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Synthesis of a C-Linked Antifreeze Glycoprotein Building Block Containing an Internal Olefin

By
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For the M.Sc. degree in Chemistry

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Abstract

Antifreeze glycoproteins (AFGPs) are polypeptide compounds found in the blood of Antarctic notothenioids and northern cod. These substances inhibit the growth and proliferation of ice in fish inhabiting sub-zero conditions. The structure of AFGPs, and the mechanism of ice growth inhibition at the molecular level have been a source of intense debate. Work in our laboratory involves the synthesis of AFGP analogues in an effort to discern the mechanism of ice growth inhibition and enhance antifreeze attributes for potential therapeutic applications.

We have previously synthesized C-linked AFGP analogues possessing antifreeze properties (TH, RI and DIS). One such analogue, [OGG]₄[Gal], has exhibited recrystallization inhibition (RI) activity comparable to native AFGPs. An important structural feature of [OGG]₄[Gal] is the presence of an amide bond. We report herein the synthesis of a C-linked AFGP building block with an internal olefin as an amide isostere, in an effort to elucidate the function of the amide bond in the recrystallization inhibition activity of [OGG]₄[Gal].
Acknowledgements

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# Table of Contents

Abstract ........................................................................................................ ii

Acknowledgements ..................................................................................... iii

Table of Contents ........................................................................................ iv

List of Figures ................................................................................................ vii

List of Graphs ............................................................................................... viii

List of Schemes ............................................................................................ ix

List of Tables ............................................................................................... xi

List of Abbreviations .................................................................................... xii

**Chapter 1 Introduction**

1.1 Overview of Biological Antifreezes ..................................................... 2

1.2 Classification of Biological Antifreezes ............................................ 5

1.2.1 Antifreeze Proteins ....................................................................... 5

1.2.2 Antifreeze Glycoproteins ............................................................... 7

1.3 In Vivo Localization of Biological Antifreezes ................................. 11

1.4 Antifreeze Properties .......................................................................... 11

1.4.1 Thermal Hysteresis ...................................................................... 11

1.4.2 Dynamic Ice Shaping ................................................................... 13

1.4.3 Recrystallization Inhibition ........................................................... 15

1.4.4 Membrane Stabilization ................................................................. 16

1.5 Mechanism of Ice Inhibition ............................................................... 16
4.4 Coupling of Carbohydrate and Amino Acid Components

Appendix

$^1$H and $^{13}$C NMR Spectra
List of Figures

Figure 1.2.1 Cross-section of Type I AFP.................................................................6
Figure 1.2.2 Structure of a typical AFGP.................................................................8
Figure 1.2.3 Structure of AFGP6-8...........................................................................9
Figure 1.2.4 Structure of Arctic and North Atlantic Cod AFGPs.............................10
Figure 1.4.1 Schematic representation of thermal hysteresis....................................12
Figure 1.4.2 Dynamic ice shaping............................................................................13
Figure 1.4.3 Ice crystals grown with AFPs...............................................................14
Figure 1.5.1 Adsorption inhibition model...............................................................17
Figure 1.5.2 AFGP hydrogen bonding with ice lattice...........................................18
Figure 1.5.3 AFP1 hydrogen bonding with ice lattice.............................................19
Figure 1.7 Early structure function studies on AFGP8.............................................22
Figure 1.8 Breyer's ice cream bars with Type III AFP.............................................24
Figure 2.2.1 AFGPs in acidic or basic media..........................................................40
Figure 2.3.1 Core components of AFGPs...............................................................42
Figure 2.3.2 Comparison of AFGPs.......................................................................42
Figure 2.3.3 Second generation C-Linked AFGP analogues.................................45
Figure 2.3.4 Third generation C-Linked AFGP analogues.....................................46
Figure 2.4.1 C-linked AFGP analogue with an internal olefin...............................48
Figure 3.1 Structure of C-linked olefin building block........................................53
Figure 3.2 Structure of Sudan Red 7B.................................................................60
Figure 3.4 Structure of 1, 10-Phenanthroline......................................................70
Figure 3.6 Cis and trans building blocks.............................................................80
List of Graphs

Graph 1.1 Freezing temperatures of various solutions........................................4

Graph 2.3 RI activity of AFGP analogues.................................................................46
List of Schemes

Scheme 2.1.1 Anderson’s convergent synthesis of O-linked AFGP..........................34
Scheme 2.1.2 Filira’s linear solid phase synthesis strategy.................................35
Scheme 2.1.3 Nishimura’s synthesis of native AFGP........................................37
Scheme 2.1.4 Chen’s SPPS approach to native AFGP........................................39
Scheme 2.3.1 Synthesis of first generation AFGP analogues.................................44
Scheme 2.4.1 Retrosynthesis of AFGP analogue 35...........................................49
Scheme 3.1.1 Bertozzi’s approach to C-glycosyl analog of β-Gal-O-Ser.................54
Scheme 3.1.2 Retrosynthesis of C-linked olefin building block............................55
Scheme 3.2.1 Bromination of β-D-galactose pentacetate......................................56
Scheme 3.2.2 Allylation of α-bromo-galactose tetracetate....................................57
Scheme 3.2.3 Deprotection and reprotction of monosaccharide 11.......................58
Scheme 3.2.4 Ozonolysis of benzyl protected olefin............................................58
Scheme 3.2.5 Cleavage of benzyl ethers with ozone...........................................59
Scheme 3.2.6 Ozonolysis in the presence of Sudan Red 7B..................................60
Scheme 3.3.1 Retrosynthesis of amino acid component.......................................61
Scheme 3.3.2 Synthesis of chiral phosphonium salt............................................63
Scheme 3.3.3 Synthetic scheme for the synthesis TBDMS aldehyde......................64
Scheme 3.3.4 Wittig reaction of TBDMS aldehyde and phosphonium salt............65
Scheme 3.3.5 Comparison between the use of one and two equivalents of base.....66
Scheme 3.3.6 Attempts at derivatization with TsCl, I₂ and MsCl..........................67
Scheme 3.3.7 Mechanism for the regeneration of starting material......................67
Scheme 3.3.8 Generation of the amino acid coupling partner..............................68
Scheme 3.4 Wittig reaction of coupling partners.................................................69
Scheme 3.5.1 Revised approach to building block.................................................72
Scheme 3.5.2 Swern oxidation.............................................................................72
Scheme 3.5.3 Test coupling with methyltriphenylphosphonium iodide ..............73
Scheme 3.5.4 Generation of second Wittig reagent .............................................74
Scheme 3.5.5 Revised coupling............................................................................74
Scheme 3.5.6 Attempts at photochemical isomerization.....................................75
Scheme 3.5.7 Deprotection and oxidation of glycoconjugate.............................76
Scheme 3.7.1 SPPS of building block.................................................................78
Scheme 3.7.2 Debenzylation of benzyl protected polymer.................................81
List of Tables

Table 1.2.1 Characteristics of antifreeze proteins.........................................................5
Table 1.2.2 Classification of AFGPs based on molecular weight.................................8
Table 1.7 Nishimura’s SAR Results..............................................................................23
Table 3.3 Conditions for oxazolidinone ring formation.................................................62
Table 3.4 Test reactions of amino acid coupling partner with benzaldehyde..............71
List of Abbreviations

[OGG]_4[Gal]  (Ornithine, glycine, glycine)_4 (galactose)

AFPs  Antifreeze proteins

AFGPs  Antifreeze glycoproteins

Ala  Alanine

Asn  Asparagine

Asx  Asparagine/ aspartic acid

Bn  Benzyl

Boc  tert-Butyloxy carbonyl

CD  Circular Dichroism

CDI  1,1-Carbonyldiimidazole

DCM  Dichloromethane

DIPEA  Diisopropylethylamine

DIS  Dynamic ice shaping

DMAP  Dimethylaminopyridine

DMF  N, N-Dimethyl formamide

DMSO  Dimethyl sulfoxide

DPPA  Diphenylphosphorylazide

ESI  Electrospray ionization

Fmoc  9-Fluorenylmethoxycarbonyl

Fmoc-OSu  Fmoc-succinimide

Gly  Glycine
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBTU</td>
<td>2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>J</td>
<td>Coupling constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>M⁺</td>
<td>Molecular Ion</td>
</tr>
<tr>
<td>MLGS</td>
<td>Mean largest grain size</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MTT</td>
<td>Methylthiazole diphenyl-tetrazolium bromide</td>
</tr>
<tr>
<td>Orn</td>
<td>Ornithine</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>RI</td>
<td>Recrystallization inhibition</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SPS</td>
<td>Solid phase synthesis</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>TBDMS</td>
<td>tert-Butyldimethylsilyl</td>
</tr>
<tr>
<td>TH</td>
<td>Thermal hysteresis</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TMSOTf</td>
<td>Trimethylsilyl trifluoromethane sulfonate</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction to Biological Antifreezes
1.1 Overview of Biological Antifreezes

Biological antifreezes are naturally occurring polypeptide compounds found in a variety of organisms. These substances are essential for the survival of organisms inhabiting sub-zero environments. Biological antifreezes have been shown to inhibit ice crystal growth and the recrystallization of ice which would otherwise prove fatal.\textsuperscript{1,2,3}

In contrast to homeotherms (warm-blooded birds and mammals), poikilotherms (cold-blooded fish, amphibians and reptiles) regulate their body temperature according to their ambient environment. The internal temperature of poikilotherms in sub-zero conditions often matches that of their surroundings. This raises an interesting question regarding fish that inhabit Arctic and Antarctic waters, which often reach -1.8 °C; why don’t they freeze? The apparent paradox was resolved by Scholander and colleagues\textsuperscript{4} who discovered that certain poikilotherms, Teleost fish for example, are able to survive at extreme temperatures (-1.8 °C) due to the unusually low freezing temperature of their blood.

The water temperature in McMurdo Sound Antarctica averages -1.87 °C throughout the year.\textsuperscript{5} The freezing point depression of seawater (1.87 °C) has been attributed to the colligative effect. Colligative properties of solutions are dependent upon the concentration of solutes rather than their composition. Despite the frigid seawater, Teleost fish such as \textit{Pathogenia hansoni}, \textit{Pathogenia bernacchii} and \textit{Pathogenia borchgrevinki} are able to survive in this habitat. DeVries and coworkers have determined that the freezing point of blood in Teleost fish is approximately -2.1 °C.\textsuperscript{5} The 0.23 °C difference between seawater and blood serum freezing point is sufficient to ensure that
fish do not freeze in ice laden water. Curiously, dialyzable solutes such as sodium chloride, urea, and free amino acids in the blood of *Pathogenia borchgrevinki* account for only 70% of the observed freezing-point depression. DeVries and coworkers discovered that biological antifreezes, antifreeze glycoproteins (AFGPs), were responsible for the remaining 30% of the observed freezing-point depression. DeVries determined that the manner in which AFGPs depress the freezing point of Teleost blood is not colligative. AFGPs are present in the blood of fish at low molar concentrations, and consequently their ability to influence the freezing point in a colligative manner is negligible. In fact, AFGPs in Teleost blood are able to depress the freezing point only 0.002 °C according to the colligative property alone. In contrast, the observed freezing point depression attributed to AFGPs in Teleost fish is 0.65 °C. Thus, the mechanism by which antifreeze glycoproteins inhibit the growth of ice is different than that of colligatively acting substances.

A comparison of the freezing points of aqueous solutions of AFGP3-5, sodium chloride, chicken lysozyme (a protein) and galactose (a carbohydrate) are illustrated on the following page.
Graph 1.1: Freezing temperatures of solutions of NaCl, galactose, lysozyme, AFGP3-5, and a mixture of AFGP + NaCl as a function of solute concentration (in mg/mL).³

According to Graph 1.1, at low concentrations AFGPs exhibit a greater ability to reduce the freezing point of aqueous solutions than sodium chloride and structurally similar components (i.e. galactose and lysozyme). It is important to note that due to the size of AFGPs (AFGP3-5 range from 10.5-21.5 kDa), the effective molar concentration of AFGPs is 500x lower than that of sodium chloride. As a result, AFGPs are more active per mole of compound than sodium chloride. Furthermore, the activity of AFGPs can not be explained solely by reference to their constituent parts (carbohydrate and peptide moieties). For example, antifreeze glycoproteins exhibit greater freezing point depression than galactose and lysozyme. Finally, the parallel nature of the plots of AFGP and AFGP + sodium chloride indicate that the effect of the salt is strictly additive; implying that sodium chloride does not interact with the glycoproteins in a manner which influences the freezing point-depressing behaviour of AFGPs.
1.2 Classification of Biological Antifreezes

Biological antifreezes are divided into two categories based upon their structures; carbohydrate containing antifreeze glycoproteins (AFGPs) and antifreeze proteins (AFPs).

1.2.1 Antifreeze Proteins (AFPs)

Antifreeze proteins are non-homologous biopolymers. They can be divided into five subtypes based upon their structural variability. AFP Types I-IV are native to fish, while Type V AFPs are insect biological antifreezes.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Type I AFP</th>
<th>Type II AFP</th>
<th>Type III AFP</th>
<th>Type IV AFP</th>
<th>Type V AFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass (kDa)</td>
<td>3.3 – 4.5</td>
<td>11.0-24.0</td>
<td>6.5-14.0</td>
<td>12.0</td>
<td>8.4-12.5</td>
</tr>
<tr>
<td>Properties</td>
<td>alanine rich, single α-helix</td>
<td>globular, random coil</td>
<td>globular, glutamine, α-helical</td>
<td>globular, 3° structure bundles</td>
<td>threonine and cysteine rich, disulfide bridges</td>
</tr>
<tr>
<td>Source</td>
<td>Sculpin and right-eyed flounder</td>
<td>sea raven, smelt, atlantic herring</td>
<td>wolfish, eel pout and ocean pout</td>
<td>longhorn sculpin</td>
<td>yellow meal worm beetle</td>
</tr>
</tbody>
</table>

Table 1.2.1 Characteristics of antifreeze proteins (adapted from Haymet).
Type I AFPs were originally discovered by DeVries\textsuperscript{9,10} and are the most documented antifreeze protein. These proteins vary in size from 3.3 – 4.5 kDa. They consist of repeating amino acid sequences characterized by an alanine rich amphiphilic $\alpha$-helix. Hew\textsuperscript{11} has shown that the $\alpha$-helix possesses a hydrophilic face with threonine hydroxyl functionalities oriented parallel to the longitudinal axis of the helix (refer to Figure 1.2.1). It has been hypothesized that the hydrophilic face is involved in the binding of the protein to ice.\textsuperscript{1,3,12}

\textbf{Figure 1.2.1} Cross-section of Type I AFP depicting the three faces of the $\alpha$-helix; the threonine-aspartic acid/asparagine face, the hydrophobic face (consisting of alanine), and the hydrophilic face (comprised of threonine) which has been associated with ice binding.

