular ice formation was seen in electron micrographs of frozen liver cells and erythrocytes.^{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.³²

Mazur et. al. suggested the two-factor hypothesis of freezing injury: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.³⁶ This has been experimentally confirmed and results are presented in characteristic upside-down *U*-shaped curves shown in **Figure 1-**

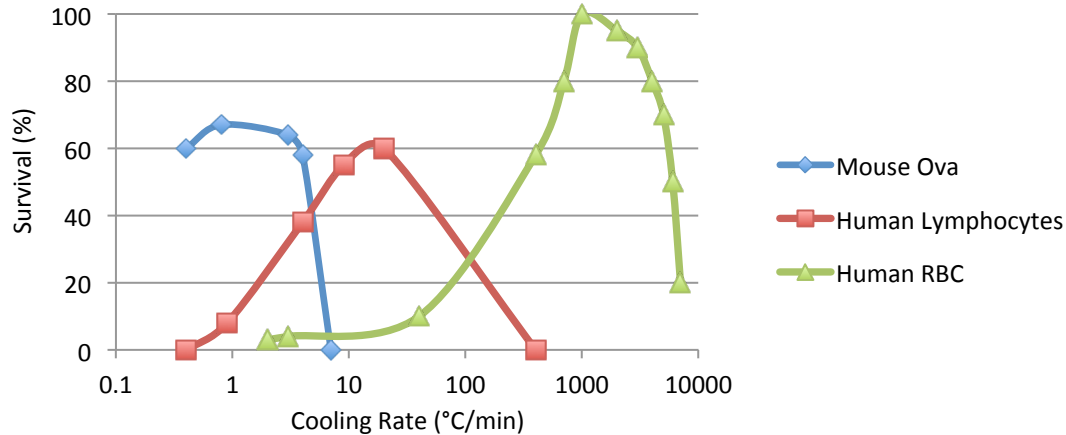


Figure 1-6 Survival rate of indicated cells at different cooling rates after freeze-thaw.³⁷

6.³⁷ 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.³⁸ 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

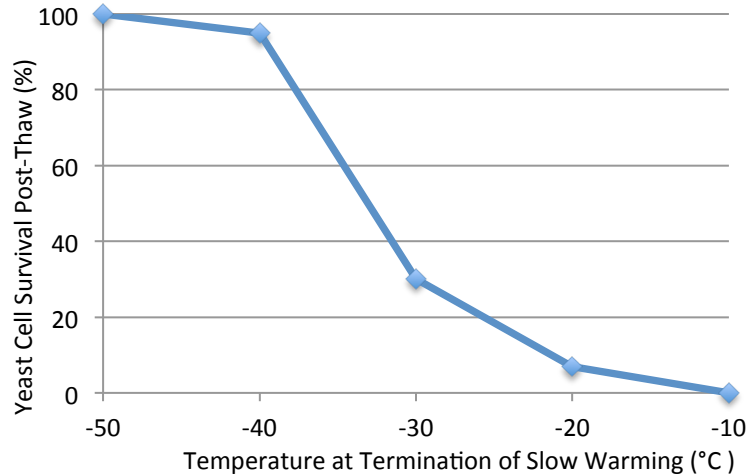


Figure 1-7 Effect of warming slowly from -196°C to various temperatures and then rapidly thawing on survival of yeast cells.

small ice crystals simply melt.³⁸ The profound effect of ice recrystallization during thawing on cell survival has been demonstrated in yeast, higher plant cells,³⁹ ascites tumor cells,⁴⁰ and hamster tissue culture cells.⁴¹ This correlation is clearly presented in a study by MacKenzie depicted in **Figure 1-7**.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.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.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.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.³² The process of migratory ice recrystallization is mediated through Ostwald ripening.^{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.⁴⁴ In Ostwald ripening, the total ice volume of this process remains constant while the average size of the ice crystals increases.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,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

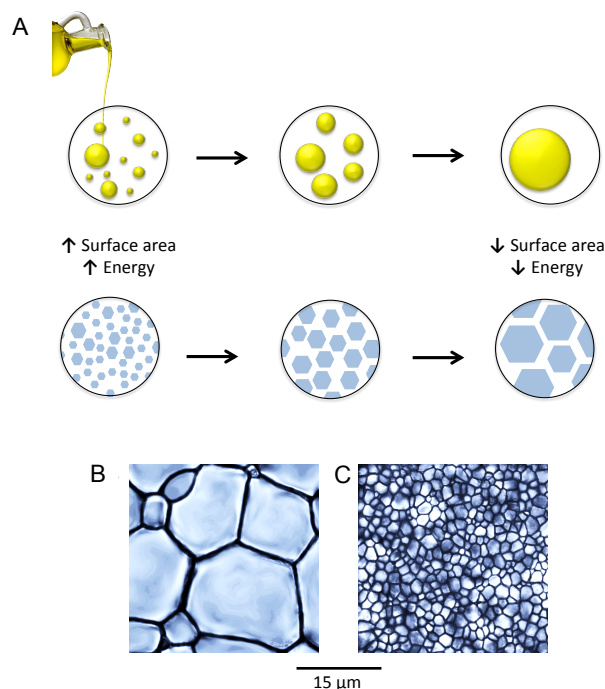


Figure 1-8 (A) The effect of Ostwald ripening with oil in water (top) and with ice crystals (bottom). Modified photographs of ice crystals from a splat cooling assay depicting: **(B)** no inhibition of ice recrystallization and **(C)** inhibition of ice recrystallization.

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.⁴⁷ Glycerol was found to protect many cell types such as human erythrocytes, human bone marrow, human renal cells, and many more.²⁸ After this, many other CPAs have been discovered and used including methanol, propylene glycol, dimethylacetamide, and dimethyl sulfoxide (DMSO).^{36,48} DMSO, discovered ten years after the first glycerol CPA publication by Polge,² was shown to be the most versatile of all CPAs by Lovelock and Bishop⁴⁹ as it was also effective at preserving cells that glycerol could not.²⁸ These CPAs are characterized as permeating CPAs as they are able to traverse the cell membrane⁴⁷ and decrease the electrolyte concentration within the cell and reduce the extent of osmotic shrinkage.^{37,50} 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.⁵ Eventually, when the temperature is sufficiently low (below -80°C), the water left in the cell will cool to a glassy matrix, also known as vitrification. Vitrification excludes intracellular ice crystals that will cause damage during thawing.⁴⁷ Additionally, the increasing viscosity of CPAs in the cells may inhibit or slow down ice growth.⁴⁷ 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.⁵¹ 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.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.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 unfrozen⁵² and may suppress or inactivate ice nuclei.⁵³

1.6 Toxicity of cryoprotectants

Since the landmark discovery of glycerol as a CPA by Polge *et al.*,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.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**).⁵⁵ At a very low DMSO concentration the

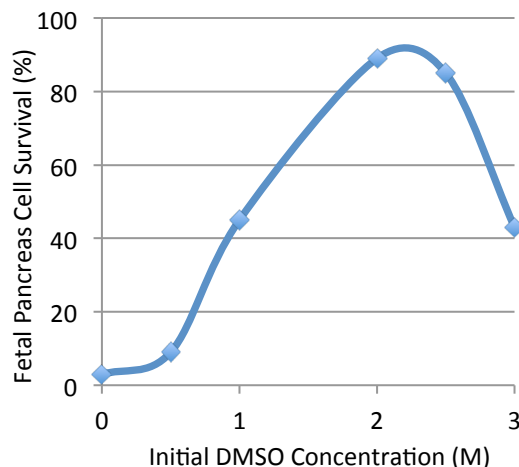


Figure 1-9 Relationship between initial concentration of DMSO after freeze-thaw of fetal pancreas.

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).⁵⁶ It was obvious that the hearts which were exposed to higher concentrations of DMSO were more damaged.⁵⁴ 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.⁵⁷ Similar toxicity results were reported with ethylene glycol, methanol and ethanol, and glycerol.⁵⁴ 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.⁵⁸ DMSO may also inhibit fructose diphosphatase which will inhibit glycolysis after freeze-thaw.^{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.⁶⁰ Finally, DMSO is able to denature proteins by binding to hydrophobic and aromatic side chains.⁶¹ Numerous other studies have been conducted on the freezing cytotoxicity of DMSO⁶⁰ 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.⁶² All studies indicate that the amount of DMSO present in the sample is related to the level of toxicity.^{63,64} The most common side effects include nausea and vomiting which is experienced by approximately 50% of patients.^{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.^{63,67,68} Another example indicates that DMSO from infusion can cause short-term amnesia and disorientation.^{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.⁶³ There are many reasons as to why DMSO is cytotoxic in humans. Studies have indicated that DMSO depresses the response of diaphragm muscles⁷¹ and decreases conduction velocity in nerves.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.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.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.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.⁷⁴⁻⁷⁶ The sea ocean temperatures that are inhabited by Arctic fish can decrease to -1.9°C yet the fish remain alive although 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 .⁷⁷ It was later discovered that antifreeze proteins in blood plasma were responsible for the remainder of the protection.^{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.⁸⁰ Additionally, many other AFPs have been found in

organisms other than fish. AFPs are present in insects such as the spruce budworm moth,^{81,82} the yellow mealworm beetle,^{83,84} the fire-coloured beetle,⁸⁵ and the snow flea.⁸⁶ AFPs have also been found in plants,⁸⁷⁻⁹⁴ fungi, and bacteria.⁹⁵⁻¹⁰⁰

Table 1-1 Mass, structural features and natural sources of antifreeze proteins⁸⁰

	AFP I ¹⁰¹⁻¹¹⁰	AFP II ^{111,112}	AFP III ¹¹²⁻¹²¹	AFP IV ¹²²⁻¹²⁴
Mass (kDa)	3.3-4.5	11-24	6.5	12
Structural Features	Single α -helix	Globular with disulfide bonds	Globular with β -sandwich	Helical bundle, alanine rich
Source	Winter flounder, Sculpins	Rainbow smelt, Atlantic herring	Ocean pout, Eel Pout	Longhorn sculpin

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.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-

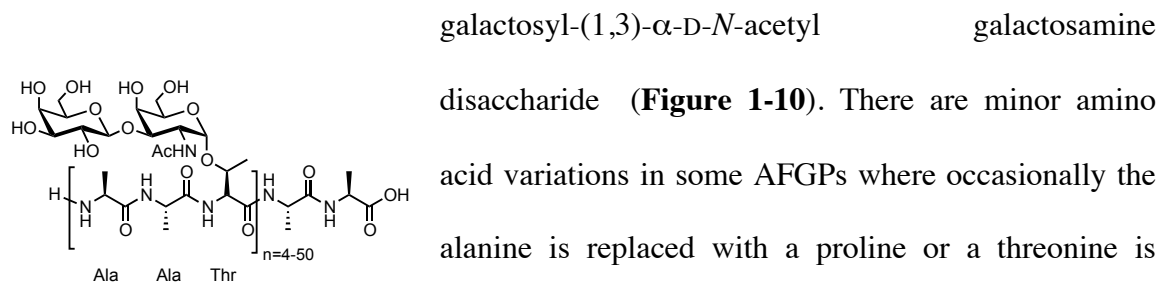


Figure 1-10 General structure of AFGP showing the disaccharide linked to the Ala-Ala-Thr peptide repeat.

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

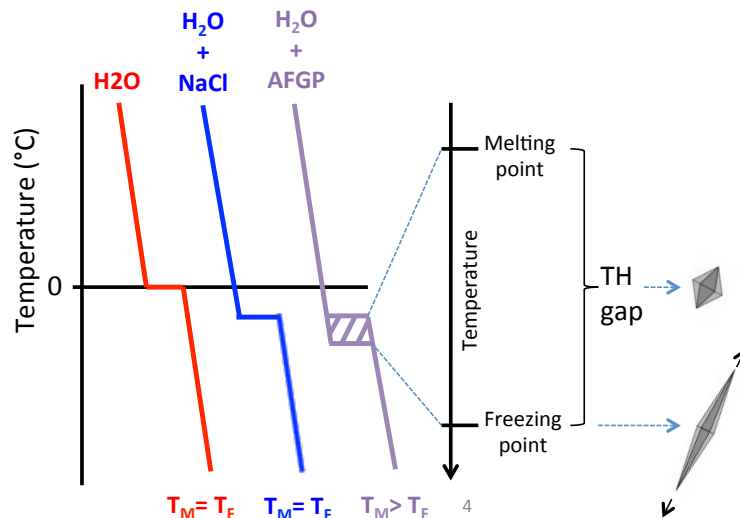


Figure 1-11 Diagram showing thermal hysteresis (TH). T_m represents the melting point and T_f represents the freezing point.

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.¹²⁵ In fish, TH gaps range from 1 to 1.5°C.¹²⁶

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 disfavoured addition of water molecules. This phenomenon is referred to as the Kelvin Effect (**Figure 1-12A**).^{127,128} 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

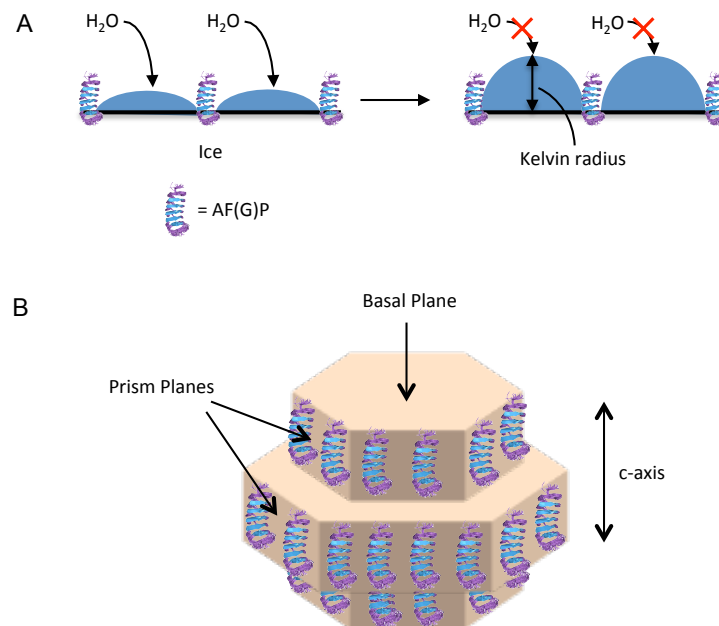


Figure 1-12 The effects of AF(G)Ps binding to ice crystals. A) Schematic representation of the Kelvin Effect. B) Diagram of the adsorption of AF(G)Ps onto the prism plane of an ice crystal causing the hexagonal bipyramidal shape.

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.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 bipyramidal shape of the ice crystal (**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 β -methyl of the threonine is crucial for the function of the antifreeze.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’.¹³² Overall, in literature the use of AF(G)Ps as cryopreservatives is considered ineffective.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 below the TH gap.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

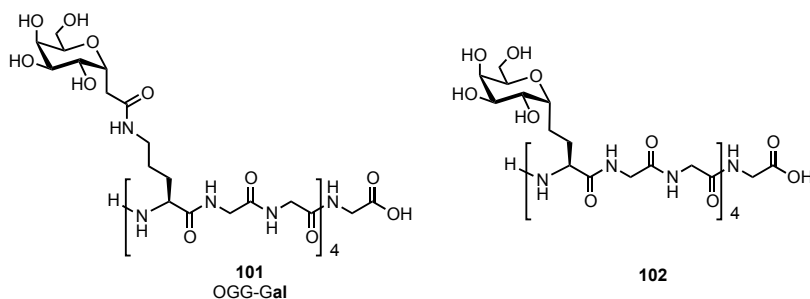


Figure 1-13 Structures of *C*-linked AFGP analogues **101** and **102**.

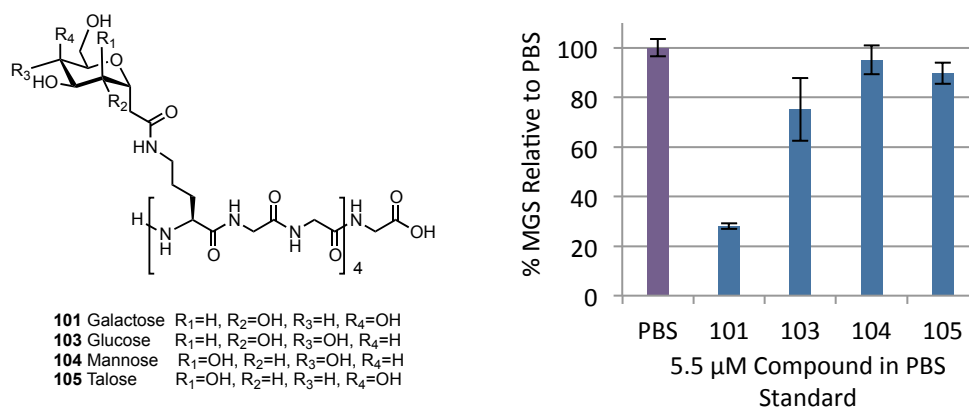


Figure 1-14 Structures of C-linked antifreeze glycoproteins with modified carbohydrate moieties. Error bars represent the standard error of the mean (SEM).

cells as well as a 2.5% DMSO solution at concentrations of 1 mg/mL.¹⁴¹ 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^{140,142} and domain recognition software¹⁴³ 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.¹⁴⁰ When the monosaccharides were examined individually, as reducing sugars,

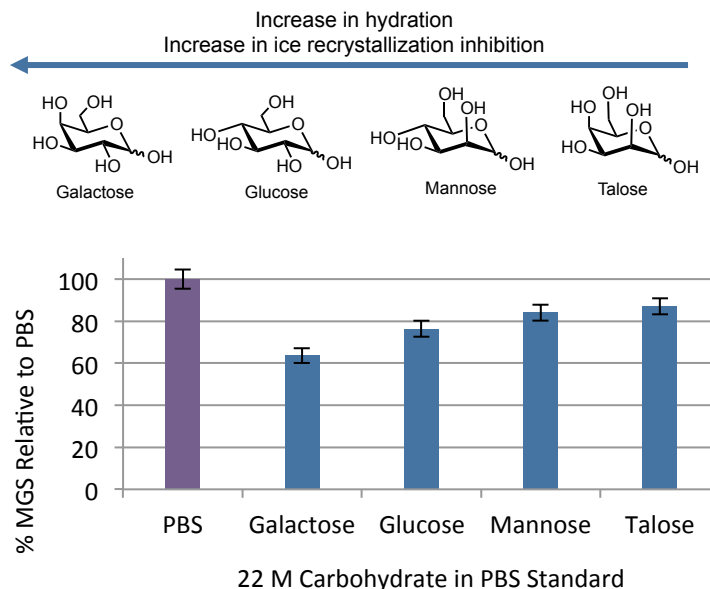


Figure 1-15 A representation of the hydration index. Compounds that are more hydrated have higher IRI activity. Error bars represent the standard error of the mean (SEM).

similarly, 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 carbohydrate¹⁴⁴⁻¹⁴⁶ and it was deduced that hydration is closely correlated to IRI activity.^{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

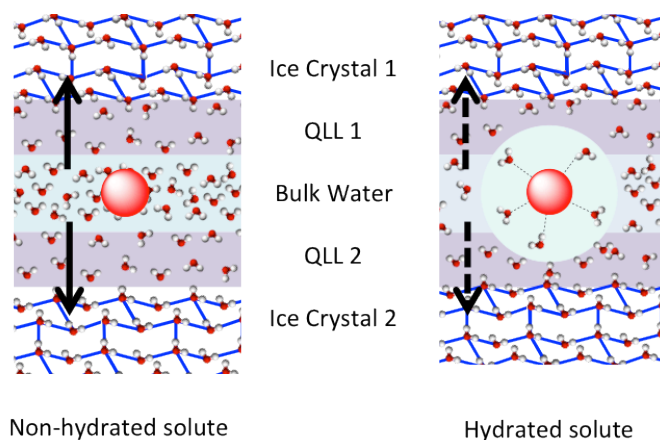


Figure 1-16 A representation of the proposed mechanism for ice recrystallization inhibition. With a non-hydrated solute, water molecules add to the ice crystals from the bulk water (solid arrows). With a hydrated solute, the movement of water molecules to the ice crystals is reduced (dashed arrows).

semi-ordered quasi-liquid layer.¹⁴⁸⁻¹⁵⁴ 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.¹⁴⁷ 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

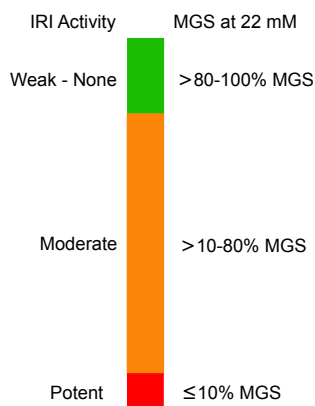


Figure 1-17 The IRI gauge. For compounds assessed with the splat cooling assay at 22 mM in PBS.

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 α -C-linked allyl group was investigated in analogues **106-109** while **110** and **111** contained a β -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}

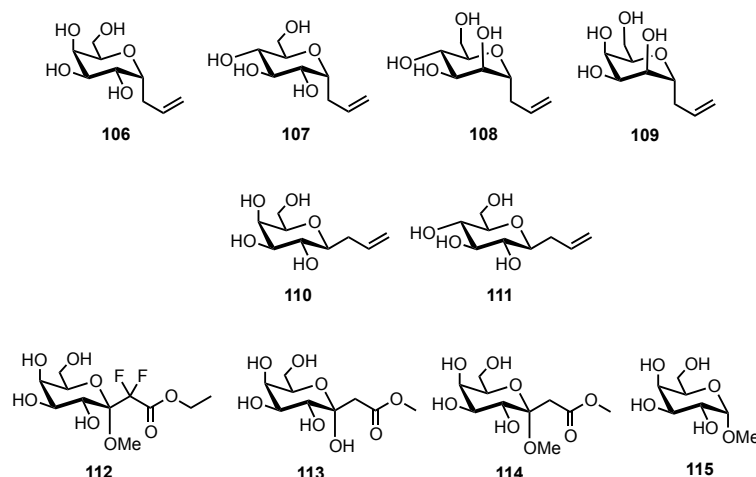


Figure 1-18 Structures of monosaccharide ice recrystallization inhibitors.

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.¹⁵⁷ 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 alkyl 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 μ M, 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 μ M concentration. The ionizable groups did

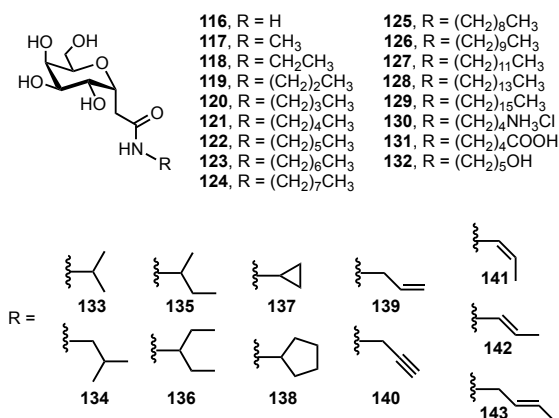


Figure 1-19 Structures of IRI active alkylated derivatives.

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 were 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.¹⁵⁷

The Ben group reported the finding of carbohydrate-based small molecule ice recrystallization inhibitors that have potent IRI activity at very low concentrations.¹⁵⁸ This was the first precedent of small molecules possessing potent IRI activity. From the previous study with the truncated analogues¹⁵⁷ 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.¹⁵⁸ The glucose-derived non-ionic surfactant β -octyl-

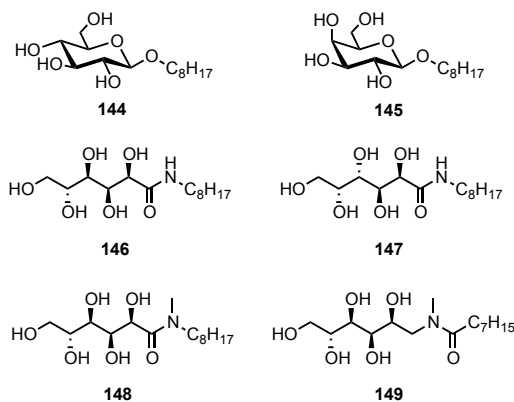


Figure 1-20 Structures of carbohydrate-based IRI active surfactants and hydrogelators.

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.¹⁴⁷ 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.¹⁴⁷ 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-aldoamides, **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

1. Polge, C. *Proc. R. Soc. Lond. B. Biol. Sci.* **1957**, *147*, 498.
2. Polge, C.; Smith, A. U.; Parkes, A. S. *Nature* **1949**, *164*, 666.
3. Chen, C. *The Lancet* **1986**, *327*, 884.
4. Lovelock, J. E. *Biochem. J.* **1954**, *56*, 265.
5. Lovelock, J. E. *Biochim. Biophys. Acta* **1953**, *10*, 414.
6. Broxmeyer, H. E.; Douglas, G. W.; Hangoc, G.; Cooper, S.; Bard, J.; English, D.; Arny, M.; Thomas, L.; Boyse, E. A. *Proc. Natl. Acad. Sci. U. S. A.* **1989**, *86*, 3828.
7. Iafolla, M. A.; Tay, J.; Allan, D. S. *Biol. Blood Marrow Transplant.* **2014**, *20*, 20.
8. Mason, C.; Dunnill, P. *Regen. Med.* **2008**, *3*, 1.
9. Saporta, S.; Kim, J.-J.; Willing, A. E.; Fu, E. S.; Davis, C. D.; Sanberg, P. R. *J. Hematopath. Stem Cell* **2003**, *12*, 271.
10. Reimann, V.; Creutzig, U.; Kögler, G. *Dtsch. Arztebl. Int.* **2009**, *106*, 831.
11. Kalra, L.; Ratan, R. R. *Stroke* **2008**, *39*, 273.
12. Moise, K. J. J. *Obstet. Gynecol.* **2005**, *106*, 1393.
13. Abrahamsen, J. F.; Wentzel-larsen, T.; Bruserud, Ø. *Cytotherapy* **2004**, *6*, 356.
14. Parato, K. A.; Senger, D.; Forsyth, P. A.; Bell, J. C. *Nat. Rev. Cancer* **2005**, *5*, 965.
15. Greiff, D.; Rightsel, W. A. *Cryobiology* **1967**, *3*, 432.
16. Merrill, D.; Wade, C.; Fahnestock, P.; Baker, R. *Long-term and short-term stability of viruses depend on storage temperature and preservation methods*, BEI Resources, 2012.

17. Brandau, D. T.; Jones, L. S.; Wiethoff, C. M.; Rexroad, J.; Middaugh, C. R. *J. Pharm. Sci.* **2003**, *92*, 218.
18. WHO; UNAIDS; Bank, W.; UNESCO; UNFPA Geneva: World Health Organization, 2000.
19. Kuhs, W.; Lehmann, M. *Water Sci. Rev.* **1986**, *2*, 1.
20. Malenkov, G. *J. Phys.: Condens. Matter* **2009**, *21*, 283101.
21. Murray, B. J.; Bertram, A. K. *Phys. Chem. Chem. Phys.* **2006**, *8*, 186.
22. Murray, B. J.; Knopf, D. A.; Bertram, A. K. *Nature*. **2005**, *434*, 202.
23. Libbrecht, K. G. *Rep. Prog. Phys.* **2005**, *68*, 855.
24. Stranski, I. N. *Z. Physik* **1942**, *119*, 22.
25. Tam, R. Y.; Ferreira, S. S.; Czechura, P.; Chaytor, J. L.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 17494.
26. Nenow, D. *Prog. Cryst. Growth Charact. Mater.* **1984**, *9*, 185.
27. Sadtschenko, V.; Ewing, G. E. *J. Chem. Phys.* **2002**, *116*, 4686.
28. Karow, A. M. *J. Pharm. Pharmacol.* **1969**, *21*, 209.
29. Mazur, P. *Ann. N. Y. Acad. Sci.* **1960**, *85*, 610.
30. Mazur, P. *J. Gen. Physiol.* **1963**, *47*, 347.
31. Lovelock, J. E. *Proc. R. Soc. Lond. B. Biol. Sci.* **1957**, *147*, 427.
32. Mazur, P.; Leibo, S. P.; Chu, E. H. Y. *Exp. Cell Res.* **1972**, *71*, 345.
33. Muldrew, K.; McGann, L. E. *Biophys. J.* **1994**, *66*, 532.
34. Trump, B. F.; Young, D. E.; E. A. Arnold; Stowell, R. E. *FASEB J.* **1965**, *24*, S144.
35. Rapatz, G. L.; Luyet, B. J. *Biodynamica* **1961**, *8*, 295.
36. Gao, D.; Critser, J. K. *ILAR J.* **2000**, *41*, 187.
37. Mazur, P. *Am. J. Physiol. Cell Physiol.* **1984**, *247*, C125.
38. Pegg, D. In *Cryopreservation and Freeze-Drying Protocols*; Day, J., Stacey, G., Eds.; Humana Press: 2007; Vol. 368, p 39.
39. Sakai, A.; Otsuka, K. *Plant Physiol.* **1967**, *42*, 1680.
40. Shimada, K.; Asahina, E. *Cryobiology* **1975**, *12*, 209.
41. Farrant, J.; Walter, C.; Lee, H.; McGann, L. *Cryobiology* **1977**, *14*, 273.
42. MacKenzie, A. In *Ciba Foundation Symposium-The Frozen Cell*; Wiley Online Library: 1970, p 89.
43. Bank, H. *Cryobiology* **1973**, *10*, 157.
44. Hagiwara, T.; Hartel, R.; Matsukawa, S. *Food Biophys.* **2006**, *1*, 74.
45. Pronk, P.; Infante Ferreira, C. A.; Witkamp, G. J. *J. Cryst. Growth* **2005**, *275*, e1355.
46. Budke, C.; Heggemann, C.; Koch, M.; Sewald, N.; Koop, T. *J. Phys. Chem. B* **2009**, *113*, 2865.
47. Fuller, B. J. *Cryo-Lett.* **2004**, *25*, 375.
48. Karlsson, J. O. M.; Toner, M. *Biomaterials* **1996**, *17*, 243.
49. Lovelock, J. E.; Bishop, M. W. H. *Nature* **1959**, *183*, 1394.
50. Woods, E. J.; Zieger, M. A. J.; Gao, D. Y.; Critser, J. K. *Cryobiology* **1999**, *38*, 403.
51. Anchordoguy, T. J.; Cecchini, C. A.; Crowe, J. H.; Crowe, L. M. *Cryobiology* **1991**, *28*, 467.
52. Körber, C.; Scheiwe, M. W.; Boutron, P.; Rau, G. *Cryobiology* **1982**, *19*, 478.

53. Franks, F.; Asquith, M. H.; Hammond, C. C.; Skaer, H. I. B.; Echlin, P. *J. Microsc.* **1977**, *110*, 223.
54. Fahy, G. M. *Cryobiology* **1986**, *23*, 1.
55. Mazur, P. In *Organ Preservation for Transplantation*; 2nd ed ed.; Marcel Dekker: New York, 1981, p 143.
56. Fahy, G. M.; Karow Jr, A. M. *Cryobiology* **1977**, *14*, 418.
57. Fahy, G. M. *Cryobiology* **1980**, *17*, 371.
58. Kendall, E. J.; McKersie, B. D. *Physiol. Plant.* **1989**, *76*, 86.
59. Baxter, S. J.; Lathe, G. H. *Biochem. Pharmacol.* **1971**, *20*, 1079.
60. Fahy, G. M.; Lilley, T. H.; Linsdell, H.; Douglas, M. S. J.; Meryman, H. T. *Cryobiology* **1990**, *27*, 247.
61. Arakawa, T.; Kita, Y.; Timasheff, S. N. *Biophys. Chem.* **2007**, *131*, 62.
62. Craig, J. I. O.; Turner, M. L.; Parker, A. C. *Blood Rev.* **1992**, *6*, 59.
63. Zambelli, A.; Poggi, G.; Da Prada, G.; Pedrazzoli, P.; Cuomo, A.; Miotti, D.; Perotti, C.; Preti, P.; Robustelli Della Cuna, G. *Anticancer Res.* **1998**, *18*, 4705.
64. Perseghin, P.; Balduzzi, A.; Bonanomi, S.; Dassi, M.; Buscemi, F.; Longoni, D.; Rovelli, A.; Uderzo, C. *Bone Marrow Transplant.* **2000**, *26*.
65. Davis, J. M.; Rowley, S. D.; Braine, H. G.; Piantadosi, S.; Santos, G. W. *Blood* **1990**, *75*, 781.
66. Stroncek, D. F.; Fautsch, S. K.; Lasky, L. C.; Hurd, D. D.; Ramsay, N. K.; McCullough, J. *Transfusion* **1991**, *31*, 521.
67. Abdelkefi, A.; Lakhal, A.; Moojat, N.; Hamed, L. B.; Fekih, J.; Ladeb, S.; Torjman, L.; Othman, T. B. *Bone Marrow Transplant.* **2009**, *44*, 323.
68. Cavaletti, G.; Oggioni, N.; Sala, F.; Pezzoni, G.; Cavalletti, E.; Marmioli, P.; Petruccioli, M. G.; Frattola, L.; Tredici, G. *Toxicol. Lett.* **2000**, *118*, 103.
69. Otrock, Z. K.; Beydoun, A.; Barada, W. M.; Masroujeh, R.; Hourani, R.; Bazarbachi, A. *Haematologica* **2008**, *93*, e36.
70. Shu, Z.; Heimfeld, S.; Gao, D. *Bone Marrow Transplant.* **2013**.
71. Sams, W. M.; Carroll, N. V.; Crantz, P. L. *Exp. Biol. Med.* **1966**, *122*, 103.
72. Sams, W. M. *Ann. N. Y. Acad. Sci.* **1967**, *141*, 242.
73. Zenhäusern, R.; Tobler, A.; Leoncini, L.; Hess, O. M.; Ferrari, P. *Ann. Hematol.* **2000**, *79*, 523.
74. Scholander, P. F.; van Dam, L.; Kanwisher, J. W.; Hammel, H. T.; Gordon, M. S. *J. Cell. Comp. Physiol.* **1957**, *49*, 5.
75. Gordon, M. S.; Amdur, B. H.; Scholander, P. F. *Biol. Bull.* **1962**, *122*, 52.
76. Scholander, P. F.; Maggert, J. E. *Cryobiology* **1971**, *8*, 371.
77. Lin, Y.; Duman, J. G.; DeVries, A. L. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 87.
78. DeVries, A. L.; Wohlschlag, D. E. *Science* **1969**, *163*, 1073.
79. DeVries, A. L.; Komatsu, S. K.; Feeney, R. E. *J. Biol. Chem.* **1970**, *245*, 2901.
80. Harding, M. M.; Anderberg, P. I.; Haymet, A. D. J. *Eur. J. Biochem.* **2003**, *270*, 1381.
81. Tyshenko, M. G.; Doucet, D.; Davies, P. L.; Walker, V. K. *Nat. Biotech.* **1997**, *15*, 887.
82. Hew, C. L.; Kao, M. H.; So, Y.-P.; Lim, K.-P. *Can. J. Zool.* **1983**, *61*, 2324.
83. Graham, L. A.; Liou, Y.-C.; Walker, V. K.; Davies, P. L. *Nature* **1997**, *388*, 727.

84. Schneppenheim, R.; Theede, H. *Comp. Biochem. Physiol. B.* **1980**, 67, 561.
85. Duman, J. G.; Li, N.; Verleye, D.; Goetz, F. W.; Wu, D. W.; Andorfer, C. A.; Benjamin, T.; Parmelee, D. C. *J. Comp. Physiol. B.* **1998**, 168, 225.
86. Graham, L. A.; Davies, P. L. *Science* **2005**, 310, 461.
87. Smallwood, M.; Worrall, D.; Byass, L.; Elias, L.; Ashford, D.; Doucet, C. J.; Holt, C.; Telford, J.; Lillford, P.; Bowles, D. J. *Biochem. J.* **1999**, 340, 385.
88. Huang, T.; Nicodemus, J.; Zarka, D. G.; Thomashow, M. F.; Wisniewski, M.; Duman, J. G. *Plant Mol. Biol.* **2002**, 50, 333.
89. Sidebottom, C.; Buckley, S.; Pudney, P.; Twigg, S.; Jarman, C.; Holt, C.; Telford, J.; McArthur, A.; Worrall, D.; Hubbard, R.; Lillford, P. *Nature* **2000**, 406, 256.
90. Pudney, P. D. A.; Buckley, S. L.; Sidebottom, C. M.; Twigg, S. N.; Sevilla, M. P.; Holt, C. B.; Roper, D.; Telford, J. H.; McArthur, A. J.; Lillford, P. J. *Arch. Biochem. Biophys.* **2003**, 410, 238.
91. Kumble, K. D.; Demmer, J.; Fish, S.; Hall, C.; Corrales, S.; DeAth, A.; Elton, C.; Prestidge, R.; Luxmanan, S.; Marshall, C. J.; Wharton, D. A. *Cryobiology.* **2008**, 57, 263.
92. John, U. P.; Polotnianka, R. M.; Sivakumaran, K. A.; Chew, O.; Mackin, L.; Kuiper, M. J.; Talbot, J. P.; Nugent, G. D.; Mautord, J.; Schrauf, G. E.; Spangenberg, G. C. *Plant, Cell Environ.* **2009**, 32, 336.
93. Griffith, M.; Ewart, K. V. *Biotech. Adv* **1995**, 13, 375.
94. Tremblay, K.; Ouellet, F. B.; Fournier, J.; Danyluk, J.; Sarhan, F. *Plant Cell Physiol.* **2005**, 46, 884.
95. Duman, J. G.; Olsen, T. M. *Cryobiology* **1993**, 30, 322.
96. Hoshino, T.; Kiriaki, M.; Nakajima, T. *Cryoletters* **2003**, 24, 135.
97. Gilbert, J. A.; Davies, P. L.; Laybourn-Parry, J. *FEMS Microbiol. Lett.* **2005**, 245, 67.
98. Sun, X.; Griffith, M.; Pasternak, J. J.; Glick, B. R. *Can. J. Microbiol.* **1995**, 41, 776.
99. Yamashita, Y.; Nakamura, N.; Omiya, K.; Nishikawa, J.; Kawahara, H.; Obata, H. *Biosci., Biotechnol., Biochem.* **2002**, 66, 239.
100. Kawahara, H.; Nakano, Y.; Omiya, K.; Muryoi, N.; Nishikawa, J.; Obata, H. *J. Biosci. Bioeng.* **2004**, 98, 220.
101. Ewart, K. V.; Lin, Q.; Hew, C. L. *CMLS, Cell. Mol. Life Sci.* **1999**, 55, 271.
102. Jia, Z.; Davies, P. L. *Trends Biochem. Sci.* **2002**, 27, 101.
103. Fletcher, G. L.; Hew, C. L.; Davies, P. L. *Annu. Rev. Physiol.* **2001**, 63, 359.
104. Davies, P. L.; Sykes, B. D. *Curr. Opin. Struc. Biol.* **1997**, 7, 828.
105. Hew, C. L.; Yang, D. S. C. *Eur. J. Biochem.* **1992**, 203, 33.
106. Duman, J. G.; DeVries, A. L. *Comp. Biochem. Physiol. B.* **1976**, 54, 375.
107. Duman, J. G.; DeVries, A. L. *Nature* **1974**, 247, 237.
108. Ananthanarayanan, V. S.; Hew, C. L. *Biochem. Biophys. Res. Commun.* **1977**, 74, 685.
109. Yang, D. S. C.; Sax, M.; Chakrabartty, A.; Hew, C. L. *Nature* **1988**, 333, 232.
110. Raymond, J. A.; Radding, W.; DeVries, A. L. *Biopolymers.* **1977**, 16, 2575.
111. Cheng, C. C. M.; DeVries, A. L. *Mol. Aspects Fish Mar. Biol.* **2002**, 1, 83.
112. Baardsnes, J.; Davies, P. L. *Biochim. Biophys. Acta* **2002**, 1601, 49.

113. Sönnichsen, F. D.; DeLuca, C. I.; Davies, P. L.; Sykes, B. D. *Structure* **1996**, *4*, 1325.
114. Madura, J. D.; Taylor, M. S.; Wierzbicki, A.; Harrington, J. P.; Sikes, C. S.; Sönnichsen, F. *J. Mol. Struct.* **1996**, 388, 65.
115. Miura, K.; Ohgiya, S.; Hoshino, T.; Nemoto, N.; Odaira, M.; Nitta, R.; Tsuda, S. *J. Biochem.* **1999**, *126*, 387.
116. DeLuca, C. I.; Davies, P. L.; Ye, Q.; Jia, Z. *J. Mol. Biol.* **1998**, *275*, 515.
117. Jia, Z.; Deluca, C. I.; Davies, P. L. *Protein Sci.* **1995**, *4*, 1236.
118. Hew, C. L.; Slaughter, D.; Joshi, S. B.; Fletcher, G. L.; Ananthanarayanan, V. S. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **1984**, *155*, 81.
119. Schrag, J. D.; Cheng, C.-H. C.; Panico, M.; Morris, H. R.; Deries, A. L. *Biochim. Biophys. Acta - Prot. Struct. Mol. Enzymol.* **1987**, *915*, 357.
120. Sönnichsen, F.; Sykes, B.; Chao, H.; Davies, P. *Science* **1993**, *259*, 1154.
121. Xue, Y. Q.; Sicheri, F.; Ala, P.; Hew, C. L.; Yang, D. S. C. *J. Mol. Biol.* **1994**, *237*, 351.
122. Deng, G.; Andrews, D. W.; Laursen, R. A. *FEBS Lett.* **1997**, *402*, 17.
123. Deng, G.; Laursen, R. A. *Biochim. Biophys. Acta* **1998**, *1388*, 305.
124. Cheng, C.-H. C. *Curr. Opin. Genet. Dev.* **1998**, *8*, 715.
125. DeVries, A. L. *Comp. Biochem. Physiol. B.* **1988**, *90*, 611.
126. Urrutia, M. E.; Duman, J. G.; Knight, C. A. *Biochim. Biophys. Acta* **1992**, *1121*, 199.
127. Raymond, J. A.; DeVries, A. L. *Proc. Natl. Acad. Sci. U. S. A.* **1977**, *74*, 2589.
128. Wilson, P. W. *Cryoletters* **1993**, *14*, 31.
129. Fairley, K.; Westman, B. J.; Pham, L. H.; Haymet, A.; Harding, M. M.; Mackay, J. P. *J. Biol. Chem.* **2002**, *277*, 24073.
130. Haymet, A.; Ward, L. G.; Harding, M. M.; Knight, C. A. *FEBS Lett.* **1998**, *430*, 301.
131. Chao, H.; Houston, M. E.; Hodges, R. S.; Kay, C. M.; Sykes, B. D.; Loewen, M. C.; Davies, P. L.; Sönnichsen, F. D. *Biochemistry (Mosc).* **1997**, *36*, 14652.
132. Wang, T.; Zhu, Q.; Yang, X.; Layne Jr, J. R.; DeVries, A. L. *Cryobiology* **1994**, *31*, 185.
133. Wang, J.-H. *Cryobiology*. **2000**, *41*, 1.
134. Pham, L.; Dahiya, R.; Rubinsky, B. *Cryobiology* **1999**, *38*, 169.
135. Knight, C. A.; Duman, J. G. *Cryobiology* **1986**, *23*, 256.
136. Liu, S.; Ben, R. N. *Org. Lett.* **2005**, *7*, 2385.
137. Eniade, A.; Purushotham, M.; Ben, R. N.; Wang, J.; Horwath, K. *Cell Biochem. Biophys.* **2003**, *38*, 115.
138. Baust, J.; Van Buskirk, R. *In Vitro Cell. Dev. Biol. Animal* **2000**, *36*, 262.
139. Baust, J. M. *Cell Preservation Technol.* **2002**, *1*, 17.
140. Czechura, P.; Tam, R. Y.; Dimitrijevic, E.; Murphy, A. V.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 2928.
141. Leclère, M.; Kwok, B. K.; Wu, L. K.; Allan, D. S.; Ben, R. N. *Bioconj. Chem.* **2011**, *22*, 1804.
142. Knight, C. A.; Hallett, J.; DeVries, A. L. *Cryobiology* **1988**, *25*, 55.

143. Jackman, J.; Noestheden, M.; Moffat, D.; Pezacki, J. P.; Findlay, S.; Ben, R. N. *Biochem. Biophys. Res. Commun.* **2007**, *354*, 340.
144. Galema, S. A.; Engberts, J. B. F. N.; Hoeiland, H.; Foerland, G. M. *J. Phys. Chem.* **1993**, *97*, 6885.
145. Galema, S. A.; Hoeiland, H. *J. Phys. Chem.* **1991**, *95*, 5321.
146. Galema, S. A.; Howard, E.; Engberts, J. B. F. N.; Grigera, J. R. *Carbohydr. Res.* **1994**, *265*, 215.
147. Tam, R. Y.; Ferreira, S. S.; Czechura, P.; Chaytor, J. L.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 17494.
148. Karim, O. A.; Haymet, A. *Chem. Phys. Lett.* **1987**, *138*, 531.
149. Hayward, J. A.; Haymet, A. *J. Chem. Phys.* **2001**, *114*, 3713.
150. Fletcher, N. *Phil. Mag.* **1962**, *7*, 255.
151. Fletcher, N. H. *Philos. Mag.* **1968**, *18*, 1287.
152. Karim, O. A.; Haymet, A. *J. Chem. Phys.* **1988**, *89*, 6889.
153. Furukawa, Y.; Yamamoto, M.; Kuroda, T. *J. Cryst. Growth* **1987**, *82*, 665.
154. Furukawa, Y.; Ishikawa, I. *J. Cryst. Growth* **1993**, *128*, 1137.
155. Chaytor, J. L.; Ben, R. N. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 5251.
156. Balcerzak, A. K.; Ferreira, S. S.; Trant, J. F.; Ben, R. N. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 1719.
157. Trant, J. F.; Biggs, R. A.; Capicciotti, C. J.; Ben, R. N. *RSC Adv.* **2013**, *3*, 26005.
158. Capicciotti, C. J.; Leclère, M.; Perras, F. A.; Bryce, D. L.; Paulin, H.; Harden, J.; Liu, Y.; Ben, R. N. *Chem. Sci.* **2012**, *3*, 1408.

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.

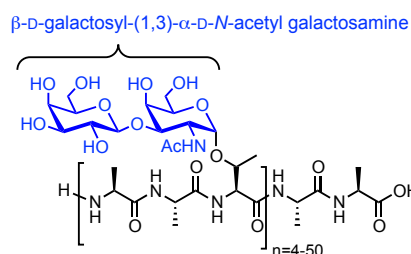


Figure 2-1 Structure of native AFGP showing the disaccharide moiety.

Structure-function studies have demonstrated that the β -D-galactosyl-(1,3)- α -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 β -(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 β -(1,3)³ and β -(1,4)³ glycosidic linkages were previously synthesized by the Ben group (**Figure 2-2a**) and the β -(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 β -(1,3)- and β -(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 β -(1,6) glycosidic linkage would have on IRI activity.

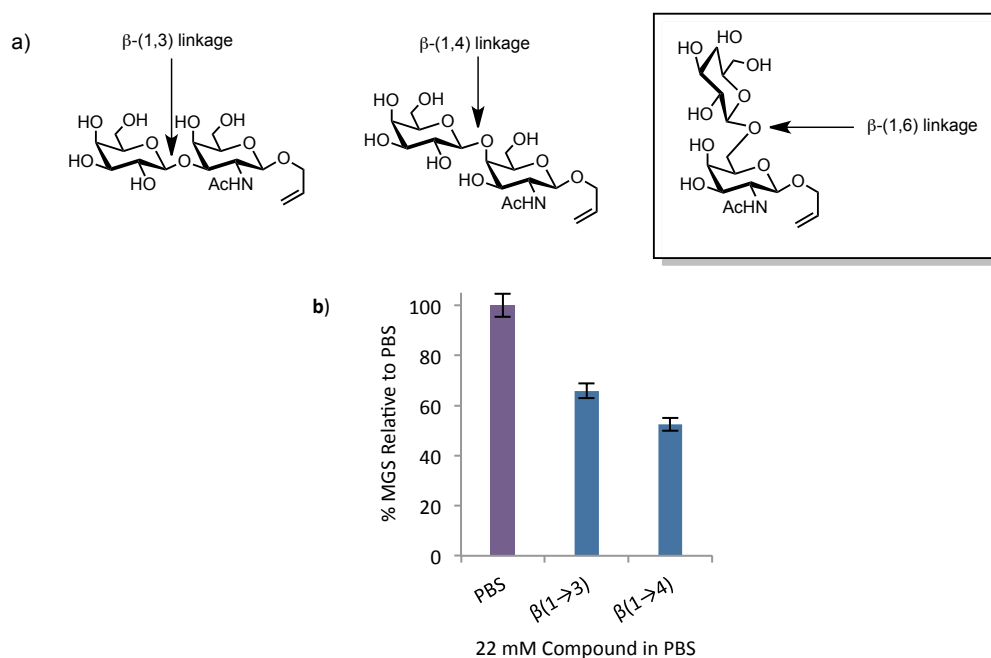


Figure 2-2 a) Structures of target disaccharides and b) IRI activity of AFGP analogues previously synthesized by the Ben group.

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**)

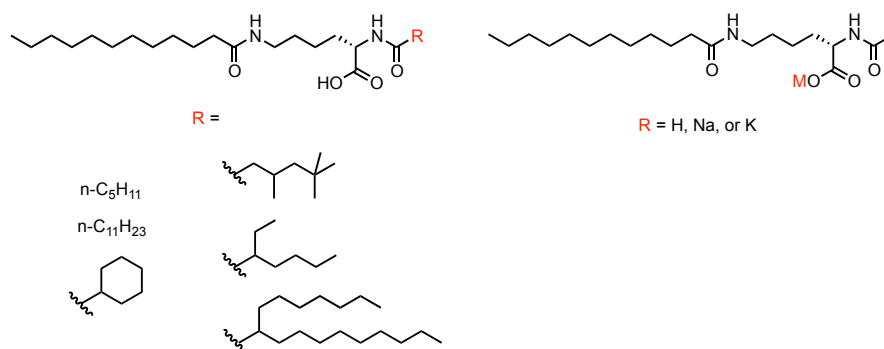


Figure 2-3 Structures of lysine-based gelators.

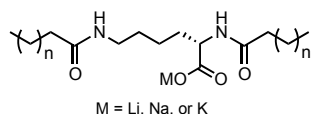


Figure 2-4 Structure of anionic di-alkyl lysine derivatives.

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,

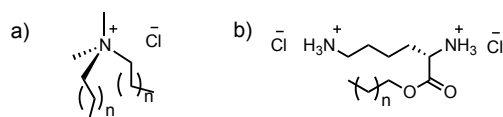


Figure 2-5 Structures of anti-ice nucleating agent surfactants/gelators: a) quaternary ammonium salts and b) cationic lysine alkyl esters.

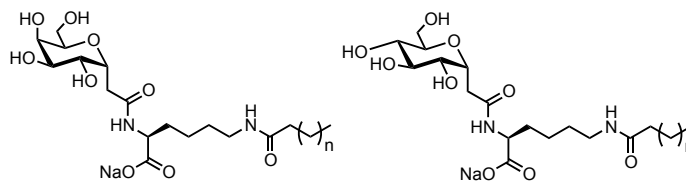


Figure 2-6 Structures of galactose and glucose glycoconjugates.

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**).¹² The gemini surfactants are good model compounds for the study of structural features important for IRI activity since they contain various ionic

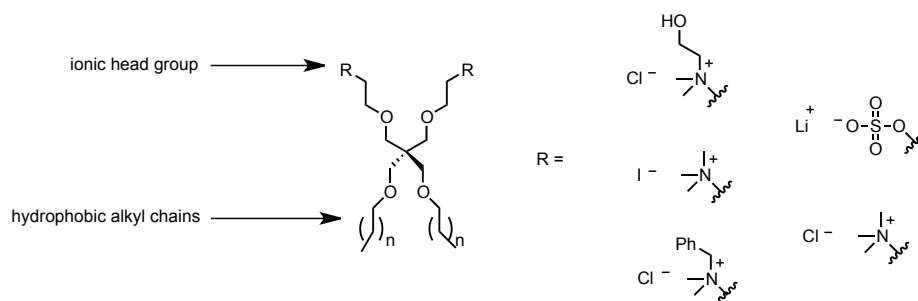


Figure 2-7 Structures of Gemini surfactants hydrocarbon chains and various ionic groups.

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,¹³ changing counterions should also modify IRI activity as anions and cations have different hydration shells.¹⁴ 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.

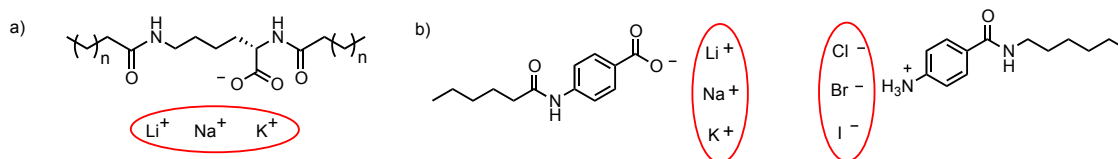


Figure 2-8 Counterions of a) di-alkyl lysine surfactants and b) *para*-amino benzoic acid derivatives.

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,¹⁵⁻¹⁸ Tris-buffered saline,¹⁹ various concentrations of NaCl,²⁰⁻²² and CaCl₂.²³ Therefore, the fourth objective was to investigate what effect the PBS salts have on IRI activity by testing the four PBS salts, NaCl, KCl, Na₂HPO₄, and KH₂PO₄ 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.²⁴ Also, vaccine distribution around the world is still not optimal and better

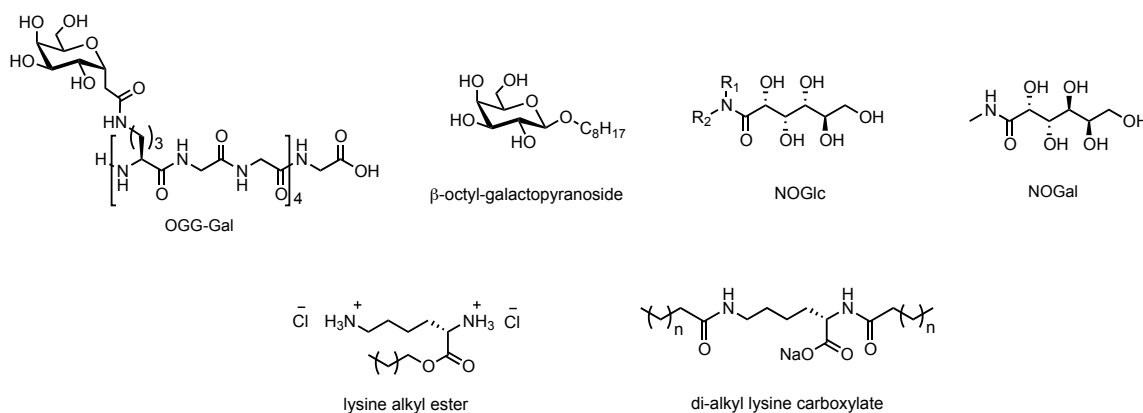


Figure 2-9 Structures of ice recrystallization inhibitors that will be tested for their protective effects with viruses.

stabilizers must be developed to prevent vaccine inactivation through 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 stomatitis 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

1. Tachibana, Y.; Matsubara, N.; Nakajima, F.; Tsuda, T.; Tsuda, S.; Monde, K.; Nishimura, S.-I. *Tetrahedron* **2002**, *58*, 10213.
2. Tachibana, Y.; Fletcher, G. L.; Fujitani, N.; Tsuda, S.; Monde, K.; Nishimura, S.-I. *Angew. Chem. Int. Ed.* **2004**, *43*, 856.
3. Ferreira, S. S., University of Ottawa, 2010.
4. Suzuki, M.; Yumoto, M.; Kimura, M.; Shirai, H.; Hanabusa, K. *Chem. Eur. J.* **2003**, *9*, 348.
5. Suzuki, M.; Owa, S.; Shirai, H.; Hanabusa, K. *Tetrahedron* **2007**, *63*, 7302.
6. Yabuuchi, K.; Tochigi, Y.; Mizoshita, N.; Hanabusa, K.; Kato, T. *Tetrahedron* **2007**, *63*, 7358.

7. Suzuki, M.; Yumoto, M.; Shirai, H.; Hanabusa, K. *Chem. Eur. J.* **2008**, *14*, 2133.
8. Suzuki, M.; Yumoto, M.; Shirai, H.; Hanabusa, K. *Tetrahedron* **2008**, *64*, 10395.
9. Suzuki, M.; Hanabusa, K. *Chem. Soc. Rev.* **2009**, *38*, 967.
10. Suzuki, M.; Abe, T.; Hanabusa, K. *J. Colloid Interface Sci.* **2010**, *341*, 69.
11. Watanabe, M.; Makino, T.; Okada, K.; Hara, M.; Watabe, S.; Arai, S. *Agric. Biol. Chem.* **1988**, *52*, 201.
12. Menger, F. M.; Littau, C. A. *J. Am. Chem. Soc.* **1993**, *115*, 10083.
13. Tam, R. Y.; Ferreira, S. S.; Czechura, P.; Chaytor, J. L.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 17494.
14. Smith, D. W. *J. Chem. Educ.* **1977**, *54*, 540.
15. Smallwood, M.; Worrall, D.; Byass, L.; Elias, L.; Ashford, D.; Doucet, C. J.; Holt, C.; Telford, J.; Lillford, P.; Bowles, D. J. *Biochem. J.* **1999**, *340*, 385.
16. Sidebottom, C.; Buckley, S.; Pudney, P.; Twigg, S.; Jarman, C.; Holt, C.; Telford, J.; McArthur, A.; Worrall, D.; Hubbard, R.; Lillford, P. *Nature*. **2000**, *406*, 256.
17. Sidebottom, C.; Buckley, S.; Pudney, P.; Twigg, S.; Jarman, C.; Holt, C.; Telford, J.; McArthur, A.; Worrall, D.; Hubbard, R.; Lillford, P. *Nature* **2000**, *406*, 256.
18. Tremblay, K.; Ouellet, F. B.; Fournier, J.; Danyluk, J.; Sarhan, F. *Plant Cell Physiol.* **2005**, *46*, 884.
19. Tomczak, M. M.; Marshall, C. B.; Gilbert, J. A.; Davies, P. L. *Biochem. Biophys. Res. Commun.* **2003**, *311*, 1041.
20. Lu, S.-S.; Inada, T.; Yabe, A.; Zhang, X.; Grandum, S. In *Proc. Symposium on Energy Engineering in the 21st Century*; DTIC Document: 2000, p 860.
21. Knight, C. A.; Wen, D.; Laursen, R. A. *Cryobiology* **1995**, *32*, 23.
22. Raymond, J. A.; Knight, C. A. *Cryobiology* **2003**, *46*, 174.
23. Inada, T.; Lu, S.-S. *Cryst. Growth Des.* **2003**, *3*, 747.
24. Parato, K. A.; Senger, D.; Forsyth, P. A.; Bell, J. C. *Nat. Rev. Cancer* **2005**, *5*, 965.

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 -

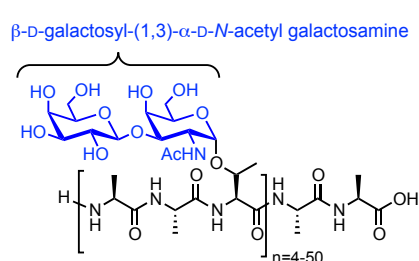


Figure 3-1 Structure of native AFGP highlighting the disaccharide moiety.

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.^{1,2} 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.³ As previous work by the Ben group showed that simple mono- and disaccharides exhibit weak to moderate IRI activity,⁴ 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**,⁵ **302**,⁶ and **303**⁵ 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.⁴ In this study, the β -C-allyl galactose derivate had less than a 10% decrease in IRI activity relative to the native *O*-linked galactose. However, β -C-allyl glucose was statistically indistinguishable to the *O*-linked glucose. Following these conclusions, it is expected that the β -*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.² Finally, **303** contains the β -(1,3)-linkage. The IRI data (**Figure 3-2**) shows that these disaccharides have moderate IRI activity. Out of these three, **303**, containing the β -(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 β -(1,6)-linked disaccharide was still unknown.

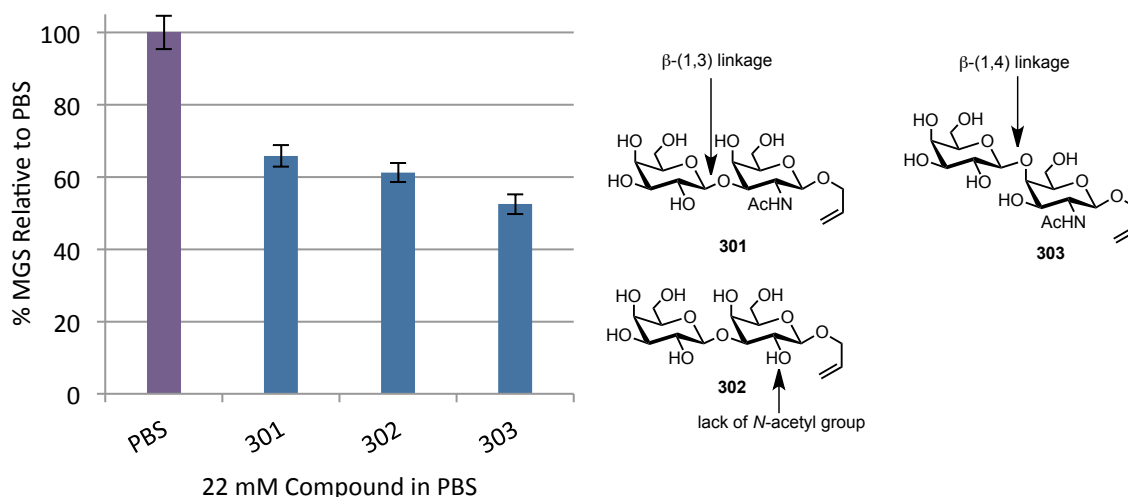


Figure 3-2 Structures and IRI activity of AFGP analogues **301-303** previously synthesized by the Ben group.

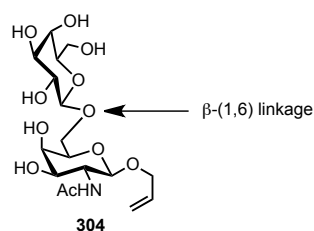
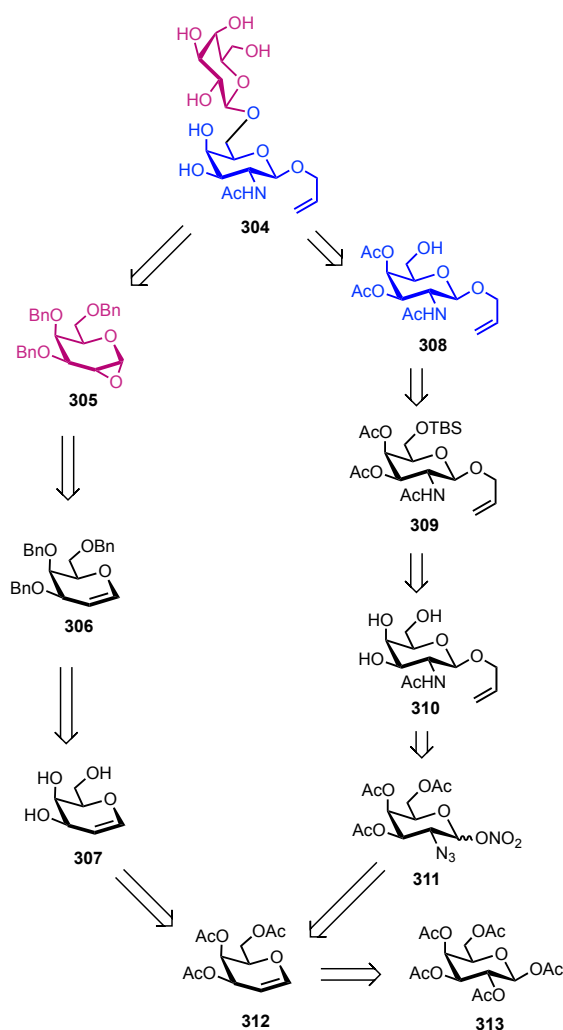


Figure 3-3 Target disaccharide **304** containing a β -(1,6)-linker.

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 galactose-amine **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

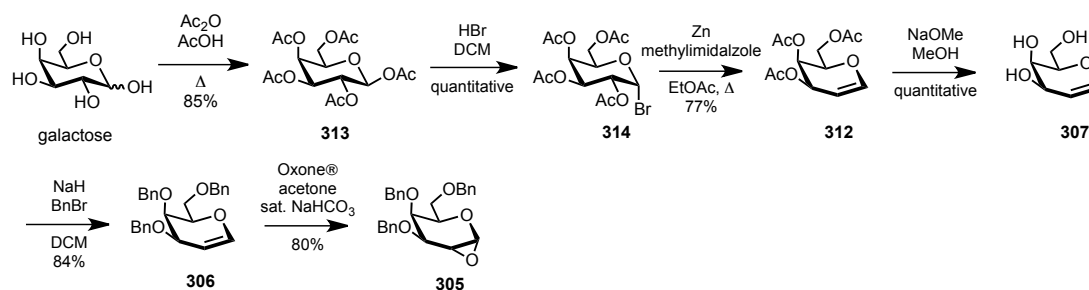


Scheme 3-1 Original divergent retrosynthetic scheme of synthesis of β -D-galactosyl-(1,6)- α -D-N-acetyl galactose-amine starting from β -D galactose pentaacetate.

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 through 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.

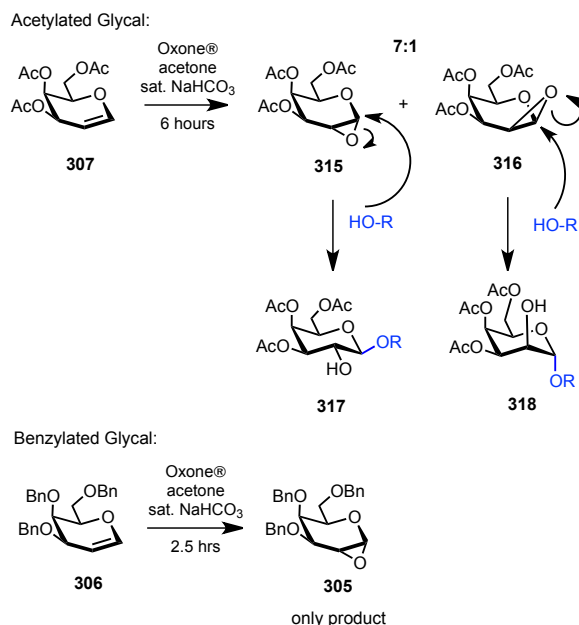
3.1.1. Synthesis of glycosyl donor **305**

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 a overall yield of 77%.⁷ Deprotection of the acetate groups, protection with benzyl groups, and oxidation



Scheme 3-2 Synthesis of glycosyl donor **305**.

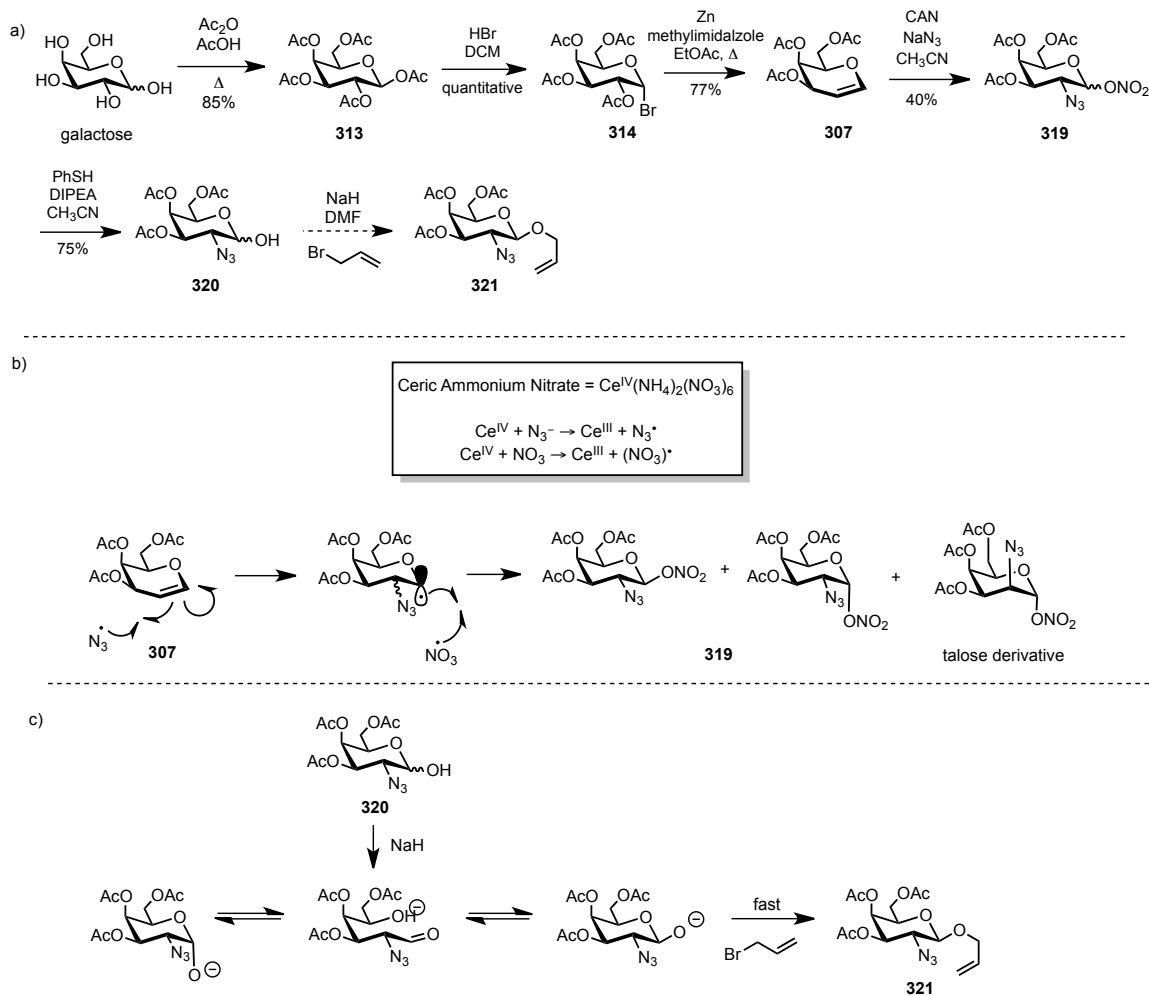
with in-situ formed dimethyldioxirane (DMDO) from Oxone® yielded glycosyl donor **305** in 80% yield. It was important to exchange the protecting groups from acetates to benzyls for the epoxidation step. Acetate groups reduce the nucleophilicity of the glycal towards DMDO. The epoxidation with an acetylated 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**)

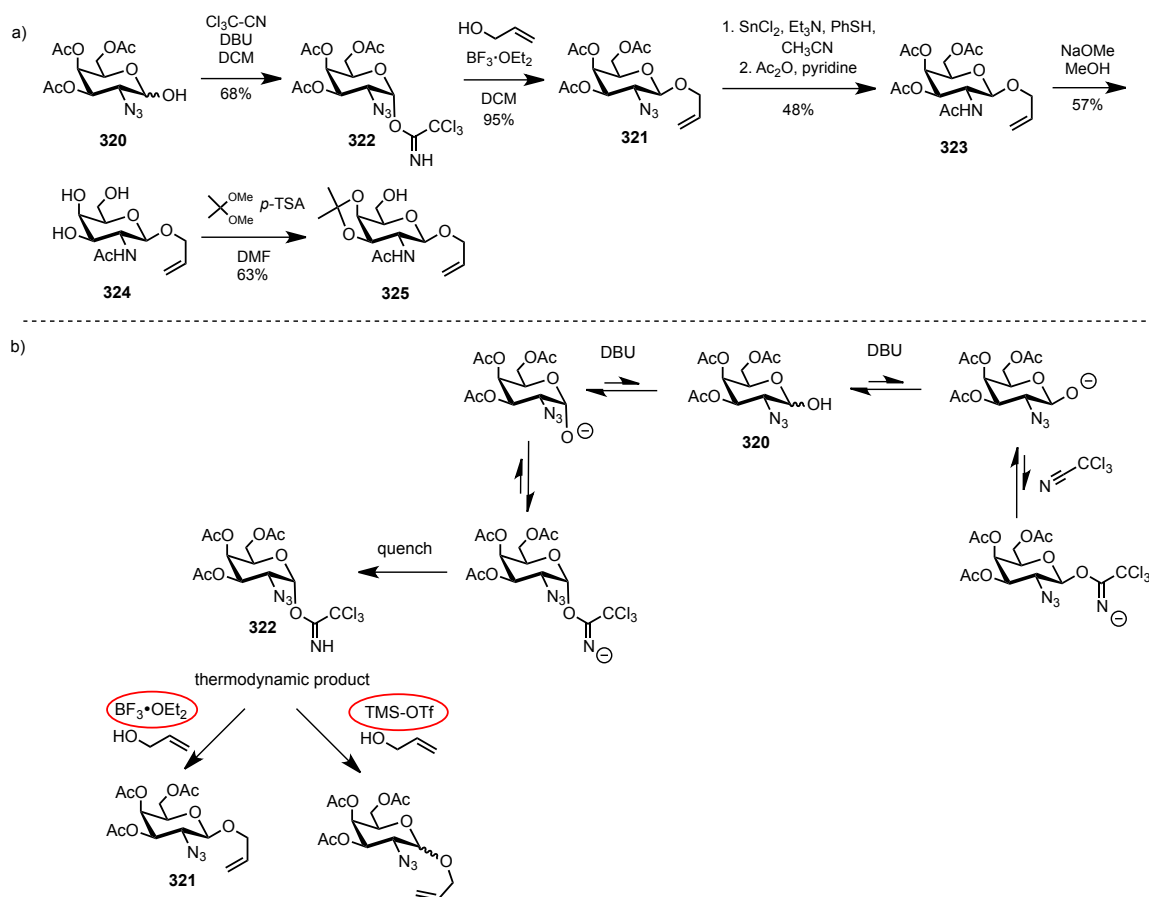
The synthesis of the glycosyl donor began with the preparation of glycal **307** in three steps from galactose. Azidonitration⁹ of **307** with sodium azide and ceric ammonium nitrate (CAN) furnished **319** in 40% yield (**Scheme 3-4a**). The proposed mechanism of the azidonitration is depicted in **Scheme 3-4b**. Ceric ammonium nitrate has the formula $\text{Ce}^{\text{IV}}(\text{NH}_4)_2(\text{NO}_3)_6$ 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



Scheme 3-4 Synthetic scheme of glycosyl acceptor **321** showing the a) general synthetic scheme, b) the mechanism for the formation of **319** by azidonitration, and c) mechanism of direct *O*-alkylation.

resulting azide radical will add to C-2 of galactose resulting in a radical at the more stabilized anomeric position.⁹ One possible mechanism for the nitration at the anomeric position proceeds by the radical addition of cerium-oxidized $\text{NO}_3^\bullet$. 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%)⁹ 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*-alkylation¹⁰ 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.¹⁰ 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.^{10,11} 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_2CO_3 as base, the kinetic β -trichloroacetimidate would be formed.^{10,11}



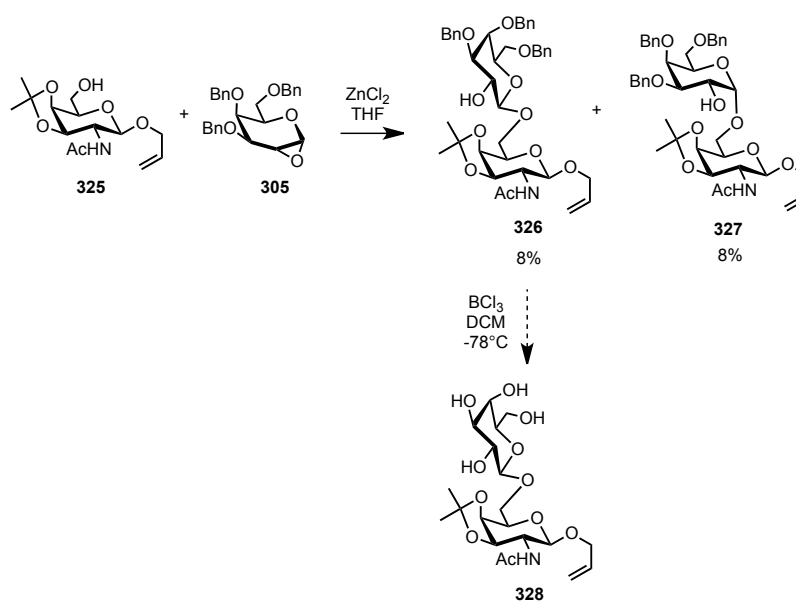
Scheme 3-5 a) Synthetic scheme for synthesis of glycosyl acceptor **325** and b) mechanism for the formation of trichloroacetimidate **322** and allylated **321**.

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 $\text{BF}_3 \cdot \text{OEt}_2$ results in the selective formation of the β -glycoside via $\text{S}_{\text{N}}2$ inversion of configuration. Displacement of the α -trichloroacetimidate moiety of **322** by allyl alcohol in the presence of $\text{BF}_3 \cdot \text{OEt}_2$ furnished β -O-allyl galactoside **321** in 95% yield. When $\text{BF}_3 \cdot \text{OEt}_2$ 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 $\rightarrow$ 6)-2-acetamido-2-deoxy- β -D-galactopyranoside (**304**)

The initial glycosylation was attempted with glycosyl acceptor **325** and glycosyl donor **305** with ZnCl_2 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

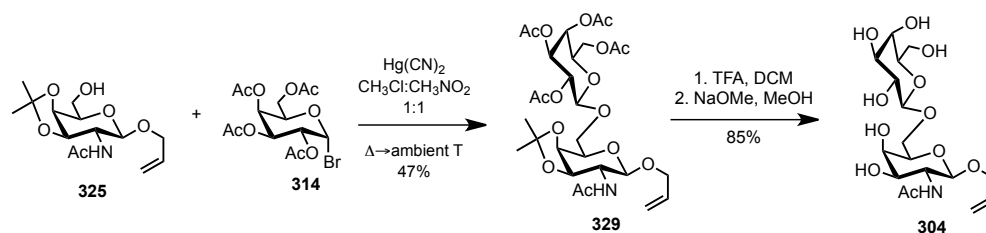


Scheme 3-6 Attempted synthesis of disaccharide **328**.

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.¹² However, BCl₃ has been shown to de-benzylate carbohydrates in the presence of a C-allyl groups.¹³ 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 $\rightarrow$ 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.¹⁴ 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.^{15,16} These mercuric salts are weaker promoters than silver carbonate.



