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Library Synthesis of Thiol-linked Hybrid Inositol Glycans

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LIBRARY SYNTHESIS OF THIOL-LINKED HYBRID INOSITOL GLYCANS

A Thesis

Presented To

The Faculty of the Department of Chemistry

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Scott Chan

August 2017

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The Designated Thesis Committee Approves the Thesis Titled

LIBRARY SYNTHESIS OF THIOL-LINKED HYBRID INOSITOL GLYCANS

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ABSTRACT

LIBRARY SYNTHESIS OF THIOL-LINKED HYBRID INOSITOL GLYCANS

by Scott Chan

Inositol phosphate glycans (IPGs) are natural pseudo-oligosaccharides that are produced in some mammalian cells upon stimulation by insulin. IPGs are capable of inducing insulin-like effects in insulin sensitive cells, making them viable drug targets for treating type II diabetes. However, the synthesis of highly insulin-mimetic synthetic IPGs is inefficient, requiring more than fifty steps. The development of hybrid inositol glycans (HIGAs) has mitigated this problem. HIGAs consists of a commercially available non-carbohydrate moiety that is coupled with a saccharide group that requires only fifteen steps to synthesize. HIGAs are thus much more efficient to synthesize on large scales. This thesis describes the attempted synthesis of a library of HIGAs. The goal was to link five non-carbohydrate moieties to an IPG-like disaccharide via thiol-linkage. Three of the five non-carbohydrate moieties were negatively charged while the other two were neutral to study the effect that the negative charge has on the insulin-like activity of the HIGAs. The insulin-like activity can be quantified using a glucose uptake assay in cultured murine adipocytes. Unfortunately, the syntheses of the HIGAs were not completed but a novel precursor compound has been synthesized.

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LIST OF ABBREVIATIONS

- 1, 5-IAEDAN - 5-(amino)-naphthalene-1-sulfonic acid
- ACL - ATP citrate lyase
- BF₃- boron triflouride
- CEBP - CCAAT enhancer binding proteins
- DACM- *N*-(7-dimethylamino-4-methylcoumarin-3-yl))maleimide
- DBU-1,8-diazabicyclo[5,4,0]undec-7-ene
- DDP-4 - dipeptidyl pepitdase 4
- DMEM- Dulbecco's Modified Eagle Medium
- EC₅₀- Half maximal effective concentration
- GLP-1 - glycogen-like peptide 1
- GLUT-4 - glucose transporter Type 4
- GPI - glycosylphosphatidylinositols
- GPI-PLC - glycoposphatidylinositol-specific phospholipase C
- GS - glycogen synthase
- GSK-3 - glycogen synthase kinase-3
- HEPES-(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- HIGA - hybrid inositol glycan
- HSL - hormone sensitive lipase
- IBMX - isobutyl-1-methylxanthine
- IG - inositol glycan
- IPG – inositol phosphate glycan

IR - insulin receptor

IRS-1/2 - insulin receptor substrates 1/2

lcDIG - low density detergent insoluble glycolipid

MIR - maximal insulin response

PDE3B - phosphodiesterase 3B

PDK1 - phosphoinositol dependent kinase 1

PIP3 - phosphatidylinositol-3,4,5-triphosphate

PKA - protein kinase A

PKB - protein kinase B

PPAR- γ - peroxisome proliferator-activated receptor gamma

PP1 - protein phosphatase 1

NMR - nuclear magnetic resonance

NRK - non-tyrosine kinase

SUR1 - sulfonylurea receptor 1

TBS- tertbutyldimethylsilyl

TMSOTf-trimethylsilyl trifluoromethanesulfonate

TLC- thin-layer chromatography

VSG - variant surface glycoprotein

CHAPTER 1

INTRODUCTION

1.1 Diabetes: Type I vs. Type II

Diabetes is a metabolic disease that results in the improper maintenance of a blood glucose level. A normal blood glucose level range from 4-7 mmol/l. To maintain this healthy range, either the peptide hormone glucagon or insulin is released¹. When blood glucose level is low, alpha cells of the pancreas secrete glucagon promoting the conversion of glycogen into glucose. This results in the elevation of blood glucose levels. When blood glucose level is high, beta cells of the pancreas secrete insulin. This causes the uptake and storage of glucose into insulin-sensitive cells. This phenomenon effectively lowers blood glucose level. Diabetes is characterized by hyperglycemia (high blood glucose levels), which is the result of faulty insulin signaling.

Diabetes is categorized into type I and type II. Type I diabetes, previously referred to as juvenile diabetes, is an autoimmune disease in which the body attacks and destroys the beta cells of the pancreas. The destruction of beta cells prevents the synthesis and secretion of insulin². Type II diabetes, the focus of this thesis, is often referred to as adult-onset diabetes. Type II diabetes is characterized by insulin resistance with a gradual desensitization of cells to insulin preventing insulin signal transduction³.

1.2 Treating Type II Diabetes

Type II diabetes currently affects 29.1 million people in the United States⁴. Patients with type II diabetes may suffer severe complications such as cardiovascular diseases, diabetic neuropathy, diabetic retinopathy, and diabetic nephropathy⁴. Early stages of type

II diabetes can be mitigated by altering diet, exercise, and insulin injections⁵. However, these methods are not sustainable solutions and life-long drug treatments are generally necessary.

There are currently six types of medications for type II diabetes which include: biguanides, sulfonylureas, meglitinides, thiazolidinediones, glycogen-like peptide-1 (GLP-1) receptor agonists, and dipeptidyl peptidase-4 (DPP-4) inhibitors⁵. Metformin, a type of biguanide is the oldest and most widely used drug in the treatment of type II diabetes. Metformin effectively reduces hyperglycemia but the exact mode of action is unknown⁵. Sulfonylureas and melitinides bind to sulfonylurea receptor 1 (SUR1) of potassium channels in pancreatic beta cells, causing the potassium channels to close resulting in a gradient. This allows an influx of calcium into the cell inciting the release of insulin⁶. Sulfonylureas bind to SUR1 with a stronger binding affinity and slower dissociation rate, thus inducing a longer lasting effect than meglitinides⁷. As for the thiazolidinediones, they are able to mitigate hyperglycemia by binding to the peroxisome proliferator-activated receptor gamma (PPAR- γ), inducing lipogenesis and converting glucose into fatty acids⁸. Finally, the GLP-1 receptor agonists and the DPP-4 inhibitors often referred to as the new generation drugs, were approved by the FDA in 2006. Both of these classes of drugs promote GLP-1 activity. GLP-1 is an incretin that, upon binding to the GLP-1 receptor, induces the release of insulin while inhibiting the secretion of glucagon. GLP-1 can be degraded by DPP-4 but DPP-4 inhibitors are able to prevent the degradation of GLP-1, therefore promoting GLP-1 activity⁹.

Unfortunately, the six classes of type II diabetes treatments have been linked to serious side effects such as lactic acidosis, cardiovascular disease, hemolytic anemia, liver failure, pancreatitis, pancreatic cancer, hypoglycemia, etc⁵. The treatments can be used as monotherapies but are more commonly used together. Although a greater anti-diabetic effect is observed when the drugs are used in tandem, the prevalence of adverse effects are increased as well. For example, when metformin was used in conjunction with a thiazolidinedione, there was a 6-fold increase in the incidence of pancreatitis⁵. Scientists are still searching for type II diabetes treatments with minimal side effects. Inositol phosphate glycans (IPGs) have been found to be a viable drug target due to their ability to induce insulin-like effects.

1.3 Inositol Phosphate Glycans (IPGS)

IPGs are naturally occurring pseudo-oligosaccharides that are derived from glycosylphosphatidylinositols (GPIs). There are two categories of GPIs consisting of GPI anchors and free GPIs. GPI anchors contain proteins that are attached at the terminal end while free GPIs lack a tethered protein. Structures of GPI anchors are highly conserved. They often contain an ethanolamine-mannose- α (1-2)-mannose- α (1-6)-mannose- α (1-4)-glucoseamine- α (1-6)-*myo*-inositol group¹⁰. GPIs are ubiquitous in higher eukaryotic cells and can also be found in some protozoan parasites, bacteria, and yeast. GPIs play critical roles in hormone, growth factor, and cytokine signal transduction¹¹. In 1986, Saltiel et al. treated bovine liver with insulin and observed that an enzyme modulator, which was later termed IPG, was released¹². It was hypothesized that upon stimulation by insulin, a glycosylphosphatidylinositol specific phospholipase C (GPI-PLC) is activated and

hydrolytically cleaves the phosphodiester bond of the GPI anchor. A protease may also come in to cleave the protein to generate the IPG¹³.

There are two structurally different types of IPGs that have been identified: IPG type A and IPG type P. IPG type A contains a *myo*-inositol group and glucosamine group while IPG type P consists of a *chiro*-inositol group and galactosamine group. The IPG type A that was isolated by Saltiel et al. in 1986 was observed to have the ability to stimulate insulin sensitive cells even in the absence of insulin¹². In 2003, the first structurally characterized putative insulin mediator IPG type P, INS-2, was isolated from beef liver by Larner et al.¹⁵ When INS-2 was administered intravenously to diabetic rats, a reduction in plasma glucose was observed. Increased levels of glucose incorporated into glycogen was also observed¹⁴. These findings from Saltiel et al. and Larner et al. strengthened the hypothesis that IPGs are secondary messengers of the insulin-signaling pathway.