Type II AFPs have been found in sea raven, smelt and herring.\textsuperscript{3,13} These cysteine and alanine rich globular proteins range from 11.0 to 24.0 kDa. The secondary structure of Type II AFPs is mainly random coil; however, other elements of the secondary structure include $\alpha$-helices and $\beta$-pleated sheets.\textsuperscript{3} Sonnichsen\textsuperscript{14} has postulated that disulfide bridges between proximal cysteine residues are essential for antifreeze activity.
Type III AFPs have been isolated from ocean pout by Hew et al.\textsuperscript{15} and are globular in structure. These proteins vary in size from 6.5-14.0 kDa and the primary structure is not dominated by any particular amino acid.\textsuperscript{3} The secondary structure of Type III AFPs consist of β-sheets arranged orthogonally, resulting in the formation of a ‘β-sandwich’ tertiary structure.\textsuperscript{3}

Type IV AFPs were first isolated by Deng and coworkers\textsuperscript{16} from longhorn sculpin. These antifreezes are approximately 12 kDa and are characterized by a glutamine rich primary structure. The tertiary structure of Type IV AFPs consist of a bundle of 4 α-helices (refer to Table 1.2).\textsuperscript{16,17}

Insect AFPs have been isolated and characterized by Graham\textsuperscript{18} from the yellow meal-worm beetle \textit{Tenebrio molitor}. These AFPs consist of 12 amino acid repeats with a cysteine moiety positioned at intervals no farther than 6 residues. These proteins vary in molecular weight from 8.4 kDa to 12.5 kDa.\textsuperscript{18}

\subsection{1.2.2 Antifreeze Glycoproteins (AFGPs)}

AFGPs were first isolated and characterized by DeVries\textsuperscript{5} from the blood serum of Antarctic notothenioids \textit{Pathogonia borchgrevinki}, \textit{hansoni}, and \textit{bernacchii}. Antifreeze glycoproteins have also been identified in Arctic and north Atlantic cod.\textsuperscript{19} Unlike AFPs which are structurally diverse, AFGPs are structurally homologous. Each AFGP consists of a peptide backbone composed of alanine-alanine-threonine (Ala-Ala-Thr) repeats, with slight sequence variation, and a \(\beta\)-d-galactosyl-(1→3)-α-N-acetyl-d-galactosamine disaccharide fused to the threonine hydroxyl\textsuperscript{3,8} (Figure 1.2.2).
AFGP8 is the smallest glycopeptide with n=4.

Notothenioid glycoproteins have been further divided into 8 classes (AFGP1-8) based upon their mobility in electrophoretic media (which is proportional to molecular weight). These proteins range in size from 2.6 kDa (n=4) to 33.7 kDa (n=50). On the basis of molecular weight, AFGP1-5 are classified as large while AFGP6-8 are classified as small (Table 1.2.2).

<table>
<thead>
<tr>
<th>Antifreeze</th>
<th>Molecular Weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFGP1</td>
<td>33.7</td>
</tr>
<tr>
<td>AFGP2</td>
<td>28.8</td>
</tr>
<tr>
<td>AFGP3</td>
<td>21.5</td>
</tr>
<tr>
<td>AFGP4</td>
<td>15.5</td>
</tr>
<tr>
<td>AFGP5</td>
<td>10.5</td>
</tr>
<tr>
<td>AFGP6</td>
<td>5.0</td>
</tr>
<tr>
<td>AFGP7</td>
<td>3.8</td>
</tr>
<tr>
<td><strong>AFGP8</strong></td>
<td><strong>2.6</strong></td>
</tr>
</tbody>
</table>

Table 1.2.2 Classification of AFGPs based on molecular weight.
In addition to variations in molecular weight, the amino acid composition can differ in small AFGPs since proline is often substituted for alanine (Figure 1.2.3).

![Diagram of AFGP6-8 structure](image)

**Figure 1.2.3** Structure of AFGP6-8 with minor sequence variation. The first repeating unit may consist of proline instead of alanine and the C-terminus is characterized by a Pro-Ala-Ala-Thr-Ala-Ala motif.

Although nototheniid AFGPs possess simple primary structures, their amino acid composition can vary slightly and their size can vary considerably.

Arctic and North Atlantic cod AFGPs are remarkably similar to their unrelated nototheniid counterparts. One notable variation in these species is replacement of threonine with arginine, and the resulting absence of a sugar moiety at this position (refer to Figure 1.2.4). However, the lack of a sugar residue at this location is under dispute, since the absence may be an artifact of isolation.
Isolation of AFGPs from the rock cod *Gadus ogac* by Fletcher and co-workers\(^{20}\) indicate that multiple isoforms of AFGPs can exist within a particular class. For example, AFGP6 was found to contain 14 different isoforms. A protein isoform is any of several different forms of the same protein arising from single nucleotide polymorphisms (SNPs). As a result, the notation AFGPx should not always be interpreted to refer to a single compound.

Fletcher and colleagues\(^{20}\) have shown that larger AFGPs are more active than their smaller counterparts.

AFGP8 has been a synthetic target of numerous groups due to its small size, and consequential ease of preparation. In addition, synthetic analogues for structure activity studies are predicated upon AFGP8.
1.3 In Vivo Localization of Biological Antifreezes

Biological antifreezes have been traditionally isolated from the blood of vertebrates; however, they have also been found in tissue. AFGPs have been discovered in the blood, peritoneal, pericardial and extradural fluid of Antarctic notothenioid fish. Secreted fluids such as urine contain low concentrations of AFGP6-8. It has been postulated that biological antifreezes are secreted by the liver and reach fluid compartments via passive distribution. AFGPs have been identified in the interstitial fluid of all fish tissues except the brain. Some researchers have suggested that AFGPs can be synthesized in the epidermis of fish. Hew has shown contrary to long held belief that AFGPs are not confined solely to extra-cellular media, but can also accumulate in cells.

1.4 Antifreeze Properties

1.4.1 Thermal Hysteresis (TH)

A characteristic property of biological antifreezes is the ability to depress the freezing point of aqueous solution without significantly affecting the melting point. This property is referred to as thermal hysteresis (TH) and is used to detect and quantify antifreeze activity. In contrast to colligatively acting solutes such as sodium chloride, which depress the freezing and melting points to the same extent, biological antifreezes do not depress the freezing and melting points to the same extent. The difference between melting temperature ($T_m$) and freezing temperature ($T_f$) is known as the thermal hysteretic gap (TH gap) (Figure 1.4.1).
The magnitude of the TH gap is dependent upon the nature and concentration of the biological antifreeze employed. Large AFGPs (AFGP1-4) have been shown to exhibit greater TH activity than smaller sized AFGPs (AFGP6-8). Due to structural differences, direct TH comparisons between AFGPs and AFPs are tenuous; however, in terms of molecular mass, Type I winter flounder AFPs exhibit greater activity than similar sized glycoproteins. With respect to concentration dependence, biological antifreezes exhibit an asymptotic concentration at which activity is saturated (i.e. increases in solution concentration of the protein beyond the saturation level will not increase TH activity appreciably, refer to Figure 1.1).
1.4.2 Dynamic Ice Shaping

The growth of ice crystals is inhibited in the TH gap. This is due to the adsorption of antifreezes on specific faces of the nascent ice crystal.\textsuperscript{31} The result of antifreeze adsorption is the formation of alternative crystal structures. This generation of ice crystals with variable morphology is referred to as dynamic ice shaping.

\textbf{Figure 1.4.2 i)} Hexagonal structure of an ice Ih lattice unit and \textbf{ii)} Dynamic ice-shaping, resulting in the formation of a hexagonal bipyramidal crystal.

In the absence of antifreezes, ice crystals grow perpendicular to the c-axis, enlarging the basal plane.\textsuperscript{31,32} In contrast, in the presence of biological antifreezes ice
grows parallel to the c-axis (Figure 1.4.2). DeVries\textsuperscript{34,35,36,37} has shown that antifreezes can selectively adsorb to the prism faces preventing growth along the a-axes. As a result, in the presence of biological antifreezes crystal growth occurs along the c-axis forming a hexagonal bipyramidal structure (Figure 1.4.2).

Within the TH gap, biological antifreezes retard the growth of ice and alter the morphology of ice crystals to hexagonal bipyridmidal. At temperatures below the TH gap, ice crystal growth has been described as “explosive,” resulting in the formation of ice spicules. While spiculation may be attractive for procedures such as cryosurgery, the shape and rapid growth of ice crystals is detrimental for cryopreservation.

\textbf{Figure 1.4.3 i)} Ice crystals grown with AFP within the TH gap showing bipyramidal morphology (left; birds eye view, right; profile).\textsuperscript{38,39} \textbf{ii)} Ice crystal grown with AFP below the TH gap, showing spiculation.\textsuperscript{38}
1.4.3 Recrystallization Inhibition

In addition to thermal hysteresis (TH) and dynamic ice shaping (DIS), recrystallization inhibition is an important property of biological antifreezes. When a frozen sample is maintained below its melting point or warmed slightly above its melting point, ice grains permeating the sample will change in shape and size. This phenomenon is referred to as ice recrystallization. In this process, smaller grains are incorporated into larger grains to yield a sample with a larger mean grain size and fewer grains. It has been postulated that the migration of the grain boundary is driven by an effort to minimize the overall grain boundary energy via an increase in grain size.³

There are two ways by which recrystallization can be inhibited; changing the boundary energy, or diminishing the diffusion of water. With regards to changing the interface energy, AFGPs are able to bind to the surface of crystals at the ice/water interface and prevent ice boundary migration. Furthermore, biological antifreezes are able to achieve this at very low concentrations.⁴⁰⁴¹ Knight⁴² has shown that even peptides devoid of antifreeze activity are able to display RI activity by decreasing the diffusion of water. This occurs due to the extrusion of solutes from the ice lattice during freezing, resulting in a highly concentrated inter-boundary region which inhibits the necessary diffusion of water to the growing crystal.

It should be noted that thermal hysteresis and recrystallization inhibition are not linked to one another, i.e. they are decoupled.⁴³
1.4.4 Membrane Stabilization

The ability of AFGPs to maintain cell membrane integrity at low physiological temperatures is an important attribute. In the absence of antifreeze glycoproteins, liposome membranes leak as they are cooled/warmed through the thermotropic phase transition.\textsuperscript{44, 45} The thermotropic phase transition is the change from a liquid state to a crystalline state. In this transitional stage both crystalline and liquid states coexist, resulting in increased membrane permeability. In the absence of AFGPs, Hayes\textsuperscript{46} has reported the leakage of trapped markers during the transition. No leakage was observed in the presence of AFGPs. Fletcher\textsuperscript{47} has proposed that AFGPs prevent disruption of the cell membrane by inserting into the lipid bilayer and stabilizing it.

1.5 Mechanism of Ice Inhibition

At The Macroscopic Level:

Currently there are two mechanisms which have been theorized to explain the macroscopic mechanism of ice inhibition, a nucleation inhibition model and an adsorption-inhibition model. The nucleation inhibition paradigm suggests that antifreezes prevent the formation of a nucleus of critical size by blocking interfacial sites and/or increasing the interfacial energy.\textsuperscript{3, 48}

The adsorption-inhibition model advocated by Knight and DeVries\textsuperscript{49, 50} suggests that antifreezes bind to the growing ice surface preventing additional water molecules from adding to where the antifreeze has adsorbed. This results in ice growth between antifreeze adsorption sites only. The shape of the ice front becomes spherical and since
the water-ice interfacial energy increases with curvature of the ice front, growth becomes thermodynamically unfavourable ($\Delta G = +$). This is known as the Kelvin Effect.$^{51}$

![Diagram of water and ice phases](image)

**Figure 1.5.1** Adsorption-inhibition model.

The exact mode of antifreeze binding in the adsorption-inhibition model is still under debate. Knight and DeVries$^{50}$ suggest that antifreeze binding is irreversible. Their assertion is predicated upon the unlikelihood of the 24 hydrogen bonds formed per AFGP8 molecule from being broken in concert. Kuroda$^{52}$ has suggested that this model is incorrect, and adsorption may be reversible. Wen and Laursen$^{53}$ have attempted to reconcile these conflicting views suggesting that antifreeze binding is concentration dependent. At low concentrations reversible adsorption occurs; however, at elevated glycoprotein concentrations, intermolecular interactions (van der Waals forces) between antifreezes dominate, resulting in greater binding affinity.
At The Molecular Level:

The mechanism of antifreeze interaction with the ice-water interface is a subject of controversy. Hydrogen bonding between the disaccharide moiety of the glycoprotein and the ice surface has been postulated to account for ice binding. Proponents of this theory advocate that antifreezes such as AFGP8 are capable of forming 24 Hydrogen bonds with the ice lattice per molecule. This figure is derived from the observation that only two carbohydrate hydroxyls are appropriately aligned to interact with the ice surface per repeating unit (n=4), and each hydroxyl forms 3 hydrogen bonds.\(^5^4\)

![Figure 1.5.2](image)

**Figure 1.5.2** i) Early model hypothesizing 1 hydrogen-bond per aligned hydroxyl. ii) Revised model, hypothesizing 3 hydrogen-bonds per aligned hydroxyl.

The hydrogen bonding hypothesis is an extension of a similar supposition proposed for AFP1. According to this model, the hydroxyl groups of the \(\alpha\)-threonine
residues and the carboxyl group of the aspartates form hydrogen-bonds with the oxygen atoms on the pyramidal faces of the ice lattice\textsuperscript{55} (Figure 1.5.3).

\[\text{Figure 1.5.3 Interaction of AFP1 with ice lattice.}\textsuperscript{55}\]

Furthermore, structure activity studies have indicated that hydrophobic interactions may be just as important as hydrophilic interactions. Replacement of \textit{L}-threonine residues on AFP1 with \textit{L}-serine and \textit{L}-valine indicate that hydrophobic methyl groups may play a larger role than previously thought.\textsuperscript{56,57} \textit{L}-Serine mutants were devoid of antifreeze activity, while \textit{L}-valine mutants showed comparable activity to native AFP1. The only difference between \textit{L}-valine, \textit{L}-threonine and \textit{L}-serine AFPs which can possibly account for the variation in activity is the presence of \(\beta\)-methyl moieties in \textit{L}-valine and \textit{L}-threonine AFPs and the absence of \(\beta\)-methyl functionalities in \textit{L}-serine mutants.
1.6 Conformational Analysis

Determining the conformation adopted by biological antifreezes is essential for elucidating how they function. The conformation adopted by AFGPs in solution has an important role in how the glycoprotein interacts with the ice lattice. In contrast to the well defined structure of AFPs, the secondary structure of AFGPs has not been definitively addressed.