Scheme 4-7 Revised glycosylation with mercury cyanide as activator and deprotection to furnish final product **304**.

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 previously^{5,6}) 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

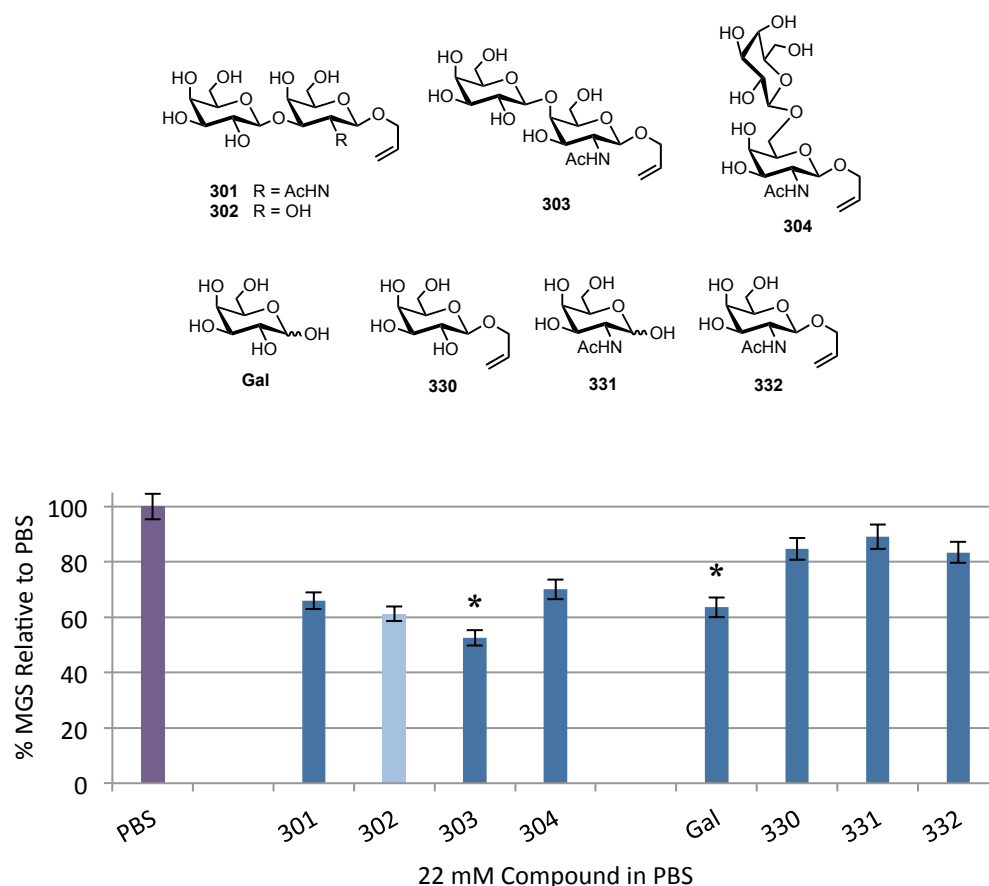


Figure 3-4 IRI activity of disaccharides **301-304** and monosaccharides Gal, and **330-332**. Error bars indicate standard error of the mean (SEM). Asterisks indicate significant difference defined by unpaired Student's *t*-test ($p < 0.05$) within each set.

in AFGPs possesses a β -(1,3) linkage, corresponding to **301** but the most active disaccharide **303**, possesses the β -(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).^{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

TH activity.² 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.⁵ 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.^{4,18} 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.² 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.² 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

1. Tachibana, Y.; Matsubara, N.; Nakajima, F.; Tsuda, T.; Tsuda, S.; Monde, K.; Nishimura, S.-I. *Tetrahedron* **2002**, *58*, 10213.
2. Tachibana, Y.; Fletcher, G. L.; Fujitani, N.; Tsuda, S.; Monde, K.; Nishimura, S.-I. *Angew. Chem. Int. Ed.* **2004**, *43*, 856.
3. Czechura, P.; Tam, R. Y.; Dimitrijevic, E.; Murphy, A. V.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 2928.
4. Tam, R. Y.; Ferreira, S. S.; Czechura, P.; Chaytor, J. L.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 17494.
5. Ferreira, S. S., University of Ottawa, 2010.
6. Trant, J. F., University of Ottawa, 2012.
7. Zhao, J.; Wei, S.; Ma, X.; Shao, H. *Green Chem.* **2009**, *11*, 1124.
8. Rani, S.; Vankar, Y. D. *Tetrahedron Lett.* **2003**, *44*, 907.
9. Lemieux, R.; Ratcliffe, R. *Can. J. Chem.* **1979**, *57*, 1244.
10. Schmidt, R. R. *Angew. Chem. Int. Ed.* **1986**, *25*, 212.
11. Schmidt, R. R.; Kinzy, W. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 21.
12. Greene, T. W.; Wuts, P. G.; Wiley, J. *Protective Groups in Organic Synthesis*; Wiley New York, 1999; Vol. 168.
13. Xie, J.; Ménand, M.; Valéry, J.-M. *Carbohydr. Res.* **2005**, *340*, 481.
14. Demchenko, A. V. *Handbook of Chemical Glycosylation* **2008**, 1.

15. Wallace, J. E.; Schroeder, L. R. *J. Chem. Soc., Perkin Trans. 2* **1976**, 1632.
16. Toshima, K.; Tatsuta, K. *Chem. Rev.* **1993**, 93, 1503.
17. Eniade, A.; Purushotham, M.; Ben, R. N.; Wang, J.; Horwath, K. *Cell Biochem. Biophys.* **2003**, 38, 115.
18. Chaytor, J. L.; Ben, R. N. *Bioorg. Med. Chem. Lett.* **2010**, 20, 5251.

Chapter 4. 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.¹ 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.^{2,3} Such compounds have a variety of applications including oil spill cleanup,^{4,5} tissue engineering,⁶ controlled-release drug formulations⁷ 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*).⁸ These bacteria have specialized membrane proteins that nucleate ice crystals.⁹ 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).¹⁰ However, these nucleation active bacteria nucleate ice at higher temperature than water would normally freeze at, causing frost damage in frost-sensitive plants.⁹ 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.^{11,12} 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.⁸ Two types of surfactants that were tested for their anti-ice nucleation activity were quaternary ammonium surfactants and cationic lysine surfactants.⁸ Since some of these compounds inhibited ice nucleation, they may also possess ice recrystallization inhibition properties.

Table 4-1 Cationic quaternary ammonium surfactants

Compound	R ₁	R ₂
401	Bn	C ₁₆ H ₃₃
402	CH ₃	C ₁₀ H ₂₁
403	CH ₃	C ₁₆ H ₃₃

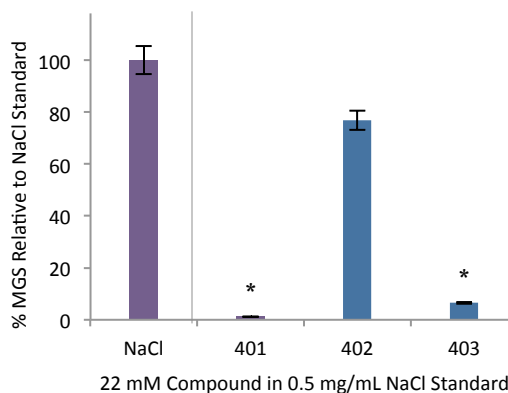


Figure 4-1 IRI activity of cationic quaternary ammonium chloride surfactants **401-403**. Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student's *t*-test ($p < 0.05$) within each set (separated by vertical lines).

Cationic quaternary ammonium salts, **401** - **403** are commercially available surfactants that are used as disinfectants, surfactants, emulsifiers, fabric softeners, and antistatic agents.¹³ 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.⁸ Therefore, ice would form below -5°C with the inhibitors and approximately at 0°C without inhibitors.⁸ 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.¹⁴ 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 osmometer¹⁵ 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.

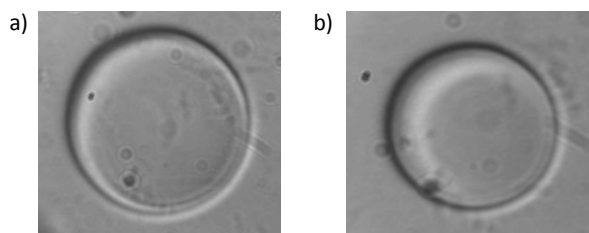


Figure 4-2 Round ice crystals of a) **401** and b) **402** showing no dynamic ice shaping.

Due to the high IRI activity of the quaternary ammonium chloride salts, compounds **401-403** were assessed for their ability to cryopreserve Tf-1 α cells (human bone marrow erythroblast) and Hep G2 (human liver carcinoma cells). Tf-1 α 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.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.¹⁶ 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-1 α 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 assay¹⁷⁻¹⁹ was performed.

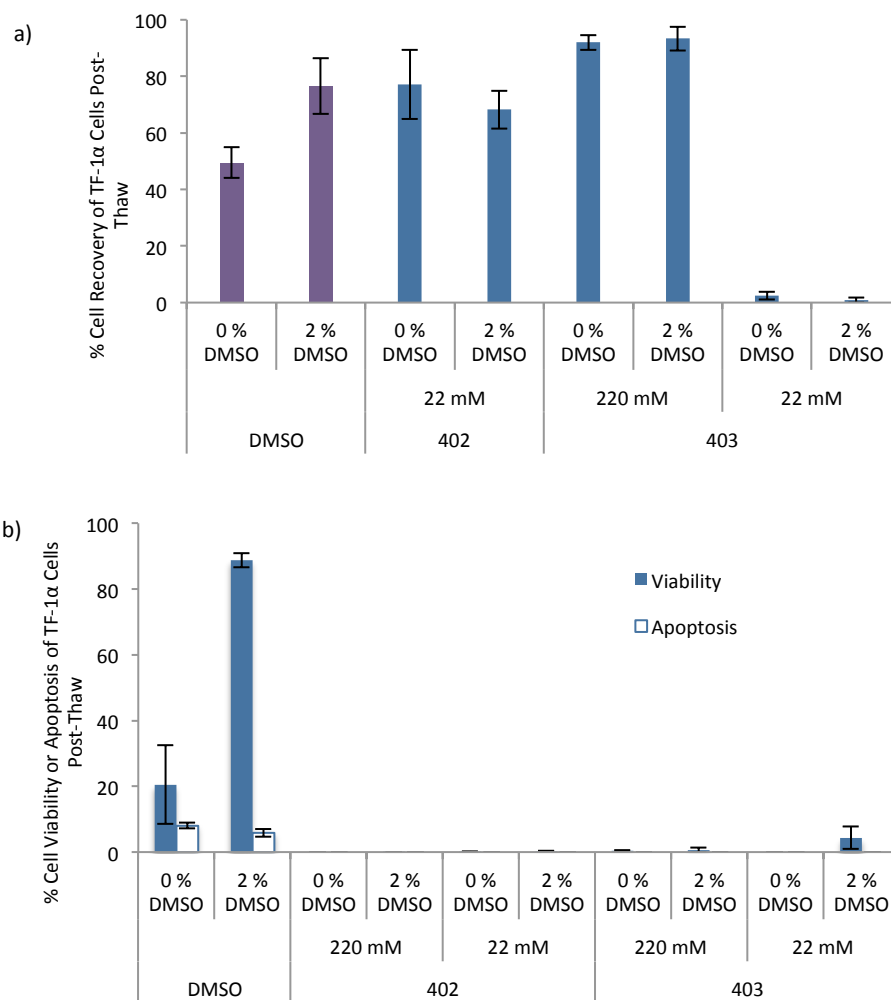


Figure 4-3 Cryopreservation of TF-1 α cells with quaternary ammonium chloride salts **402** and **403** showing **a)** percent recovery and **b)** percent viability and apoptosis. Error bars represent the standard error of the mean (SEM).

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.¹⁸ 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.

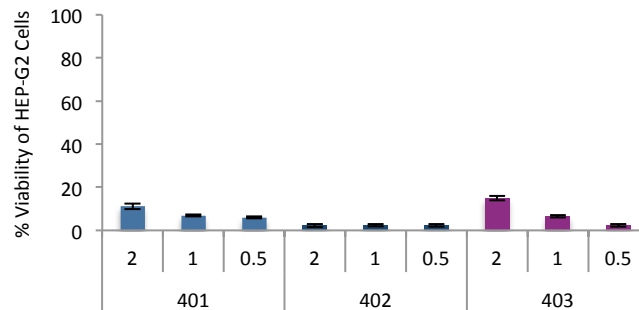


Figure 4-4 Cell viability of Hep-G2 cells incubated with quaternary ammonium chloride salts **401-403** in a MTT Assay. Concentrations are in mM.

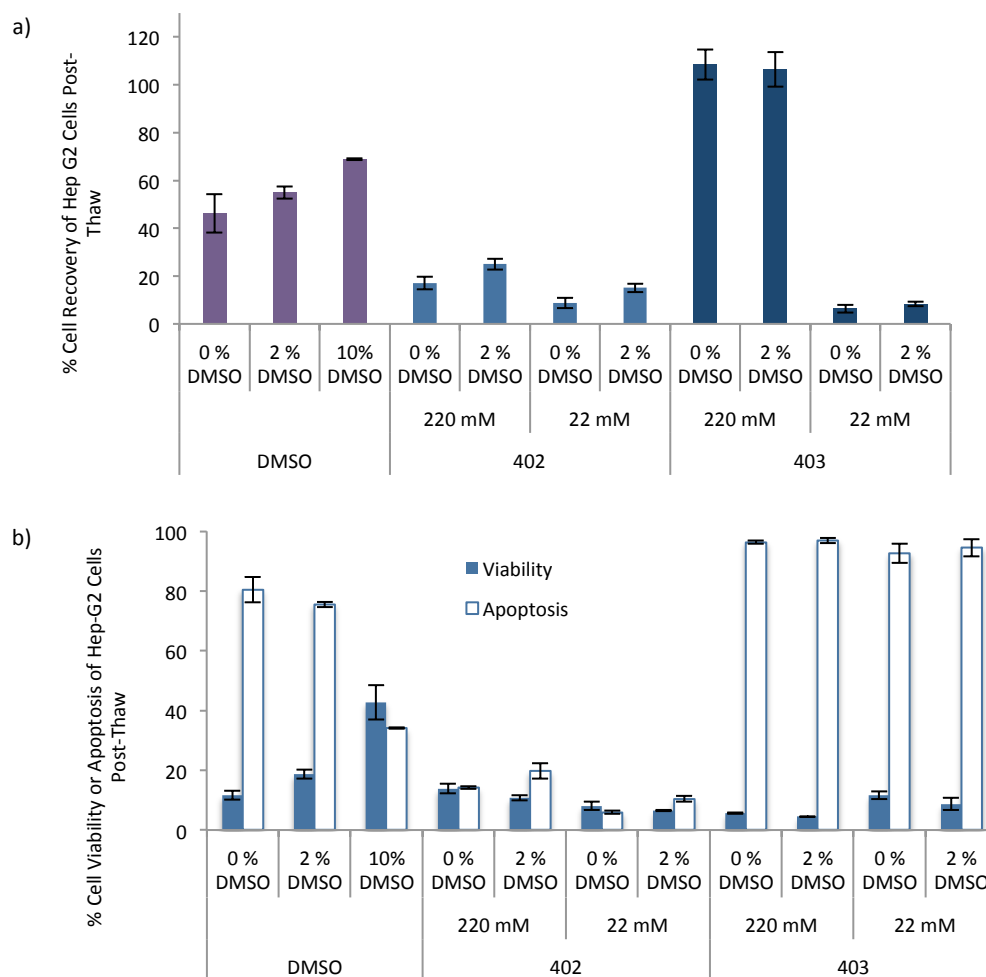
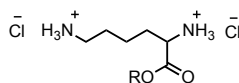


Figure 4-5 Cryopreservation of Hep G2 cells with quaternary ammonium chloride salts **402** and **403** showing **a)** percent recovery and **b)** percent viability and apoptosis. Error bars represent the standard error of the mean (SEM).

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*.

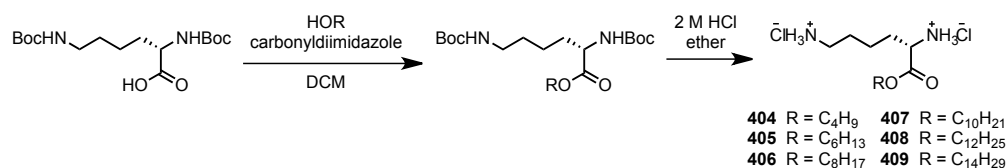
The second class of cationic surfactants that were examined as potential anti-INAs to protect crops from frost damage is lysine derivatives **404-409** (**Table 4-2**).⁸

Table 4-1 Structures of cationic lysine surfactants



Compound	R	Compound	R
404	C ₄ H ₉	407	C ₁₀ H ₂₁
405	C ₆ H ₁₃	408	C ₁₂ H ₂₅
406	C ₈ H ₁₇	409	C ₁₄ H ₂₉

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 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**).



Scheme 4-1 Synthesis of cationic lysine surfactants.

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 =

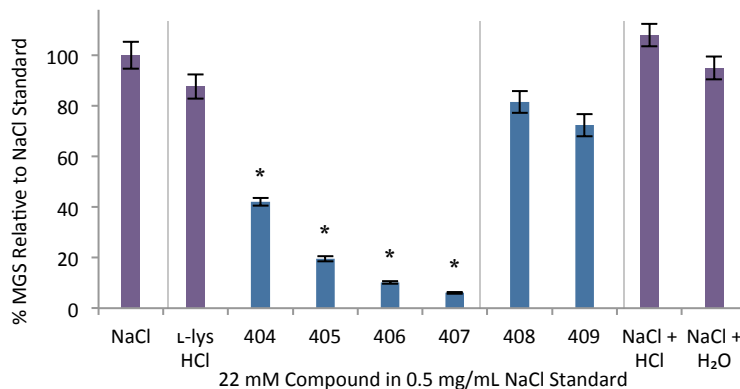


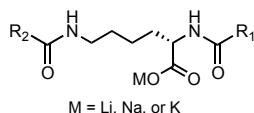
Figure 4-6 IRI activity of cationic lysine surfactants **404–409**. Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student's *t*-test ($p < 0.05$) within each set (separated by vertical lines).

7). Statistical analysis¹⁷ of these results indicated that the IRI activity of the standard 0.5 mg mL NaCl solution was indistinguishable from both the 0.5 NaCl mg mL 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.

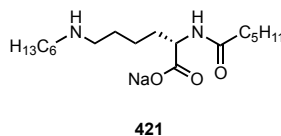
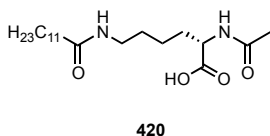
4.2. Anionic lysine-based surfactants

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.²⁰⁻²³ The physical and material properties

Table 4-3 Structures of anionic lysine surfactants



Compound	R ₁	R ₂	Compound	R ₁	R ₂
410	C ₅ H ₁₁	C ₃ H ₇	415	CH ₃	C ₁₁ H ₂₃
411	C ₅ H ₁₁	C ₅ H ₁₁	416	C ₇ H ₁₅	C ₁₁ H ₂₃
412	C ₅ H ₁₁	C ₇ H ₁₅	417	C ₁₁ H ₂₃	C ₁₁ H ₂₃
413	C ₅ H ₁₁	C ₉ H ₁₉	418	C ₁₁ H ₂₃	C ₃ H ₇
414	C ₅ H ₁₁	C ₁₁ H ₂₃	419	C ₁₁ H ₂₃	C ₇ H ₁₅



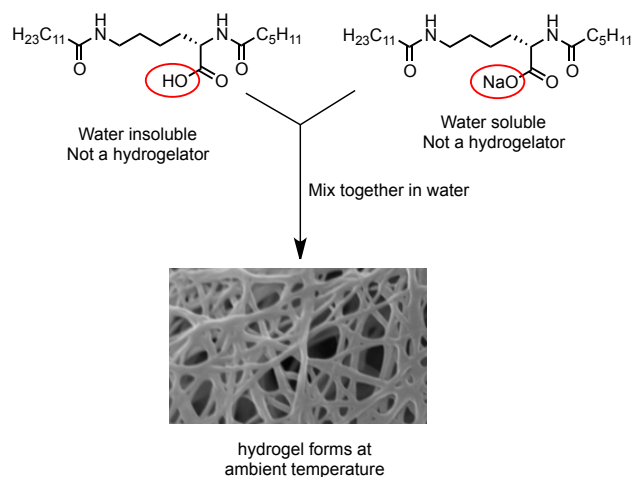
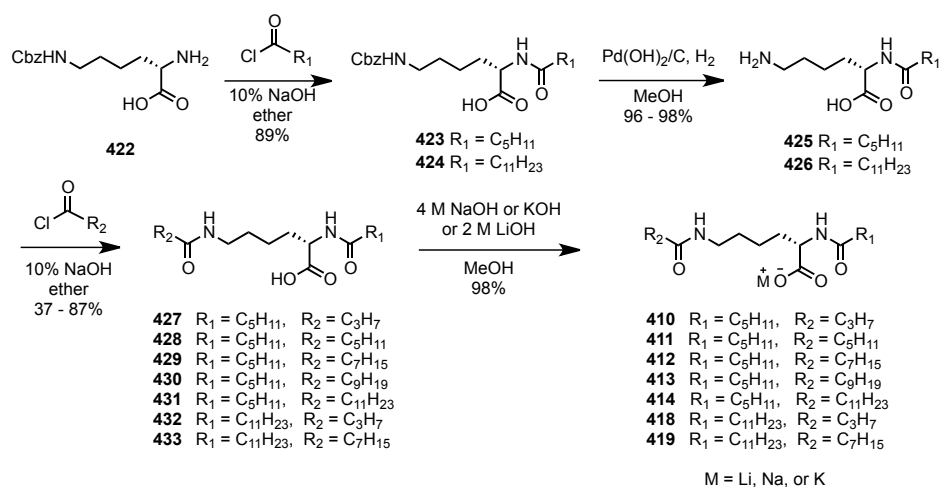


Figure 4-7 The water insoluble and water soluble surfactants will only form hydrogels if mixed together.

of the lysine-based gelators have been extensively studied.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 α - and ϵ -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.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.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



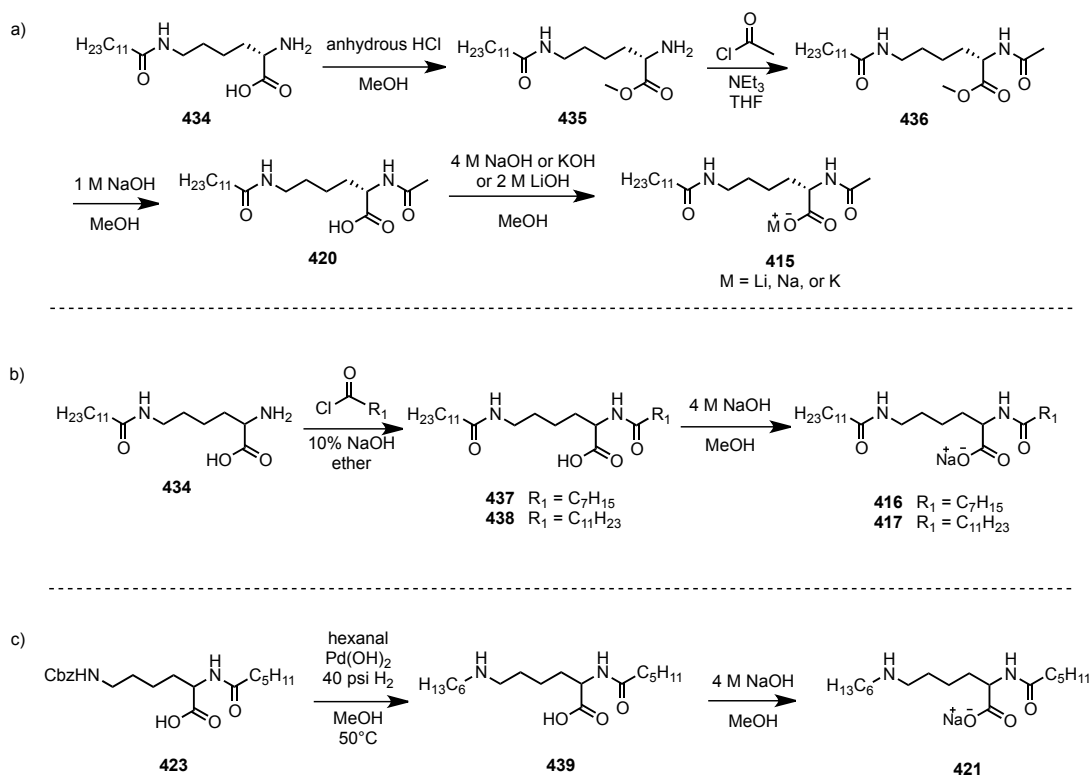
Scheme 4-2 Synthesis of lithium, sodium, or potassium L-lysinate derivatives **410-414**, **418**, **419**.

tested as a carboxylic acid since it was reported that it was soluble in aqueous NaCl at lower concentrations.²⁰

4.2.1. Synthesis of anionic lysine surfactants

The synthesis of most of the anionic lysine surfactants is shown in **Scheme 4-2**. ϵ -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.

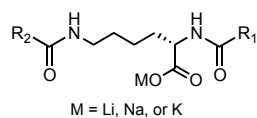
The synthesis of **415**, containing an acetamide, was not successful (**Scheme 4-2**) since acetyl chloride is more water-soluble compared to the other alkyl chlorides, and thus more easily hydrolyzed. The biphasic reactions with acetyl chloride resulted in yields lower than 20%. Therefore, carboxylic acid **434** was first protected as a methyl ester under anhydrous acidic conditions and the acetate group was installed at the α -



Scheme 4-3 Synthesis of anionic lysine surfactants a) **415**, b) **416**, **417**, and c) **421**

amino terminus (**435**) (**Scheme 4-3a**).^{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



Compound	R ₁	R ₂	Compound	R ₁	R ₂
410	C ₅ H ₁₁	C ₃ H ₇	415	CH ₃	C ₁₁ H ₂₃
411	C ₅ H ₁₁	C ₅ H ₁₁	416	C ₇ H ₁₅	C ₁₁ H ₂₃
412	C ₅ H ₁₁	C ₇ H ₁₅	417	C ₁₁ H ₂₃	C ₁₁ H ₂₃
413	C ₅ H ₁₁	C ₉ H ₁₉	418	C ₁₁ H ₂₃	C ₃ H ₇
414	C ₅ H ₁₁	C ₁₁ H ₂₃	419	C ₁₁ H ₂₃	C ₇ H ₁₅

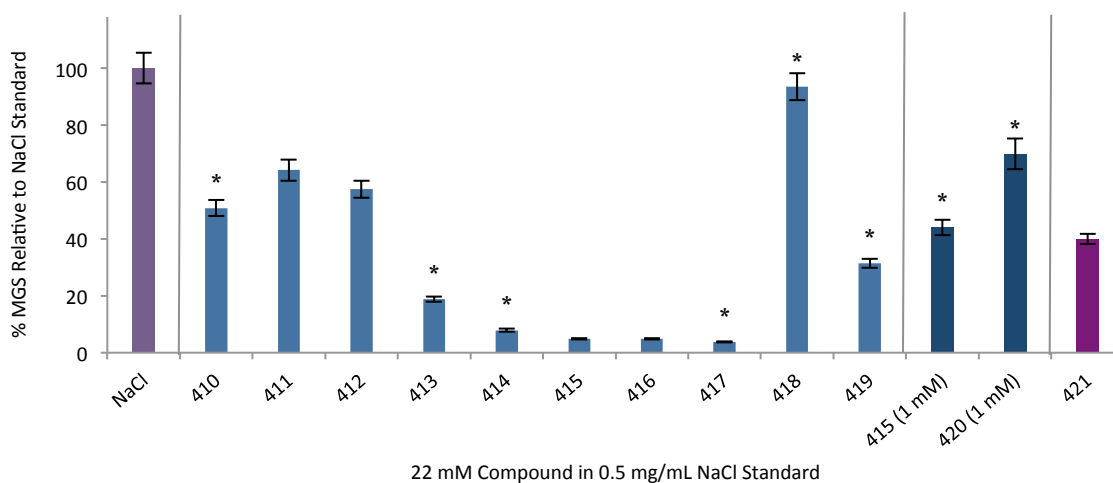
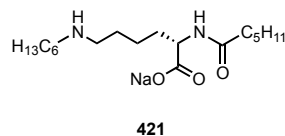
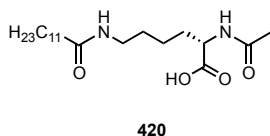


Figure 4-8 IRI activity of anionic lysine surfactants as sodium salts (**410-421**). Error bars indicate that standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student's *t*-test ($p < 0.05$) within each set (separated by vertical lines).

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 R₁ was kept constant at five carbons while R₂ was varied between three and eleven carbon atoms. The most IRI active analogue of this series (**414**) had the longest R₂ chain of eleven carbons. R₂ chain lengths of three to seven carbons (**410-412**) resulted in only moderate IRI activity whereas **413**, having an R₂ length of

nine carbons was much more active. Collectively, these data suggest that from these surfactants, an alkyl chain of eleven carbons at R₂ is optimal for IRI activity. Analogues **415-417** demonstrate whether the length of the R₁ alkyl chain influenced the IRI activity of **414**. In these analogues, R₂ contained eleven carbons and R₁ was varied with one (**415**), seven (**416**), or eleven (**417**) carbon units. The results in **Figure 4-8** indicate that the length of R₁ had less of an affect on IRI activity than R₂. Although analogue **417**, possessing an eleven-carbon alkyl chain at R₁, 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 R₁ is not an essential structural feature. The importance of a long alkyl chain at R₂ was further demonstrated in analogues **418** and **419** which both have eleven carbons at R₁. Analogue **418** with a three-carbon alkyl chain at R₂ was not active, however, as R₂ was lengthened to seven carbons (**419**), the activity increased. However, this analogue was still not as active as **417** where R₂ contained eleven carbons. These results further support the fact that a long alkyl chain at the R₂ 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 R₂ 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.¹ 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

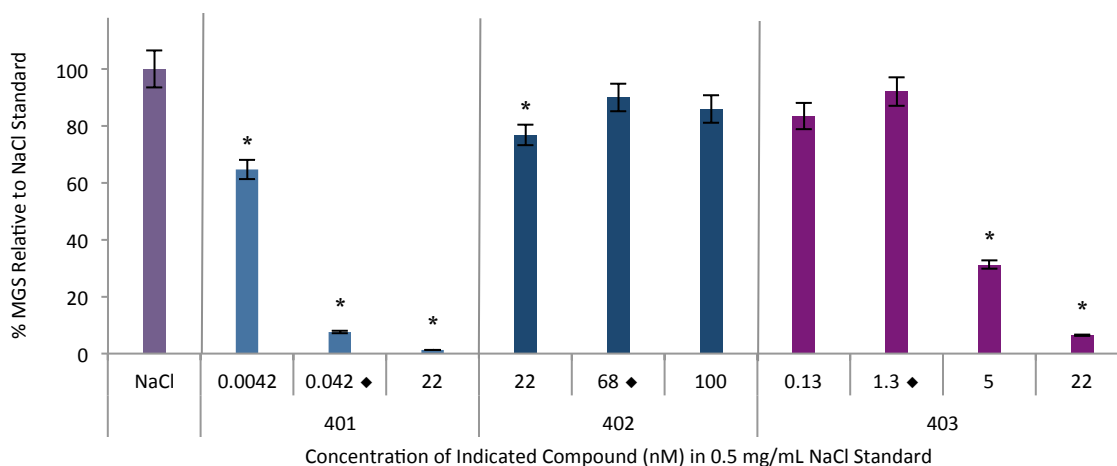


Figure 4-9 IRI activity of cationic anti-INAs (**401–403**) below, at, and above the CMC (CMC is indicated with $\blacklozenge$). Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student's *t*-test ($p < 0.05$) within each set (separated by vertical lines).

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

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.²⁰ 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

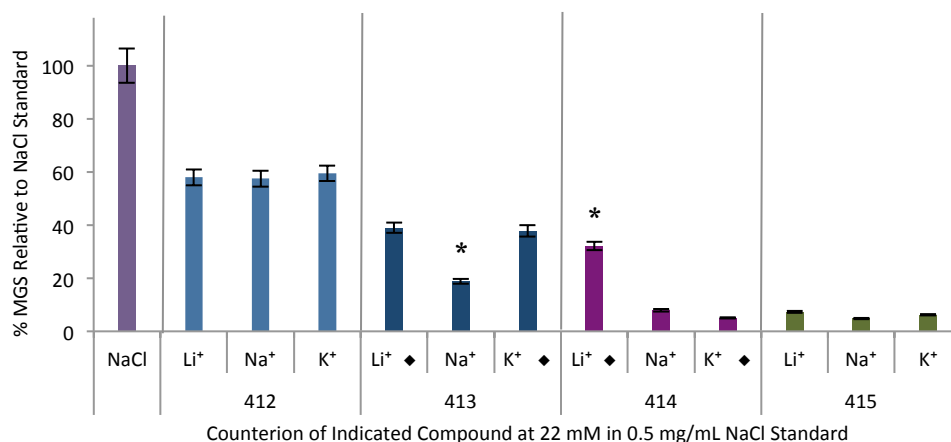


Figure 4-10 IRI activity of anionic lysine surfactants/gelators (**412–415**) as lithium, sodium, and potassium salts. Compounds which gelled at 22 mM in a NaCl standard are indicated with ♦. Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student's t-test ($p < 0.05$) within each set (separated by vertical lines).

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

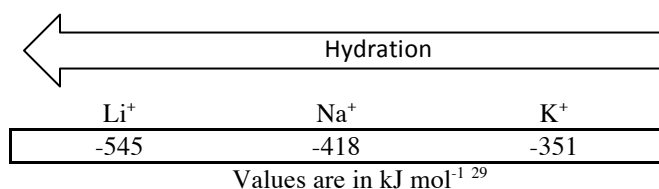
IRI activity since it has the longest R_2 alkyl chain. Surfactant **415**, containing eleven carbons at R_2 and one carbon at R_1 , in which the sodium salt has the highest IRI activity out of this set, also had the highest IRI activity when including the average of its salts. From these observations, 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 R_2 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,²⁸



Li ⁺	Na ⁺	K ⁺
-545	-418	-351

Values are in kJ mol⁻¹ ²⁹

Figure 4-11 Enthalpies of hydration of Li, Na, and K.

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-1 α cells from cryoinjury. The results in **Figure 4-12a** indicated that the recovery of cells (intact cells,

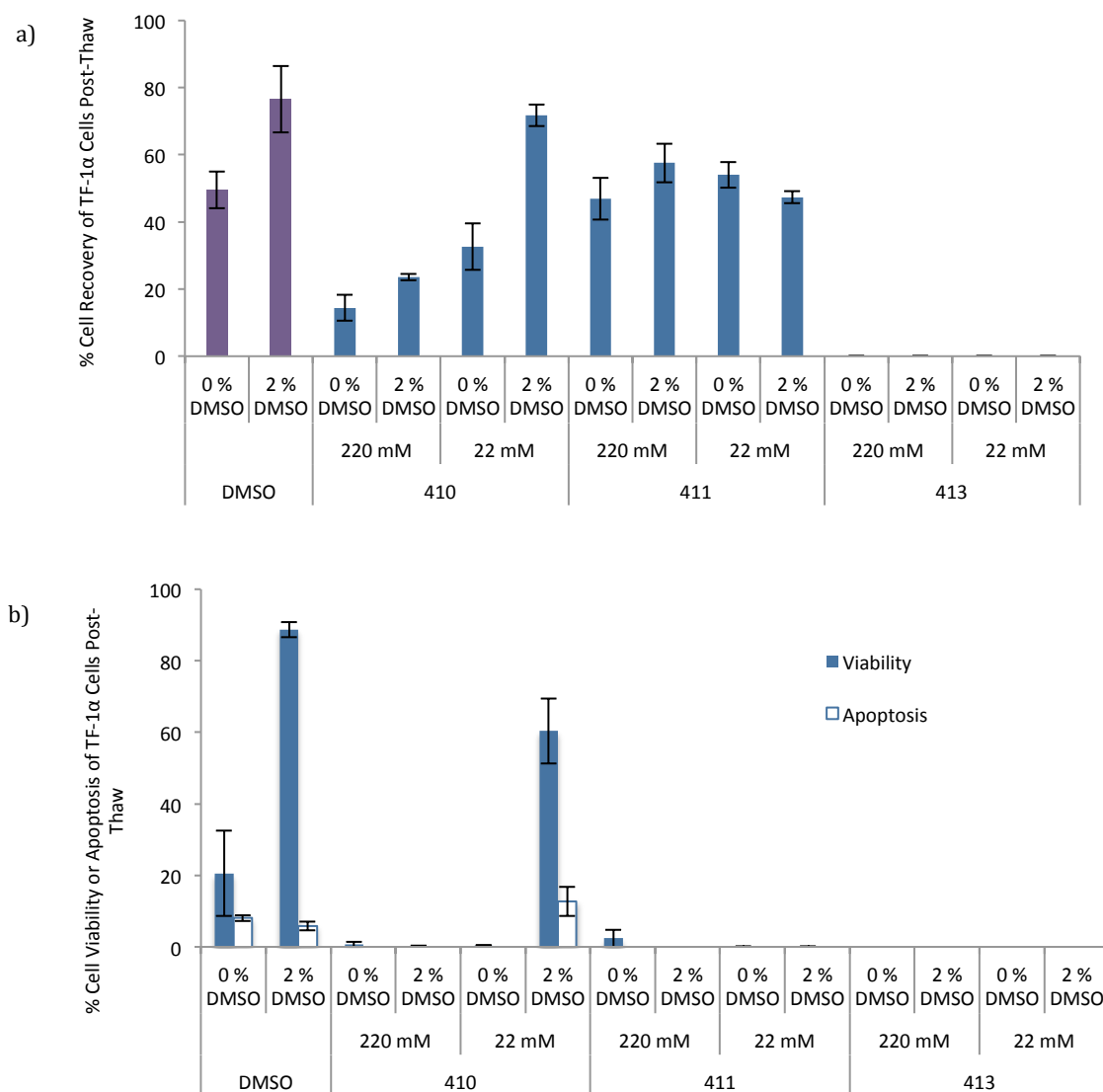


Figure 4-12 Cryopreservation of TF-1α cells cells with anionic lysine surfactants **410**, **411**, and **413** showing a) percent recovery and b) percent viability and apoptosis. Error bars represent the standard error of the mean (SEM).

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

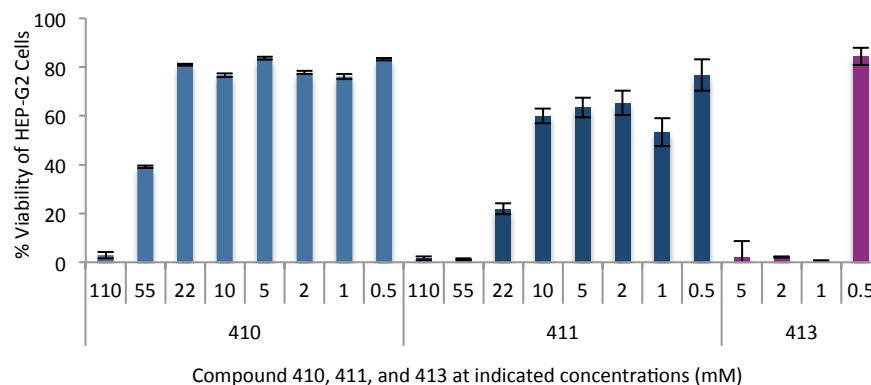


Figure 4-13 Cell viability of Hep-G2 cells incubated with anionic lysine surfactants **410**, **411**, and **413** in a MTT Assay.

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 cytotoxicity 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-1 α 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.

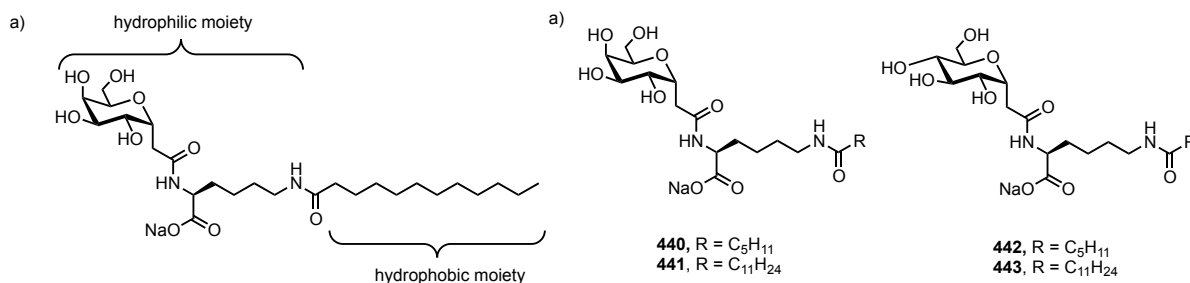
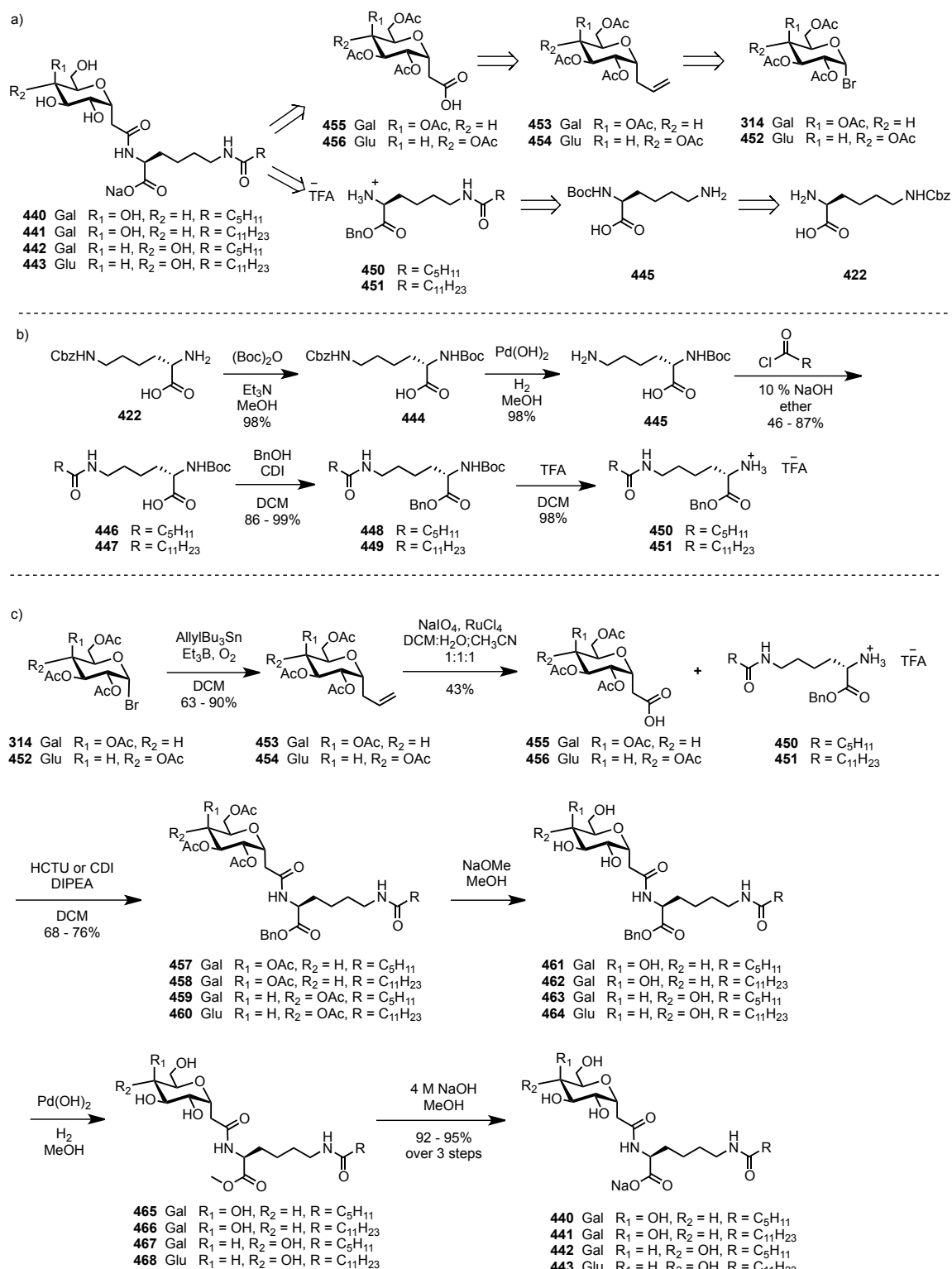


Figure 4-14 a) Carbohydrate glycoconjugate with hydrophilic and hydrophobic moieties. b) Structures of glycoconjugates **440-443**.

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*-butyl 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*-butyl 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 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.



Scheme 4-4 Synthesis of lysine glycoconjugates. **a)** Retrosynthesis of glycoconjugates, **b)** Synthesis of lysine component **450** and **451**. **c)** Synthesis of glycoconjugates **440-443**.

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

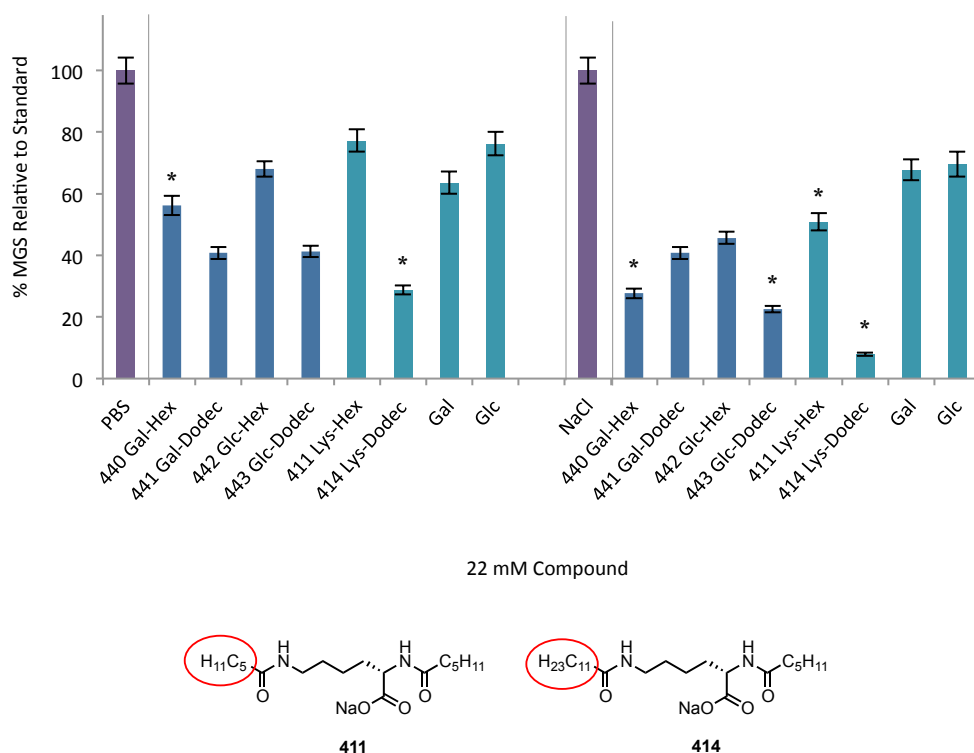


Figure 4-15 IRI activity of lysine glycoconjugates **440-443** in PBS and NaCl Standard solutions, and lysine surfactants **411** and **414**. In **440 – 443** the notation (for example Gal-Hex) represents the carbohydrate (Gal) and the R chain length (Hex) of the glycoconjugate. Error bars indicate 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

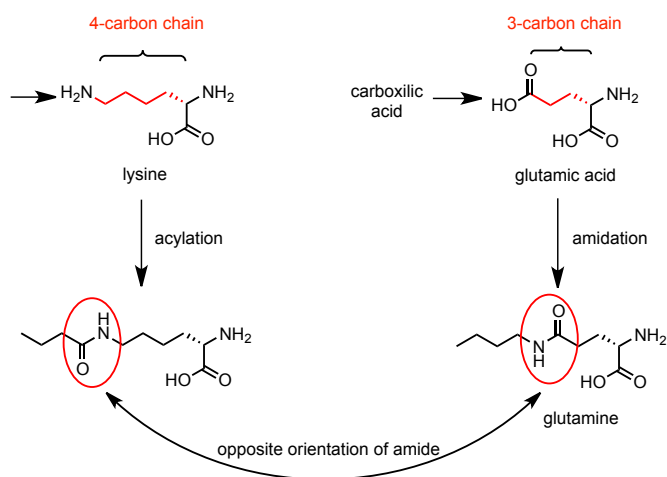
dodecanoyl-lysine **441**, had higher IRI activity than galactose (**Gal**). This was similarly seen with the glucosylated glycoconjugates, where **442** and **443** had higher IRI activity than glucose (**Glc**). It was also expected that the carbohydrates glycosidated with dodecanoyl-lysine, **441** 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

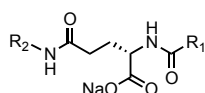


As the anionic lysine surfactants demonstrated potent IRI activity, we wanted to determine if other amino acid-based surfactants would also yield the same results, and therefore glutamine surfactants were synthesized and analyzed for IRI

Figure 4-16 Structural features of glutamine compared to lysine.

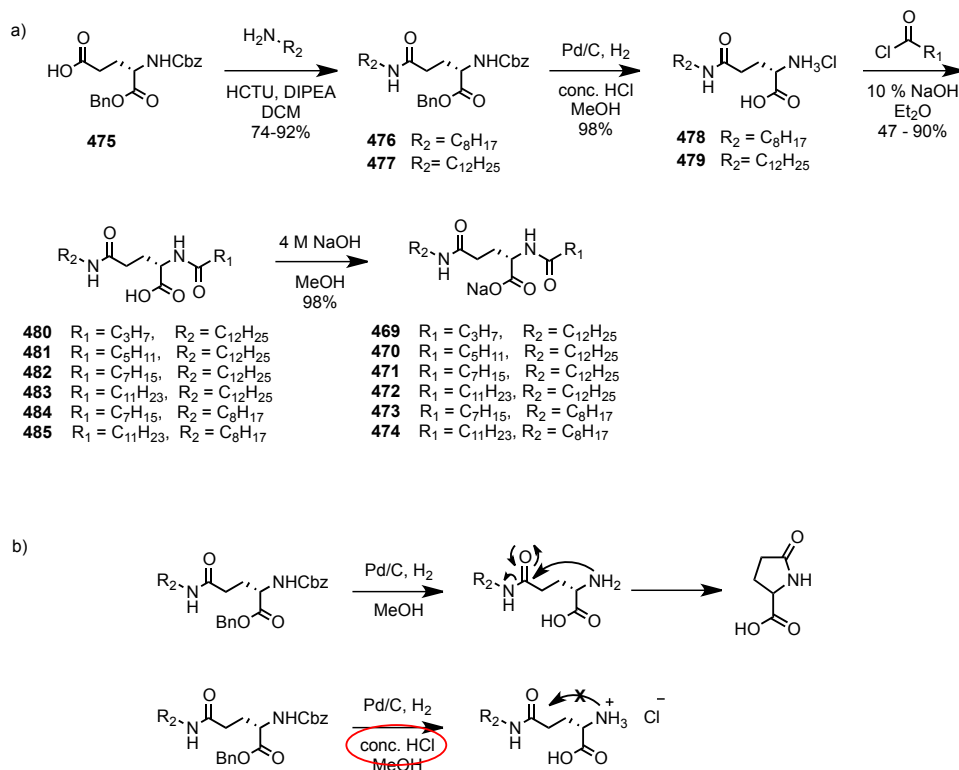
activity. It is important to note that the starting material for the glutamine surfactants is glutamic acid, containing a δ -carboxylic acid, not a δ -amide. In comparison to lysine, glutamic acid contains a carboxylic acid at the δ -position, while lysine contains an amine at the ϵ -position (**Figure 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



Compound	R ₁	R ₂	Compound	R ₁	R ₂
469	C ₃ H ₇	C ₁₂ H ₂₅	472	C ₁₁ H ₂₃	C ₁₂ H ₂₅
470	C ₅ H ₁₁	C ₁₂ H ₂₅	473	C ₇ H ₁₅	C ₈ H ₁₇
471	C ₇ H ₁₅	C ₁₂ H ₂₅	474	C ₁₁ H ₂₃	C ₈ H ₁₇

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

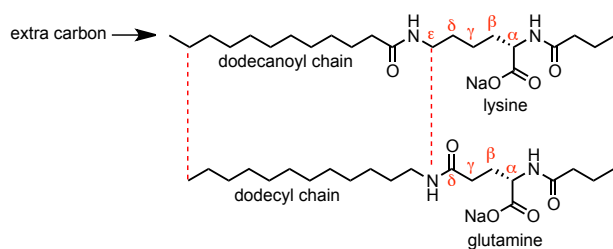
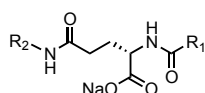


Figure 4-17 The total length of the lysine surfactant is 1 carbon longer than the comparable glutamine surfactant.

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 R_1 and R_2 alkyl chain lengths. The dotted line indicates the similar glutamine and lysine surfactants.

Table 4-4 Structures of glutamine surfactants



Compound	R ₁	R ₂	Compound	R ₁	R ₂
469	C ₃ H ₇	C ₁₂ H ₂₅	472	C ₁₁ H ₂₃	C ₁₂ H ₂₅
470	C ₅ H ₁₁	C ₁₂ H ₂₅	473	C ₇ H ₁₅	C ₈ H ₁₇
471	C ₇ H ₁₅	C ₁₂ H ₂₅	474	C ₁₁ H ₂₃	C ₈ H ₁₇

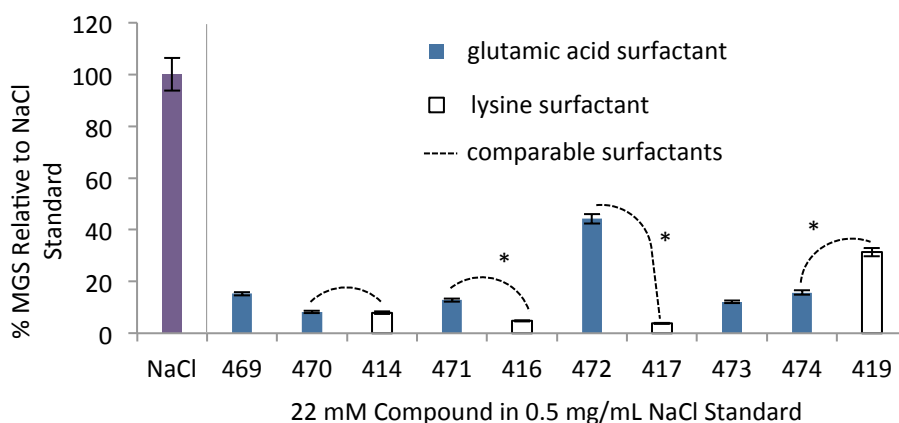


Figure 4-18 IRI activity of glutamic acid surfactants (**469-474**). Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student's *t*-test ($p < 0.05$) within each set.

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 δ -carbon rather than the ϵ -carbon (in lysine). The distance between the amide bond and the carboxylate and α -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

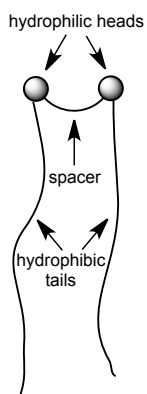


Figure 4-19
Structural
features of
gemini
surfactants

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**).³¹ 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** possess 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.

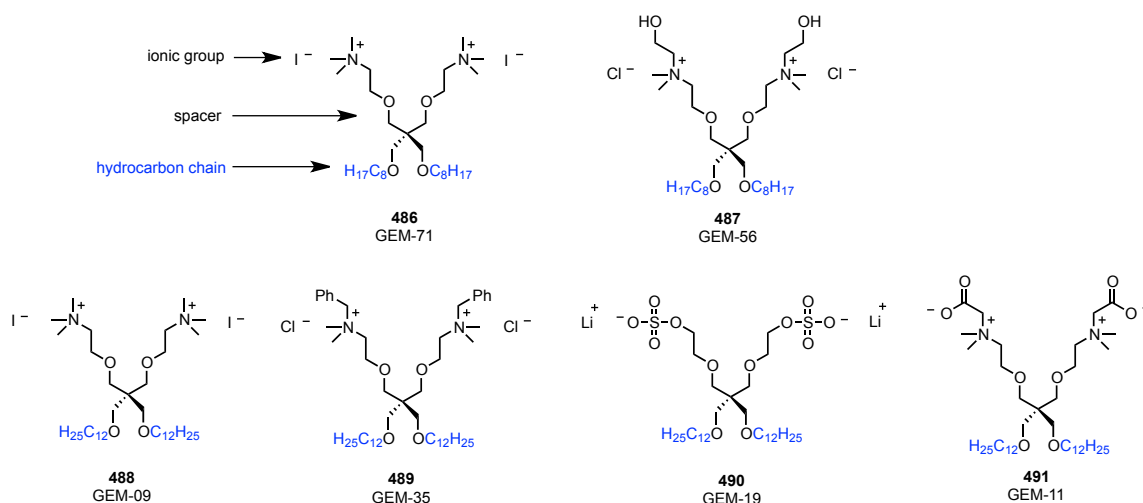


Figure 4-20 Structures of gemini surfactants.

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.

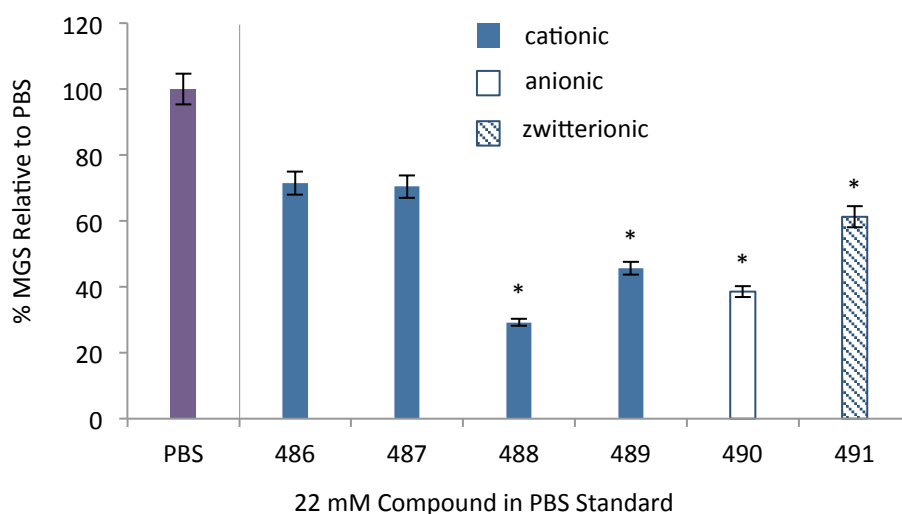


Figure 4-21 IRI activity of gemini surfactants **486-490**. The different bar colours indicate the class of gemini surfactants (cationic, anionic, or zwitterionic). Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student's *t*-test ($p < 0.05$) within each set (separated by vertical lines).

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**

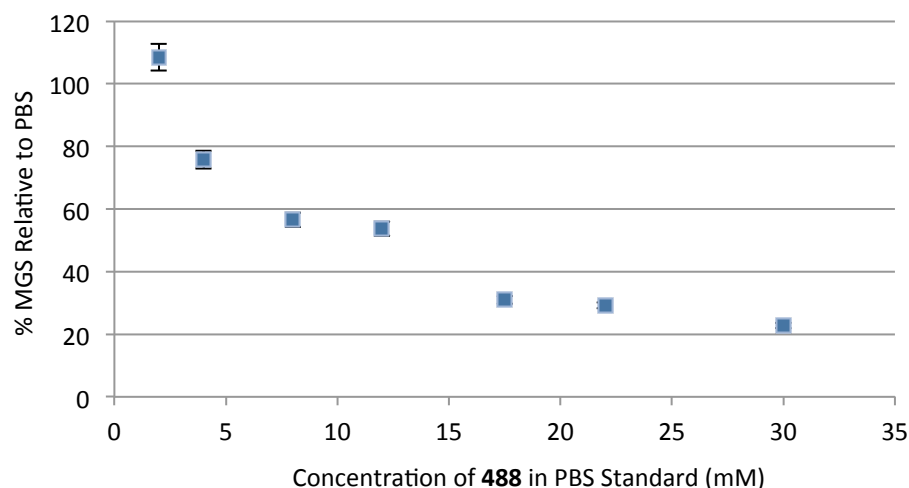


Figure 4-22 IRI activity of gemini surfactant **488** in a concentration scan. Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student's *t*-test ($p < 0.05$) within each set.

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

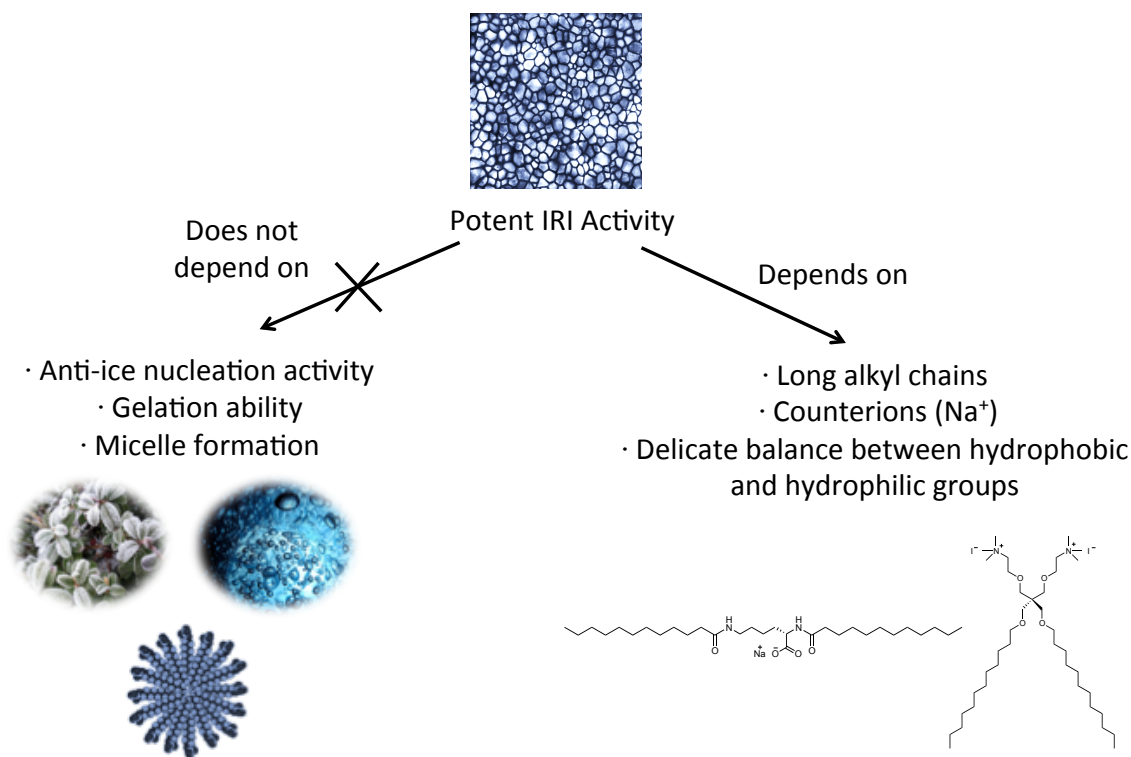


Figure 4-23 Graphical summary of the influences on IRI activity.

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

1. Capicciotti, C. J.; Leclère, M.; Perras, F. A.; Bryce, D. L.; Paulin, H.; Harden, J.; Liu, Y.; Ben, R. N. *Chem. Sci.* **2012**, *3*, 1408.
2. Estroff, L. A.; Hamilton, A. D. *Chem. Rev.* **2004**, *104*, 1201.
3. Suzuki, M.; Hanabusa, K. *Chem. Soc. Rev.* **2009**, *38*, 967.
4. Bhattacharya, S.; Krishnan-Ghosh, Y. *Chem. Commun.* **2001**, 185.
5. Jadhav, S. R.; Vemula, P. K.; Kumar, R.; Raghavan, S. R.; John, G. *Angew. Chem.* **2010**, *122*, 7861.
6. Lee, K. Y.; Mooney, D. J. *Chem. Rev.* **2001**, *101*, 1869.
7. Friggeri, A.; Feringa, B. L.; van Esch, J. J. *Controlled Release* **2004**, *97*, 241.
8. Watanabe, M.; Makino, T.; Okada, K.; Hara, M.; Watabe, S.; Arai, S. *Agric. Biol. Chem.* **1988**, *52*, 201.
9. Lindow, S. E.; Arny, D. C.; Upper, C. D. *Plant Physiol.* **1982**, *70*, 1084.
10. Inada, T.; Koyama, T.; Goto, F.; Seto, T. *J. Phys. Chem. B* **2011**, *115*, 7914.
11. Wowk, B.; Fahy, G. M. *Cryobiology* **2002**, *44*, 14.
12. Yankofsky, S.; Levin, Z.; Moshe, A. *Curr. Microbiol.* **1981**, *5*, 213.
13. Rahn, O.; Van Eseltine, W. P. *Annu. Rev. Microbiol.* **1947**, *1*, 173.
14. Rubinsky, B.; Arav, A.; DeVries, A. L. *Cryobiology* **1992**, *29*, 69.
15. Chakrabartty, A.; Hew, C. L. *Eur. J. Biochem.* **1991**, *202*, 1057.
16. Biosciences, B. San Jose, California, 2000; Vol. 11-11032-01.
17. Chaytor, J. L.; Tokarew, J. M.; Wu, L. K.; Leclère, M.; Tam, R. Y.; Capicciotti, C. J.; Guolla, L.; von Moos, E.; Findlay, C. S.; Allan, D. S.; Ben, R. N. *Glycobiology* **2012**, *22*, 123.
18. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55.
19. Liu, S.; Wang, W.; von Moos, E.; Jackman, J.; Mealing, G.; Monette, R.; Ben, R. N. *Biomacromol.* **2007**, *8*, 1456.
20. Suzuki, M.; Abe, T.; Hanabusa, K. *J. Colloid Interface Sci.* **2010**, *341*, 69.
21. Suzuki, M.; Yumoto, M.; Shirai, H.; Hanabusa, K. *Tetrahedron* **2008**, *64*, 10395.
22. Suzuki, M.; Yumoto, M.; Shirai, H.; Hanabusa, K. *Chem. Eur. J.* **2008**, *14*, 2133.
23. Suzuki, M.; Yumoto, M.; Kimura, M.; Shirai, H.; Hanabusa, K. *Chem. Eur. J.* **2003**, *9*, 348.
24. Cardwell, P. H. *J. Colloid Interface Sci.* **1966**, *22*, 430.
25. Rosen, M. J. In *Surfactants and Interfacial Phenomena*; 3rd ed.; Wiley-Interscience: Hoboken NJ, 2004, p 105.
26. Ray, A.; Nemethy, G. *J. Am. Chem. Soc.* **1971**, *93*, 6787.
27. Helenius, A.; Simons, K. *Biochim. Biophys. Acta* **1975**, *415*, 29.
28. Tam, R. Y.; Ferreira, S. S.; Czechura, P.; Chaytor, J. L.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 17494.
29. Smith, D. W. *J. Chem. Educ.* **1977**, *54*, 540.

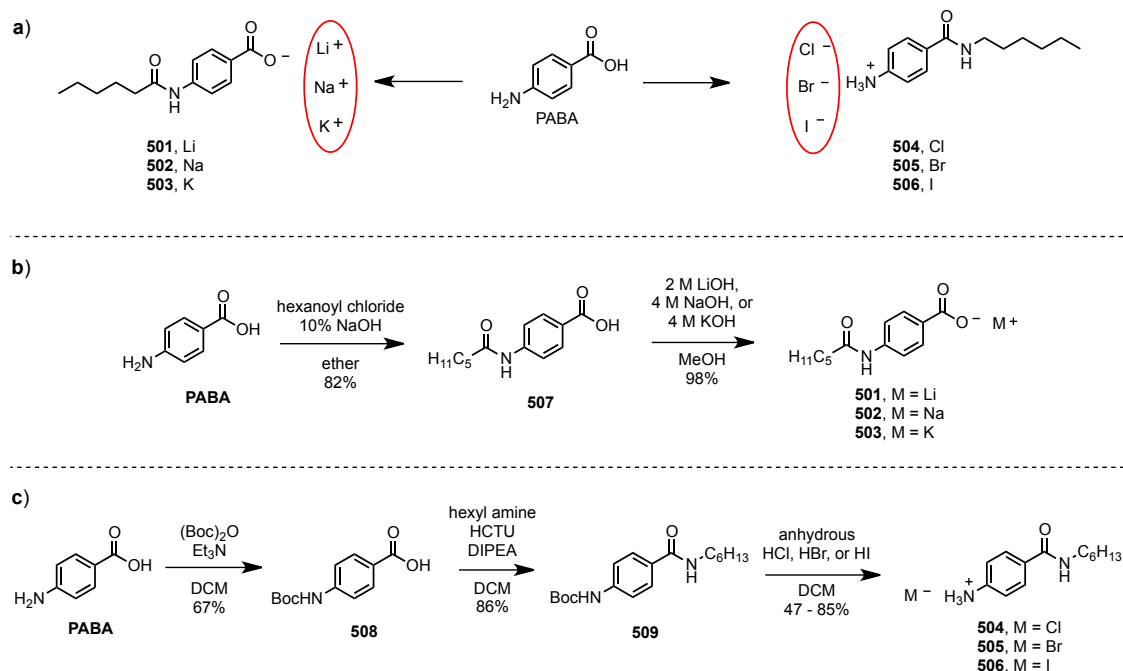
30. Czechura, P.; Tam, R. Y.; Dimitrijevic, E.; Murphy, A. V.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 2928.
31. Menger, F. M.; Littau, C. A. *J. Am. Chem. Soc.* **1993**, *115*, 10083.

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



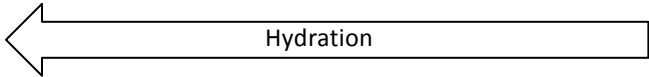
Scheme 5-1 PABA-based compounds to study counterion effects. **a)** The use of PABA allows the study of cationic and anionic counterions. **b)** Synthesis of PABA compounds with cationic counterions **501-503**. **c)** Synthesis of PABA compounds with anionic counterions **504-506**.

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 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



Li ⁺	Na ⁺	K ⁺
-545	-418	-351
Cl ⁻	Br ⁻	I ⁻
-338	-304	-261

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

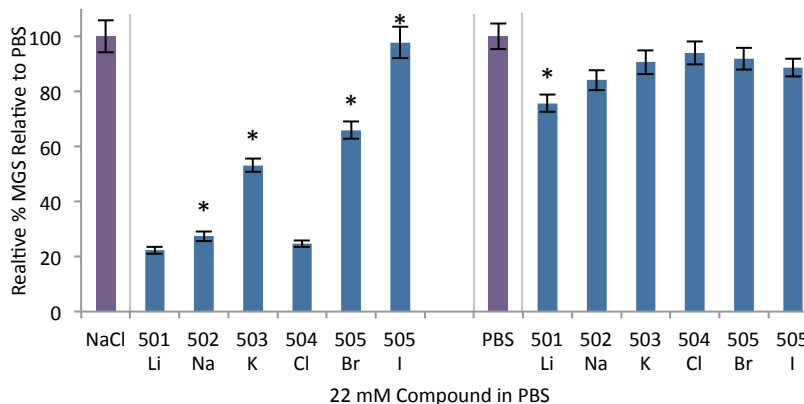


Figure 5-2 IRI activity of PABA-based compounds **501** – **505** with different counterions. Error bars indicate the standard error of the mean (SEM). Asterisks indicate a significant difference defined by unpaired Student's *t*-test ($p < 0.05$) within each set (separated by vertical lines). Note: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

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.

5.3. Determining the “best” IRI assay solutions for standardization of the assay

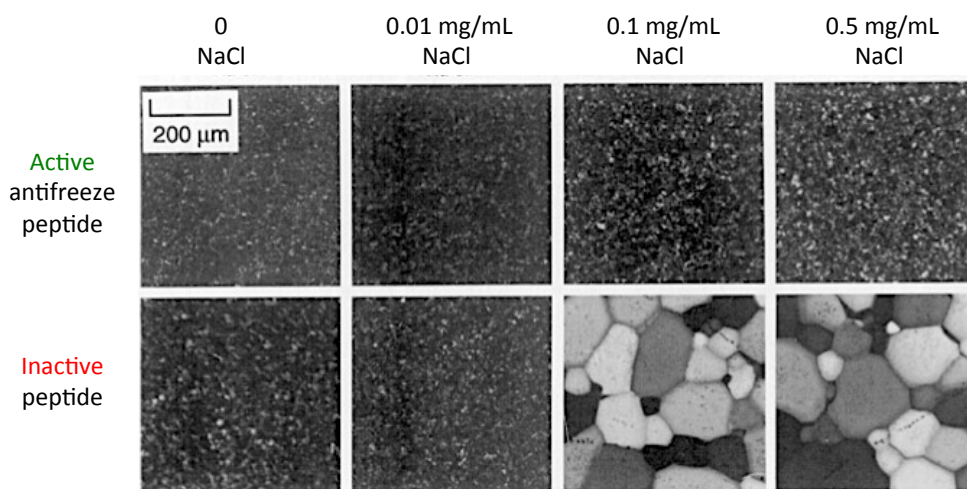


Figure 5-3 Effect of NaCl on recrystallization inhibition by an active and inactive antifreeze protein. Protein concentration is 0.02 mg/mL. Only NaCl 0.1 and 0.5 mg/mL destroy false-positives. Picture from Knight, 1995.³

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.

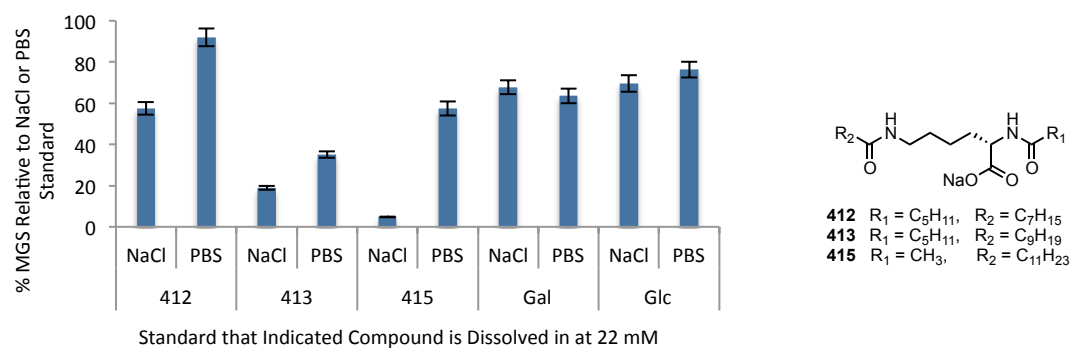


Figure 5-4 IRI activity of anionic lysine surfactants and monosaccharides in NaCl or PBS solution. Error bars indicate the standard error of the mean (SEM).

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.

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 Na₂HPO₄, and 0.25 mg/mL KH₂PO₄, 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 + Na₂HPO₄, NaCl + KCl, Na₂HPO₄ + KH₂PO₄, and NaCl + Na₂HPO₄ + KH₂PO₄.

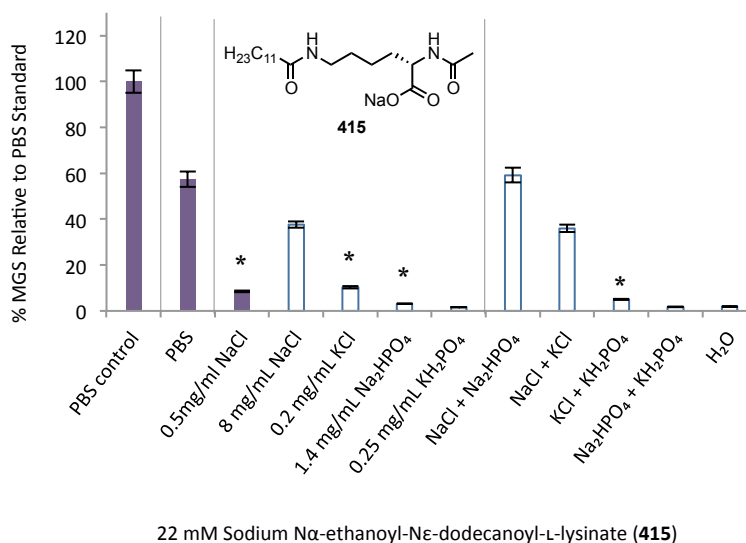


Figure 5-5 IRI activity of individual PBS salts at the concentration found in PBS with 22 mM sodium N^{α} -ethanoyl- N^{ϵ} -dodecanoyl-L-lysinate (**415**). Salt combinations with no indicated concentrations are at the concentration found when as an individual salt. Error bars represent the standard error of the mean. Asterisks indicate a significant difference defined by unpaired Student's t -test ($p < 0.05$) in each set (separated by vertical lines). Note that PBS and NaCl+Na₂HPO₄ are statistically different than 8 mg mL⁻¹ NaCl and NaCl+KCl.

KCl + KH₂PO₄, and Na₂HPO₄ + KH₂PO₄. The salts were grouped together by either similar cations (Na and K) or anions (Cl and PO₄) and are at concentrations that exist in PBS. **415** in the NaCl + Na₂HPO₄ 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 + Na₂HPO₄ combination contains a total of 9.4 mg/mL of salt (8 mg/mL of NaCl and 1.4 mg/mL Na₂HPO₄) 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 + KH₂PO₄ is less IRI active than Na₂HPO₄ + KH₂PO₄ 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.¹² This is defined by the formula:¹²

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

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

0.25 mg/mL KH ₂ PO ₄	0.2 mg/mL KCl	KCl + KH ₂ PO ₄	0.5 mg/mL NaCl	1.4 mg/mL Na ₂ HPO ₄	Na ₂ HPO ₄ + KH ₂ PO ₄	8 mg/mL NaCl	NaCl + KCl	NaCl + Na ₂ HPO ₄	PBS
1.84	2.68	4.52	8.56	29.6	31.4	137	140	167	171

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*^α-ethanoyl-*N*^ε-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

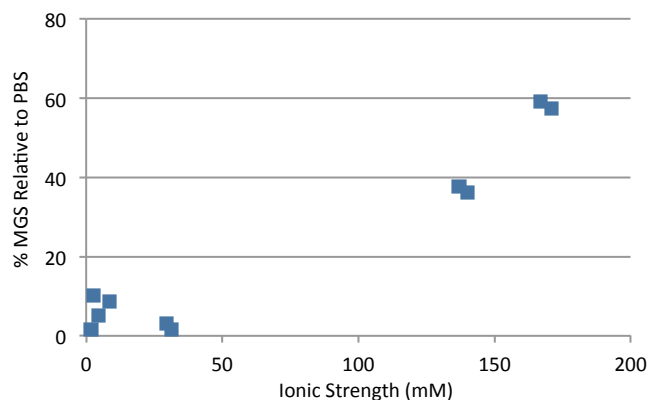


Figure 5-6 Correlation between ionic strength and IRI activity of 22 mM sodium *N*^α-ethanoyl-*N*^ε-dodecanoyl-L-lysinate (**415**) compared to a PBS control.