1.4 Insulin Signaling Pathways

Insulin is a hormone that controls major biological functions of the cell such as: the activation of pyruvate dehydrogenase phosphatase, protein synthesis, cell proliferation, cell differentiation, etc. The biological functions induced by insulin that affect diabetes are glucose transport, activation of glycogen synthase, lipogenesis, and glycolysis, which are ways insulin sensitive cells store and breakdown glucose. Insulin, upon binding to the α -subunit of the insulin tyrosine kinase receptor (IR), induces a conformational change in the β -subunit of the IR. The conformational change in the β -subunit leads to the cross phosphorylation of cytosolic regions of the IR, recruiting insulin receptor substrates 1 and

2 (IRS-1 and IRS-2). IRS-1 and IRS-2 are then phosphorylated by the cytosolic kinase domain of the IR leading to the phosphorylation of phosphatidylinositol-3,4-5-triphosphate(PIP3) by IRS-1 and IRS-2. This leads to a phosphorylation cascade of phosphoinositol dependent kinase 1 (PDK1) and protein kinase B (PKB) in that order. After the activation of PKB, the insulin-signaling pathway diverges, resulting in glucose transport, activation of glycogen synthase, lipogenesis, and glycolysis¹⁵.

1.4.1 Glucose transport

Glucose transporter type 4 (GLUT-4) is a transporter protein that is responsible for the uptake of glucose through the cell membrane. GLUT-4 is phosphorylated by PKB directly. Alternatively, GLUT-4 may also be activated by insulin receptor substrate complex Cbl-CAP. Cbl-CAP, in tandem with adaptor protein CrkII and guanine exchange nucleotide exchange factor C3G, activates Tc10. Tc10 then induces GLUT-4 to be transferred in vesicles to the cell surface where GLUT-4 will then transport glucose into the cell¹⁵.

1.4.2 Glycogen synthesis

Glycogen synthesis is induced through the activation of PIP3 and PKB. Phosphorylated PIP3 activates protein phosphatase 1 (PP1), leading to the activation of glycogen synthase (GS) via dephosphorylation. GS is also alternatively activated via the inactivation of glycogen synthase kinase-3 (GSK-3). The phosphorylation of GSK is catalyzed by PKB and in turn deactivates GSK, leading to GS activity. These metabolic steps promote the conversion of glucose into glycogen¹⁵.

1.4.3 Lipogenesis

Lipogenesis is promoted by the phosphorylation of PKB. Activated PKB binds to ATP citrate lyase (ACL), activating the enzyme via phosphorylation. ACL converts citrate into acetyl-CoA which then acts as a building block for lipogenesis in the cytosol. Lipogenesis can be promoted indirectly through the decrease in lipolysis. PKB activates a series of proteins upstream, leading to the phosphorylation of phosphodiesterase 3B (PDE3B), which reduces the levels of cyclic adenosine monophosphate. This results in a decrease in protein kinase A (PKA) activity as well as preventing the activation of hormone sensitive lipase (HSL). Lipolysis would thus fail to occur. Induction of lipogenesis causes glucose to be converted into fatty acids and by preventing lipolysis; fatty acids are not degraded¹⁵.

1.5 IPGs as Secondary Messengers of the Insulin Signaling Pathway

Multiple groups have done extensive research in attempts to elucidate the IG (inositol glycan) signaling pathway, but the pathway has yet to be fully understood. In 2000, Müller et al. used immune complex kinase assays to observe the non-tyrosine kinases (NRTK) pp59Lyn and pp125Fak. These experiments revealed that these two NRTKs were heavily involved in IG signaling, which led Müller et al. to propose a plausible signaling pathway¹⁶. Initially, binding of insulin to the insulin receptor causes the release of glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC). GPI-PLC then cleaves GPIs, releasing IPGs from the cell surface. Upon the release of IPGs, GPI anchored proteins on the outer membrane are displaced from the IG receptor and relocated to a low density detergent insoluble glycolipid (ldDIG) region. Similarly, pp59Lyn is also

displaced to lcDIG regions in the cytosolic regions of the cell when IPGs bind to the IG receptor. The translocation of the pp59Lyn causes it to phosphorylate pp125Fak. Insulin receptor substrates 1 and 2 (IRS1/2) are then recruited by pp125Fak and phosphorylated by pp59Lyn. At this point, the IPG pathway and insulin signaling pathways converge, inducing glycogen synthase, lipogenesis, glycolysis, and glucose uptake¹⁵.

1.6 Synthetic IPGs

Since natural IPGs are extremely difficult to purify, scientists began synthesizing IPGs. In 1992, d'Alarcao and coworkers synthesized the first IPG to elicit an insulin response¹⁷. The goal was to synthesize an IPG modeled after the GPI anchor motif of mannose- α (1-2)-mannose- α (1-6)-mannose- α (1-4)-glucosamine- α (1-6)-*myo*-inositol. This led to the synthesis of the disaccharide IPG 1 and various analogs (Figure 1). When the analogues were tested using the lipogenesis assay, they were completely inactive except for IPG 1. IPG 1 was found to have a 30-40% maximal insulin response (MIR). It was concluded by d'Alarcao and coworkers that an unacylated glucosamine and a cyclic phosphate were essential for synthetic IPGs to yield insulin mimetic responses¹⁷.

Currently, the synthetic IPG to elicit the greatest MIR of >90% was synthesized by Frick et al. in 1998¹⁸. In the same paper, it was reported that 45 other synthetic IPGs had promising insulin mimetic properties. It was confirmed that a synthetic IPG requires a cyclic phosphate present on the inositol and an unacylated glucosamine group to elicit insulin mimetic responses. It was also observed that anionic charges on the distal mannose enhance insulin-mimetic effects. Having established that these characteristics

are essential motifs for synthetic IPGs, various synthetic strategies have been developed to achieve the desired glycosidic linkages, and glucosamine and inositol connectivity.

Figure 1. IPG 1 and various analogues synthesized by d'Alarcao and coworkers in 1992.

1.6.1 Synthetic strategies for glycosidic linkages

It is crucial that the glycosidic linkages are formed in a stereoselective and regioselective manner. This can be accomplished through various linkage methods and a series of protection and deprotection strategies. Commonly used strategies for stereoselective glycosylation involve thioglycosides, glycosyl halides, and imidate intermediates.

1.6.1.1 Thioglycoside Strategy for Glycosylation

The thioglycoside method is commonly used for stereoselective glycosidic linkages. This strategy involves the replacement of the anomeric hydroxyl group with a protected thiol group. To activate the thiol group for glycosylation, a metal salt is often used. The mechanism proposed by Ito and Ogawa was that a sulfonium salt is formed when the 1-methylthiomannoside reacts with phenylselenenyl triflate. The complex then rearranges into the more reactive selenonium salt (Scheme 1)¹⁹. The selenonium salt relies on the 1,2 position-directing groups present on 1-thiomannoside to influence stereoselectivity.

Scheme 1. Proposed mechanism of 1-Thiomannoside glycosylation catalyzed by selenium salt by Ito and Ogawa.

A synthetic scheme was developed by Jaworek et al. in 2001 was designed to be modular. The mannoses can be quickly linked into varying sized oligosaccharides (Scheme 2)²⁰. The two main building blocks were the 1-methylthiomannoside and *p*-methoxyphenyl-2,3,4-tri-*O*-benzyl- α -*D*-mannopyranoside. Utilizing Ito and Ogawa's phenylselenenyl triflate method, α -glycosylation was achieved by the 1,2-trans directing groups present on the 1-methylthiomannoside. This allows the 6-position hydroxyl present on *p*-methoxyphenyl-2,3,4-tri-*O*-benzyl- α -*D*-mannopyranoside to attack and attach favorably at the α -position. Once the glycosylation has occurred, the *p*-methoxyphenyl group is removed and the 1-methylthiomannoside is formed to allow the subsequent glycosylation. This method can be repeated many times until the desired oligosaccharide length is formed.

Scheme 2. Formation of dimannoside utilizing thioglycoside glycosyl donor method by Jaworek et al.

Another example of thioglycoside-promoted glycosylation is the synthesis of a *Trypanosomacruzi* derivative by Yashunsky et al. (Scheme 3)²¹. This was a (3+3) strategy where a tri-mannoside was formed and coupled to a pseudotrisaccharide. To form the tri-mannoside, debenzoylation of the 2- and 6-positions followed by selective phenoxylation of the 6-position converted the dimannoside into a glycosyl acceptor. Coupling the glycosyl acceptor with another 1-thiomannoiside was catalyzed with methyl trifluoromethanesulfonate. The 1,2-trans directing group present on the 1-ethylthiomannoside afforded predominantly α glycosylation forming the desired tri-mannoside.

Scheme 3. Formation of a trimannoside derivative utilizing the thioglycoside method of glycosylation by Yashunsky et al.