DeVries has reported that AFGPs possess minimal α-helix content.\textsuperscript{6} Early circular dichroism (CD) studies suggest that the secondary structure of AFGPs in solution is random coil.\textsuperscript{58,59} In contrast, Morris\textsuperscript{60} has suggested that AFGPs form an extended 3-fold left-handed helix. This supposition was made on the basis that sugar residues align on only one side of the helix, affording a stable hydrogen-bonding face capable of interacting with the ice lattice. Rao\textsuperscript{61} has hypothesized that the helical arrangement represents an energy minima.

Feeney\textsuperscript{62} has indicated that AFGP conformation is temperature dependent. At low temperatures AFGP\textsubscript{8} has been reported to adopt a rod-like conformation, reminiscent of a three fold polyproline type II helix. At higher temperatures, a flexible coil structure has been observed. Using CD, Ben\textsuperscript{63} has indicated that at room temperature random coil is the predominant conformation with minor α-helix and β-sheet contributions. These results suggest that AFGPs are highly flexible, and are capable of adopting various conformations depending on the conditions.
1.7 Structure Activity Relationships

Detailed structure-activity relationship studies have been conducted on AFPs and AFGPs. Early structure-activity work performed by Feeney on native AFGPs has shown a correlation between TH activity and length of the polypeptide. Small glycopeptides obtained via subtilisin hydrolysis of AFGP8 exhibited no discernable antifreeze activity. These penta-Ala-Ala-Thr-Ala-Ala peptides were devoid of any TH activity. This is not surprising considering earlier data shows that larger AFGPs(1-4) are more active than smaller AFGPs.

Sugar residues on AFGP8 have proven essential for TH activity. Cleavage of the base labile glycosidic bond with 0.5M NaOH results in β-elimination of the disaccharide moiety and loss of activity. In addition to highlighting the importance of the sugar residue, racemization was also observed, indicating the significance of the amino acid side chain.

Periodate oxidation of the terminal galactosyl hydroxyls resulted in a loss of TH activity (Figure 1.7). Furthermore, acetylation of the sugar hydroxyls inactivated the glycoprotein as well. These results suggest that certain hydroxyl groups are essential for TH activity. Oxidation of the disaccharide C6 hydroxyls with galactose oxidase did not impact activity. This suggests that the C6 hydroxyls are not essential for TH. However, generation of C6 carboxylates and bisulfite adducts (Figure 1.7) has shown that a negative charge on the carbohydrate is not amenable for activity. Formation of borate esters at C3 and C4, or C4 and C6 of the terminal galactose moiety also exhibited a lack of TH, indicating that the functional groups on the sugar are important.
Recent SAR work by Nishimura\(^72\) has confirmed previous assumptions and led to the development of additional hypotheses. The independent synthesis and testing of a series of AFGP8 analogues by Nishimura has confirmed the necessity of hydrophobic functionalities such as N-acetyl and \(\beta\)-methyl groups (Table 1.7; Entries 2 & 3). Furthermore, Nishimura has shown that the \(\alpha\)-glycosidic-peptide linkage is essential for TH activity (Table 1.7; Entry 4). In addition, the configuration of sugar hydroxyls was also found to be crucial, since lactose analogues were devoid of activity (Table 1.7; Entry 5). Curiously, the galactosamine monosaccharide exhibited comparable activity to AFGP8 (Table 1.7; Entry 6), suggesting that disaccharide and terminal galactose moieties are not essential for TH activity.
<table>
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</tr>
<tr>
<td>2</td>
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</tr>
<tr>
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</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Structure" /></td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 1.7 Nishimura's SAR results.
1.8 Applications of Biological Antifreezes

The properties of biological antifreezes make them valuable tools for a variety of applications, ranging from cryopreservation, to ice cream.

The preservation of perishable foods such as frozen meats and ice cream is important to the frozen food industry. The concern regarding frozen foods is the loss of texture and the detrimental effect on taste. This is due to the formation of large ice crystals via improper storage and multiple freezing-thawing cycles. The ability of AFPs and AFGPs to inhibit ice recrystallization and therefore prevent the loss of ice cream texture make them valuable tools in the ice cream industry. Recently, Goff and Regand\textsuperscript{75} have shown that AFPs from cold-acclimated winter wheat extract (AWWE) can be successful in improving ice cream texture, making the frozen dessert more palatable. Furthermore, the American Food and Drug Administration has recently approved the use of biological antifreezes in food products.\textsuperscript{76} Isolation of AFPs from ocean pout, and large scale cloning in yeast cell lines has been accomplished by Unilever. These AFPs have been incorporated in Breyer's Light ice cream bars with great success.

\textbf{Figure 1.8} Breyer's Light ice cream bars containing Type III AFPs from ocean pout.
Payne and colleagues\textsuperscript{77} have also shown that AFGPs administered to lamb prior to slaughter, followed by storage at -20 °C for 2-16 weeks, results in meat samples with smaller ice crystals and decreased drip (water retention). These examples illustrate the utility of biological antifreezes in the burgeoning frozen food industry.

The application of antifreezes to cryosurgery is an area of significant interest. Cryosurgery is a procedure which employs subzero temperatures to destroy specific tissues. The destruction of cancer cells via cryosurgery has the benefits of being minimally invasive and cost effective.\textsuperscript{78, 79} \textit{In vivo} tumor treatment with AFP1 by Rubinsky\textsuperscript{80, 81} was found to be extremely effective (mortality rate of 100 %). This is due to the dynamic ice shaping (DIS) properties of biological antifreezes which result in the formation of membrane damaging ice spicules. In contrast, cryosurgery without these chemical adjuvants is problematic due to the survival of cancerous tissue.

The formation of intracellular and/or extracellular ice is a problem for cryopreservation. While intracellular ice formation results in mechanical damage to organelles and is almost always lethal, accumulation of extracellular ice can result in mechanical damage to the cell membrane, and cellular dehydration. Cellular dehydration is due to the formation of ice crystals outside the cell, resulting in an expulsion of solutes from the ice lattice and an increase in extracellular solute concentration. This results in an osmotic imbalance, with the cell hyperosmotic relative to the extracellular fluid. The subsequent movement of water out of the cell to the extracellular fluid results in dehydration. Permeable cryoprotectants such as DMSO can alleviate the osmotic imbalance, but are incapable of inhibiting extracellular ice formation. A possible solution
to this challenge is a procedure called *vitrification*. Vitrification is a fast-cooling technique and can be coupled with high concentrations of cryoprotectants to convert a sample into a glass like amorphous solid. A drawback of this technique is the toxicity associated with conventional cryoprotectants. One way to overcome these challenges is to use biological antifreezes. Seren\(^82\) has shown that AFGPs regulate the osmotic pressure and inhibit the growth of ice during vitrification of pig oocytes.

Biological antifreezes are also useful for the hypothermic storage of organs. The ability of AFGPs to stabilize the cellular membrane through the thermotropic transition makes them ideal for conventional storage protocols.
References:


Chapter 2

Goals and Objectives
2.1 Synthesis of AFGPs and AFGP Analogues

Antifreeze glycoproteins are obtained primarily from the blood of teleost fish. Due to the low natural abundance of these “O-linked” compounds and the difficulty in purifying them, traditional methods of isolation have proven inadequate. In order to obtain large quantities of analytically pure glycoproteins for commercial applications and research, alternative strategies are required. Unlike AFPs which have been readily cloned with yeast expression vectors, cloning of AFGPs has not been accomplished due to the incompatibility of the carbohydrate functionality with established cloning techniques. Laboratory synthesis of antifreeze glycoproteins is an attractive alternative. Synthetic approaches allow for the construction of AFGPs and AFGP analogues for structure-activity relationship (SAR) studies.

The most common antifreeze target for chemical synthesis is AFGP8. The relative simplicity of AFGP8 makes it amenable to synthetic approaches and as a result, analogues for SAR investigations are predicated upon this glycoprotein.

The first reported synthesis of an AFGP analogue was Anderson’s tripeptide glycoprotein. This synthesis employed a convergent approach consisting of separate construction of the disaccharide and peptide components (Scheme 2.1.1). In order to achieve this, benzoyl protected galactose bromide 1 was coupled with a benzyl protected galactose derivative 2, via AgOTf to afford the core disaccharide 3. This was followed by conversion of the anomeric substituent to the corresponding chloride 4, and AgOTf mediated coupling with orthogonally protected tripeptide 5. The coupling was achieved
in 60 % yield. Finally, removal of protecting groups with Pd/C and H₂ afforded O-linked AFGP analogue 6.

Scheme 2.1.1 Anderson’s convergent synthesis of O-linked AFGP.

Drawbacks inherent to Anderson’s convergent approach are poor diastereoselectivity during coupling (resulting in α/β mixtures), low reactivity of the threonine hydroxyl and poor solubility of the peptide chain under glycosylation conditions.⁵,⁶ The stepwise assembly of glycosylated amino acids in a linear fashion is an alternative strategy which can overcome these limitations. Linear approaches to
AFGP synthesis include Filira's continuous flow solid phase synthesis (SPS) of β-linked galactose. In contrast to AFGP8 which possess an α-glycosidic linkage and a disaccharide, Filira's synthesis incorporates a β-glycosidic linkage and a galactose residue. This modification was performed in order to mimic the terminal galactose moiety of native AFGP8.

Scheme 2.1.2 Filira's linear solid phase synthesis strategy.

Filira’s approach (Scheme 2.1.2) involved treatment of commercially available D-galactose pentaacetate 7 with selectively protected L-threonine 8 in the presence of BF$_3$·OEt$_2$, followed by deprotection of the amino acid moiety to afford 9. N-terminus reprotetion with base labile Fmoc succinimide furnished the building block 10. Stepwise addition of the amino acids was then accomplished on a Pepsyn KA solid-phase
resin using continuous flow solid-phase peptide synthesis, followed by TFA mediated cleavage from the resin and acetyl deprotection to afford the glycoprotein 11.

The first synthesis of native O-linked AFGPs was accomplished by Nishimura and Tsuda\(^8\) (Scheme 2.1.3). The disaccharide moiety was synthesized by coupling a galactosyl trichloroacetimidate donor 12 with a glycosyl fluoride acceptor 13 in the presence of TMSOTf. The product, 14, was then coupled with orthogonally protected tripeptide 15 via cyclopentadienylzirconium dichloride and silver perchlorate to afford 16 in 64 % yield. Reduction of the azide with \(\text{NiCl}_2\) and \(\text{NaBH}_4\), followed by acetylation with \(\text{Ac}_2\text{O}\) furnished the protected disaccharide moiety. Deprotection of the carbohydrate and peptide functionalities afforded the AFGP monomer. Finally, diphenylphosphorylazide (DPPA) promoted polymerization of the unprotected monomer afforded a mixture of glycopeptides (17) in 69 % yield. The low yields of the coupling step and subsequent polymerization, in addition to the generation of a mixture of glycopeptides (with \(n= 10, 11\) and 12) requiring laborious separation make this approach inexpedient.
Scheme 2.1.3 Nishimura’s first synthesis of native AFGP.

Chen and colleagues\(^9\) reported the second synthesis of native \(O\)-linked AFGP via an Fmoc based solid phase peptide synthesis approach (Scheme 2.1.4). An important feature of this synthesis is the ability to control the length of the polymer chain using SPS. Reaction of selenyl glycoside 18 with selectively protected L-threonine afforded a monoglycoside. Treatment of the monoglycoside with thiourea to liberate the C3-hydroxyl for subsequent coupling afforded 19 in 83 % yield. Coupling of the glycoconjugate with thioglycoside 20 in the presence of an electrophilic iodine promoter (NIS) furnished the disaccharide 21 in 93 % yield. Deprotection of the C-terminus and concomitant reduction of the azide, followed by acetylation afforded Gal-NHAc.
disaccharide building block 22. SPPS followed by cleavage from the resin and NaOMe
deprotection yielded 23 with 4 and 8 repeating units (Scheme 2.1.4.).

An advantage of Chen’s solid-phase approach is direct control over the amino
acid composition, allowing for the construction of a polypeptide with the desired amino
acid sequence. As a consequence, the cumbersome separation of a complex mixture of
glycoproteins required by Nishimura’s approach is avoided.
Scheme 2.1.4 Chen’s SPPS approach to native AFGPs.
2.2 C-Linked AFGP Analogues

The l-threonine-disaccharide motif exhibited by native AFGPs is an example of an “O-linkage.” Native O-linked glycoproteins are unstable due to the sensitivity of the glycosidic linkage to enzymes, acids and bases. Hydrolysis in acidic media results in the formation of an oxocarbenium cation, while cleavage of the glycosidic bond via β-elimination occurs in basic media (Figure 2.2.1).

**Figure 2.2.1** Hydrolysis of the labile glycosidic linkage in acidic media yielding the oxocarbenium cation, and cleavage in basic media via β-elimination.

The instability of O-linked glycopeptides and the challenges in synthesizing these compounds have led to the development of less labile C-linked analogues. C-glycoproteins are less prone to enzymatic cleavage and are stable to acidic and basic
conditions. Furthermore, these variants are important for structure-function elucidation and drug discovery. Synthesis of these C-linked compounds is predicated upon common synthetic strategies and numerous syntheses have been reported.\textsuperscript{10,11,12}

Despite the significant change in electronics upon replacement of an oxygen atom with a methylene group, C-linked glycopeptides exhibit similar conformation to their O-linked counterparts.\textsuperscript{13,14,15} Kishi\textsuperscript{16} has shown that C-glycoproteins adopt similar solution conformation as their O-linked counterparts. These results have been corroborated by receptor-ligand binding studies. X-Ray crystallographic analysis of C-lactose bound to peanut lectin reveals “an almost perfect exo-anomeric conformation” of the saccharide, and an overall conformation which is “virtually identical to its parent O-lactose.”\textsuperscript{17}

The conformational similarity of C-linked glycopeptides with their O-linked counterparts and the added stability of C-glycoproteins make them ideal synthetic targets for structure-function studies and drug discovery. Work in our laboratory is focused on the exploration of C-glycopeptides as biological antifreezes.

\textbf{2.3 C-Linked AFGP Analogues in the Ben Laboratory}

For the purpose of our analyses, antifreeze glycoproteins can be reduced to three variable regions, the carbohydrate moiety, the linker region and the amino acid side chain (Figure 2.3.1). Work in our laboratory involves manipulation of these three regions to produce analogous compounds with custom tailored RI activity and no TH activity.
Figure 2.3.1 Core components of AFGPs. X= O in native systems, and X= CH$_2$ in C-linked analogues.

The main differences between native AFGPs and our first generation analogues are substitution of d-galactose for the disaccharide, l-lysine for l-threonine and the replacement of l-alanine in the peptide backbone with l-glycine (Figure 2.3.2).