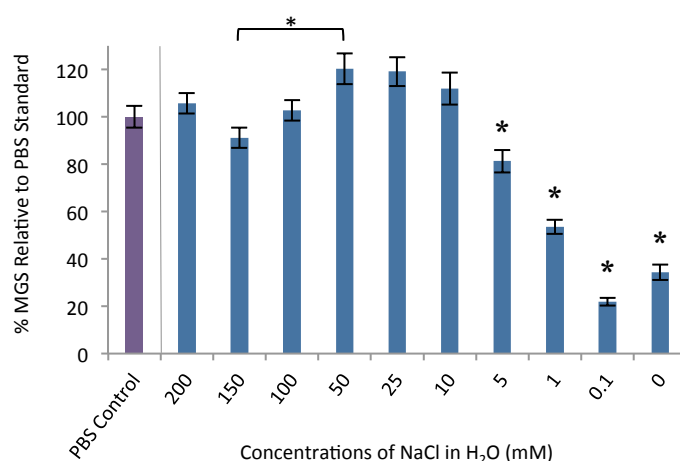


Figure 5-7 The effect of NaCl concentration on IRI activity. Error bars represent the standard error of the mean. Asterisks indicate a significant difference defined by unpaired Student's *t*-test ($p < 0.05$) within the set (separated by vertical lines).

(**Figure 5-7**). 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. Na₂HPO₄ and KH₂PO₄ 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 Na₂HPO₄ as this salt at 137 mM has

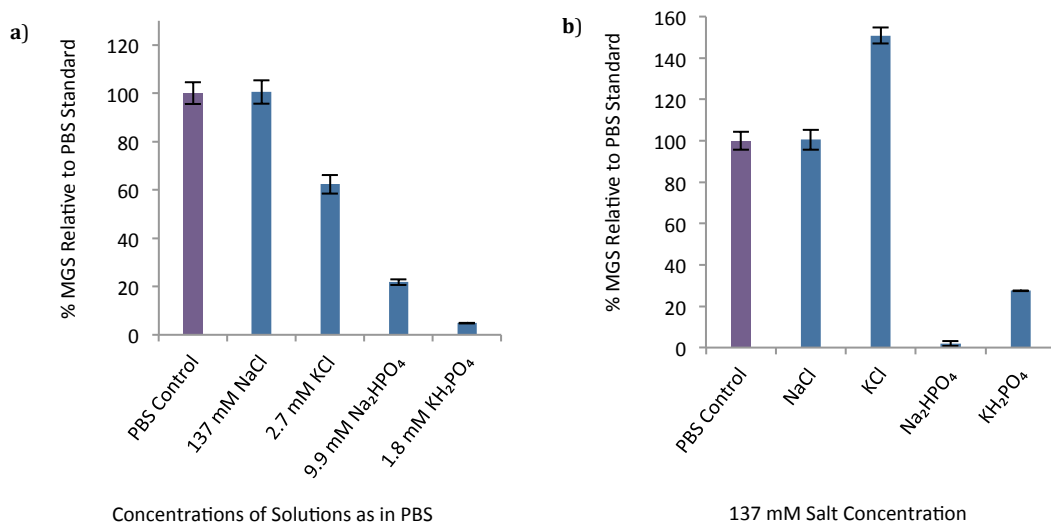


Figure 5-8 Effect of different PBS salt concentrations on IRI activity. **a)** The solutions are at the concentrations that they exist in PBS. **b)** The PBS salts at 137 mM (original concentration of NaCl in PBS).

much higher IRI activity than at 9.9 mM. However, with KCl and KH₂PO₄, 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 Na₂HPO₄, 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 H₂PO₄⁻ to PO₃⁻.¹³ The Hofmeister series ranks cations and anions according to how well they precipitate proteins out of solution,¹⁴⁻¹⁶ 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, KH_2PO_4 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 (Na_2HPO_4 is more active than NaCl, and Na_2HPO_4 is more

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

← Hydration	
Na^+	K^+
-418	-351
phosphate	Cl^-
?	-338
Values are in kJ mol^{-1}	

More IRI Active	vs.	Less IRI Active
<u>NaCl</u>		<u>KCl</u>
<u>Na_2HPO_4</u>		<u>KH_2PO_4</u>
<u>KH_2PO_4</u>		<u>KCl</u>
<u>Na_2PO_4</u>		<u>NaCl</u>

The variable ions are underlined.

active than KH_2PO_4). 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 Na_2HPO_4 so in the comparison between KH_2PO_4 , 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 Na_2HPO_4 and KH_2PO_4 is large, so that if the concentration of KH_2PO_4 was doubled, Na_2HPO_4 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.

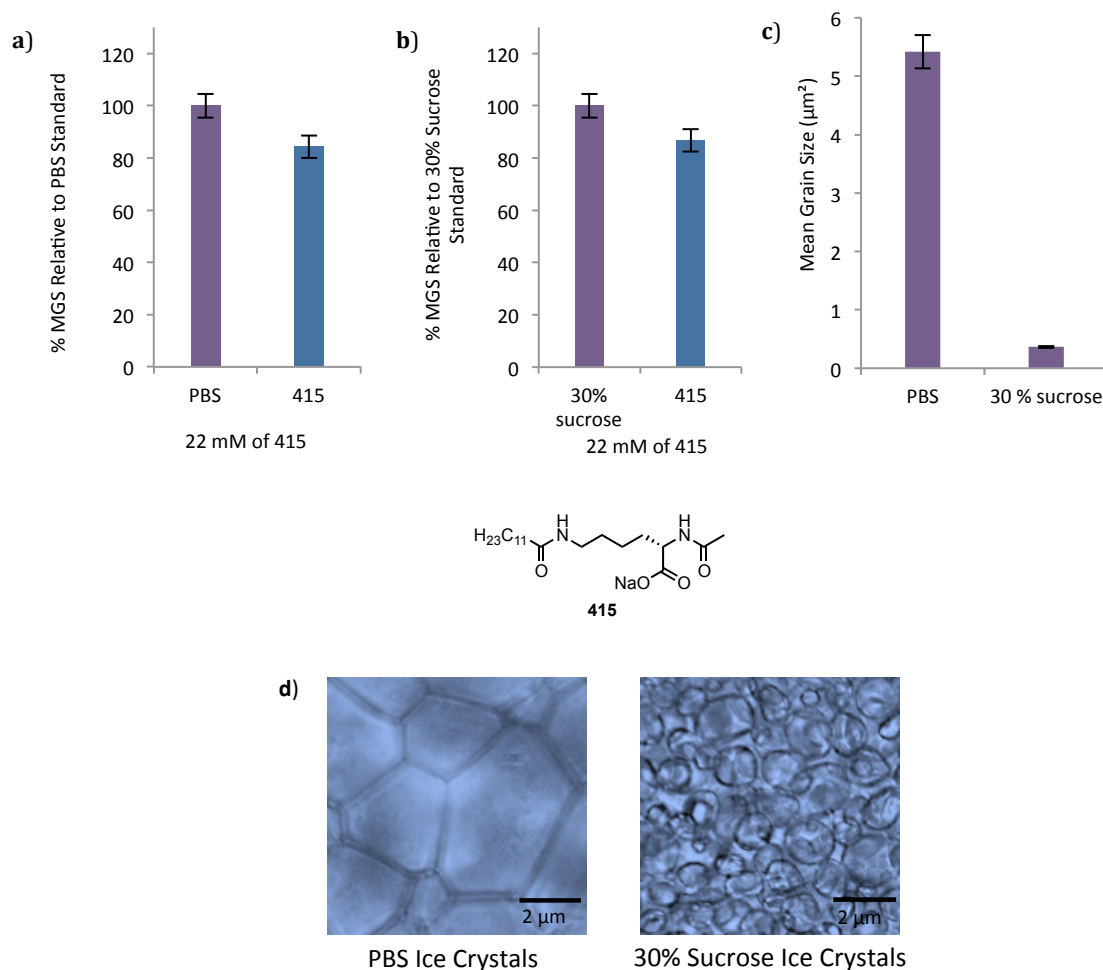


Figure 5-9 Comparison between PBS and 30% sucrose IRI assay solutions. **a)** IRI activity of **415** in PBS. **b)** IRI activity of **415** in 30% sucrose. **c)** Comparison of the mean grain sizes of PBS and 30% sucrose. **d)** Picture of PBS and 30% sucrose ice crystals.

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

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

1. Smith, D. W. *J. Chem. Educ.* **1977**, *54*, 540.
2. Tam, R. Y.; Ferreira, S. S.; Czechura, P.; Chaytor, J. L.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, *130*, 17494.
3. Knight, C. A.; Wen, D.; Laursen, R. A. *Cryobiology* **1995**, *32*, 23.
4. Smallwood, M.; Worrall, D.; Byass, L.; Elias, L.; Ashford, D.; Doucet, C. J.; Holt, C.; Telford, J.; Lillford, P.; Bowles, D. J. *Biochem. J.* **1999**, *340*, 385.
5. Sidebottom, C.; Buckley, S.; Pudney, P.; Twigg, S.; Jarman, C.; Holt, C.; Telford, J.; McArthur, A.; Worrall, D.; Hubbard, R.; Lillford, P. *Nature*. **2000**, *406*, 256.
6. Sidebottom, C.; Buckley, S.; Pudney, P.; Twigg, S.; Jarman, C.; Holt, C.; Telford, J.; McArthur, A.; Worrall, D.; Hubbard, R.; Lillford, P. *Nature* **2000**, *406*, 256.
7. Tremblay, K.; Ouellet, F. B.; Fournier, J.; Danyluk, J.; Sarhan, F. *Plant Cell Physiol.* **2005**, *46*, 884.
8. Tomczak, M. M.; Marshall, C. B.; Gilbert, J. A.; Davies, P. L. *Biochem. Biophys. Res. Commun.* **2003**, *311*, 1041.
9. Lu, S.-S.; Inada, T.; Yabe, A.; Zhang, X.; Grandum, S. In *Proc. Symposium on Energy Engineering in the 21st Century*; DTIC Document: 2000, p 860.
10. Raymond, J. A.; Knight, C. A. *Cryobiology* **2003**, *46*, 174.
11. Inada, T.; Lu, S.-S. *Cryst. Growth Des.* **2003**, *3*, 747.
12. Solomon, T. *J. Chem. Educ.* **2001**, *78*, 1691.
13. Ruben, E. A.; Chapman, M. S.; Evanseck, J. D. *J. Phys. Chem. A* **2007**, *111*, 10804.
14. Cacace, M. G.; Landau, E. M.; Ramsden, J. J. *Q. Rev. Biophys.* **1997**, *30*, 241.
15. Hofmeister, F. *Arch. Exp. Pathol. Pharmacol* **1888**, *24*, 10.
16. Kunz, W.; Lo Nostro, P.; Ninham, B. W. *Curr. Opin. Colloid Interface Sci.* **2004**, *9*, 1.

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.^{1,2} The advantages of using oncolytic viruses as cancer therapeutics include the ability to rapidly modify the viruses with recombinant DNA technology to create ‘designer viruses’³ and the natural effect that the viruses have to stimulate tumor-specific inflammation.¹ These treatments are in the stages of clinical trials presently.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.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.⁵ Over three million deaths are prevented in developing countries due to vaccination programs.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

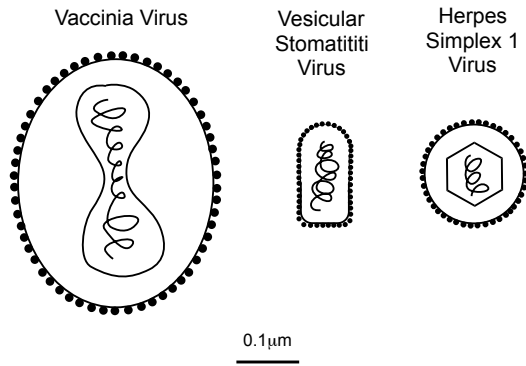


Figure 6-1 Structures and sizes of oncolytic viral vectors.

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 temperatures⁷ or freezing temperatures.⁸ 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.^{9,10} 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**).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.¹¹ VV is naturally attracted to tumor cells¹² 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.^{13,14} 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.^{15,16} VSV is a small bullet-

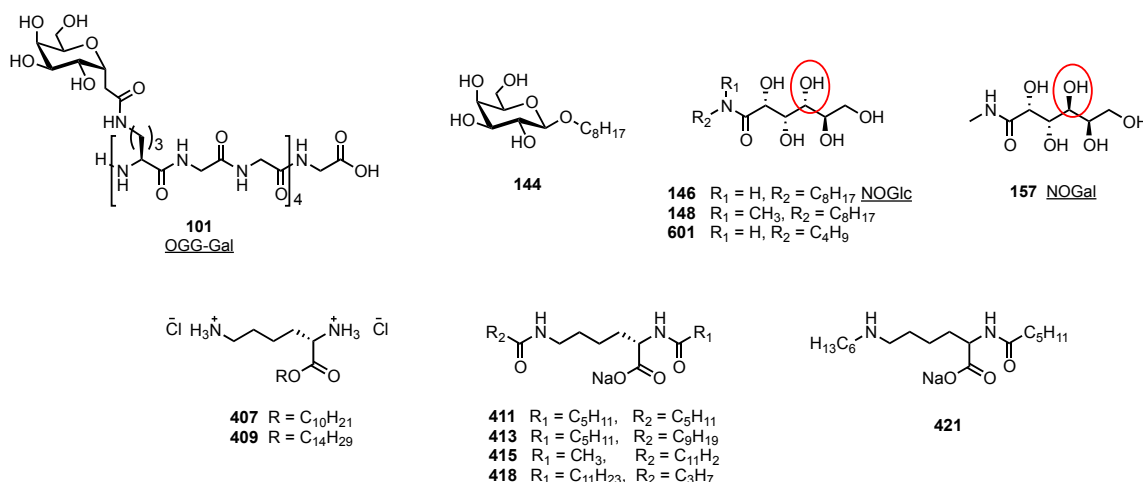


Figure 6-2 Structures of ice recrystallization inhibitors assessed for their ability to protect viral vectors at various conditions: room temperature, freeze-thaw, and lyophilization.

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.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.¹⁸ As well, VSV is being developed as a vector for vaccines against HIV-1,¹⁹ Ebola virus,²⁰ hepatitis B²¹ and C.²² Finally, HSV-1 is a double-stranded DNA virus and causes cold sores in humans.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 2013.²³

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,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

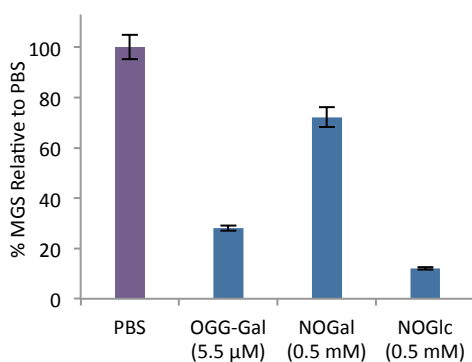


Figure 6-3 IRI activity of ice recrystallization inhibitors that protect viral vectors. Error bars indicate standard error of the mean.

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.²⁵ 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.²⁶ In this assay, virus titers in varying dilutions are added to monolayers of Vero cells (kidney epithelial cells)

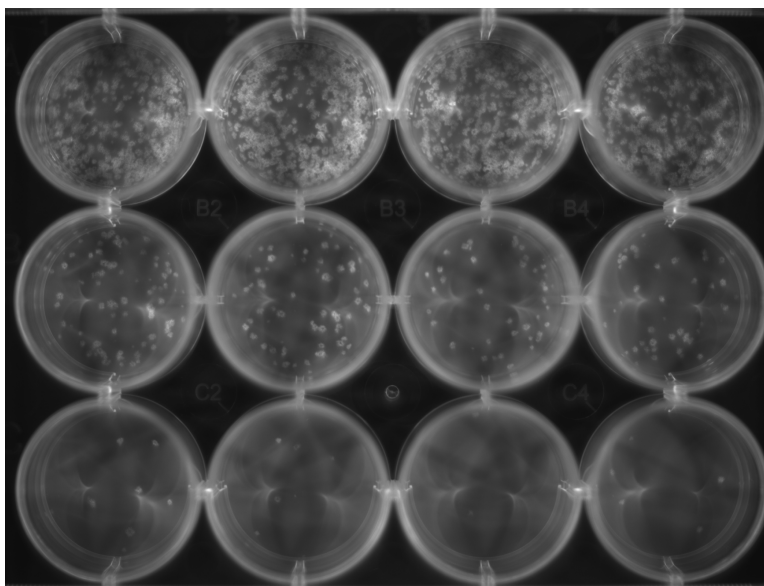


Figure 6-4 Plaque forming assay depicting wells containing plaques with Vero cells at high (top) to low (bottom) concentrations. White spots are plaques.

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

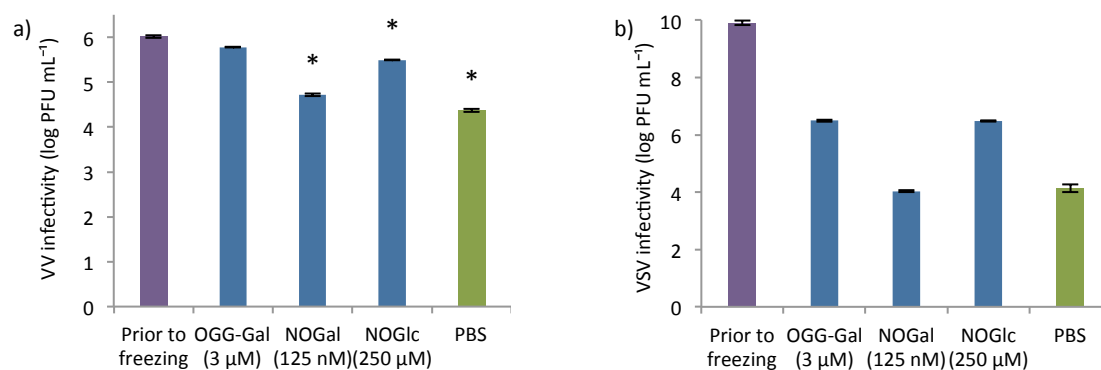


Figure 6-5 Effect of ice recrystallization inhibitors on the infectivity of **a)** VV and **b)** VSV after 10 freeze-thaw cycles. Error bars indicated that standard error of the mean. Asterisks indicate a significant difference defined by unpaired Student's *t*-test ($p < 0.05$). Note: in VSV, OGG-Gal and NOGlc are statistically different than NOGal and PBS.

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.²⁴ 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.^{27,28} Enveloped viruses are sensitive to chemical or physical conditions that disrupt the membrane including desiccation, heat, detergents, and extreme pH's.²⁷ 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.²⁹ Since lyophilization is the sublimation of water from a frozen sample at low pressure, ice recrystallization would occur during the time the ice sublimates. 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 Log₁₀ PFU mL⁻¹ while VV in the PBS control lost its infectivity by 2 Log₁₀ 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

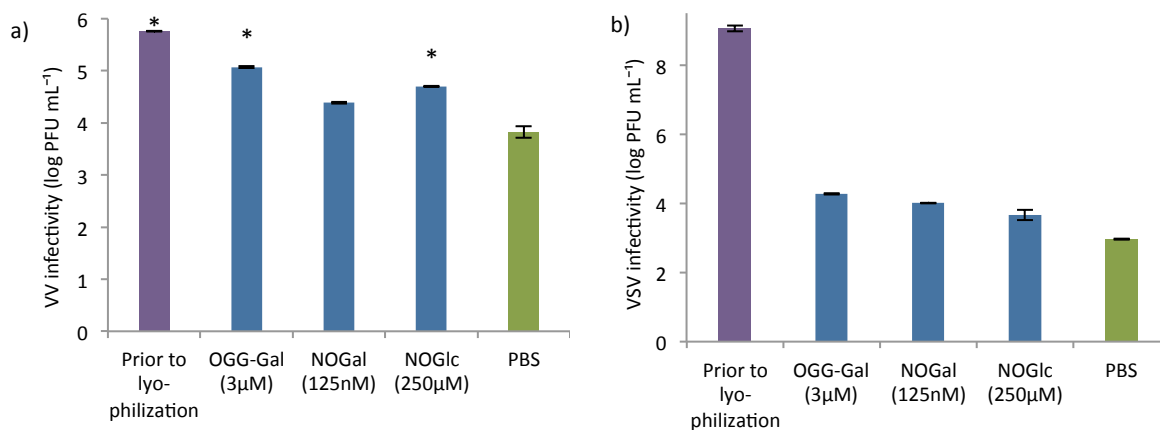


Figure 6-6 Effect of ice recrystallization inhibitors on **a)** VV and **b)** VSV after lyophilization.

by almost 5 Log₁₀ 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.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 Log₁₀ PFU mL⁻¹ of the original infectivity while VV in PBS loses more than 1 Log₁₀ infectivity within eight days. This is also seen with HSV-1 (**Figure 6-7b**)

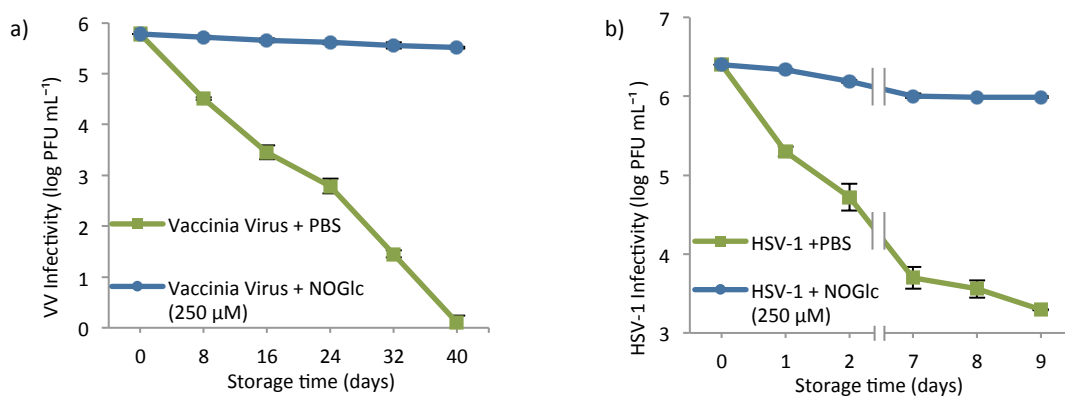


Figure 6-7 Effect of NOGlc on viral vectors at 22°C. **a)** VV infectivity during 40 days. **b)** HSV-1 infectivity during 9 days. Error bars indicate the standard error of the mean.

when at nine days, the virus with NOGlc loses only 0.4 Log₁₀ PFU mL⁻¹ infectivity, while the control loses more than 1 Log₁₀ 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

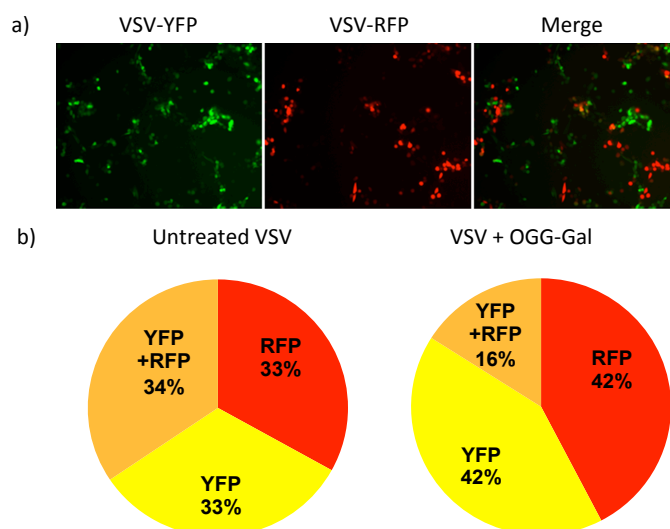


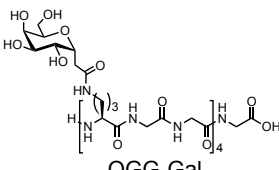
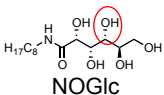
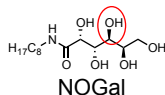
Figure 6-8 Cells infected with a mixture consisting of equal amounts of VSV expressing YFP and RFP after one freeze-thaw cycle; **a)** cells expressing YFP, RFP and both YFP and RFP, respectively. **b)** Percentage of cells infected by YFP, RFP, and both YFP and RFP for untreated, and OGG-Gal treated VSV.

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

Table 6-1 Summary table.

		 OGG-Gal	 NOGlc	 NOGal
Room Temperature Stability	VV			↑↑↑↑↑↑↑↑↑↑↑↑
	VSV			
	HSV-1			↑↑↑↑↑
Cryopreservation	VV	↑↑	↑	↑↑
	VSV	↑↑↑↑		↑↑↑↑
	HSV-1			
Lyophilization	VV	↑↑	↑	↑↑
	VSV	↑↑	↑↑	↑
	HSV-1			

↑ - represents recovered infectivity in 0.5 Log₁₀ PFU mL⁻¹ compare with PBS control.

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 activity³² and protection of viruses through 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 Health Organizations, vaccines can be utilized if they lose less than 1 Log₁₀ of the original titer amount.^{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

1. Parato, K. A.; Senger, D.; Forsyth, P. A.; Bell, J. C. *Nat. Rev. Cancer* **2005**, *5*, 965.
2. Miest, T. S.; Cattaneo, R. *Nat. Rev. Microbiol.* **2014**, *12*, 23.
3. Ottolino-Perry, K.; Diallo, J.-S.; Lichty, B. D.; Bell, J. C.; McCart, J. A. *Mol. Ther.* **2010**, *18*, 251.
4. Gould, E. *Mol. Biotechnol.* **1999**, *13*, 57.
5. Brandau, D. T.; Jones, L. S.; Wiethoff, C. M.; Rexroad, J.; Middaugh, C. R. *J. Pharm. Sci.* **2003**, *92*, 218.
6. Galazka, A.; Milstien, J.; Zaffran, M. *Thermostability of vaccines*; Global Programme for Vaccines and Immunization, World Health Organization, 1998.
7. Cherian, E. *Arch. Dis. Child.* **1993**, *69*, 600.
8. Jeremijenko, A.; Kelly, H.; Sibthorpe, B.; Attewell, R. *BMJ* **1996**, *312*, 1651.
9. Galazka, A.; Milstien, J.; Zaffran, M. *World Health Organization. Geneva* **1998**.
10. Knezevic, I. *Biologicals* **2009**, *37*, 357.
11. Isaacs, S. N. *Vaccinia Virus and Poxvirology, Methods in Molecular Biology* Totowa, NJ, 2004.
12. Whitman, E. D.; Tsung, K.; Paxson, J.; Norton, J. A. *Surgery* **1994**, *116*, 183.
13. Puhlmann, M.; Brown, C. K.; Gnant, M.; Huang, J.; Libutti, S. K.; Alexander, H. R.; Bartlett, D. L. *Cancer Gene Ther.* **2000**, *7*, 66.
14. Gnant, M. F.; Noll, L. A.; Irvine, K. R.; Puhlmann, M.; Terrill, R. E.; Alexander, H. R.; Bartlett, D. L. *J. Natl. Cancer Inst.* **1999**, *91*, 1744.
15. McCart, J. A.; Ward, J. M.; Lee, J.; Hu, Y.; Alexander, H. R.; Libutti, S. K.; Moss, B.; Bartlett, D. L. *Cancer Res.* **2001**, *61*, 8751.
16. Thorne, S. H.; Hwang, T.-H. H.; O’Gorman, W. E.; Bartlett, D. L.; Sei, S.; Kanji, F.; Brown, C.; Werier, J.; Cho, J.-H.; Lee, D.-E. *J. Clin. Invest.* **2007**, *117*, 3350.

17. King, A. M.; Adams, M. J.; Lefkowitz, E. J.; Carstens, E. B. *Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses*; Elsevier, 2012; Vol. 9.
18. Balachandran, S.; Barber, G. N. *IUBMB Life* **2000**, *50*, 135.
19. Clarke, D.; Cooper, D.; Egan, M.; Hendry, R. M.; Parks, C.; Udem, S. *Springer Semin. Immunopathol.* **2006**, *28*, 239.
20. Geisbert, T. W.; Daddario-DiCaprio, K. M.; Lewis, M. G.; Geisbert, J. B.; Grolla, A.; Leung, A.; Paragas, J.; Matthias, L.; Smith, M. A.; Jones, S. M.; Hensley, L. E.; Feldmann, H.; Jahrling, P. B. *PLoS Path.* **2008**, *4*, e1000225.
21. Cobleigh, M. A.; Buonocore, L.; Uprichard, S. L.; Rose, J. K.; Robek, M. D. *J. Virol.* **2010**, *84*, 7513.
22. Majid, A. M.; Ezelle, H.; Shah, S.; Barber, G. N. *J. Virol.* **2006**, *80*, 6993.
23. Amgen melanoma drug meets Phase III endpoint. *Genetic Engineering & Biotechnology News* [Online Early Access]. Published Online: 2013. (accessed March 9, 2014).
24. Baust, J. M. *Cell Preservation Technol.* **2002**, *1*, 17.
25. Krajden, M.; Minor, J. M.; Rifkin, O.; Comanor, L. *J. Clin. Microbiol.* **1999**, *37*, 1683.
26. Jerne, N. K.; Henry, C.; Nordin, A. A.; Fuji, H.; Koros, A.; Lefkovits, I. *Immunol. Rev.* **1974**, *18*, 130.
27. Lucas, W. In *eLS*; John Wiley & Sons, Ltd: 2001.
28. Cosset, F.-L.; Lavillette, D. In *Adv. Genet.*; Theodore Friedmann, J. C. D., Stephen, F. G., Eds.; Academic Press: 2011; Vol. Volume 73, p 121.
29. Adams, G. D. In *Vaccine Protocols*; Springer: 2003, p 223.
30. Adams, G. D. 2003; Vol. 87, p 223.
31. Bergey, E. J.; Cho, M.-I.; Hammar skjöld, M.-L.; Rekosh, D.; Levine, M. J.; Blumberg, B. M.; Epstein, L. G. *Crit. Rev. Oral Biol. Medicine* **1993**, *4*, 467.
32. Chaytor, J. L.; Tokarew, J. M.; Wu, L. K.; Leclère, M.; Tam, R. Y.; Capicciotti, C. J.; Guolla, L.; von Moos, E.; Findlay, C. S.; Allan, D. S.; Ben, R. N. *Glycobiology* **2012**, *22*, 123.

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.

^1H (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_3OD),

or deuterium oxide (D₂O) 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 μ L/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/H₂O as the MALDI matrix. High resolution mass spectrometry (HRMS) data was acquired on Applied Biosystems/Sciex QStar (Concord, ON). Samples in MeOH or H₂O were mixed with Agilent ES tuning mix for internal calibration, and infused into the mass spectrometer at 5 μ L/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 μ L 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 μ m 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.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 *t*-tests were performed to a 95% confidence level.

7.3. Assessing Thermal Hysteresis (TH)

Nanoliter osmometry was performed using a Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, NY), as described by Chakrabartty and Hew.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-1 α 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-1 α 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 centrifuged 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 cyro 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 100 μ L 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-1 α post cryopreservation flow cytometry

Samples were quickly thawed in a 37°C water bath. 900 μ L 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 μ L of the solution in cryo tube was transferred to flow tubes. 7 μ L of 7-AAD and 7 μ L 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 μ L). 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

V⁺. 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 cyro 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 100 μ L 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 μ L of 1X dilution of the Annexin 5 binding buffer was added to each cryo tube and 100 μ L of this solution was transferred to flow tubes. 5 μ L of 7-AAD and 4 μ L of PE Annexin 5 was added to flow tubes and the tubes were agitated and incubated in the dark for 15 min. 10 μ L of Countbright® beads was added and the solution in flow tubes was diluted with 1 x Annexin 5 binding buffer to 1 mL (381 μ L). 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.

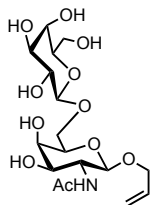
Counting beads were measured with 610 nm optical filter (FL3). Time between thawing and analysis by flow cytometry was less than 1 h. All samples were tested in triplicate.

7.4.7. MTT assay with HepG2 Cells

The MTT assay was performed as described previously.⁴ HepG2 cells were plated in 96-well plates and treated with 100 μ L 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 μ L of fresh media and 50 μ L of MTT solution (5 mg/mL) in HBSS (Hank's balanced salt solution) were added and the plates were incubated at 37°C with 5% CO₂ for 3 h. The plates were then centrifuged, the media aspirated and 200 μ L of MTT solubilization solution (10% Triton X-100, 0.1 N HCl in isopropanol) was added to each well. The plates were incubated at room temperature in the dark for 2-4 h and the absorbance of each well was then read at a wavelength of 570 nm with a 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 $\rightarrow$ 6)-2-acetamido-2-deoxy- β -D-galactopyranoside (304)



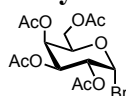
331 (25 mg, 0.03 mmol) was dissolved in CH₂Cl₂ (0.5 mL) and TFA was added until pH reached approx. 2. The solution was stirred at room temperature for 30 min after which triethyl amine was added until pH 7 and the solvent was removed. The residue was diluted in CH₂Cl₂ and washed with H₂O. The aqueous phase was washed twice with CH₂Cl₂ and the combined organic phases were dried with MgSO₄, 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:H₂O) to yield white product **204** (14 mg, 85%).

¹H NMR (500 MHz, D₂O) δ 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 (ddd, 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).

¹³C NMR (500 MHz, D₂O) δ 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.

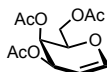
ESI-MS m/z calcd for C₁₇H₂₉NO₁₁ [M + Na]⁺: 446.16. Found: 446.37.

2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl bromide (**314**)



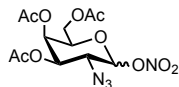
Compound was prepared from **313** as previously described.⁵ All spectral data was consistent with that reported in the literature.⁵

3,4,6-tri-*O*-acetyl-D-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-D-galactopyranosyl nitrate (**319**)

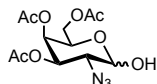


Compound was prepared from **307** as previously described.⁷

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 H₂O two times and a third time with brine. The organic phase was dried with Mg SO₄, 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

¹H NMR (500 MHz, CDCl₃) δ 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). ¹³C NMR (500 MHz, CDCl₃) δ 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 C₁₂H₁₆KN₄O₁₀⁺ [M+K]⁺: 415.05. Found: 415.32.

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

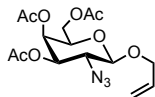


Compound was prepared from **319** as previously described.⁸

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 NaHCO₃, and brine and the organic phase was dried with MgSO₄ 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

¹H NMR (500 MHz, CDCl₃) δ 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). ¹³C NMR (400 MHz, CDCl₃) δ 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 C₁₂H₁₇N₃O₈ [M + K]⁺: 370.06. Found: 370.16.

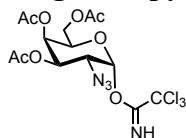
Allyl 3,4,6-tri-O-acetyl-2-azido-2-deoxy-β-D-galactopyranoside (**321**)



To a solution of α -D-trichloroacetimidate **322** (0.155 g, 0.326 mmol) in anhydrous CH_2Cl_2 (6 mL) cooled to -20°C , allyl alcohol (67 μL , 0.98 mmol, 3 eq) and $\text{BF}_3 \cdot \text{OEt}_2$ (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.⁹

^1H NMR (400 MHz, CDCl_3) δ ppm 5.95 (dddd, $J = 17.2, 10.5, 6.2, 5.3$, 1H), 5.38-5.33 (m, 2H), 5.26 (ddd, $J = 10.4, 1.4$, 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). **^{13}C NMR** (400 MHz, CDCl_3) 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. **^{13}C NMR** (400 MHz, CDCl_3) δ ppm 170.39, 170.10, 169.85, 133.02, 118.35, 101.12, 71.69, 70.68, 70.65, 66.35, 61.24, 60.82, 20.65, 20.62, 60.62. **ESI-MS** m/z calcd for $\text{C}_{15}\text{H}_{21}\text{N}_3\text{O}_8$ $[\text{M} + \text{K}]^+$: 410.10. Found: 410.17.

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

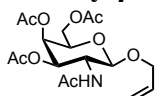


Compound was prepared from **320** as previously described.⁸

320 (1.9 g, 5.7 mmol) was dissolved in CH_2Cl_2 (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 for 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% Et_2N) 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, CDCl_3) 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). **^{13}C NMR** (400 MHz, CDCl_3) 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 $\text{C}_{14}\text{H}_{17}\text{Cl}_3\text{N}_4\text{O}_8$ $[\text{M} + \text{K}]^+$: 512.97. Found: 513.11.

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

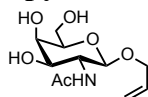


To a stirred solution of SnCl_2 (0.86 g, 3.8 mmol) in CH_3CN (8 mL), the following were added consecutively: thiophenol (1.68 g, 15.2 mmol, 1.6 mL), Et_2N (1.6 mL, 11.4

mmol), and **321** (0.94 g, 2.53 mmol) in CH₃CN (30 mL). The reaction mixture was stirred at room temperature for 30 min and then diluted with CH₂Cl₂ and washed with 10% NaOH. The aqueous phase was extracted with CH₂Cl₂ and the combined organic extracts were dried with MgSO₄ and concentrated under reduced pressure. The residue was dissolved in pyridine (23 mL) and Ac₂O (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.^{10,11}

¹H NMR (400 MHz, CDCl₃) δ 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), 3.91 (ddd, J = 0.7, 6.4 Hz, 1H), 2.13 (s, 1H), 2.04 (s, 1H), 1.99 (s, 1H), 1.95 (s, 1H). **¹³C NMR** (400 MHz, CDCl₃) δ 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 C₁₇H₂₅NO₉ [M + K]⁺: 426.12. Found: 426.10.

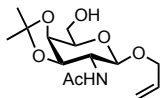
Allyl 2-acetamido-2-deoxy-β-D-galactopyranoside (**324**)



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 CH₂Cl₂:MeOH) to yield white product **324** (0.05 g, 57%). All spectral data was consistent with that reported in the literature.¹²

¹H NMR (300 MHz, D₂O) δ 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 (dddd, J = 13.2, 5.1, 1.4 Hz, 1H), 4.16 (dddd, 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). **¹³C NMR** (400 MHz, D₂O) δ 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 C₁₁H₁₉NNaO₆ [M + Na]⁺: 284.11. Found: 284.20.

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

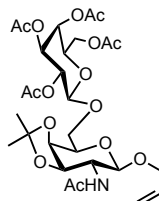


To a solution of **324** (0.16 g, 0.62 mmol) in DMF (3 mL), 2,2-dimethoxy propane (0.30 mL, 2.5 mmol) and p- toluenesulfonic acid (12 mg, 0.062 mmol) were added and the mixture was stirred at room temperature for 15 hrs. The solution was neutralized with triethyl amine, concentrated, and purified by silica column chromatography (100% EtOAc, 1% triethyl amine) to yield two major fractions, the first fraction being **325** (0.11

g, 60%).

¹H NMR (500 MHz, D₂O) δ 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 NMR** (500 MHz, D₂O) δ 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 C₁₄H₂₃NO₆ [M + H]⁺: 302.16, C₁₄H₂₃NNaO₆ [M + Na]⁺: 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)



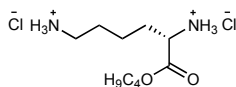
To a suspension of **325** (74 mg, 0.25 mmol) in a 1:1 chloroform: nitromethane mixture (5 mL) with 3 Å molecular sieves (0.23 g), galactopyranosyl bromide **314** (0.15 g, 0.37 mmol) was added and the mixture was stirred for 30 min at room temperature. Hg(CN)₂ (0.11 g, 0.42 mmol) was added and refluxed for 1 hr. The solution was brought to room temperature and stirred for 15 hrs after which the solution was diluted with CH₂Cl₂, washed with saturated NaHCO₃, brine, dried with MgSO₄, 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^α,*N*^ε-Bis(*t*-butyloxycarbonyl)-L-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*^α,*N*^ε-bis(*t*-butyloxycarbonyl)-L-lysine alkyl ester as a yellowish oil. The oil was suspended in 2 M HCl in anhydrous Et₂O (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 Et₂O x 4 and residual Et₂O was removed under reduced pressure to yield L-lysine alkyl ester dihydrochloride as a yellow oil.

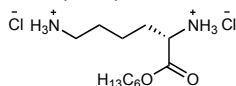
L-Lysine butyl ester dihydrochloride (404)



The procedure above was used to give compound **404** in an isolated yield of 62 %.

¹H NMR (400 MHz, *D*₂O) 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). **¹³C NMR** (400 MHz, *D*₂O) 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 C₁₀H₂₂N₂O₂ [M+H]⁺: 203.18. Found: 203.26.

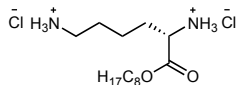
L-Lysine hexyl ester dihydrochloride (405)



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

¹H NMR (500 MHz, *D*₂O) 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, 3H). **¹³C NMR** (400 MHz, *D*₂O) 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 C₁₂H₂₆N₂O₂ [M+H]⁺: 231.21. Found: 231.24.

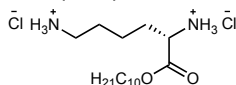
L-Lysine octyl ester dihydrochloride (406)



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

¹H NMR (400 MHz, *D*₂O) 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). **¹³C NMR** (400 MHz, *D*₂O) 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 C₁₄H₃₀N₂O₂ [M+H]⁺: 259.41. Found: 259.34.

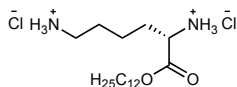
L-Lysine decyl ester dihydrochloride (407)



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

¹H NMR (400 MHz, *D*₂O) 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). **¹³C NMR** (400 MHz, *D*₂O) 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 C₁₆H₃₄N₂O₂ [M+H]⁺: 287.27. Found: 287.35.

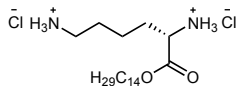
L-Lysine dodecyl ester dihydrochloride (408)



N^{α},N^{ϵ} -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 N^{α},N^{ϵ} -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, *D*₂O) 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, *D*₂O) 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, 22.10, 21.47, 13.46. **ESI-MS** *m/z* calcd for C₁₈H₃₈N₂O₂ [M+H]⁺: 315.30. Found: 315.38.

L-Lysine tetradecyl ester dihydrochloride (**409**)



N^{α},N^{ϵ} -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 N^{α},N^{ϵ} -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, *D*₂O) δ 4.23 (m, 2H), 4.11 (t, *J* = 6.4 Hz, 1H), 2.97 (t, *J* = 7.6 Hz, 2H), 1.96 (m, 2H), 1.67 (m, 4H), 1.47 (m, 2H), 1.36-1.17 (m, 22H), 0.82 (t, *J* = 6.4 Hz, 3H). **¹³C NMR** (400 MHz, *D*₂O) δ 169.96, 67.02, 52.64, 38.97, 31.72, 29.55, 29.55, 29.48, 29.46, 29.37, 29.37, 29.37, 29.17, 28.96, 27.93, 26.30, 25.39, 22.45, 21.60, 13.75. **ESI-MS** *m/z* calcd for C₂₀H₄₂N₂O₂ [M+H]⁺: 343.32. Found: 343.41.

(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 %.

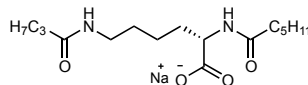
Lithium Salts:

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.

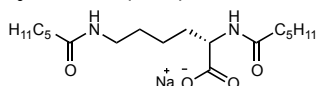
Sodium *N*^α-hexanoyl-*N*^ε-butanoyl-L-lysinate (410)



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

¹H NMR (300 MHz, D₂O) δ 4.00 (dd, *J*=9.0, 4.2 Hz, 1 H), 3.04 (t, *J*= 6.7 Hz, 2 H), 2.14 (td, *J*= 4.1, 7.4 Hz, 2 H), 2.06 (t, *J*= 7.3 Hz, 2H), 1.66 (m, 1H), 1.53-1.24 (m, 7H), 1.24-1.02 (m, 6H), 0.76 (t, *J*= 7.10 Hz, 3H), 0.85 (t, *J*= 6.5 Hz, 3H). **¹³C NMR** (300 MHz, D₂O) δ 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. **ESI-MS** *m/z* calcd for C₁₆H₂₉N₂O₄⁻ [M]⁻: 313.21. Found: 313.17.

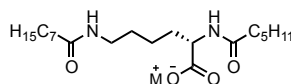
Sodium *N*^α,*N*^ε-bis(hexanoyl)-L-lysinate (411)



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

¹H NMR (500 MHz, D₂O) δ 4.10 (dd, *J*= 9.2, 4.4 Hz, 1H), 3.13 (t, *J*= 6.8 Hz, 2H), 2.24 (m, 2H), 2.17 (t, *J*= 7.4 Hz, 2H), 1.76 (m, 1H), 1.68-1.41 (m, 7H), 1.38-1.17 (m, 11H), 0.83 (t, *J*= 7.0 Hz, 3H), 0.82 (t, *J*= 7.0 Hz, 3H). **¹³C NMR** (300 MHz, D₂O) δ 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. **ESI-MS** *m/z* calcd for C₂₀H₃₇N₂O₄⁻ [M]⁻: 341.24. Found: 341.25.

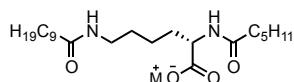
Lithium, Sodium, or Potassium *N*^α-hexanoyl-*N*^ε-octanoyl-L-lysinate (412)



The procedures above were used from the di-alkyl L-lysine **429**.

¹H NMR (500 MHz, *D*₂O) δ 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). **¹³C NMR** (400 MHz, *D*₂O) δ 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 C₂₀H₃₇N₂O₄[−] [M][−]: 369.28. Found: 369.25.

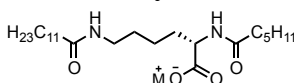
Lithium, Sodium, or Potassium *N*^α-hexanoyl-*N*^ε-decanoyl-L-lysinate (413)



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

¹H NMR (500 MHz, *D*₂O) δ 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), 1.26-0.96 (m, 19H), 0.85 (bt, 3H), 0.81 (bt, 3H). **¹³C NMR** (500 MHz, *D*₂O) δ 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 C₂₂H₄₁N₂O₄[−] [M][−]: 397.31. Found: 397.24.

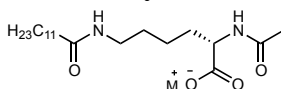
Lithium, Sodium, or Potassium *N*^α-hexanoyl-*N*^ε-dodecanoyl-L-lysinate (414)



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

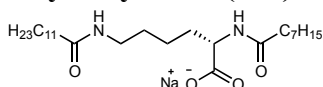
¹H NMR (400 MHz, *D*₂O) δ 4.15 (dd, *J* = 9.4, 3.8 Hz, 1H), 3.16 (m, 2H), 2.28 (m, 2H), 2.19 (t, *J* = 7.4 Hz, 2H), 1.80 (m, 1H), 1.73-1.42 (m, 7H), 1.42-0.12 (m, 22H), 0.87 (t, *J* = 6.9 Hz, 3H), 0.83 (t, *J* = 6.9 Hz, 3H). **¹³C NMR** (500 MHz, *D*₂O) δ 179.27, 175.26, 174.96, 54.64, 38.82, 36.17, 36.07, 31.94, 31.77, 31.42, 30.00, 29.90, 29.79, 29.50, 29.03, 29.01, 28.26, 26.08, 25.93, 22.80, 22.57, 22.55, 13.92, 13.67. **ESI-MS** *m/z* calcd for C₂₄H₄₅N₂O₄[−] [M][−]: 425.34. Found: 425.33.

Lithium, Sodium, or Potassium *N*^α-ethanoyl-*N*^ε-dodecanoyl-L-lysinate (415)



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

Sodium *N*^α-octanoyl-*N*^ε-dodecanoyl-L-lysinate (416)

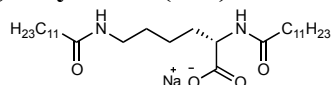


The procedure above was used from the di-alkyl L-lysine **437**.

¹H NMR (500 MHz, *D*₂O) δ 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_2O) δ 179.27, 175.26, 174.96, 54.64, 38.82, 36.17, 36.07, 31.94, 31.77, 31.42, 30.00, 29.90, 29.79, 29.50, 29.50, 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 $\text{C}_{26}\text{H}_{49}\text{N}_2\text{O}_4^-$ [M] $^-$: 453.37. Found: 453.37.

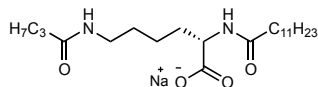
Sodium N^α, N^ϵ -bis(dodecanoyl)-L-lysinate (417)



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

^1H NMR (300 MHz, $D_2O + 10\%$ CD_3OD) 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, $D_2O + 10\%$ CD_3OD) δ 179.19, 176.23, 175.40, 55.99, 40.35, 37.48, 37.25, 33.87, 33.14, 30.84, 30.82, 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 $\text{C}_{30}\text{H}_{57}\text{N}_2\text{O}_4^-$ [M] $^-$: 509.43. Found: 509.42.

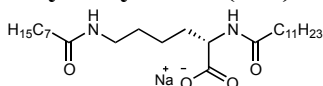
Sodium N^α -dodecanoyl- N^ϵ -butanoyl-L-lysinate (418)



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

^1H NMR (500 MHz, D_2O) δ ppm 4.14 (dd, $J = 8.8, 3.8$ Hz, 1H), 3.14 (m, 2H), 2.25 (t, $J = 7.4$ Hz, 2H), 1.77 (m, 1H), 1.55-1.24 (m, 7H), 1.25-0.96 (m, 18H), 0.87 (t, $J = 7.4$ Hz, 3H), 0.82 (t, $J = 6.6$ Hz, 3H). ^{13}C NMR (300 MHz, D_2O) δ ppm 179.15, 175.59, 175.22, 54.66, 38.87, 37.83, 36.06, 31.81, 31.47, 29.67, 29.61, 29.61, 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 $\text{C}_{22}\text{H}_{41}\text{N}_2\text{O}_4^-$ [M] $^-$: 397.31. Found: 397.34.

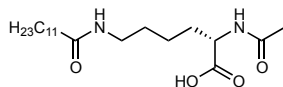
Sodium N^α -dodecanoyl- N^ϵ -octanoyl-L-lysinate (419)



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

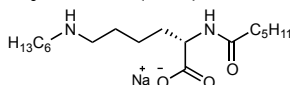
^1H NMR (500 MHz, D_2O) δ 4.13 (dd, $J = 8.7, 3.3$ Hz, 1H), 3.15 (m, 2H), 2.26 (m, 2H), 2.18 (t, $J = 7.4$ Hz, 2H), 1.79 (m, 1H), 1.70-1.40 (m, 7H), 1.40-1.10 (m, 26H), 0.85 (t, $J = 7.0$ Hz, 3H), 0.82 (t, $J = 6.6$ Hz, 3H). ^{13}C NMR (500 MHz, D_2O) δ 179.25, 175.30, 175.10, 54.62, 38.75, 36.16, 36.07, 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 $\text{C}_{26}\text{H}_{49}\text{N}_2\text{O}_4^-$ [M] $^-$: 453.37. Found: 453.43.

N^α -ethanoyl- N^ϵ -dodecanoyl-L-lysine (420)



Compound was prepared as previously described.¹³ All spectral data was consistent with that reported in the literature.¹³

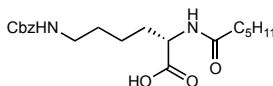
Sodium *N*^α-hexanoyl-*N*^ε-hexyl-L-lysinate (421)



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

¹H NMR (500 MHz, *D*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). **¹³C NMR** (500 MHz, *D*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 C₁₈H₃₅N₂O₃[−] [*M*][−]: 327.27. Found: 327.21.

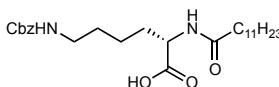
N^α-Hexanoyl-*N*^ε-carboxybenzyl-L-lysine (423)



N^α-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 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, *CDCl*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). **¹³C NMR** (400 MHz, *CDCl*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 C₂₀H₃₀N₂O₅ [*M*+*H*]⁺: 379.23. Found: 379.33.

N^α-Dodecanoyl-*N*^ε-carboxybenzyl-L-lysine (424)

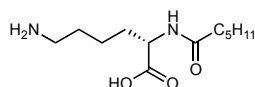


N^ε-Carboxybenzyl-L-lysine **422** (0.31 g, 1.09 mmol) was dissolved in 10 % NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0 °C and dodecanoyl chloride (0.38 mL, 1.64 mmol, 1.5 eq) was added slowly to the ether layer with no mixing. The biphasic 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, (CD₃)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), 1.57 (m, 1H), 1.49 (m, 2H), 1.42 (m, 2H), 1.35-1.20 (m, 18H), 0.86 (t, *J* = 6.89 Hz, 3H). **¹³C NMR** (400 MHz, CDCl₃) δ 174.99, 174.51, 156.95, 136.44, 128.53, 128.14, 128.00, 127.91, 66.74, 52.14, 40.45, 36.37, 31.93, 31.59, 31.45, 29.66, 29.65, 29.56, 29.37, 29.37, 29.30, 25.70, 22.70, 22.24, 22.05, 14.14. **ESI-MS** *m/z* calcd for C₂₆H₄₂N₂O₅ [M+H]⁺: 363.32. Found: 463.47.

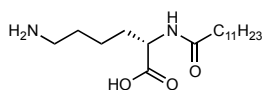
***N*^α-Hexanoyl-L-lysine (425)**



N^α-Hexanoyl-*N*^ε-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, 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.

***N*^α-Dodecanoyl-L-lysine (426)**



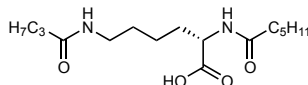
N^α-Dodecanoyl-*N*^ε-carboxybenzyl-L-lysine (**424**) (0.12 g, 0.25 mmol) was dissolved in MeOH (final concentration of 0.1 M) and Pd(OH)₂ on carbon (5 mg) was added. H₂ atmosphere was introduced into the flask and the mixture was stirred for 16 hours at room temperature after which the solution was filtered through a Celite pad and concentrated under reduced pressure to yield a white powder (**426**) (0.079 g, 0.21 mmol, 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, (CD₃)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^α-Alkyl-L-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 MgSO₄, 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.

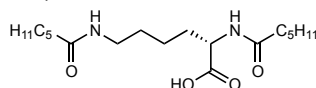
N^α-Hexanoyl-*N*^ε-butanoyl-L-lysine (**427**)



The procedure above was used with *N*^α-hexanoyl-L-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%).

¹H NMR (300 MHz, CDCl₃) δ 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 Hz, 2H), 2.18 (t, *J* = 7.5 Hz, 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). **¹³C NMR** (400 MHz, CDCl₃) δ 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 C₁₆H₃₀N₂O₄ [M + H]⁺: 315.23. Found: 315.25.

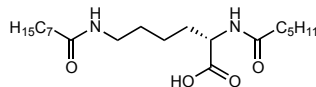
N^α,*N*^ε-Bis(hexanoyl)-L-lysine (**428**)



The procedure above was used with *N*^α-hexanoyl-L-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%).

¹H NMR (300 MHz, CDCl₃) δ 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). **¹³C NMR** (400 MHz, CDCl₃) δ 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 C₁₈H₃₄N₂O₄ [M + H]⁺: 343.26. Found: 343.3.

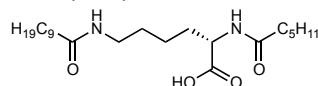
N^α-Hexanoyl-*N*^ε-octanoyl-L-lysine (**429**)



The procedure above was used with *N*^α-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 NMR (500 MHz, *CDCl*₃) δ 6.80 (d, *J* = 6.5 Hz, 1H), 5.82 (m, 1H), 4.52 (td, *J* = 7.3, 4.8, 4.8 Hz, 1H), 3.36 (m, 1H), 3.18 (m, 1H), 2.35 (t, *J* = 7.5 Hz, 1H), 2.27 (td, *J* = 7.1, 1.1 Hz, 2H), 2.19 (t, *J* = 7.6 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). **¹³C NMR** (400 MHz, *CDCl*₃) δ 174.59, 174.59, 174.32, 52.13, 38.93, 36.68, 36.37, 31.70, 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.

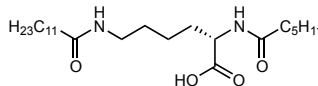
***N*^α-Hexanoyl-*N*^ε-decanoyl-L-lysine (**430**)**



The procedure above was used with *N*^α-hexanoyl-L-lysine (**425**) (0.022 g, 0.090 mmol) decanoyl chloride (28 μL, 0.13 mmol) to yield **430** (0.027 g, 0.067 mmol, 75%).

¹H NMR (500 MHz, *CDCl*₃) δ 6.83 (bd, *J* = 5.2 Hz, 1H), 6.18 (bs, 1H), 4.45 (bd, *J* = 4.8 Hz, 1H), 3.57 (q, *J* = 7.2, 7.2, 7.2 Hz, 2H), 3.21 (m, 2H), 2.22 (t, *J* = 7.7, 7.7 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 Hz, 3H), 0.87 (t, *J* = 6.7 Hz, 3H). **¹³C NMR** (400 MHz, *CDCl*₃) δ 173.69, 173.41, 173.37, 57.94, 39.18, 36.78, 36.73, 31.88, 31.51, 29.51, 29.43, 29.31, 28.61, 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.

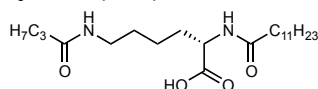
***N*^α-Hexanoyl-*N*^ε-dodecanoyl-L-lysine (**431**)**



The procedure above was used with *N*^α-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 NMR (300 MHz, *CDCl*₃) δ 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 Hz, 6H). **¹³C NMR** (300 MHz, *CDCl*₃) δ 174.29, 174.13, 174.13, 38.93, 36.64, 36.28, 31.87, 31.47, 29.65, 29.61, 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.

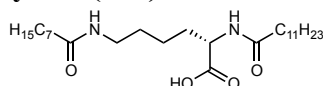
***N*^α-Dodecanoyl-*N*^ε-butanoyl-L-lysine (**432**)**



The procedure above was used with *N*^α-dodecanoyl-L-lysine (**426**) (0.010 g, 0.030 mmol) and butyryl chloride (5 μL, 0.046 mmol) to yield **432** (0.011 g, 0.027 mmol, 87%).

¹H 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). **¹³C 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 C₂₂H₄₂N₂O₄ [M + H]⁺: 399.32. Found: 399.42.

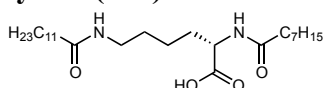
***N*^α-Dodecanoyl-*N*^ε-octanoyl-L-lysine (433)**



The procedure above was used with *N*^α-dodecanoyl-L-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%).

¹H 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). **¹³C NMR** (400 MHz, *CDCl*₃) δ 174.74, 174.58, 173.96, 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 C₂₆H₅₀N₂O₄ [M + H]⁺: 455.39. Found: 455.52.

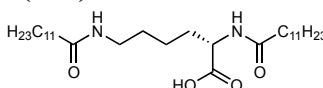
***N*^α-Octanoyl-*N*^ε-dodecanoyl-L-lysine (437)**



The procedure above was used with *N*^ε-dodecanoyl-L-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%).

¹H 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). **¹³C NMR** (400 MHz, *CDCl*₃) δ 174.60, 174.51, 174.29, 52.10, 38.83, 36.69, 36.40, 31.87, 31.68, 31.39, 29.66, 29.62, 29.59, 29.51, 29.36, 29.31, 29.27, 28.99, 28.86, 25.83, 25.68, 22.64, 22.58, 22.11, 14.08, 14.03. **ESI-MS** *m/z* calcd for C₂₆H₅₀N₂O₄ [M + H]⁺: 455.39. Found: 455.44.

***N*^α,*N*^ε-Bis(dodecanoyl)-L-lysine (438)**

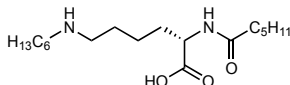


The procedure above was used with *N*^ε-dodecanoyl-L-lysine (**434**) (33 mg, 0.10 mmol) and dodecanoyl chloride (35 μL, 0.15 mmol) to yield **438** (0.045 g, 0.088 mmol, 87%).

¹H 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). ^{13}C NMR (300 MHz, CDCl_3) δ 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, 29.36, 29.35, 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 $\text{C}_{30}\text{H}_{58}\text{N}_2\text{O}_4$ $[\text{M} + \text{H}]^+$: 511.45. Found: 511.58.

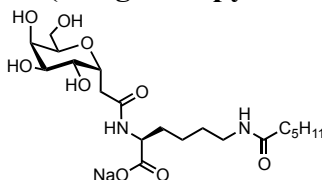
***N*^α-Hexanoyl-*N*^ε-hexyl-L-lysine (439)**



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), $\text{Pd}(\text{OH})_2$ on carbon (3 mg). The mixture was subjected to 40 bar of H_2 in a Parr Bomb while stirring at 50 $^\circ\text{C}$ for 18 hrs. The mixture was cooled to room temperature for 1 hr before opening Parr Bomb to atmosphere. The $\text{Pd}(\text{OH})_2$ 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_2O : CH_3CN , R_f = 0.3) to yield **439** as a yellowish oil (8.6 mg, 0.026 mmol, 33%).

^1H NMR (300 MHz, CDCl_3) δ 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) δ 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 $\text{C}_{18}\text{H}_{36}\text{N}_2\text{O}_3$ $[\text{M} + \text{H}]^+$: 329.28. Found: 329.29.

Sodium *N*^ε-hexanoyl-*L*-lysinate-1-(α -D-galactopyranoside) (440)

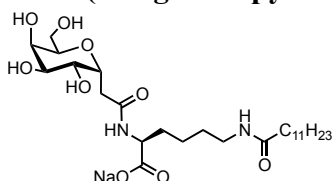


Benzyl *N*^ε-hexanoyl-*L*-lysine-1-(2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranoside) (**457**) (0.19 g, 0.26 mmol) was dissolved in MeOH (2 mL) and 0.1 M NaOMe was added dropwise until the solution was ca. pH = 12. The reaction mixture was stirred at room temperature for 14 hrs after which the reaction was neutralized with Dowex 50 (H^+) resin, filtered, and concentrated under reduced pressure to yield benzyl *N*^ε-dodecanoyl-*L*-lysine-1-(α -D-galactopyranoside). The residue was dissolved in MeOH (5 mL) and $\text{Pd}(\text{OH})_2$ 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*^ε-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).

^1H NMR (500 MHz, D_2O) δ 4.51 (dt, J = 10.0, 4.9, 1H), 4.18 (dd, J = 7.8, 4.9, 1H), 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_2O) δ 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. ESI-MS m/z calcd for $\text{C}_{20}\text{H}_{35}\text{N}_2\text{O}_9^-$ $[\text{M}]^-$: 447.23. Found: 447.34.

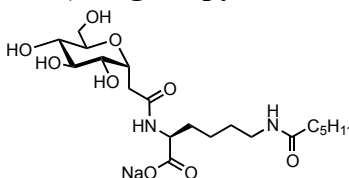
Sodium *N*^ε-dodecanoyl-*L*-lysinate-1-(α -D-galactopyranoside) (441)



Benzyl *N*^ε-dodecanoyl-*L*-lysine-1-(2,3,4,6-tetra-*O*-acetyl- α -D-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*^ε-dodecanoyl-*L*-lysine-1-(α -D-galactopyranoside). The residue was dissolved in MeOH (3 mL) and $\text{Pd}(\text{OH})_2$ 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*^ε-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 **441** as a white powder (0.19 g, 0.034 mmol, 87% yield).

^1H NMR (300 MHz, D_2O) δ 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) δ 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. ESI-MS m/z calcd for $\text{C}_{26}\text{H}_{48}\text{N}_2\text{O}_9$ $[\text{M} + \text{H}]^+$: 532.34. Found 532.17.

Sodium *N*^ε-hexanoyl-*L*-lysinate-1-(α -D-glucopyranoside) (442)

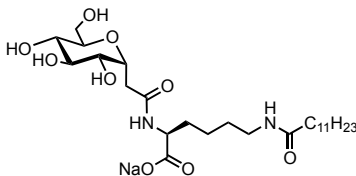


Benzyl *N*^ε-hexanoyl-*L*-lysine-1-(2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranoside) (**459**) (0.12 g, 0.17 mmol) was dissolved in MeOH (2 mL) and 0.1 M NaOMe was added dropwise until the solution was ca. pH = 12. The reaction mixture was stirred at room temperature for 14 hrs after which the reaction was neutralized with Dowex 50 (H^+) resin, filtered, and concentrated under reduced pressure to yield benzyl *N*^ε-hexanoyl-*L*-lysine-1-(α -D-glucopyranoside). The residue was dissolved in MeOH (5 mL) and $\text{Pd}(\text{OH})_2$ 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*^ε-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).

¹H NMR (300 MHz, *MeOD*) δ 4.33 (dq, *J* = 11.8, 3.8, 1H), 4.18 (dd, *J* = 7.5, 4.8, 1H), 3.71 (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). **¹³C NMR** (500 MHz, *CDCl*₃) δ 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. **¹³C NMR** (300 MHz, *MeOD*): δ 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. **ESI-MS** *m/z* calcd for C₂₀H₃₅N₂O₉⁻ [*M*]⁻: 447.23. Found: 446.99.

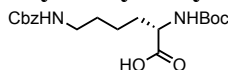
Sodium *N*^ε-dodecanoyl-*L*-lysinate-1-(α-D-glucopyranoside) (443**)**



Benzyl *N*^ε-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 benzyl *N*^ε-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 hours at room temperature after which the solution was filtered through a Celite pad and concentrated under reduced pressure to yield *N*^ε-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).

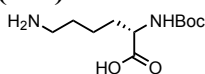
¹H NMR (500 MHz, *MeOD*) δ 4.37-4.33 (m, 2H), 3.71 (dd, *J* = 11.8, 2.5, 1H), 3.63 (dd, *J* = 11.8, 5.4, 1H), 3.56 (dd, *J* = 9.3, 5.9, 1H), 3.50 (ddd, *J* = 8.9, 5.6, 2.9, 1H), 3.43 (t, *J* = 8.9, 1H), 3.10 (t, *J* = 6.9, 2H), 2.63 (dd, *J* = 15.0, 10.6, 1H), 2.53 (dd, *J* = 14.9, 3.9, 1H), 2.10 (t, *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). **¹³C NMR** (500 MHz, *MeOD*) δ 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. **ESI-MS** *m/z* calcd for C₂₆H₄₇N₂O₉⁻ [*M*]⁻: 531.33. Found: 531.17

***N*^α-(*tert*-Butoxycarbonyl)-*N*^ε-carboxybenzyl-L-lysine (444)**



N^ε-Carboxybenzyl-L-lysine **422** (8.2 g, 39.4 mmol) was suspended in 10% Et₃N in MeOH (200 mL) and di-*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.

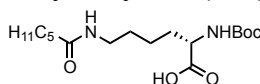
***N*^α-(*tert*-Butoxycarbonyl)-L-lysine (445)**



N^α-(*tert*-Butoxycarbonyl)-*N*^ε-carboxybenzyl-L-lysine (**444**) (11 g, 28.8 mmol) was dissolved in MeOH (final concentration of 0.1 M) and Pd(OH)₂ on carbon (1.1 g) was added. H₂ atmosphere was introduced into the flask and the mixture was stirred for 16 hours at ambient temperature after which the solution was filtered through a Celite pad and concentrated under reduced pressure to yield **445** (7.0 g, 28.2 mmol, 98% yield).

¹H NMR (300 MHz, CDCl₃) δ 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). ¹³C NMR (300 MHz; CDCl₃) δ 176.6, 157.7, 81.4, 53.6, 39.2, 30.1, 27.7, 27.7, 27.7, 26.2, 22.1. ESI-MS *m/z* calcd for C₁₁H₂₃N₂O₃⁺ [M+H]⁺: 247.17. Found: 247.20.

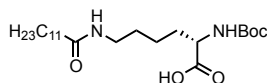
***N*^α-(*tert*-Butoxycarbonyl)-*N*^ε-hexanoyl-L-lysine (446)**



N^α-(*tert*-Butoxycarbonyl)-L-lysine (**445**) (1.0 g, 4.1 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 (0.85 mL, 6.1 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 CH₂Cl₂ 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 (5:5 petroleum ether:EtOAc + 1% AcOH to 6:3:1 EtOAc:petroleum ether:MeOH + 1% AcOH) to yield **446** as a white product (1.4 g, 3.9 mmol, 87% yield).

¹H NMR (400 MHz, CDCl₃) δ 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). ¹³C NMR (400 MHz, CDCl₃) δ 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. ESI-MS *m/z* calcd for C₁₇H₃₃N₂O₅⁺ [M+H]⁺: 345.24. Found: 345.27.

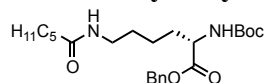
***N*^α-(*tert*-Butoxycarbonyl)-*N*^ε-dodecanoyl-*L*-lysine (447)**



N^α-(*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 CH₂Cl₂ 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 (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).

¹H NMR (500 MHz, CDCl₃) 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). ¹³C NMR (400 MHz, CDCl₃) 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, 29.48, 29.45, 29.0, 28.5, 28.5, 26.0, 22.8, 22.5, 14.3. ESI-MS *m/z* calcd for C₂₃H₄₄N₂O₅ + NH₄-CH₃CN⁺ complex (ammonia-acetonitrile) [M+NH₄-CH₃CN]⁺: 487.39. Found: 487.48.

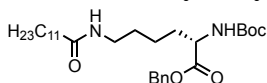
Benzyl *N*^α-(*tert*-butoxycarbonyl)-*N*^ε-hexanoyl-*L*-lysine (448)



N^α-(*tert*-Butoxycarbonyl)-*N*^ε-hexanoyl-*L*-lysine (**446**) (1.4 g, 4.0 mmol) was dissolved in anhydrous CH₂Cl₂ (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).

¹H NMR (300 MHz, CDCl₃) 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). ¹³C NMR (300 MHz, CDCl₃) 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 C₂₄H₃₈N₂NaO₅⁺ [M+Na]⁺: 457.27. Found: 457.33.

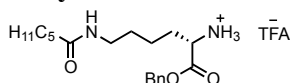
Benzyl *N*^α-(*tert*-butoxycarbonyl)-*N*^ε-dodecanoyl-*L*-lysine (449)



N^α-(*tert*-Butoxycarbonyl)-*N*^ε-dodecanoyl-*L*-lysine (**447**) (0.84 g, 1.6 mmol) was dissolved in anhydrous CH₂Cl₂ (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).

¹H NMR (300 MHz, CDCl₃) δ 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), 1.52-1.36 (m, 11H), 1.39-1.18 (m, 18H), 0.87 (t, J = 6.6, 3H). ¹³C NMR (500 MHz, CDCl₃) δ 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, 29.5, 29.5, 29.1, 28.5, 28.5, 28.5, 26.0, 22.84, 22.67, 14.3 ESI-MS *m/z* calcd for C₃₀H₅₁N₂O₅⁺ [M+H]⁺: 519.38. Found: 419.23.

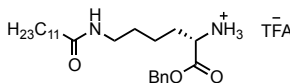
Benzyl *N*^ε-hexanoyl-*L*-lysine monohydrotrifluoro acetate (**450**)



Benzyl *N*^α-(*tert*-butoxycarbonyl)-*N*^ε-hexanoyl-*L*-lysine (**448**) (1.5 g, 3.4 mmol) was dissolved in CH₂Cl₂ (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.

¹H NMR (500 MHz, CDCl₃) δ 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). ¹³C NMR (400 MHz, CDCl₃) δ 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. ESI-MS *m/z* calcd for C₁₉H₃₁N₂O₃⁺ [M+H]⁺: 335.23. Found: 335.26.

Benzyl *N*^ε-dodecanoyl-*L*-lysine monohydrotrifluoro acetate (**451**)

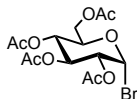


Benzyl *N*^α-(*tert*-butoxycarbonyl)-*N*^ε-hexanoyl-*L*-lysine (**449**) (0.015 g, 0.035 mmol) was dissolved in CH₂Cl₂ (5 mL), and trifluoroacetic acid was added dropwise until the solution became ca. pH = 1. The mixture was stirred at ambient temperature for 2 hours or until completion of reaction was 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).

¹H NMR (300 MHz, MeOD) δ 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). ¹³C NMR (500 MHz, CDCl₃) δ 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, 29.62, 29.46, 29.46, 29.44, 29.35, 28.5, 26.0, 22.8, 21.8, 14.2. ESI-MS *m/z* calcd for C₂₅H₄₃N₂O₃⁺ [M]⁺:

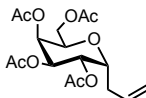
419.33. Found: 419.19.

2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranosyl bromide (452)



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

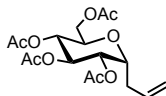
Allyl 2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranoside (453)



Allyl 2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranoside (**314**) (14.3 g, 34.8 mmol) was dissolved in anhydrous CH₂Cl₂ under argon and allyl tributyl tin (27 mL, 86.9 mmol, 2.5 eq) and 1M Et₂B (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 CH₃CN and pentanes. The CH₃CN 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).

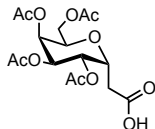
¹H NMR (500 MHz, CDCl₃) δ 5.69 (ddt, *J* = 16.8, 10.0, 6.8, 1H), 5.28 (t, *J* = 9.1, 1H), 5.15-5.01 (m, 3H), 4.92 (t, *J* = 9.1, 1H), 4.22 (dt, *J* = 10.7, 5.3, 1H), 4.15 (dd, *J* = 12.2, 5.3, 1H), 4.02 (dd, *J* = 12.1, 2.1, 1H), 3.81 (ddd, *J* = 8.9, 5.6, 2.7, 1H), 2.53-2.47 (m, 1H), 2.28 (dt, *J* = 15.3, 5.1, 1H), 2.02 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.98 (s, 3H). **¹³C NMR** (400 MHz, CDCl₃): 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 C₁₇H₂₄NaO₉⁺ [*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.⁵ All spectral data was consistent with that reported in the literature.⁵

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

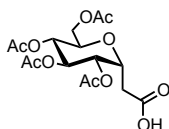


Allyl 2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranoside (**453**) was dissolved in CH₂Cl₂ and CH₃CN and H₂O was added in the following proportions: 2:2:3 CH₂Cl₂:CH₃CN:H₂O for a total volume of 32 mL. Sodium periodate (7.7 g, 36.1 mmol, 6 eq) and ruthenium trichloride (0.037 g, 0.18 mmol, 0.03 eq) were 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).

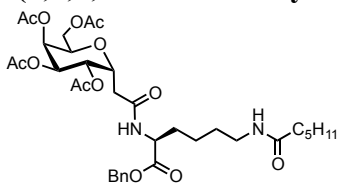
¹H NMR (500 MHz, CDCl₃) δ 5.39 (dd, J = 3.0, 1H), 5.30 (dd, J = 9.0, 5.1, 1H), 5.15 (dd, J = 9.0, 3.3, 1H), 4.68 (ddd, J = 8.8, 5.3, 1H), 4.20 (dd, J = 10.9, 7.1, 1H), 4.13 (td, J = 6.0, 2.7, 1H), 4.08 (dd, J = 10.9, 5.0, 1H), 2.69 (dd, J = 15.6, 8.9, 1H), 2.61 (dd, J = 15.6, 5.6, 1H), 2.10 (s, 3H), 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, 9H). **¹³C NMR** (500 MHz, CDCl₃) δ 175.6, 170.9, 170.2, 169.66, 169.53, 169.53, 70.16, 70.06, 69.6, 69.3, 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 *N*^ε-hexanoyl-*L*-lysine-1-(2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranoside) (**457**)

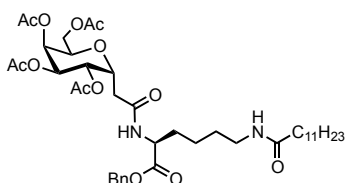


2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl acid (**455**) (0.73 g, 1.8 mmol) was dissolved in anhydrous CH₂Cl₂ (18 mL) under argon, and 1,1'-Carbonyldiimidazole (CDI) (0.32 g, 2.0 mmol, 1.1 eq) was added, and the mixture was stirred at ambient temperature for 30 min. Diisopropylethylamine (0.66 mL, 3.8 mmol, 2.1 eq) and benzyl *N*^ε-hexanoyl-*L*-lysine monohydrotrifluoro acetate (**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 $\text{C}_{35}\text{H}_{51}\text{N}_2\text{O}_{13}^+$ $[\text{M}+\text{H}]^+$: 707.34. Found: 707.44.

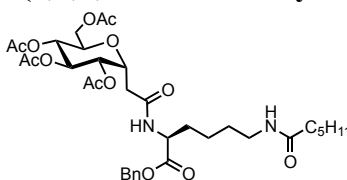
Benzyl N^ϵ -dodecanoyl-*L*-lysine-1-(2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranoside) (458)



2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl acid (**455**) (0.156 g, 0.40 mmol) was dissolved in anhydrous CH_2Cl_2 (5 mL) under argon, and diisopropylethylamine (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. Diisopropylethylamine (0.14 mL, 0.88 mmol, 2.1 eq) and benzyl N^ϵ -dodecanoyl-*L*-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).

^1H 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 173.9, 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, 29.74, 29.72, 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 $\text{C}_{41}\text{H}_{63}\text{N}_2\text{O}_{13}^+$ $[\text{M}+\text{H}]^+$: 791.43. Found: 791.21.

Benzyl N^ϵ -hexanoyl-*L*-lysine-1-(2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranoside) (459)

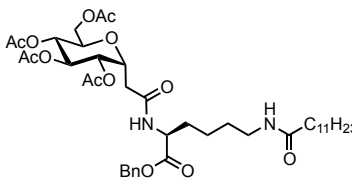


2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranosyl acid (**456**) (0.24 g, 0.61 mmol) was dissolved in anhydrous CH_2Cl_2 (5 mL) under argon, and 1,1'-Carbonyldiimidazole (CDI) (0.11 g, 0.67 mmol, 1.1 eq) was added, and the mixture was stirred at ambient temperature for 30 min. Diisopropylethylamine (0.44 mL, 1.2 mmol, 2.1 eq) and benzyl N^ϵ -hexanoyl-*L*-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).

