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Synthesis of Inhibitors for the Cholera Family of Toxins

By

Suzanne Hof ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

DEPARTMENT OF CHEMISTRY

Edmonton, Alberta

Fall 2000

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Synthesis of Inhibitors for the Cholera Family of Toxins** by **Suzanne Hof** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy.**

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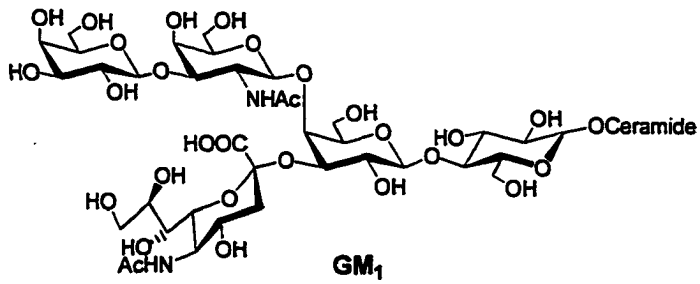
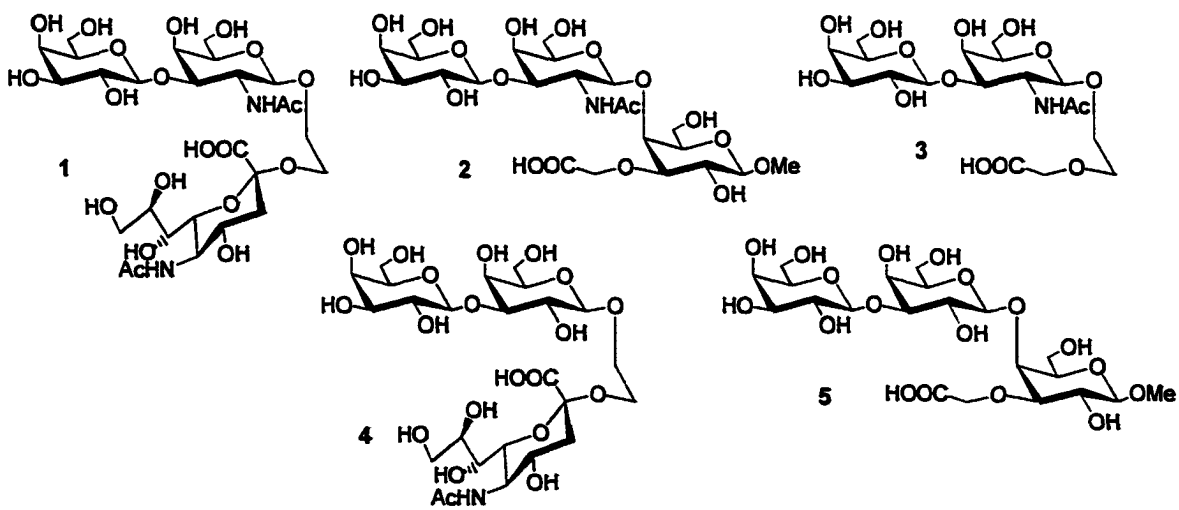
ABSTRACT

A number of recognition events at the cell-surface are mediated by carbohydrate structures. An example of this interaction is found when bacterial toxins such as cholera toxin (*Vibrio cholerae*) and heat-labile enterotoxin (*Escherichia coli*) bind to intestinal epithelial cells. The functional receptor for this interaction is the ganglioside GM_1.