Figure 2.3.2 i) Native AFGP8 and ii) First Generation C-Linked AFGP Analogues.
Substitution of the disaccharide moiety with galactose was performed to simplify the synthesis. Early SAR work by Feeney indicates that the terminal galactose residue is essential for antifreeze activity, and later studies by Nishimura reveal that antifreeze activity is retained upon substitution of the β-D-galactosyl-(1→3)-α-N-acetyl-D-galactosamine disaccharide subunit with a monosaccharide. The replacement of L-lysine for L-threonine was predicated upon the fact that AFGPs from Arctic and North Atlantic cod possess L-arginine in place of L-threonine. The amide bond in our analogues mimic the guanidine functionality in L-arginine based AFGPs. Finally, the amino acid composition of the peptide backbone was altered from L-alanine to L-glycine to avoid possible racemization during amino acid coupling sequences. No racemization has been reported from the use of solid phase peptide synthesis in our lab.

Polypeptides possessing tripeptide repeats of 3, 6 and 9 amino acid units were among the first generation of C-glycopeptides synthesized. 3-mer polymers (n=3) were found to be devoid of RI activity (Graph 2.3, pg. 46) In contrast, longer biopolymers, 6 and 9-mers were found to exhibit weak RI activity. The lack of activity observed in 3-mers, coupled with the fact that native AFGP8 possess 4 repeating units, led to the hypothesis that the minimum length for activity may be 4 repeating units.

The synthesis of AFGP analogue was accomplished by Ben and Enaide via a CDI mediated coupling of the carboxylic acid with the protected tripeptide to yield building block. Deprotection with Pd/C and H₂ followed by SPPS furnished biopolymer. TFA cleavage from the Wang resin followed by NaOMe deprotection afforded the 3-mer, the 6-mer and the 9-mer (Scheme 2.3.1).
Scheme 2.3.1 Synthesis of our first generation C-linked AFGP analogues.

Second generation analogues were constructed with the same features of first generation AFGP analogues; however, the polypeptide core contained 4 repeating units (Figure 2.3.3). 4-mers, \([\text{LGG}]_4[\text{Gal}]\) analogue 29, and \([\text{LAA}]_4[\text{Gal}]\) analogue 30 did not exhibit measurable antifreeze activity. In contrast, when a methylene unit was removed from the linker region, analogue 31 exhibited significant recrystallization inhibition activity (Graph 2.3, pg. 46). Replacement of \(\text{L-lysine}\) with \(\text{L-ornithine}\) in 31 indicates that there may be a correlation between the length of the linker and recrystallization inhibition activity.
Recently, third generation analogues have been synthesized in our lab with interesting results (Figure 2.3.4). These analogues possess an all carbon linker (no amide bond) between the sugar and the polypeptide backbone. C-serine analogue 34 exhibited greater recrystallization inhibition (RI) activity than our [OGG]Gal benchmark 31 (Graph 2.3, pg. 46). Furthermore, 34 displays comparable RI activity to AFGP8. Curiously, analogues 32 and 33 exhibited no RI activity. The lack of recrystallization inhibition activity in 32 and 33 may be due solely to the length of the linker, or may be due to enhanced flexibility of the longer side chains in 32 and 33. Further study on the linker is required to establish the importance of rigidity in this region.
Figure 2.3.4 Third generation C-linked AFGP analogues.

Graph 2.3 RI activity of AFGP analogues measured by mean largest grain size.
2.4 Goals and Objectives

Results from our second generation analogues suggest that there may be a specific linker configuration for optimal RI activity, while third generation analogues suggest that an all carbon linker is a suitable replacement for the O-linkage in native AFGP8.

An important question which has yet to be answered is the role of the amide bond in our [OGG]₄[Gal] analogue 31. Since our third generation analogues indicate that activity can be retained with a linker consisting strictly of methylene units, the possibility of an all carbon ornithine analogue intrigued us.

In order to elucidate the function of the amide bond in 31, we proposed the synthesis of 35, an [OGG]₄[Gal] analogue possessing an internal olefin (Figure 2.4.1). This amide isostere would help to uncover the role of the amide moiety in the RI activity of [OGG]₄[Gal]. An isostere is a mimic of a parallel functional group/compound. The amide bond in 31 exhibits partial double bond character (trans) and thus, an analogue with an internal trans-double bond would serve as a mimic. Observation of RI activity in 35 would indicate whether the antifreeze activity expressed by 31 is due to the rigidity imparted by the amide bond, or perhaps due to some other phenomenon. The partial double bond character of the amide in 31 may limit the extent of free rotation of the carbohydrate moiety and consequently stabilize it in certain orientations, maximizing carbohydrate-ice interactions. Another possible explanation for the function of the amide bond in 31 is hydrogen bonding directly with the ice lattice itself, or perhaps with the sugar hydroxyls.
Figure 2.4.1 C-linked AFGP analogue containing an all carbon backbone and an internal olefin as an amide isostere.

In order to discern the role of the amide bond in the recrystallization inhibition activity of 31, we decided to synthesize 35 according to Scheme 2.4.1. Our strategy consists of the solid phase peptide synthesis of the N-terminus protected building block 36. We hypothesized that the building block could be obtained from the ring opening and subsequent Corey-Schmidt oxidation of 37. We envisioned synthesis of glycoconjugate 37 via a Wittig reaction of the benzyl protected phosphonium salt of α-galactose 38 with chiral aldehyde 39. The chiral aldehyde was envisaged via coupling of a 2 carbon linker 40 with chiral oxazolidin-2-one 41. The chiral reagent can be obtained from l-serine via the approach described by Sibi and Renhowe.24
Scheme 2.4.1 Retrosynthetic analysis of AFGP analogue 35.
References:


Chapter 3

Preparation of C-Linked AFGPs

Figure 3.1 Structure of C-linked [OGG]$_4$[Gal] olefin monomer.

An important structural feature of our building block is the presence of an internal trans-double bond. The significance of the double bond has been highlighted in the previous section. This amide isostere can be incorporated using a number of synthetic strategies such as the Peterson Reaction, the Julia Olefination, Olefin Cross Metathesis and the Wittig Reaction. Synthesis of 1 via the Peterson Reaction would require a preassembled glycoconjugate tethered to a β-hydroxysilane. There is no readily accessible route to such a compound. Furthermore, removal of sugar protecting groups such as benzyl ethers by reducing metals employed in a typical Julia Olefination make this approach unfavourable. Finally, problems associated with cross-product selectivity, including the presence of homo-coupled products make olefin cross metathesis a poor choice towards the synthesis of 1.
Our original approach towards the synthesis of 1 was based upon Bertozzi and Bednarski’s synthesis of a C-glycosyl analog of β-Galactose-O-Serine 2. This strategy employed the Wittig reaction of benzyl protected d-galactose aldehyde 4 and a phosphonium salt derived from l-serine 5. The β-stereochemistry at the anomeric center of 2 is due to the aldehyde, while the l-stereochemistry of the α-amino acid component of 2 arises from the Wittig reagent. The key step involved coupling of aldehyde 4 with the ylide generated from phosphonium salt 5 to afford 3 in 34 % yield, and a 15:1 trans/cis mixture of olefins. Reduction of the double bond, followed by ring opening, Fmoc protection and Jones oxidation furnished 2 (49 % from 3). Considering the structural similarity of our target with glycoconjugate 2, we decided to adopt this approach.

Scheme 3.1.1 Bertozzi and Bednarski’s approach to C-glycosyl analog of β-Gal-O-Ser 2.

The retrosynthesis of our approach to 1 is described in Scheme 3.1.2 and is based upon a Wittig reaction of aldehyde 7 with the ylide generated from phosphonium salt 8.
3.2 Preparation of Carbohydrate Coupling Partner 7

The requisite aldehyde was synthesized according to previously established protocols in our lab.\textsuperscript{13} This involved bromination of commercially available \(\beta\)-\(\alpha\)-galactose pentacetate 9 with HBr in acetic acid. The resulting \(\alpha\)-bromo-pyranose 10 was obtained in 98 % yield (\textbf{Scheme 3.2.1 i}). The \(\alpha\)-stereochemistry of the bromo sugar arises from nucleophilic attack of the bromide anion on the oxocarbenium intermediate. Although both epimers are possible, the \(\alpha\)-product is preferred\textsuperscript{14, 15} Overlap of the antiperiplanar oxygen lone pair with the \(\sigma^*\text{C-Br}\) orbital stabilizes the \(\alpha\)-anomer. (\textbf{Scheme 3.2.1 ii}).

\begin{itemize}
  \item[i] Bromination of \(\beta\)-\(\alpha\)-galactose pentacetate 9 to afford \(\alpha\)-\(\beta\)-bromo galactose tetratacetate 10.
  \item[ii] Preference of the \(\alpha\)-anomer due to the anomeric effect.\textsuperscript{14, 15}
\end{itemize}
α-Allyl-D-galactose tetracetate 11 was generated from the reaction of allyl reagents with anomeric glycosyl radical precursors. The labile C-Br bond is ideal for homolytic cleavage, resulting in the generation of stable anomeric radicals. According to the protocol established by Magnusson, photochemical mediated allylation of α-bromo-D-galactose tetracetate was achieved with stoichiometric quantities of bis-tributyl tin and phenyl allyl sulfone to afford exclusively α-allyl-D-galactose tetracetate in 88 % yield (Scheme 3.2.2).

![Scheme 3.2.2 Allylation of α-bromo-galactose tetracetate](image)

Deprotection of the monosaccharide with NaOMe in methanol was achieved cleanly and quantitatively to afford 12. Treatment of 12 with NaH, followed by addition of BnBr via cannula furnished benzyl protected allyl sugar 13 in 85 % yield. The choice of benzyl protecting groups for the carbohydrate component was predicated upon the incompatibility of acetate protecting groups with standard Wittig conditions.
**Scheme 3.2.3** Deprotection and re-protection to furnish 13.

Benzyl protected α-C-allyl-α-galactose was subject to ozonolysis in dichloromethane followed by reductive workup with triphenylphosphine. However, the reaction did not proceed cleanly (Scheme 3.2.4). In addition to the desired aldehyde, a mixture of unidentifiable aldehydes were also produced, ostensibly due to overoxidation. Integration of the $^1$H NMR (9-10 ppm region) indicated the presence of numerous aldehydes. Attempts at separation and purification via flash chromatography and glass backed analytical TLC proved fruitless.

**Scheme 3.2.4** Ozonolysis of olefin 13

A possible explanation for the formation of byproducts in such reactions has been proposed by Defaye and Uillle. They reported the mild deprotection of benzyl ethers in the presence of ozone. Cleavage of benzyl ethers via ozonolysis was observed for benzyl protected methyl α-glucopyranosides, yielding the corresponding alcohols.
Defaye and Utille suggested that debenzylation involves insertion of ozone into the methylene bond of benzyl ethers, followed by decomposition and the formation of alcohol/polyol species (Scheme 3.2.5 i).

An alternate explanation may involve anomeric epimerization. Zou and coworkers reported the epimerization of 2'-carbonyl-α-C-glycopyranosides (including 7) to their corresponding β-anomers\(^{19}\) via treatment with 4 % NaOMe. Epimerization occurs via an acyclic α,β-unsaturated aldehyde formed from enolization of the sugar and subsequent β-elimination, followed by ring closure through an intramolecular 1,4-addition (Scheme 3.2.5 ii). Scala and Byram have reported similar results.\(^{20,21}\)

Scheme 3.2.5 i) Cleavage of benzyl ether protecting groups by ozone and ii) epimerization of 2-(2,3,4,6-Tetra-O-benzyl-α-D-galactopyranosyl)-ethyl aldehyde with 4 % NaOMe.
In order to overcome the problem of over oxidation during ozonolysis we decided to use the azo-dye Sudan Red 7B (Figure 3.2) as an indicator. The colourimetric change of a Sudan Red-aldehyde solution is indicative of excess ozone and therefore completion of ozonolysis. Using the indicator Sudan Red 7B, aldehyde 7 was obtained cleanly in near quantitative yield (Scheme 3.2.6).

Scheme 3.2.6 Ozonolysis in the presence of Sudan Red 7B.
3.3 Preparation of Amino Acid Coupling Partner 8

The next step in our synthesis of building block 1 was construction of the amino acid moiety 8. We envisioned 8 via Wittig reaction of silyl protected aldehyde 15 and the ylide generated from chiral phosphonium salt 16. The chiral phosphonium salt is accessible from commercially available l-serine 17.

Scheme 3.3.1 Retrosynthesis of amino acid component.

The synthesis of 16 has been previously described by Sibi and Renhowe. The key step in the synthesis of this Wittig reagent was oxazolidinone ring formation from 17. In order to achieve this, Sibi and Renhowe combined l-serine methyl ester hydrochloride with phosgene (20% solution in toluene) in the presence of aqueous potassium carbonate. We attempted ring closure using alternatives to phosgene. The results are summarized in Table 3.3.
Our first attempts at ring closure with Et₂CO as a phosgene substitute proved fruitless (Entries 1 and 2). Only L-serine methyl ester hydrochloride was recovered. This was followed by attempts with solid (CCl₃)₂CO (triphosgene) (Entry 3), but this also failed to produce acceptable results. Similarly, adjusting the solvent system to a biphasic aqueous/THF system, or an aqueous/THF/Toluene system was also unsuccessful (Entry 4 and 5). We serendipitously discovered an alternate protocol involving the free amino acid published by Falb and Nudelman.²² This procedure involved treatment of L-serine in NaOH with triphosgene dissolved in dioxane, followed by stirring overnight in methanol. This was successful, furnishing 18 as yellow oil in 83 % yield. The product was identical

---

**Table 3.3** Conditions for oxazolidinone ring formation.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>R'</th>
<th>Conditions</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CO₂Me</td>
<td>NH₂HCl</td>
<td>Et₂CO, aq K₂CO₃, 0°C, 3 hrs</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>CO₂Me</td>
<td>NH₂HCl</td>
<td>Et₂CO, aq K₂CO₃, 0°C/140°C, 3 hrs</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>CO₂Me</td>
<td>NH₂HCl</td>
<td>(CCl₃)₂CO, aq K₂CO₃, 0°C, 3 hrs</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>CO₂Me</td>
<td>NH₂HCl</td>
<td>(CCl₃)₂CO, aq K₂CO₃, THF, 0°C, 3 hrs</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>CO₂Me</td>
<td>NH₂HCl</td>
<td>(CCl₃)₂CO, aq K₂CO₃, THF/Tol. 0°C, 3 hrs</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>CO₂⁻</td>
<td>NH₃⁺</td>
<td>(CCl₃)₂CO in dioxane, 1M NaOH, 0°C, 3/overnight stirring in MeOH</td>
<td>83</td>
</tr>
</tbody>
</table>

---

²² This procedure involved treatment of L-serine in NaOH with triphosgene dissolved in dioxane, followed by stirring overnight in methanol. This was successful, furnishing 18 as yellow oil in 83 % yield. The product was identical.
(\textsuperscript{1}H NMR, \textsuperscript{13}C NMR, mass spectrum, IR, and boiling point) to previous reports by Sibi and Renhowe.