¹H NMR (500 MHz, *CDCl*₃) δ 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). **¹³C NMR** (500 MHz, *CDCl*₃) δ 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 **ESI-MS** *m/z* calcd for C₃₅H₅₀N₂O₁₃⁺ [M+H]⁺: 707.33. Found: 707.21.

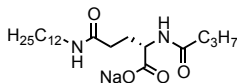
Benzyl *N*^c-dodecanoyl-*L*-lysine-1-(2,3,4,6-tetra-*O*-acetyl-α-*D*-glucopyranoside) (460)



2,3,4,6-tetra-*O*-acetyl-α-*D*-glucopyranosyl acid (**456**) (0.14 g, 0.36 mmol) was dissolved in anhydrous CH₂Cl₂ (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*^c-dodecanoyl-*L*-lysine monohydrotrifluoro acetate (**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).

¹H NMR (500 MHz, *CDCl*₃) δ 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), 2.03 (s, 3H), 1.83 (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). **¹³C NMR** (500 MHz, *CDCl*₃) δ 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, 29.49, 29.45, 29.45, 29.43, 29.0, 26.0, 22.8, 22.3, 20.88, 20.81, 20.79, 14.2. **ESI-MS** *m/z* calcd for C₄₁H₆₃N₂O₁₃⁺ [M+H]⁺: 791.43. Found: 791.30.

Sodium *N*^α-butanoyl-*N*^δ-dodecyl-*L*-glutamate (469)

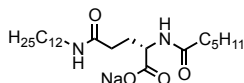


N^α-butanoyl-*N*^δ-dodecyl-*L*-glutamine (**480**) (0.023 g, 0.06 mmol) was dissolved in MeOH

(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 NMR (500 MHz, *D*₂O) δ 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, *D*₂O) 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.

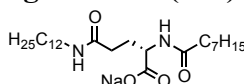
Sodium *N*^α-hexanoyl-*N*^δ-dodecyl-*L*-glutamate (**470**)



N^α-hexanoyl-*N*^δ-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 NMR (500 MHz, *D*₂O) δ 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, *D*₂O) δ 178.4, 174.6, 167.9, 54.6, 39.5, 35.9, 32.6, 31.9, 31.1, 30.21, 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.

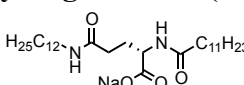
Sodium *N*^α-octanoyl-*N*^δ-dodecyl-*L*-glutamate (**471**)



N^α-octanoyl-*N*^δ-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 NMR (500 MHz, *D*₂O) δ 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; *D*₂O): δ 178.3, 175.3, 174.5, 54.6, 39.5, 36.0, 32.6, 32.0, 31.7, 30.10, 30.04, 30.04, 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*^α-dodecanoyl-*N*^δ-dodecyl-*L*-glutamate (**472**)

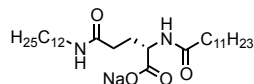


N^α-dodecanoyl-*N*^δ-dodecyl-*L*-glutamine (**483**) (0.032 g, 0.064 mmol) was dissolved in

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).

¹H NMR (500 MHz, *CDCl*₃) δ 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). **¹³C NMR** (500 MHz, *MeOD*) δ 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, 30.73, 30.68, 30.53, 30.56, 30.50, 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 C₂₉H₅₅N₂O₄[−] [*M*][−]: 495.42. Found: 495.44.

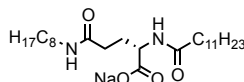
Sodium *N*^α-dodecanoyl-*N*^δ-dodecyl-*L*-glutamate (**473**)



N^α-dodecanoyl-*N*^δ-dodecyl-*L*-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).

¹H NMR (500 MHz, *D*₂*O*) δ 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* = 6.2 Hz, 3H), 0.71 (t, *J* = 7.0 Hz, 3H). **¹³C NMR** (400 MHz, *D*₂*O*) δ 178.3, 175.4, 174.7, 54.7, 39.6, 36.1, 32.7, 31.91, 31.71, 29.50, 29.38, 29.06, 29.06, 29.01, 28.0, 27.0, 25.7, 22.57, 22.55, 13.82, 13.70. **ESI-MS** *m/z* calcd for C₂₁H₃₉N₂O₄[−] [*M*][−]: 383.29. Found: 383.29.

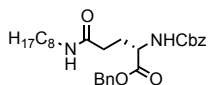
Sodium *N*^α-dodecanoyl-*N*^δ-octyl-*L*-glutamate (**474**)



N^α-dodecanoyl-*N*^δ-octyl-*L*-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 (0.075 g, 0.016 mmol, 98% yield).

¹H NMR (500 MHz, *D*₂*O*) δ 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). **¹³C NMR** (400 MHz, *D*₂*O*) δ 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 C₂₅H₄₇N₂O₄[−] [*M*][−]: 439.35. Found: 439.36.

Benzyl *N*^α-(carboxybenzyl)-*N*^δ-octyl-*L*-glutamine (**476**)

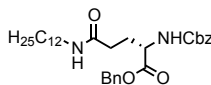


To a solution of anhydrous CH₂Cl₂ and benzyl *N*^α-(carboxybenzyl)-*L*-glutamic acid (**475**)

(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 NMR (300 MHz, *CDCl*₃) δ 7.27 (m, 10H), 5.90 (m, *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 NMR** (400 MHz, *CDCl*₃) δ 171.91, 171.79, 156.4, 136.3, 135.3, 128.67, 128.54, 128.52, 128.37, 128.20, 128.08, 67.3, 67.0, 53.8, 39.7, 32.5, 31.8, 29.6, 29.30, 29.26, 28.5, 27.0, 22.7, 14.2. **ESI-MS** *m/z* calcd for C₂₈H₃₉N₂O₅⁺ [M+H]⁺: 483.29. Found: 483.31.

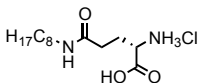
Benzyl *N*^α-(carboxybenzyl)-*N*^δ-dodecyl-*L*-glutamine (**477**)



To a solution of anhydrous CH₂Cl₂ and benzyl *N*^α-(carboxybenzyl)-*L*-glutamic acid (**475**) (0.061 g, 0.16 mmol) under argon and *N,N'*-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 NMR (500 MHz, *CDCl*₃) δ 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 NMR** (300 MHz, *CDCl*₃) δ 171.91, 171.73, 156.4, 136.3, 135.3, 128.76, 128.64, 128.51, 128.30, 128.19, 67.4, 67.2, 53.8, 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 C₃₂H₄₇N₂O₅⁺ [M+H]⁺: 539.35. Found: 539.31.

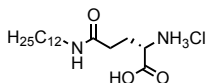
N^δ-octyl-*L*-glutamine monohydrochloride (**478**)



Benzyl *N*^α-(carboxybenzyl)-*N*^δ-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 H₂. 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 NMR (300 MHz, *MeOD*) δ 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 NMR** (400 MHz, *MeOD*) δ 174.0, 171.5, 53.6, 40.6, 33.0, 32.6, 30.39, 30.37, 30.30, 28.0, 27.4, 23.7, 14.4. **ESI-MS** *m/z* calcd for C₁₃H₂₇N₂O₃⁺ [M+H]⁺: 259.20. Found: 259.22.

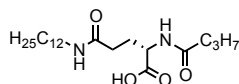
N^δ-dodecyl-*L*-glutamine monohydrochloride (**479**)



Benzyl *N*^α-(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 H₂. 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).

¹H NMR (300 MHz, MeOD) δ 3.90 (t, J = 6.2, 1H), 3.18 (dt, J = 3.2, 1.6, 1H), 3.04 (t, J = 7.0, 2H), 2.35 (t, J = 7.2, 2H), 2.13-1.96 (m, 2H), 1.37 (t, J = 6.4, 2H), 1.10-1.08 (m, 18H), 0.77 (t, J = 6.6, 3H). ¹³C NMR (300 MHz, CDCl₃) δ 170.72, 170.63, 51.6, 31.3, 30.7, 29.1, 29.1, 29.0, 29.0, 29.0, 28.80, 28.75, 26.5, 26.1, 22.1, 14.0. ESI-MS *m/z* calcd for C₁₇H₃₅N₂O₃⁺ [M+H]⁺: 315.26. Found: 315.15.

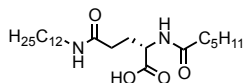
N^α-butanoyl-*N*^δ-dodecyl-*L*-glutamine (**480**)



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 μL, 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 MgSO₄, and concentrated under reduced pressure to yield **480** (0.022 g, 0.056 mmol, 90%).

¹H NMR (500 MHz, CDCl₃) δ 7.45 (d, J = 5.3, 1H), 6.22 (m, 1H), 4.40 (q, J = 5.6, 1H), 3.26 (m, 2H), 2.66 (m, 1H), 2.45 (m, 1H), 2.24 (t, J = 7.5, 2H), 2.13 (m, 1H), 2.06 (m, 1H), 1.67 (sextet, J = 7.4, 2H), 1.51 (t, J = 6.9, 2H), 1.29-1.21 (m, 18H), 0.95 (t, J = 7.4, 3H), 0.88 (t, J = 7.0, 3H). ¹³C NMR (500 MHz, CDCl₃) δ 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 C₂₁H₄₀N₂O₄⁺ + NH₄-CH₃CN⁺ complex (ammonia-acetonitrile) [M+NH₄-CH₃CN]⁺: 443.36. Found: 443.46.

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

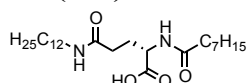


N^δ-dodecyl-*L*-glutamine monohydrochloride (**479**) (0.018 g, 0.057 mmol) was dissolved in 10 % NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0 °C and hexanoyl chloride (12 μL, 0.085 mmol, 1.5 eq) was added slowly to the ether layer with 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).

¹H 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). **¹³C 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 C₂₃H₄₄N₂NaO₄⁺ [M+Na]⁺: 435.32. Found: 435.37.

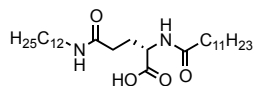
***N*^α-octanoyl-*N*^δ-dodecyl-*L*-glutamine (482)**



N^δ-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%).

¹H 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). **¹³C NMR** (500 MHz, CDCl₃) δ 174.8, 174.0, 173.4, 52.6, 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 C₂₅H₄₇N₂O₄⁻ [M]⁻: 439.35. Found: 439.18.

***N*^α-dodecanoyl-*N*^δ-dodecyl-*L*-glutamine (483)**



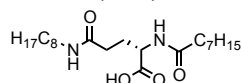
N^δ-dodecyl-*L*-glutamine monohydrochloride (**479**) (0.030 g, 0.096 mmol) was dissolved in 10 % NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0 °C and dodecanoyl chloride (35 μL, 0.15 mmol, 1.5 eq) was added slowly to the ether layer with 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 MgSO_4 , and concentrated under reduced pressure. The crude product was purified by silica column chromatography (9.5:0.5 CH_2Cl_2 :MeOH) to yield **483** (0.040 g, 0.081 mmol, 84% yield).

^1H NMR (500 MHz, CDCl_3) 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). **^{13}C NMR** (500 MHz, CDCl_3) 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 $\text{C}_{29}\text{H}_{56}\text{N}_2\text{NaO}_4^+$ $[\text{M}+\text{Na}]^+$: 519.41. Found: 519.44.

ZZ

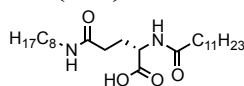
N^α -dodecanoyl- N^δ -dodecyl-*L*-glutamine (484)



N^δ -octyl-*L*-glutamine monohydrochloride (**478**) (0.019 g, 0.066 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 , 0.061 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 **484** (0.018 g, 0.047 mmol, 72% yield).

^1H NMR (300 MHz, CDCl_3) δ 7.32 (d, J = 5.6, 1H), 6.47 (s, 1H), 4.40 (q, J = 5.8, 1H), 3.26 (q, J = 6.6, 2H), 2.60 (m, 1H), 2.44 (m, 1H), 2.25 (t, J = 7.6, 2H), 2.20-1.99 (m, 2H), 1.62 (m, 2H), 1.52 (m, 2H), 1.36-1.22 (m, 18H), 0.88 (t, J = 6.5, 6H). **^{13}C NMR** (400 MHz, CDCl_3) δ 174.8, 174.0, 173.4, 52.6, 40.2, 36.6, 32.8, 31.92, 31.80, 29.43, 29.37, 29.34, 29.31, 29.12, 28.5, 27.1, 25.7, 22.77, 22.74, 14.22, 14.20. **ESI-MS** m/z calcd for $\text{C}_{21}\text{H}_{41}\text{N}_2\text{O}_4^+$ $[\text{M}+\text{H}]^+$: 385.31. Found: 385.35.

N^α -dodecanoyl- N^δ -octyl-*L*-glutamine (485)

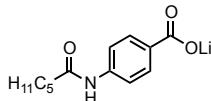


N^δ -octyl-*L*-glutamine monohydrochloride (**478**) (0.010 g, 0.035 mmol) was dissolved in 10 % NaOH (3 parts) and ethyl ether (2 parts) was added (final concentration of 0.1 M). The mixture was cooled to 0 °C and octanoyl chloride (6 μL , 0.025 mmol, 0.7 eq) was added slowly to the ether layer with 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 **485** (0.0073 g, 0.017 mmol, 47% yield).

^1H NMR (500 MHz, CDCl_3) δ 7.34 (d, J = 5.4, 1H), 6.38 (bs, 1H), 4.40 (q, J = 5.8, 1H), 3.26 (q, J = 6.7, 2H), 2.62 (m, 1H), 2.44 (m, 1H), 2.25 (t, J = 7.6, 2H), 2.14 (m, 1H), 2.04 (m, 1H), 1.62 (m, 2H), 1.52 (m, 2H), 1.35-1.21 (m, 26H), 0.88 (t, J = 6.9, 6H). **^{13}C NMR**

(400 MHz, $CDCl_3$) 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 calcd for $C_{25}H_{47}N_2O_4^-$ [M] $^-$: 439.35. Found: 439.16.

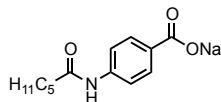
Lithium 4-hexanamidobenzoate (**501**)



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, D_2O) 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). **^{13}C 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 calcd for $C_{13}H_{16}NO_3^-$ [M] $^-$: 234.11. Found: 234.06.

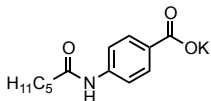
Sodium 4-hexanamidobenzoate (**502**)



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, D_2O) δ 7.85 (d, J = 8.6, 2H), 7.46 (d, J = 8.5, 2H), 2.32 (m, 2H), 1.61 (m, 2H), 1.27 (m, 4H), 0.84 (d, J = 3.9, 3H). **^{13}C 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 calcd for $C_{13}H_{16}NO_3^-$ [M] $^-$: 234.11. Found: 234.05.

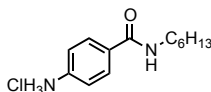
Potassium 4-hexanamidobenzoate (**503**)



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, D_2O) 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). **^{13}C 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 calcd for $C_{13}H_{16}NO_3^-$ [M] $^-$: 234.11. Found: 234.06.

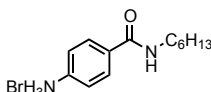
4-(Hexylcarbamoyl)benzenaminium chloride (504)



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).

^1H NMR (500 MHz, D_2O) δ 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) δ 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 $\text{C}_{13}\text{H}_{21}\text{N}_2\text{O}^+ [\text{M}]^+$: 221.16. Found: 221.13.

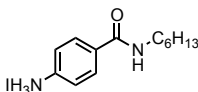
4-(Hexylcarbamoyl)benzenaminium bromide (505)



tert-Butyl (4-(hexylcarbamoyl)phenyl)carbamate (**507**) (0.024 g, 0.074 mmol) was dissolved in anhydrous CH_2Cl_2 (5 mL) and anhydrous HBr was bubbled through 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).

^1H NMR (400 MHz, D_2O) δ 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) δ 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 $\text{C}_{13}\text{H}_{21}\text{N}_2\text{O}^+ [\text{M}]^+$: 221.16. Found: 221.13.

4-(Hexylcarbamoyl)benzenaminium iodide (506)

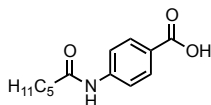


tert-Butyl (4-(hexylcarbamoyl)phenyl)carbamate (**510**) (0.040 g, 0.12 mmol) was dissolved in anhydrous CH_2Cl_2 (5 mL) and anhydrous HI was bubbled through the solution until a precipitate formed. The solution was stirred at ambient temperature for an additional hour. The white precipitate and solvent 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 **506** (0.036 g, 0.11 mmol, 85% yield).

¹H NMR (500 MHz, *D*₂O) δ 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). **¹³C NMR** (500 MHz, *D*₂O) δ 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 C₁₃H₂₁N₂O⁺ [*M*]⁺: 221.16. Found: 221.13.

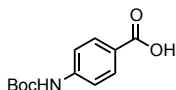
4-hexanamidobenzoic acid (**507**)



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 H₂O 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%).

¹H NMR (300 MHz, *DMSO*) δ 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). **¹³C NMR** (300 MHz, *DMSO*) δ 171.8, 167.0, 143.4, 130.4, 124.9, 118.2, 36.5, 30.9, 24.7, 21.9, 13.9. **ESI-MS** *m/z* calcd for C₁₃H₁₆NO₃⁻ [*M*]⁻: 234.11. Found: 234.01.

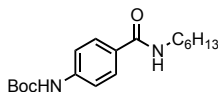
4-((*tert*-Butoxycarbonyl)amino)benzoic acid (**508**)



4-Aminobenzoic acid was dissolved in a mixture of 1:2 H₂O:dioxane for a total volume of 2.5 mL and Et₃N (0.2 mL, 1.4 mmol, 2 eq) was added. After 5 min of stirring at ambient temperature, di-*tert*-butyl dicarbonate (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 **508** as a white powder (0.12 g, 0.49 mmol, 67% yield).

¹H NMR (300 MHz, *CDCl*₃) δ 8.04 (d, *J* = 8.8, 2H), 7.46 (d, *J* = 8.8, 2H), 1.54 (s, 9H). **¹³C NMR** (300 MHz, *CDCl*₃) δ 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 C₁₂H₁₄NO₄⁻ [*M*]⁻: 236.09. Found: 236.02.

tert-Butyl (4-(hexylcarbamoyl)phenyl)carbamate (**509**)



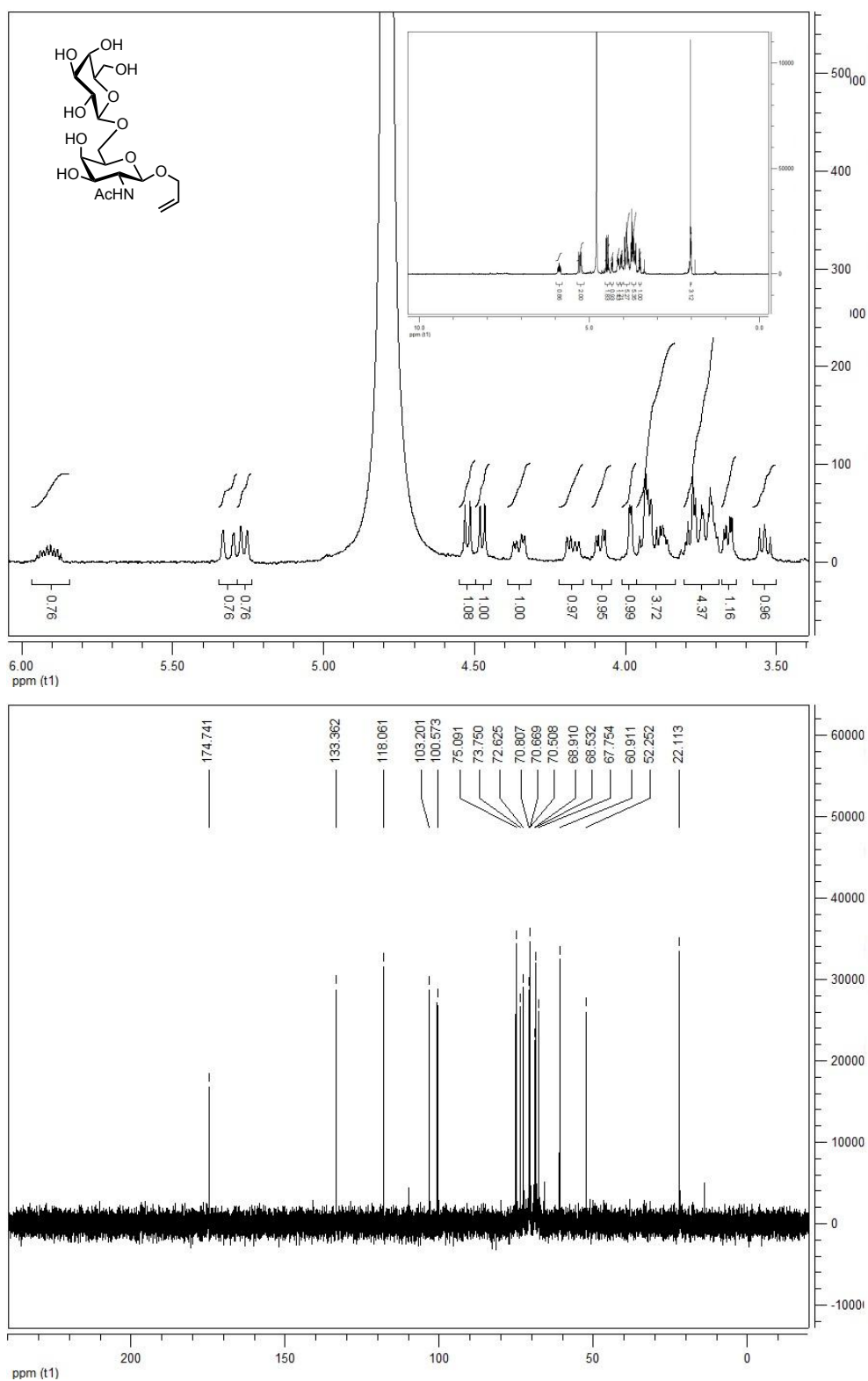
To a solution of anhydrous CH₂Cl₂ and 4-((*tert*-Butoxycarbonyl)amino)benzoic acid

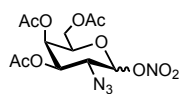
(**508**) (0.048 g, 0.20 mmol) under argon, diisopropylethylamine (0.054 mL, 0.20 mmol, 1.1 eq) and HCTU (0.093 g, 0.22 mmol, 1.1 eq) was added and the mixture was stirred for 30 min. Hexyl amine (0.023 mL, 0.22 mmol, 1.1 eq) was added and after 16 hrs, the solvent was removed under reduced pressure 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).

¹H NMR (300 MHz, *CDCl*₃) δ 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). **¹³C NMR** (300 MHz, *CDCl*₃) δ 167.1, 152.6, 141.5, 128.9, 128.0, 117.9, 81.0, 40.2, 31.6, 29.8, 28.4, 28.4, 28.4, 26.8, 22.7, 14.1. **ESI-MS** *m/z* calcd for C₁₈H₂₉N₂O₃⁺ [M+H]⁺: 321.22. Found: 321.17.

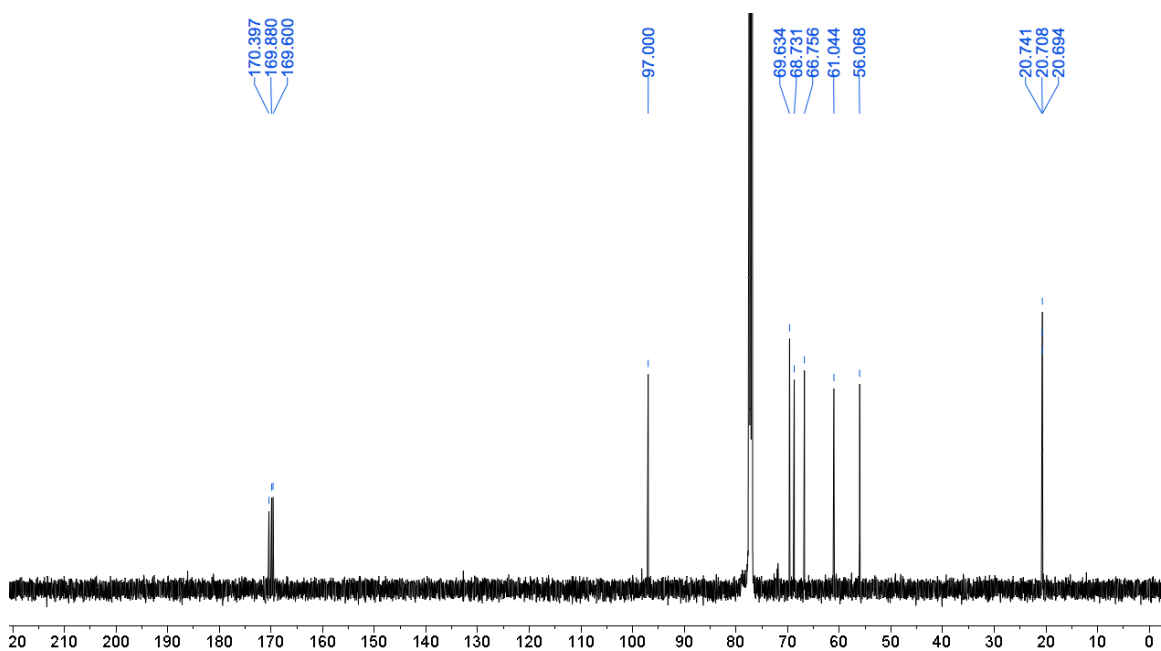
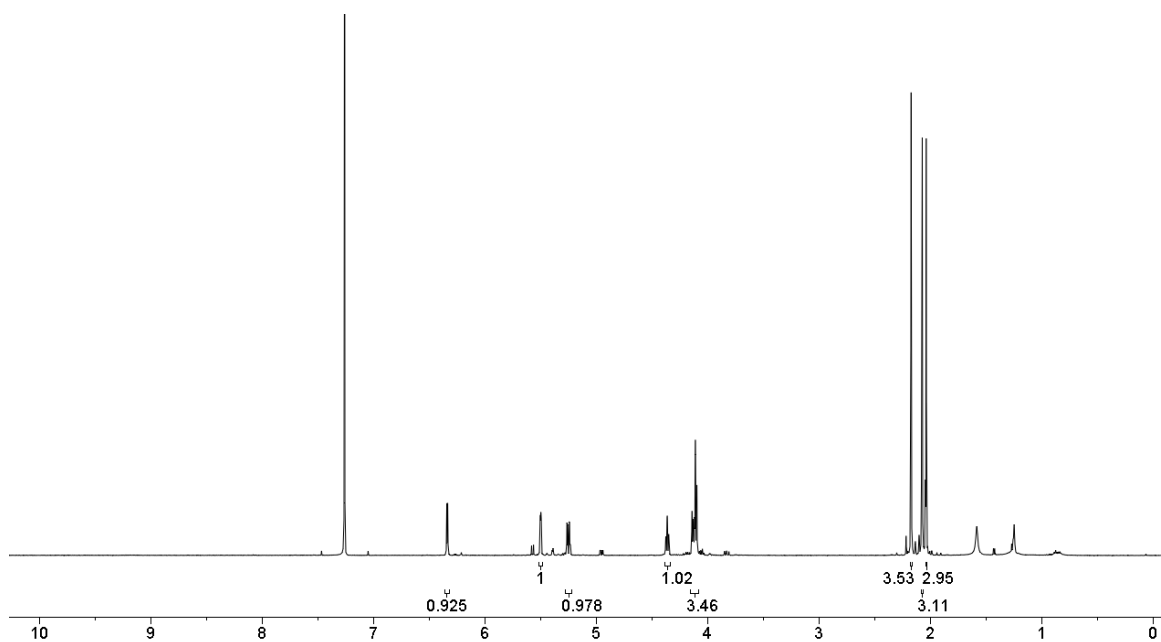
7.6. Spectroscopic Data

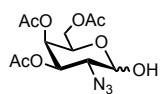
304



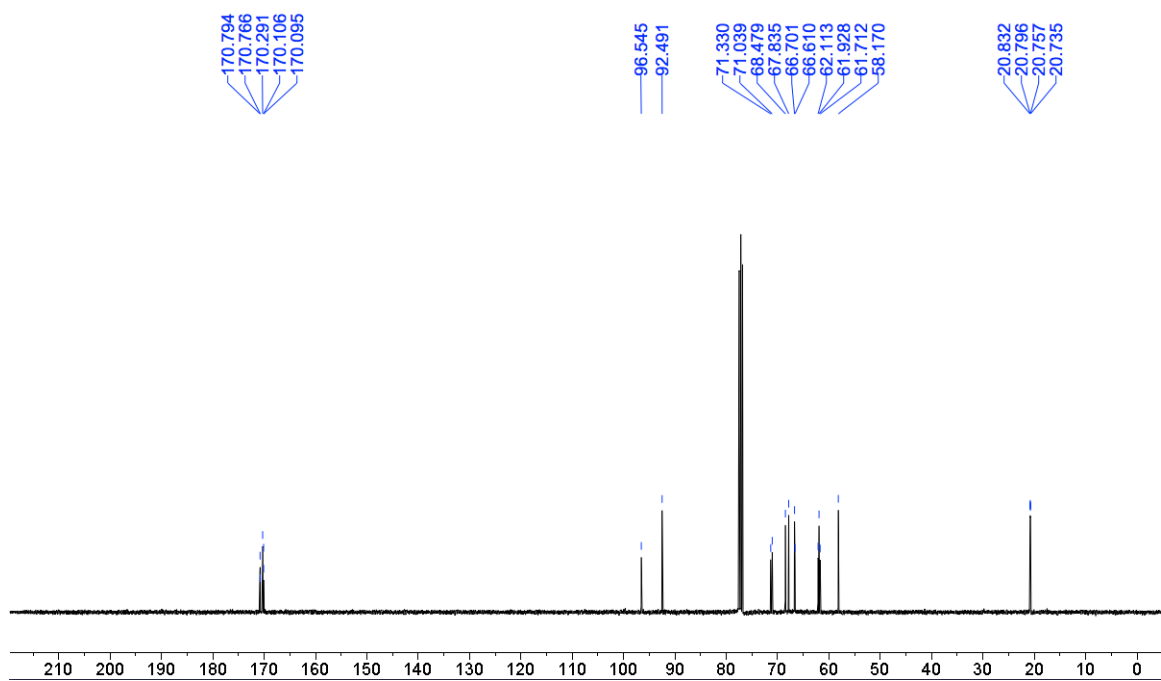
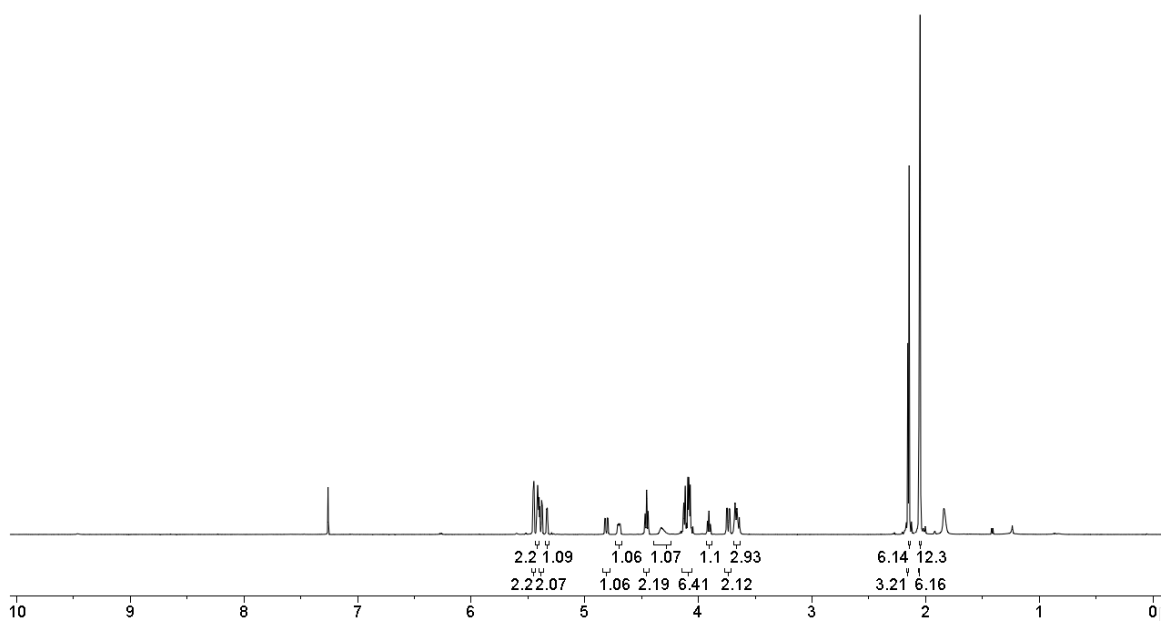


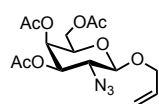
319



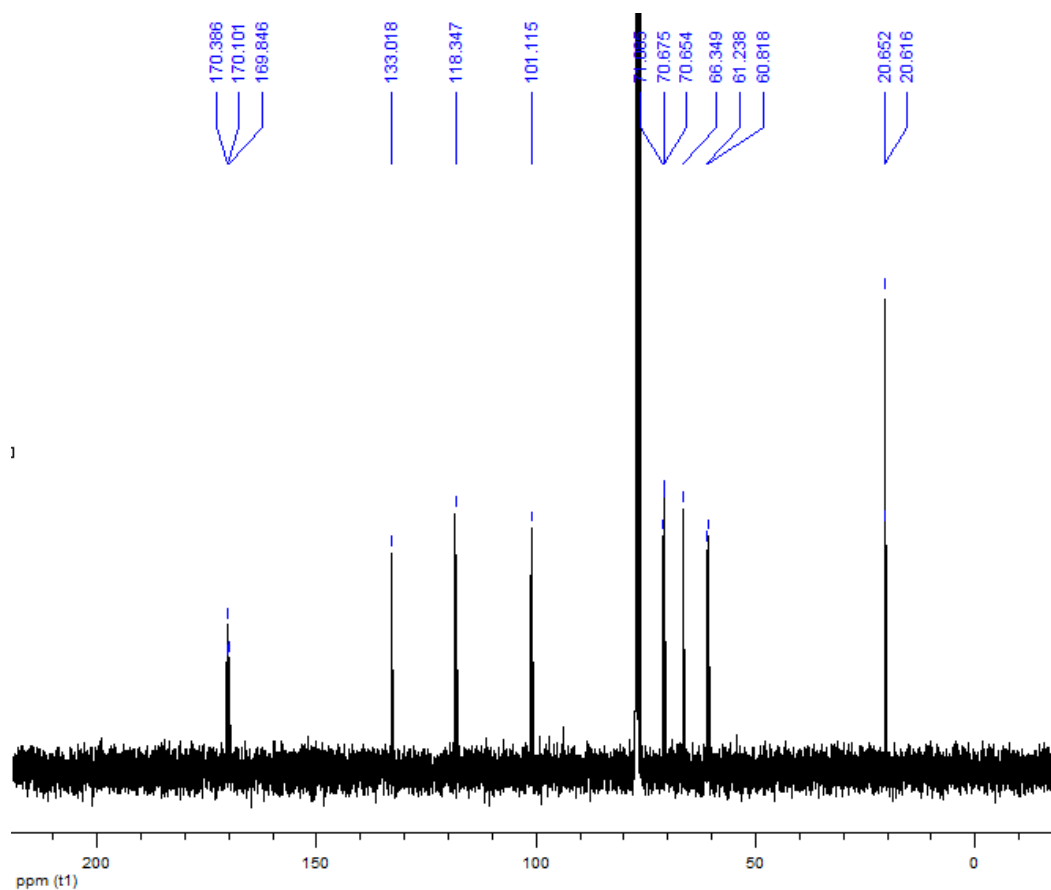
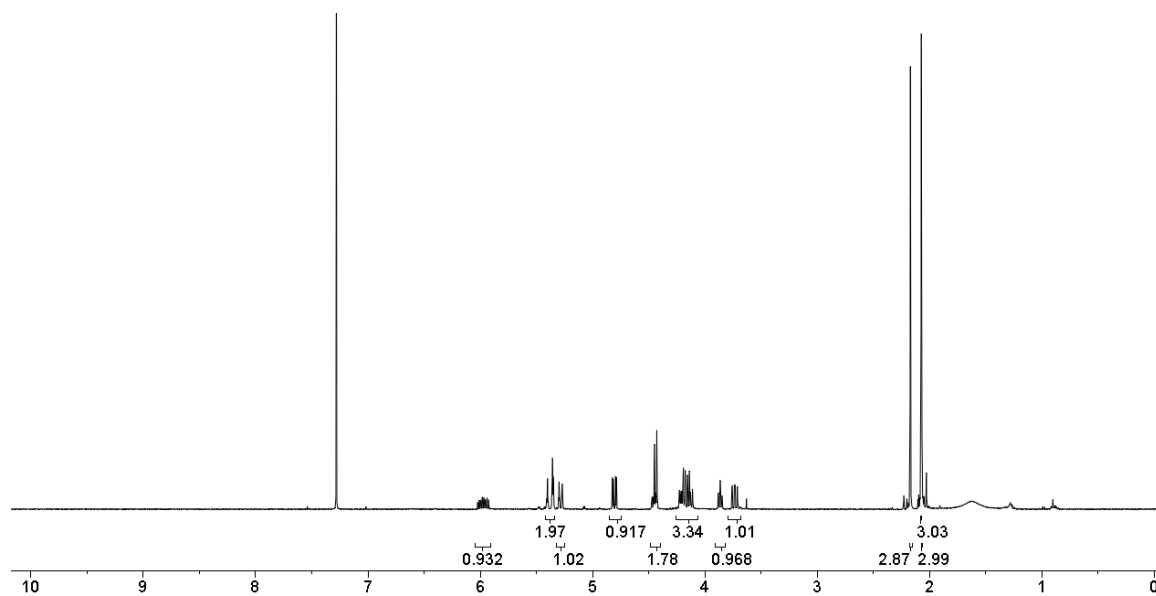


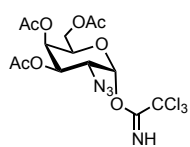
320



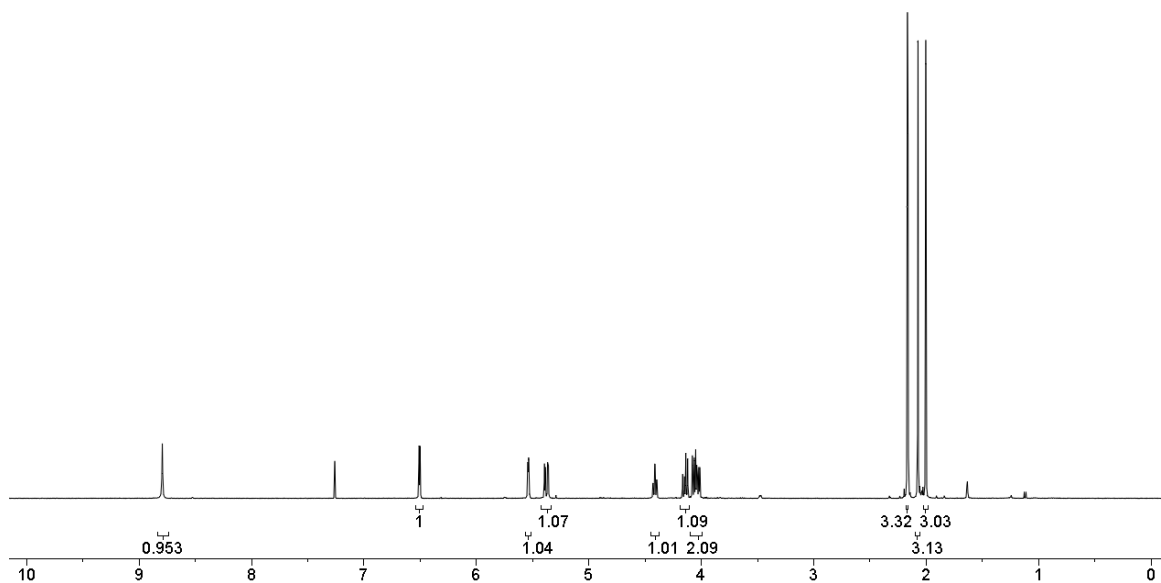


321





322

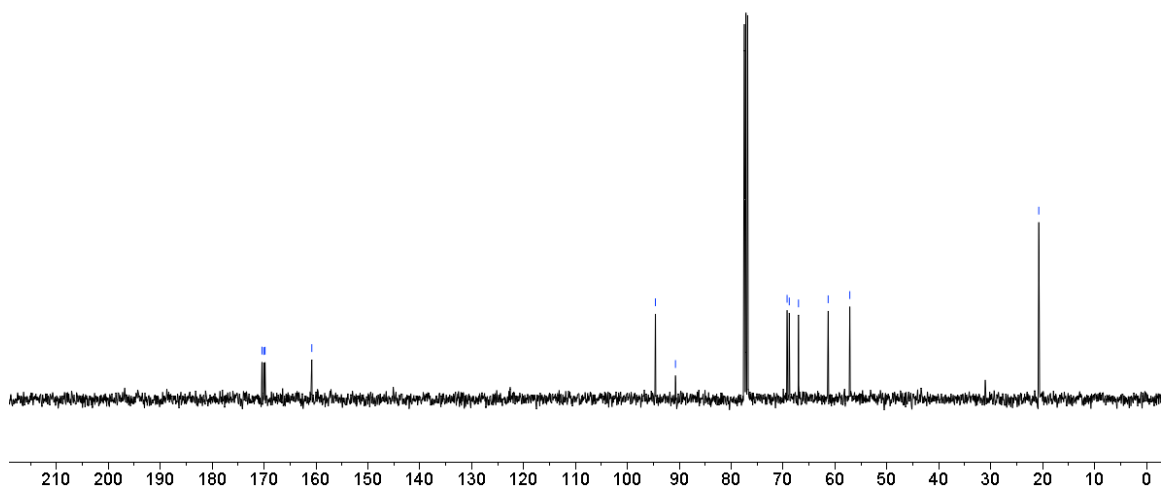


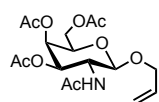
170.397
170.041
169.807
160.801

94.601
90.734

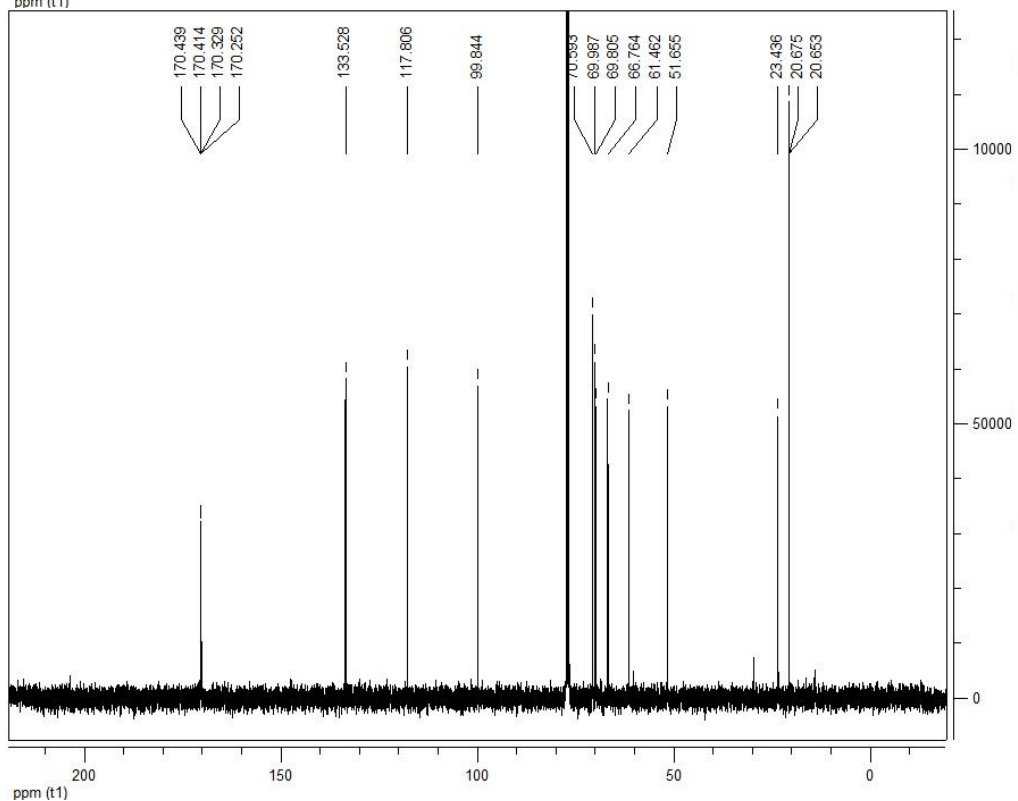
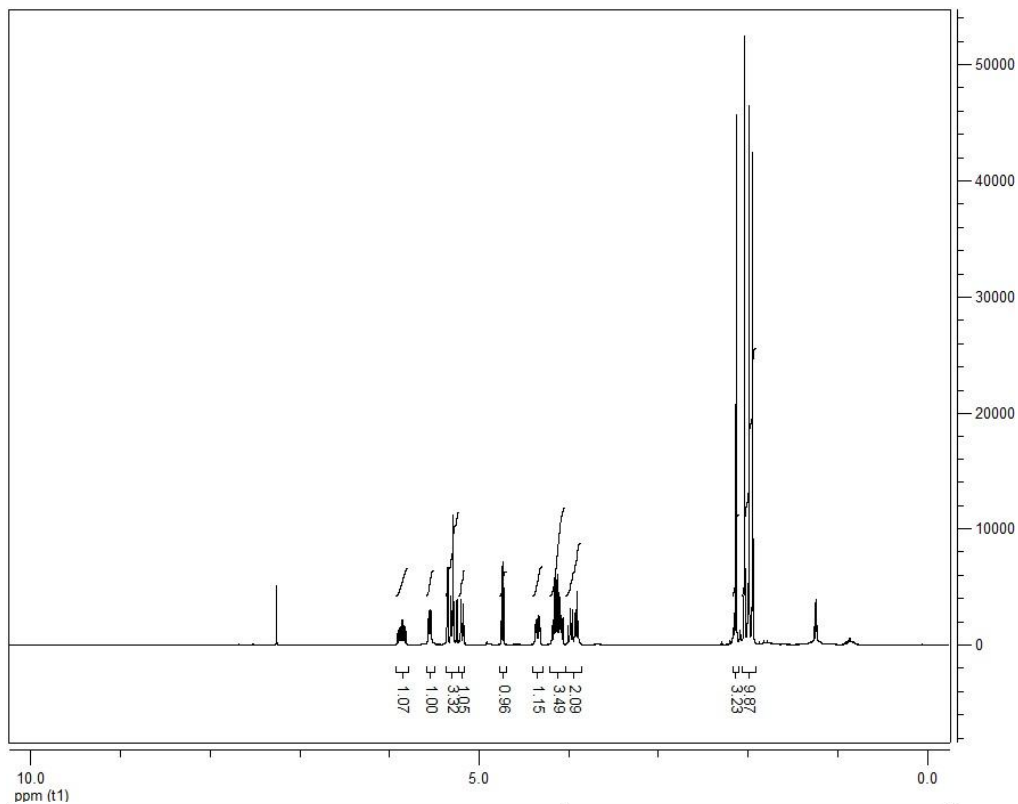
69.233
68.807
67.035
61.317
57.153

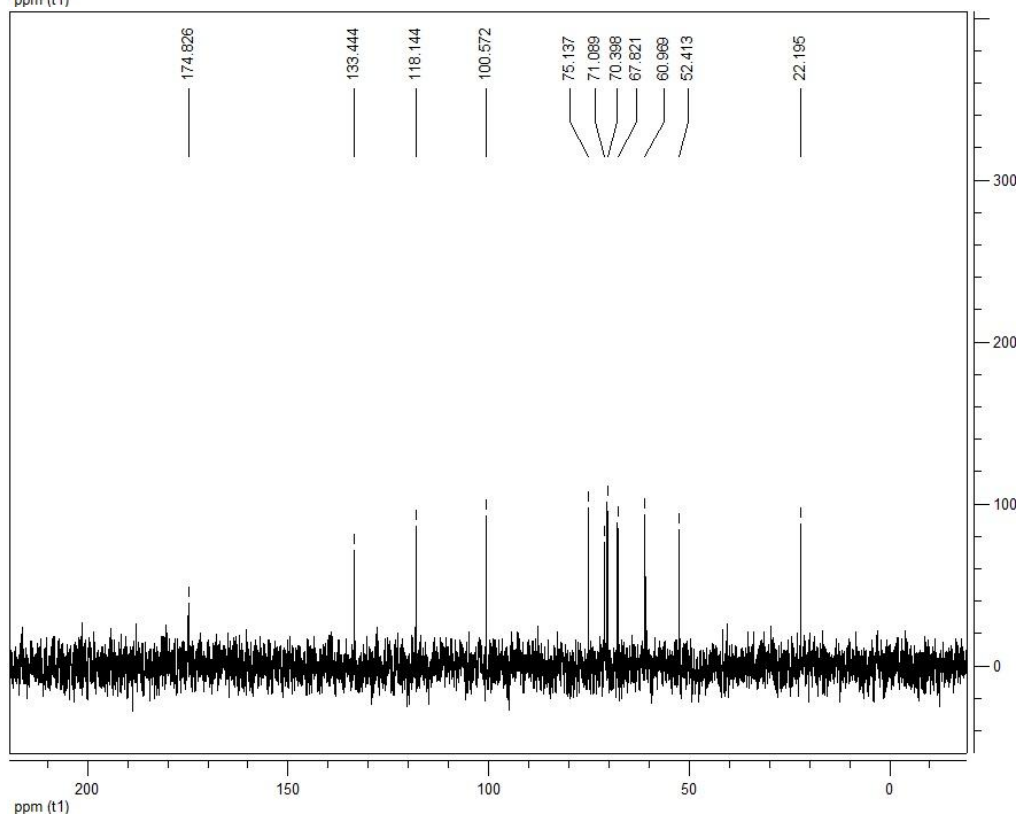
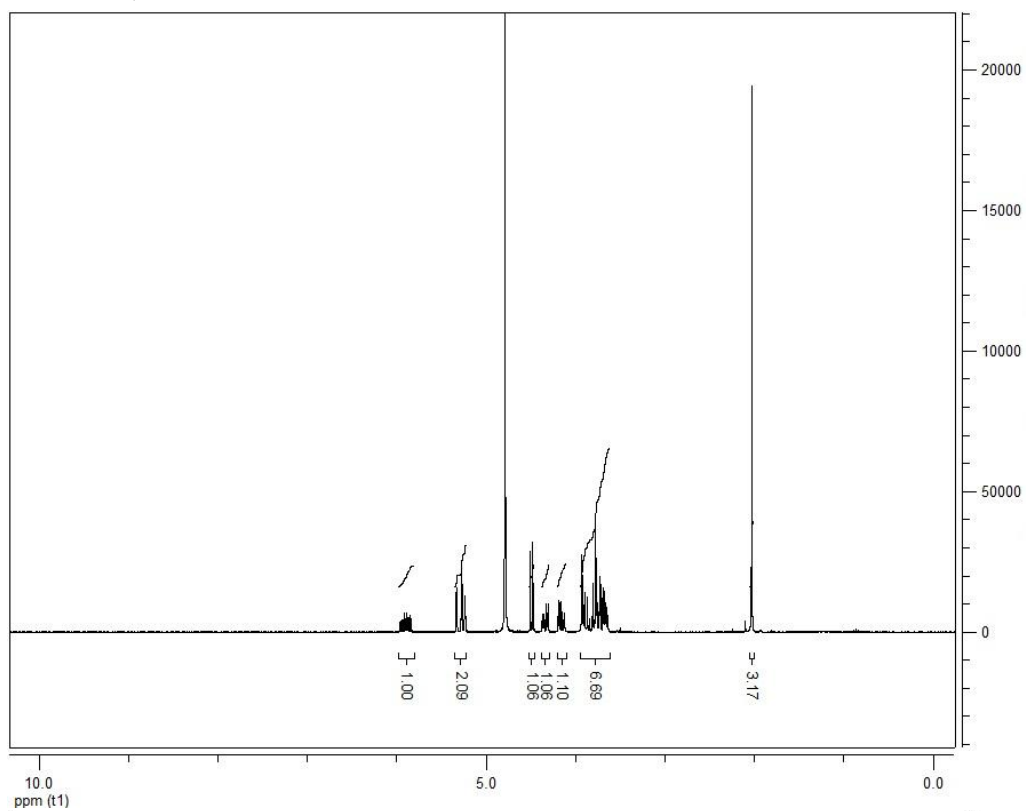
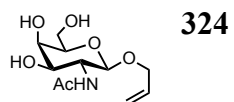
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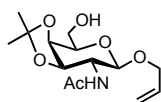




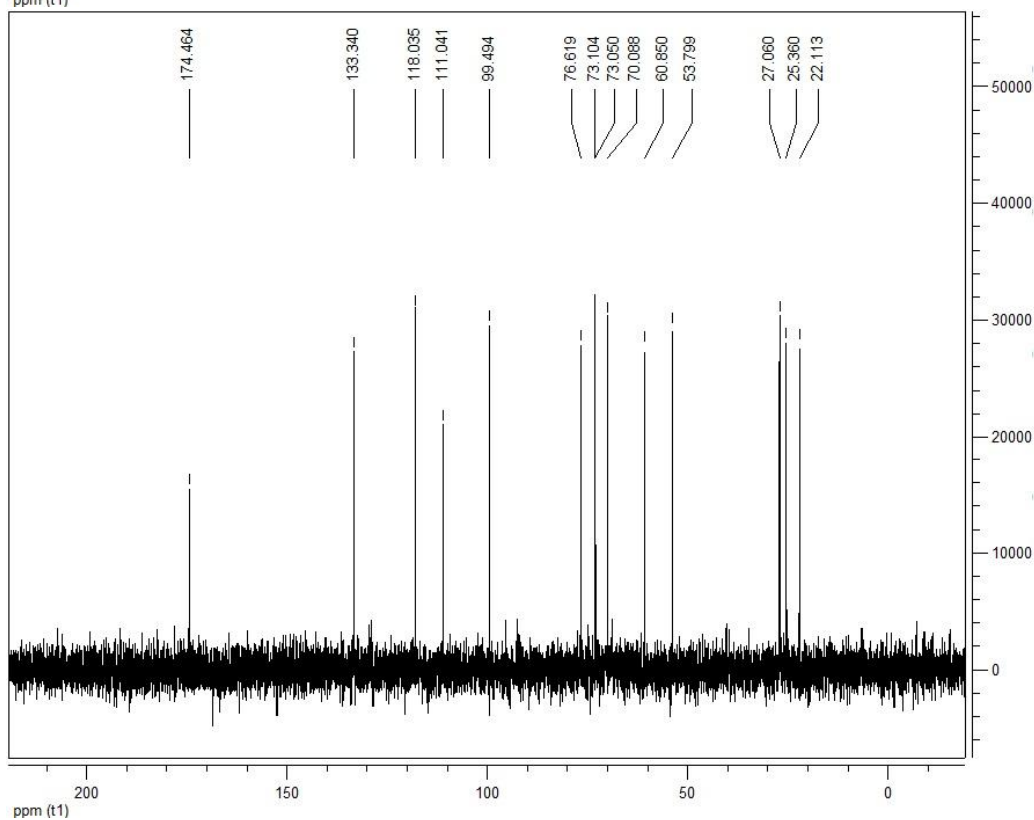
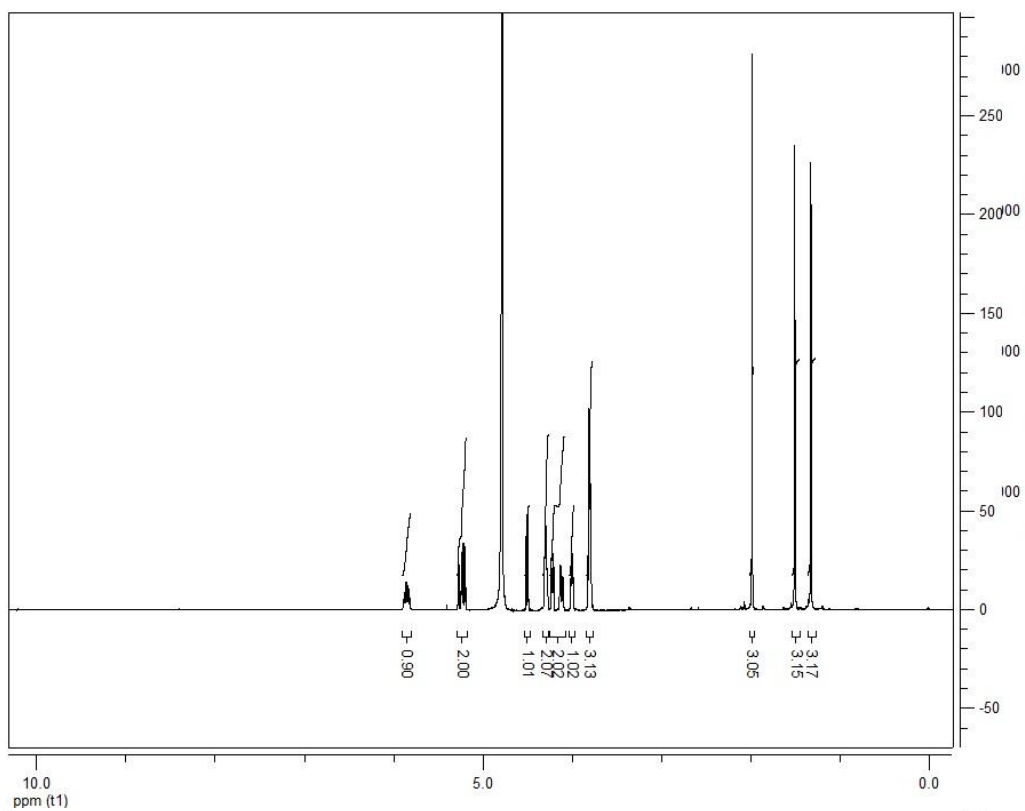
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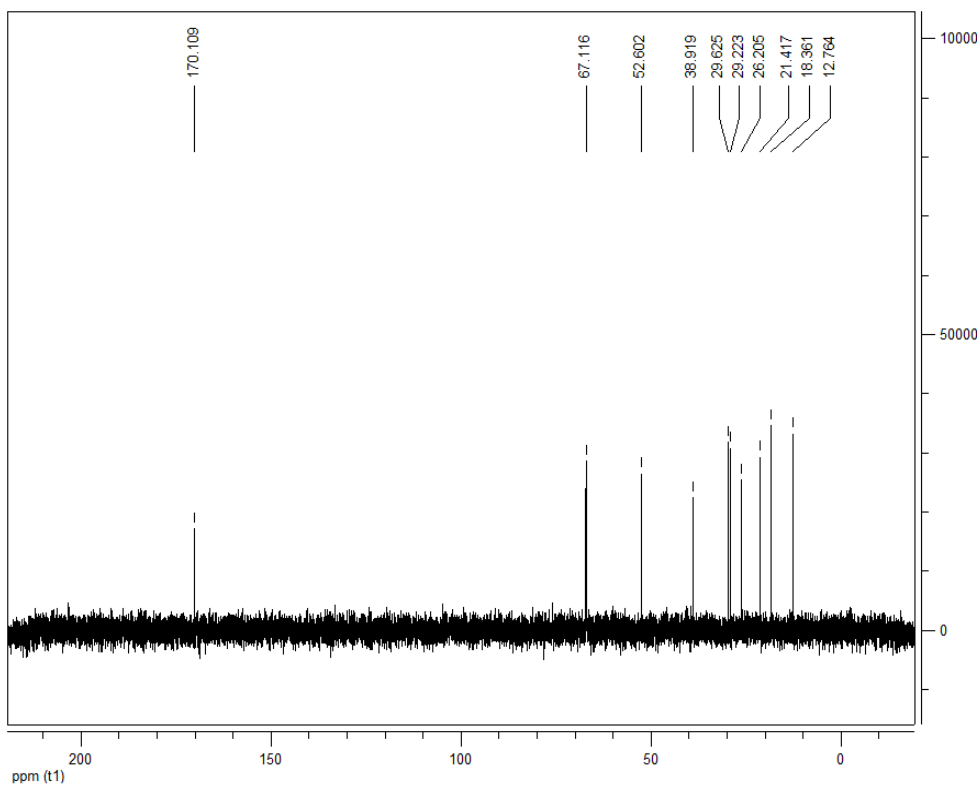
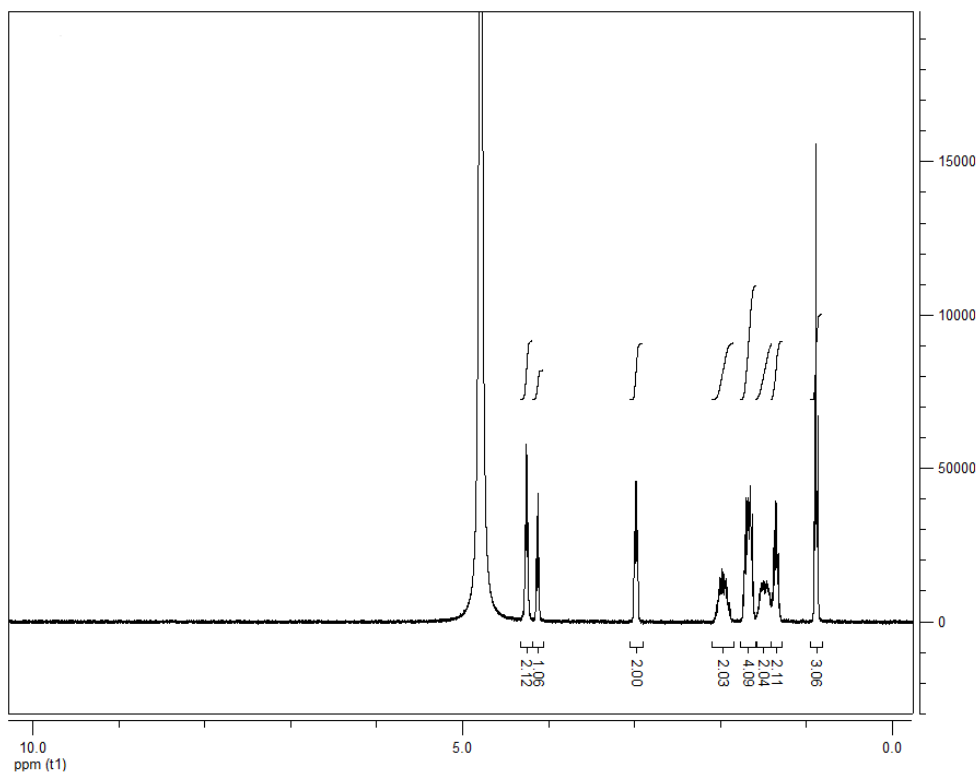
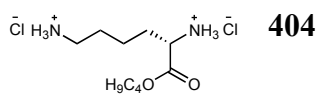


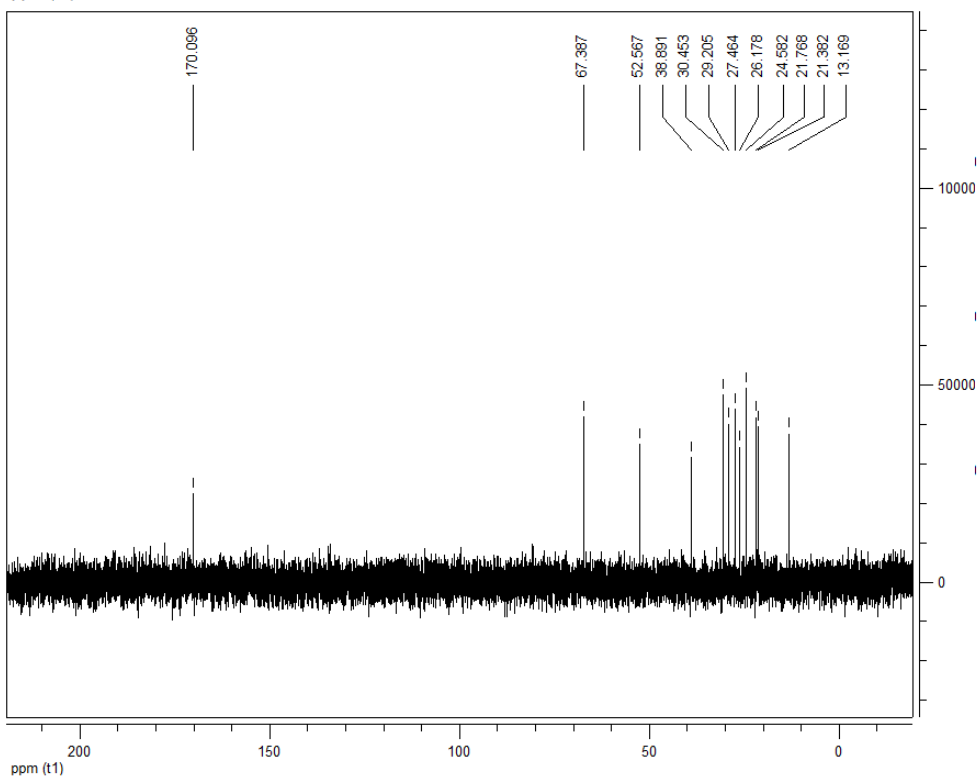
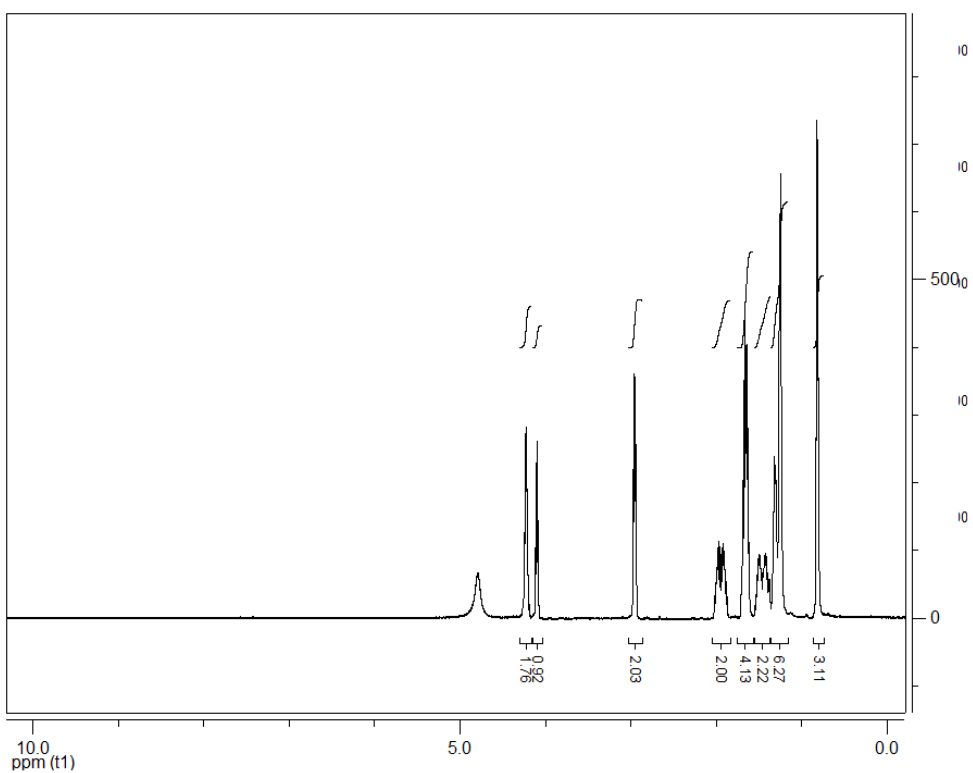
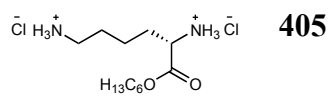


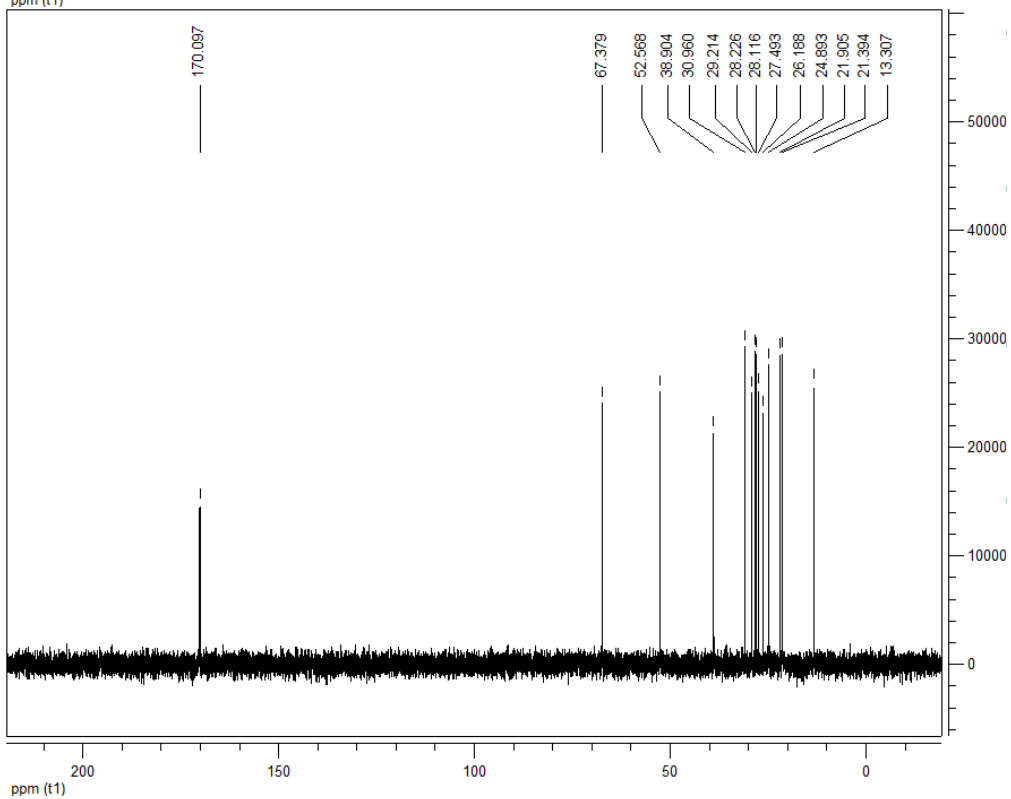
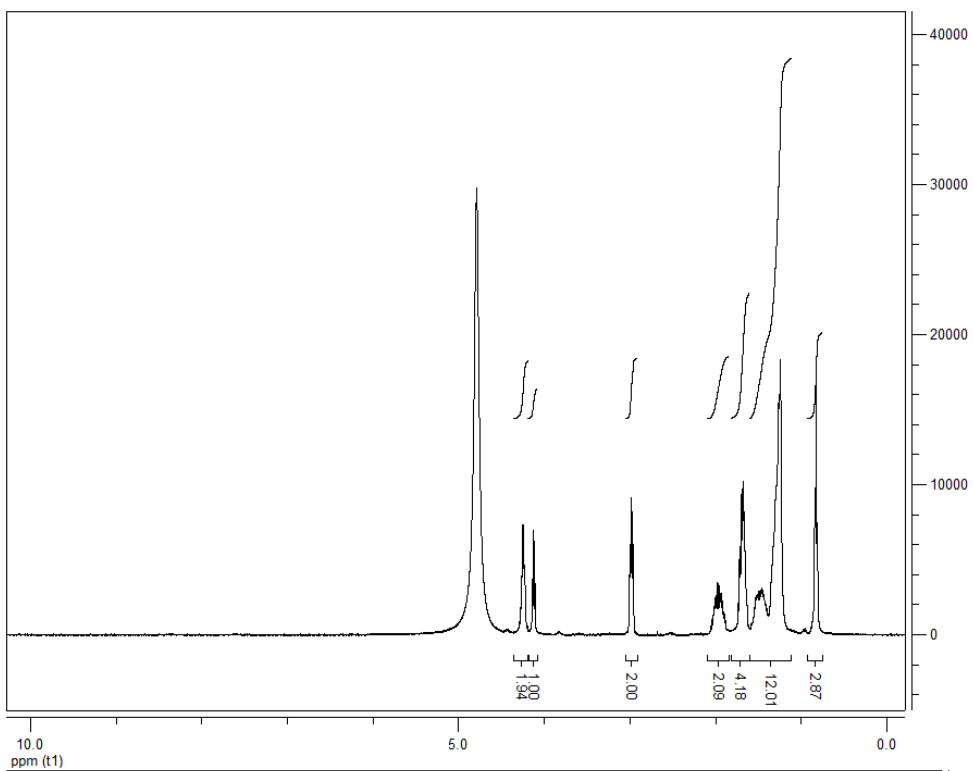
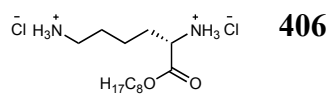


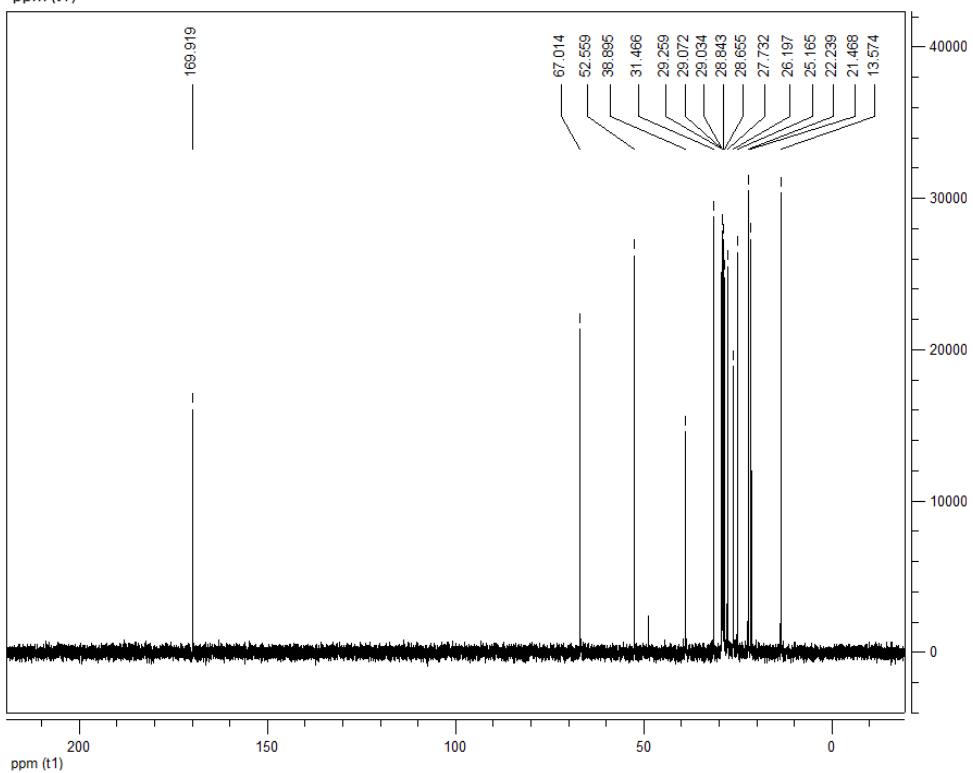
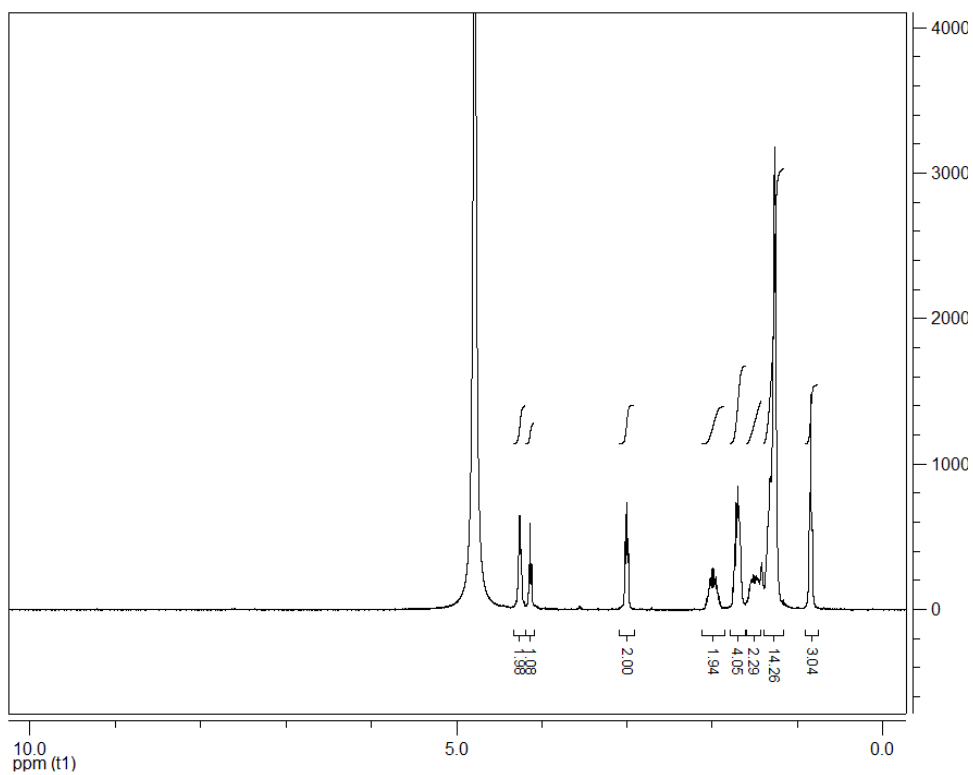
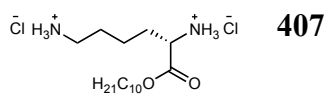
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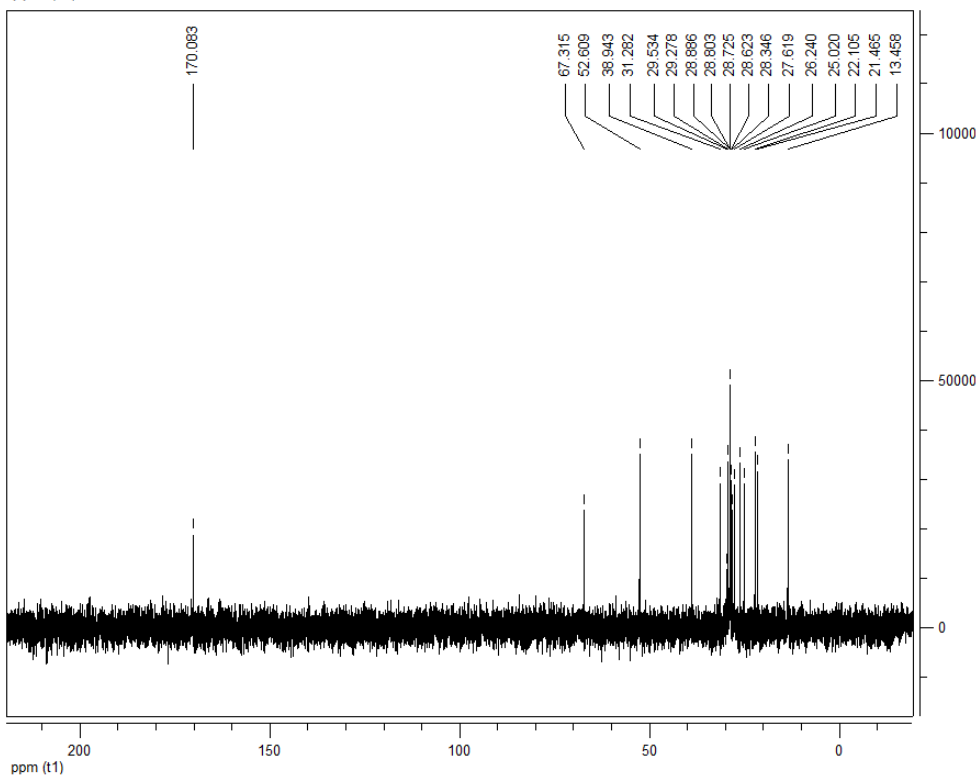
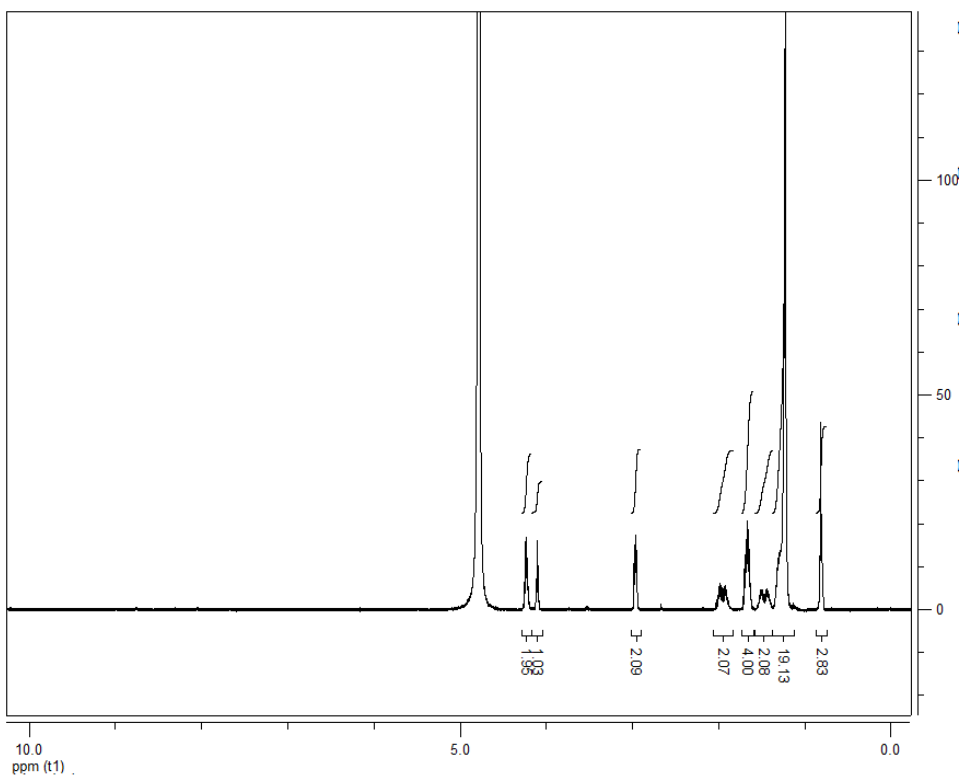
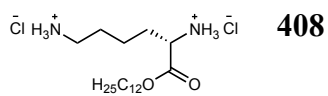