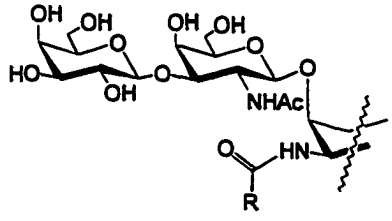
GM_1

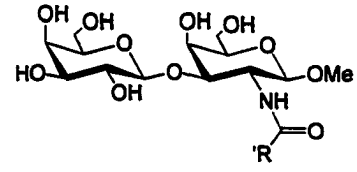
To investigate the minimum carbohydrate structure required for tight toxin binding, a series of GM_1 analogues were designed and synthesized. The analogues contained the terminal galactose and the carboxylate of sialic acid present in GM_1. The target molecules incorporated either a flexible two-carbon linker in the place of the branching galactose residue (**1**), a carboxymethyl fragment in place of the sialic acid residue (**2**), or both of these modifications (**3**). Two trisaccharide targets (**4** and **5**) were prepared wherein the acetamido of the GalNAc residue was replaced by a hydroxyl group.



The GM_1 analogues were tested for activity using an enzyme-linked competitive inhibition assay adapted for both heat-labile enterotoxin and cholera toxin. Results suggested that only the molecule containing the intact sialic acid and GalNAc residues (**1**) was an effective inhibitor for both LT and CT, with IC_{50} values in the low micromolar range. When frontal affinity chromatography coupled with mass spectrometric detection (FAC-MS) was used as the assay, compound **1** was again a strong binder, with K_d values in the low micromolar range. Its galactose analogue **4** was a slightly weaker ligand than **1** for both LT and CT.

The second part of the thesis focused on the generation of small libraries of potential ligands for LT and CT using solid-phase synthetic methods. The first approach was





based on the use of cyclic 1,2 *–cis* amino alcohol linkers to mimic the branching galactose of GM_1. Unfortunately, the amine proved too hindered to allow the efficient synthesis of a library using this method. The second library approach involved derivatization of the galactosamine nitrogen of the GM_1 terminal disaccharide Galβ(1→3)GalNβOMe. Results of the FAC-MS assay of the library components did not reveal any active compounds.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Ac	acetyl
Ala	alanine
Aliquat 336	tricaprylylmethylammonium chloride
All	allyl
anal.	analysis
aq.	aqueous
Ar	aromatic
Asp	aspartic acid
Bn	benzyl
BSA	bovine serum albumin
Bu	butyl
Bz	benzoyl
calcd	calculated
CAN	ceric (IV) ammonium nitrate
CMC	critical micellar concentration
CSA	camphorsulfonic acid
d	doublet or day(s)
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMTST	dimethyl(methylthio)sulfonium triflate

equiv.	equivalent
Et	ethyl
FAC	frontal affinity chromatography
FAB	fast atom bombardment
Fuc	fucose
Gal	galactose
GalNAc	D-2-acetamido-2-deoxy-galactopyranose
gem	geminal
Glc	glucose
GlcNAc	D-2-acetamido-2-deoxy-glucopyranose
Gly	glycine
h	hour(s)
Hz	Hertz
HMBC	heteronuclear multiple bond coherence
HR-ESMS	high-resolution electrospray mass spectrometry
J	coupling constant
Lys	lysine
m	multiplet
m/z	mass to charge ratio
Man	mannose
Me	methyl
Met	methionine
mg	milligram(s)

MHz	megahertz
min	minute(s)
ml	millilitre(s)
mmol	millimole(s)
mol	mole(s)
Ms	methanesulfonyl
MS	mass spectrometry or molecular sieves
NeuAc	*N*-acetyl neuraminic acid, sialic acid
NIS	*N*-iodosuccinimide
NMR	nuclear magnetic resonance
PBS	phasphate buffered saline
Ph	phenyl
Phe	phenylalanine
Phth	phthaloyl
pMBn	*p*-methoxybenzyl
PNZ	*p*-nitrobenzyloxycarbonyl
ppm	parts per million
py	pyridine
q	quartet
quant	quantitative
rt	room temperature
s	singlet
satd	saturated

Ser	serine
t	triplet
TESOTf	triethylsilyl trifluoromethanesulfonate
Tf	trifluoromethansulfonyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Thr	threonine
TIC	total ion chromatogram
TLC	thin layer chromatography
TMSOTf	trimethylsilyl trifluoromethanesulfonate
Ts	*p*-toluenesulfonyl
Tyr	tyrosine
Val	valine
vic	vicinal
Xyl	xylose