**Scheme 3.3.2** Synthesis of chiral phosphonium salt 16.

Reduction of the ester with NaBH\textsubscript{4} in ethanol followed by tosylation of the primary alcohol furnished 20. Finklestein reaction of the tosylate with NaI in refluxing acetone yielded iodo auxiliary 21. Displacement of the iodine moiety in an S\textsubscript{N}\textsuperscript{2} fashion with excess triphenylphosphine afforded Wittig reagent 16. The phosphonium salt was found to be hygroscopic and was consequently dried in a Kugelrohr and stored under Ar (g) in a dessicator.
Next, we attempted the synthesis of a 2-carbon unit as described by Pirrung and Webste\textsuperscript{23} (Scheme 3.3.3). Coupling of 16 directly with the aldehyde 7 was not feasible since the product of such a coupling would lack two methylene units in the linker region and therefore would be structurally dissimilar to amide \([\text{OGG}_4\text{Gal}].\) In addition, there is no convenient route directly to 8 or to a synthetic equivalent of 8. As a result, TBDMS protection of commercially available but-3-en-1-ol furnished the protected olefin 23 in 95\% yield. Ozonolysis of the olefin, followed by reductive workup with dimethyl sulfide afforded the protected aldehyde 24 as a colourless oil. The aldehyde was found to be unstable and was stored under an inert atmosphere at \(-20\,^\circ\text{C}.\)

![Scheme 3.3.3 Synthetic scheme for the synthesis 24.](image)

\textbf{Scheme 3.3.3} Synthetic scheme for the synthesis 24.
**Scheme 3.3.4** Wittig reaction of 16 and 24 to furnish 25 as a mixture of E/Z olefins.

Wittig reaction of 16 and 24 proceeded smoothly to afford 25 as a 4:1 mixture of E/Z olefins in 88% yield. Coupling required treatment of the salt with 1.95 equivalents of base. This was performed to ensure double deprotonation of 16, since the pKa of the amide proton (NH) is approximately 20.6, while the pKa of the α-methylene protons (RCH₂PPh₃) are approximately 21. Addition of a single equivalent of base would result in competitive deprotonation of NH versus CH₂, resulting in a dramatic reduction in yield. In addition, Sibi and Renhowe have theorized that treatment of the salt with a single equivalent of base results in loss of chirality via β-elimination. Bis-deprotonation with 2 equivalents of base precludes β-elimination and therefore prevents racemization (**Scheme 3.3.5**).
Scheme 3.3.5 Comparison between the use of one and two equivalents of base in the deprotonation of phosphonium salt 16.

Removal of the silyl protecting group with TBAF furnished the free alcohol 26. This was followed by catalytic reduction of the double bond with Pd/C and H$_2$ (g) to afford 27. We hypothesized that Garreg reaction of 27 would yield iodo auxiliary 28 and convenient access to phosphonium salt 29. However, this was not the case. Instead, starting material (27) was recovered in addition to an unexpected polycyclic product. Attempts at tosylation and mesylation of the primary alcohol also proved unsuccessful (Scheme 3.3.6).
Scheme 3.3.6 Attempts at derivatization of 27 with TsCl, I$_2$ and MsCl.

We speculated that the unhindered and slightly nucleophilic amide nitrogen in 27 may be responsible for the absence of product. We hypothesized that formation of a 6-membered intramolecular ring, followed by ring opening upon aqueous workup was responsible for the regeneration of starting material and the polycyclic product (Scheme 3.3.7).

Scheme 3.3.7 Possible mechanism for the regeneration of 27.
We tested this hypothesis by protecting the amide with di-tert-butyl dicarbonate to afford t-BOC derivative \(30\). This was followed by TBAF mediated silyl deprotection to yield \(31\) and reduction with Pd/C and \(H_2\) (g) to furnish alcohol \(32\). We attempted the Garreg reaction again, but this time with the amide nitrogen protected as a tert-butoxy carbonyl functionality. The reaction proceeded smoothly, yielding \(33\) and supporting our assertion of amide nitrogen involvement. Stirring the primary iodide overnight with excess triphenylphosphine in DMF afforded the second coupling partner, phosphonium salt \(8\).

\[
\begin{align*}
25 & \quad \text{N\textsubscript{Boc}} \\
\text{(BOC}_2\text{)O} & \quad \text{DMAP, } \text{Et}_3\text{N} \\
\text{THF} & \quad 92\% \\
\text{ } & \quad \text{THF} \\
\text{TBAF} & \quad 84\% \\
\text{77\%} & \quad \text{H}_2, \text{Pd/C} \\
\text{MeOH} & \quad 93\% \\
\text{I}_2, \text{PPh}_3, \text{Imidazole, THF} & \quad 85\% \\
\text{8} & \quad \text{93\%} \\
\text{33} & \quad \text{85\%} \\
\text{32} & \quad \text{OH}
\end{align*}
\]

\textbf{Scheme 3.3.8} Generation of the amino acid coupling partner \(8\).
3.4 Coupling of Carbohydrate and Amino Acid Components

With coupling partners 7 and 8 available, we sought to couple the two components using a Wittig reaction (Scheme 3.4). Unfortunately, this proved unsuccessful. Initial attempts were made with nBuLi and the more hindered tertiary anion, tBuLi. In both instances phosphonium salt 8 and aldehyde 7 were recovered quantitatively. In addition, the absence of any triphenylphosphine oxide byproduct indicated that condensation failed to occur. It was unclear precisely why the coupling did not proceed. Possible explanations for the lack of product were speculated to be either; 1) failure to generate the ylide (although unlikely, especially in the presence of strong BuLi bases), or 2) failure to generate the betaine intermediate/oxaphosphetane ring, due to steric hindrance from the bulky benzyl protected aldehyde.

Scheme 3.4 Wittig reaction of aldehyde 7 and salt 8.
We performed a series of test reactions with a well known aldehyde to determine why the reaction failed. Phosphonium salt 8 was treated with a variety of bases and quenched with benzaldehyde. The results are summarized in Table 3.4. Attempts at ylide generation with nBuLi (Entry 1), followed by quench with benzaldehyde proved unsuccessful. The reaction was then performed under the same conditions, but with 1,10-phenanthroline (Entry 2). Phenanthroline is an indicator often used in the titration of alkyl lithiums (Figure 3.4). The presence of a deep red colour in solutions containing 1,10-phenanthroline is indicative of excess base. We added the indicator to a solution of 8 in THF and added sufficient quantities of nBuLi to impart a visible red hue. The solution was stirred for 1 hour at -78 °C and quenched again with benzaldehyde. No product was detected, and only starting materials were recovered. Attempts with tBuLi (Entry 3), NaH (Entry 4), LDA (Entry 5) and LiHMDS (Entry 6) also proved unsuccessful. In all cases, starting material 8 and benzaldehyde accounted for the mass balance. These results indicated that the problem lay with the phosphonium salt.
1. Base, -78 °C 1 hr
2. Benzaldehyde, -78 °C 2 hr

<table>
<thead>
<tr>
<th>Entry</th>
<th>Base</th>
<th>Yield of 34 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>nBuLi</td>
<td>0*</td>
</tr>
<tr>
<td>2</td>
<td>nBuLi with 1, 10-Phenanthroline</td>
<td>0*</td>
</tr>
<tr>
<td>3</td>
<td>tBuLi</td>
<td>0*</td>
</tr>
<tr>
<td>4</td>
<td>NaH</td>
<td>0*</td>
</tr>
<tr>
<td>5</td>
<td>LDA</td>
<td>0*</td>
</tr>
<tr>
<td>6</td>
<td>LiHMDS</td>
<td>0*</td>
</tr>
</tbody>
</table>

*Starting material recovered.

Table 3.4 Test reactions of 8 with Benzaldehyde.

3.5 Revised Approach

Our failure to generate 6, and our inability to couple 8 with benzaldehyde prompted a change in strategy. It was evident that the difficulty in coupling the two components was due to the amino acid coupling partner. Consequently, we decided to ‘switch’ coupling partners by converting 8 into its corresponding aldehyde 35, and 7 into its corresponding phosphonium salt 36 (Scheme 3.5.1). This approach was based on the strategy elucidated by Kihlberg and colleagues.25
Before implementing the revised approach, we decided to explore the reactivity of 35 to Wittig olefination. Therefore, we attempted oxidation of 31 to its corresponding aldehyde. Swern oxidation cleanly furnished coupling partner 35 in over 90% yield (Scheme 3.5.2).

Next, we tried coupling 35 with methyltriphenylphosphonium iodide, 37. Methyltriphenylphosphonium iodide was readily precipitated from a stirred solution of CH₃I and PPh₃, dried in Kugelrohr and stored under argon in a dessicator until ready for
use. Coupling of 37 and the aldehyde with nBuLi was successful, furnishing the monosubstituted olefin 38 in 61% yield. 

![Chemical structure](image)

**Scheme 3.5.3** Test coupling of aldehyde 35 with methyltriphenylphosphonium iodide.

Encouraged by these results, we sought convenient access to phosphonium salt 36. With 13 already available, we surmised that the synthetic handle on 13 could be exploited for easy access to the salt. In fact, the precursor to 36, benzyl protected α-iodoethyl C-galactose (40), has already been reported from 13 by Nolen and coworkers. Ozonolysis of 13 was performed again, but this time followed by reductive workup with NaBH₄ to furnish 39 cleanly and in excellent yield. This was followed by Garreg reaction with PPh₃, I₂ and imidazole according to the procedure described by Nolen to afford iodo sugar 40 in 86% yield. However, α-iodoethyl C-glycosides are prone to forming bicyclic structures with concomitant loss of the C2 O-benzyl group upon storage for extended periods of time. Therefore, we moved quickly, stirring the iodide in excess PPh₃ overnight to furnish phosphonium salt 36 in quantitative yield.
Scheme 3.5.4 Generation of Wittig reagent 36.

Next, we attempted coupling monosaccharide 36 with amino acid 35 under the same conditions as our previous attempts. This time coupling was successful, yielding 41 as a 1:4 mixture of E:Z isomers in 38 % yield. In addition, we obtained the ring opened product 42 in 15 % yield. The in situ ring opening of 41 was unexpected.

Scheme 3.5.5 Coupling of phosphonium salt 36 with aldehyde 37.
Although the coupling was successful, our initial target was the trans diastereomer. Attempts to photochemically isomerize the olefin with I$_2$ in chloroform failed. Photochemical isomerization with Ph$_2$S$_2$ based upon established protocols was also attempted and proved unsuccessful.\textsuperscript{27} In both cases, decomposition of the starting material was observed upon exposure to a 450 W Hg lamp.

\begin{align*}
\text{Scheme 3.5.6} & \text{ Attempts at photochemical isomerization of 41.} \\
& \text{Although the cis diastereomer was not what we originally set out to produce, we hypothesized that a cis double bond in the linker region would still allow for assessment of the amide functionality on AFGP activity.} \\
& \text{Oxazolidinone ring opening with CsCO}_3 \text{ in MeOH afforded 42 in 87 % yield. Numerous approaches were considered for the oxidation of 42 to its corresponding carboxylic acid. Popular single step techniques include chromium based strategies such}
\end{align*}
as the Jones Oxidation\textsuperscript{28} and Corey Schmidt Oxidation\textsuperscript{29}. Corey-Schmidt Oxidation with PDC in DMF furnished building block 43 in 78\% yield.

\begin{center}
\begin{tikzpicture}
    \node (41) at (0,0) {\includegraphics[width=0.9\textwidth]{s3_5_7.png}};
    \node at (0,-2) {41};
    \node at (2,-2) {42};
    \node at (4,-2) {43};
    \node at (0,-3) {CsCO\_3}
    \node at (0,-3.3) {MeOH}
    \node at (0,-3.6) {87\%}
    \node at (2,-3) {PDC}
    \node at (2,-3.3) {DMF}
    \node at (2,-3.6) {78\%};
\end{tikzpicture}
\end{center}

\textbf{Scheme 3.5.7} Deprotection and oxidation of glyconjugate 41 to afford building block 43.
3.6 Conclusion

Although we were unable to synthesize exclusively the trans \([OGG]_4[Gal]\) building block we originally planned, we were able to obtain the Z-diastereomer by modifying the coupling partners. A *cis*-double bond in the linker region still allows for assessment of amide rigidity on AFGP activity.

![Figure 3.6](image)

**Figure 3.6** Original *trans* target (left), and *cis* isomer actually obtained (right).

3.7 Future work

Solid Phase Peptide Synthesis (SPPS) of the building block according to the approach elucidated by Merrifield\(^{30}\) is the next step (**Scheme 3.7.1**). SPPS involves immobilization of the substrate on an insoluble, yet porous support, and stepwise assembly of the peptide. Classical techniques for peptide synthesis (i.e. solution phase) are often labour, time and skill intensive. Furthermore, solution phase techniques suffer from low yields due to the difficulties involved in separating and purifying often poorly
soluble intermediates. In contrast, SPPS is high yielding (individual coupling and
deprotection steps are virtually quantitative), allows for easy purification (requires simply
washing off excess reagents, since the peptide is immobilized) and is amenable to
automation.

Scheme 3.7.1 SPPS of building block 43.

Debenzylation of polymer 44 can be accomplished with BCl₃, followed by
reverse phase HPLC purification to furnish 45, [OGG]₄[Gal] analogue with an internal
double bond (Scheme 3.7.2).
Scheme 3.7.2 Debenzylation of benzyl protected AFGP analogue with an internal double bond 44.

Assessment of the analogue for thermal hysteresis (TH) activity and dynamic ice shaping (DIS) can be conducted with nanolitre osmometry. Recrystallization inhibition (RI) activity can be assessed via the “splat assay” described by Knight et al. and the secondary structure can be determined by circular dichroism (CD) spectroscopy.

Finally, the analogue can be evaluated for cytotoxicity and cryotoxicity by in vitro testing in a human embryonic cell line using the MTT assay.
References:


Chapter 4

Experimental: Materials and Methods
4.1 General

Dichloromethane, acetonitrile and triethylamine were distilled over calcium hydride prior to use. Benzene, ether and tetrahydrofuran (THF) were distilled over sodium-benzophenone before use. N,N-dimethylformamide (DMF), methanol and acetone were dried with activated molecular sieves (4 Å) overnight prior to use. Solution phase reactions were conducted under an inert atmosphere (N₂ (g) or Ar (g)), unless specified otherwise. Reactions were monitored by thin layer chromatography; visualized with U.V. light (254 nm), and/or i) aqueous ceric ammonium molybdate (CAM), or ii) aqueous potassium permanganate (KMnO₄) stains. Purification via flash chromatography was conducted with silica gel (Merck) of 240-400 mesh and columns fixed with fritted glass filters. Titration of organolithium reagents was conducted with commercially available N-benzylbenzamide in THF according to established protocols.¹ Ozonolysis was performed with a Yanco Industries ozonolysis apparatus.