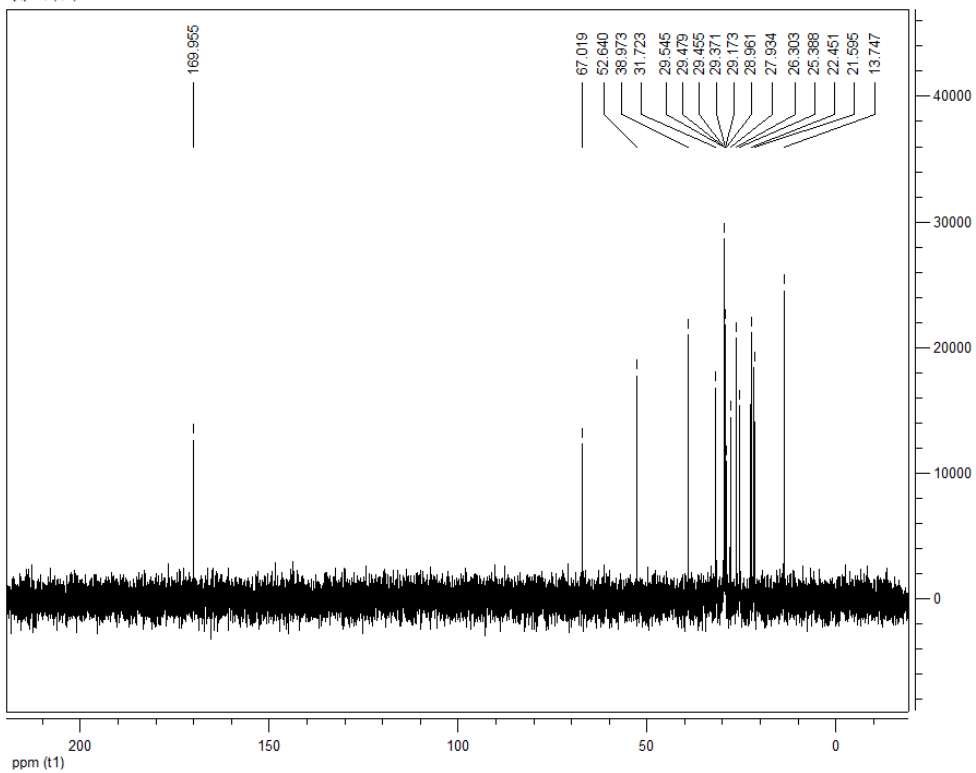
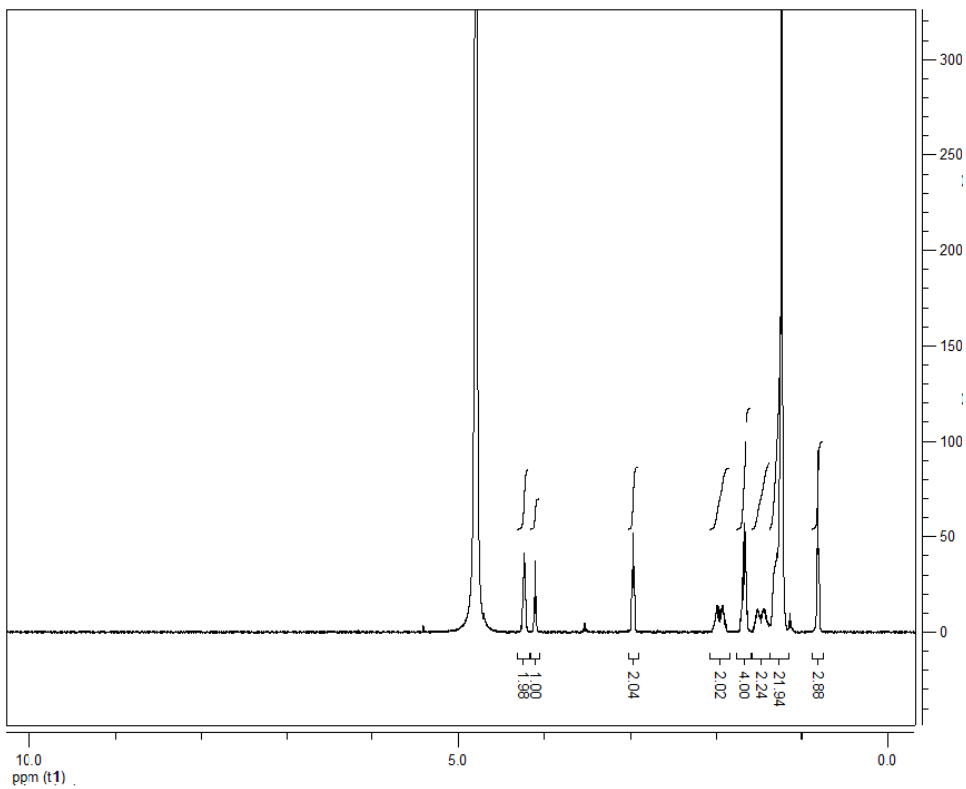
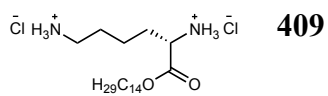


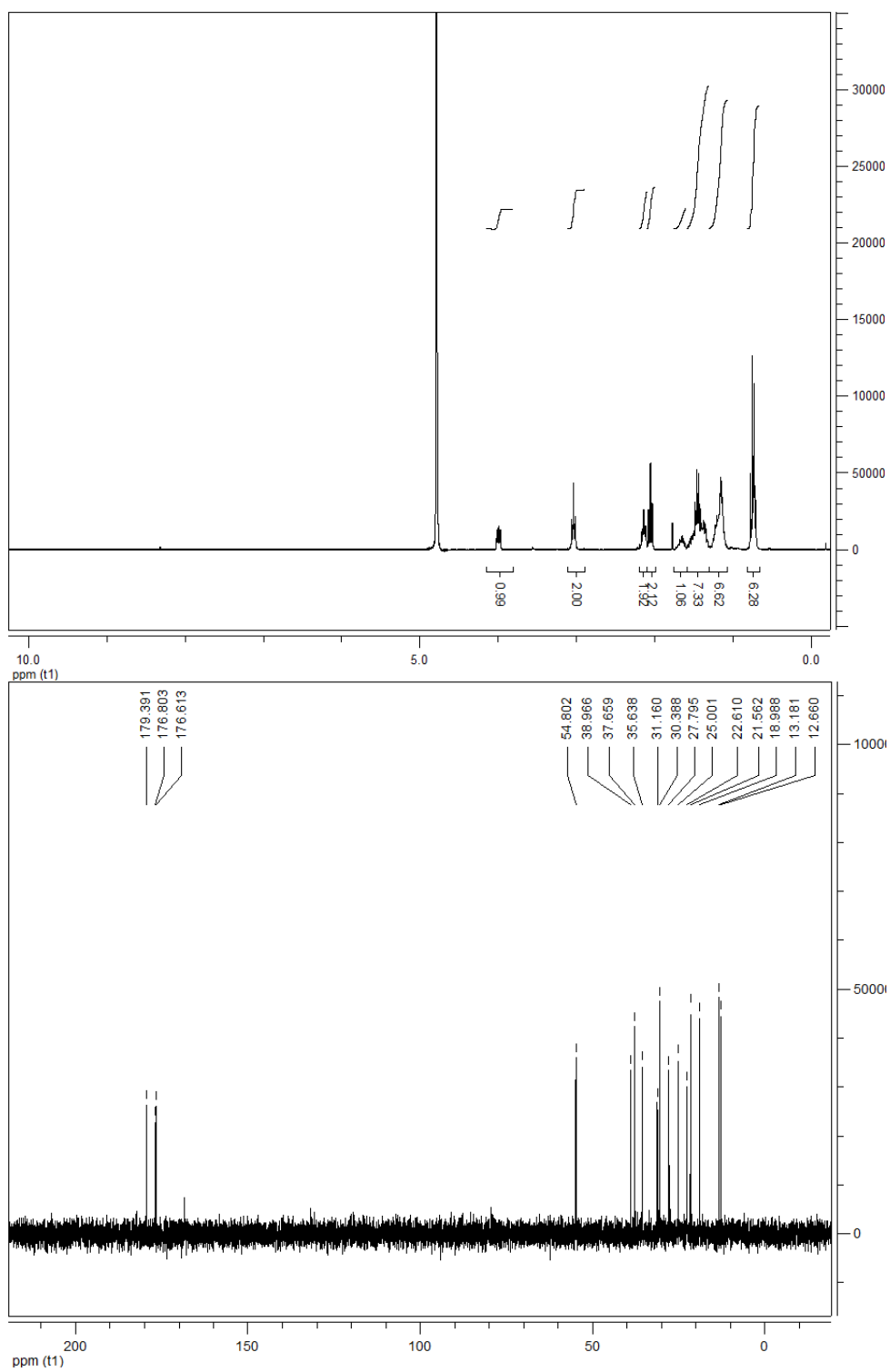
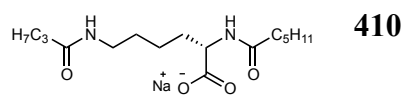


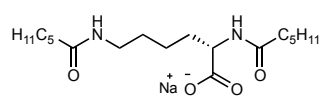




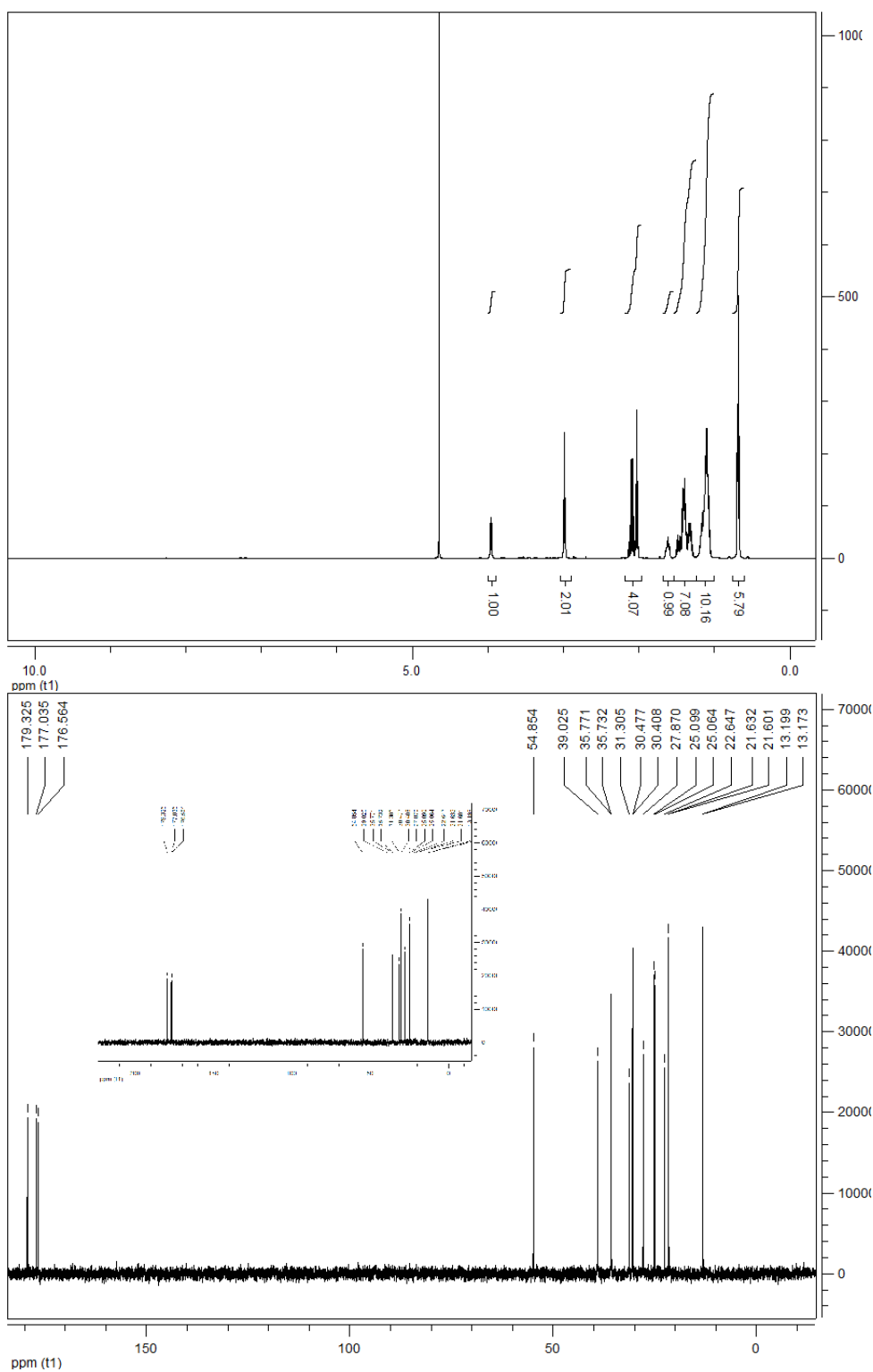


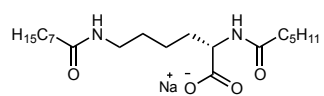




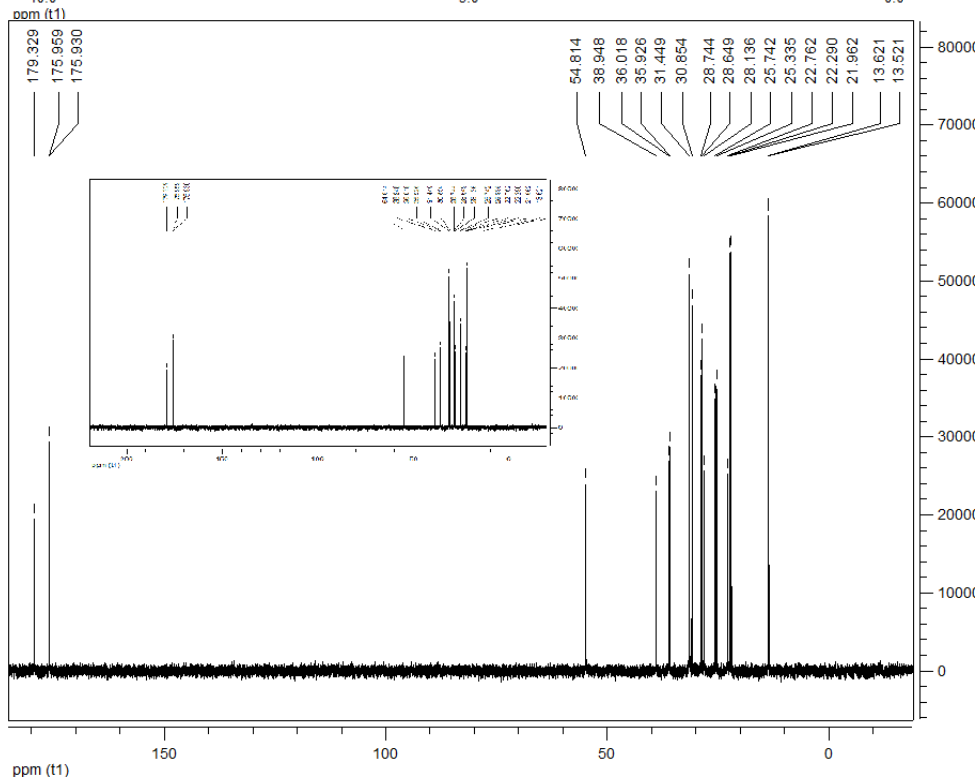
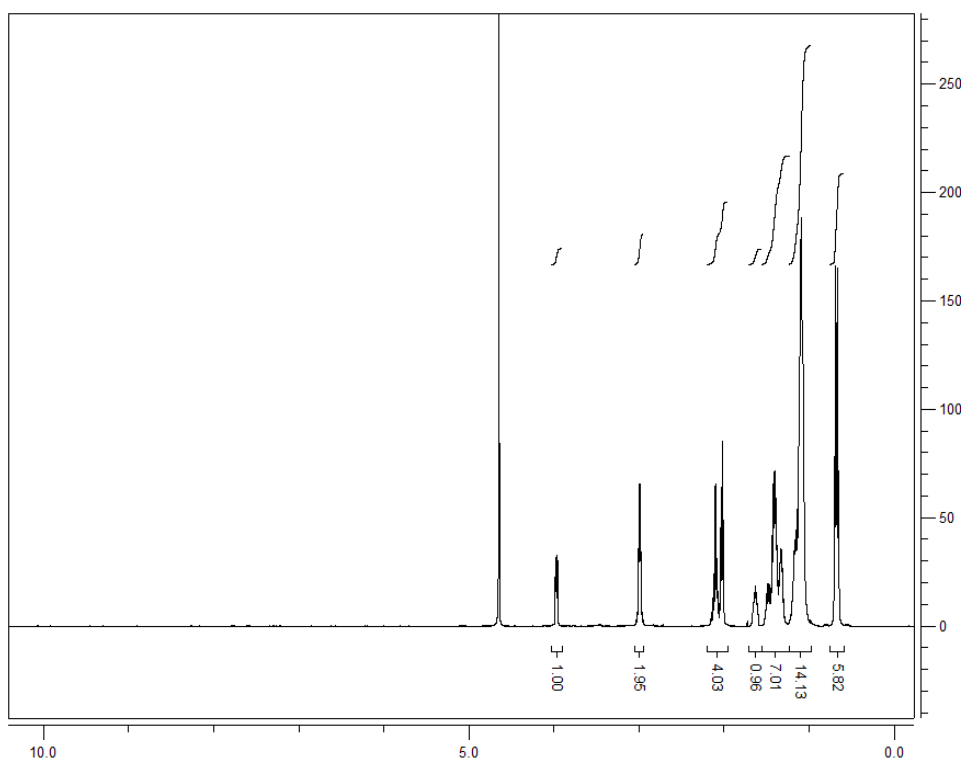


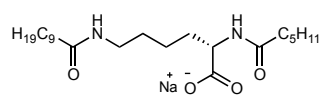
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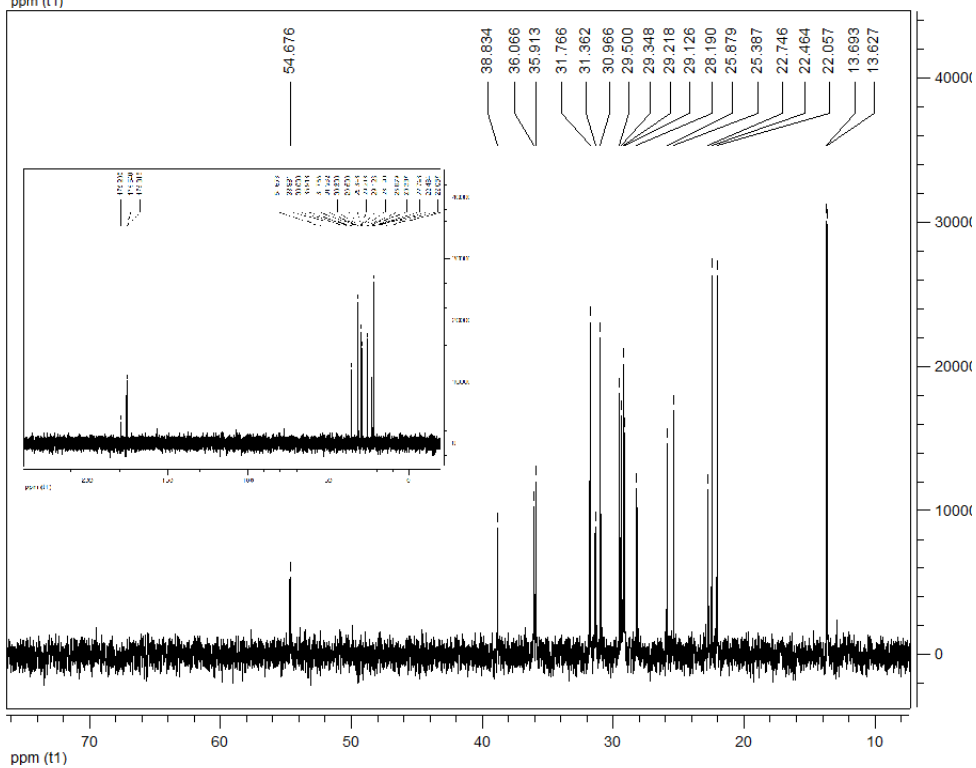
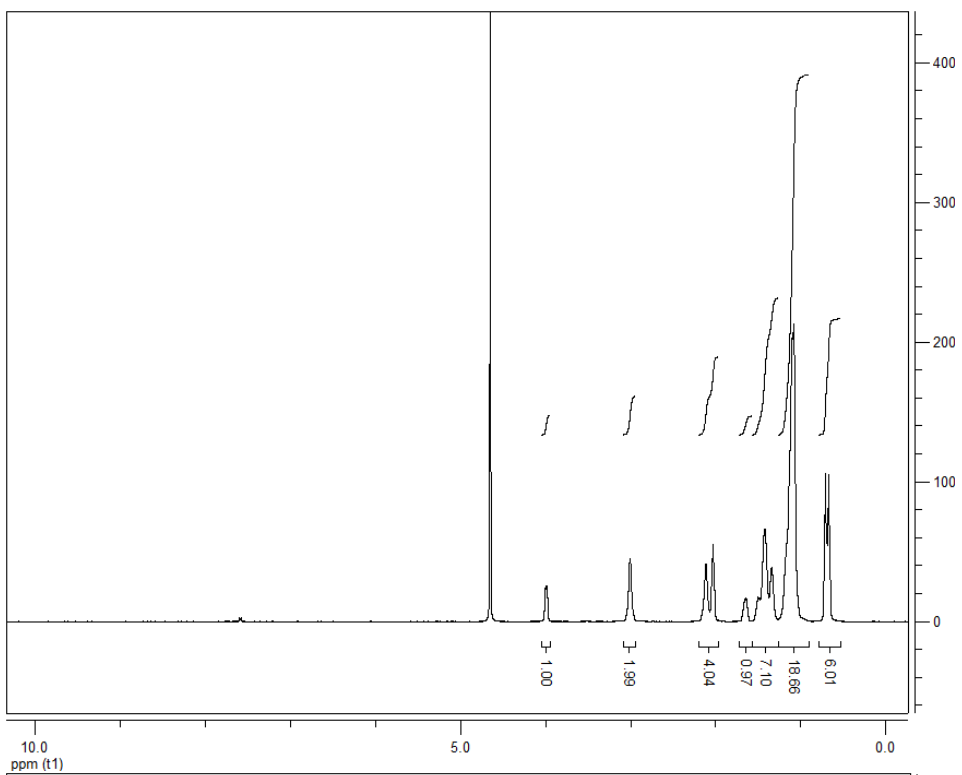


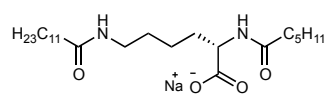
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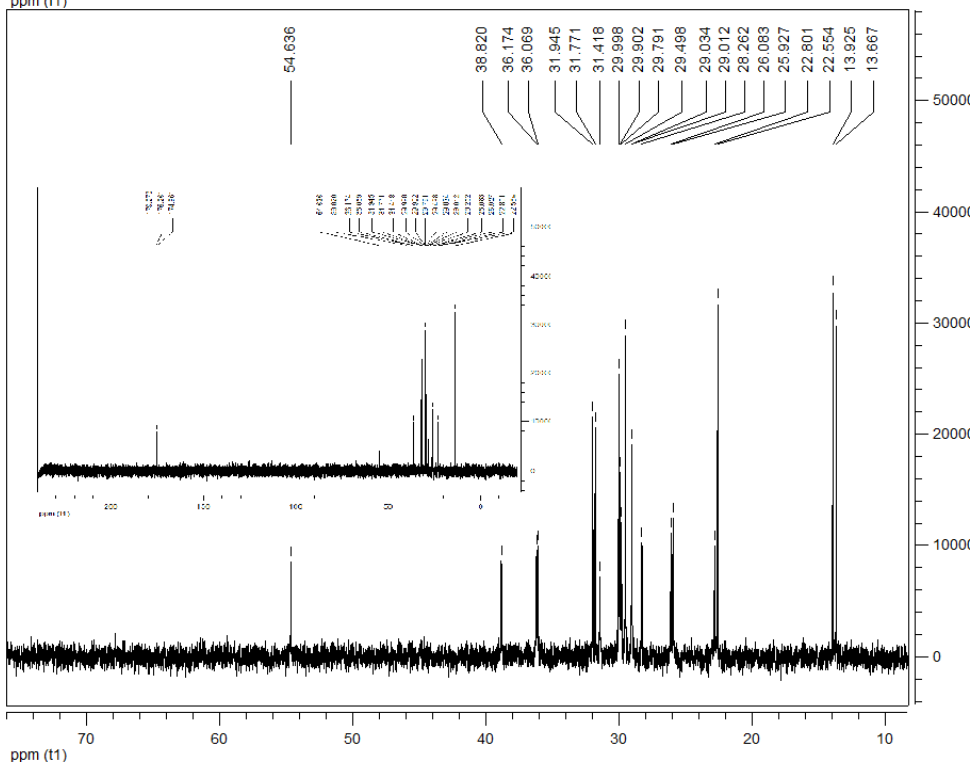
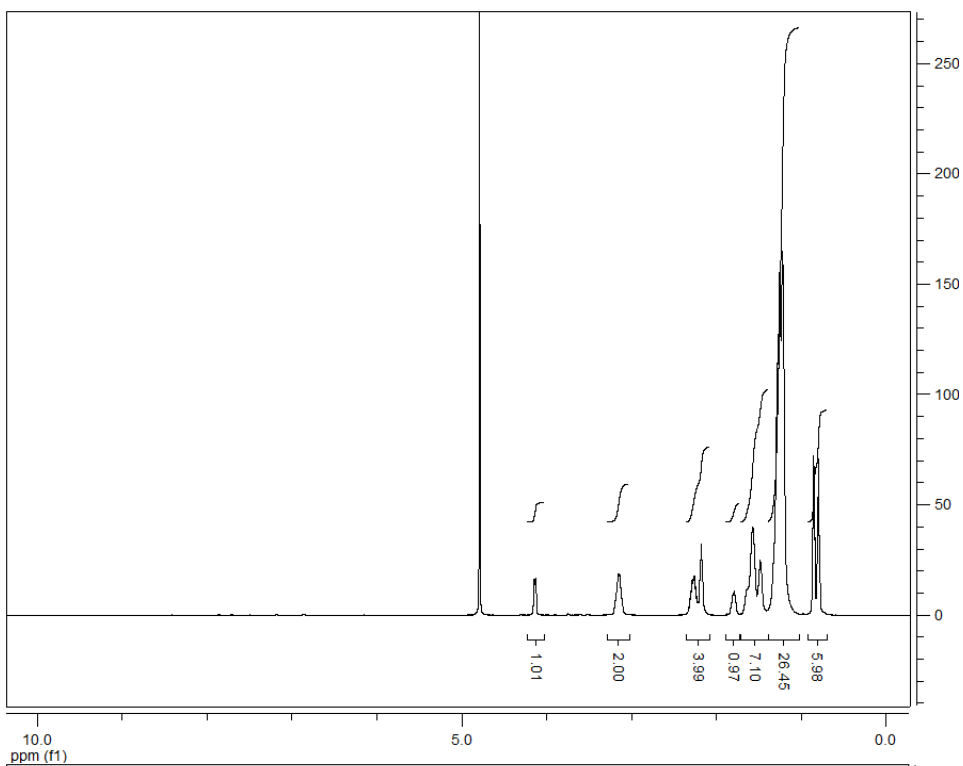


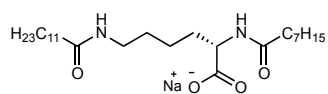
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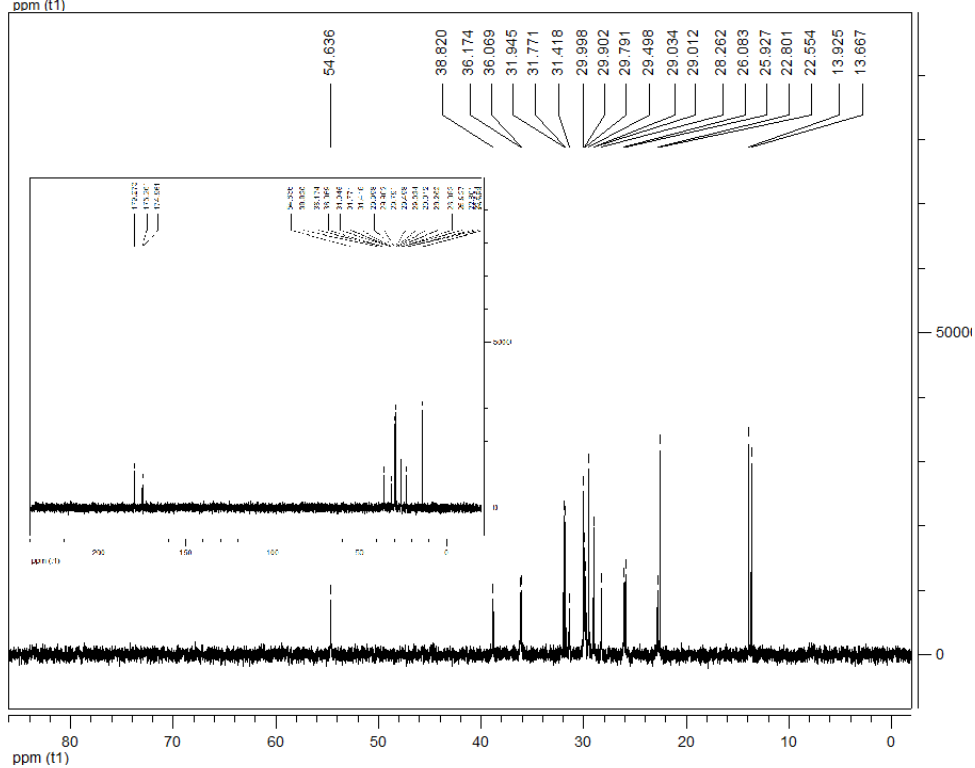
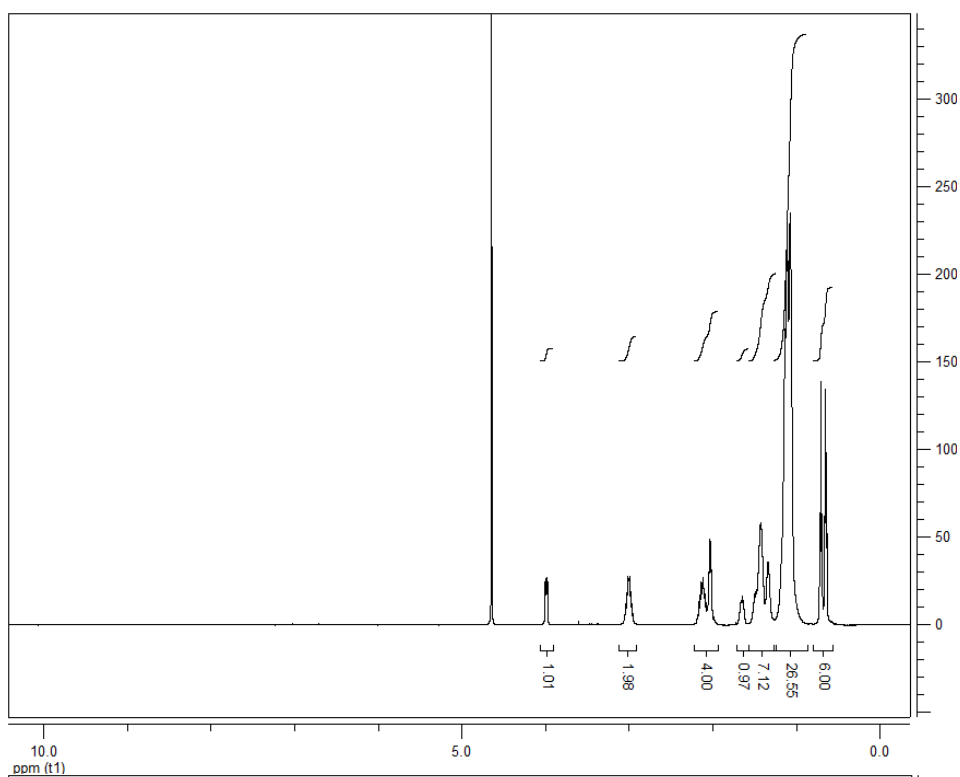


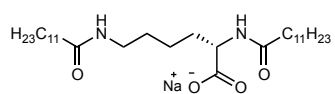
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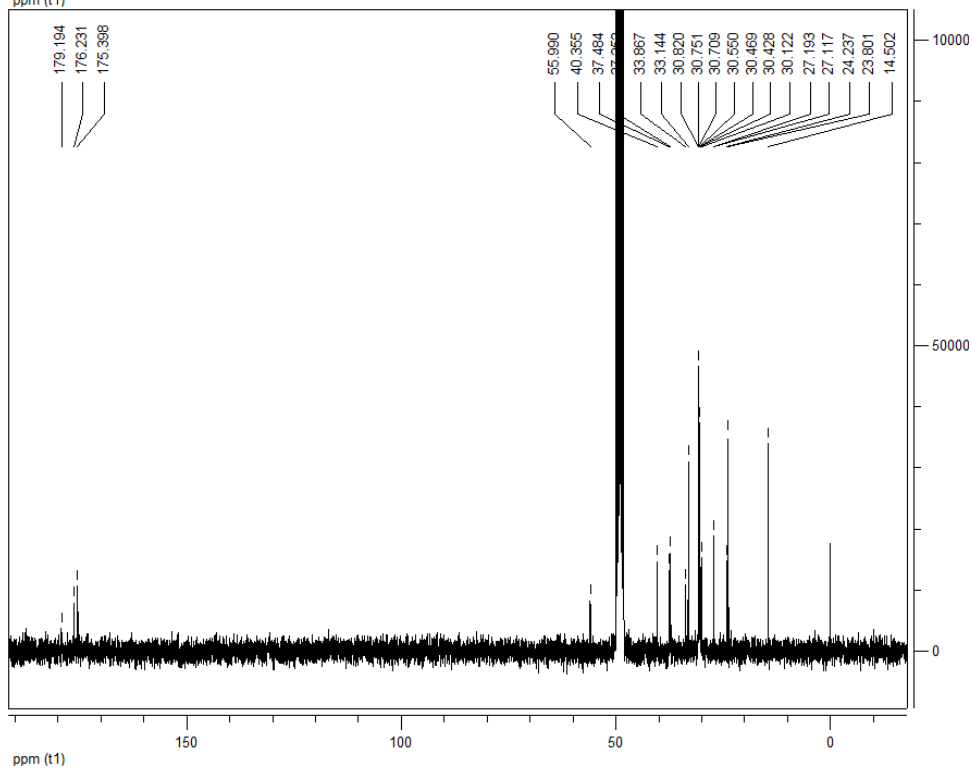
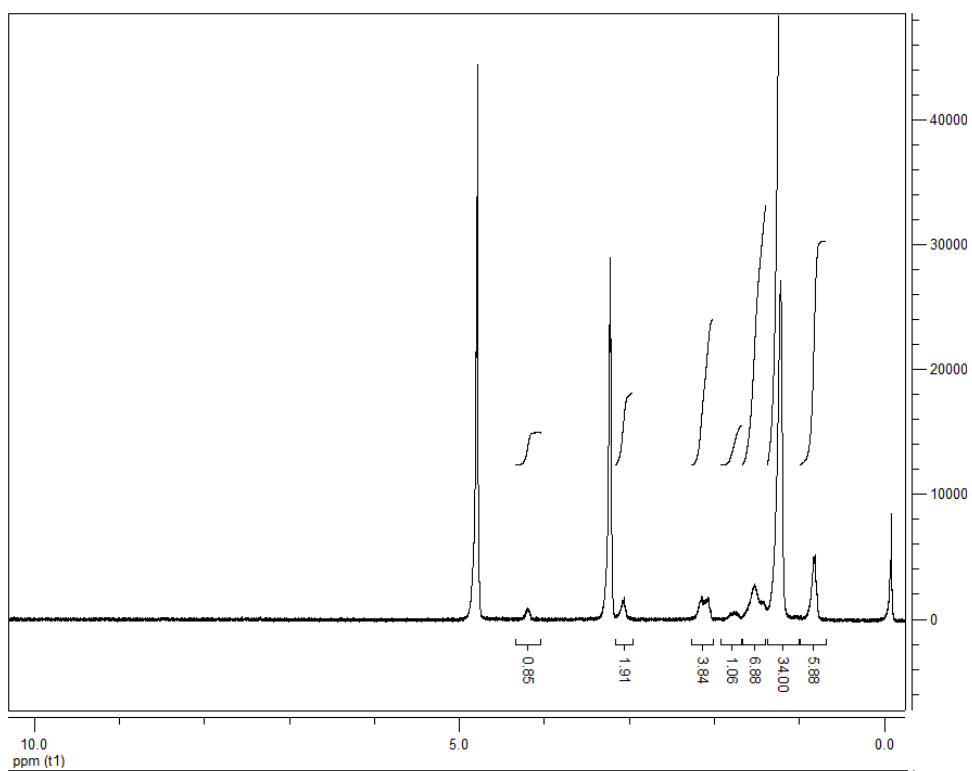


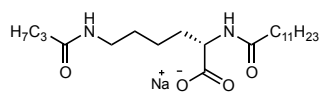
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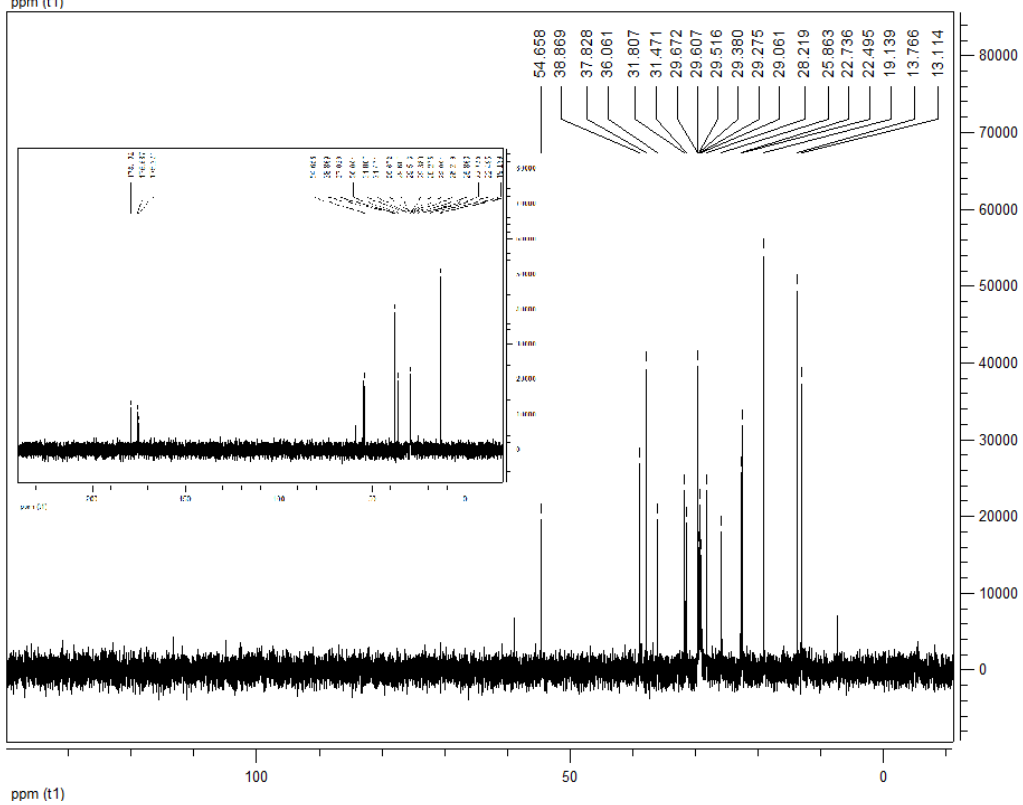
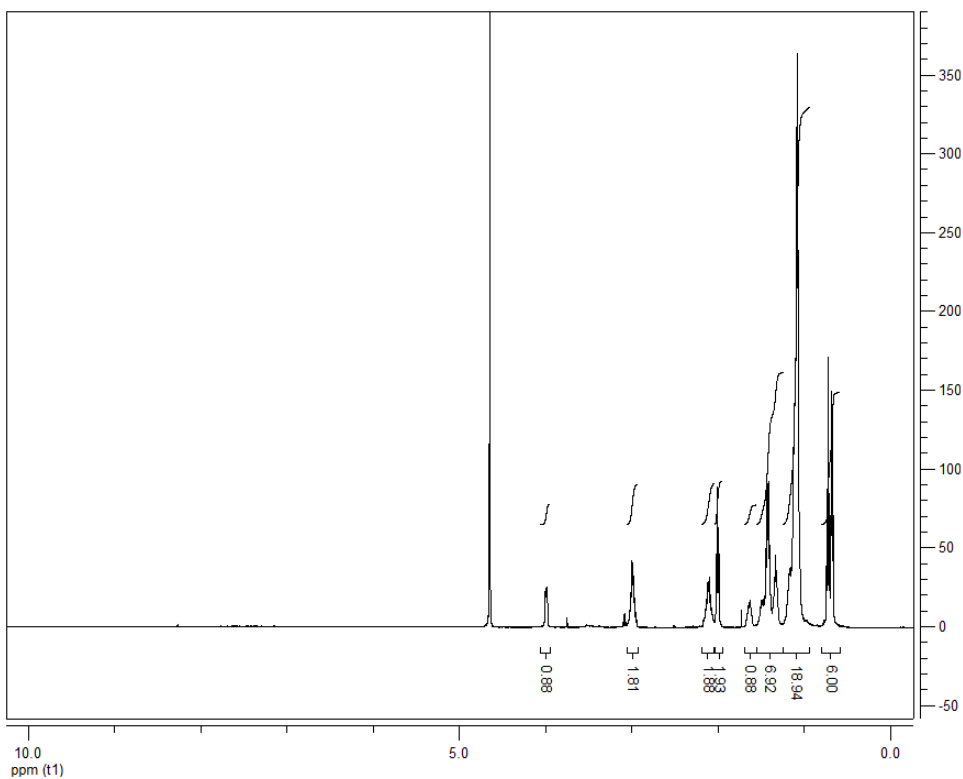


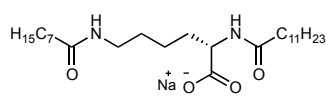
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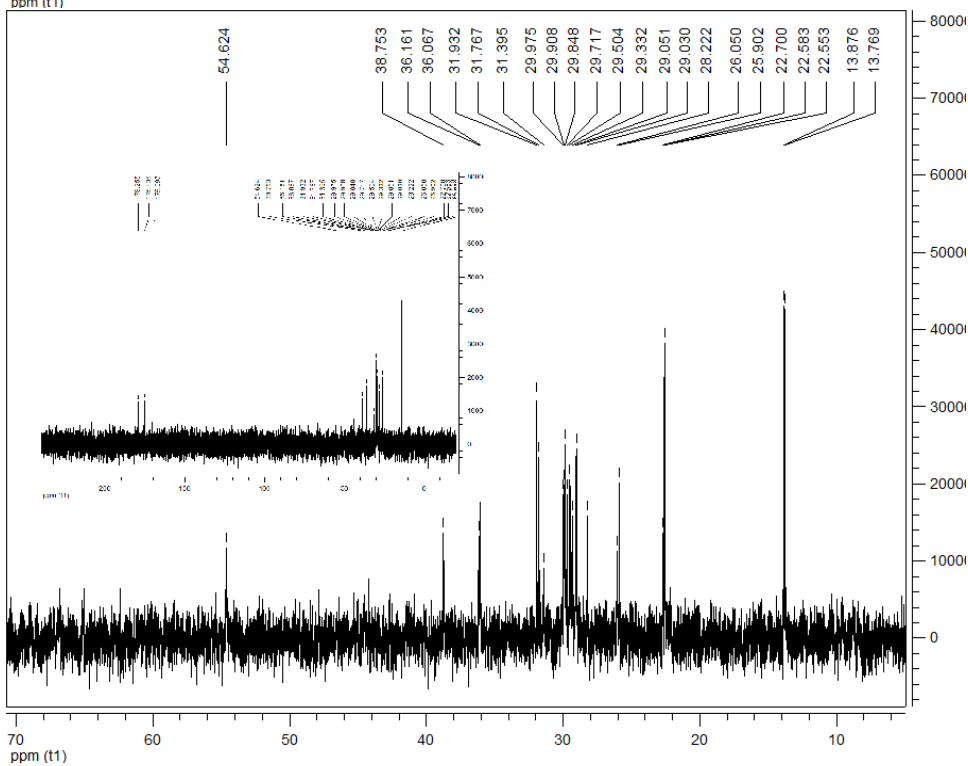
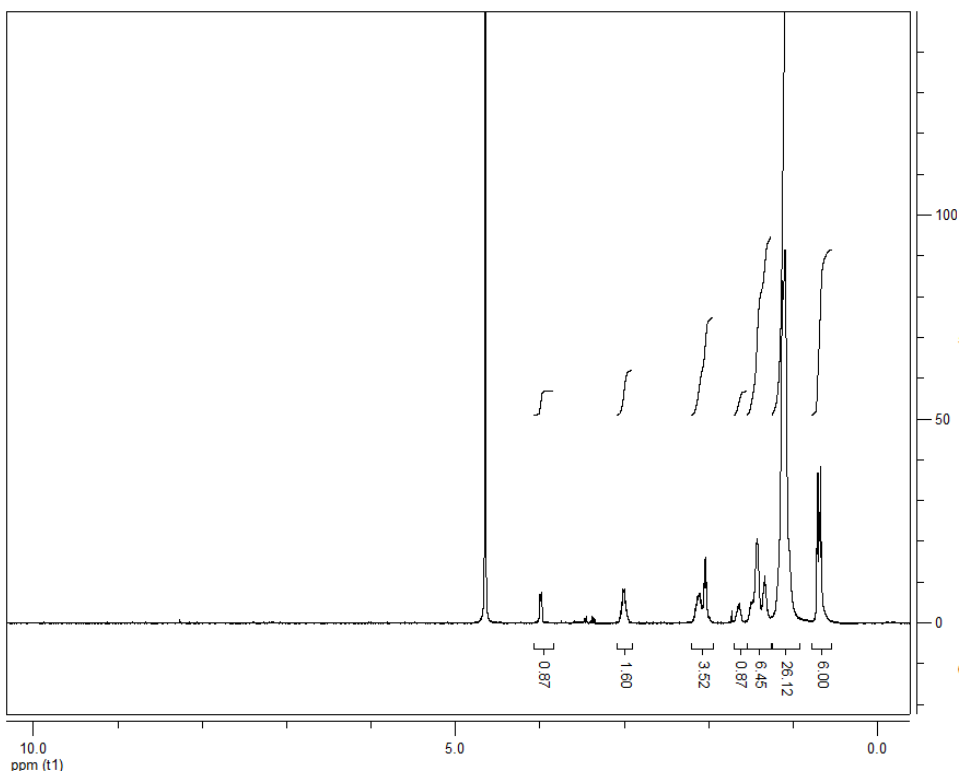


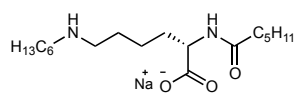
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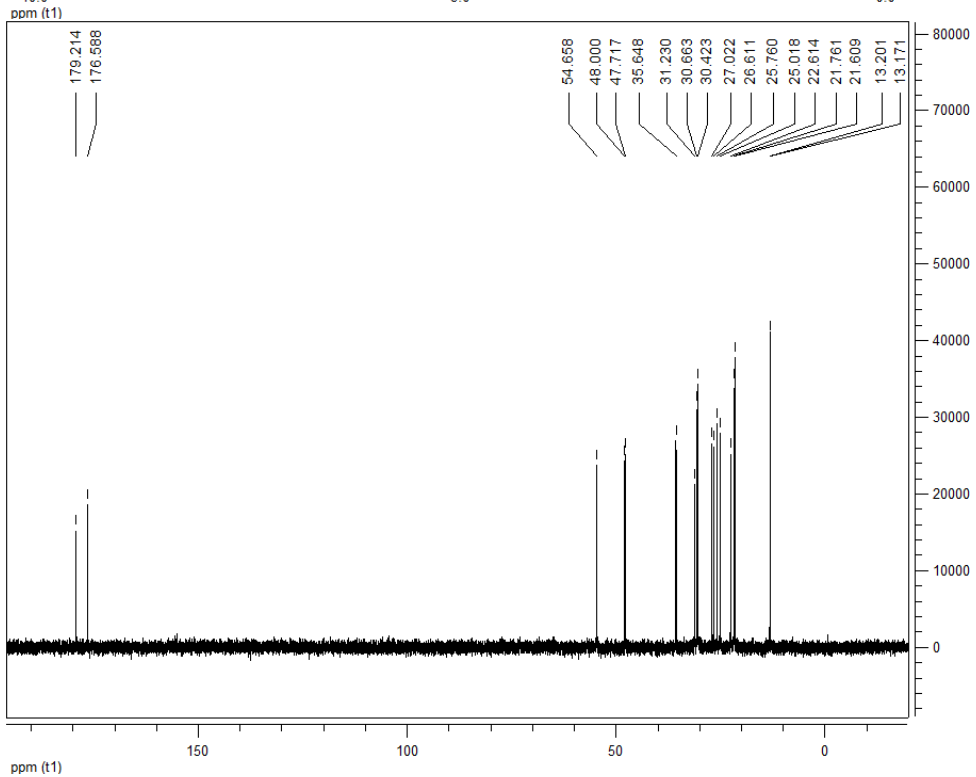
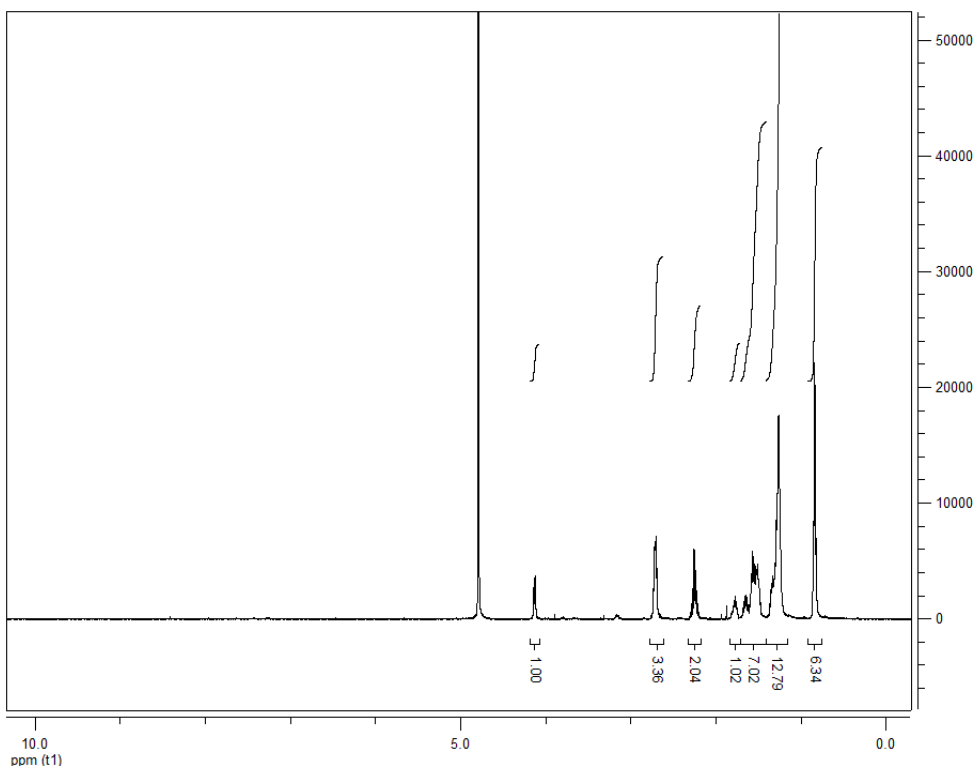


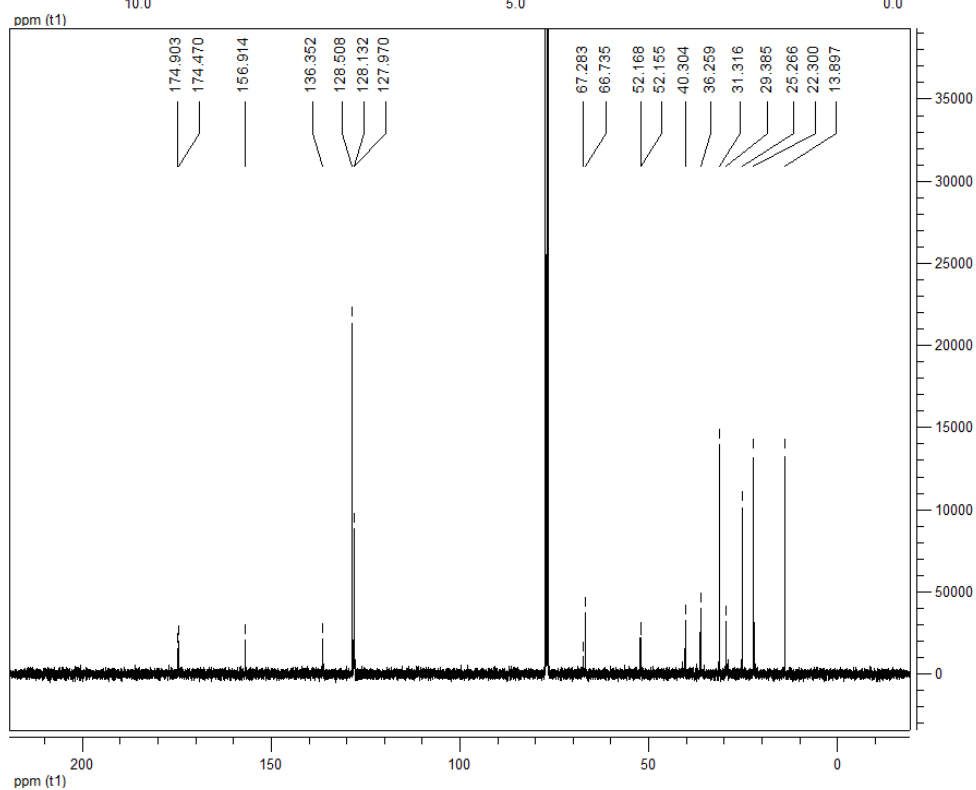
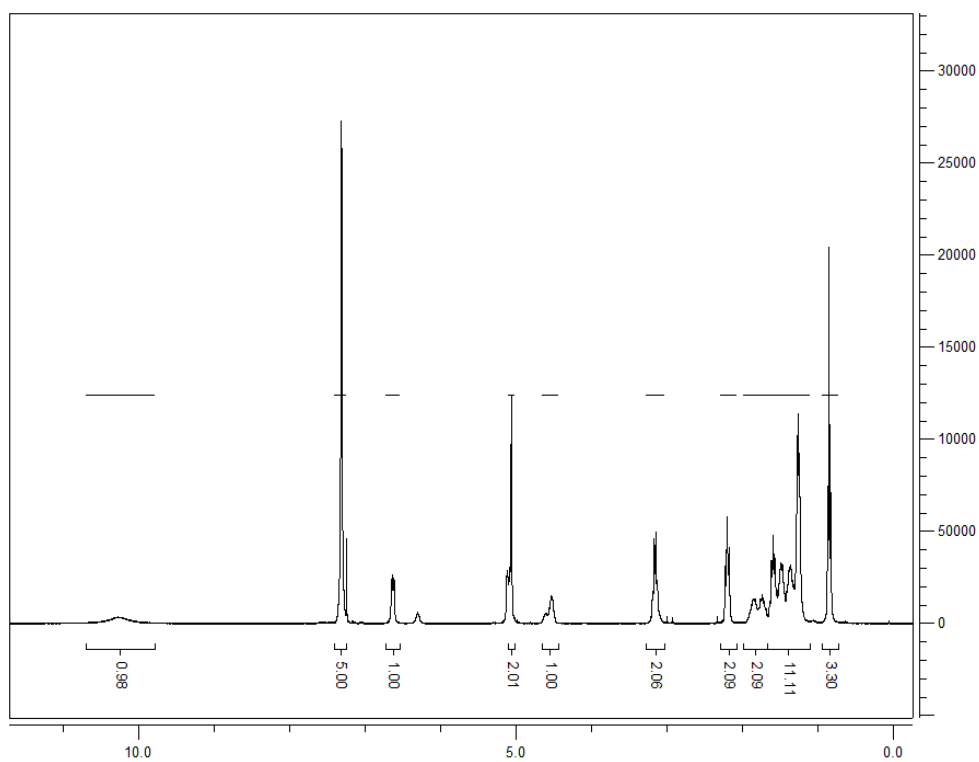
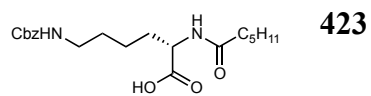
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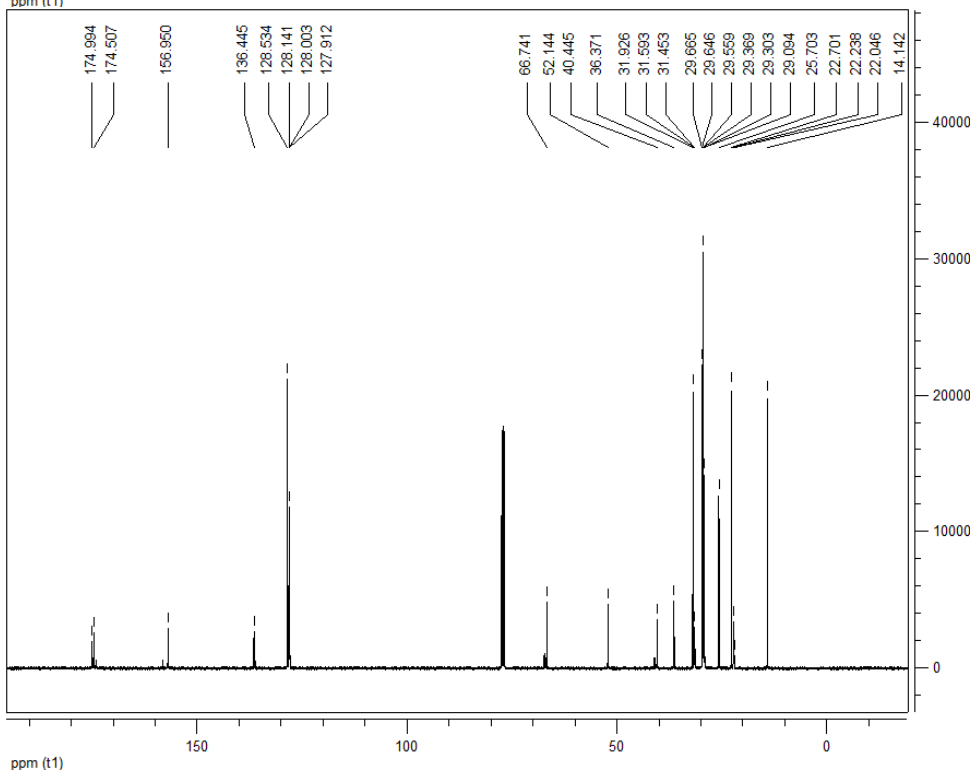
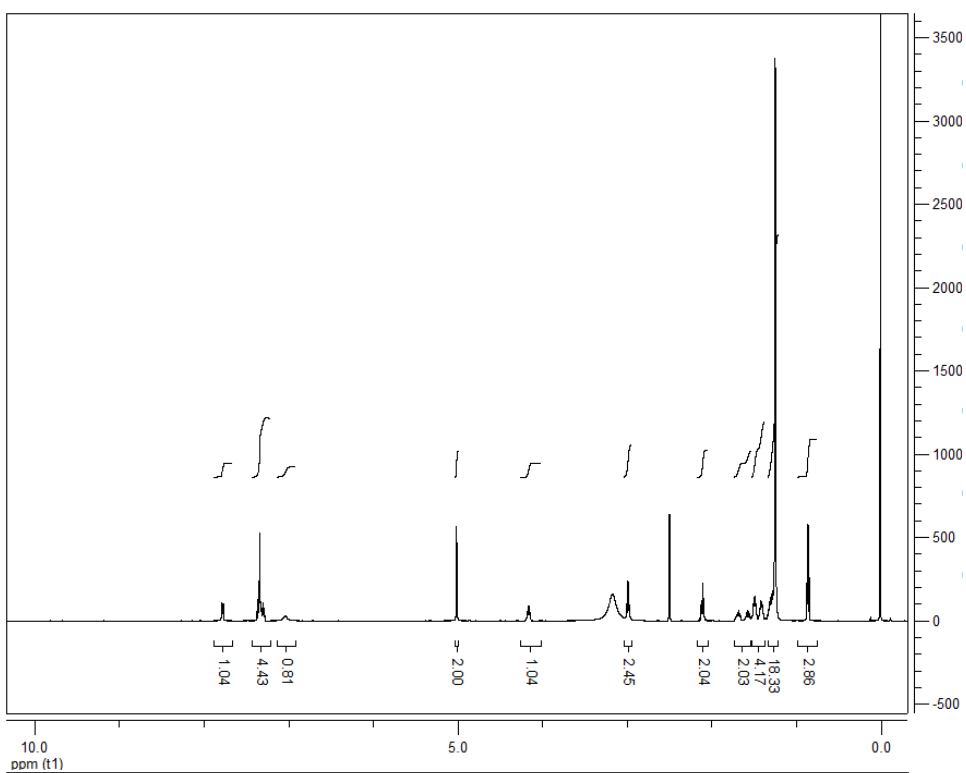
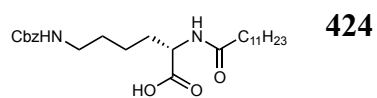


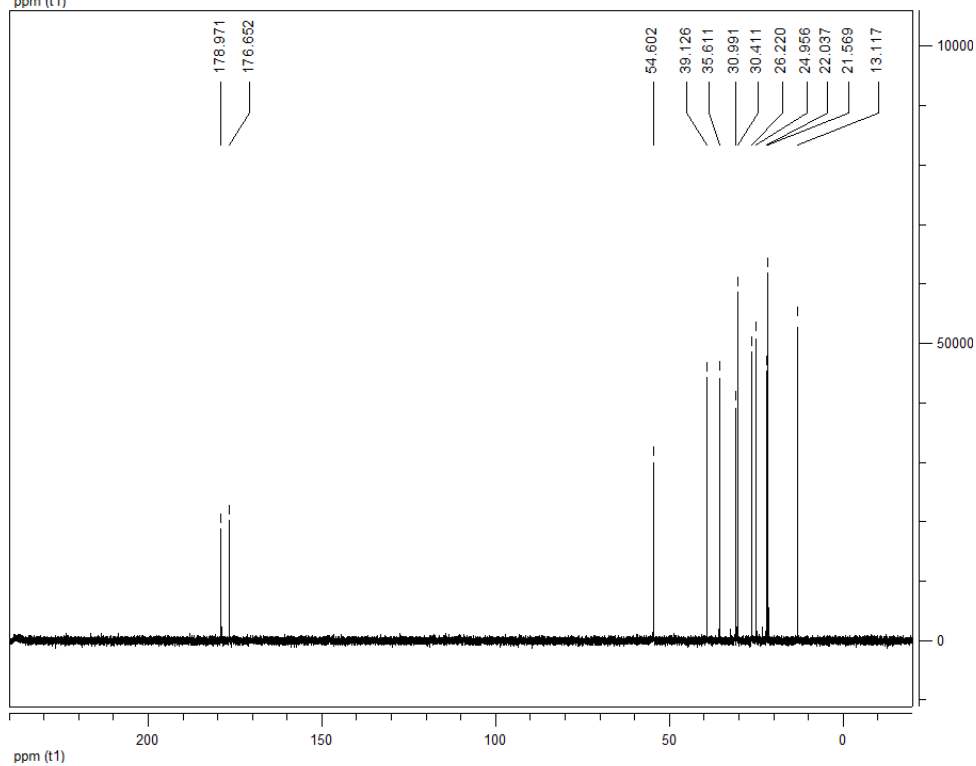
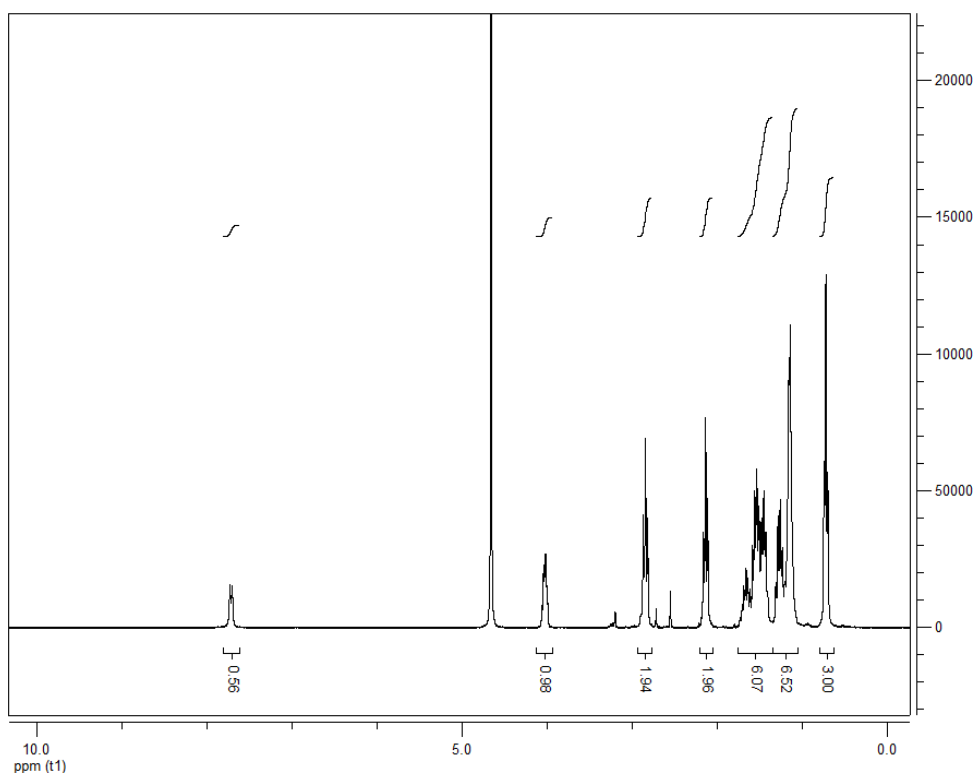
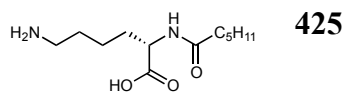


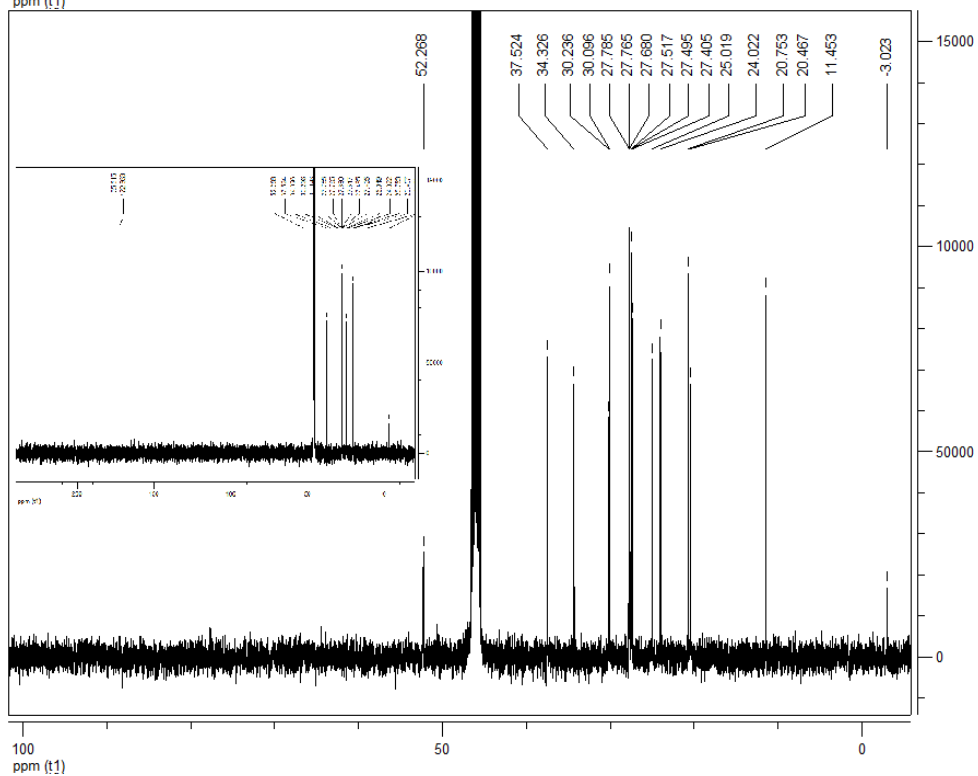
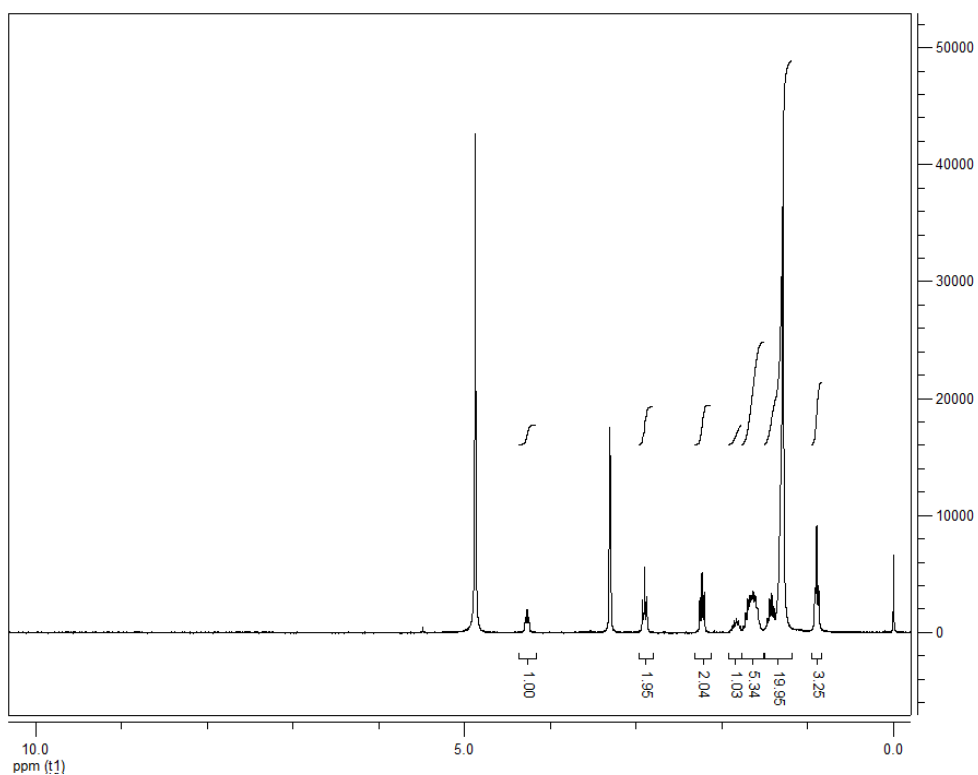
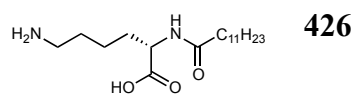
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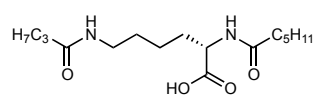