1.6.1.2 Glycosyl halide strategy for glycosylation

An alternative method of stereoselective glycosylation is through the usage of glycosyl halides. In a synthesis performed by Chakraborty et al., α -selective glycosylation was desired to link the mannosides and glucosamine. However, a mixture of α and β anomers were desired at the final coupling step of the inositol to the glucosamine (Scheme 5)²². To achieve α -selective glycosylation of the mannosides it is essential to have a glycosyl fluoride donor that is a D-mannoside derivative. Synthesis of the mannosyl fluoride donor was achieved by first deprotecting the 1-position hydroxyl by removing the methyl orthoester group. The 1-position was then selectively fluorinated using the diethylaminosulfur trifluoride/THF method. Extensive studies carried out by Posner et al. for this fluorination method revealed that the 2-position directing group is vital for stereoselectivity. It was also noted that utilizing THF as a solvent optimizes the reactivity²³. The 2-position was then deacetylated and subsequently orthogonally protected with allyl bromide to allow selective manipulation after inositol coupling. Coupling of the mannosyl fluoride donor to the glycosyl acceptor was promoted with silver triflate and bis(cyclopentadienyl)zirconium dichloride. Suzuki proposed that a zirconium perchlorate salt forms to activate the C-F bond, generating a highly reactive oxonium species to allow the hydroxyl group of the glycosyl acceptor to readily couple (Scheme 4)²⁴. After coupling, the 1-position of the glucosamine is deprotected and fluorinated to convert it into a glycosyl donor for the inositol group.

Scheme 4. Proposed mechanism of zirconium catalyzed glycosylation by Suzuki et al.

Scheme 5. Glycosylation utilizing glycosyl fluoride precursor by Chakraborty et al.

Glycosyl bromides and chlorides are also widely used for glycosyl halide promoted glycosylation. In the synthesis of a GPI anchor by Wu et al., a trimannoside precursor was required²⁵. Starting from the D-mannose 1,2-orthoester, the 3,4,5 positions were deacetylated. This was followed by benzylation orthogonal to the 2-position. The 1-position was then deprotected to allow the chlorination at the anomeric position forming the glycosyl donor. The glycosyl chloride was then treated with silver triflate to activate the C-Cl bond in the presence of the 1-methylthiomannoside to allow α -glycosylation. The dimannoside was subsequently deacetylated for the next glycosylation step. To form the glycosyl bromide donor, the 1-methyl- α -D-mannoside was perbenzylated followed by

acetolysis of the 6-position. The 1-position was then brominated in the presence of HBr. Finally, the α -glycosylation with the Koenigs-Knorr method was catalyzed with silver triflate to form the trimannoside (Scheme 6).

Scheme 6. Glycosylation using glycosyl bromide and glycosyl chloride donors.

1.6.1.3 Imidate intermediate strategy for glycosylation

The method of glycosylation that will be utilized in this thesis is the trichloroacetimidate strategy. This method was developed by Schimdt et al. in 1994 with proposed mechanism and conditions for stereoselectivity (Scheme 7)²⁶. It was reported that base-promoted activation of the anomeric hydroxyl-imidate intermediate traps stereochemistry for the mild acid catalyzed glycosylation in the presence of the glycosyl acceptor. It was observed that from the 1-oxide ion, the β -imidate intermediate is favored. However, through retro-reaction and anomerization of the 1-oxide in a base catalyzed fashion, the α -position imidate is produced. This led Schmidt et al. to optimize conditions with various bases such as K_2CO_3 , NaH, and 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) to favor stereoselectivity.

Scheme 7. Proposed mechanism of imidate formation by Schmidt et al.

An example of the imidate intermediate method is the synthetic scheme used by Muller et al. in 2002 for YCN-PIG^{18,27}. The first step in this approach was to attach the first mannose to the 4' hydroxyl present on the glucosamine. Facilitated by the α -imideate present on the D-mannoside, α -glycosylation was predominantly favored. To prime the compound for the next glycosylation step, the 1-position TBS group present on the glucosamine was removed with tetrabutylammonium fluoride. Next, the imideate intermediate was installed in the presence of K_2CO_3 . The β -anomeric imideate promoted β glycosylation attaching the glucosamine to the inositol in the presence of trimethylsilyl tri-flouro-methansulfonate (TMSOTf). The glycosidic bonds of the last three mannoses were formed by the same strategy of deacylating the hydroxyls with sodium methoxide at the desired positions and stereoselectively attaching the subsequent mannoses via the trichloroacetimidate method (Scheme 8).

Scheme 8. Formation of a pseudotrisaccharide utilizing imidate-promoted glycosylation.

An alternative method to installing the imidate intermediate is observed in the synthesis of a GPI anchor by Seeberger et al. that consisted of a hexasaccharide. The precursor 1,2-orthoester derivative of D-mannoside was initially deprotected to give an acetylated 2-position hydroxyl and a free anomeric hydroxyl^{28,29}. Next, the imidate was selectively added at the α -anomeric position utilizing DBU as the base to form the glycosyl donor. The α imidate then drove α glycosylation in the presence of the mild acid TMSOTf coupling the two D-mannoside derivatives. The acetal located on the distal mannose was then removed to allow the next α -selective glycosylation with the same conditions to form the tri-mannoside building block (Scheme 9).

Scheme 9. Utilizing stereoselective imidate formation to promote α glycosylation.

In the attempts to synthesize a *Trypanosoma cruzi*-derived GPI, Nikolaev et al. used the strategy of forming the tri-mannoside first. The tri-mannoside will then be coupled to a pseudodisaccharide containing the glucosamine and inositol group³⁰. To form the glycosidic linkages, an alternative method of forming α imidates was used. The formation of the tetra-mannoside required a 1,2-orthoester D-mannoside derivative. The anomeric hydroxyl group was initially deprotected with trifluoroacetic acid to allow the formation of the imidate at the α position catalyzed by the base Cs_2CO_3 . The α glycosylation was promoted by the α imidate in the presence of TMSOTf and the 2-position was subsequently deacylated with methanol and HCl to form a glycosyl acceptor. This method is repeated to promote α glycosylation forming the tri-mannoside building block (Scheme 10)^{31,32}.

Scheme 10. Cs_2CO_3 promoted installation of imidate to form glycosidic linkages of a trimannoside.

1.6.2 Formation of glucosamine

The 2-deoxy-glucosamine derivatives are essential to IPGs and can be synthesized utilizing a variety of strategies. These methods may involve precursors with pre-existing 2-position amine functionality or 2-position amine functionality can be introduced to the precursor compound. Through a series of selective protections and deprotections, the desired glucosamine donor can be achieved.

In the synthesis of a *Plasmodium falciparum* GPI derivative by Guo et al. the glucosamine was achieved by first forming 1,6-anhydro-2-azido-2-deoxy- β -glucopyranose. This was carried out using the method proposed by Beau et al. in 1992 (Scheme 11)^{33,34}. From D-glucal, O-stannylation was afforded by reflux in presence of bis-tributylstannyl oxide. The compound was then subsequently treated with iodine. It was proposed that this compound isomerizes into an epoxide yielding a free hydroxyl at the 4-position which was protected with allyl bromide. Next, the epoxide allowed the trans-diaxial azido substitution of the 2-position with sodium azide in alkaline conditions

while also exposing the hydroxyl at the 3-position. The 3-position hydroxyl was subsequently orthogonally protected via benzylation, and ring-opening was achieved by acetolysis. To convert the glucosamine into a glycosyl donor, the 1-position O-acetyl was selectively deprotected and subsequently fluorinated achieving the desired glucosamine precursor (Scheme 12).

Scheme 11. Proposed mechanism for epoxide formation by Beau et al.

Scheme 12. Formation of glucosamine donor by Guo et al.

Another method of forming the glucosamine was described by Berlin et al. in the synthesis of various glycosyl-inositol derivatives (Scheme 13)³⁵. Starting with a peracetylated D-glucal precursor, the compound was deprotected with sodium methoxide and subsequently O-benzylated with benzyl bromide. Azidonitration was then carried out with ceric ammonium nitrate and sodium azide. This formed the 2-azido nitro-glucoside via double bond addition. The nitro group was then removed using sodium nitrite to afford the free hydroxyl at the anomeric position. The desired glucosamine donor was then achieved by treating the glucosamine with trichloroacetimidate in the presence of DBU.

Scheme 13. Glucosamine derivative afforded by azido nitration.

Alternative modifications to achieve a desired glucosamine precursor can be observed in the synthesis of an IPG mediator by Martín-Lomas et al. in 2000³⁶. This method utilizes commercially available 2-dexoy-D-glucosamine hydrochloride (Scheme 14). The amine was converted into an azide by diazo transfer catalyzed by copper sulfate in the presence of triflic azide. Wong et al. proposed that copper sulfate initially forms a complex with the amine present on the glucosamine to release a protonated ligand³⁷. The nucleophilic amine group present on the copper complex then attacks the triflic azide group followed by deprotonation from the copper ligand. This results in the copper stabilized tetrazine. Breakdown of the complex results in the release of the desired azide and copper-triflyl imido complex (Scheme 15). After the diazo transfer, the masked glucosamine was subsequently peracetylated with acetic anhydride and protected this functionality was installed at the anomeric position with thiophenol in the presence of BF_3 -etherate. The compound was then subsequently deacylated and the 4- and 6-positions were protected by the formation of the benzylidene acetal followed by O-benzoylation of the 3-position. Selective ring opening utilizing sodium cyanoborohydride resulted in the 6-position O-benzyl and the free hydroxyl at the 4-position. The 4-position hydroxyl was then