¹H and ¹³C NMR analyses were performed with an INOVA 500 MHz spectrometer and Bruker Avance 400 and 500 MHz spectrometers. NMR experiments were performed mainly with deuterated chloroform. Deuterated methanol, water and benzene were also employed. Chemical shifts are reported in parts per million (ppm) and are referenced to the appropriate deuterated solvent. ¹H integration is denoted in parentheses and coupling constants are reported in Hz. ¹H signal splitting patterns are indicated by s-singlet, br-broad singlet, d-doublet, t-triplet, m-multiplet and dd-doublet of doublets. Infrared (IR) spectra were obtained from an FTIR-8400 Fourier Transform

Infra Red Spectrophotometer (Simadzu). Mass spectral analyses were conducted with an Electrospray mass spectrometer. All spectral analyses were performed at facilities located at the University of Ottawa.
4.2 Protocol for the Preparation of Carbohydrate Components

Preparation of 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide (10)

According to established protocols, a flame dried 250 ml round bottom flask with a \( \text{N}_2 \) (g) feed was charged with a stir bar and commercially available \( \beta \)-D-galactose pentacetate (10 g, 25.12 mmol). The flask was cooled in an ice bath and a solution of 33 % HBr in acetic acid (50 ml) was added. The ice bath was removed and the solution stirred for 2hrs. The solution was diluted with 150 ml of DCM and added to a separatory funnel. The organic layer was washed repeatedly with water until the pH of the aqueous layer was \( \approx 7 \). The organic layer was dried over MgSO\(_4\), filtered through Whatman paper and concentrated \textit{in vacuo} to yield a white paste. Coevaporation with toluene furnished a white solid. The solid was recrystallized from Et\(_2\)O to afford the title compound (10.29 g, 98 %).

\(^1\text{H NMR} \) (400 MHz, CDCl\(_3\)), \( \delta \): 6.69 (1H, d, \( J=4.0 \) Hz), 5.51 (1H, dd, \( J=1.2, 3.3 \) Hz), 5.40 (1H, dd, \( J=3.3, 10.6 \) Hz), 5.04 (1H, dd, \( J=4.0, 10.6 \) Hz), 4.48 (1H, t, \( J=6.7 \) Hz ), 4.18 (1H, dd, \( J=6.3, 11.4 \) Hz), 4.10 (1H, dd, \( J=6.8, 11.4 \)), 2.15 (3H, s), 2.11 (3H, s), 2.06 (3H, s), 2.01 (3H, s);

---


\[ \text{^13C NMR (400 MHz, CDCl}_3\), } \delta: 170.1, 169.8, 169.6, 169.5, 87.9, 70.8, 67.7, 67.5, 66.7, 60.6, 20.5, 20.4, 20.4, 20.3: \]

**MS (ESI, MeCN)** calcd. For C\(_{14}\)H\(_{15}\)BrO\(_9\), m/z: (M\(^+\)) 410.02, found 433.0 [(M+Na\(^+\)].

**Preparation of 2,3,4,6-tetra-O-acetyl-\(\alpha\)-1-allyl-\(\delta\)-galactopyranoside (11)**

![Chemical structure of 11](image)

Compound 10 (1.50 g, 3.41 mmol) was added to a flame dried flask equipped with an Ar (g) inlet and a stir bar according to established protocols\(^4\). The flask was charged with benzene and flushed for 30 minutes, followed by addition of allyl phenyl sulfone (1.5 mL, 8.0 mmol) and bis-tributyl tin (2.2 mL 4.4 mmol). The resulting solution was sonicated under Ar (g) for an additional 40 min. The Ar (g) feed and sonicator were removed and the flask sealed with parafilm. The flask was irradiated with a 450 W mercury lamp for \(\approx\) 18 hours and monitored by TLC. Upon completion, the reaction mixture was loaded directly onto a silica gel column and flushed with hexanes to remove excess bis-tributyl tin. The column was then eluted with 10:1, 8:1 and 6:1 hexanes: ethyl acetate until phenyl allyl sulfone started to elute. Upon elution of phenyl allyl sulfone, the column was subject to 3:1 hexanes: ethyl acetate until the title compound was

recovered. Removal of the solvent in vacuo afforded 11 as a crystalline solid (1.20 g, 88 %).

$^1$H NMR (400 MHz, CDCl$_3$), $\delta$: 5.70- 5.81 (1H, m), 5.42 (1H, dd, $J$=2.4, 0.7 Hz), 5.27 (1H, dd, $J$=5.0, 9.3 Hz), 5.21 (1H, dd, $J$=3.2, 9.3 Hz), 5.09- 5.16 (2H, m), 4.26-4.33 (1H, m), 4.20 (1H, dd, $J$ = 8.9, 12.8 Hz), 4.06-4.11 (2H, m), 2.41- 2.55 (1H, m), 2.21- 2.39 (1H, m), 2.12 (3H, s), 2.07 (3H, s), 2.04 (3H, s), 2.00 (3H, s);

$^{13}$C NMR (400 MHz, CDCl$_3$), $\delta$: 170.6, 170.1, 169.9, 169.8, 133.3, 117.6, 71.4, 68.3, 67.9, 67.6, 61.4, 30.9, 20.8, 20.7, 20.7, 20.6:

MS (ESI, MeCN) calcd. For C$_{17}$H$_{24}$O$_9$, m/z: (M$^+$) 374.12, found 411.1 [(M+K$^+$)].

**Preparation of α-1-allyl-d-galactose (12)**

2.0 g, 5.31 mmol of 11 was added to a solution of freshly prepared NaOMe (pH $\approx$ 10) and stirred overnight. The reaction was monitored by TLC, and upon completion treated with Amberlite IR 120(H) ion-exchange resin. Sufficient resin was added to ensure a pH of 7. The beads were filtered off with Whatman paper and the solution concentrated to yield α-1-allyl-d-galactose as a white solid in 99 % yield (1.10 g).
\[^1\text{H NMR}\] (500 MHz, MeOD), \(\delta\): 5.83- 5.94 (1H, m), 5.09- 5.15 (1H, m), 5.02- 5.06 (1H, m), 3.87- 4.03 (3H, m), 3.66- 3.89 (4H, m), 2.34- 2.50 (2H, m);

\[^{13}\text{C NMR}\] (500 MHz, MeOD), \(\delta\): 136.8, 116.9, 75.7, 74.2, 72.0, 70.2, 70.0, 63.0, 31.1;

\[\text{MS (ESI, MeOH) calcd. For } C_{9}H_{16}O_{5}, m/z: (M^+) 204.10, \text{ found 205.1 [(M + H\(^{+}\)]})];

\[\text{IR (MeOH) cm}^{-1}: 3323, 1074 \text{ and 1024.}\]

Preparation of 2,3,4,6-tetra-O-benzyI-\(\alpha\)-1-allyl-\(\beta\)-galactopyranoside (13)

A flame dried flask equipped with a \(N_2\) (g) feed and stir bar was charged with 400 mg, 2.0 mmol, of \(\alpha\)-1-allyl-\(\beta\)-galactose and 5 ml of dry DMF. 500 mg (5.0 mmol) of NaH and catalytic \(\text{tBuNH}_2 \text{+T}\) were quickly transferred to a second round bottom flask containing 5 mL of dry DMF and a stir bar. The \(\alpha\)-1-allyl-\(\beta\)-galactose solution (first flask) was transferred to the second flask via cannula. The flask was then submerged in an ice bath and BnBr (2.5g, 13.0 mmol) was added dropwise via syringe. Additional DMF was added to the flask to maintain a stirable slurry. The ice bath was removed and the slurry stirred overnight. The solution was diluted with water and extracted repeatedly with ethyl acetate (8x). The combined organic extracts were dried over MgSO\(_4\) and concentrated under vacuum. Flash chromatography (8:1, hexanes:ethyl acetate) yielded the title compound as a colourless oil (0.95g, 85%).
\(^1\text{H NMR}\) (400 MHz, CDCl\(_3\)), \(\delta\): 7.25-7.34 (20H, m), 5.70-5.81 (1H, m), 5.01-5.09 (2H, m), 4.47-4.72 (8H, m), 4.04-4.09 (1H, m), 3.98-4.02 (2H, m), 3.81-3.87 (2H, m), 3.69-3.73 (2H, m), 3.66 (1H, dd, \(J=\) 4.7, 10.6), 2.29-2.42 (2H, m);

\(^{13}\text{C NMR}\) (400 MHz, CDCl\(_3\)), \(\delta\): 138.5, 138.4, 138.2, 137.7, 135.1, 133.7, 128.3, 128.2, 127.9, 127.8, 127.7, 127.5, 127.4, 116.7, 76.4, 74.2, 73.1, 73.3, 73.0, 72.9, 72.5, 70.8, 70.7, 67.2, 32.2;

\textbf{MS} (ESI, MeCN) calcd. For C\(_{37}\)H\(_{40}\)O\(_5\), \(m/z\): (M\(^+\)) 564.29, found 587.3 [(M+Na\(^+\)];

\textbf{IR} (CH\(_2\)Cl\(_2\)) cm\(^{-1}\): 3050, 1501 and 1454.

\textbf{Preparation of 2,3,4,6-tetra-O-benzyl-\(\alpha\)-d-galactopyranosyl-ethyl aldehyde (7)}

1.0 g, 1.95 mmol, of 13 was added to a 25 mL round bottom flask containing freshly distilled DCM, \(\approx\) 1 mg of Sudan Red 7B and a stir bar. The solution was cooled to -78 °C in an acetone/dry ice bath and treated with ozone while stirring until the reddish colour disappeared \((\approx 20\text{ min})\). The solution was then flushed with nitrogen for 20 min and quenched with triphenylphosphine. The bath was removed and the solution was stirred overnight. Column chromatography (6:1 hexanes: ethyl acetate) furnished the aldehyde 7 as a colourless oil (0.96 g, 96%).
\(^1\)H NMR (400 MHz, CDCl\(_3\)), \(\delta\): 9.68 (1H, t, \(J=2.2\) Hz), 7.27 - 7.37 (18H, m), 7.20 - 7.24 (2H, m), 4.44 - 4.72 (9H, m), 3.98 - 4.07 (2H, m), 3.76 - 3.87 (2H, m), 3.62 - 3.72 (2H, m), 2.63 (2H, dd, \(J=2.2, 6.6\) Hz), 2.14 (3H, s), 2.11 (3H, s), 2.05 (3H, s), 2.00 (3H, s);

\(^{13}\)C NMR (400 MHz, CDCl\(_3\)), \(\delta\): 200.6, 138.3, 138.3, 138.2, 137.7, 128.4, 128.4, 128.3, 128.1, 127.9, 127.8, 127.7, 127.6, 127.5, 76.1, 73.8, 73.3, 73.3, 73.1, 73.0, 67.0, 42.9;

MS (ESI, MeCN) calcd. For C\(_{366}H_{380}O_6\), \(m/z\): (M\(^+\)) 566.27, found: 567.3 [(M+H\(^+\))];

IR (CH\(_2\)Cl\(_2\)) \(cm^{-1}\): 2671, 1741, 1503, and 1451.

**Preparation of 2,3,4,6-tetra-O-benzyl-\(\alpha\)-D-galactopyranosyl-ethanol (39)**

![Diagram of molecule 39]

50 mg, 0.117 mmol, of \(\text{13}\) was added to a 5 mL round bottom flask containing 1.0 ml of freshly distilled DCM, Sudan Red 7B (\(\approx\) 2 mg) and a stir bar. The solution was cooled to \(-78^\circ\)C in an acetone/dry ice bath and treated with ozone while stirring until the reddish hue disappeared (\(\approx\) 5 min). The solution was then flushed with nitrogen for 20 min and treated with 15 mg, 0.340 mmol, of NaBH\(_4\). The bath was removed and the solution was stirred for 2 hours. Column chromatography (3:1 hexanes: ethyl acetate) furnished the alcohol 39 as a colourless oil (0.47 g, 94%).
\textbf{\textsuperscript{1}H NMR} (400 MHz, CDC\textsubscript{3}), \(\delta\): 7.24-7.36 (20H, m), 4.70 (1H, dd, J= 5.08, 11.8 Hz), 4.63 (1H, dd, J=4.2, 11.8 Hz), 4.49-4.56 (4H, m), 4.16-4.22 (1H, m), 4.08-4.13 (1H, m), 3.95 (2H, m), 3.74 (4H, m), 3.50 (1H, dd, J= 3.7, 10.6 Hz), 2.78 (1H, br), 1.93-2.03 (1H, m) 1.63-1.70 (1H, m);

\textbf{\textsuperscript{13}C NMR} (400 MHz, CDC\textsubscript{3}), \(\delta\): 138.4, 138.2, 138.0, 137.9, 128.4, 128.3, 128.0, 127.9, 127.8, 127.7, 127.6, 127.6, 127.5, 77.0, 76.7, 74.2, 73.3, 73.2, 73.1, 73.0, 72.1, 67.7, 61.2, 29.8;

\textbf{MS} (ESI, MeCN) calcd. For C\textsubscript{36}H\textsubscript{40}O\textsubscript{6}, m/z: (M\(^+\)) 568.28, found 569.3 [(M\(^+\)].

\textbf{Preparation of 2,3,4,6-tetra-\textit{O}-benzyl-\textit{\(\alpha\)}-\textit{\(\beta\)}-galactopyranosyl-ethyl iodide (40)}

\begin{center}
\includegraphics[width=0.5\textwidth]{image}
\end{center}

350 mg (0.62 mmol) of 2,3,4,6-tetra-\textit{O}-benzyl-\textit{\(\alpha\)}-\textit{\(\beta\)}-galactopyranosyl-ethyl ethanol was added to a 10 mL round bottom flask containing 3.0 ml of freshly distilled THF and a stir bar as described by Nolen.\textsuperscript{5} 240 mg (0.90 mmol) of triphenylphosphine, 170 mg (2.5 mmol) of imidazole and 170 mg (0.70 mmol) of I\(_2\) were sequentially added to the solution. The solution was stirred for 30 minutes, concentrated under reduced pressure and dissolved in 5 ml of EtOAc. The organic layer was washed successively


92
with sodium sulfite (until the yellow colour dissipated), distilled water (2x) and brine (1x). The organic phase was dried over MgSO₄, filtered and concentrated in vacuo. Column chromatography (10:1 hexanes: ethyl acetate) furnished 40 as a colourless oil (360 mg, 86%).