Part I

Synthesis of Analogues of GM_1 and their Evaluation as Inhibitors of Cholera Toxin and Heat-Labile Enterotoxin

Chapter 1:

Introduction

1.1 Introduction to Carbohydrates

Every undergraduate student in the sciences will, at some point in the course of their studies, be exposed to the subject of carbohydrates and their apparent ubiquity in life processes. A glance into a biochemistry text will quickly reveal a chapter discussing carbohydrates which predictably begins with a discussion of the conversion of water and atmospheric CO_2 to sugars during photosynthesis by plants [1]. This chapter then goes on to recite the different mono-and polysaccharides present in nature. The polysaccharides described invariably include those that play structural roles in the form of cellulose (plants), chitin (lobster and crab shell, insect exoskeleton) and the peptidoglycan layer of bacterial cell walls, as well as those that participate in energy storage in the form of starch and glycogen. One will also find an entire chapter devoted to this energy storage and the subsequent liberation of this energy through oxidative metabolism.

These structural and energetic functions have been recognized for many years and are quite well understood. Our undergraduate student may be forgiven for thinking that she has been taught everything there is to know about carbohydrates. It has recently become apparent, though, that the roles described above only scratch the surface. Carbohydrates are responsible for a much wider range of operations than ever previously imagined [2].

The field of "Glycobiology" has come into existence through ongoing investigations into the many other intriguing roles that carbohydrates play in biological events [3].

1.2 Roles of Cell-Surface Carbohydrates

Cell membranes are more than simply passive phospholipid bilayers providing a barrier between the interior and exterior of a cell. Rather, cell membranes are fluid and dynamic, playing an active role in the function of the cell with their ability to regulate the transport of specific materials both in and out of the cell. It is also now recognized that the surface of mammalian cells is coated with an array of carbohydrate structures [4]. These carbohydrates are implicated in an increasing number of critical cell-cell recognition events, some of which include fertilization of an ovum by sperm [5], recruitment of leukocytes to areas of injured tissue [6] and the organization of cells during embryonic development [7]. In addition, carbohydrate antigens often elicit immune responses [8]. While these are all normal and necessary biological functions, cell-surface oligosaccharides have also been found to mediate a number of pathological processes as well. Carbohydrates can act as anchors for the adhesion of viruses and bacteria to the cell surface [9]. Plant and bacterial toxins can also bind to carbohydrate receptors [10]. Changes in glycosylation patterns have been observed in cells transformed by cancer, and may be implicated in the metastatic process, although the mechanism by which this occurs is not fully understood [11].

It is apparent from the diverse nature of the recognition events involving carbohydrates that oligosaccharides must have unique structural characteristics in order to satisfy the exact specificities required. When compared to nucleotides and amino acids (the other two major building materials used in biological systems) it becomes apparent that carbohydrates are well suited to the task of conveying highly specific information [12]. Nucleic acids, the nucleotide polymers responsible for carrying genetic information, and proteins, which are comprised of amino acids, are linear in their primary structures. This is because amino acids are connected with only amide bonds and nucleotides with only 3'-5' phosphodiester linkages. Carbohydrates, on the other hand, are capable of great structural complexity due to their ability to form intersaccharidic linkages between the different hydroxyls present on each monomer, leading to highly branched and variable structures. Other structural elements that also contribute to the variety in structure include α vs. β anomeric configurations, and pyranose vs. furanose rings. This extraordinary potential for diversity in structure allows carbohydrates to play an important role in the transfer of information at the cell surface.