[illegible]
$$\begin{array}{c}
 \text{L}_4\text{Cu} \xrightleftharpoons[\text{-HL}]{\text{RNH}_2} \left[\text{L}_3\text{Cu}-\text{N} \begin{array}{c} \text{H} \\ \diagup \diagdown \\ \text{R} \end{array} \right] \xrightleftharpoons{\text{TiN}_3} \left[\begin{array}{c} \text{Ti} \\ \diagup \diagdown \\ \text{N}^- \text{---} \text{N} \\ \diagup \diagdown \\ \text{N} \text{---} \text{N}^+ \\ \diagup \diagdown \\ \text{L}_3\text{Cu} \text{---} \text{N} \\ \diagup \diagdown \\ \text{R} \end{array} \right] \\
 \updownarrow \text{RNH}_2 \qquad \qquad \qquad \updownarrow \text{-HL} \\
 \left[\text{L}_2\text{Cu}=\text{N} \begin{array}{c} \text{Ti} \\ \diagup \diagdown \\ \text{R} \end{array} \right] \xrightleftharpoons{\text{-RN}_3} \left[\begin{array}{c} \text{Ti} \\ \diagup \diagdown \\ \text{N} \text{---} \text{N} \\ \diagup \diagdown \\ \text{N} \text{---} \text{N} \\ \diagup \diagdown \\ \text{L}_2\text{Cu} \text{---} \text{N} \\ \diagup \diagdown \\ \text{R} \end{array} \right]
 \end{array}$$

1.6.3 Formation of inositol

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Methods of obtaining inositol derivatives from underivatized *myo*- or *chiro*- inositol can be difficult to achieve due to the challenge of regioselective protection of the hydroxyl groups. A method developed by Vacca et al. to synthesize phosphorylated inositol glycosyl acceptors involved an underivatized *myo*-inositol precursor³⁸. The *myo*-inositol was protected with ethoxycyclohexane resulting in three variously protected racemic mixtures (Scheme 16). After separation, the ketal derivative of *myo*-inositol was tosylated at the 3-position and subsequently treated with (1*S*)-(-)-camphanic acid to form the 6-camphanate esters. It was noted that at this point that separation of the two diastereomers was achievable. Upon triflate inversion of the undesired isomer utilizing the tetrabutylammonium nitrate method, the desired optically pure product is afforded. Deprotection of the triflate and the camphante, followed by the installation of the protected phosphate group resulted in the desired inositol glycosyl acceptor³⁹ (Scheme 17) .

Scheme16. Racemic Products of inositol protected by ethoxycyclohexene.

Scheme 17. Utilizing D-camphanate to achieve resolution in the formation of inositol derivative.

A similar method was used by Ogawa et al. in the synthesis of a *Trypanosoma brucei* variant^{40,41}. Starting from the dicyclohexylidene inositol derivative, the 6-position was selectively p-methoxybenzylated assisted by Bu_2SnO and subsequently 1-O-allylated with vinyl bromide. Ring opening was achieved with acidic conditions followed by perbenzylation of the free hydroxyls to afford a racemic mixture. Similar to Vacca et al., deallylation of the 1-position followed by treatment of (1S)-(-)-camphanic acid afforded enantiomeric resolution and the desired diastereomer was isolated. To convert the inositol into a glycosyl donor, the p-methoxybenzyl group was removed from the 6-position and subsequently orthogonally protected via vinylation. The camphanate was removed by hydrolysis followed by 1-O-p-methoxybenzylation. Finally, the vinyl ether protecting group was removed to give a free 4-position hydroxyl for glycosylation (Scheme 18).

Scheme 18. Formation of an inositol glycosyl acceptor by Ogawa et al.

A widely use strategy is to utilize a D-xylose precursor to form the desired derivative inositol. Starting from the perbenzylated D-xylose, anomeric Wittig methylenation was carried out. Oxidation of the free hydroxyl group utilizing the Swern oxidation results in the formation of the aldehyde. The Grignard reaction yields the optically impure allyic alcohols. It was observed that, when the Grignard reaction was carried out in the presence of $\text{MgBr}_2\text{-OEt}_2$, the anti-diastereomer was favored 8:1. The alcohol was then protected with the TBS group and the epimers were separated followed by ozonolysis to afford the dialdehyde. The ring was then closed to favor the cis-diol using samarium diiodide^{19,42} (Scheme 19) .

Scheme 19. Formation of inositol derivative from D-xylose utilizing ozonolysis.

An alternative method to the D-xylose strategy was proposed by d'Alarcao et al. to achieve the desired optically pure glycosyl acceptor. The methods were identical starting from the D-xylose precursor to the Grignard reaction forming the allylic alcohols (Scheme 20). Ring closing was alternatively achieved through the Grubbs metathesis method to afford the optically pure conduritols⁴³. The diastereomers of the conduritols can be separated at this step to afford the desired *myo*- or *chiro*-inositol with better yield and one fewer step than the ozonolysis method. The free hydroxyl can then be protected with TBS and the diol was installed using osmium tetroxide. The cyclic carbonate can be installed followed by the deprotection of TBS to afford the desired glycosyl acceptor.

Scheme 20. Formation of inositol derivative utilizing Grubb's catalyst.

1.6.4 Hybrid inositol glycans (HIGAs)

In 2005, d'Alarcao et al. attempted to use IPGs as a novel drug delivery system in insulin mimetic cells⁴⁴. A commercially available non-carbohydrate fluorescent moiety, the iodoacetamide derivative of Lucifer yellow, was attached to a thiol derivative of IPG 1 forming the first hybrid inositol glycan (HIGA)(Figure 2). Lucifer yellow served both as a hypothetical drug and as a probe to determine whether or not IPGs could be successfully delivered into the cells. It was observed that the HIGA did not cross the cell membrane; therefore this particular HIGA was not a viable drug delivery system¹³. When the HIGA was subjected to the lipogenesis assay, a 47% MIR was recorded⁴⁴. The increase in insulin activity between IPG 1 and the HIGA was accredited to the negative charges present on the Lucifer yellow derivative. Thus confirming the findings by Frick et al. The HIGA was synthesized in only 15 steps, which is significantly more efficient compared to the highly insulin mimetic IPGs synthesized by Frick et al.

Figure 2. The first hybrid inositol glycan synthesized.

HIGAs are a growing area of interest because they are relatively simple to synthesize compared to synthetic IPGs, which led to the synthesis of compound 16 reported in Fulzele thesis⁴⁵. It was presumed that the placement of the charges on the HIGA synthesized by d'Alarcao et al. was critical for to the insulin mimetic activity. The goal was to design an efficient synthesis for a compound containing a cyclic phosphate that can be easily coupled to a non-carbohydrate moiety containing an ionic charge. The precursor tri-O-benzyl-6-azido-deoxy-*myo*-inositol was formed initially. The azido group present on the precursor allows Huisgen 1, 3-dipolar-cycloaddition (“click” reaction) which is an efficient method for the addition of various functional groups. Utilizing the “click” reaction, a protected thiol functionality was introduced to act as a link to the non-carbohydrate moiety, and the cyclic phosphate group was added to the diol. After global deprotection, the thiol linkage was formed with the non-carbohydrate moiety 1,5-IADEN to form the desired HIGA. Various other non-carbohydrate moieties can be added to further investigate the correlation of insulin mimetic activity and positioning of ionic

charges on the non-carbohydrate moieties. HIGAs is a versatile group of synthetic IPGs that can be readily synthesized and manipulated to study potential insulin mimetic effects (Scheme 21).

Scheme 21. Retrosynthetic scheme of HIGA compound 16 from Fulzele Thesis.

CHAPTER 2

RESEARCH GOAL AND SYNTHETIC SCHEME

2.1 Library Synthesis of Thio-linked HIGAs

The goal was to synthesize a library of five novel HIGAs by attaching various commercially available thiol reactive non-carbohydrate moieties to a thiol derivative of the disaccharide synthesized by d'Alarcao et al. Three of the five non-carbohydrate moieties are negatively charged (Fluorescein-5-maleimide, IAEDANS, and Alexa Fluor 350 C5-maleimide), and the other two are net neutral (DACM and N-(1-pyrene)maleimide) (Figure 3). The purpose of using both charged and non-charged moieties was to determine whether the negative charges contribute to greater insulin activity.

Figure 3. The five fluorescent non-carbohydrate moieties that are linked to the disaccharide to form the desired HIGAs. The three on the top are negatively charged at pH 7 while the two on the bottom are neutral at pH 7.

An efficient synthetic scheme to the disaccharide containing the glucosamine and *myo*-inositol has been well established by d'Alarcao et al. at Tufts University (Scheme 22). The final steps to the synthesis of the disaccharide require the formation of a cyclic phosphate followed immediately by dissolving-metal reduction to globally remove the protecting groups. This step, however, exposes the disaccharide to harsh conditions. The cyclic phosphate group is susceptible to hydrolysis below pH 5 or above pH 8. The thiol group was chosen as the linker group for this reason. At pH 7, the cyclic phosphate group would be safe while the thiol group would be far more nucleophilic at pH 7 than the free hydroxyls and the amine group present in the disaccharide yielding a selective linkage at the 6-position (Figure 4). Another advantage of the thiol linkage is its ability to react readily with fluorescent moieties that are commercially available.

Scheme 22. Retrosynthesis of IPG 1 depicting the global deprotection followed by the introduction of the cyclic phosphate.

Figure 4. The thiol derivative of IPG 1 target used as a linker for non-carbohydrate attachment.