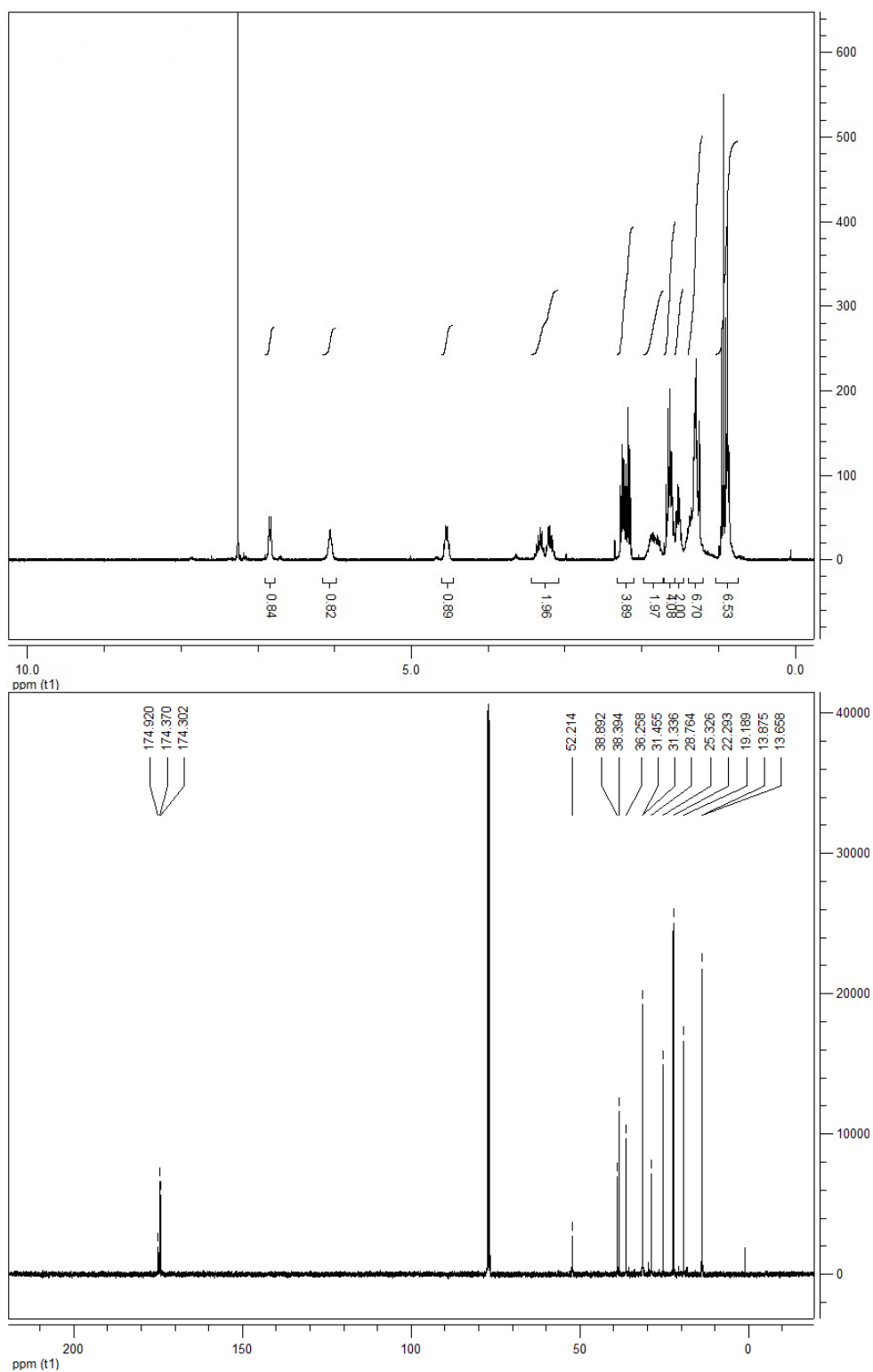


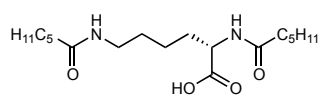




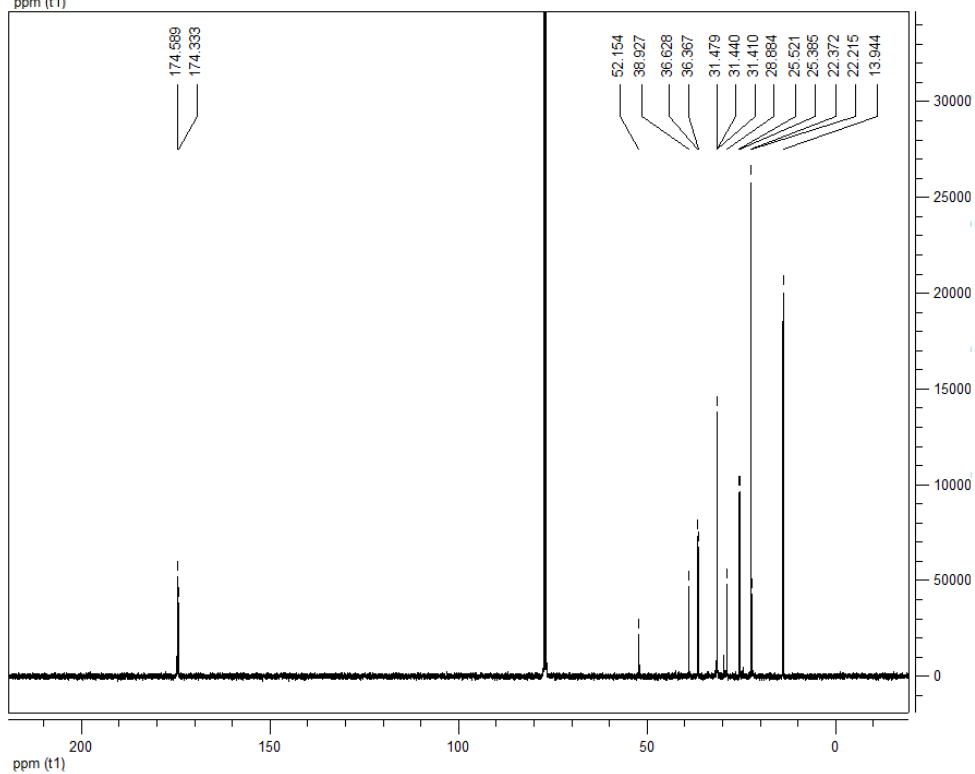
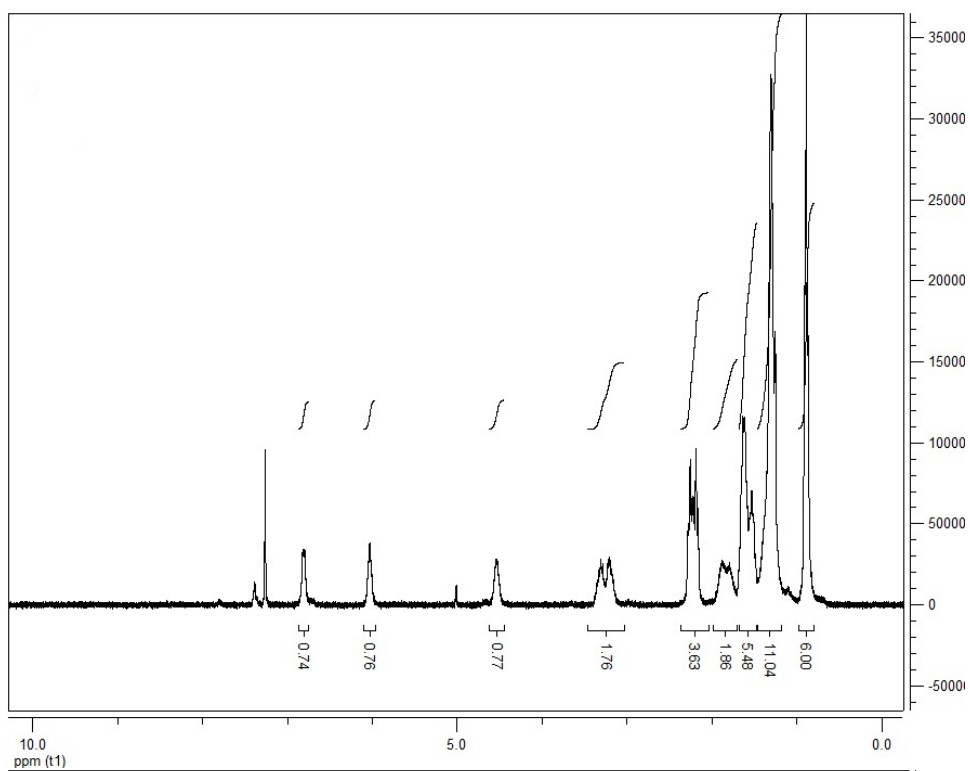


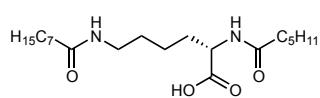
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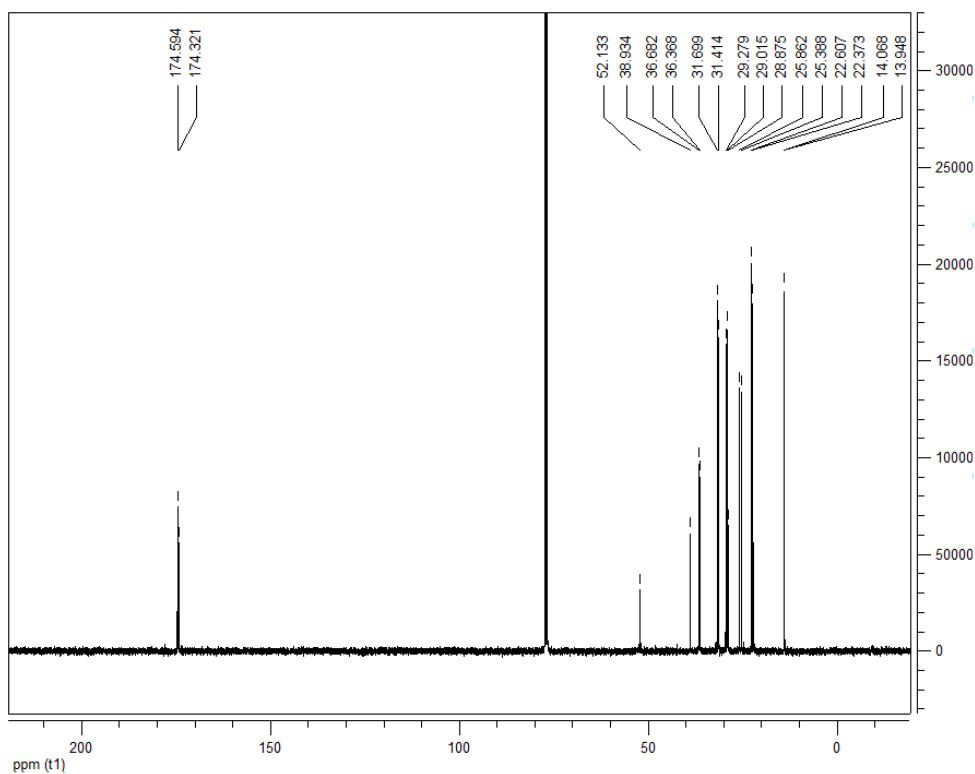
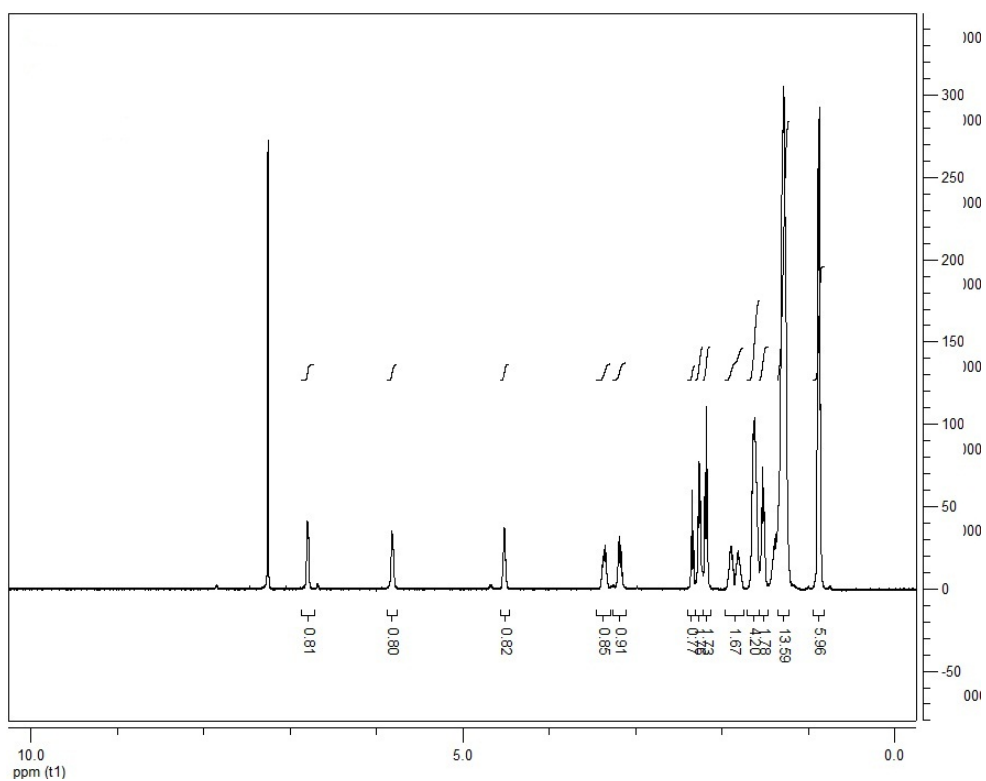


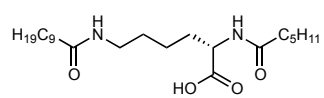
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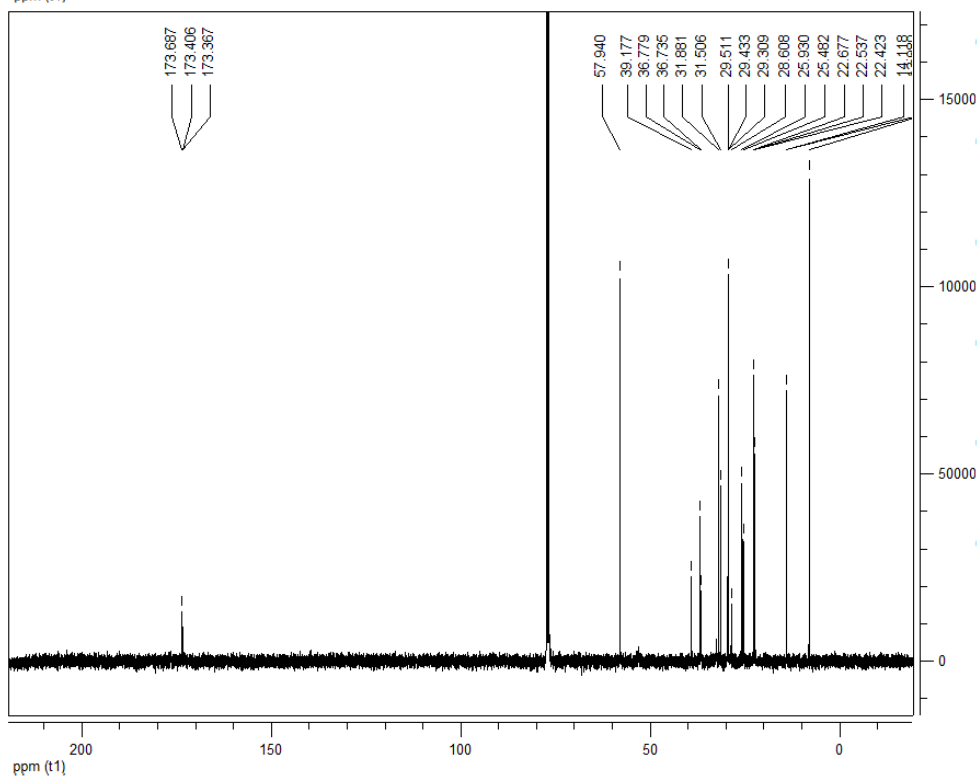
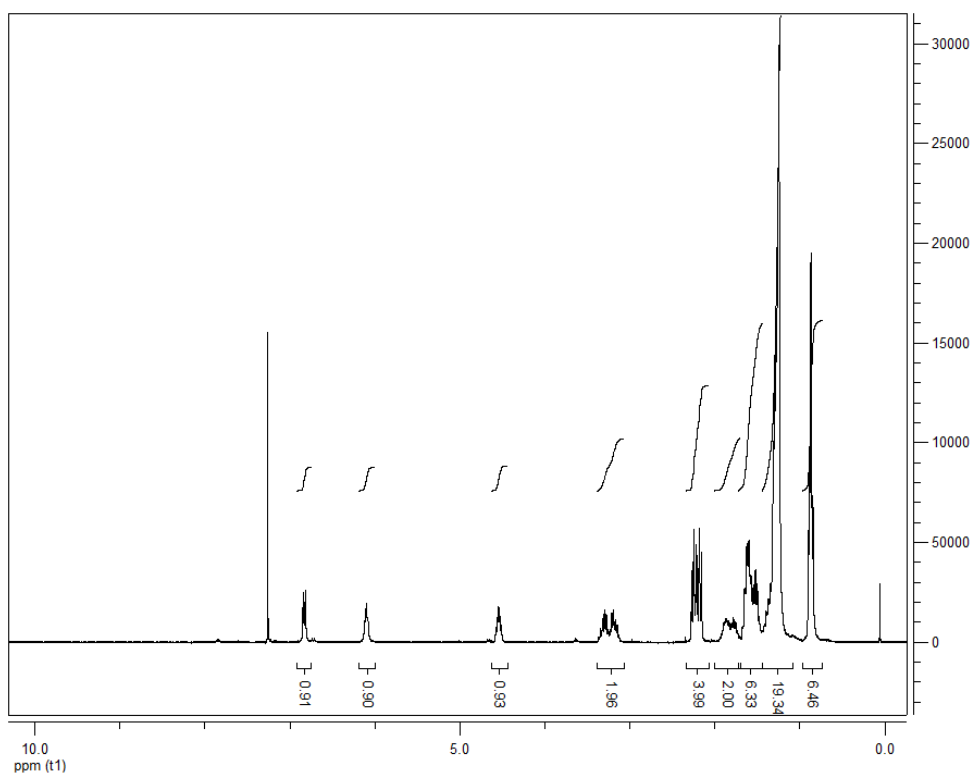


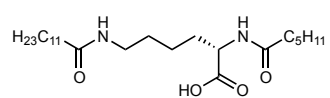
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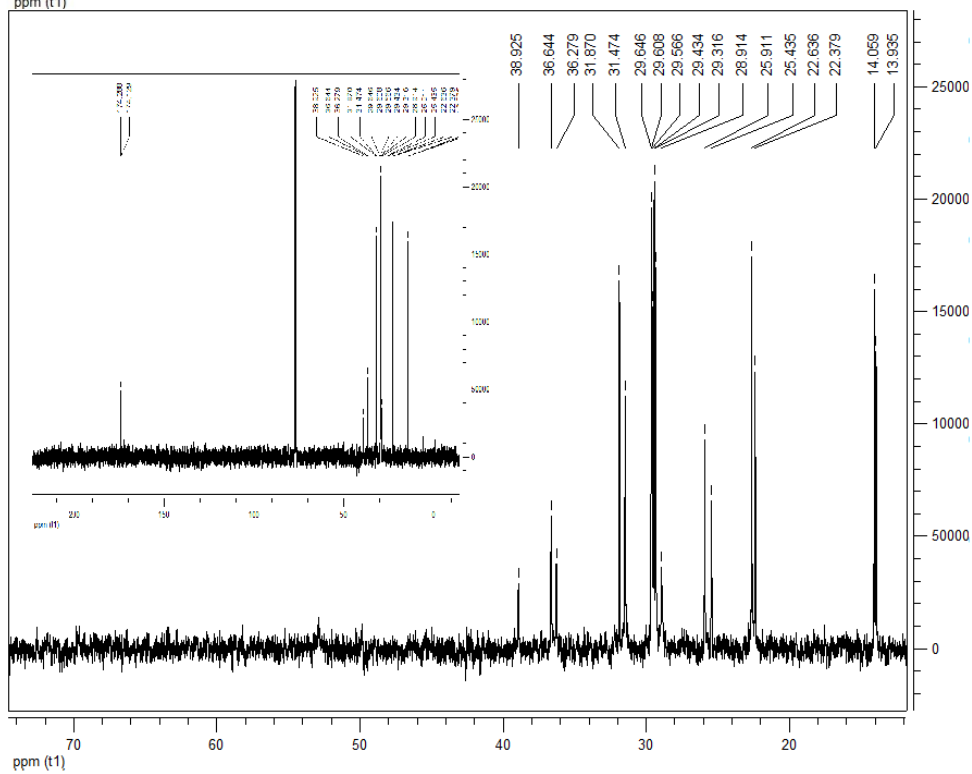
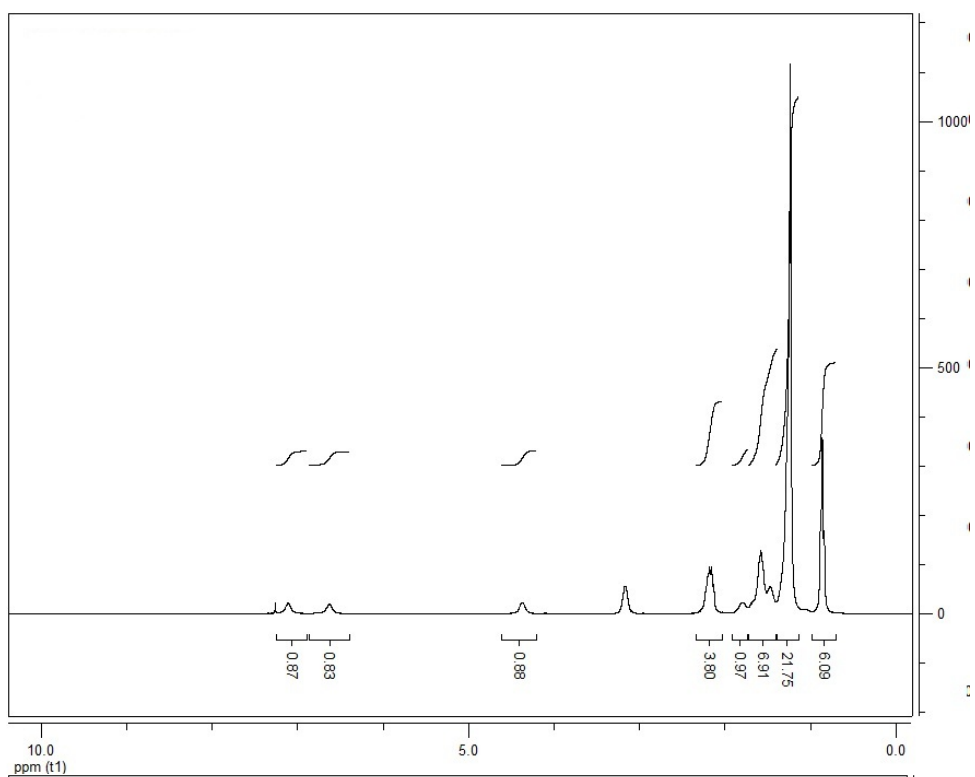


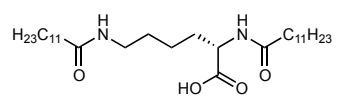
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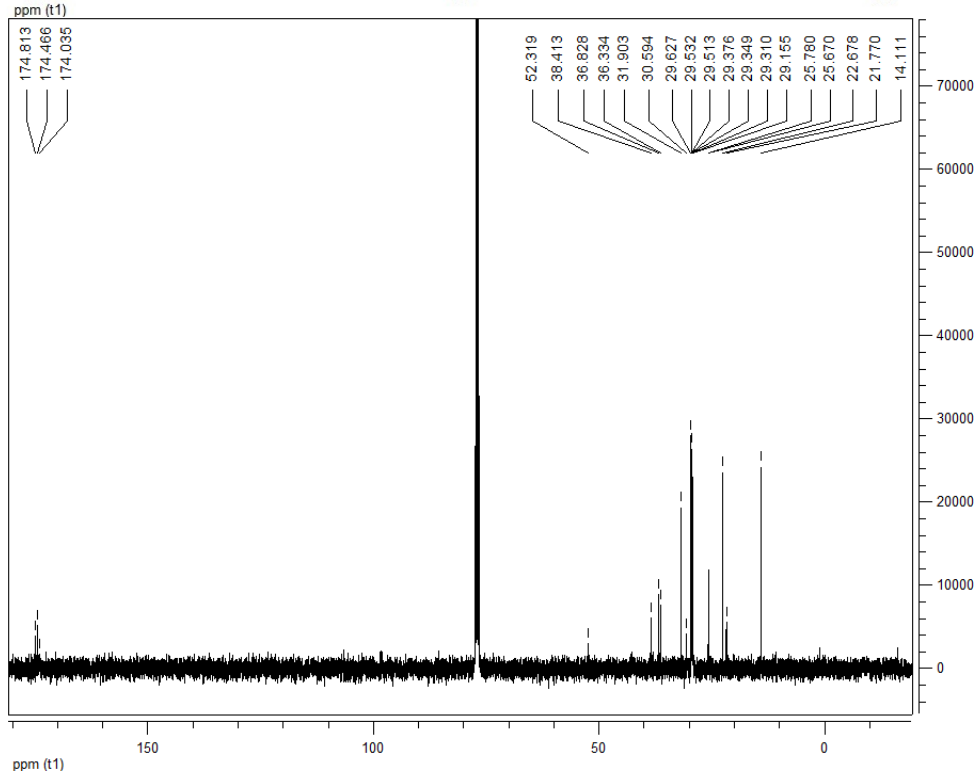
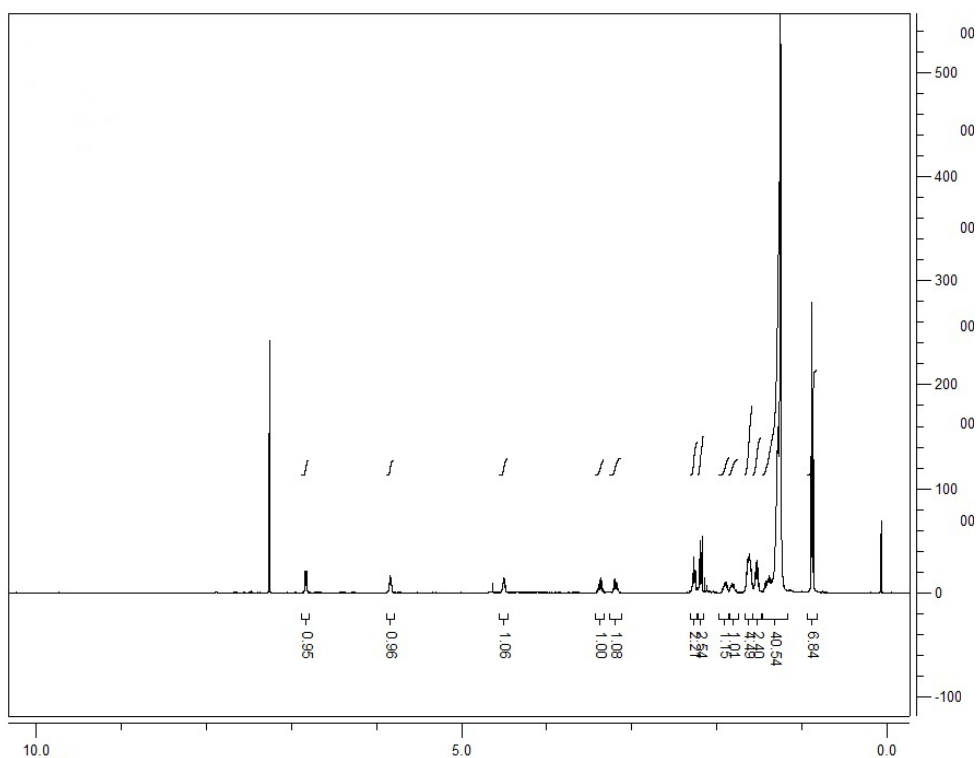


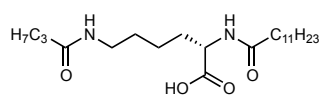
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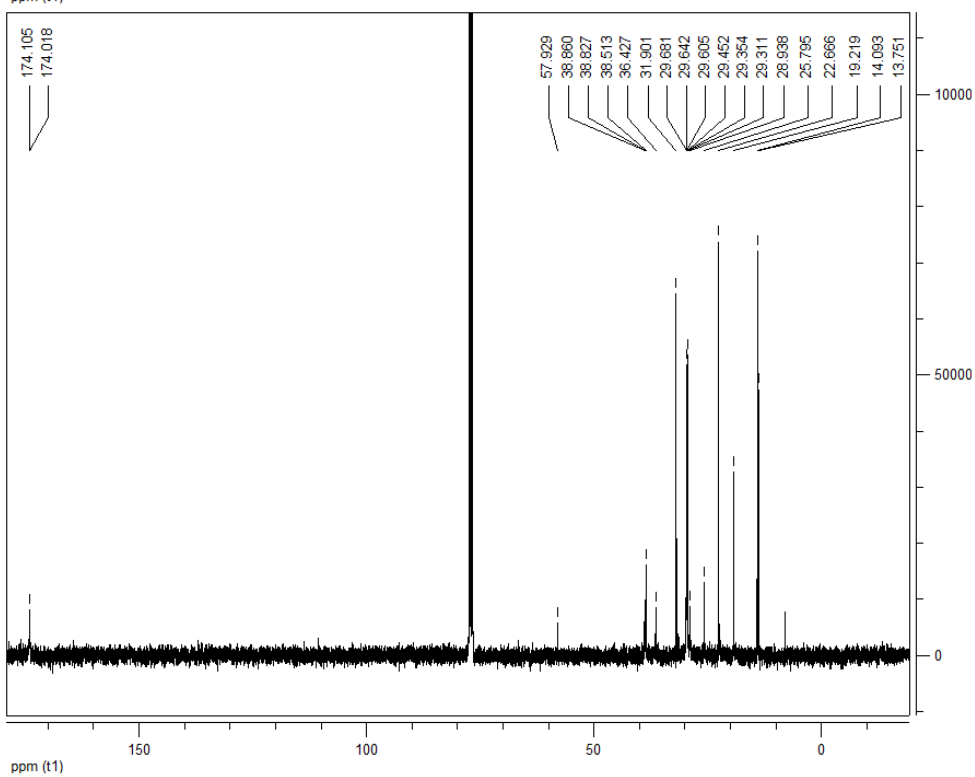
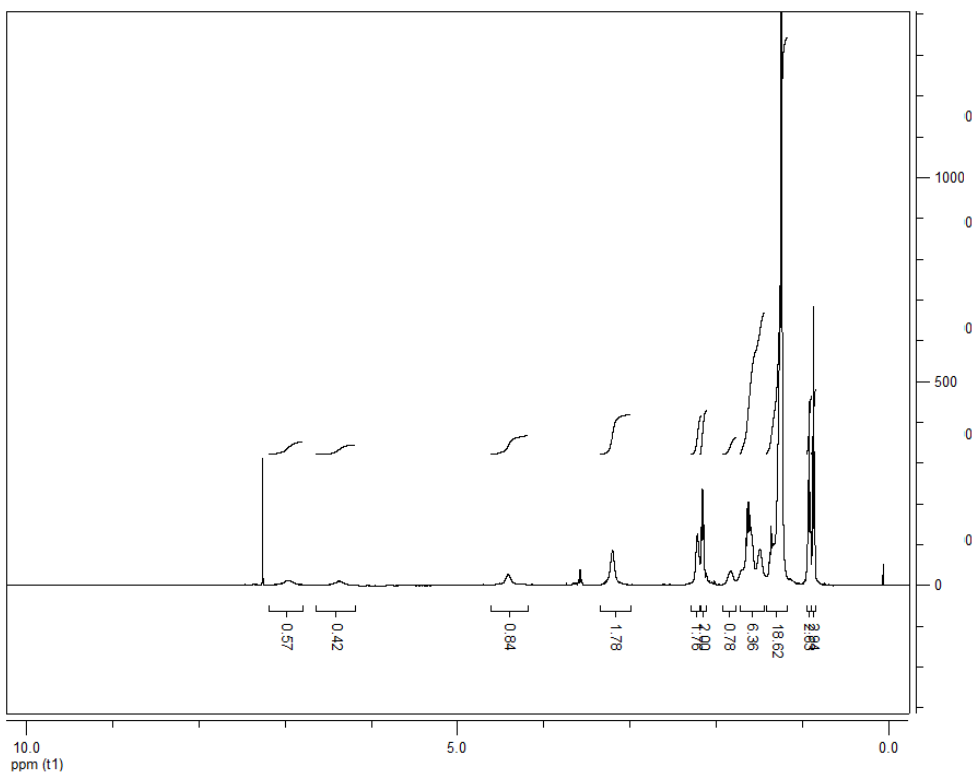


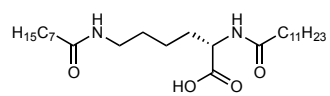
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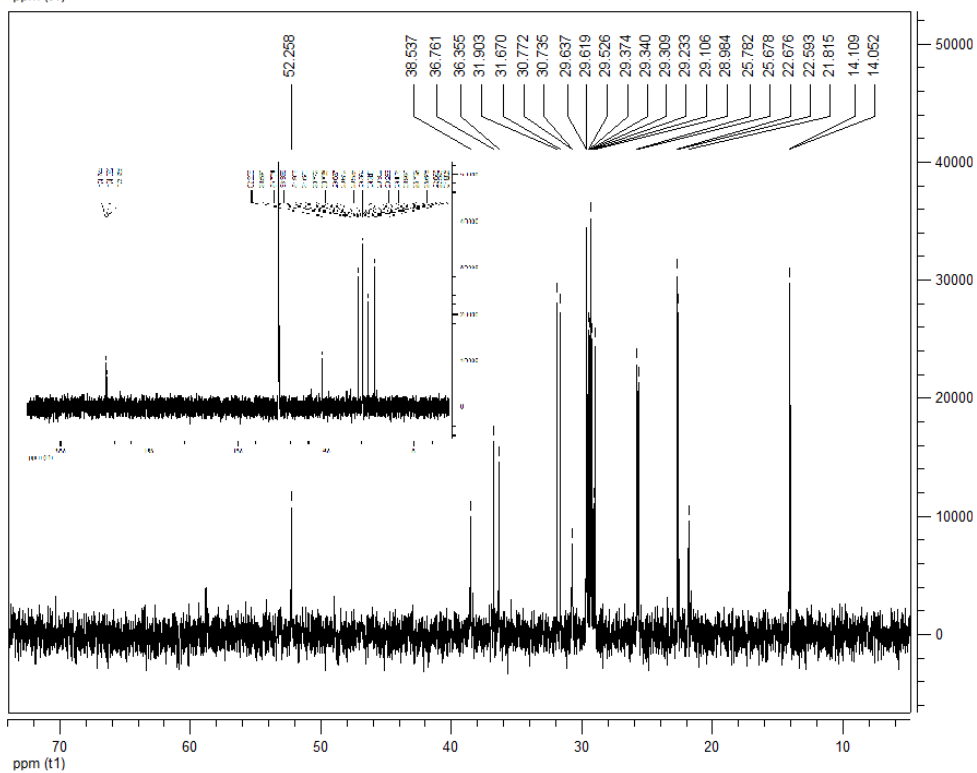
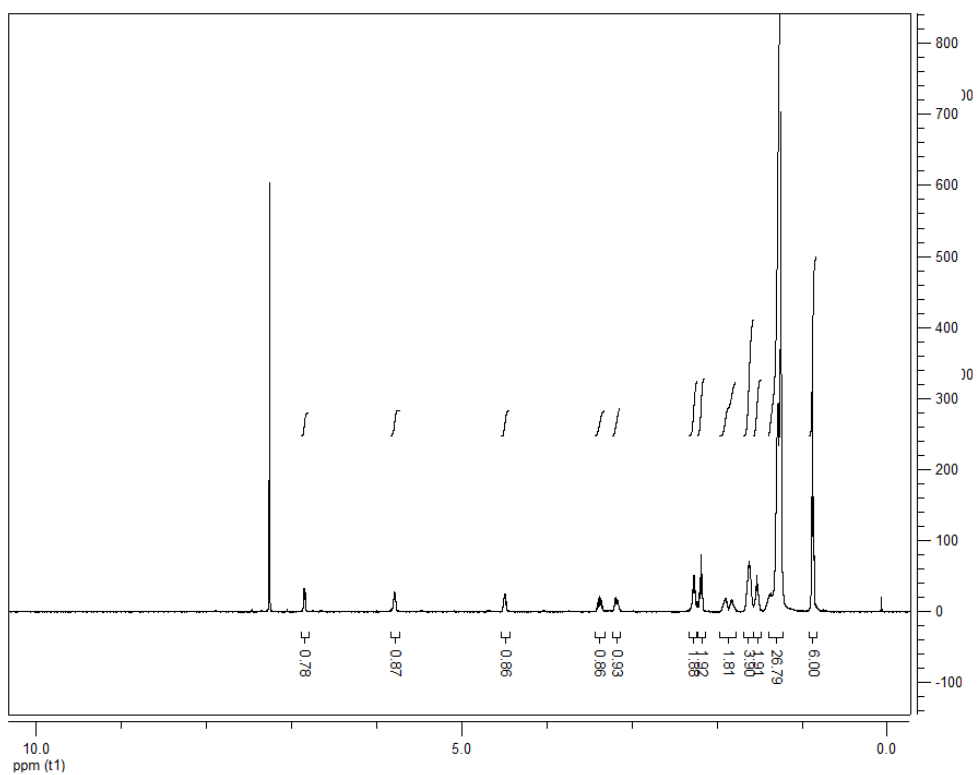


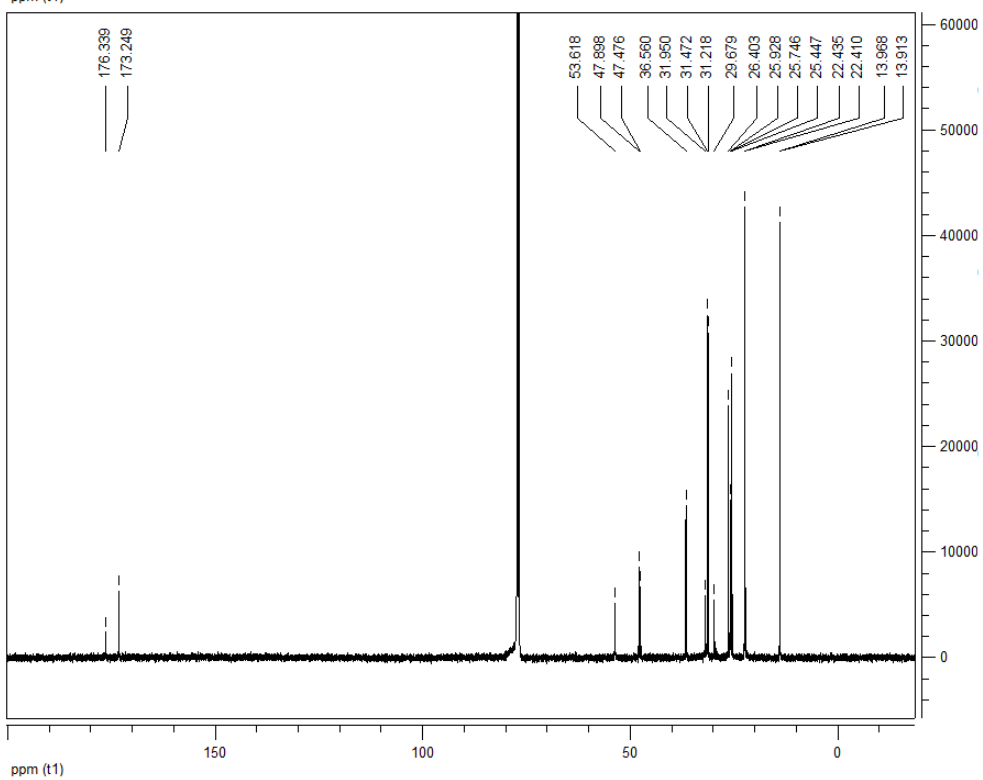
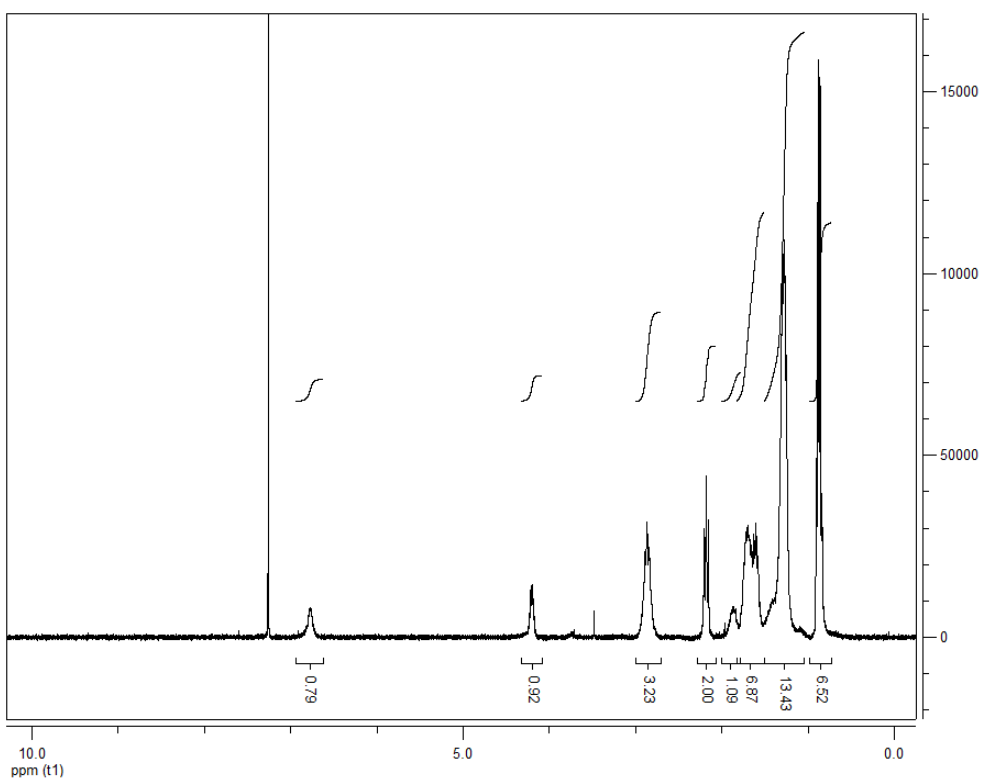
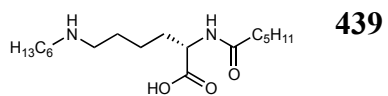
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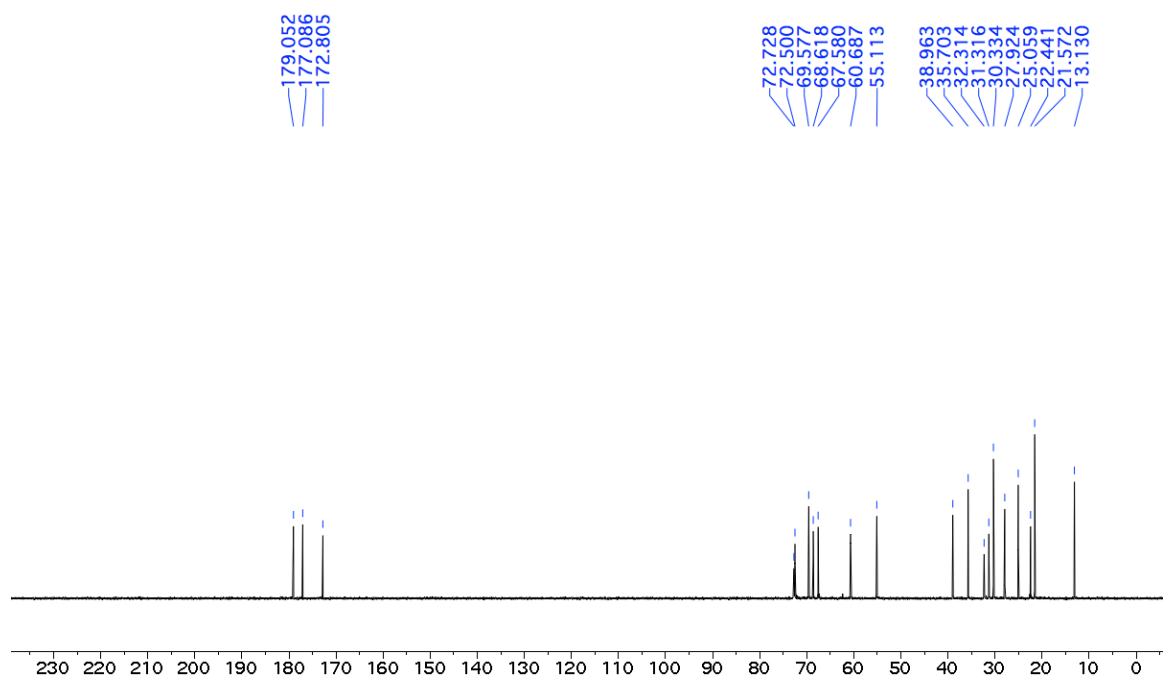
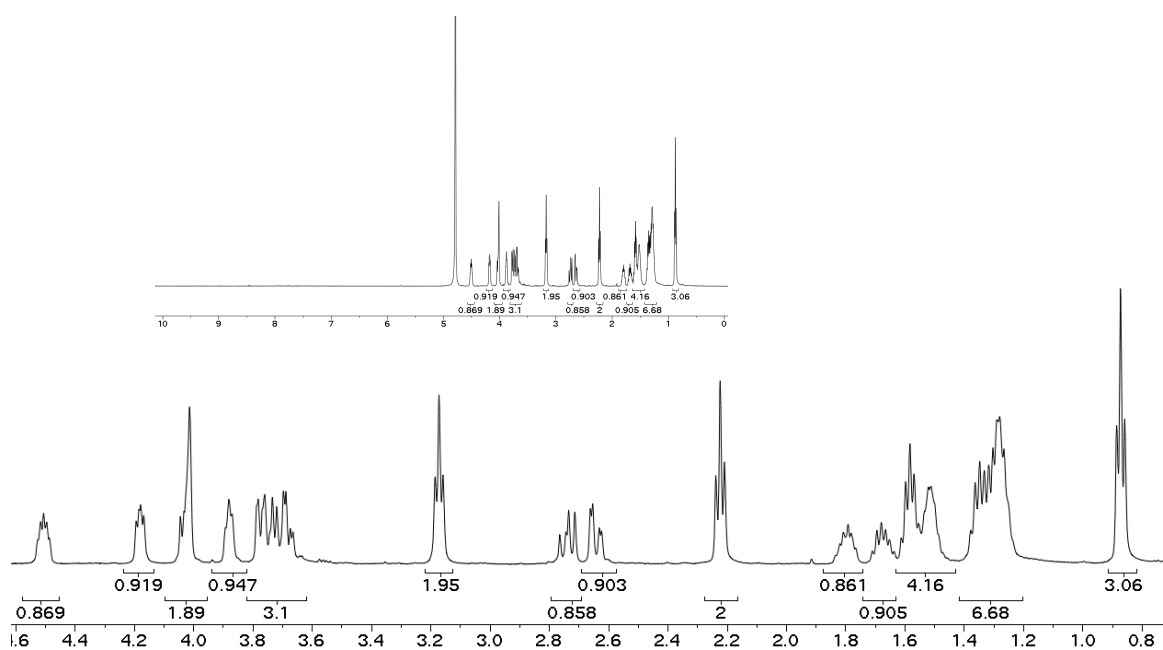
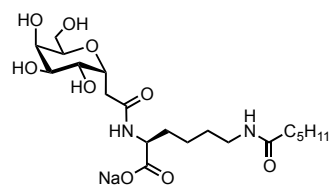


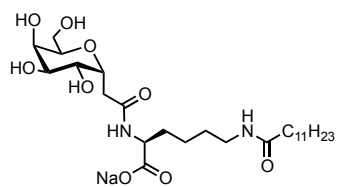
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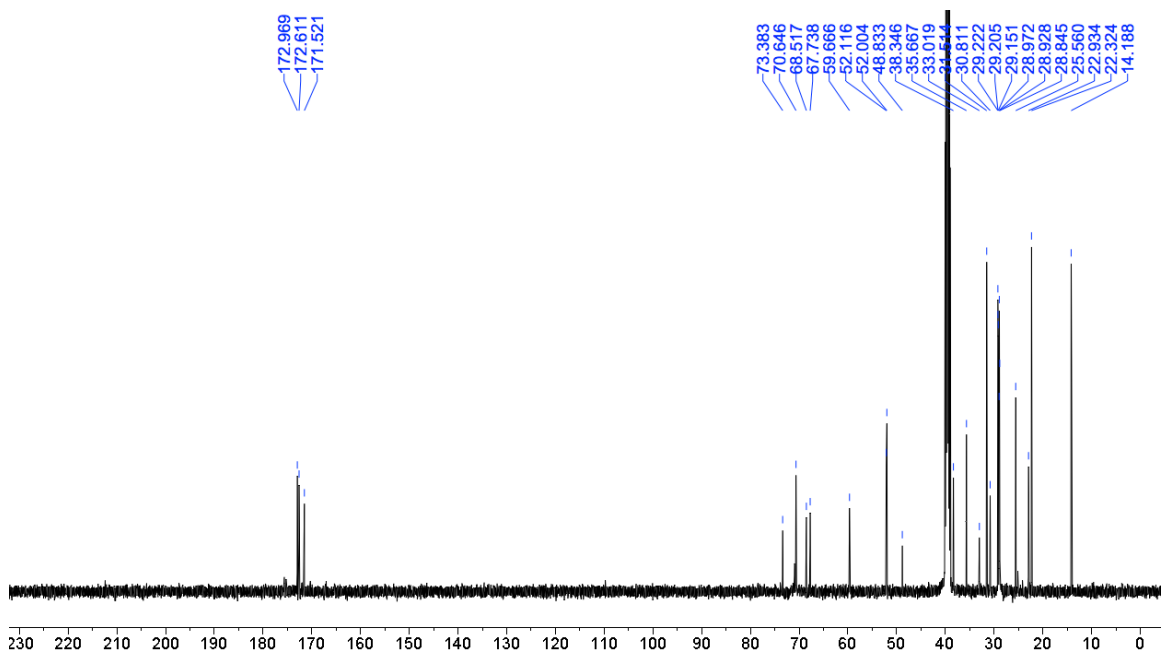
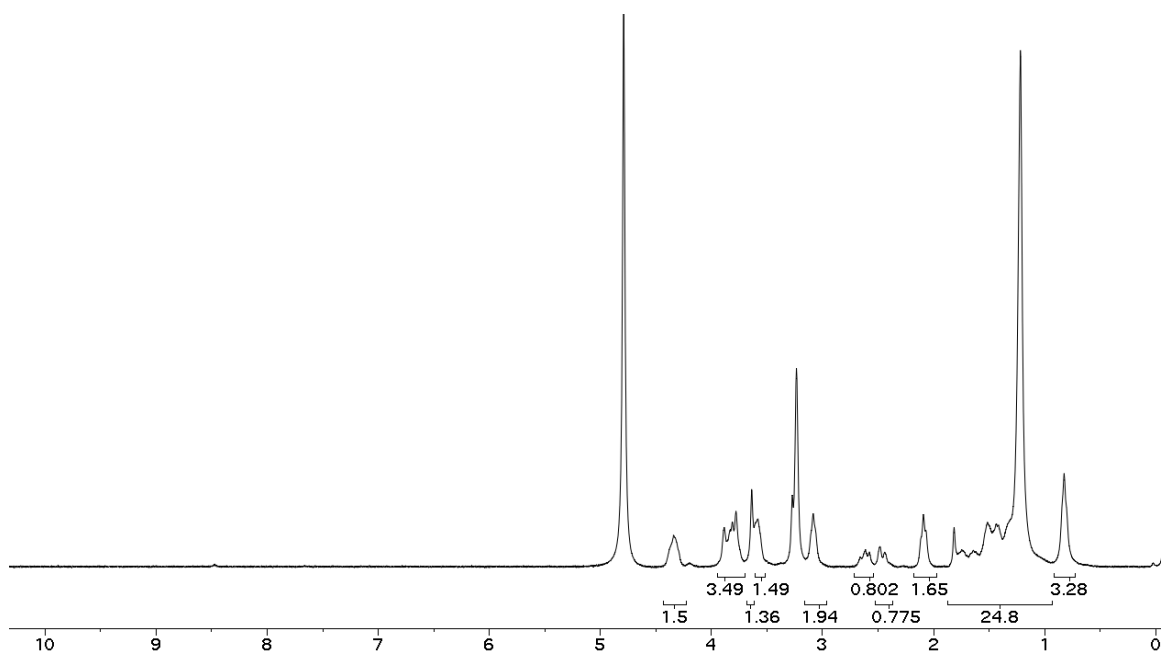


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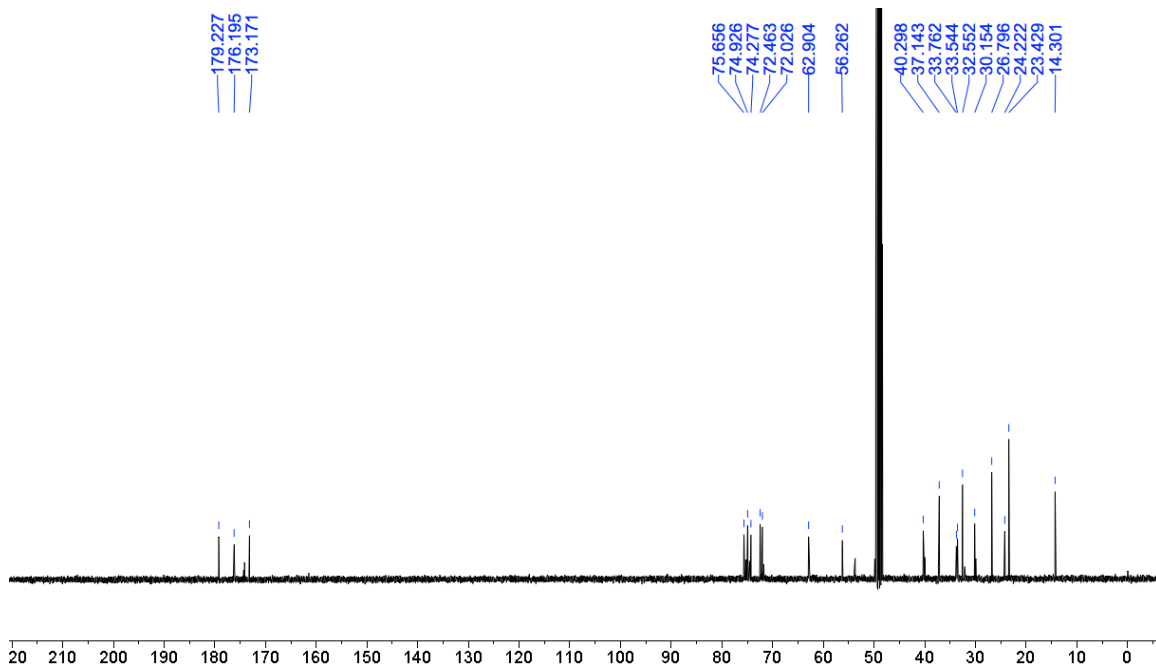
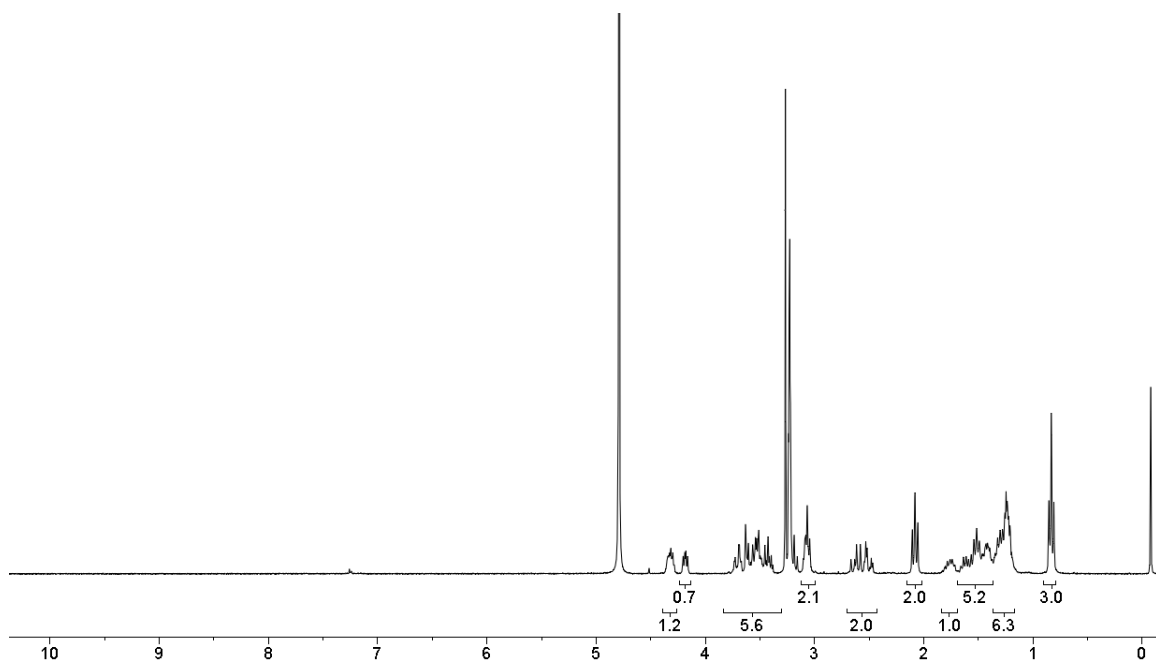
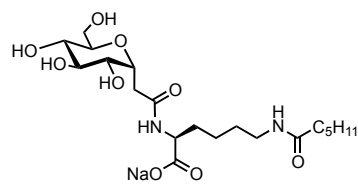




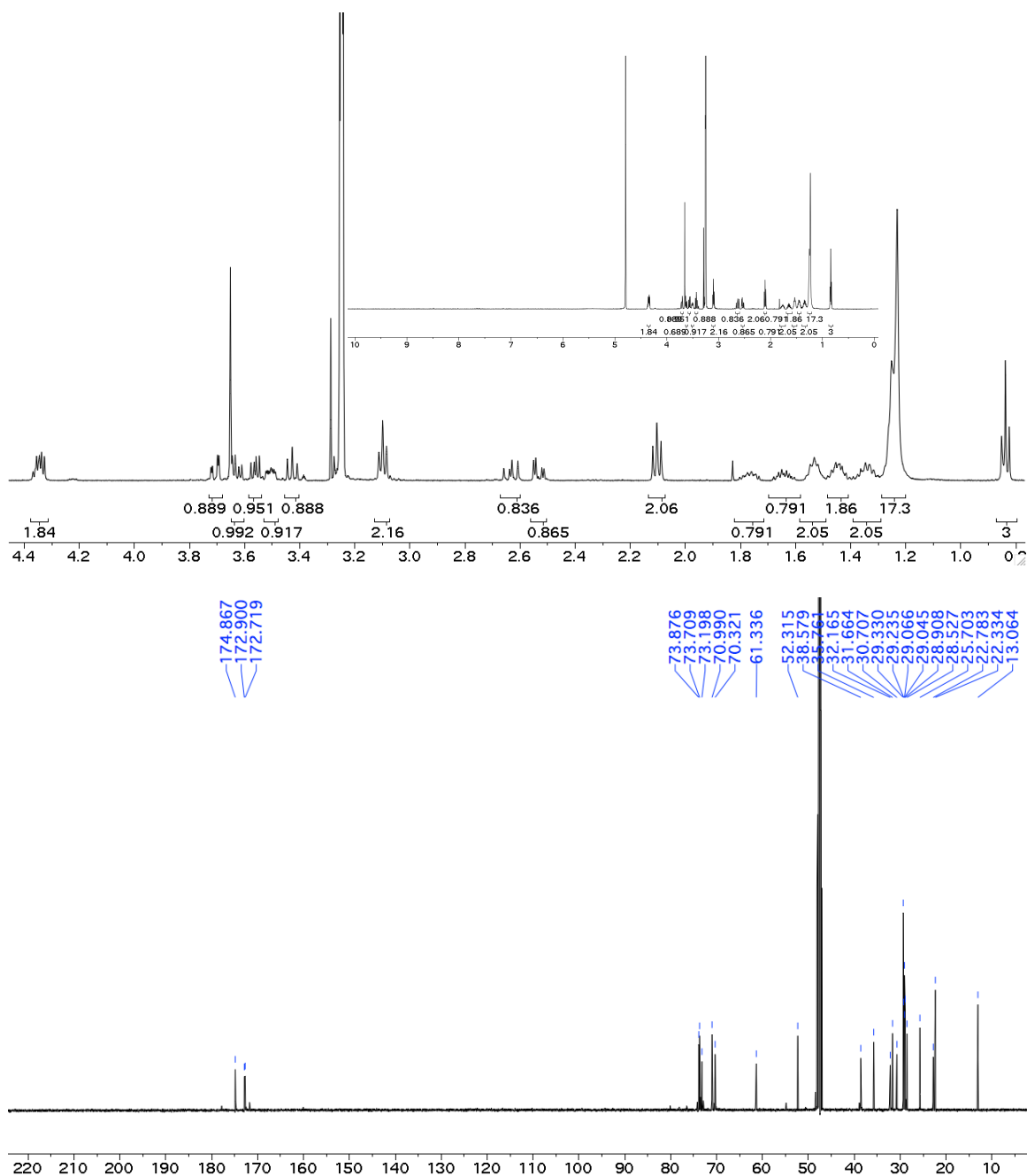
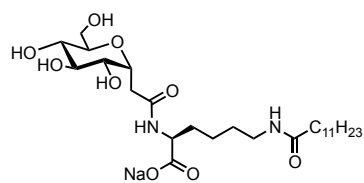
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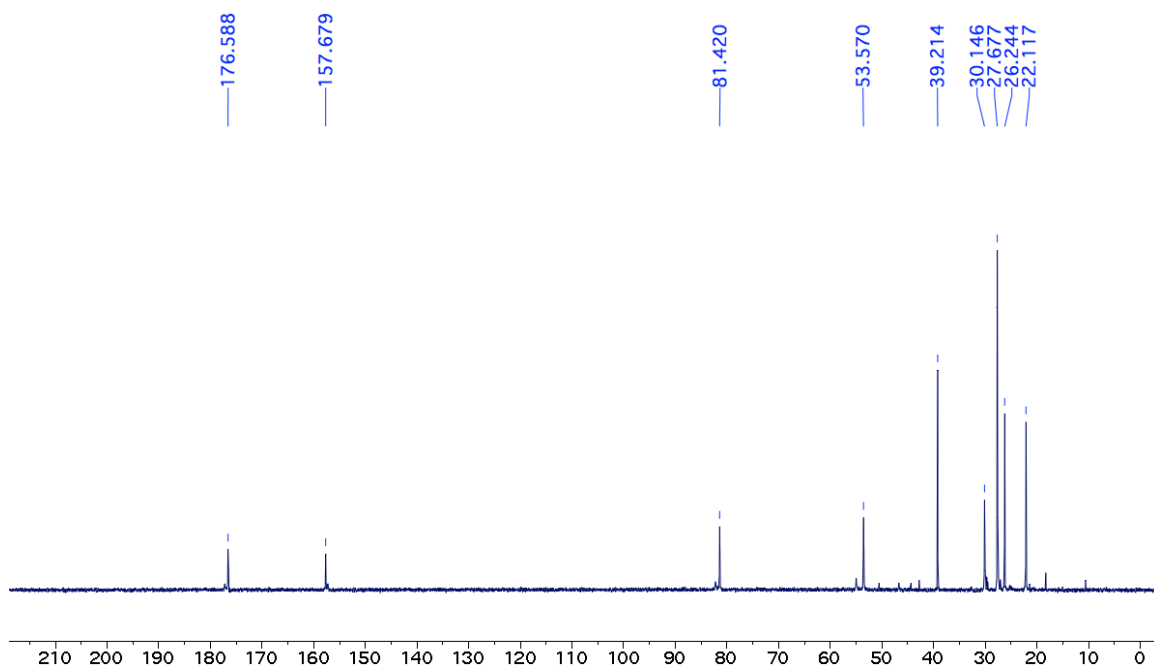
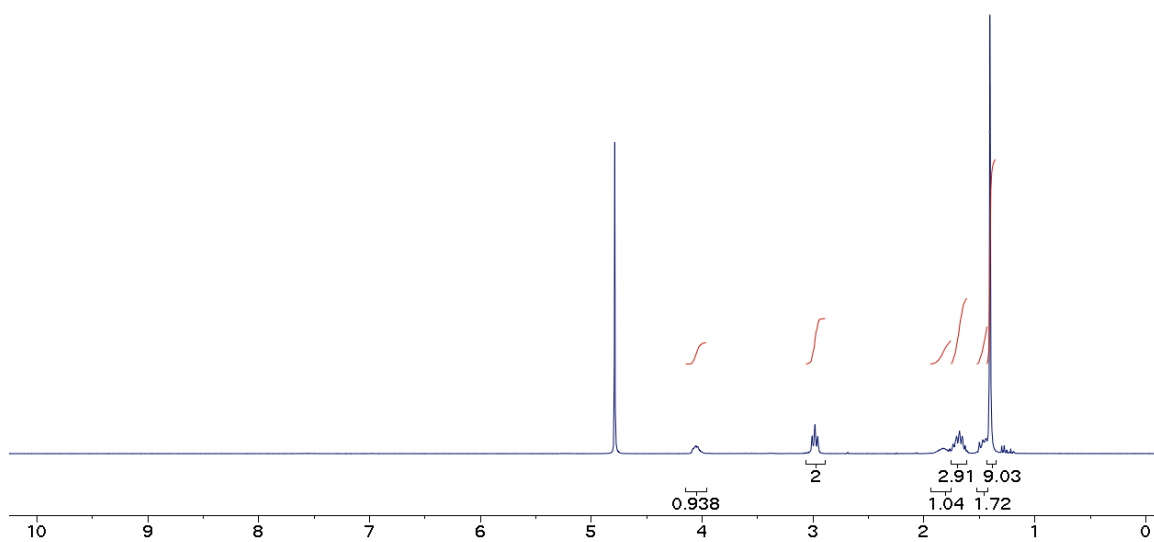
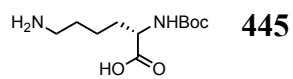


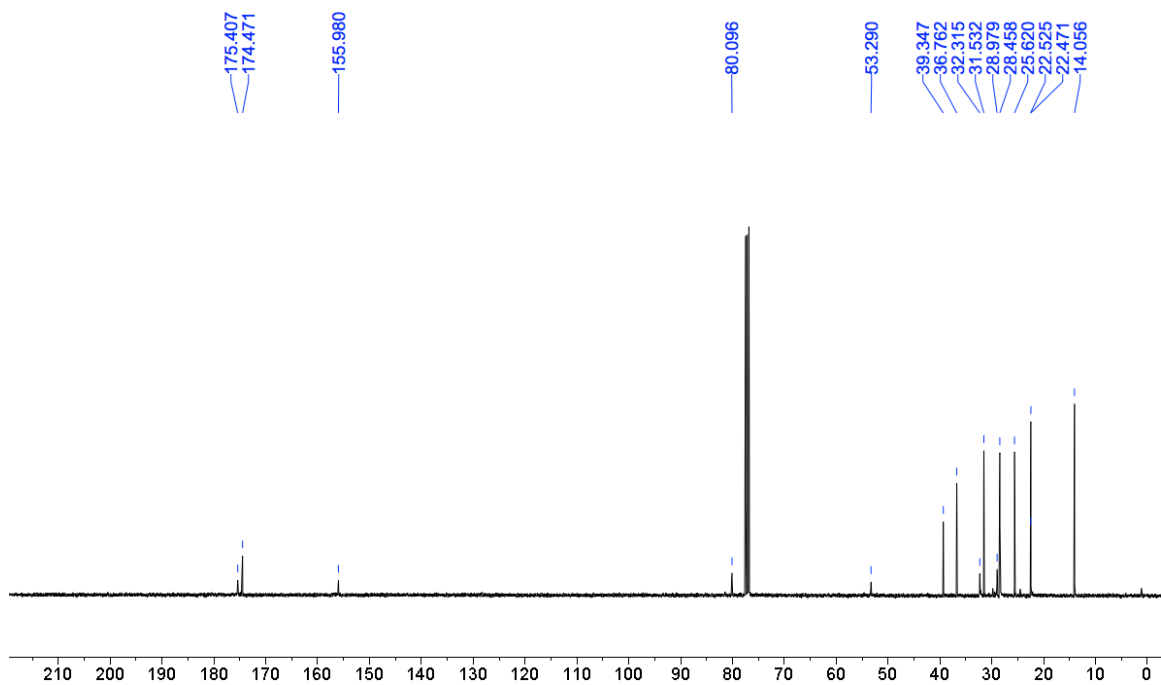
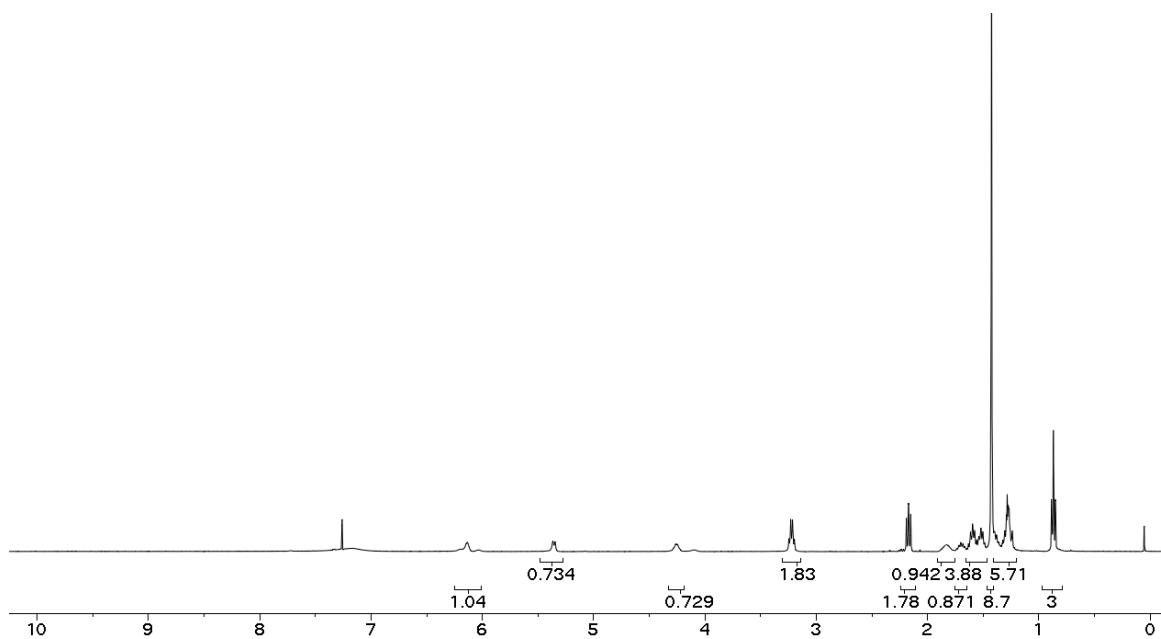
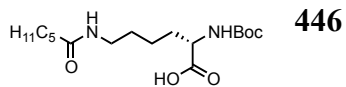
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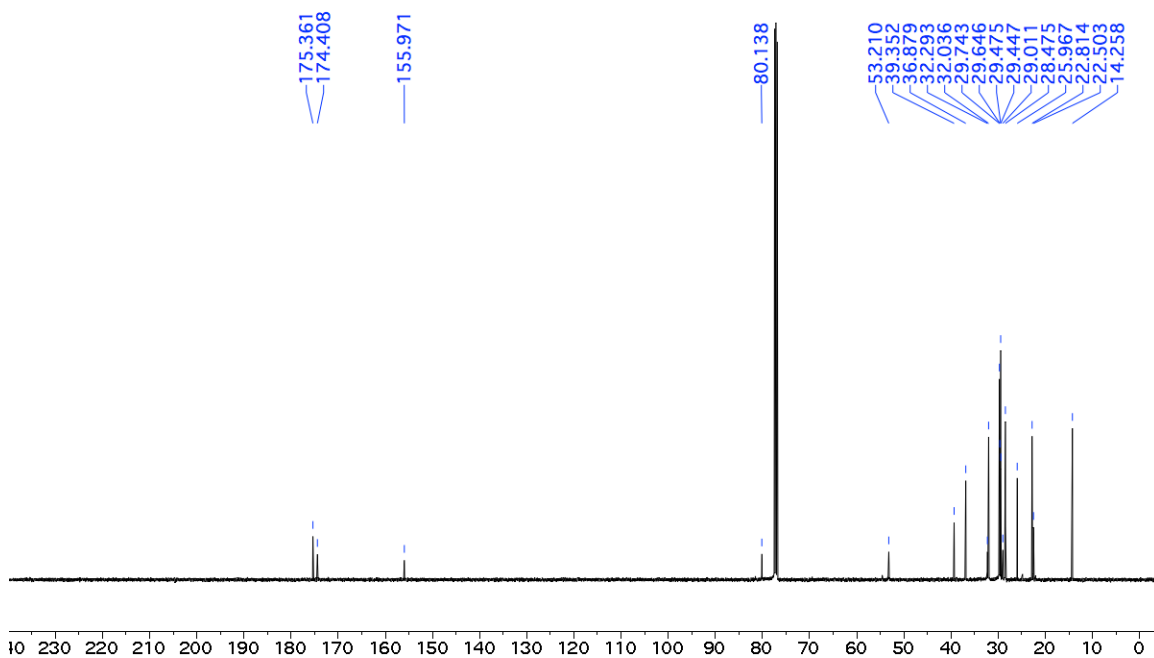
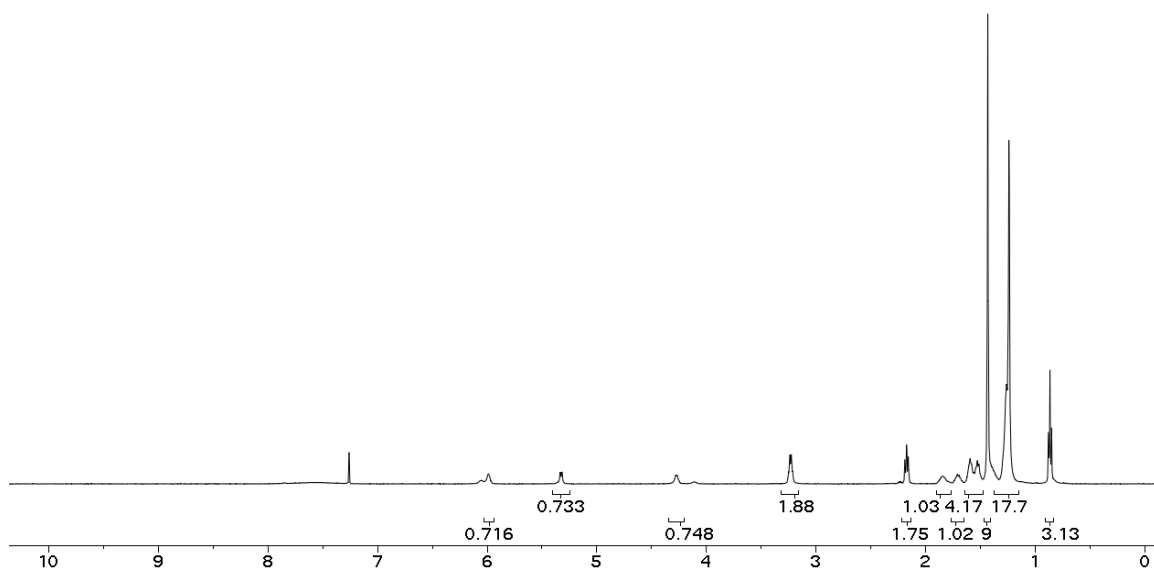
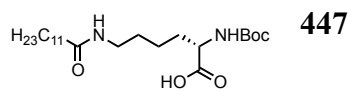


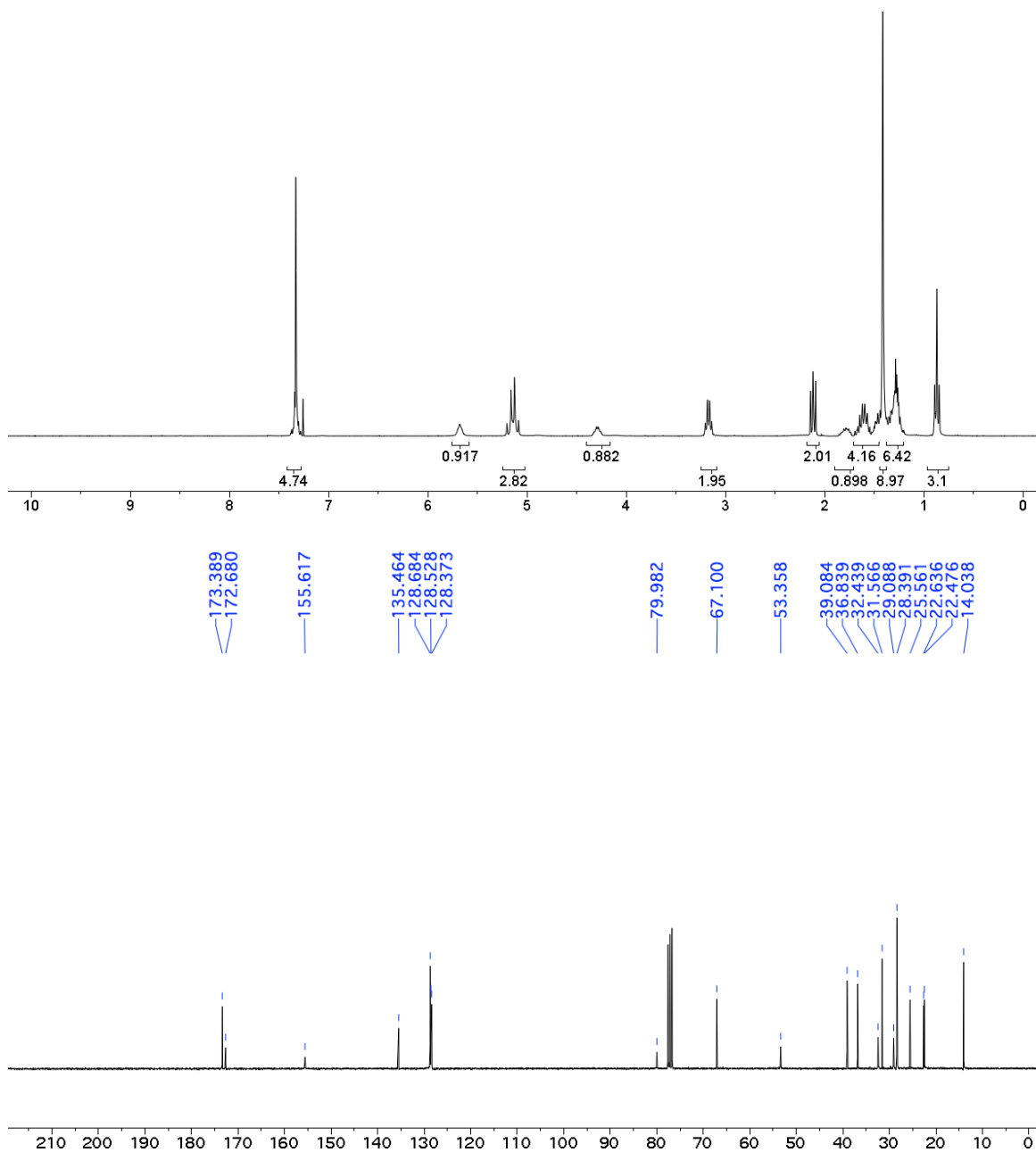
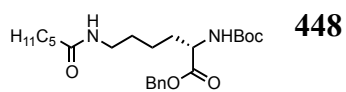
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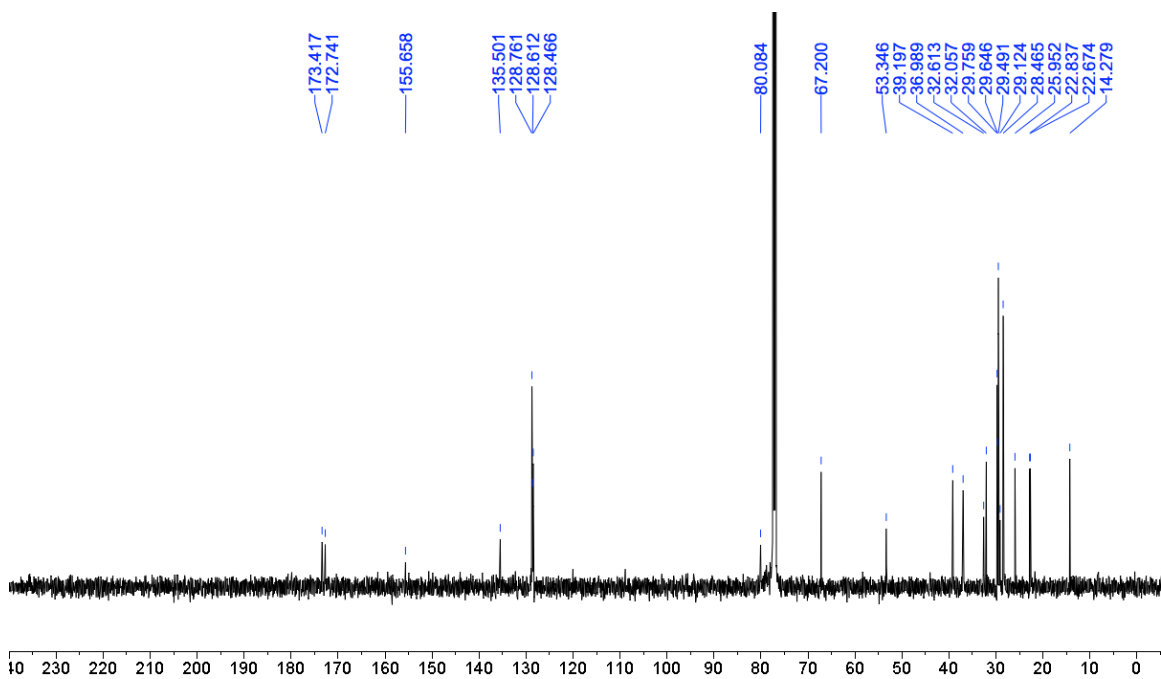
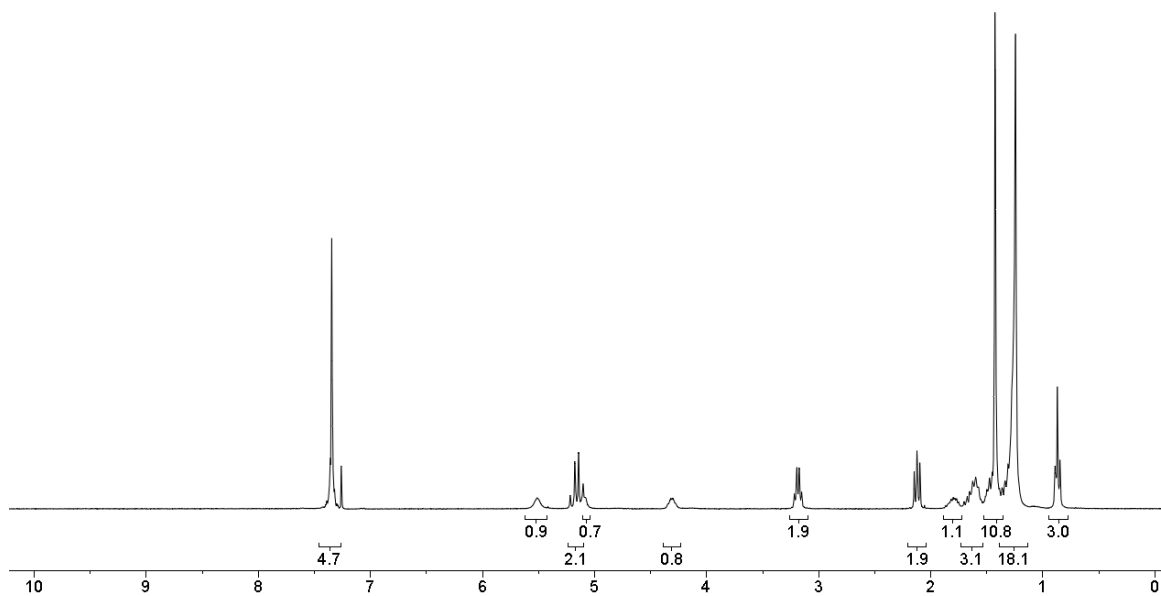
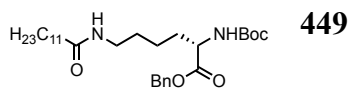