**¹H NMR** (400 MHz, CDCl₃), δ: 7.26-7.36 (18H, m), 7.15-7.25 (2H, m), 4.45-4.75 (8H, m), 4.09 (1H, m), 3.97 (1H, m), 3.83 (1H, m), 3.76 (1H, m), 3.64-3.70 (2H, m), 3.12-3.28 (2H, m), 2.12-2.22 (1H, m), 1.90-2.00 (1H, m);

**¹³C NMR** (400 MHz, CDCl₃), δ: 138.5, 138.4, 138.3, 138.0, 128.5, 128.4, 128.4, 128.3, 128.0, 127.9, 127.9, 127.7, 127.7, 127.6, 127.5, 77.2, 76.8, 76.4, 74.1, 73.4, 73.2, 73.0, 72.6, 67.7, 61.2, 31.6, 3.1;

**MS** (ESI, MeCN) calcd. For C₃₆H₃₉O₅, m/z: (M⁺) 678.18, found 701.2 [(M+Na⁺)].

### Preparation of 2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-ethyl triphenylphosphonium iodide (36)

![Chemical Structure](image)

To a flame dried round bottom flask equipped with a stir bar, 262 mg, 1.00 mmol, of triphenylphosphine was added to a solution of 40 (100 mg, 0.143 mmol) in 2 ml of dry
DMF. The solution was stirred at 40 °C overnight under a nitrogen atmosphere. Upon completion of the reaction (as judged by TLC), DMF was removed under vacuum to yield a thick oil. Excess triphenylphosphine was removed by repeated ether and THF washes to furnish a white paste. Recrystallization from toluene furnished 36 as a white amorphous solid (133 mg, 98 %). The solid was dried in a Kugelrohr and stored in a dessicator under argon.

\(^1\)H NMR (400 MHz, CDCl\(_3\)), \(\delta\): 7.58-7.77 (15H, m), 7.10-7.35 (20H, m), 4.43-4.73 (8H, m), 4.18 (1H, m), 3.87-4.05 (4H, m), 3.73 (1H, dd. J= 2.7, 6.1 Hz), 3.56-3.68 (1H, m), 3.33 (1H, m), 1.93 (2H, m);

\(^{13}\)C NMR (400 MHz, CDCl\(_3\)), \(\delta\): 138.4, 138.3, 138.1, 138.0, 135.0, 134.9, 133.5, 133.4, 130.5, 130.3, 128.9, 128.2, 128.2, 127.7, 127.6, 127.6, 127.5, 127.4, 127.4, 125.2, 118.2, 117.4, 76.3, 73.6, 73.2, 73.1, 72.8, 72.7, 72.4, 67.7, 21.9, 21.3, 19.7, 19.1;

MS (ESI, H\(_2\)O) calcd. For C\(_{54}\)H\(_{34}\)IO\(_3\)P, m/z: (M\(^+\)) 940.28, found 940.3 [M\(^+\)];

IR (CH\(_2\)Cl\(_2\)) cm\(^{-1}\): 3048, 1581 and 1279.
4.3 Protocol for the Preparation of Amino Acid Components

Preparation of (S)-4-methoxycarbonyloxazolidin-2-one (18)

Commercially available L-serine (0.525 g, 5 mmol) was dissolved in 1M NaOH (15 ml, 15 mmol) and added to triphosgene (1.5 g, 5 mmol) in p-dioxane (10 ml) as described by Falb. The mixture was stirred at room temperature for 2 hrs. The solvent was evaporated, and the crude solids were dissolved in methanol (30 ml) and the mixture stirred overnight. The solvent was decanted and evaporated to yield a viscous oil which was washed with acetonitrile (15 ml) and filtered. The filtrate was subject to rotovaporation to afford 18 as a yellow oil (0.61 g, 83%).

\[^1^H\text{NMR}\] (400 MHz, CDCl\(_3\)), \(\delta: 6.45 (1H, br), 4.61 (1H, dd, J=9.55, 8.95 \text{ Hz}), 4.52 (1H, dd, J=8.95, 4.50), 4.43 (1H, dd, J=9.55, 4.50), 3.81 (3H, s);

\[^{13}\text{C NMR}\] (400 MHz, CDCl\(_3\)), \(\delta: 170.6, 159.2, 66.8, 53.8, 53.1;\)

\(^{\text{MS}}\) (ESI, MeCN) calcd. For C\(_5\)H\(_7\)NO\(_4\), m/z: (M\(^+\)) 145.04, found: 146.0 [(M+H\(^+\)]), 86.0 [(M+H\(^+\))- CO\(_2\)Me];

\(^{\text{IR}}\) (CH\(_2\)Cl\(_2\)) cm\(^{-1}\): 3327 and 1765.

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Preparation of (R)-4-hydroxymethyloxazolidin-2-one (19)

According to the procedure described by Sibi, a dry 100 ml round-bottom flask equipped with a stirbar, 1.0 g (50 mmol) of NaBH₄ was added in portions to a solution of 18 (3.5 g, 0.025 mmol) in ethanol (50 ml). The solution was stirred for 2 hours, after which it was treated with saturated ammonium chloride, and stirred for an additional 10 minutes. The solids (mostly salt) were filtered off and the filtrate was concentrated to yield a crude white paste (2.80 g, 98 %). Flash chromatography with 9:1 ethyl acetate:methanol afforded the title compound as white crystals (2.2 g, 78 %).

**¹H NMR** (400 MHz, D₂O), δ: 4.57 (1H, t, J=9.01 Hz), 4.30 (1H, dd, J=8.93, 5.15 Hz), 4.11-4.04 (1H, m), 3.68 (1H, J=12.01, 3.76 Hz), 3.60 (1H, dd J=12.01, 4.40 Hz);

**¹³C NMR** (400 MHz, D₂O), δ: 164.9, 70.5, 65.0, 56.3;

**MS** (ESI, MeOH) calcd. For C₄H₇N0₃, m/z: (M⁺) 117.04, found:118.1 [(M+ H⁺)], 117.0 [M⁺];

**IR** (MeOH) cm⁻¹: 3372 and 1741.

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Preparation of (S)-4-(4'-tolylsulfonyl)oxymethyloxazolidin-2-one (20)

According to the procedure described by Sibi,\(^8\) TsCl (8.6g, 45.1 mmol) was added to a round bottom flask containing 19 (3.4 g 29.3 mmol) dissolved in dry pyridine (15 ml). The solution was stirred for 4 hours and monitored periodically by TLC. Upon completion of the reaction, excess pyridine was removed via rotoevaporation to yield a dark red gel. The gel was dissolved in dichloromethane (50 ml) and rinsed with 1.5 M HCl (6 x 33 ml). The combined organic extracts were dried over MgSO\(_4\), filtered and concentrated in vacuo. The resulting white solid was rinsed repeatedly with boiling hexanes to remove residual TsCl and afford 20 (87 %, 7.04 g) as a fluffy white solid.

\(^1\)H NMR (400 MHz, CDCl\(_3\)), \(\delta\): 7.79 (1H, d, J=8.31 Hz), 7.38 (1H, d, J=8.36 Hz), 5.8 (1H, br), 4.47 (1H, t, J=8.01Hz), 4.07 (4H, m), 2.46 (3H, s);

\(^{13}\)C NMR (400 MHz, CDCl\(_3\)), \(\delta\): 158.8, 145.6, 132.2, 130.2, 127.9, 69.7, 66.3, 50.9, 21.7;

MS (ESI, MeCN) calcd. For C\(_{11}\)H\(_{13}\)NO\(_5\)S, m/z: (M\(^+\)) 271.05, found: 294.0 [(M+ Na\(^+\))], 271.1 [M\(^+\)];

IR (CHCl\(_3\)) cm\(^{-1}\): 3437, 3181 and 1756.

Preparation of (S)-4-iodomethyloxazolidin-2-one (21)

A mixture of 3.75 g (12.0 mmol) of tosylate 20, and 9.0 g (56 mmol) of NaI were refluxed in 50 ml dry acetone under a N₂ (g) atmosphere as described by Sibi and Renhowe. The reaction was stirred overnight under reflux, cooled to room temperature and excess NaI removed by filtration. The solids were rinsed with ethyl acetate and concentrated on a rotary evaporator to yield a crude red solid. The solid was dissolved in ethyl acetate and washed with a saturated Na₂SO₃ (50 ml) solution until the organic layer became colourless. The aqueous sodium sulfite layer was back-extracted repeatedly with ethyl acetate (10x 25 ml). The organic extracts were combined and dried over MgSO₄, filtered and the solvent removed by rotovaporation to furnish a crude orange solid. Column chromatography (3:1 ethyl acetate:hexanes) afforded the product, 21, as a pale white solid (2.62 g, 87%).

¹H NMR (500 MHz, CDCl₃), δ: 6.79 (1H, br), 4.50 (1H, dd, J=8.729, 8.41 Hz), 4.13 (1H, dd, J=4.87, 8.99), 4.07 (1H, m), 3.22 (2H, m);

¹³C NMR (500 MHz, CDCl₃), δ: 159.4, 70.4, 53.2, 8.1;

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MS (ESI, MeCN) calcd. For C₄H₆INO₂, m/z: (M⁺) 226.94, found 250.0, [(M+Na⁺)], 227.0 [M⁺];

IR (CH₂Cl₂) cm⁻¹: 3472 and 1764.

Preparation of (S)-4-(2-oxazolidinyl)-methyltriphenylphosphonyl Iodide (22)

According to the protocol established by Sibi and Renhowe,¹⁰ a flame dried round bottom flask equipped with a stir bar and N₂ (g) feed, 15.0 g (57.25 mmol) of triphenylphosphine was added to a solution of 21 (1.5 g, 6.6 mmol) in 8 ml of dry DMF. The solution was stirred at 100 °C overnight under a nitrogen atmosphere. Upon completion of the reaction (as judged by TLC), DMF was removed under vacuum to yield a viscous translucent yellow oil. The oil was triturated repeatedly with ether and THF to furnish a white solid. Mixed solvent recrystallization with ethyl acetate and methanol afforded 22 (3.07 g, 95 %), as a white solid which was subsequently dried in a Kugelrohr and stored in a dessicator under argon.

¹H NMR (400 MHz, D₂O), δ: 7.80 (15H, m), 4.50 (1H, m), 4.35 (1H, dd J=9.22, 0.92 Hz), 4.01 (1H, dd, J= 5.01, 9.12 Hz), 3.94 (1H, m) 3.79 (1H, m);

$^{13}$C NMR (400 MHz, D$_2$O), $\delta$: 157.8, 132.4, 130.6, 127.3, 114.4, 67.8, 67.7, 44.3;

MS (ESI, H$_2$O) calcd. For C$_{22}$H$_{21}$INO$_4$P, m/z: (M$^+$) 489.04, found: 489.0 (M$^+$);

IR (CH$_2$Cl$_2$) cm$^{-1}$: 3324 and 1776.

**Preparation of 1(-tert-butyldimethylsiloxy)-3-butene (23)**

![23]

Commercially available 3-buten-1-ol (5.00 g, 69.4 mmol) was added to a solution of tert-butyldimethylsilylchloride (TBDMSCl) (11.0 g, 72.0 mmol) and imidazole (5.0 g, 73.5 mmol) in 100 ml of dichloromethane as described by Webste and Pirrung. The solution was stirred for 2 hours at room temperature, followed by repeated washes with 10% HCl, water and brine. The organic phase was dried over MgSO$_4$ and concentrated on a rotary evaporator to yield the product as a clear oil (12.32 g, 95%).

$^1$H NMR (400 MHz, CDCl$_3$), $\delta$: 5.78- 5.86 (1H, m), 4.99- 5.09 (1H, m), 3.65 (1H, $\text{t}$ $J$= 6.78 Hz), 2.24- 2.30 (1H, m), 0.90 (9H, s) 0.05 (6H, s);

$^{13}$C NMR (400 MHz, CDCl$_3$), $\delta$: 135.3, 116.2, 62.8, 37.5, 25.9, 18.3,-5.3;

MS (ESI, MeCN) calcd. For C$_{10}$H$_{22}$OSi, m/z: (M$^+$) 186.14, found: 209.1 [(M+ Na$^+$)].

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Preparation of (3-tert-butyldimethylsiloxy)propanal (24)

\[
\text{OTBDMS}
\]

1.00 g (5.4 mmol) of 23 was added to a round bottom flask containing 15 ml of MeOH and a stir bar. The solution was cooled to -78 °C in a dry ice/acetone bath and a stream of ozone was bubbled through the solution. Bubbling was stopped once a pale blue colour developed (~ 15 minutes), and the solution was flushed with dry nitrogen. Dimethyl sulfide (5 ml) was added and the solution was allowed to warm to room temperature (overnight). The solvent was removed under reduced pressure, and the crude oil was purified via flash chromatography (2:1, dichloromethane: hexanes) to afford the title compound as a colourless oil. The oil was stored under an inert atmosphere in the freezer.

\(^1\)H NMR (400 MHz, CDCl\(_3\)), \(\delta\): 9.80 (1H, t, \(J=2.0\) Hz), 3.98 (2H, t, \(J=6.03\)), 2.60 (1H, td, \(J=2.0, 6.03\) Hz), 0.88 (9H, s), 0.06 (6H, s);

\(^{13}\)C NMR (400 MHz, CDCl\(_3\)), \(\delta\): 201.8, 57.1, 46.3, 25.6, 18.0,-5.7;

MS (ESI, MeCN) calcd. For C\(_9\)H\(_{20}\)O\(_2\)Si, \(m/z\): (M\(^+\)) 188.12, found: 211.1 [M+ Na\(^+\)], 189.1 [(M + H\(^+\))];

IR (CH\(_2\)Cl\(_2\)) cm\(^{-1}\): 2627, 1757, and 963.
Preparation of (S)-4-(4-\(\text{tert}-\text{butyldimethylsiloxy}\)but-1-enyl)oxazolidin-2-one (25)

In a flame dried flask equipped with an Ar (g) inlet and a stir bar, a suspension of 22 (490 mg, 1 mmol) in 15 ml of freshly distilled THF was cooled to -78 °C. The suspension was treated with 1.95 mmol (0.80 ml) of nBuLi over a period of 10 minutes. The resulting orange-red suspension was stirred for 1 hour, followed by quench with 24 (179 mg, 0.95 mmol) in THF (2 ml). The solution was stirred for an additional 2 hours, followed by quench with saturated aqueous NH\(_4\)Cl (10 ml). THF was removed by rotovaporation and the aqueous layer extracted with ethyl acetate (5 x 15 ml). The combined organic extracts were dried over MgSO\(_4\), filtered, and concentrated to afford a viscous dark yellow oil. Column chromatography with 1:1 ethyl acetate: hexanes furnished 25 as a viscous pale yellow oil (239 mg, 88%).