2.2 Formation of HIGA

The HIGA consists of three main groups: the thiol derivative glucosamine group, the cyclic phosphate containing *myo*-inositol, and the non-carbohydrate moiety. The thiol derivative glucosamine was synthesized initially from a glucosamine precursor while the *myo*-inositol group was synthesized separately in our lab. The *myo*-inositol moiety originates in the glycosyl acceptor **18**, which was attached via an alpha linkage to the glucosamine glycosyl donor **13** (Scheme 23). Finally, the non-carbohydrate moiety was attached forming the desired HIGA.

Scheme 23. Retrosynthetic analysis of the thiol derivative of IPG-1 leading to the glucosamine glycosyl donor and then *myo*-inositol glycosyl acceptor.

The synthesis of the glycosyl donor **13** was carried out by first protecting the amine group present on the glucosamine precursor **1** via diazotransfer to give **2** (Scheme 24). Compound **2** was then peracetylated in presence of acetic anhydride to afford **3**. Selective deacetylation of the anomeric position was carried out using gaseous ammonia to yield **4**. The free hydroxyl group was then protected using tertbutylsilyl chloride forming **5**.

Global deacetylation with sodium methoxide resulted in the triol **6**. The 4' and 6' hydroxyl groups were protected using benzylidene dimethyl acetal to yield **7**, and the benzylidene ring was then selectively opened to the free 6'-hydroxyl compound **8**. The 6'-hydroxyl group was made into a good leaving group via treatment with tosyl chloride, which led to **10**. The tosyl group was then replaced with lithium benzylthiolate to afford **11**. The anomeric position was deprotected using tetrabutyl ammonium fluoride, giving **12**. The glucosamine group was converted into a glycosyl donor by treatment with trichloroacetonitrile to produce trichloroacetimidate **13** (Scheme 23).

Trimethylsilyl trifluoromethanesulfonate-promoted glycosylation of *myo*-inositol **18** with the glucosamine donor **13** selectively yielded the α product **14**. The cyclic carbonate group present on the *myo*-inositol group was subjected to hydrolysis with lithium hydroxide to yield the diol **15**. The diol group was converted into a cyclic phosphate group in the presence of methylpyridinium phosphorodichloridate followed by a global deprotection using dissolving metal reduction method to yield disulfide **16**, presumably because of air oxidation upon work-up. The disulfide was cleaved by treating **16** with tris(2-carboxyethyl)phosphine, and the fluorescent non-carbohydrate moieties are attached via thiol linkage in the presence of 25mM HEPES buffer (Scheme 24).

Scheme 24. Synthesis of Thiol-linked HIGAs. Reagents and conditions: (A) TiN_3 , H_2O , CuSO_4 , Et_3N (B) Ac_2O , pyr (C) NH_3 , THF, MeOH (D) TBSCl, Im, CH_2Cl_2 (E) NaOMe, MeOH (F) BDMA, p-TsOH, CH_3CN (G) 1. NaH, THF 0°C to rt, 2. BnBr, TBAI, 0°C to rt (H) BH_3NHMe_2 , $\text{BF}_3 \cdot \text{OEt}_2$, CH_2Cl_2 (I) TsCl, Pyr (J) BnSLi, THF (K) TBAF, AcOH, THF (L) Cl_3CCN , K_2CO_3 (M) 18, TMSOTf, Et_2O (N) LiOH, H_2O , THF (O) 1. $\text{PCl}_2\text{O}_2^- \text{MePy}^+$, 2. Na, NH_3 , THF -78°C , 3. NH_4Cl (s), -78°C , 4. CH_3OH , -78°C to rt (P) "X", 25mM HEPES, TCEP pH 7.1, rt (X= non-carbohydrate moiety).

CHAPTER 3

DISCUSSION

3.1 Alternative Method to Protecting 6' Hydroxyl

Scheme 25: Dimethoxytritylation as alternative route to protecting the 6' hydroxyl.

Dimethoxytritylation of the 6' hydroxyl was pursued initially to form the protected 6' glucosamine. The tritylation method was thought to be a more favorable route compared to the benzylidene reaction due to the inefficient ring-opening step that occurs later on in the synthetic scheme. However, after attempting the reaction, the product obtained was unstable during analytical techniques. Degradation was observed on the TLC plates. This was most likely due to the acidic nature of silica, and during NMR analysis the product did not dissolve in CDCl_3 . Due to poor yield and stability of the tritylated product, the benzylidene route was pursued instead.

3.2 Benzylidene Ring Opening

Scheme 26: Possible regio-isomers that results from the benzylidene ring opening reaction.

There are many possible complications that may arise with the benzylidene ring opening reaction. The acidic nature of the BF_3 -etherate reagent may cause degradation due to the sensitivity of the silyl group present at the anomeric position (which was observed). The ring may also open to the unwanted 4'-OH product (Scheme 26). To further complicate the situation, the NMR spectrum of the 4'-OH isomer and the 6'-OH isomer are nearly identical. To verify that the desired compound was synthesized, the product was acylated. Theoretically, if the compound were the desired 6'-acylated isomer, the two geminal 6' hydrogen peaks should shift further downfield and if it were the 4'-acylated product, only one peak would shift significantly further downfield. Upon acylation of the recovered compound, it was observed that two peaks had shifted downfield confirming that the desired product was synthesized.

3.3 Alternative Route to Protecting 3' Hydroxyl

Scheme 27. Paramethoxybenzylation as an alternate route to protecting the 3'hydroxyl.

The 3' hydroxyl group can be alternatively paramethoxybenzylated. The paramethoxybenzylation reaction required the formation of a paramethoxybenzyl-trichloroacetimidate reagent, which proved difficult to form. The reaction was extremely inconsistent, resulting in poor yields. It was observed during TLC monitoring of the reaction that the paramethoxybenzyl-trichloroacetimidate reagent was decreasing in

concentration while a spot at the baseline began to form. It was evident that the reagent was degrading before it could react with the starting material to form the desired product. The paramethoxybenzylation reaction also required the acidic reagent BF_3 -etherate, which may cleave the TBS group, resulting in further unwanted degradation products. This reaction was inefficient, resulting in poor yields and a high degree of degradation of the starting material and the reagent was observed. Therefore, this route was not pursued any further.

3.4 Alternative 6' Linkage

Scheme 28. Using amine as an alternative 6' linkage group.

An alternative 6' linkage was sought after due to the difficulty of preparing the BnSLi solution. Also, due to the sensitivity of the phosphorylated compound to hydrolyze at pH

values less than 5 and greater than 8, neutral conditions are ideal when adding the non-carbohydrate moiety. The neutral conditions led to disulfide formation of the thiol compound at the 6' position, and as previously reported by Turner, it was difficult to separate the dimer and purify the desired product.

The strategy proposed to circumvent these problems was to attach phthalimide at the 6' position, which would then be readily hydrazinolized to generate an amine nucleophile (Scheme 28). The 6'tosyl group was expected to be readily displaced using potassium phthalimide. After coupling of the glucosamine with the inositol acceptor, lithium hydroxide and water can hydrolyze the cyclic carbonate function to yield a diol, and the phthalimide can be hydrazinolized to release a nucleophilic amine ready for non-carbohydrate linkage. The diol can then be phosphorylated, and subsequently, the non-carbohydrate moiety can be added. The major downside occurs at this step because the non-carbohydrate moiety is added prior to global deprotection. These conditions might be too harsh for the non-carbohydrate moiety. Also, compared to thiol linkage, the amine linkage requires three additional steps to form the HIGA from a common precursor, while the thiol linkage only requires one additional step.

The method used was adopted from Coxon et al. with modifications to the reaction conditions⁴⁶. Due to the relatively bulky nature of potassium phthalimide, this reaction did not go to completion at room temperature. To push this reaction forward, the amount of potassium phthalimide and heating were adjusted to find optimal conditions. The first attempt at this reaction was carried out with a 1:1 molar ratio of the glucosamine and potassium phthalimide while incrementally increasing the temperature by 10°C per hour

starting at 45°C. No reaction was observed in the first attempt. Degradation was noted that at 80°C as a baseline spot began to form on the TLC plate. Degradation was most likely due to the instability of the TBS group, and a combination of slight exposure of the reaction mixture to air and heat may have promoted hydrolysis. For the second attempt, a 1:2 molar ratio of the glucosamine and potassium phthalimide was added to the reaction mixture. The temperature was increased by 5°C incrementally every hour starting at 45°C. After an hour at 65°C, it was observed the reaction began to move forward and degradation began to occur. After increasing the temperature to 75°C, degradation was occurring at a greater rate than desired product formation. The conditions for this experiment were optimized at 65°C for 3 hours with a 1:2 glucosamine to potassium phthalimide molar ratio. The yield for this reaction was 46%.

CHAPTER 4

GLUCOSE UPTAKE ASSAY

The glucose uptake assay is a method used to measure the effectiveness of the HIGAs *in vitro*. The activity of glucose transporter isoform 4 (GLUT4) will reflect the level of insulin-like activity produced by the HIGAs. GLUT4 is unique compared to other glucose transporters. They are present in vesicles within insulin sensitive tissues such as skeletal tissues and adipose tissue while other glucose transporters are most commonly found on the plasma membrane⁴⁷. The cell line chosen to carry out the glucose uptake assay was the 3T3-L1 mouse fibroblast cell line. Due to ability of the 3T3-L1 cell line to differentiate from their pre-adipocyte fibroblast phenotype to the adipocyte phenotype, this cell line is widely used to study glucose transport.