1.2.1 Glycoproteins and Glycolipids

Oligosaccharides are presented on the cell surface as either *glycoproteins* or *glycolipids*. Glycoproteins are generally membrane-bound proteins and are described as either *N*-linked or *O*-linked with reference to the type of amino acid at the site of the sugar's connection to the protein [13]. *N*-linked glycoproteins have the carbohydrate moiety, most commonly *N*-acetylglucosamine, attached to the terminal amide of an asparagine

residue which is part of a consensus sequence Asn-X-Ser/Thr, where X may be any amino acid except proline (Figure 1.1a). *O*-linked glycoproteins are glycosylated at the hydroxyl of either a serine or threonine residue. There are many different types of sugars found at *O*-linkages, but the most common sugar found in many animal glycoproteins is *N*-acetylgalactosamine (Figure 1.1b).

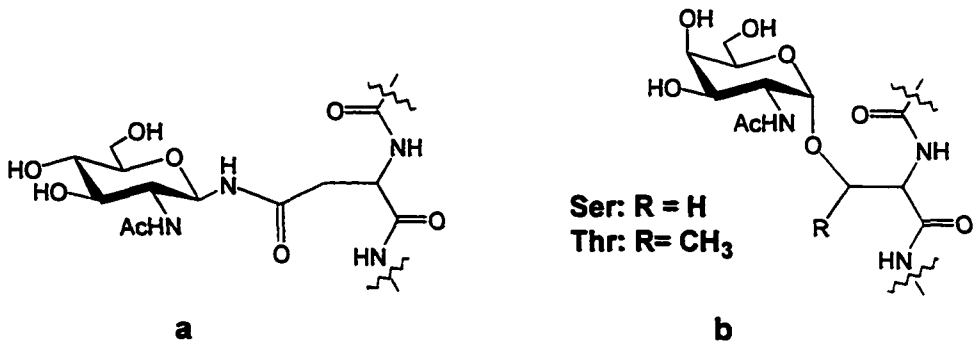


Figure 1.1: *N*- and *O*-linkages to glycoproteins

Glycolipids fall into two major categories: glycoglycerolipids and glycosphingolipids. Glycoglycerolipids are found predominantly in plants and bacteria and have the general

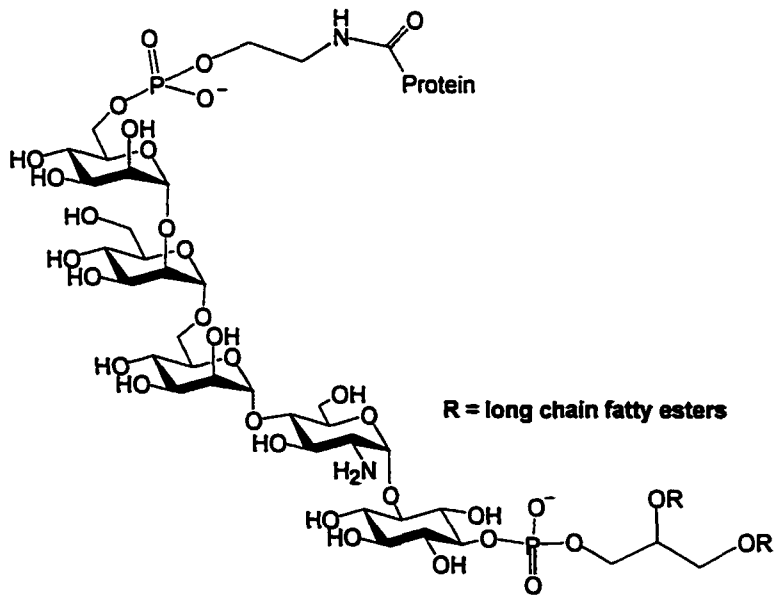


Figure 1.2: Glycerophosphatididylinositol (GPI) anchors

core structure shown in Figure 1.2 [14]. Glycosyl phophatidylinositols (GPIs) are glycoglycerolipids known to play a tethering role for certain proteins at the cell surface.