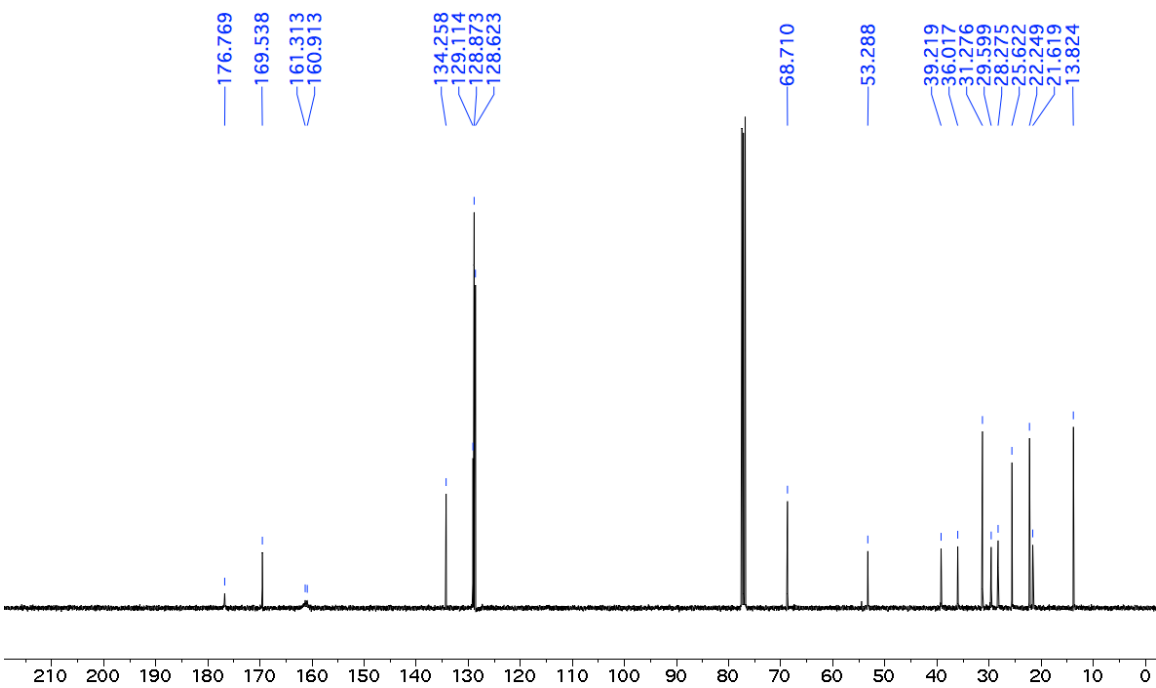
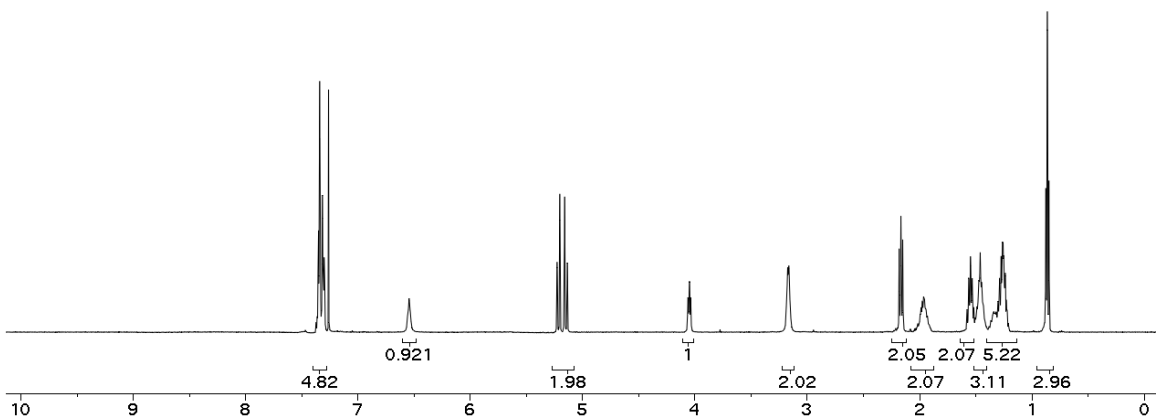
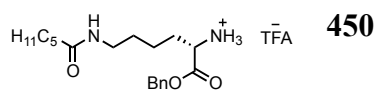


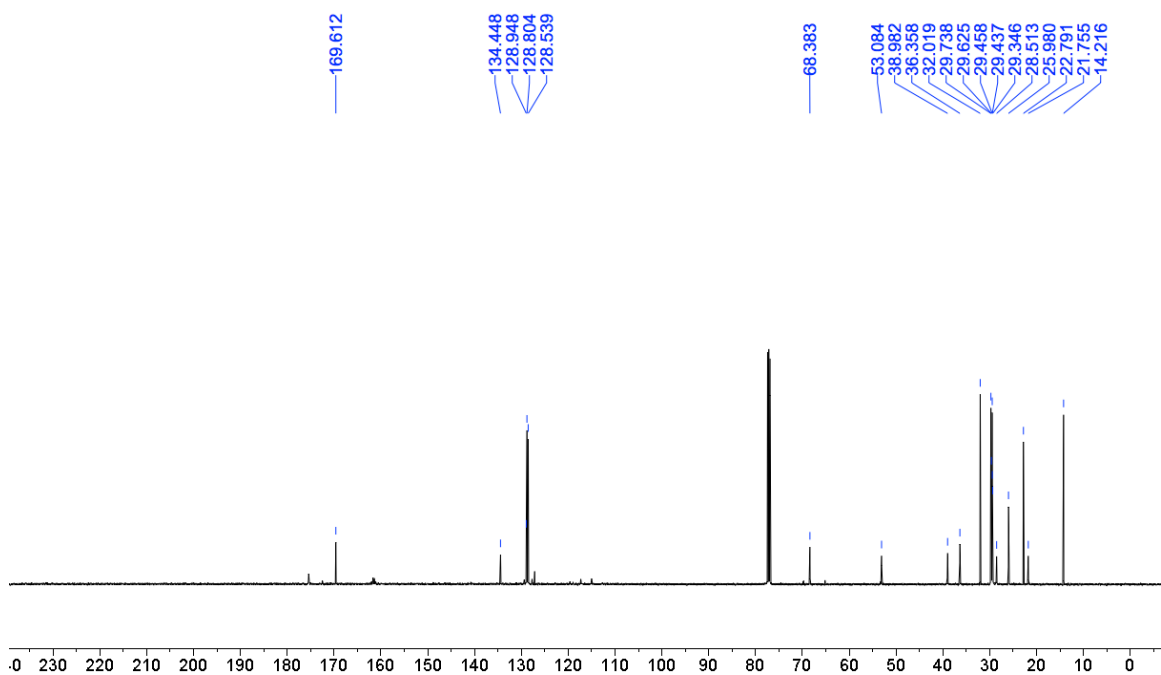
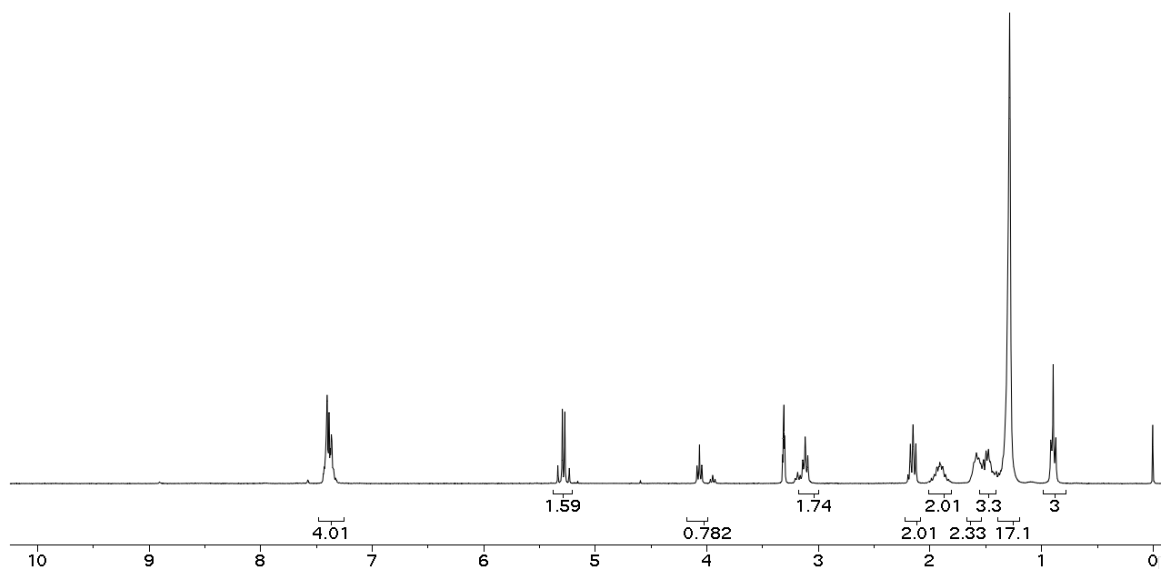
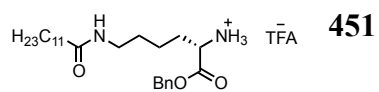


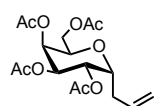




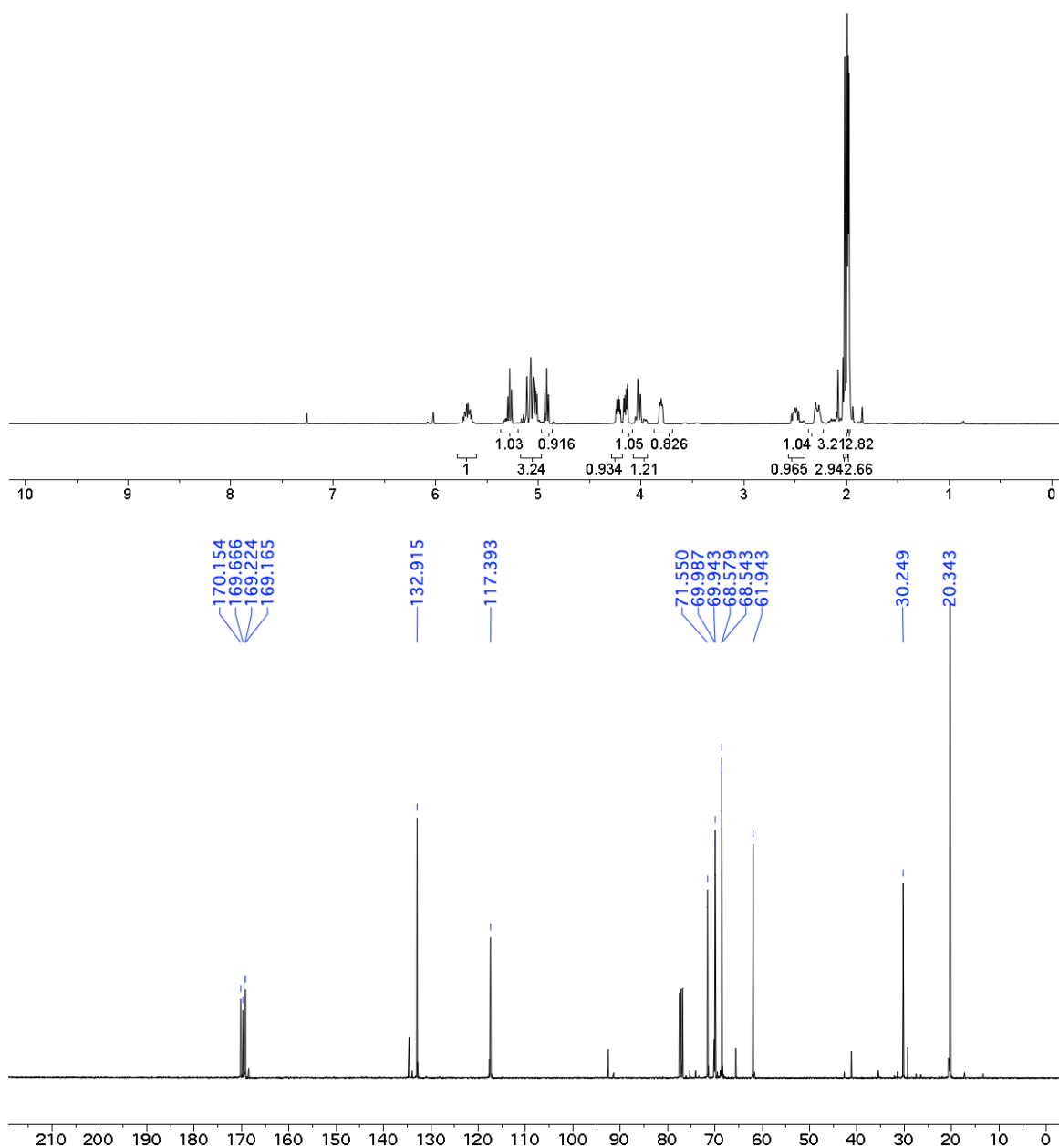


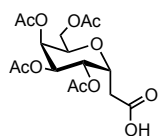




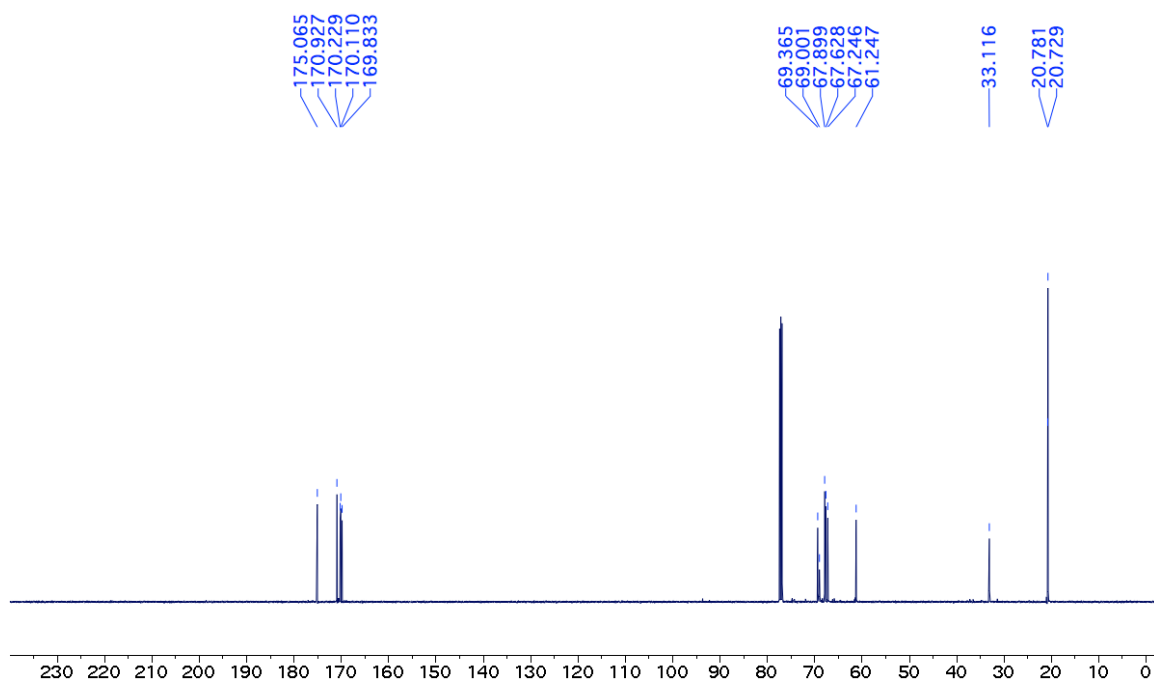
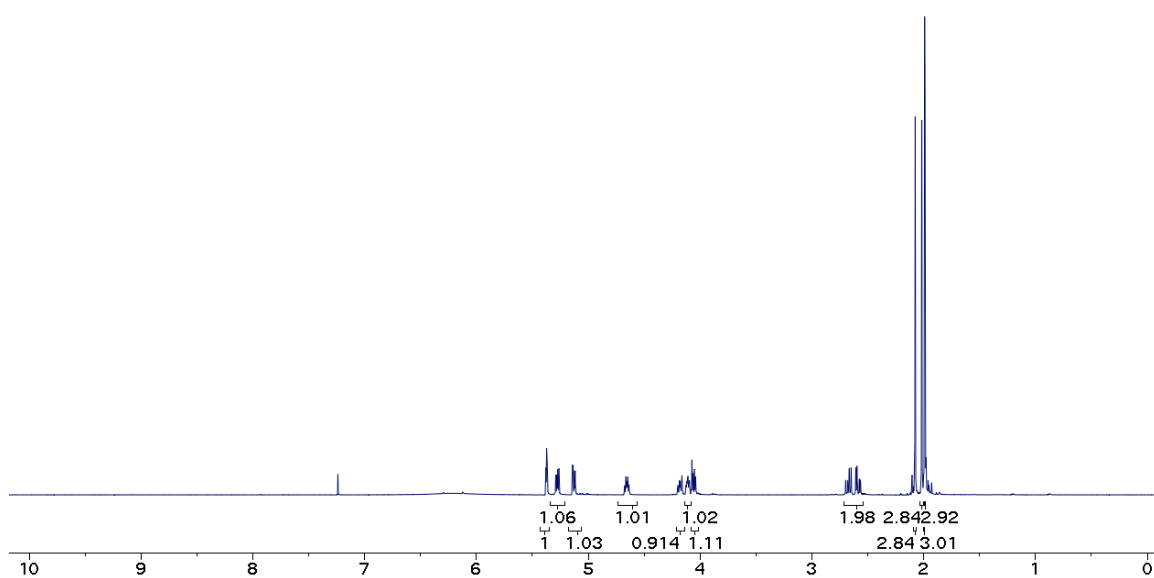


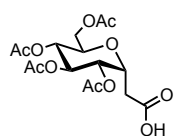
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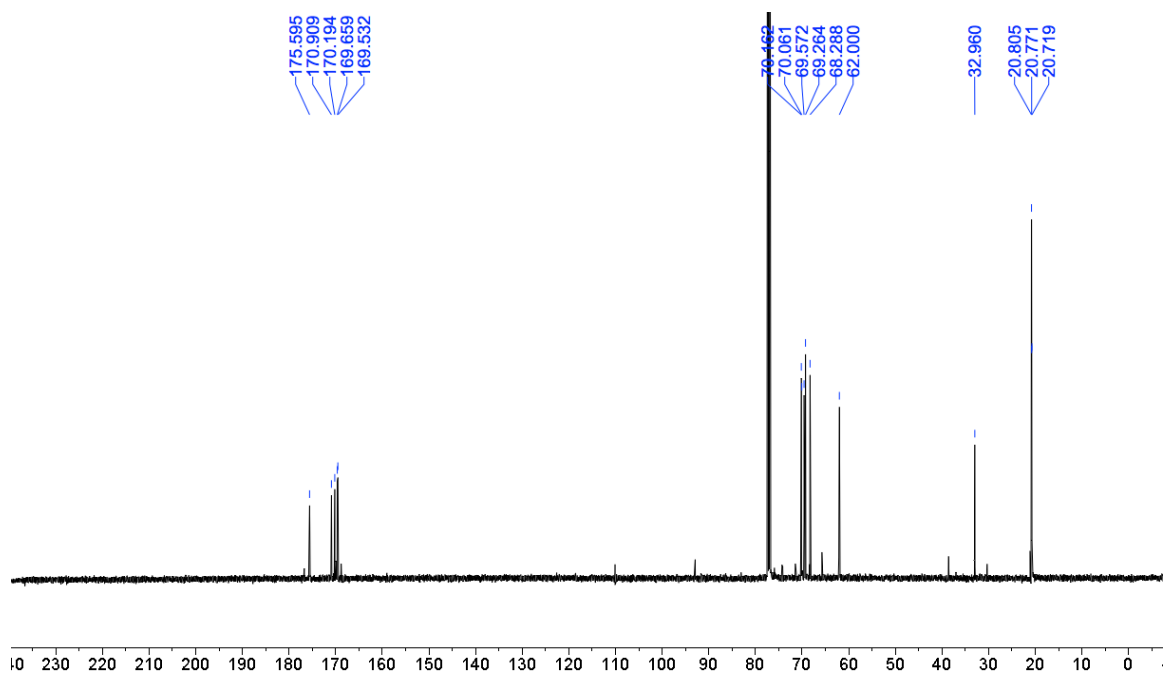
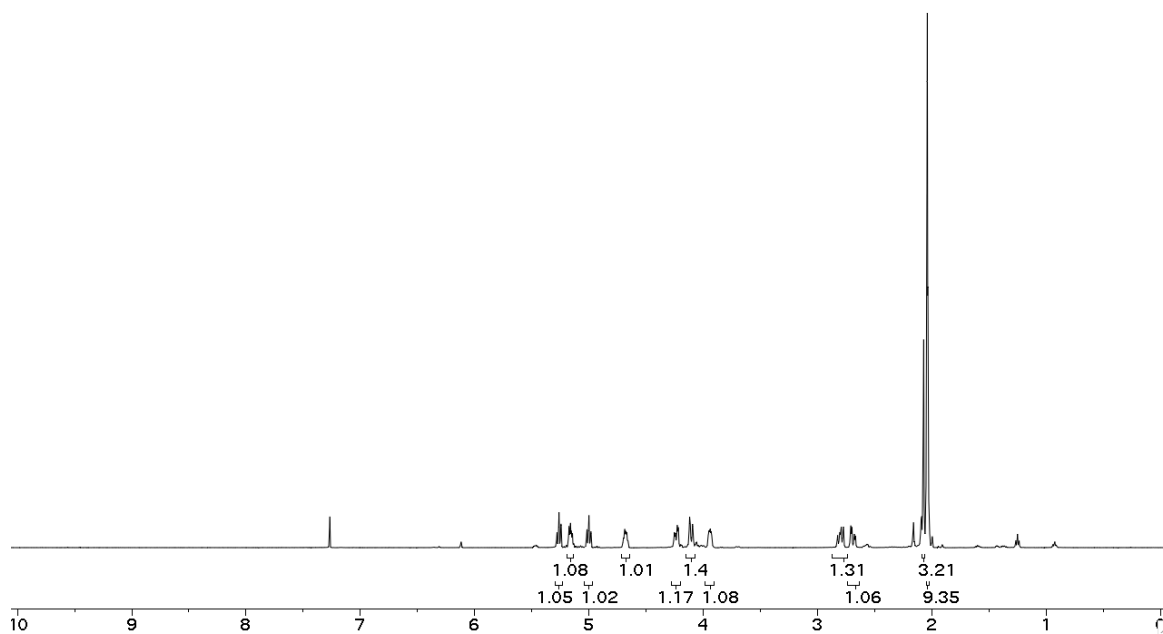


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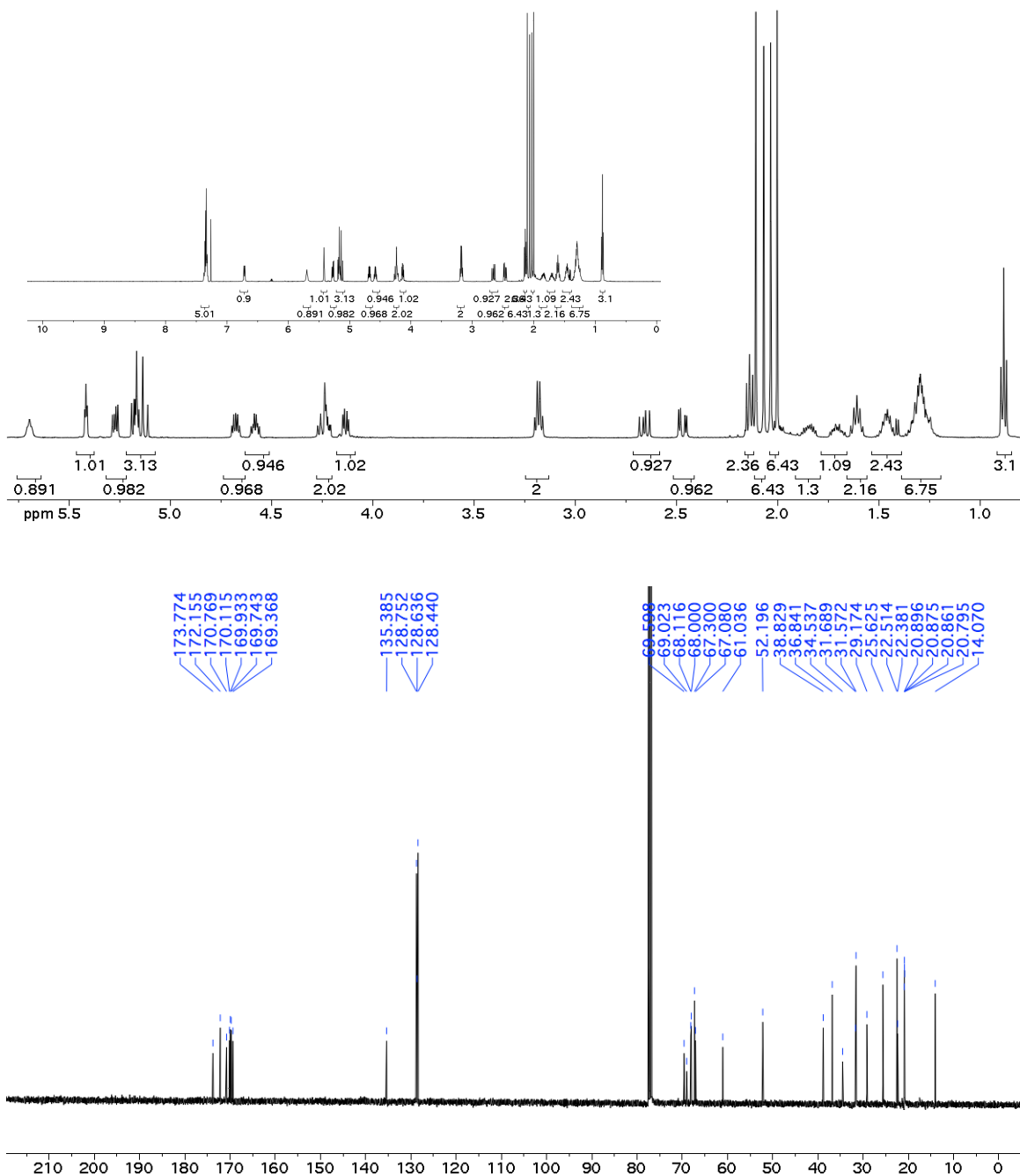
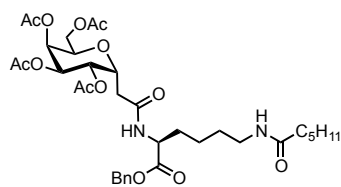




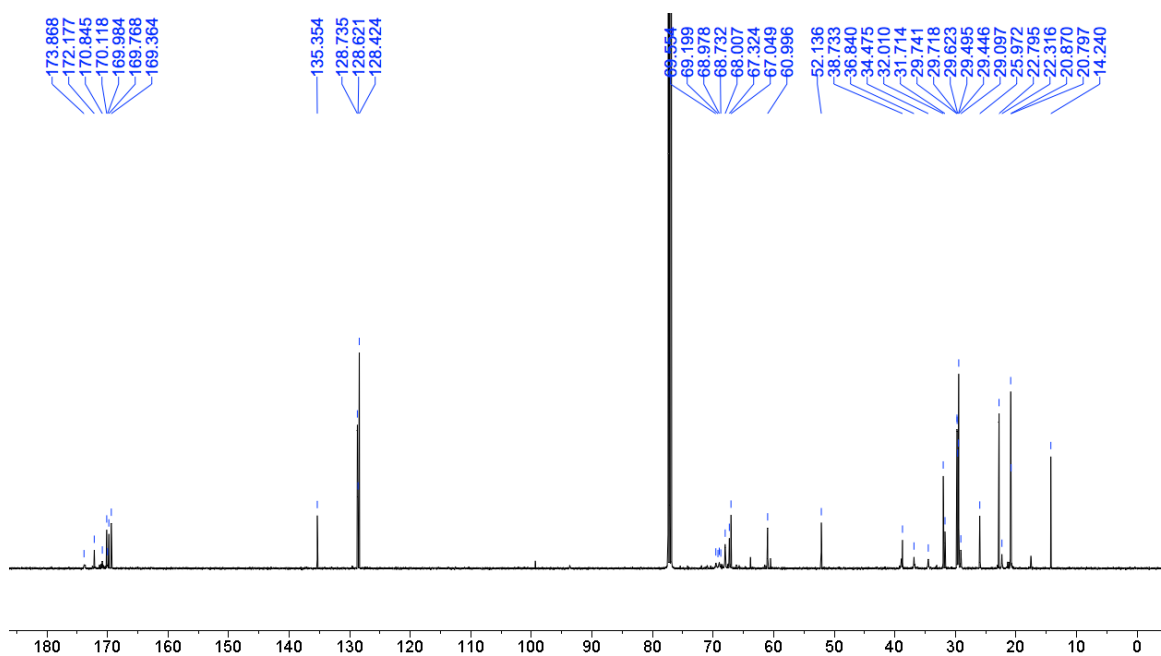
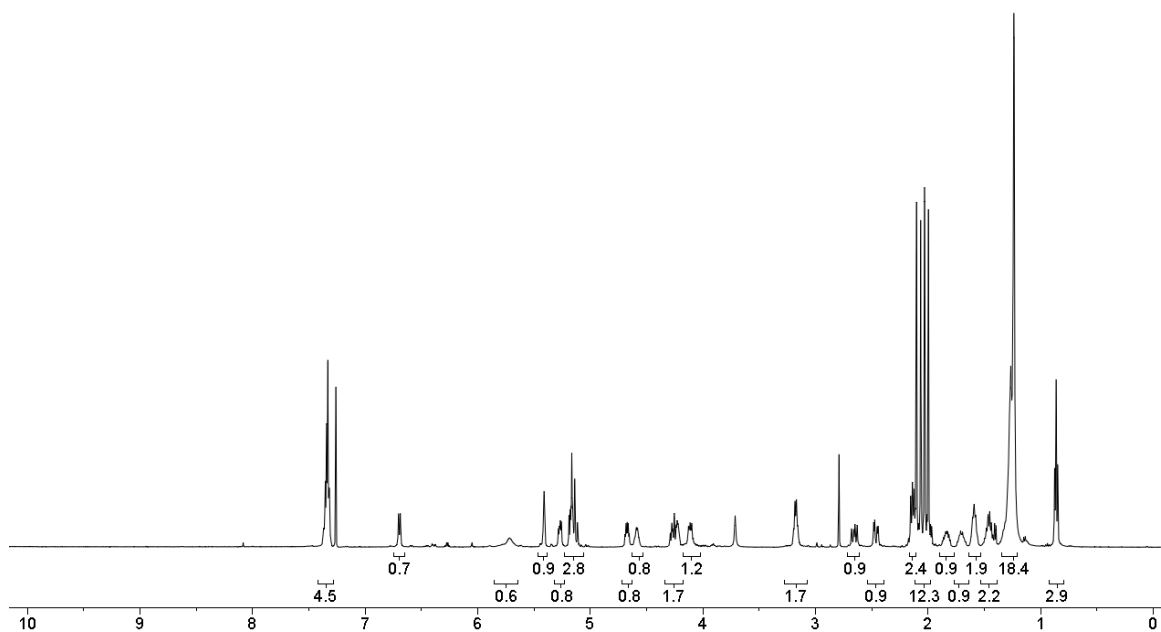
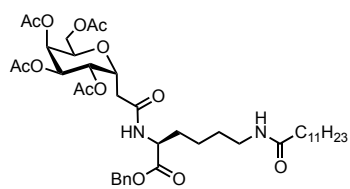
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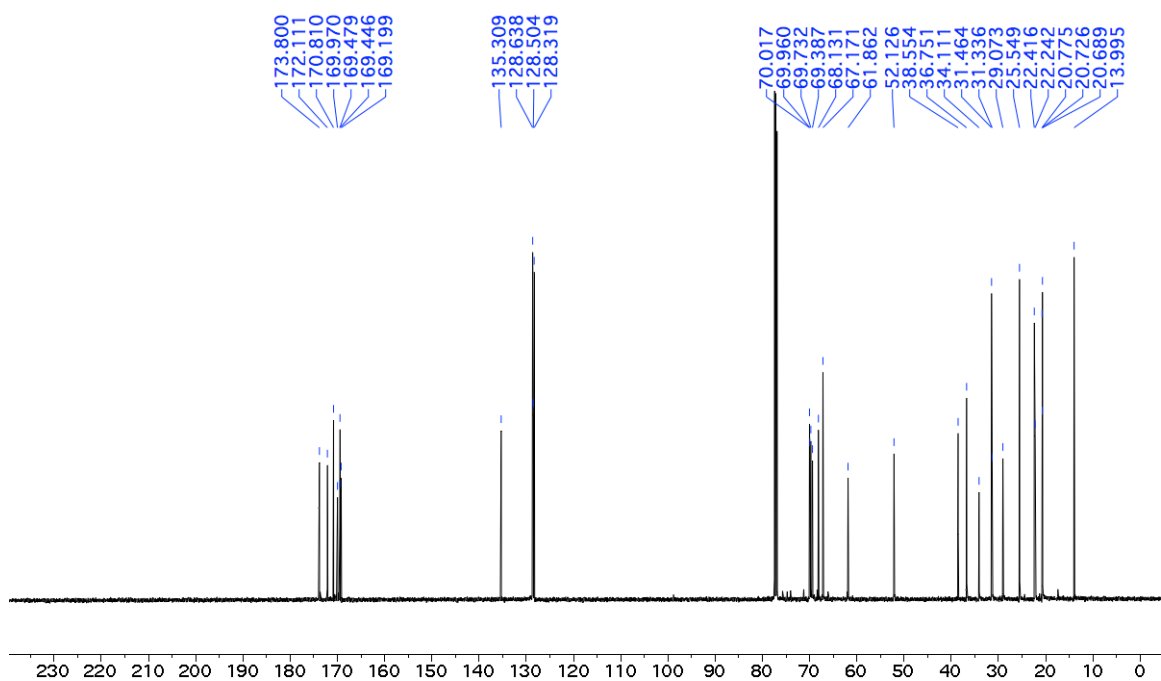
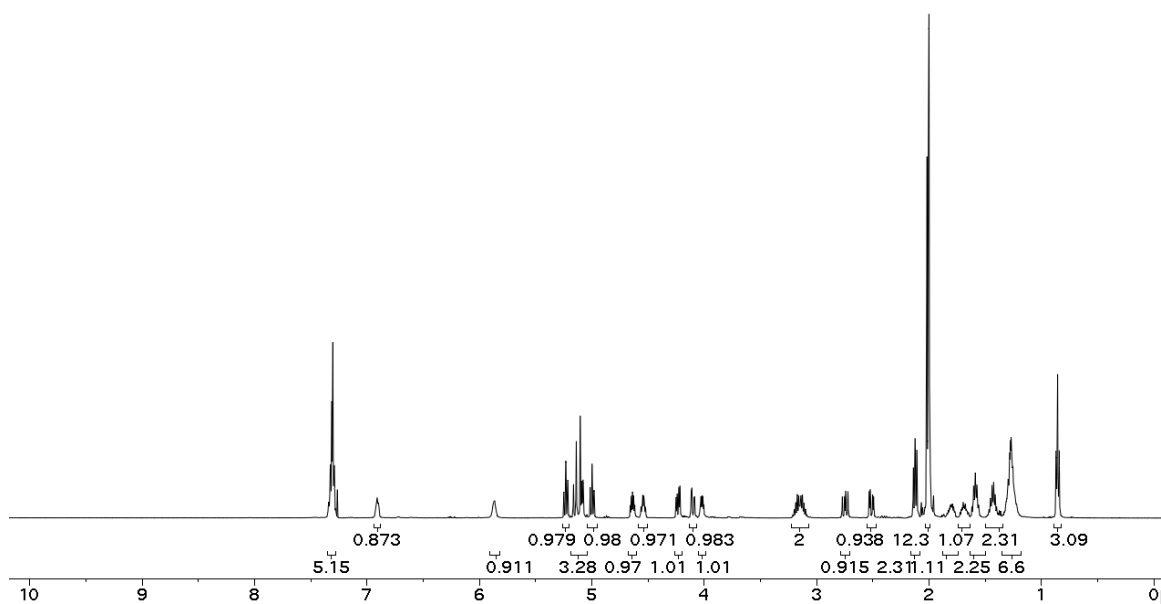
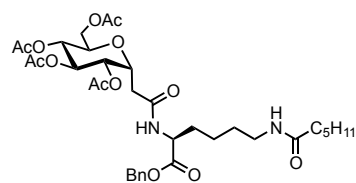
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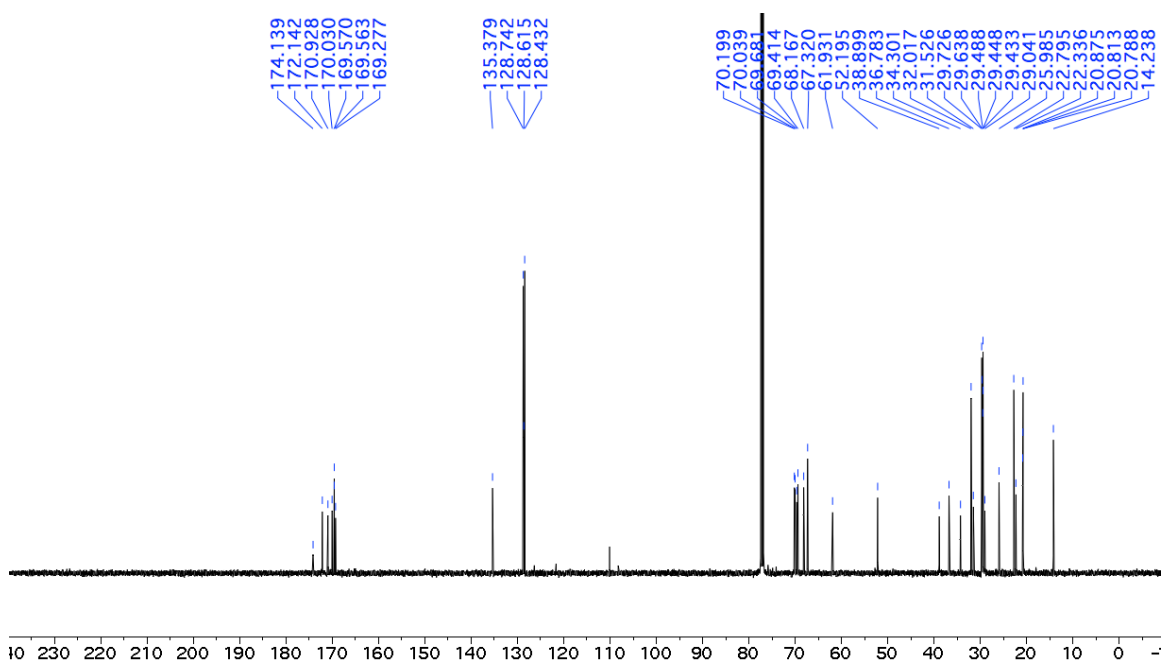
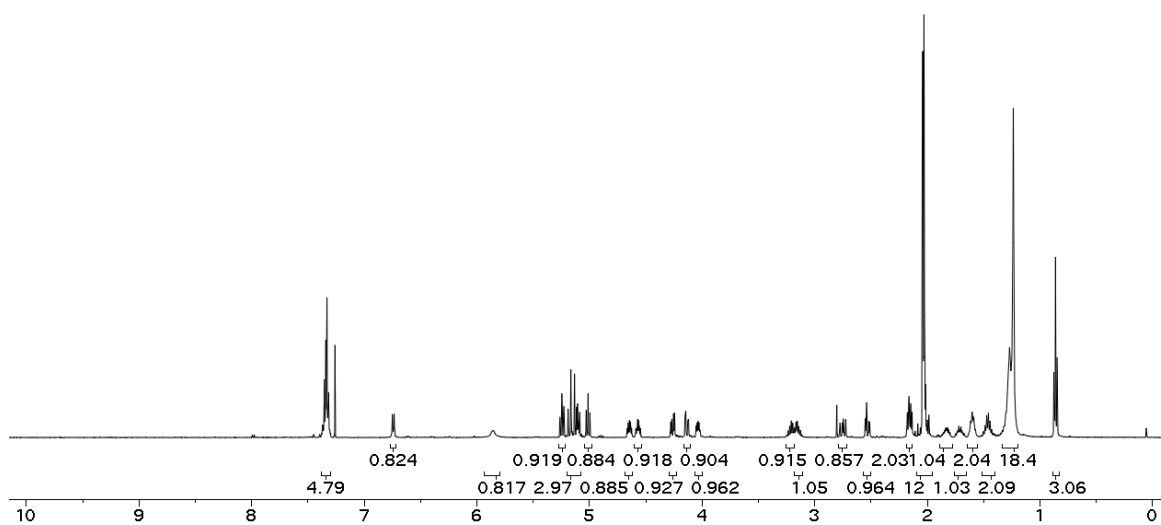
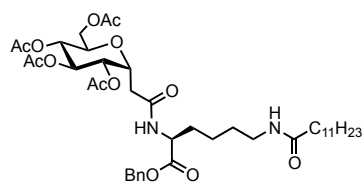
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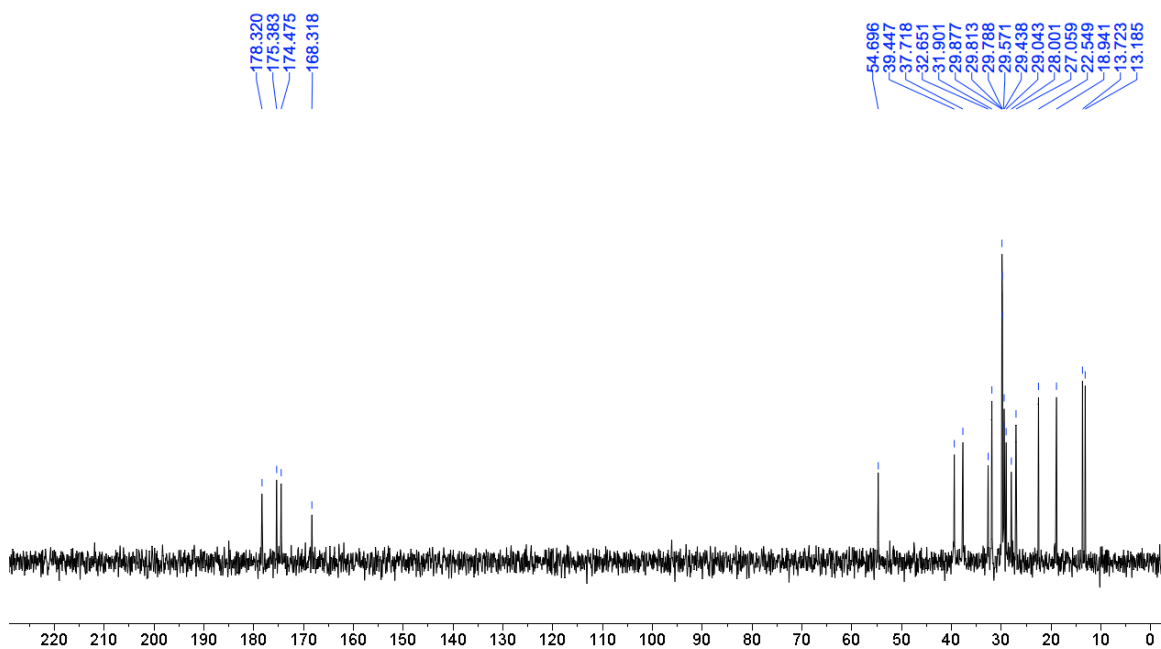
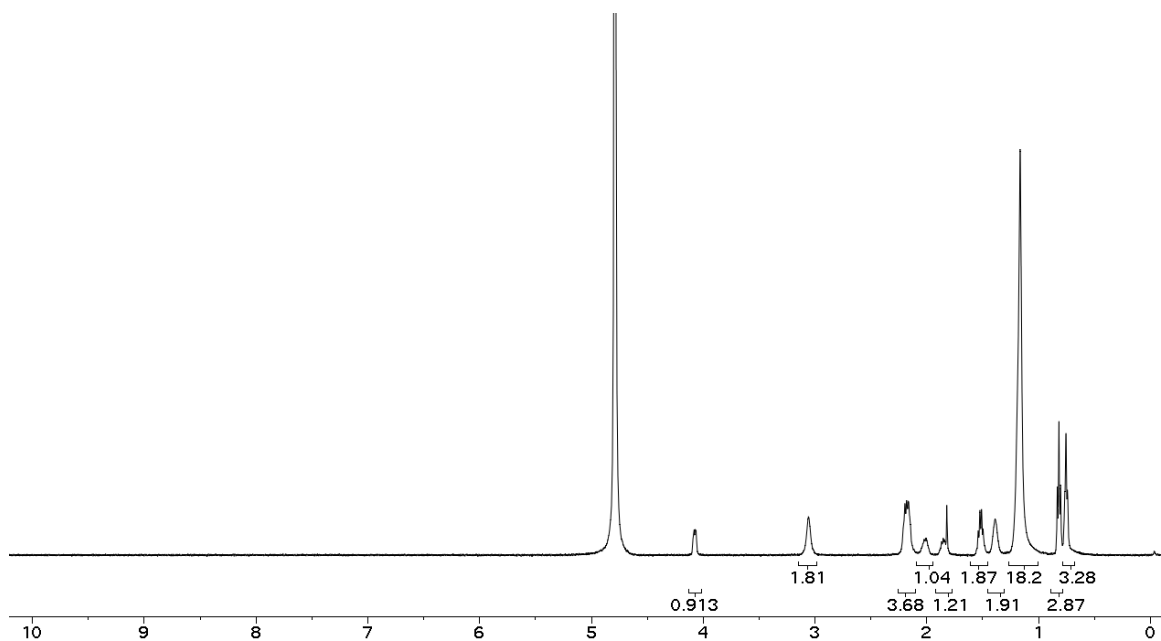
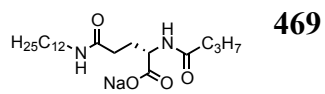


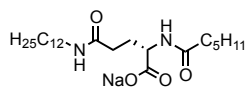
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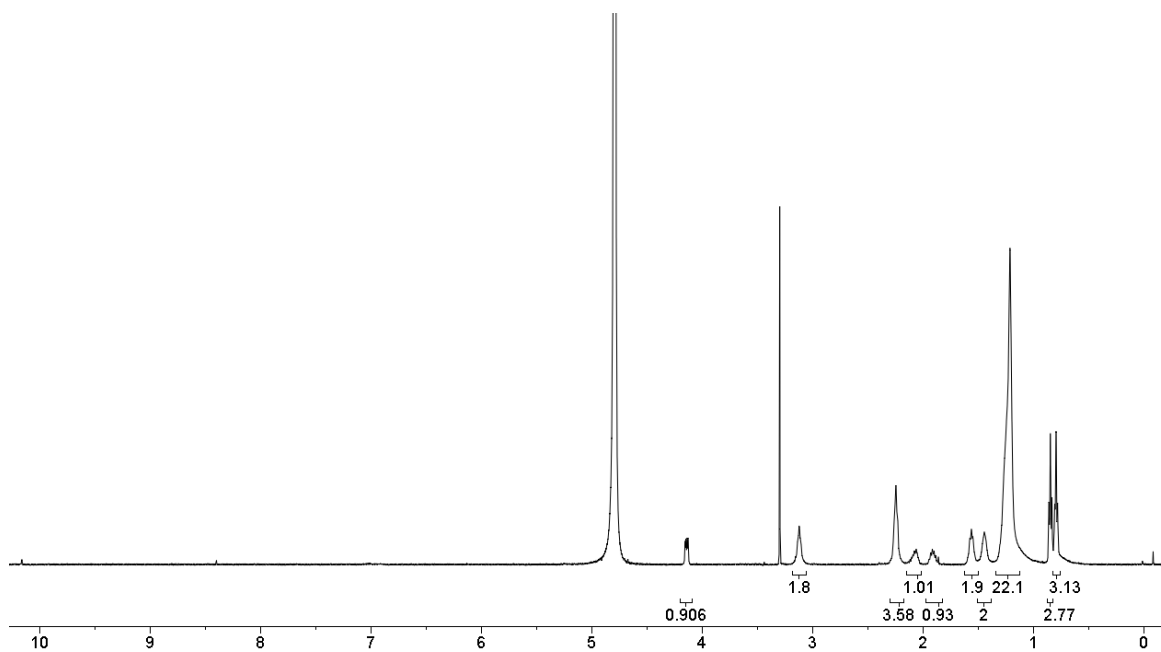
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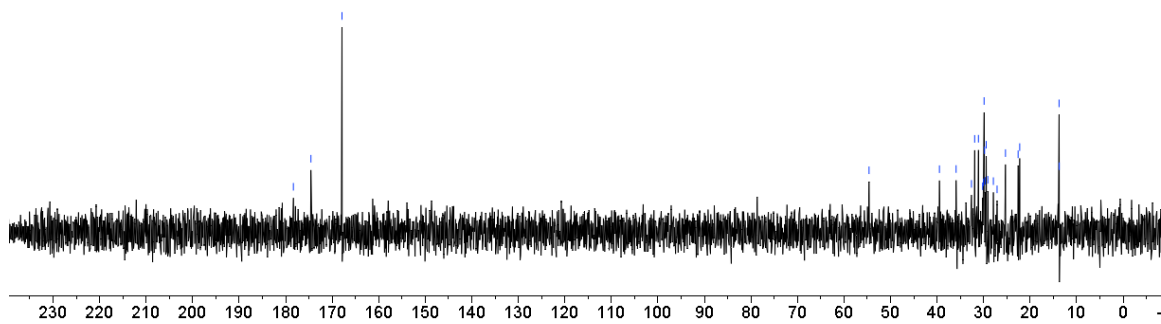


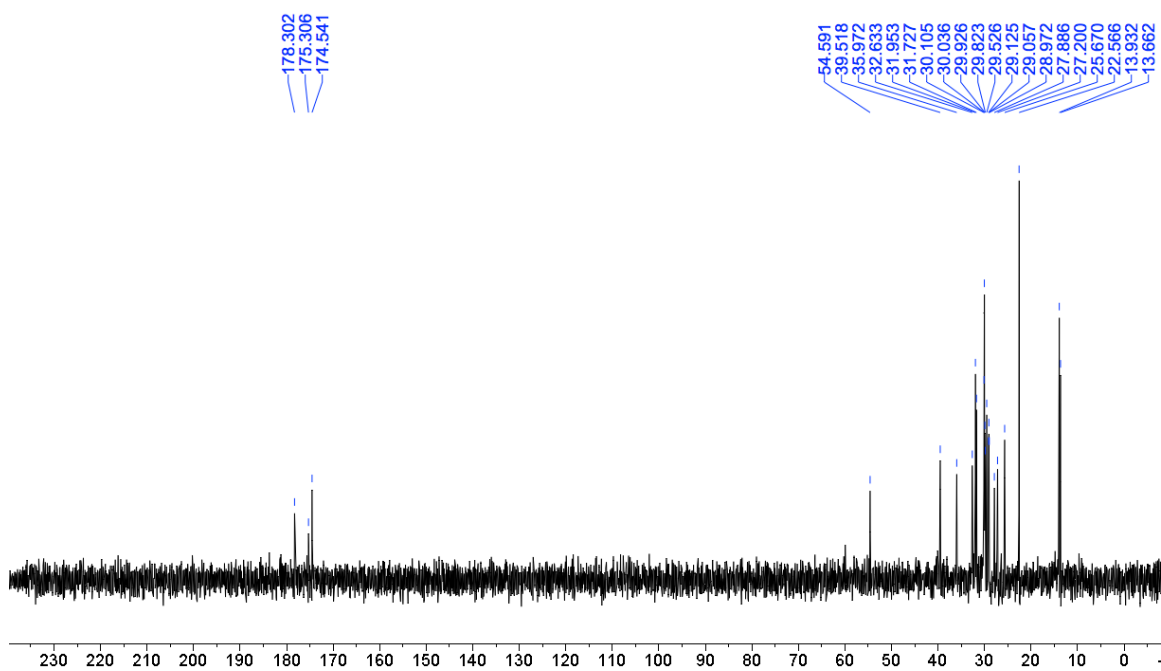
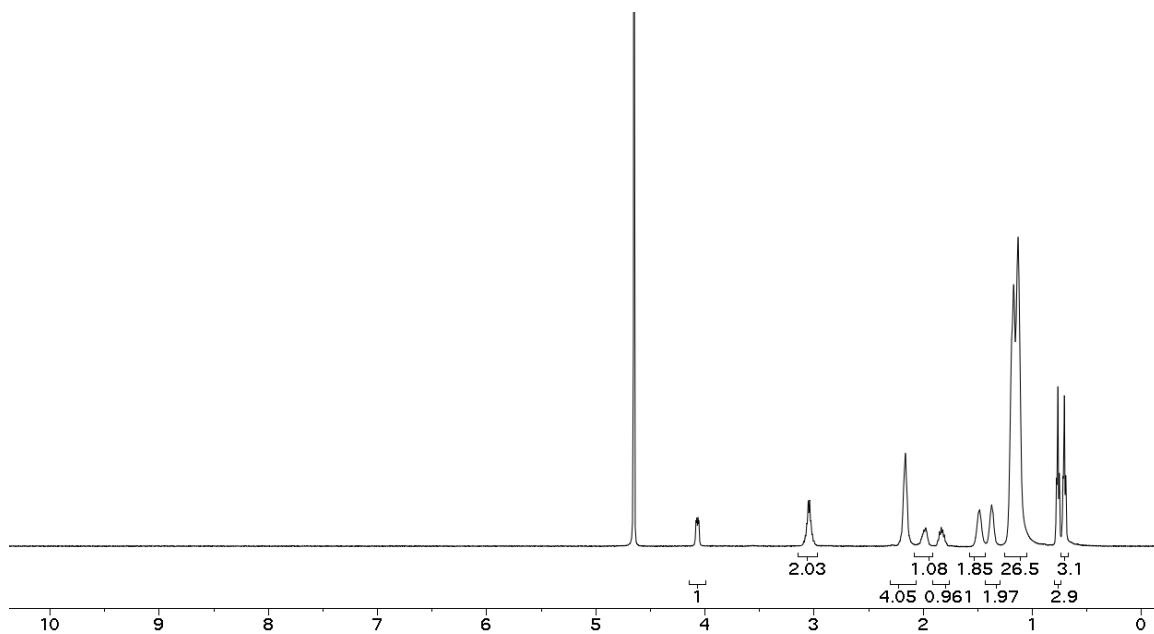
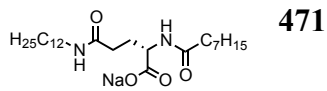
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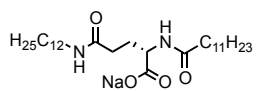


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167.901

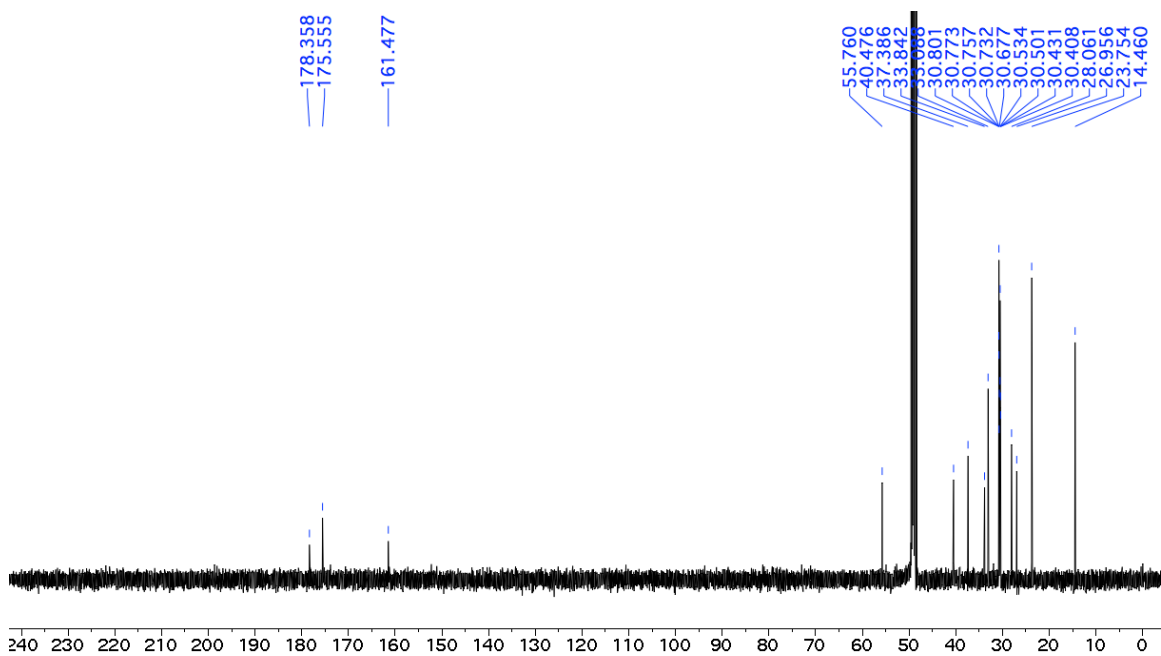
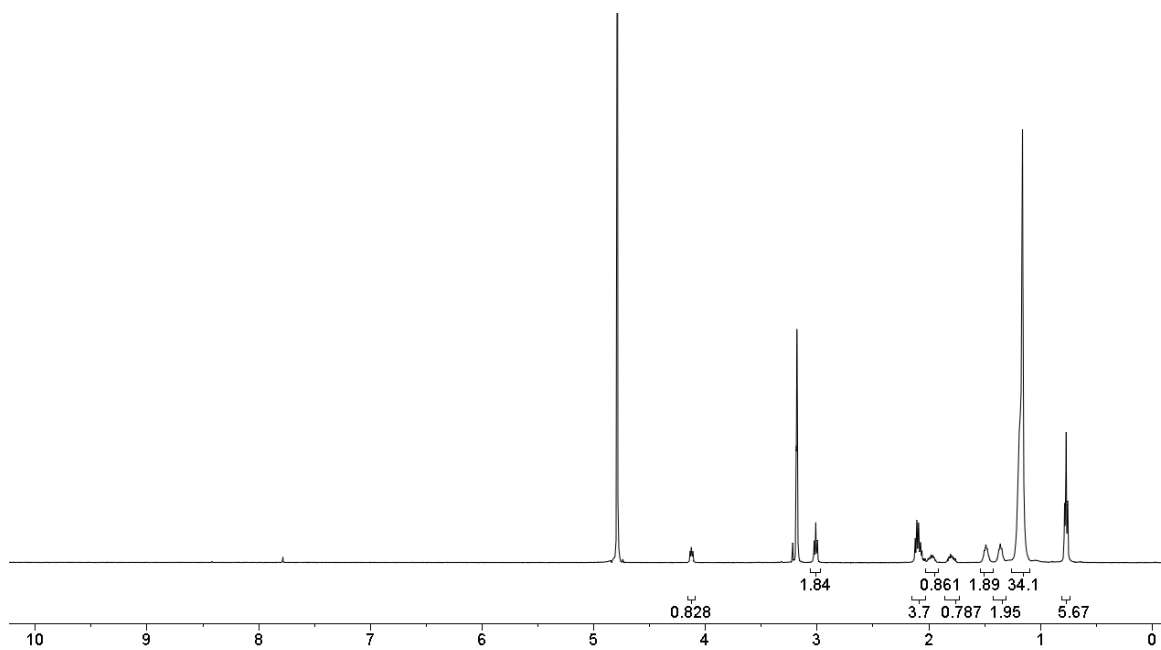
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31.100
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30.037
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27.892
27.095
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13.770
13.731

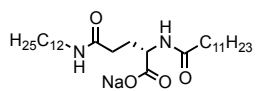




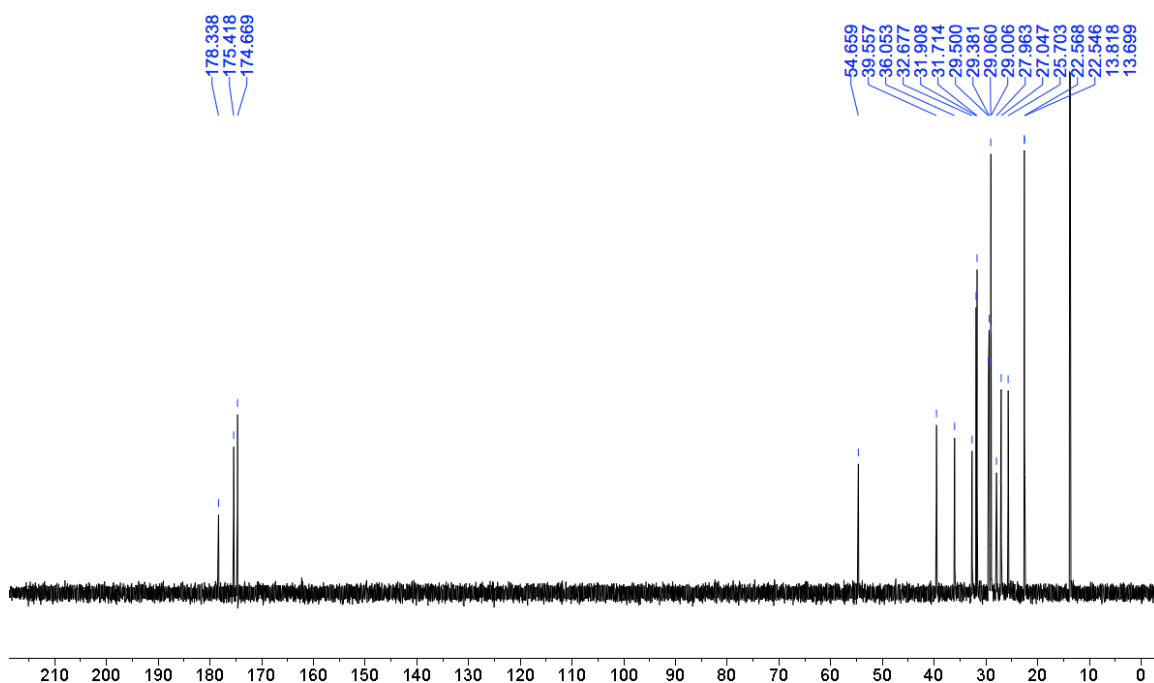
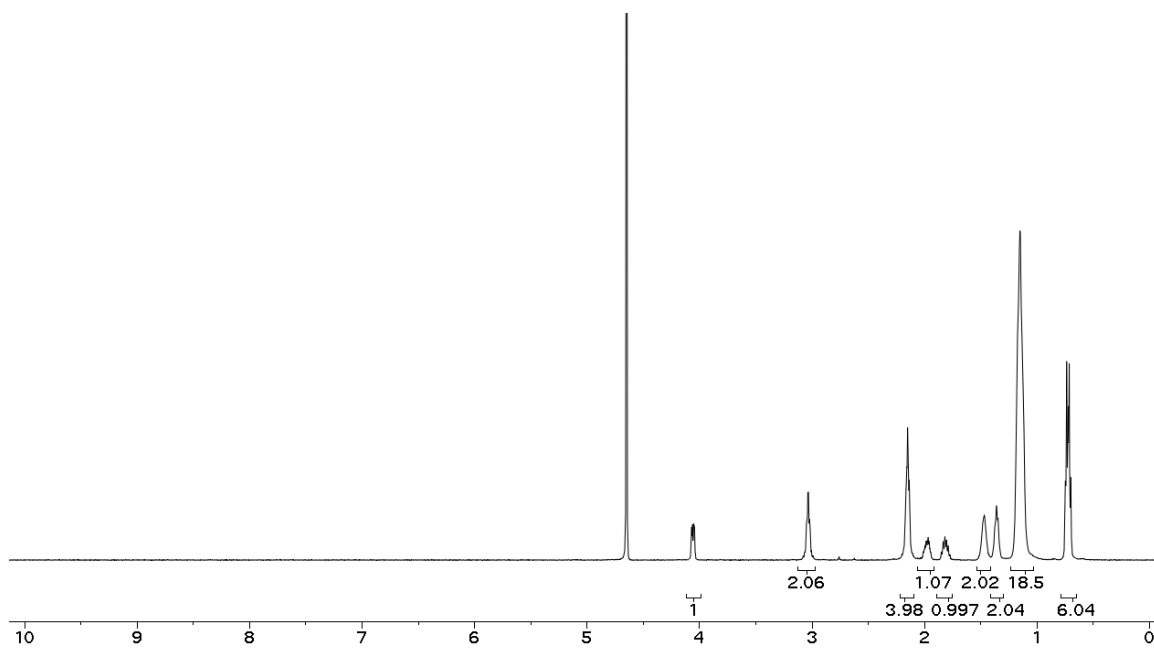


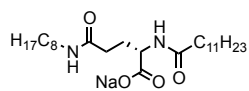
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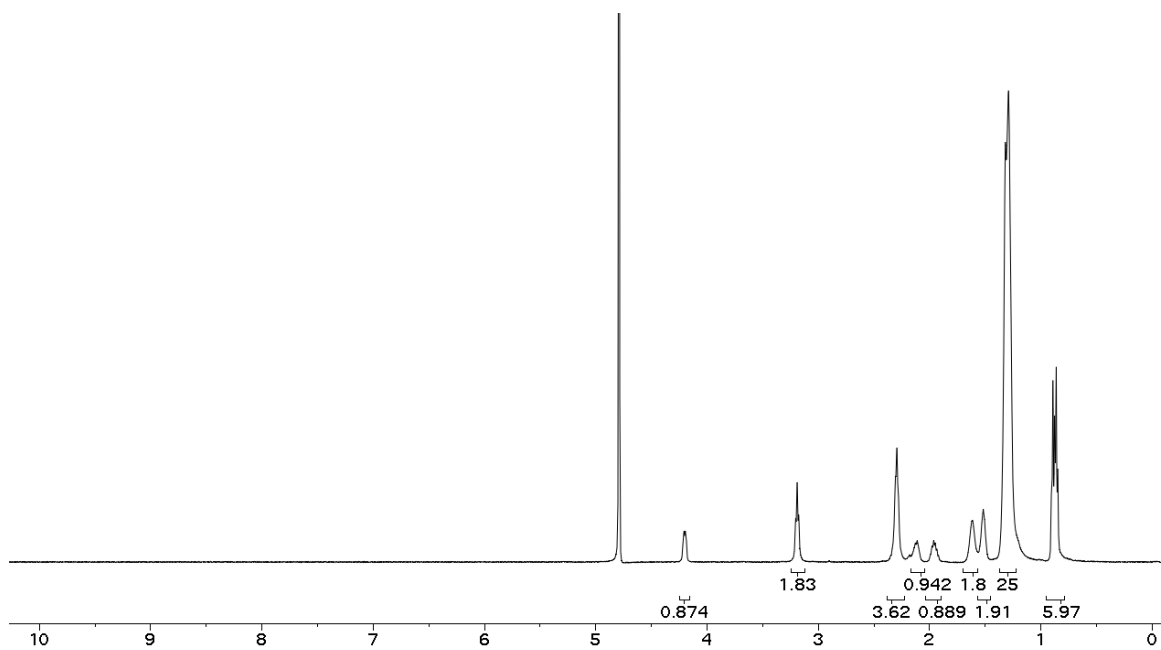


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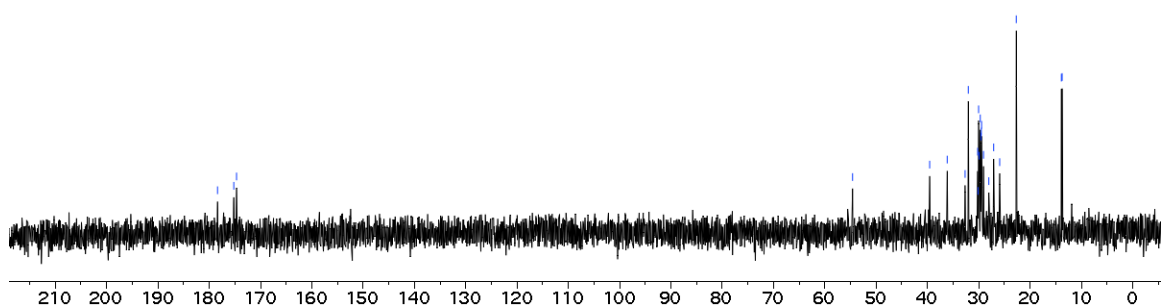


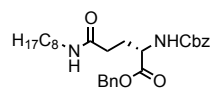
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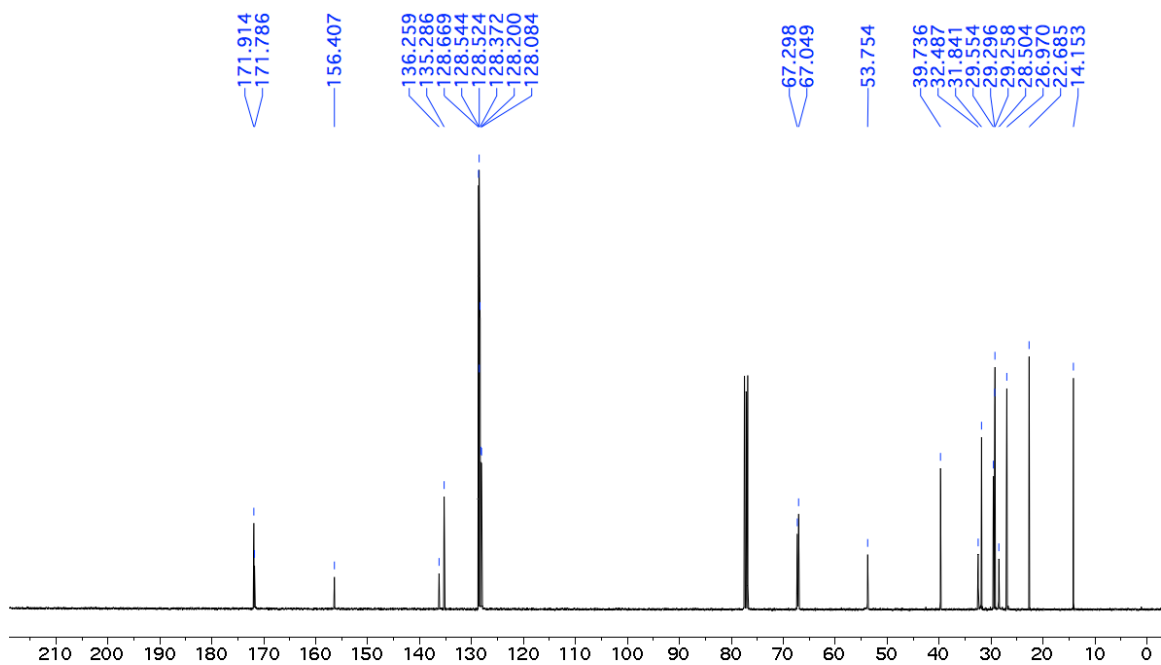
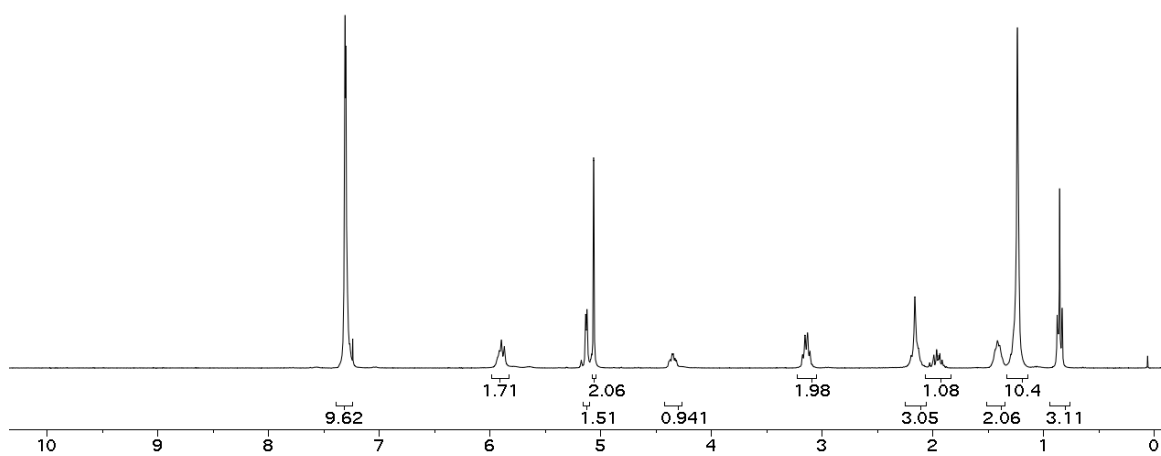
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174.674

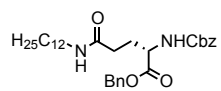
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30.132
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29.066
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13.891
13.740



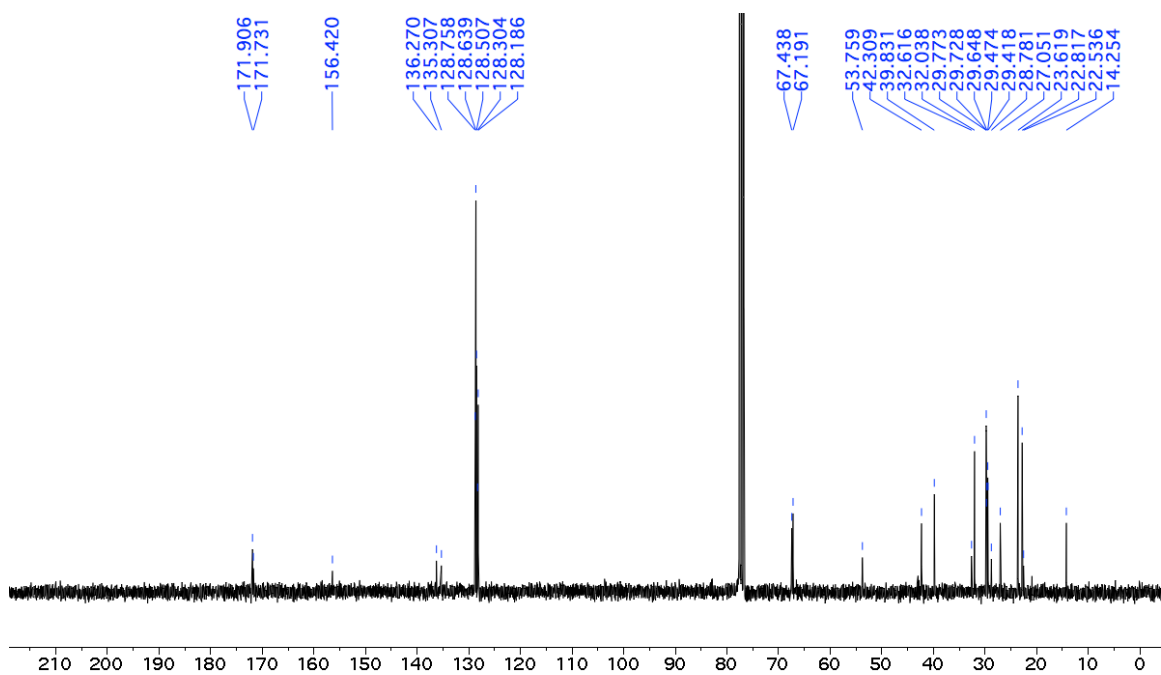
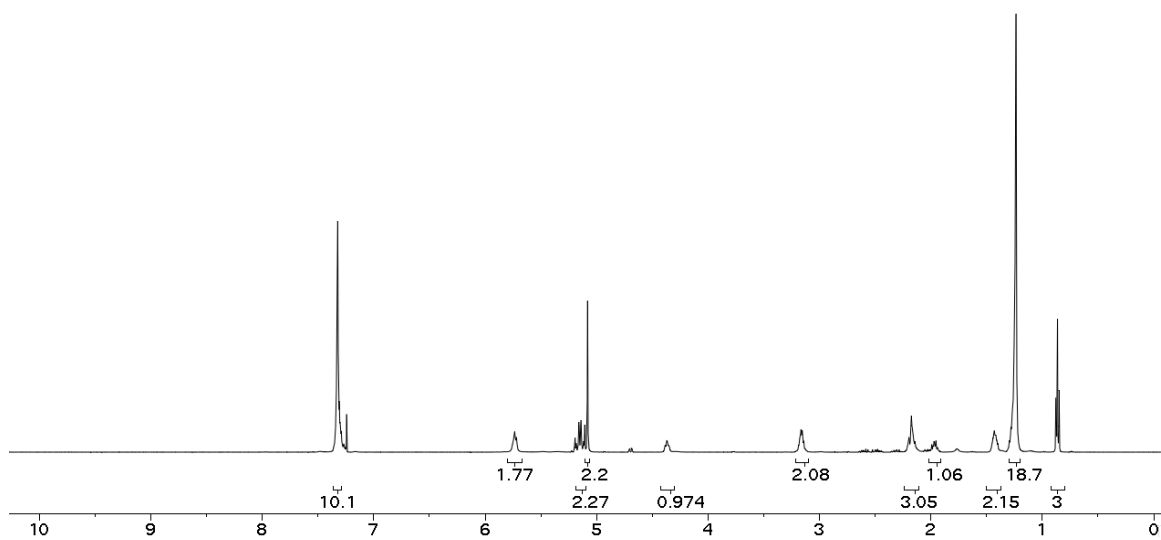