4.1 3T3-L1 Differentiation

For the experiment, frozen 3T3-L1 preadipocyte cells were purchased from ZenBio Inc. Upon thawing of the cells, the cells were distributed into cell culture dishes at approximately 5,000 cells/cm² with the cells suspended in preadipocyte medium containing DMEM, HEPES, bovine calf serum, penicillin, streptomycin and amphotericin B. The cell cultures were maintained in an incubator at 37°C with 10% CO₂ until the cells reached confluency⁴⁸. After an additional two days of incubation, differentiation of the fibroblasts into adipocyte cells was initiated by the introduction of differentiation medium containing DMEM, HEPES, fetal bovine serum, biotin, pantothenate, human insulin, dexamethasone, penicillin, streptomycin, amphotericin B, 3-isobutyl-1-methylxanthine (IBMX), and PPAR- γ agonist. Dexamethasone and IBMX are

vital to the differentiation medium because they are both regulators of transcription factors associated with the CCAAT/enhancer binding proteins (C/EBPs). Dexamethasone is a synthetic glucocorticoid with enhanced binding affinity to the glucocorticoid receptor and promotes C/EBP γ gene expression upon binding. IBMX is a phosphodiesterase inhibitor that induces C/EBP β gene expression, leading to differentiation of adipocyte cells⁴⁹. Fully differentiated cells were maintained for another seven days in adipocyte medium containing DMEM, HEPES, fetal bovine serum, biotin, pantothenate, human insulin, dexamethasone, penicillin, streptomycin, and amphotericin B.

4.2 Developing the Glucose Uptake Assay

The glucose uptake assay methodology was adopted from Müller et al. with slight modifications⁴⁹. Differentiated adipocyte cells were dislodged from the petri dish by treatment of trypsin and subsequently washed twice with PBS. Next, the dislodged cells were treated with 20 μ M cytochalasin B to disrupt any prior tubulin in order to measure specific glucose uptake activity promoted by insulin or the HIGA. As a control, another set of cells was not treated with cytochalasin B. Cells were subsequently incubated in 200nM insulin for 30 minutes at 37°C and, as a control, another set of cells was not treated with insulin. After the stimulation, the cells were incubated with 0.33 μ Ci/ml of 2-deoxy tritiated glucose for 20 minutes. It is important to use 2-deoxy tritiated glucose because once glucose has entered the cell, it may be broken down by various metabolic pathways 2-deoxy glucose will however, bypass these metabolic pathways. The cells were subsequently washed three times with ice cold PBS. Glucose uptake activity was measured by initially lysing the cells with 0.2M NaOH. A 30 μ L volume aliquot of cell

lysate was taken for Bradford assay to measure the amount of protein present while the rest of the cell lysate was added to 8 mL of scintillation cocktail. Results from the scintillator gave counts of tritiated 2-dexoy glucose per minute.

To develop the glucose uptake assay, the idea was to conduct the experiment in three phases (Table 1). In the first phase, cells were either treated with insulin or without insulin. The purpose of this phase was to confirm if the presence of insulin would result in an increase in glucose uptake activity. As well as provide information on basal glucose uptake activity in the absence of insulin to act as the negative control.

The next phase involved cells that were not treated with insulin, cells that were treated with insulin, cells that were treated with cytochalasin B, and cells treated with both cytochalasin B and insulin. GLUT-4 transporters are present on the cell membrane and within vesicles in the cytoplasm at basal level. The treatment with cytochalasin B would disrupt tubulin activity, preventing further translocation of GLUT-4 to the cell membrane. Cells treated solely with cytochalasin B would exhibit glucose uptake activity by pre-existing GLUT-4 on the cell membrane. Under the treatment of cytochalasin B in conjunction with insulin, cells would exhibit glucose uptake activity of GLUT-4 present on the cell membrane and activity induced by insulin. The cells treated with only insulin would exhibit glucose uptake activity by the GLUT-4 already present on the cell membrane, GLUT-4 in the cytoplasm, and glucose uptake activity induced by insulin. Therefore, taking the difference in activity from cells that were treated with only insulin by the activity from the cells that were treated with both insulin and cytochalasin B would yield information on specific glucose uptake activity induced by insulin.

In the final phase, the conditions were replicated from the second phase. The difference between the second phase and the final phase is the additional set of cells that are treated with HIGA in the presence or absence of cytochalasin B. This data will give the specific glucose uptake activity induced by the HIGA of interest and by comparing the specific glucose activity induced by the HIGA with specific glucose activity induced by insulin, this will determine the maximal insulin response exhibited by the HIGA.

Table 1. Planned Phases to Develop Glucose Uptake Assay

	2[3H]-DG	Cytochalasin B	Insulin	HIGA
Phase 1				
Plate 1	✓			
Plate 2	✓		✓	
Phase 2				
Plate 1	✓			
Plate 2	✓	✓		
Plate 3	✓		✓	
Plate 4	✓	✓	✓	
Phase 3				
Plate 1	✓			
Plate 2	✓	✓		
Plate 3	✓	✓	✓	
Plate 4	✓		✓	
Plate 5	✓	✓		✓
Plate 6	✓			✓

Note: Check marks represent what each plated with be treated

4.3 Results of Glucose Uptake Assay

Unfortunately, a reproducible glucose uptake assay was not established. One of the problems occurred during the Bradford Assay, which relies on a standard curve based on absorbance readings when coomassie blue dye reacts with known concentrations of

bovine serum albumin. The colorimeter that was used failed to give consistent absorbance readings of a single sample leading to an unreliable standard curve as well as unreliable readings for cell lysate samples. The major problem was contamination occurring frequently during the cell differentiation, and mature adipocyte cells were only achieved on two separate occasions. With these problems, only phase one and phase two were attempted.

Phase one of the glucose uptake assay was carried out with two sets of cells treated with insulin (WI1/WI2) and with two sets of cells that were not treated with insulin (WOI1/WOI2). Results obtained were as expected as cells that were treated with insulin on averaged had about 5 times greater count per min to microgram of protein than the cells that were not treated with insulin. There was some variation, however, since the count per min to microgram of protein for WI1 was about 2 times greater than WI2 (Table 2). It is ideal to have roughly the same count per min to microgram of protein with in the sets. This variation was also seen with the cells that were not treated with insulin.

Table 2. Results From Phase 1 of Glucose Uptake Assay

Phase 1 Results				
Sample	Concentration(μ g of protein/mL)	μ g of protein	Counts per minute(cpm)	cpm/ μ g of protein
WI1	79	2.4	4950	2100
WI2	130	4.0	4904	1300
WOI1	120	3.7	963	260
WOI2	110	3.3	1242	370

The second phase of the experiment was attempted with four sets of cells (WOI, WOI/WC, WI, and WI/WC). It would be ideal to have at least duplicates of each set of cells, however, contamination occurred in eight out of the twelve plates. Another problem occurred when the colorimeter was giving sporadic absorbance readings and the amount

of protein for each plate was unreliable. The scintillation data showed that cells that were treated with insulin had a greater count per minute than cells that were treated with both insulin and cytochalasin B (Table 3). However, cells without insulin had higher cpm than those with insulin. This could be attributed to differences in cell number, but we could not confirm this because of the problems with the absorbance measurements in the Bradford assay.

Table 3. Results From Phase 2 of Glucose Uptake Assay

Phase 2 Results	
Sample	cpm
WOI	23066
WOI/WC	17148
WI	18866
WI/WC	8505

CHAPTER 5

CONCLUSION / FUTURE WORK

Hybrid inositol glycans have been previously shown to elicit insulin mimetic effects. The goal of this study was to synthesize a library of novel HIGAs to explore the effects of the various non-carbohydrate moieties in hopes of increased potency of the insulin mimetic effects. Another goal of this study was to establish a glucose uptake assay that is capable of measuring the insulin-like effects of the HIGA. Unfortunately, a library of the desired HIGAs was not completed and a reliable glucose uptake assay was not established. A novel strategy was proposed to replace the 6'-thiol linkage with an amine linkage to avoid dimerization. However, further steps were not attempted on the 6'-amine linkage to determine whether this method is a more convenient way to form the glycosyl donor. The furthest point in the synthetic scheme achieved was the formation of the 6'-phthalimide compound.

Future work for this project would be to form the inositol acceptor starting from the conduritol and attempt the coupling of the glycosyl donor and inositol acceptor. Once that has been completed, reduction of phthalimide and linkage to a non-carbohydrate moiety can be carried out. It would be interesting to find optimal conditions for the global deprotection while also finding non-carbohydrate moieties that are able to sustain those conditions. After the successful synthesis of the HIGAs, it is important to establish a reproducible glucose uptake assay. The most important factor is to reduce contamination during cell culturing and to have a reliable colorimeter at hand.

CHAPTER 6

EXPERIMENTAL

6.1 General Methods

All reagents and solvents were purchased from Sigma Aldrich or Fischer Scientific and were not further purified unless specified. All non-aqueous reactions were carried under argon or nitrogen atmosphere. Starting materials for the non-aqueous reactions were evaporated three times with toluene at 10-15 Torr and 45°C. Reactions were monitored by using thin layer chromatography on Baker glass backed silica plates (0.25mm) with a 254nm fluorescent indicator. Purification of the compounds was carried out via TLC with Baker glass backed silica plates (0.25mm) or flash chromatography with Baker silica gel (particle size 40-50 nm). ¹H NMR data were obtained using Inova400 MHz spectrometer and samples were dissolved in CDCl₃. Mass spectrometry data were obtained using electrospray ionization.