Glycosphingolipids are the principal form of glycolipids found on mammalian cells, and are defined as those in which the lipid is a ceramide moiety (*N*-acyl sphingosine) (Figure

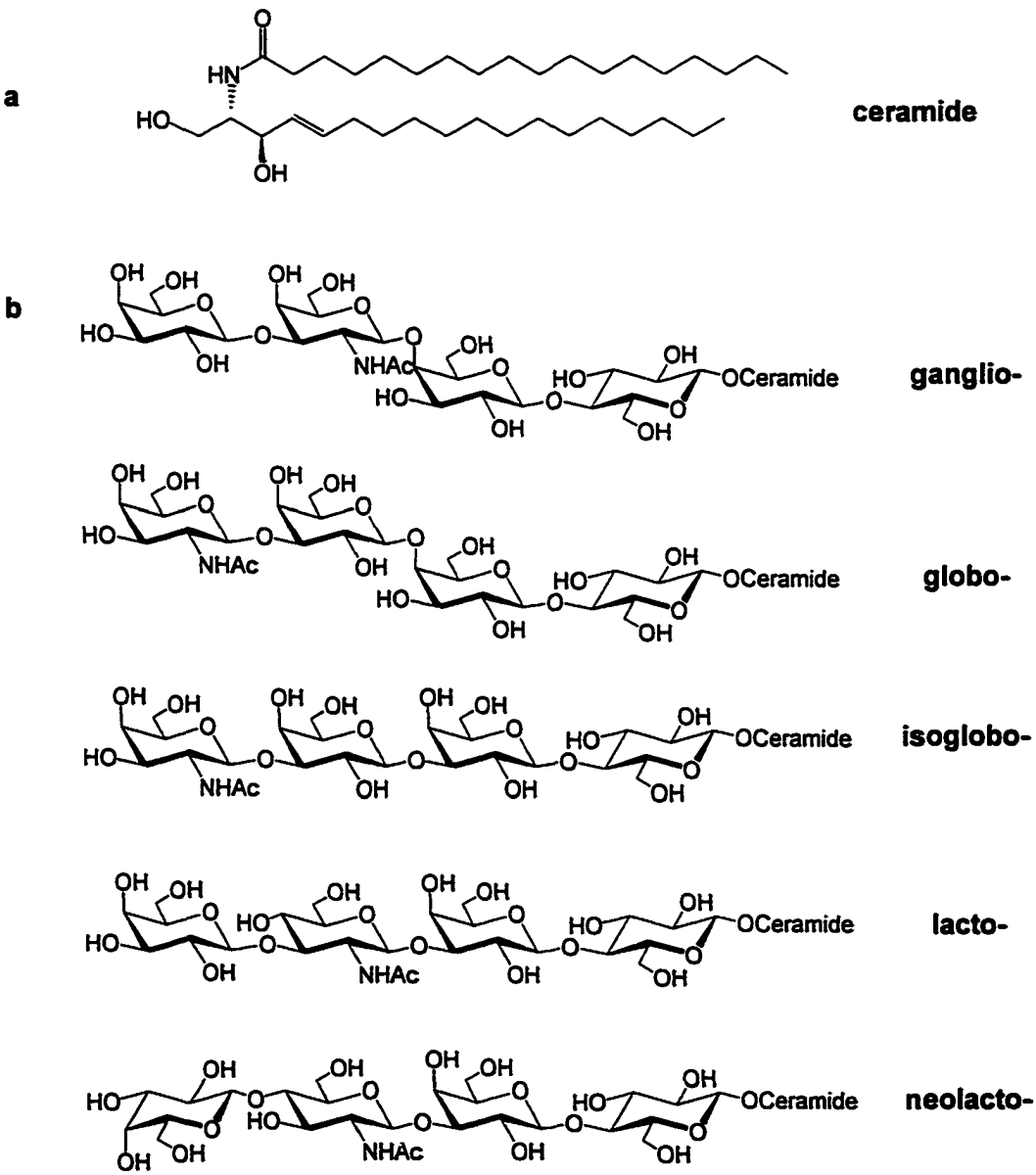


Figure 1.3: Glycerosphingolipid core tetrasaccharides

1.3a) [15]. Although the saccharide substituents can be comprised of a number of different sugars, there is a series of consensus core tetrasaccharides designated ganglio-, globo-, isoglobo-, lacto-, and neolacto-, all of which are connected to the ceramide via a glucosyl β-linkage (Figure 1.3b) [16].