\(^1\text{H NMR}\) (400 MHz, CDCl\(_3\)), \(\delta\): 5.64- 5.79 (1H, m), 5.45- 5.56 (1H, m), 5.38 (1H, br), 4.72 (1H, dd, \(J=15.88, 8.32\) Hz), 4.51 (1H, t, \(J=8.46\)) 4.02 (1H, t, \(J=8.48\)), 3.64 (2H, t, \(J=6.14\) Hz), 2.20- 2.35 (2H, m), 0.89 (9H, m), 0.06 (6H, m);

\(^{13}\text{C NMR}\) (400 MHz, CDCl\(_3\)), \(\delta\): 159.4, 132.3, 129.4, 70.3, 62.1, 49.5, 31.2, 26.0, 18.5, -5.3;

\(\text{MS}\) (ESI, MeCN) calcd. For C\(_{13}\)H\(_{25}\)NO\(_3\)Si, m/z: (M\(^+\)) 271.16, found: 310.1 [(M+K\(^+\))];

\(\text{IR}\) (CH\(_2\)Cl\(_2\)) cm\(^{-1}\): 3321, 1743, and 935.
Preparation of (S)-tert-butyl 4-(4-(tert-butyldimethylsilyloxy)but-1-enyl)-2-oxooxazolidine-3-carboxylate (30)

In a dry round bottom flask equipped with a stir bar, di-tert-butyl dicarbonate (100 mg, 0.46 mmol) was added to a solution of 25 (100 mg, 0.36 mmol), triethylamine (250 μl, 2.0 mmol) and catalytic dimethylaminopyridine (DMAP) in THF (2.0 ml). The resulting yellow solution was stirred for 2 hours at room temperature, followed by concentration in vacuo. The yellow residue obtained after rotovaporation was dissolved in 5 ml of ethyl acetate, and extracted repeatedly with 10% HCl (3x), water (2x) and brine (1x). The organic layer was dried over MgSO₄, filtered and concentrated to yield a dark yellow oil. Flash column chromatography (1:5 ethyl acetate: hexanes) furnished the title compound as white needles (122 mg, 92%).

\[ 1^H \text{NMR} (400 \text{ MHz, CDCl}_3), \delta: 5.64-5.73 (1H, m), 5.50-5.57 (1H, m), 4.98-5.06 (1H, m), 4.41 (1H, t, J= 8.6 Hz), 3.93 (1H, dd, J= 4.9, 8.7 Hz), 3.66-3.74 (1H, m), 3.55-3.64 (1H, m), 2.26-2.44 (1H, m), 1.50 (9H, s), 0.88 (9H, s), 0.43 (6H, s); \]

\[ 13^C \text{NMR} (400 \text{ MHz, CDCl}_3), \delta: 152.2, 149.1, 131.0, 128.5, 83.8, 67.3, 62.1, 52.4, 31.3, 28.0, 25.9, 18.4, -5.3; \]
**MS** (ESI, MeCN) calcd. For C_{18}H_{33}NO_{5}Si, m/z: (M⁺) 371.21, found: 371.2 [M⁺], 271.2 [(M⁺) - Boc];

**IR** (CH₂Cl₂) cm⁻¹: 1795 and 1728.

**Preparation of (S)-tert-butyl 4-(4-hydroxybut-1-enyl)-2-oxooxazolidine-3-carboxylate (31)**

![Chemical Structure of 31]

In a dry round bottom flask, TBAF·H₂O (310 mg, 1.42 mmol) was added to a stirred solution of 30 (371 mg, 1.0 mmol) in 4 ml of tetrahydrofuran. The solution was stirred at room temperature for 1 hour. THF was promptly removed by rotovaporation to yield a thick yellow oil which was dissolved in ethyl acetate (5 ml) and extracted successively with water (3 x 5 ml) and brine (2 x 5 ml). The organic layer was dried over MgSO₄, filtered and concentrated to yield a translucent yellow oil. Column chromatography (1:1 ethylacetate: hexanes) afforded 31 as a colourless oil (215 mg, 84%).

**¹H NMR** (400 MHz, CDCl₃), δ: 5.69- 5.82 (1H, m), 5.52- 5.62 (1H, m), 5.05- 5.13 and 4.65- 4.73 (total 1H, m) 4.43 (1H, m), 3.90- 4.04 (1H, m), 3.69 (2H, m), 2.30- 2.54 (2H, m), 2.22 and 1.66 (total 1H, br), 1.51 (9H, s);
\[^13\]C NMR (400 MHz, CDCl\textsubscript{3}), \(\delta\): 152.0, 149.7, 132.3, 129.3, 84.3, 67.1, 61.4, 57.0, 35.3, 27.9;

MS (ESI, MeOH) calcd. For C\textsubscript{12}H\textsubscript{19}NO\textsubscript{5}, \(m/z\): (M\textsuperscript{+}) 257.13, found: 257.1 [M\textsuperscript{+}], 157.1 [(M\textsuperscript{+}) - Boc].

**Preparation of (S)-tert-butyl 4-(4-hydroxybutyl)-2-oxooxazolidine-3-carboxylate (32)**

To a round bottom flask equipped with an inlet, 100 mg (0.386 mmol) of 31 was added to a stirred suspension of Pd/C (10 mg, 10 % w/w) in methanol (2.0 ml). The mixture was degassed with dry N\textsubscript{2} (g) for 20 minutes with stirring. H\textsubscript{2} (g) was bubbled through the suspension for 25 minutes, the inlet removed and solid Pd/C filtered off with Whatman paper. The filtrate was concentrated on a rotary evaporator to furnish a crude colourless oil. Flash chromatography (1:1 ethyl acetate: hexanes) furnished the product as a white crystalline solid (78 mg, 77 %).

\[^1\]H NMR (400 MHz, CDCl\textsubscript{3}), \(\delta\): 4.34 (1H, t, J = 8.14 Hz), 4.20- 4.28 (1H, m), 4.05 (1H, dd, J = 8.39 , 2.74 Hz), 3.65 (2H, t, J = 6.26 Hz), 1.80- 1.92 (1H, m), 1.69- 1.78 (1H, m), 1.60 (2H, m), 1.53 (9H, s), 1.42 (2H, m);
$^{13}$C NMR (400 MHz, CDCl$_3$), δ: 152.2, 149.5, 83.9, 66.4, 62.3, 54.9, 32.7, 32.2, 28.0, 20.6;

MS (ESI, MeOH) calcd. For C$_{12}$H$_{21}$NO$_5$, m/z: ($M^+$) 259.14, found: 282.1 [(M+ Na$^+$)];
IR (CH$_2$Cl$_2$) cm$^{-1}$: 3423, 1797 and 1720.

**Preparation of (S)-tert-butyl 4-(4-hydroxybutyl)-2-oxooxazolidine-3-carboxylate (37)**

![Chemical Structure of 37](image)

To a dry round bottom flask equipped with a stir bar and dichloromethane (1.0 ml), DMSO (45 mg, 0.58 mmol) was added and the solution cooled to -78 °C in an acetone/dry ice bath. Oxalyl chloride (38 mg, 0.290 mmol) was added dropwise over 5 minutes and the solution stirred for 15 minutes. 32 (50 mg, 0.19 mmol) was diluted in dichloromethane and added via syringe over 5 minutes. The solution was stirred for an additional 30 minutes. Triethylamine (78 mg, 107 mmol) was diluted in dichloromethane and added to the solution over 5 minutes by syringe, and the solution stirred for an additional 30 minutes. The solution was allowed to warm to room temperature, extracted with 10 % HCl (2 ml x 3), water (2ml x 3) and brine (2ml x 1). The organic phase was dried over MgSO$_4$, filtered and concentrated. Column chromatography (2:1 hexanes: ethyl acetate) afforded 37 as a colourless oil (45 mg, 91 %).
$^1$H NMR (400 MHz, CDCl$_3$), $\delta$: 9.88 (1H, t, J= 1.04 Hz), 4.35 (1H, t, J= 8.15 Hz), 4.25 (1H, m), 4.08 (1H, m), 2.55 (1H, t, J= 6.82 Hz), 2.45 (1H, t, J= 6.98 Hz), 1.73 (4H, m), 1.54 (9H, s);

$^{13}$C NMR (400 MHz, CDCl$_3$), $\delta$: 201.1, 152.0, 149.2, 83.9, 66.2, 54.6, 43.0, 32.2, 27.9, 16.5; MS (ESI, MeCN) calcd. For C$_{12}$H$_{21}$NO$_5$, m/z: (M$^+$) 257.13, found: 280.1 [(M+Na$^+$)], 257.1 [M$^+$] and 157.1 [(M$^+$)-Boc];

IR (CH$_2$Cl$_2$) cm$^{-1}$: 2761, 1802, 1761 and 1713.

4.4 Coupling of Carbohydrate and Amino Acid Components

Preparation of (4S)-tert-butyl 2-oxo-4-((Z)-6-((2R,3R,4R,5R)-3,4,5-tris(benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-2-yl)hex-4-enyI)oxazolidine-3-carboxylate (41)

To a flame dried flask equipped with an Ar (g) inlet and a stir bar, a suspension of 36 (202 mg, 0.215 mmol) in 2 ml of freshly distilled THF was cooled to -78 °C. The suspension was treated with 0.215 mmol (127 µl) of nBuLi over a period of 5 minutes.
The resulting orange suspension was stirred for 20 minutes, followed by quench with 37 (60 mg, 0.230 mmol) in THF (1 ml). The solution was stirred for an additional 30 minutes at -78 °C, followed by quench with saturated aqueous NH₄Cl (1 ml). THF was removed on a rotary evaporator and the aqueous layer extracted with ethyl acetate (5 x 2 ml). The combined organic extracts were dried over MgSO₄, filtered, and concentrated to afford a viscous yellow oil. Column chromatography with 4:1 hexanes: ethyl acetate furnished 41 as a viscous yellow oil (65 mg, 38 %).

**¹H NMR** (500 MHz, CDCl₃), δ: 7.25-7.35 (20H, m), 5.40 (2H, m), 4.48- 4.75 (8H, m) 4.25 (1H, t, J=8.2), 4.15 (1H, m), 3.98- 4.05 (2H, m), 3.92- 3.96 (2H, m), 3.81- 3.87 (2H, m), 3.66 (1H, dd, J= 4.2, 10.4 Hz), 2.28- 2.37 (2H, m), 2.08- 2.02 (2H, m), 1.58-1.82 (4H, m), 1.53 (9H, s);

**¹³C NMR** (500 MHz, CDCl₃), δ 152.3, 149.4, 149.4, 138.6, 138.5, 138.4, 138.3, 130.3, 128.4, 128.3, 128.3, 127.9, 127.8, 127.7, 127.6, 127.6, 127.5, 126.9, 83.8, 76.7, 74.3, 73.2, 73.2, 73.1, 72.8, 67.4, 66.4, 54.9, 32.5, 28.0, 27.0, 24.2;

**MS** (ESI, MeCN) calcd. For C₄₈H₅₇NO₉, m/z: (M⁺) 791.40, found: 814.4 [(M+ Na⁺)];

**IR** (CH₂Cl₂) cm⁻¹: 1791, 1720 and 696.
Preparation of tert-butyl (2S,Z)-1-hydroxy-8-((2R,3R,4R,5R)-3,4,5-tris(benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-2-yl)oct-6-en-2-ylcarbamate (42)

25 mg (0.032 mmol) of 42 was dissolved in 1 ml of MeOH and stirred at room temperature. Catalytic CsCO₃ (3 mg) was added and the solution stirred until the reaction was judged complete (TLC, 1 hr). MeOH was removed in vacuo and the residue was dissolved in 1 ml of DCM. The solution was washed with 2 ml of 10 % citric acid (2x) and dried over MgSO₄, filtered and concentrated. Flash chromatography with 2:1 hexanes: ethyl acetate furnished the alcohol as a clear oil (21 mg, 87 %).

**¹H NMR** (500 MHz, CDCl₃), δ: 7.16-7.26 (20H, m), 5.31 (2H, m), 4.42-4.66 (8H, m) 3.86-4.00 (3H, m), 3.78 (1H, m), 3.64-3.70 (2H, m), 3.60 (1H, m), 3.44-3.58 (2H, m), 3.32-3.38 (1H, m), 2.18-2.36 (2H, m), 1.88-2.04 (2H, m), 1.54 (1H, br), 1.37 (9H, s), 1.18-1.36 (4H, m);

**¹³C NMR** (500 MHz, CDCl₃), δ 156.5, 138.5, 138.4, 138.3, 138.2, 131.0, 128.3, 128.3, 128.3, 128.2, 127.9, 127.8, 127.8, 127.7, 127.5, 127.5, 127.4, 126.3, 79.5, 76.6, 74.2, 73.2, 73.1, 73.0, 66.0, 52.8, 31.0, 28.4, 27.2, 26.0;

**MS** (ESI, MeOH) calcd. For C₄₇H₅₉NO₈, m/z: (M⁺) 765.42, found: 788.4 [(M+ Na⁺)].
Preparation of $(2S,Z)$-2-(tert-butoxycarbonylamino)-8-$(2R,3R,4R,5R)$-3,4,5-tris(benzyloxy)-6-(benzyloxymethyl)tetrahydro-2H-pyran-2-yl)oct-6-enoic acid (43)

50 mg (0.0654 mmol) of 42 was added to a 5 ml round bottom flask containing a stir bar and 0.5 ml of dry DMF. 200 mg (0.65 mmol) of PDC was added and the solution stirred overnight. The solution was diluted with 3 ml of water and extracted with EtOAc (5 x 3 ml). The organic extracts were washed successively with 10 % HCl, distilled water and brine. The organic phase was dried over MgSO₄, filtered and concentrated to yield a crude yellow oil. Column chromatography with 13:1 DCM: MeOH furnished the product as a colourless oil (40 mg, 78%).

$^1$H NMR (500 MHz, CDCl₃), δ: 7.22-7.33 (20H, m), 5.31-5.42 (2H, m), 5.08 (1H, br), 4.46-4.70 (8H, m), 4.20-4.28 (1H, m), 4.02-4.08 (1H, m), 3.98 (1H, m), 3.89-3.94 (1H, m), 3.71 (2H, m), 3.64 (1H, dd, J= 3.2, 8.1 Hz), 2.38 (1H, m), 2.25 (1H, m), 1.92-2.11 (2H, m), 1.50-1.88 (4H, m), 1.42 (9H, s);
$^{13}$C NMR (500 MHz, CDCl$_3$), $\delta$ 176.0, 155.6, 138.4, 138.4, 138.3, 138.2, 131.0, 128.3, 128.3, 128.2, 127.9, 127.8, 127.8, 127.7, 127.5, 127.5, 127.4, 126.3, 79.5, 76.6, 74.2, 73.2, 73.1, 73.0, 66.0, 52.8, 31.0, 28.4, 27.2, 26.0;

MS (ESI, MeCN) calcd. For C$_{47}$H$_{57}$NO$_9$, m/z: (M$^+$) 779.40, found: 818.4 [(M+ K$^+$)], 802.4 [(M+ Na$^+$)];

IR (CH$_2$Cl$_2$) cm$^{-1}$: 3342, 1721 and 1703.
Appendix

$^1$H and $^{13}$C NMR Spectra