6.2 Procedures

2-azido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (3)

Triflic azide was prepared by dissolving sodium azide (10.857 g, 0.167 mol) in 200 mL of dry acetonitrile at 0°C and was allowed to stir for 15 min. Through a dropping funnel, trifluoromethane sulfonic anhydride (23.4 ml, 0.139 mol) was added to the solution drop wise for 30 min. Once all the contents were added to the reaction flask, the

reaction was maintained at 0°C to yield a yellow solution. The diazotransfer was carried out by dissolving **1** (24.99 g, 0.116 mol) in 120 mL of water. Cu(II)SO₄ (0.2005 g, 0.125 mmol) and triethylamine (32.4 mL, 0.236 mol) was added to the glucosamine solution and stirred at 0°C. Triflicazide was then added drop wise through a dropping funnel for 10 min and the ice bath was removed. The reaction was allowed to run for 16 h before adding glycine (20.36 g, 0.271 mol) to quench the reaction and allowed to stir for another 12 h. The reaction mixture was filtered and solvent was removed using rotary evaporation. The yellow crude was used for the next reaction without purification. The peractylation reaction was carried out by transferring 235 mL of dry pyridine into the reaction flask containing the diazo compound via cannula. Acetic anhydride was added through a dropping funnel over 45 min and the solution was allowed to stir at 0°C. Once all of the acetic anhydride was added, the reaction was allowed to stir for another 15 min and the ice bath was removed. The reaction went to completion after another 18 h of stirring at room temperature. The reaction was worked up by initially washing the crude with NaHCO₃. The solution was filtered and transferred to a separatory funnel to be extracted with dichloromethane (5 x 40 mL). The organic layer was collected and washed with 1 M HCl (2 x 500 mL) to remove residual pyridine. The organic solution was dried with MgSO₄. Flash chromatography was carried with a 2.5% EtOAc : 97.5% (R_f=0.18) dichloromethane solvent system to yield 30.83 g (71% yield over two steps). ¹H NMR δ 2.02 (s, 3H, Ac), 2.07(s, 3H, Ac), 2.09 (s, 3H, Ac), 2.18 (s, 3H, Ac), 3.66 (m, 1H, H-6a), 3.79 (dd, 1H, H-5), 4.07 (dd, 1H, H-6b), 4.29 (dd, 1H, H-2), 5.12-4.99 (m, 2H, H-3, H-4), 5.54 (d, 1H, H-1).

3,4,6-Tri-O-acetyl-2-Azido-2-deoxy-1-O-tert-butylidimethylsilyl-β-D-glucopyranoside (5)

Compound **3** (4.33 g, 11.60 mmol) was dissolved in 43 mL of a dry THF/methanol solvent mixture (7:3) at 0°C. Gaseous anhydrous ammonia was then bubbled through for 20 min. Shortly after, argon was bubble through to remove residual gaseous ammonia and the solvent was removed using rotary evaporation. The brown oil crude was used to carry out the silylation reaction without purification. The brown oil was evaporated 2x with toluene and dissolved in 22 mL of dry dichloromethane. Imidazole (1.44 g, 21.15 mmol) was added followed by tert-butyldimethylsilyl chloride (1.90 g, 12.66 mmol) and the reaction was allowed to run for 2h to completion. The reaction was worked up by initially diluting the reaction with 10 mL of EtOAc. Next, the mixture was washed with water and extracted with 1M HCl (3x 35 mL). Finally, the organic layer was washed one more time with water and dried with MgSO₄. The solvent was removed using rotary evaporation and the compound was purified using flash chromatography with a 30% methanol: 70% dichloromethane solvent system (R_f=0.20) to yield 2.88 g of pure compound (55.7% yield). ¹H NMR δ 0.15 (s, 6H, TBS), 0.95 (s, 9H, TBS) 2.00 (s, 3H, Ac), 2.08(s, 3H, Ac), 2.10 (s,3H,Ac), 3.40(m, 1H, H-6a), 3.64(dd, 1H, H-5), 4.09(dd, 1H, H-6b), 4.20(dd, 1H, H-2), 4.63(d, 2H, H-1) , 4.90(m, 1H, H-3,H-4)(Figure 5).

2-Azido-2-deoxy-1-O-tert-butylidimethylsilyl-β-D-glucopyranoside (6)

A 25% sodium methoxide solution was prepared by dissolving sodium metal (0.095 g, 4.1 mmol) in 5 mL of dry methanol. In a separate flask, **5** (0.1035 g, 0.232 mmol) dissolved in 0.1 mL of dry methanol. 0.9 mL of the sodium methoxide solution was transferred into the reaction flask containing **5** and allowed to stir for 16 min. The reaction was worked up by adding DOWEX-50 acidic resin to the solution until the pH reached 4-6. The DOWEX resin was then filtered out and the solvent was removed by rotary evaporation to yield a yellow oil. The crude was subjected to flash chromatography with a 12% methanol: 88% dichloromethane solvent system ($R_f = 0.23$) to yield 65 mg of pure product (86% yield).

2-Azido-2-deoxy-6-O-(4,4'-dimethoxytrityl)-1-O-tertbutyldimethylsilyl-β-D-glucopyranoside

Compound **6** (50.1 mg, 0.157 mmol) was dissolved in 1.1 mL of dry pyridine. DMTCl (66 mg, 0.195 mmol) was then added to the solution and allowed to stir for 6 h. An additional amount of DMTCl (66 mg, 0.195 mmol) was added and after 30 h. The reaction failed to go to completion and was stopped by adding 1 mL of ice-cold water. The solution was extracted with chloroform (5 x 1 mL) and the combined organic layers

were then washed with brine and evaporated by rotary evaporation. The contents were then triturated with heptane and the recovered crude was subjected to flash chromatography with a 30% EtOAc: 70% hexanes solvent system ($R_f=0.25$). 46.4 mg of pure product was recovered (44% yield). ^1H NMR δ 0.15 (s, 6H, TBS), 0.95 (s, 9H, TBS), 2.6 (bs, 1H), 2.65 (bs, 1H), 3.2-3.7 (m; 6H; H-2, H-3, H-4, H-5, H6a, H-6b), 3.8 (s, 3H, DMT), 4.55 (d, 1H, H-1), 6.7-7.5 (m, 13H, DMT).

2-Azido-2-deoxy-4,6-O-benzylidene-1-O-tert-butyldimethylsilyl- β -D-glucopyranoside (7)

Compound **6** (753 mg, 2.36 mmol) was coevaporated with toluene and then dissolved in 16.8 mL of dry acetonitrile in the presence 4-Angstrom molecular sieves. In a separate flask, p-toulenesulfonic acid monohydrate (11 mg, 0.058 mmol) was evaporated 2x with toulene and dissolved in 5 mL of dry acetonitrile. 0.3 mL of the p-toulene sulfonic acid solution was transferred into the flask containing **6** and the reaction was allowed to stir for 36 h. The reaction was worked up by adding sodium carbonate (105 mg) to quench the reaction for 30 min. Sodium bicarbonate was filtered out and solvent was evaporated. The crude was purified using flash chromatography with a 0.1% triethylamine: 19.9% ethylacetate: 80% hexanes solvent system($R_f=0.20$). 850 mg of pure product was recovered (88% yield). ^1H NMR δ 0.17 (s, 3H, TBS), 0.18 (s, 3H, TBS), 0.96 (s, 9H, TBS), 2.90 (bs, 1H, OH), 3.22-3.82 (m; 5H; H-2, H-3, H-4, H-5, H-6), 4.29 (dd; 1H; H-

6), 4.61 (d, 1H, H-1), 5.41 (s, 1H, acetal), 7.20-7.55 (m, 5H, Ph)(Figure 6).

Figure 6. ¹H NMR spectra of 2-Azido-2-deoxy-4,6-O-benzylidene-1-O-tert-butylidimethylsilyl-β-D-glucopyranoside.

2-Azido-2-deoxy-4,6-O-benzylidene-3-O-p-methoxybenzyl-1-O-tert-butylidimethylsilyl- β -D-glucopyranoside

P-methoxybenzyltrichloroacetimidate was formed by dissolving 4-methoxybenzyl alcohol (1.500 g, 10.86 mmol) in 15 mL of dry dichloromethane. 1.5 mL of a 50% KOH solution was then added along with tetra-n-butyl ammonium hydrogen sulphate (15 mg, 0.044 mmol) and stirred at -15 °C. After 5 min, trichloroacetonitrile was added (9 mL, 0.090 mol) and the reaction was allowed to stir for another 30 min at -15 °C. After 30 min, the dried ice bath was removed and the reaction was allowed to run for 24 h. The solution was extracted with dichloromethane (5 x 10 mL) and the combined organic layer was dried over MgSO_4 . MgSO_4 was filtered off and the resulting solution was passed through a thin silica pad and washed with 20 mL of dichloromethane. In a separate flask containing **7** (315 mg, 0.77 mmol), p-methoxybenzyltrichloroacetimidate (1.5 mL, 7.22 mmol) was added in the presence of 4-Ångström molecular sieves. The contents were then dissolved in 4.7 mL of dry dichloromethane and BF_3 etherate was added at 0°C. The ice bath was then immediately removed. After 18 h, additional amounts of p-methoxybenzyltrichloroacetimidate (1.5 mL, 7.22 mmol) was added but the reaction still failed to go to completion. After 36 h, the reaction was stopped by adding 3 mL of triethylamine. The solvent was then evaporated using rotary evaporation to yield a yellow oil. The crude was purified using flash chromatography with a 0.1% triethylamine: 6.9%

EtOAc : 93% hexanes solvent system ($R_f = 0.18$) to yield 94 mg of pure product (23% yield). ^1H NMR δ 0.12 (s, 3H, TBS), 0.13 (s, 3H, TBS), 0.91 (s, 9H, TBS), 3.29-3.42 (m; 2H; H-5, H-6a), 3.49 (t, 1H, H-4), 3.68 (t, 1H, H-2), 3.76 (t, 1H, H-3), 3.80 (s, 3H, PMB), 4.26 (dd; 1H; $J=4.99, 10.5\text{Hz}$, H-6b), 4.57 (d, 1H, $J=7.59\text{Hz}$, H-1), 4.78 (d, 1H, PMB), 4.88 (d, 1H, $J=11.39\text{Hz}$, PMB), 5.51 (s, 1H, acetal), 6.8-7.5 (m, 10H, Ph/PMB).