The gangliosides are acidic glycosphingolipids found in most, if not all, animal tissue, although they are more highly concentrated in brain and nervous tissue [17]. They are characterized as acidic due to the presence of one or more sialic acid residues at the terminal positions. There are two systems of nomenclature used to describe gangliosides, the Svennerholm designation being the most popular (Figure 1.4) [18]. This employs the use of letters and numbers to describe the number and position of sialic acid residues, as well as the number of neutral sugars on the molecule. Gangliosides are designated by G and the number of sialic acid residues by M = monosialo, D = disialo, T = trisialo, etc. The subscript denotes the length of the neutral sugar chain according to the formula 5-n,

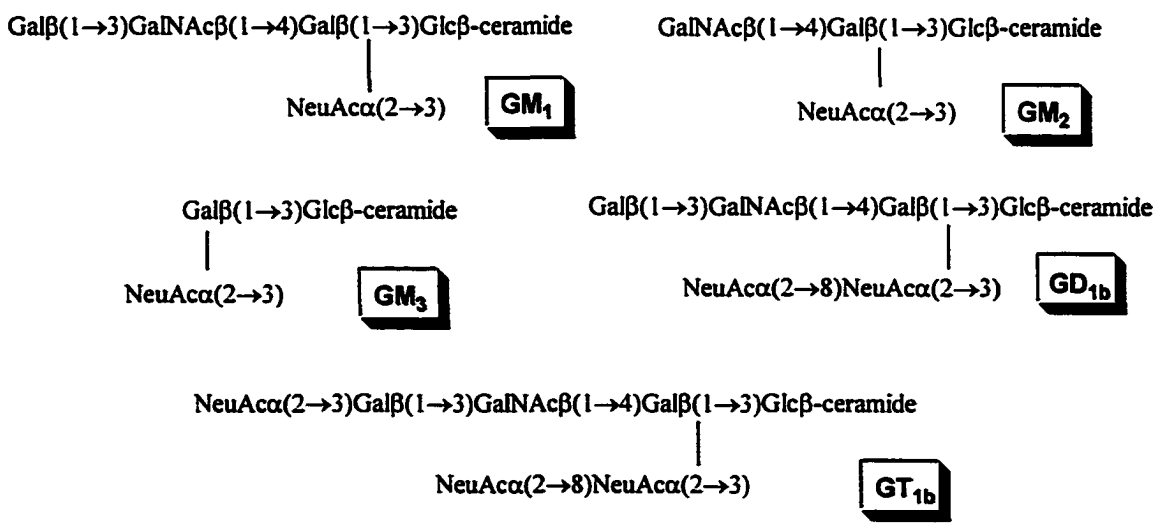


Figure 1.4: Examples of Svennerholm nomenclature

where n equals the number of neutral sugar residues. The letter subscript indicates the position of the sialic acid residues.

1.2.2 Lectins

The glycoconjugates described above do not work in isolation at the cell surface. In order for a recognition event to take place, the carbohydrate must be recognized by a protein specific to that function. Lectins are one of three classes of proteins known to bind carbohydrates, the other two classes being immunoglobulins and carbohydrate-binding enzymes [19]. Lectins were first isolated from plant tissues over one hundred years ago and it was once thought that plants were the only source of these carbohydrate-binding proteins. It is now known that these carbohydrate-binding proteins play significant roles in animal and bacterial systems as well.