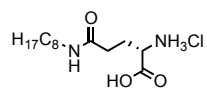
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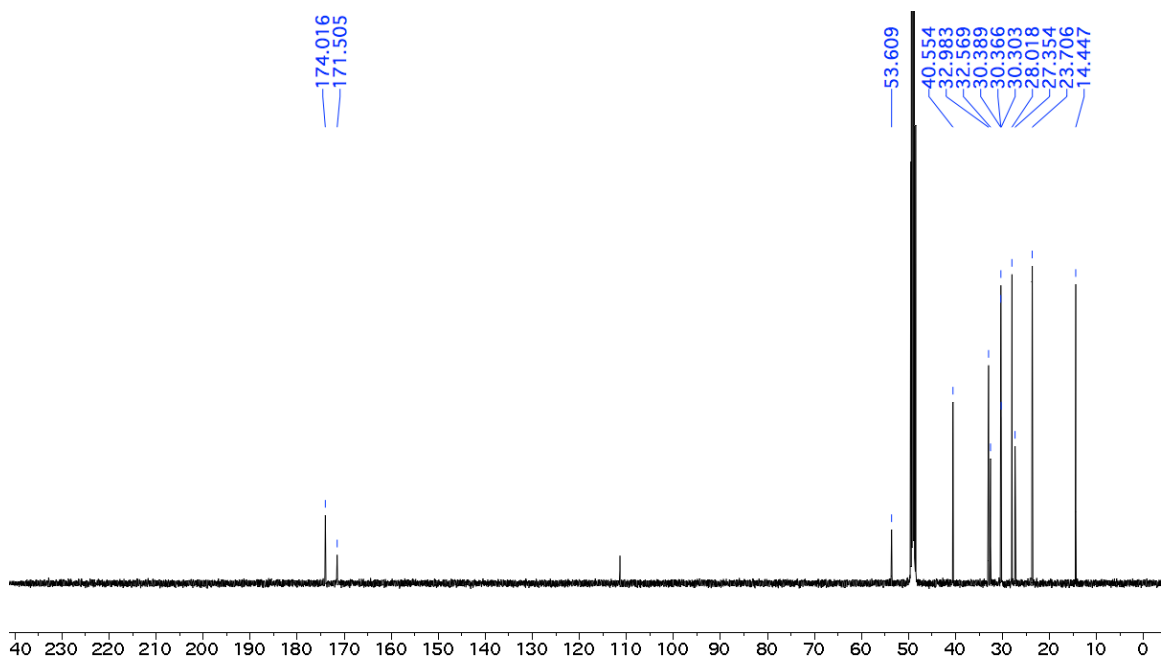
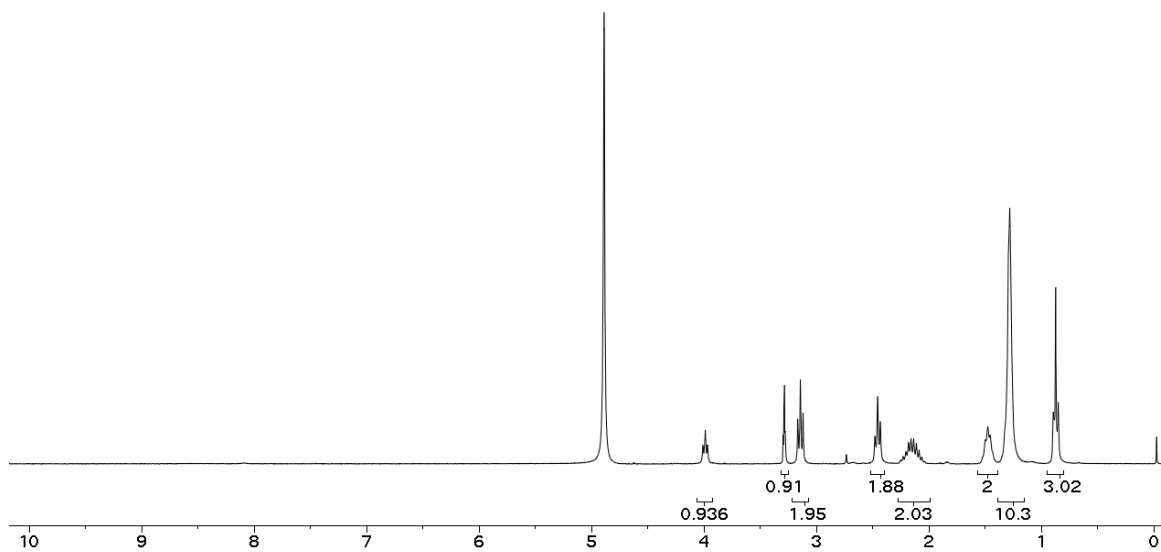


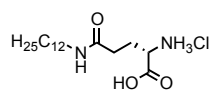
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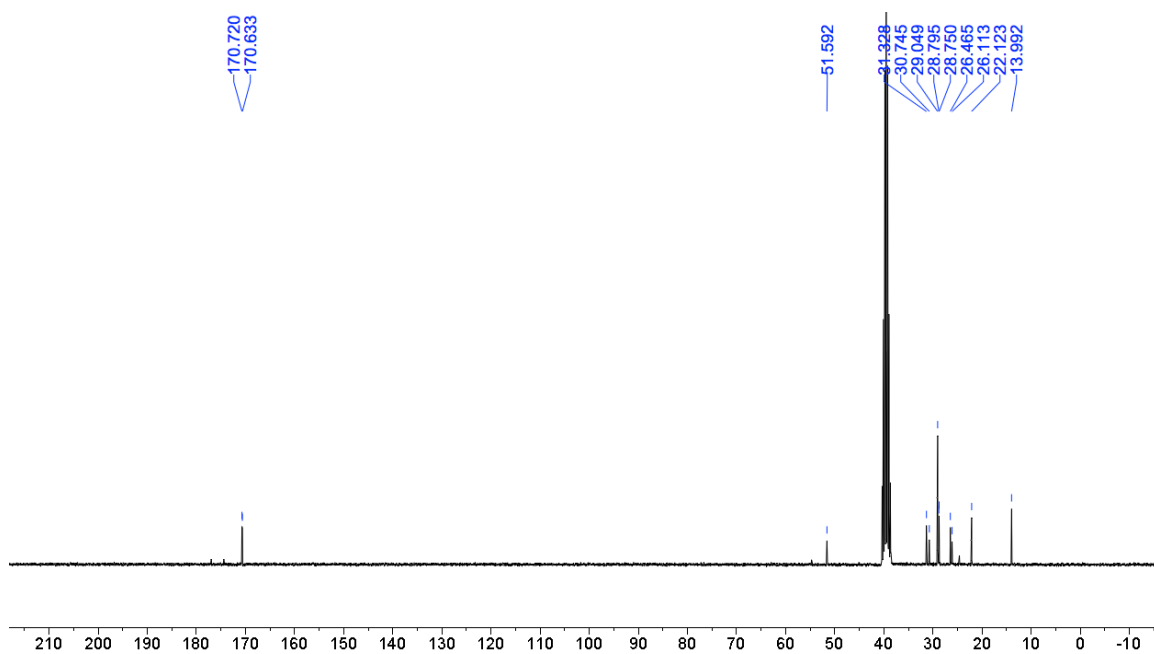
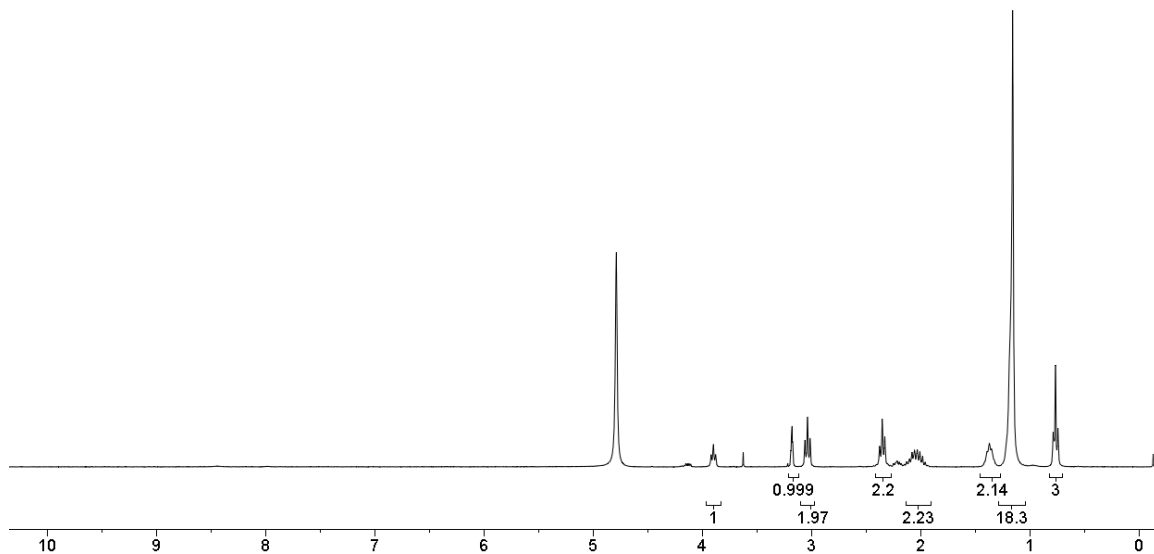


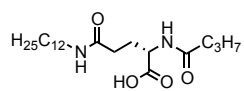
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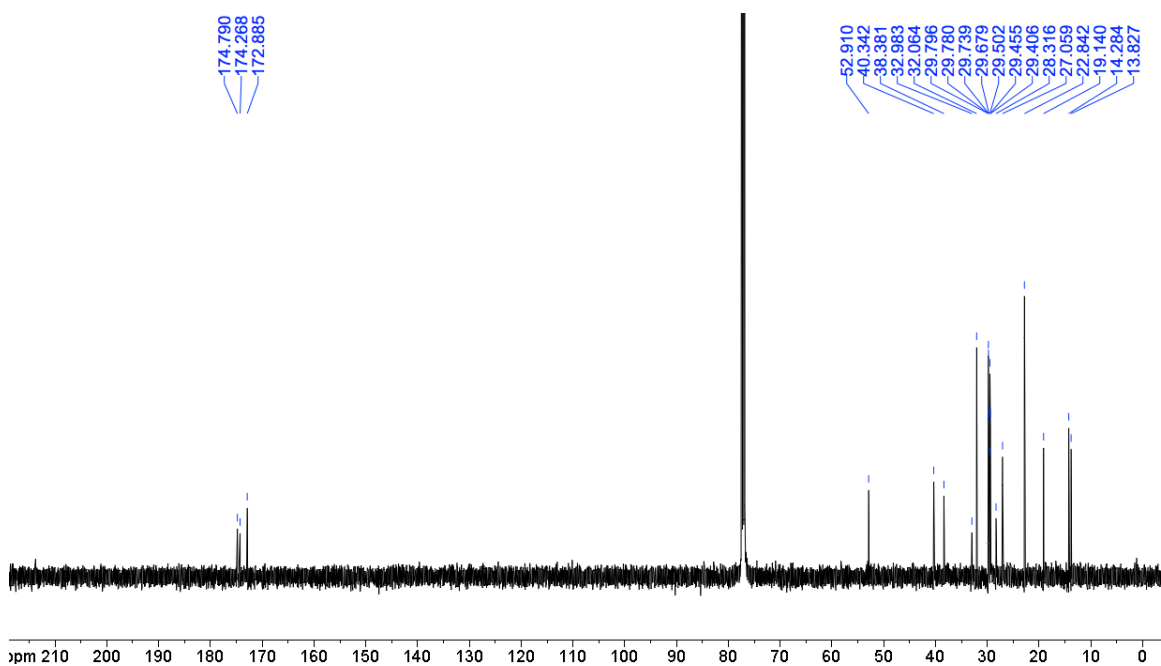
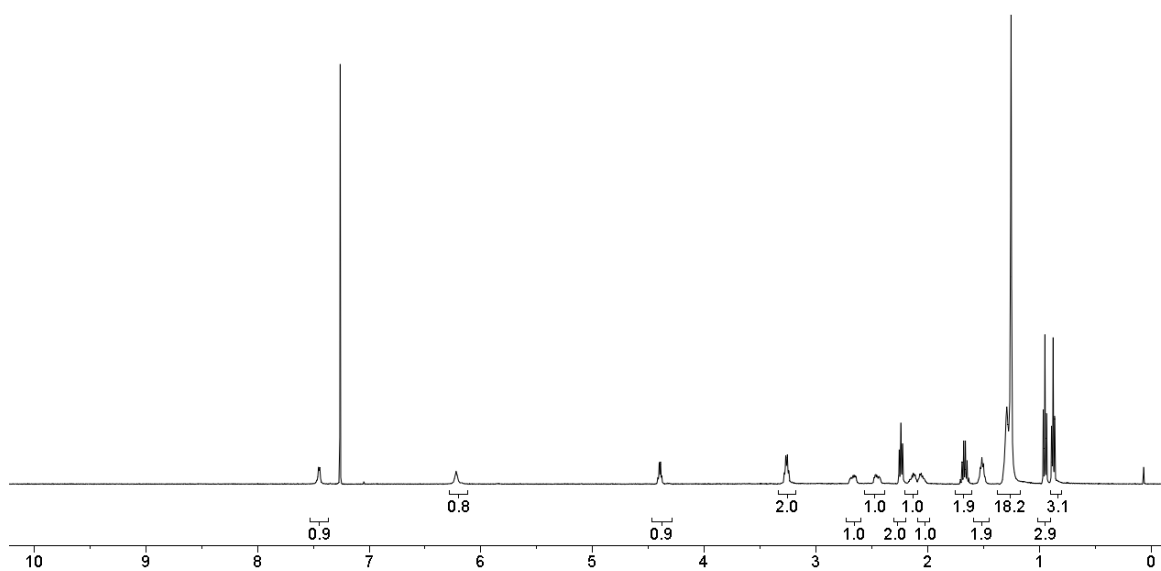


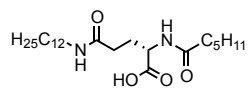
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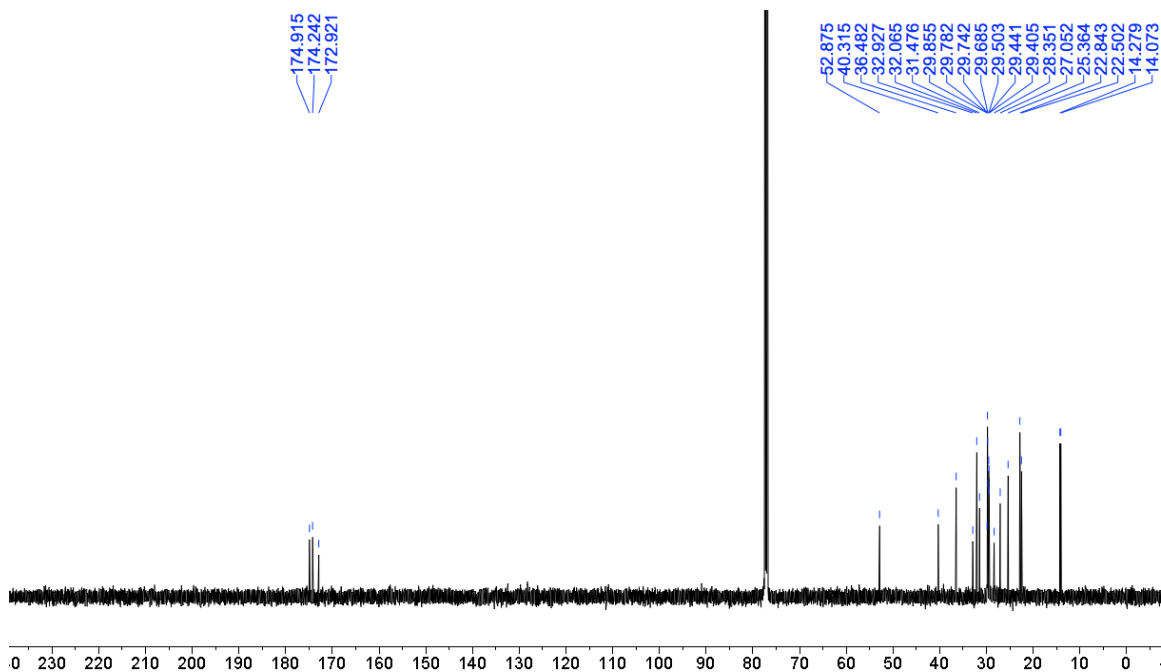
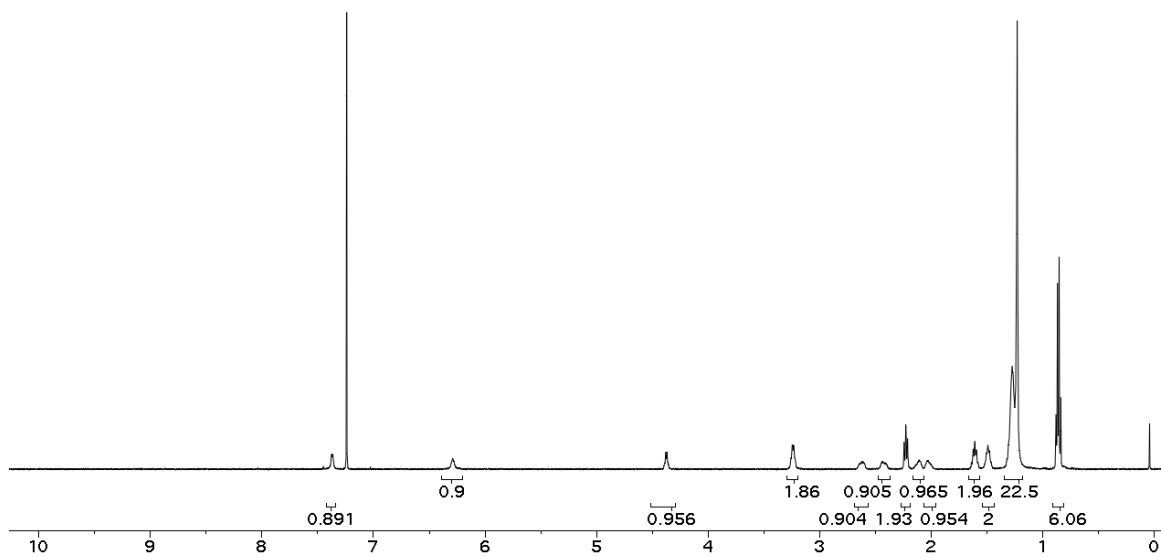


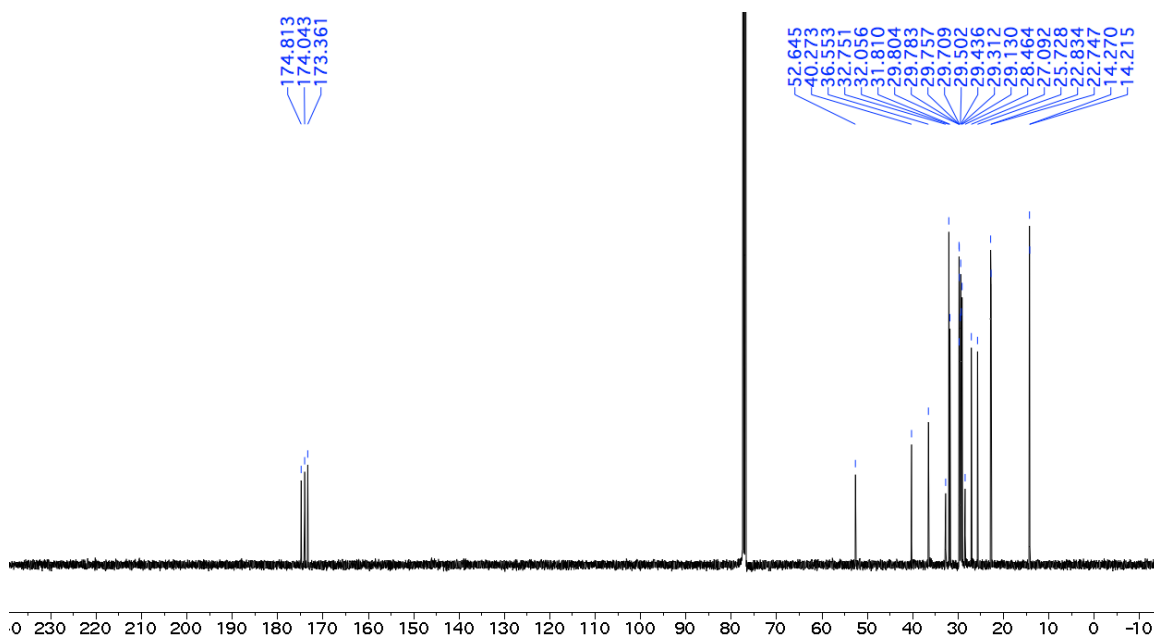
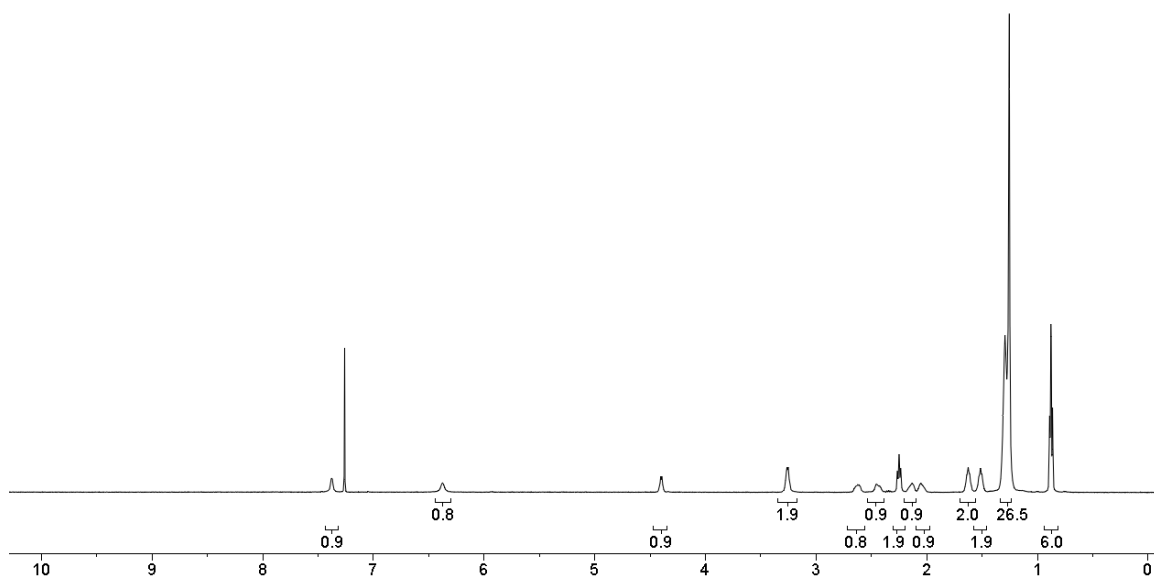
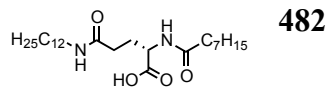
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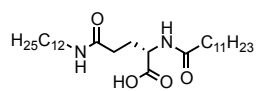




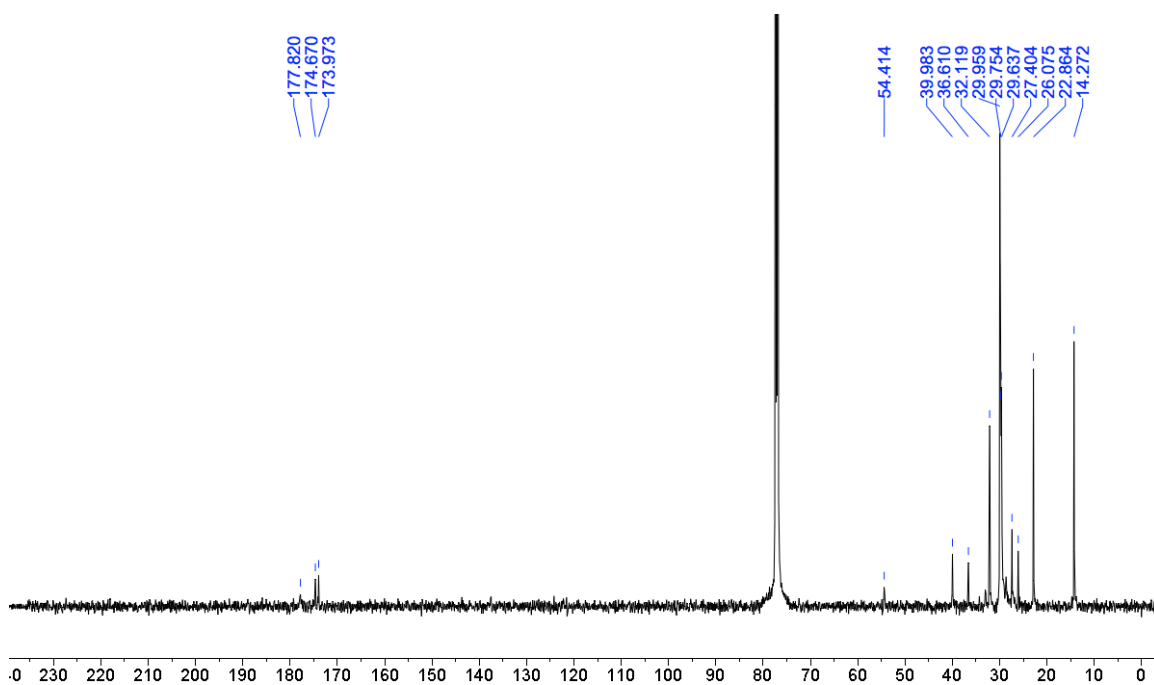
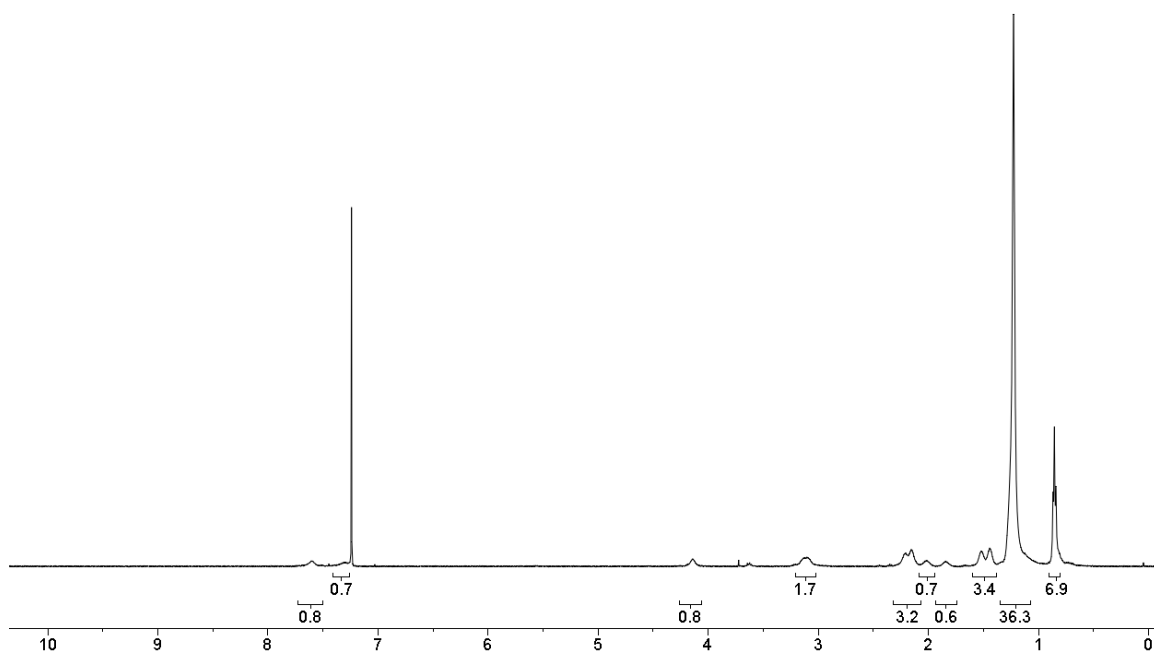
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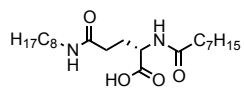




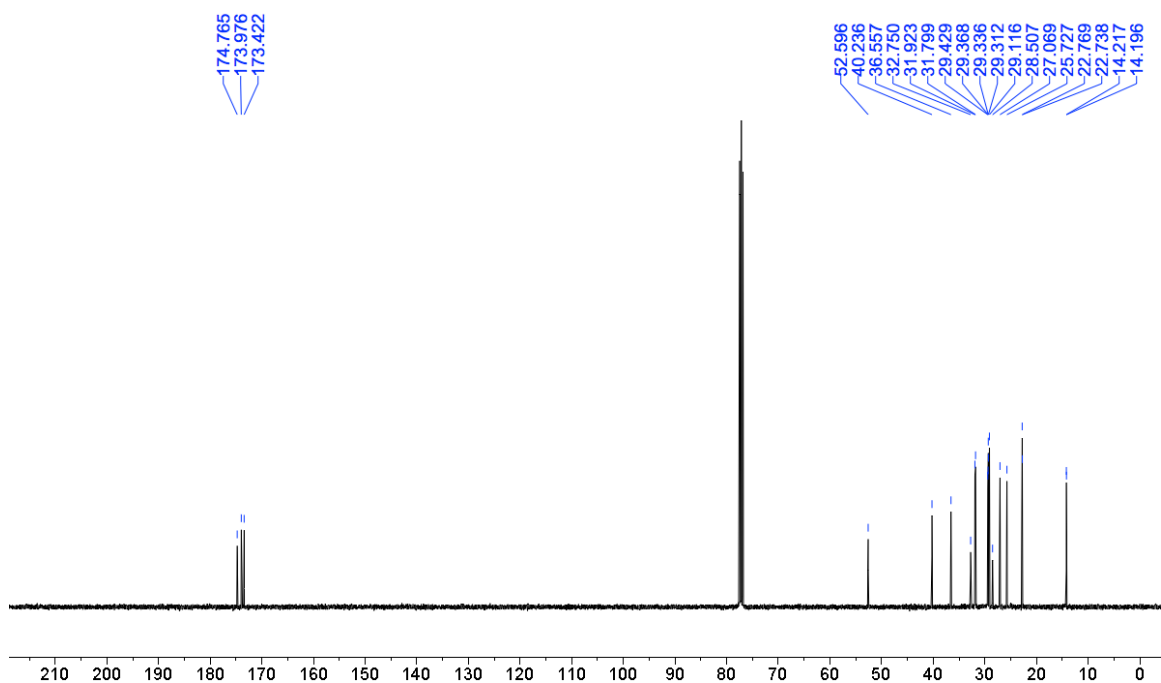
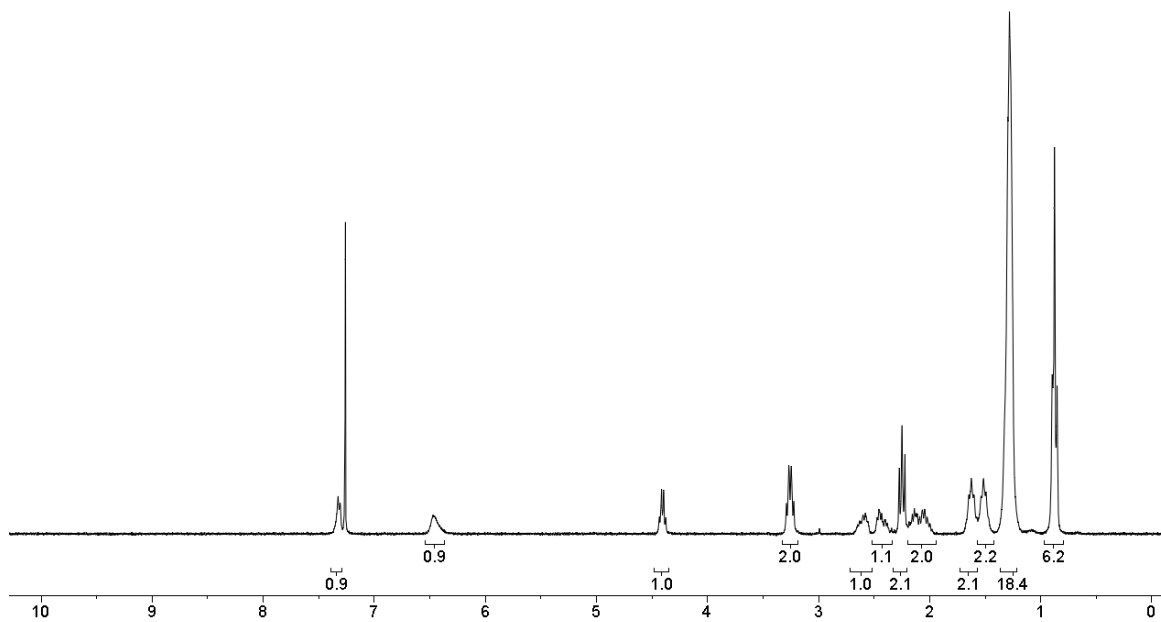


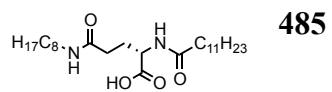
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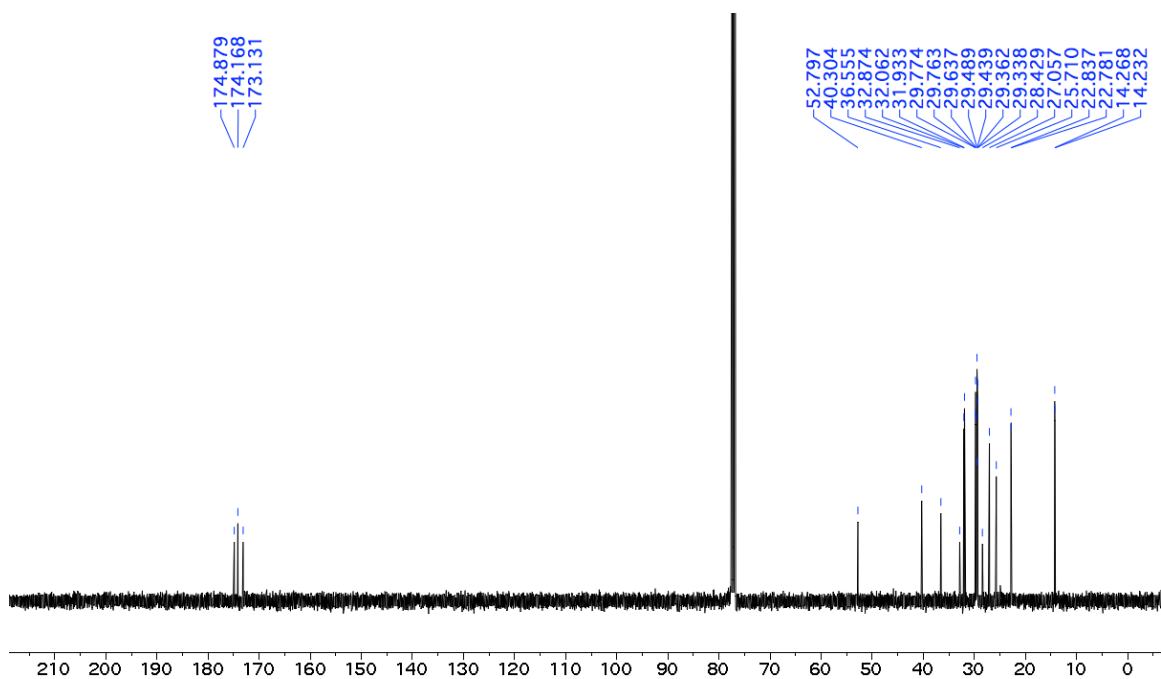
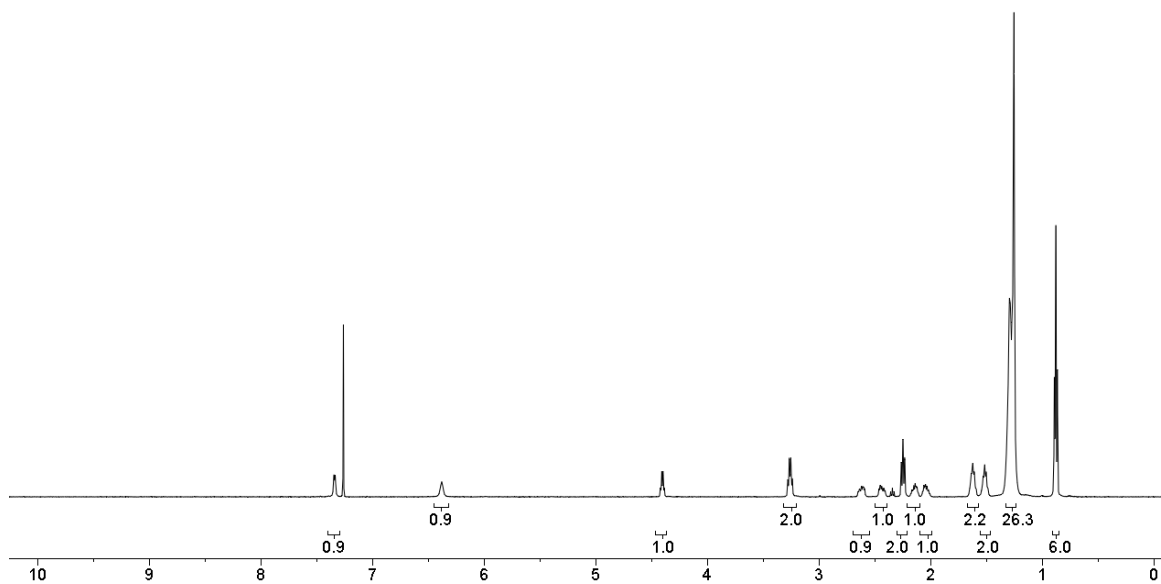


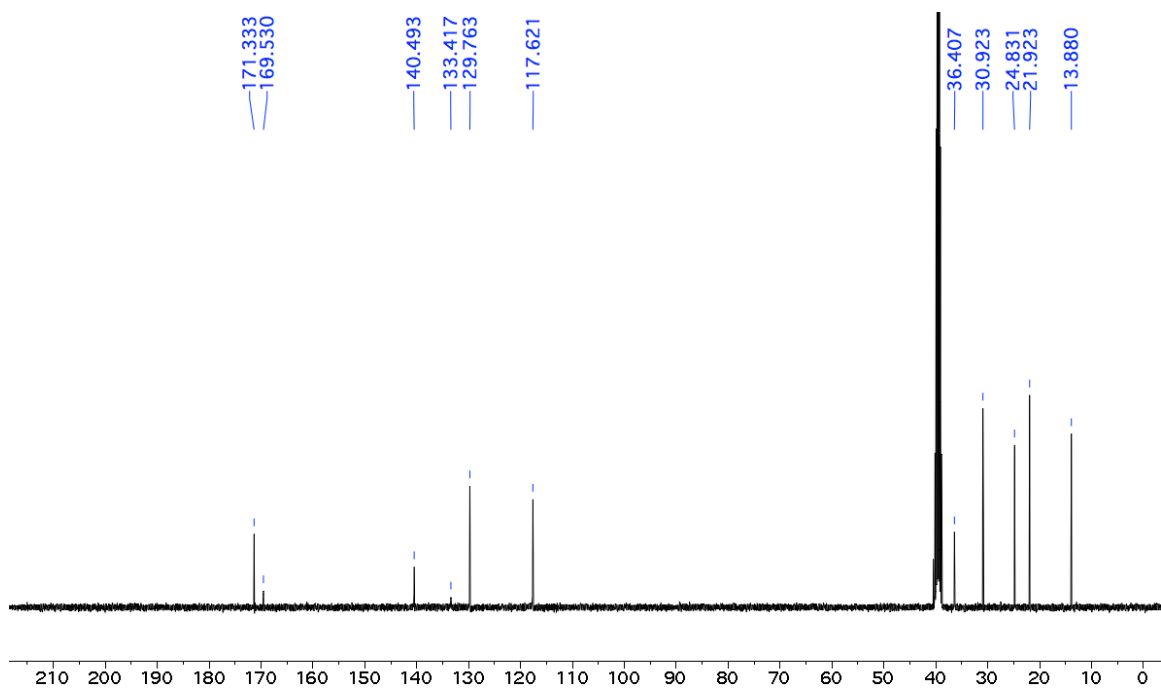
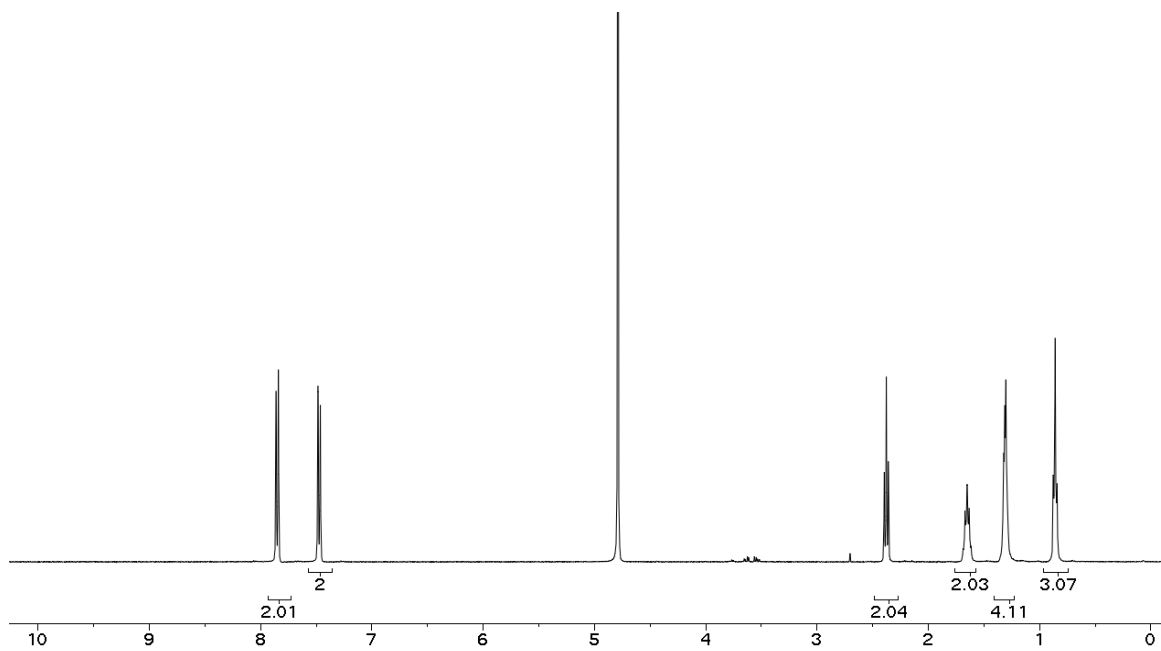
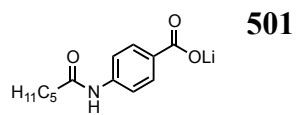
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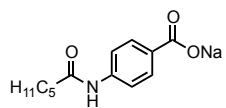




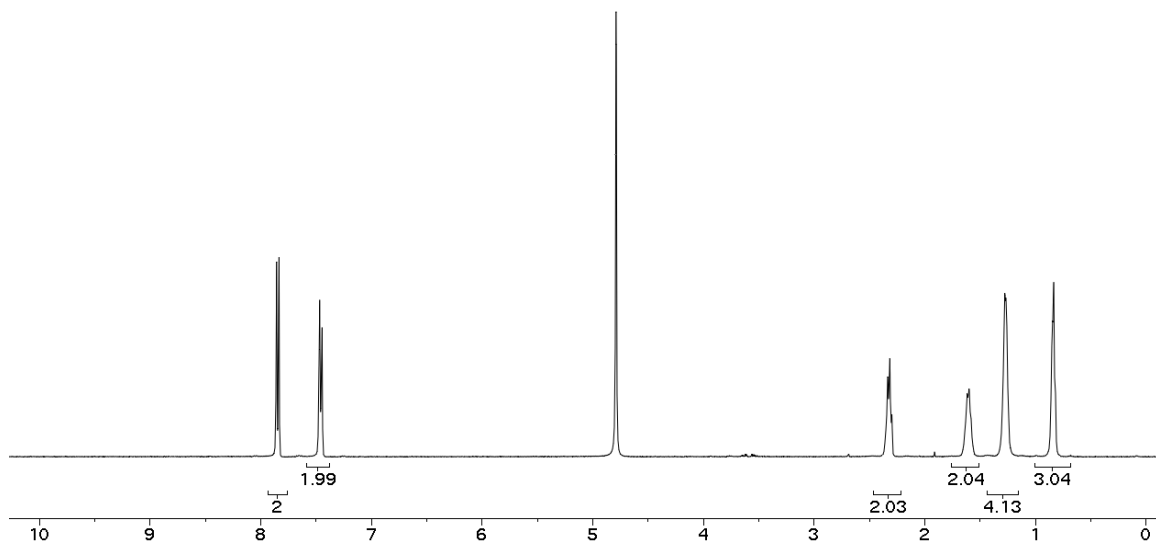
485







502



171.301
170.321

140.100
134.570

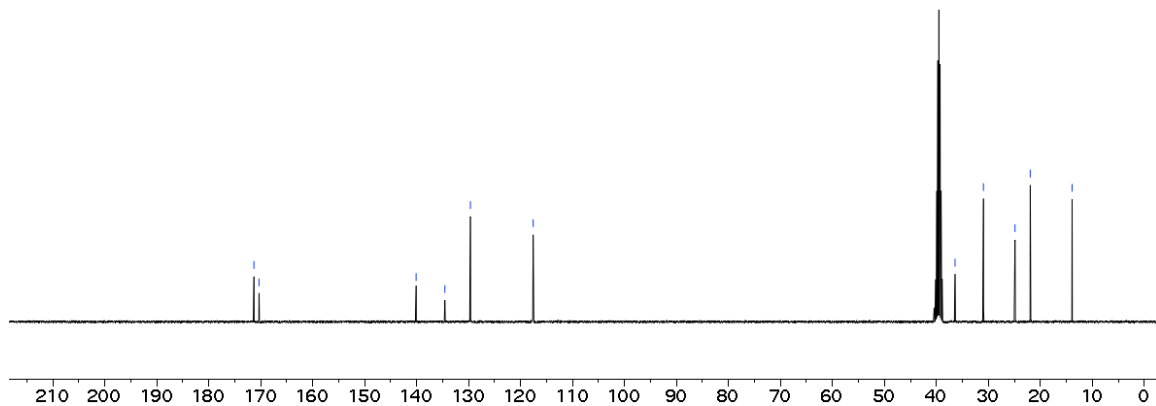
129.653

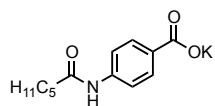
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36.407
30.950

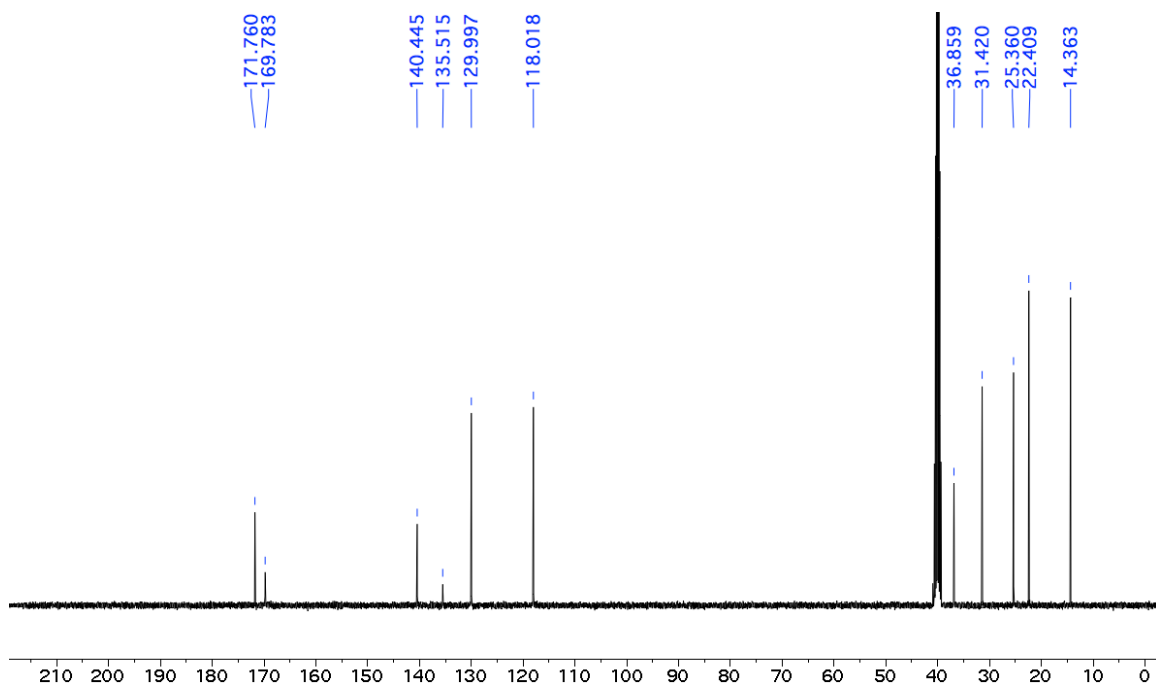
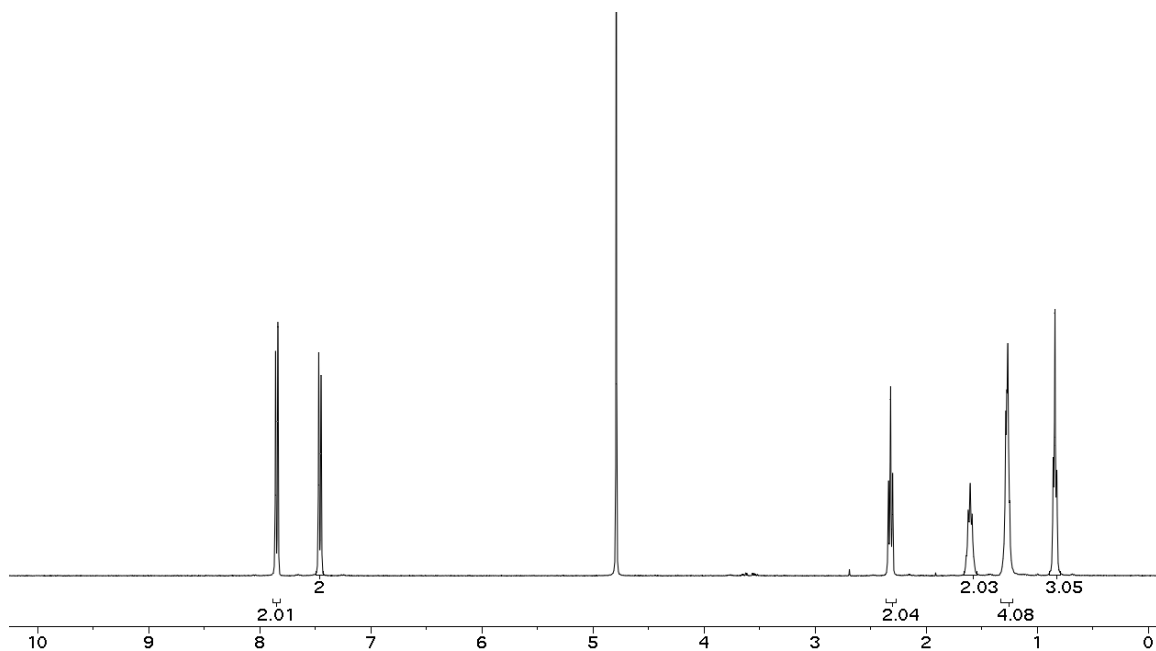
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21.943

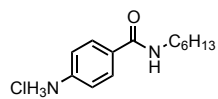
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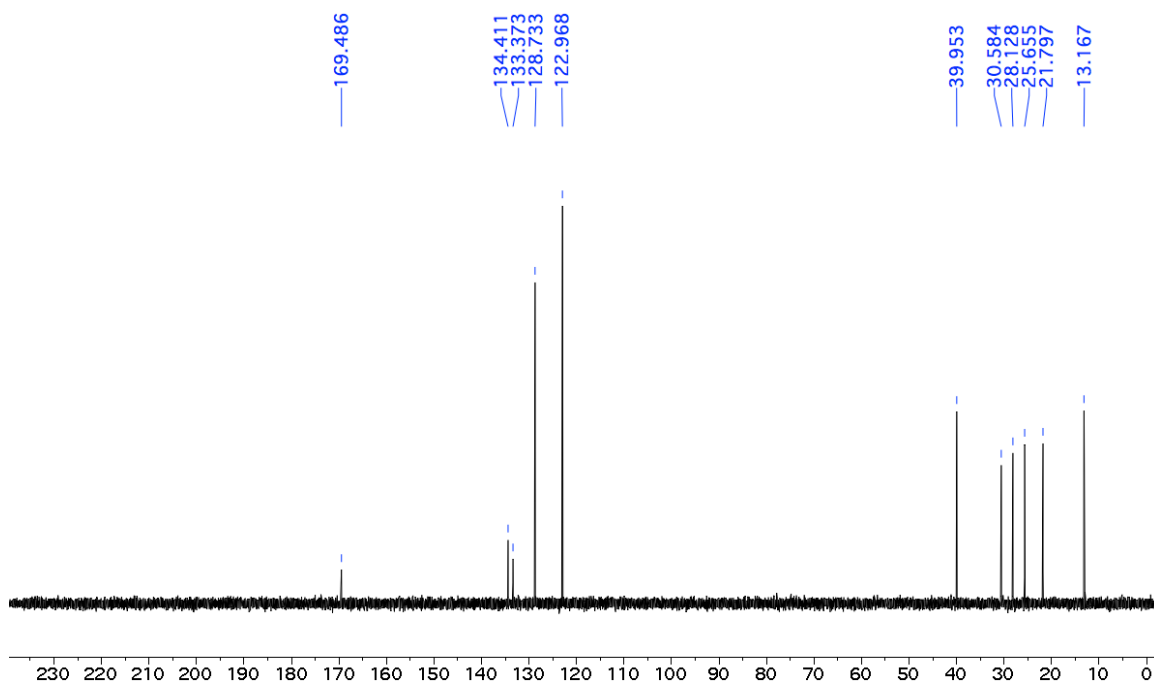
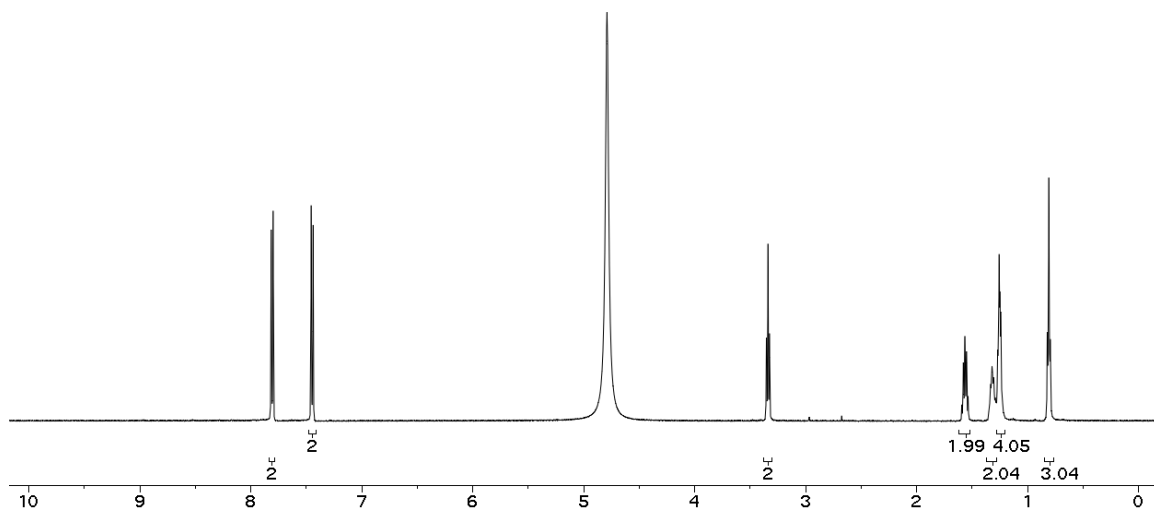


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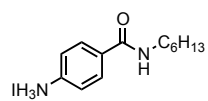




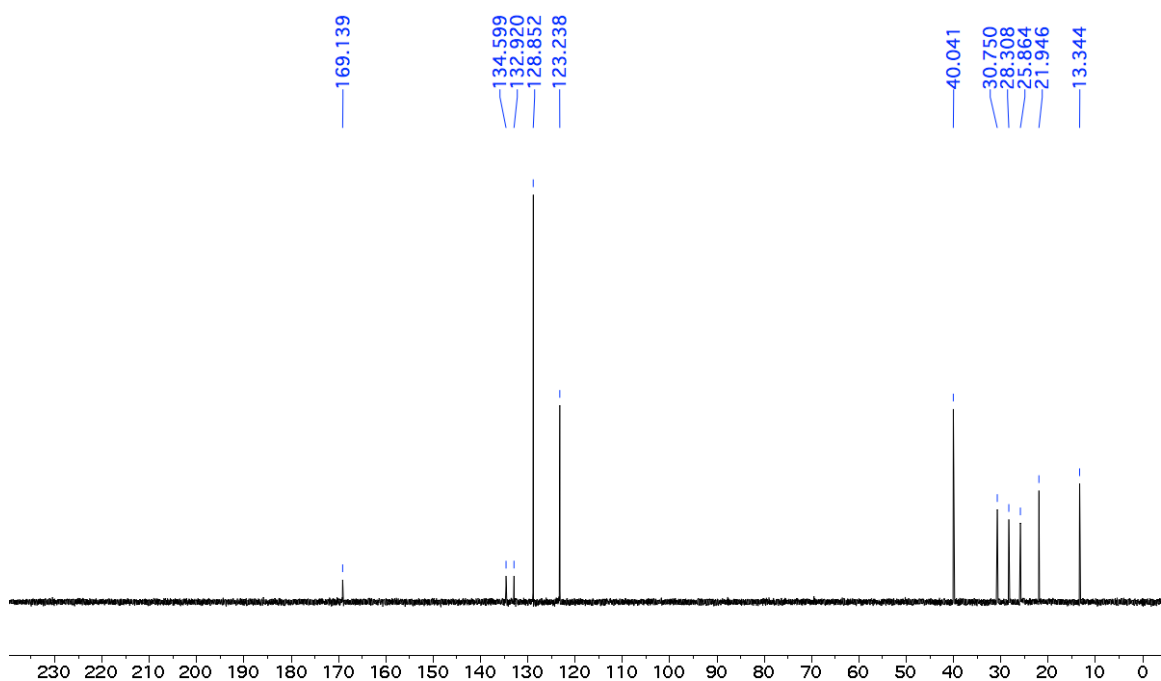
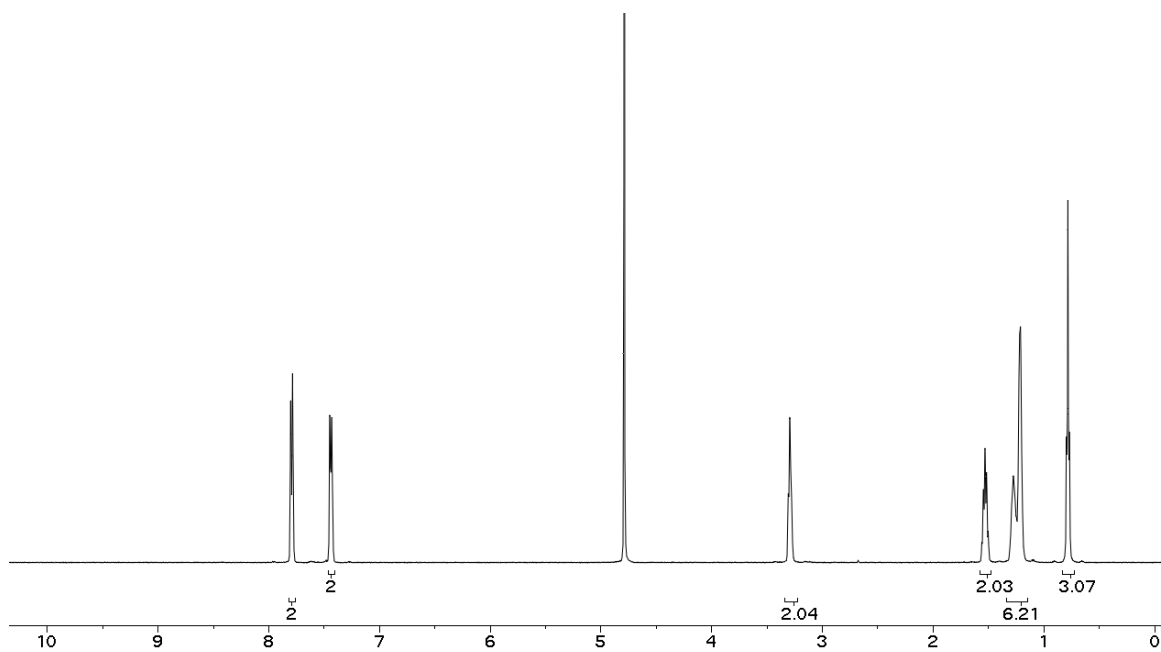
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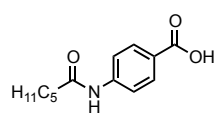




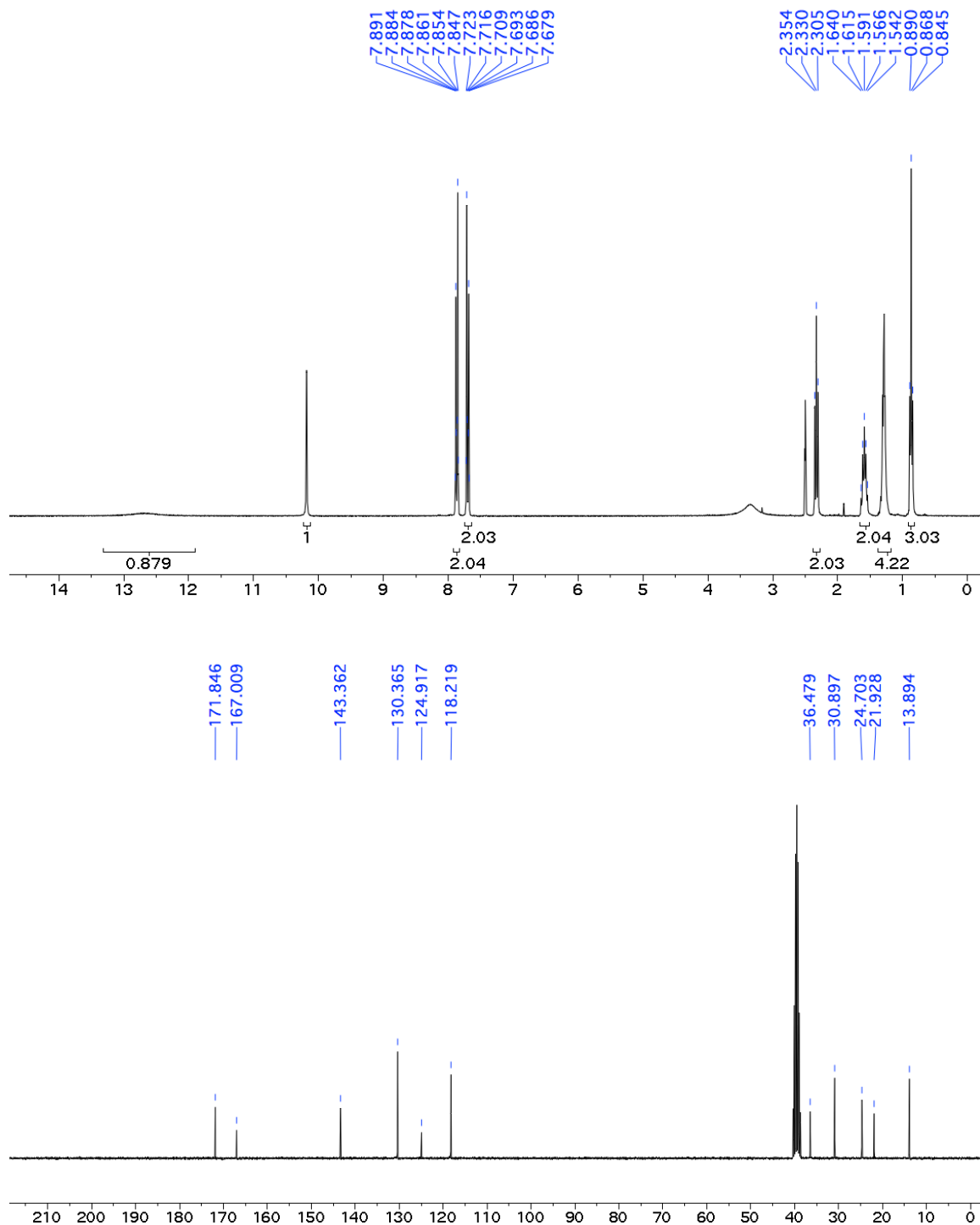


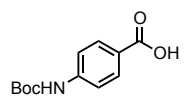
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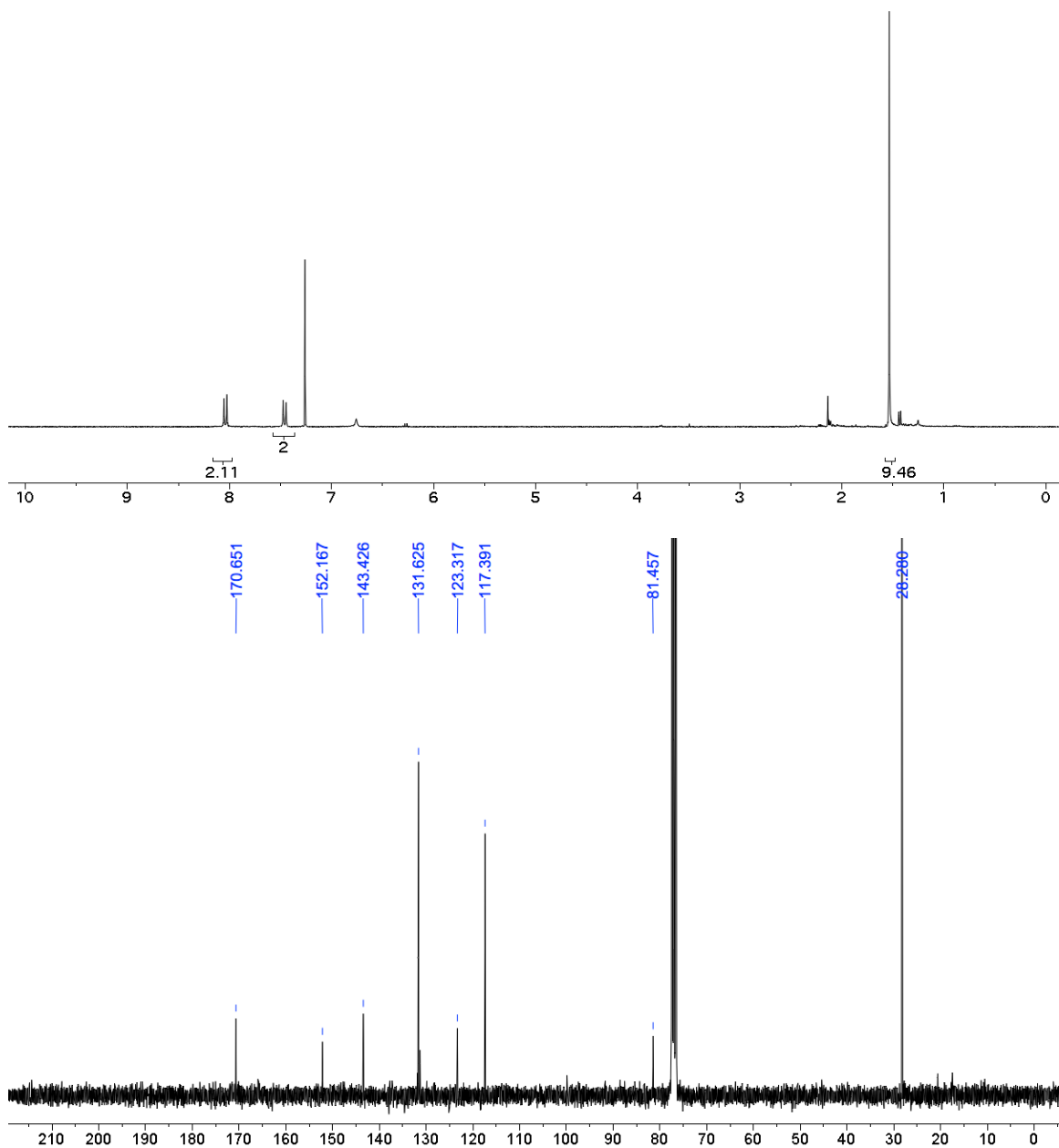


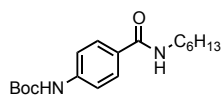
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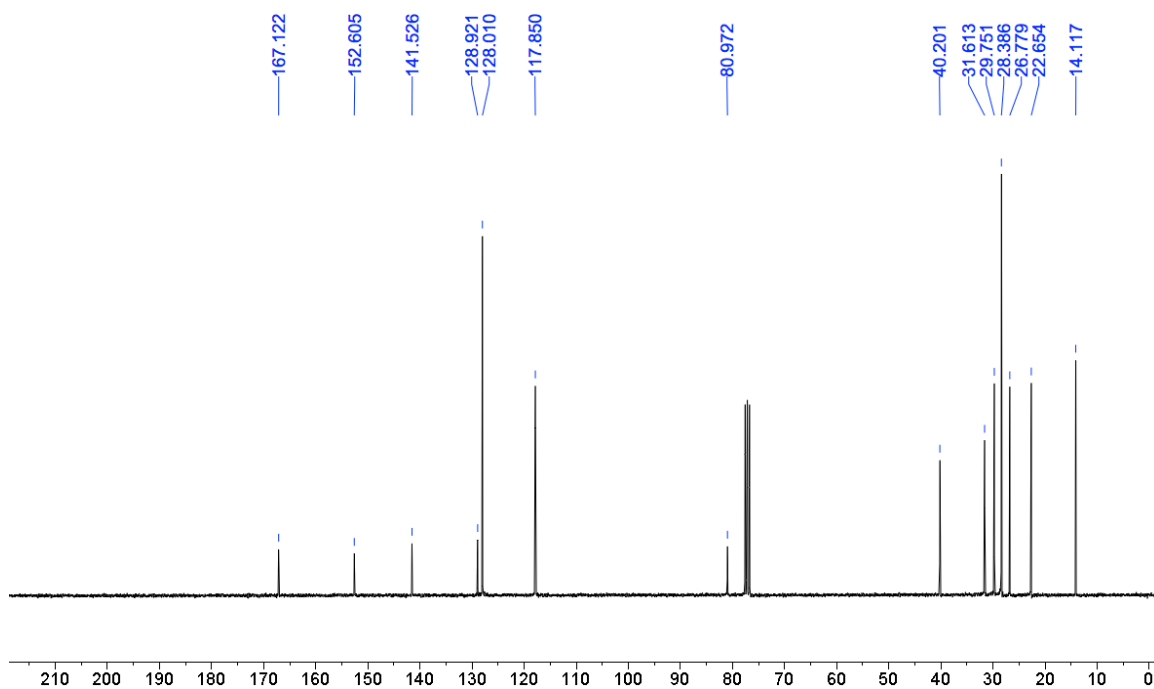
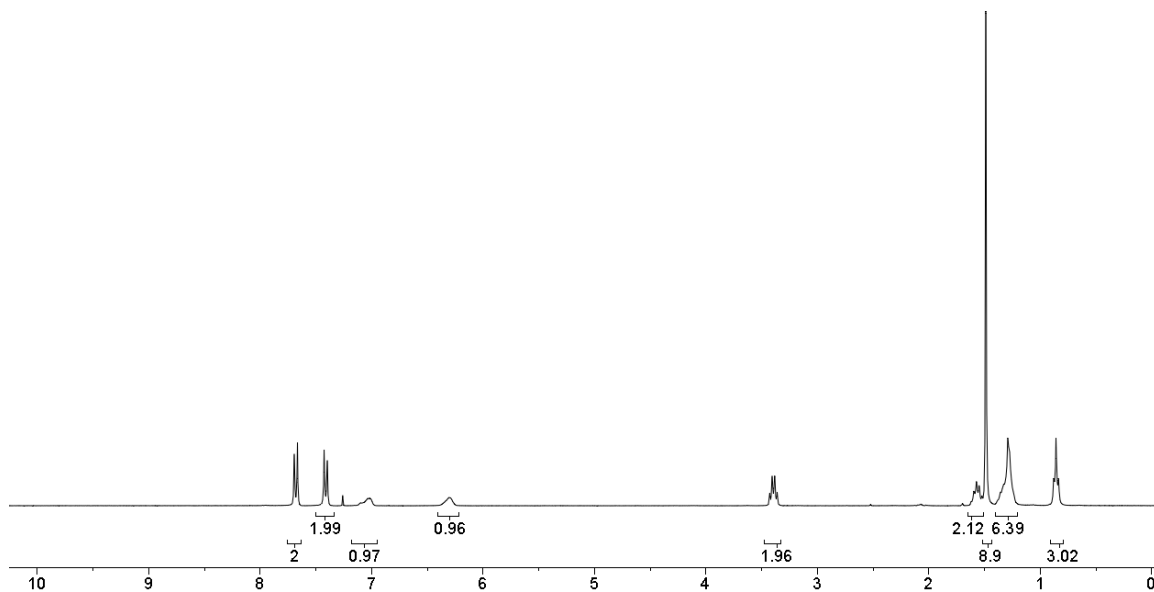


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7.7. References

1. Knight, C. A.; Hallett, J.; DeVries, A. L. *Cryobiology* **1988**, 25, 55.
2. Jackman, J.; Noestheden, M.; Moffat, D.; Pezacki, J. P.; Findlay, S.; Ben, R. N. *Biochem. Biophys. Res. Commun.* **2007**, 354, 340.
3. Chakrabartty, A.; Hew, C. L. *Eur. J. Biochem.* **1991**, 202, 1057.
4. Tam, R. Y.; Ferreira, S. S.; Czechura, P.; Chaytor, J. L.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, 130, 17494.
5. Czechura, P.; Tam, R. Y.; Dimitrijevic, E.; Murphy, A. V.; Ben, R. N. *J. Am. Chem. Soc.* **2008**, 130, 2928.
6. Broddefalk, J.; Nilsson, U.; Kihlberg, J. *J. Carbohydr. Chem.* **1994**, 13, 129.
7. Lemieux, R.; Ratcliffe, R. *Can. J. Chem.* **1979**, 57, 1244.
8. Grundler, G.; Schmidt, R. R. *Liebigs Ann. Chem.* **1984**, 1984, 1826.
9. Sabesan, S.; Lemieux, R. U. *Can. J. Chem.* **1984**, 62, 644.
10. Vermeer, H. J.; Halkes, K. M.; van Kuik, J. A.; Kamerling, J. P.; Vliegthart, J. F. *G. J. Chem. Soc., Perkin Trans. I* **2000**, 2249.
11. Wong, T. C.; Townsend, R. R.; Lee, Y. C. *Carbohydr. Res.* **1987**, 170, 27.
12. Fang, T. T.; Bendiak, B. *J. Am. Chem. Soc.* **2007**, 129, 9721.
13. Suzuki, M.; Abe, T.; Hanabusa, K. *J. Colloid Interface Sci.* **2010**, 341, 69.

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 *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 β -(1,6)-linked AFGP disaccharide analogue and assessing it for IRI activity. When compared to the native β -(1,3)- and β -(1,4)-linked AFGP disaccharide, the derivative that had the highest IRI activity was the β -(1,4)-linked disaccharide. This was not the disaccharide that is found in the native AFGP, which contains the β -(1,3)-linkage. Additionally, it was found that the C2 *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 ϵ -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 surfactants 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-1 α) 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 known 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 through 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 β -(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 α).

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.

Ghobadloo, S. M.; Balcerzak, A. K.; Gargaun, A.; Muharemagic, D.; Mironov, G. G.; Capicciotti, C. J.; Briard, J. G.; Ben, R. N.; Berezovski, M. V. Carbohydrate-Based Ice Recrystallization Inhibitors Increase Infectivity and Thermostability of Viral Vectors, *Sci. Rep.* **2014**, 4.

Balcerzak, A. K.; Febbraro, M.; Ben, R. N. The importance of hydrophobic moieties in ice recrystallization inhibitors *RSC Advances* **2013**, 3, 3232.

Balcerzak, A. K.; Ferreira, S. S.; Trant, J. F.; Ben, R. N. Structurally diverse disaccharide analogs of antifreeze glycoproteins and their ability to inhibit ice recrystallization *Bioorg. Med. Chem. Lett.* **2012**, 22, 1719.