2-Azido-3-O-benzyl-4,6-O-benzylidene-2-deoxy-1-O-tert-butyldimethylsilyl- β -D-glucopyranoside (8).

Compound **7** (474 mg, 1.16 mmol) was evaporated 2x with toluene and dissolved in 23 mL of dry THF. At 0°C , NaH (278.9 mg, 11.62 mmol) was added and the ice bath was then immediately removed. After the reaction was allowed to reach room temperature, BnBr (350 μL , 2.95 mmol) was added at 0°C . Once BnBr was added, the ice bath was once again removed allowing the reaction to reach room temperature and TBAI (16.7 mg, .045 mmol) was subsequently added. The reaction was allowed to run for 5.5 h to completion and the reaction was stopped by adding 25 mL of ice cold water at 0°C . The solution was extracted with EtOAc (5 x 25mL) and the combined organic layers were dried using MgSO_4 . MgSO_4 solvent was removed using rotary evaporation to yield a yellow/white heterogeneous solid. Dry packing method was necessary when loading the compound into the column for flash chromatography. A 4% EtOAc: 96% hexanes solvent system was used to recover 500 mg of pure compound (86.3% yield). ^1H NMR 0.15 (s, 3H, TBS), 0.16 (s, 3H, TBS), 0.91 (s, 9H, TBS), 3.3-3.41 (m; 2H; H-5, H-6a), 3.50 (t,

1H, H-4), 3.69 (t, 1H, H-2), 3.78 (t, 1H, H-3), 4.28 (dd; 1H; H-6b), 4.57 (d, 1H, H-1), 4.78 (d, 1H, Bn), 4.84 (d, 1H, J=11.4Hz, Bn), 5.55 (s, 1H, Acetal), 7.2-7.5 (m, 10H, Bn/Ph).

2-Azido-3,4-di-O-benzyl-2-deoxy-1-O-tert-butyldimethylsilyl- β -D-glucopyranoside (9).

Compound **8** (134 mg, 0.269 mmol) was evaporated 2x with toluene. BH_3NH_3 was added to reaction flask and dissolved in 2.8 mL of dry dichloromethane. BF_3 etherate was then added in a dropwise fashion and the reaction was allowed to run for 15 min. After the reaction reached completion, 4 mL of EtOAc was added to dilute the reaction and the solution was then washed with NaHCO_3 (3 x 5 mL). The organic layers were combined and then extracted with NaCl (2 x 5mL) and dried with MgSO_4 . Flash chromatography was used to purify the crude (yellow oil) with a 4% EtOAc: 96% hexanes solvent system ($R_f=0.10$) to recover 0.103 mg of pure product (77% yield). ^1H NMR 0.155 (s, 3H, TBS), 0.163 (s, 3H, TBS), 0.94 (s, 9H, TBS), 3.2-3.9 (m; 6H; H-2, H-3, H-4, H-5, H6a, H-6b), 4.54 (d, 1H, H-1), 4.63 (d, 1H, Bn), 4.95 (d, 1H, Bn), 4.84 (d, 1H, Bn), 4.89 (d, 1H, J=7.8Hz, Bn), 7.20- 7.45 (m, 10H, Bn).

2-Azido-2-deoxy-3,4-di-O-benzyl-6-tosyl-6-deoxy-1-O-tert-butyldimethylsilyl- β -D-glucopyranoside (10).

Compound **9** (25.8 mg, 0.052 mmol) was evaporated 2x with toluene and dissolved in 1.2 mL of dry pyridine. P-toulenesulfonyl chloride (0.128 g, 0.671 mmol) was added at 0°C and the ice bath was removed immediately after. The reaction was allowed to run for 21 h to completion and was quenched with 2 mL of ice cold water at 0°C. The solution was then extracted with chloroform (5 x 4 mL) and the combined organic layers were dried with MgSO₄. The solvent was evaporated using rotary evaporation and the yellow oil crude was purified using preparatory thin layer chromatography with a 3% EtOAc: 97% hexanes solvent system (R = 0.23). The purification yielded 22 mg of a yellow oil (65% yield). ¹H NMR δ 0.095 (s, 3H, TBS), 0.114 (s, 3H, TBS), 0.903 (s, 9H, TBS), 2.43 (s, 3H, Ts), 3.2- 3.5 (m; 4H; H-2, H-3, H-4, H-5), 4.06 (dd, 1H, H-6a), 4.16 (dd, 1H, H-6b), 4.45 (d, H-1), 4.50 (d, 1H, Bn), 4.75 (d, 1H, J=10.8Hz, Bn), 4.80 (d, 1H, Bn), 4.90 (d, 1H, Bn), 7.1-7.4 (m, 14H, Bn).

2-Azido-2-deoxy-3,4-di-O-benzyl-6-thiobenzyl-6-deoxy-1-O-tert-butyldimethylsilyl- β -D-glucopyranoside (10).

66 μ L of BnSH (0.556 mmol) was dissolved in 0.450ml of dry THF and at 0°C. 1.03 mL

of n-butyllithium in hexanes (0.556 mmol) was added to prepare the BnSLi solution. The ice bath was removed after the addition of n-butyllithium and the reaction was stirred at room temperature for 90 min. Separately, compound **10** (36.3 mg, 0.0556 mmol) was evaporated 2x with toluene and at 0°C, all of the BnSLi solution was added to the reaction flask. After 5 min, the ice bath was removed and allowed to react for 46 h at room temperature. The reaction flask was cooled to 0°C and 1 mL of ice cold water was added to quench the reaction. The mixture was then extracted with chloroform (3 x 1 mL) and the combined organic layer was dried with MgSO₄. The mixture was concentrated *in vacuo* to yield a yellow oil. The oil was purified using preparatory thin layer chromatography using 80% hexanes: 10% ethyl acetate solvent system leading to the recovery of 15 mg (45% yield) of pure compound. ¹H NMR δ 0.21 (s, 6H, TBS), 0.95 (s, 9H, TBS), 2.53 (dd; 1H; H-6a), 2.79 (dd; 1H; H-6b), 3.3-3.5 (m; 4H; H-2, H-3, H-4, H-5), 3.71 (d, 1H, SBn) 3.77 (d, 1H, J=13.4Hz, SBn), 4.49 (d, 1H, H-1), 4.52 (d, 1H, Bn), 4.76 (d, 1H, Bn), 4.82 (d, 1H, Bn), 4.84 (d, 1H, Bn), 7.1-7.4 (m, 15H, Bn).

2-Azido-2-deoxy-3,4-di-O-benzyl-6-phthalimide-6-deoxy-1-O-tert-butylidimethylsilyl-β-D-glucopyranoside.

Compound **10** (12.8 mg, 0.020 mmol) was evaporated 3x with toluene and dissolved in 110 μL of hexamethylphosphoramide. Potassium phthalimide (8.89 mg, 0.048 mmol) was added to the reaction flask and allowed to react at 65°C for 3 h. Once the reaction

was complete, the mixture was cooled using ice bath and quenched with 3 mL of dichloromethane. The mixture was then washed with saturated NaSO₄ (1 x 3 mL) and the resulting organic layer was washed with water (5x 3mL). The combined organic layers were washed once again with saturated NaSO₄ (1x 3mL) and dried with NaSO₄. After concentration *in vacuo*, a yellow crude oil was obtained. The crude oil was purified using preparatory thin layer chromatography with a 80% hexanes: 20% ethylacetate solvent system (R_f = 0.18) to yield 5.6mg(46%) of pure compound. ¹H NMR δ 0.21 (s, 6H, TBS), 0.95 (s, 9H, TBS), 3.2-3.3 (dd; 1H; H-6a), 3.31-3.42 (dd; 1H; H-6b), 3.3-3.5 (m; 4H; H-2, H-3, H-4, H- 5), 4.39 (d, 1H, H-1), 4.65 (d, 1H, Bn), 4.76 (d, 1H, Bn), 4.82 (d, 1H, Bn), 4.92 (d, 1H, Bn), 7.1-7.2 (m, 10H, Bn) 7.6-7.8(dd, 4H, phthalimide).

Figure 7. ^1H NMR spectra of 2-Azido-2-deoxy-3,4-di-O-benzyl-6-phthalimide-6-deoxy-1-O-tert-butyldimethylsilyl- β -D-glucopyranoside.

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