Plant lectins tend to be soluble, multimeric proteins with multiple binding sites. Lectins from animal sources on the other hand, have been found to possess a greater variety in structure. Some are membrane bound while others are soluble; some are monomeric and monovalent, yet others are multimeric and multivalent [20].

Bacteria and viruses have taken advantage of highly specific carbohydrate-protein interactions at different stages of their pathogenic course of action. The first step in the initiation of many infections is the adhesion of bacterial or viral proteins to cell-surface glycoconjugates that serve as points of attachment to the target cell. Once a bacterial

infection has been established, toxins secreted by the bacteria can in turn use carbohydrate-protein interactions to anchor to the surface of the cell prior to invasion of the cell proper.

1.2.2.1 Common Interactions in Lectin-Carbohydrate Recognition

Carbohydrate-binding proteins have been classified into two groups [21]. Group I proteins are characterized by their deep, cleft-like binding sites, which leave very little of the bound carbohydrate solvent exposed. Binding by group I proteins is considered "high affinity", meaning dissociation constants fall in the sub-micromolar range. Lectins, on the other hand, are group II proteins, which contain shallow binding sites located on the surface of the protein, meaning a larger proportion of the bound ligand remains exposed to bulk solvent. Group II proteins generally have dissociation constants in the millimolar range.

In spite of the topographical differences between the binding sites of group I and II proteins, the many different features of carbohydrate-protein interactions remain common to both groups (Figure 1.5) [22]. Among the different interactions found to play important roles in carbohydrate-protein recognition are hydrogen bonding interactions (both direct and water mediated) and nonpolar interactions, such as van der Waals contacts. Divalent cations, such as Ca^{2+} and Mn^{2+}, are also known to coordinate between amino acid residues and the carbohydrate ligand. It is also possible for charged sugars,

i.e. sialic acids or sulfated sugars, to interact with charged amino acid residues forming salt-bridges, although this is not as common as one may expect.

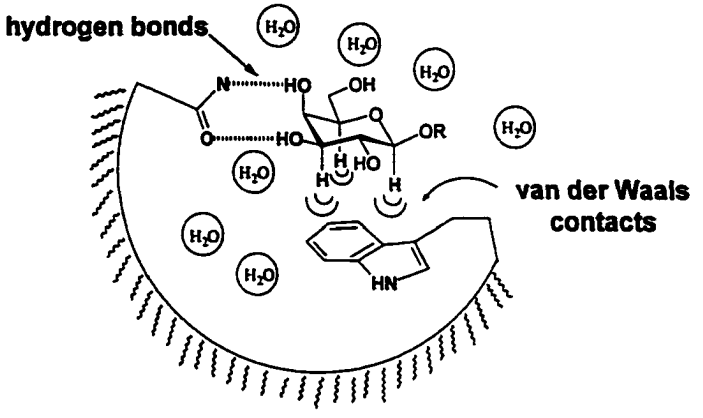


Figure 1.5: General binding motifs in protein-carbohydrate recognition

1.3 Carbohydrate Interactions with Bacterial Toxins

As mentioned in Section 1.2.2.1, some bacteria are known to secrete toxins that recognize cell-surface carbohydrate receptors. Heat-labile enterotoxin, secreted by *Escherichia coli*, and cholera toxin produced by *Vibrio cholerae* are two toxins whose interactions with carbohydrate ligands will be investigated in this work. A brief background of the diseases caused by these pathogens will be presented, as well as a description of the toxin structure and the role that each component of the toxin plays in the development of disease symptoms. Interactions detected in the published crystal structure of the sugar:CT complex [23] will be discussed and compared to binding motifs observed throughout many different lectin-carbohydrate complexes [22].

1.3.1 Introduction to Cholera and Traveler's Diarrhea

Cholera is a potentially epidemic and life-threatening diarrheal disease. It is generally associated with poor sanitation and contaminated water supplies, and remains a significant public health concern in the Third World and any area where sources of clean drinking water have been compromised by untreated sewage [24].

Cholera is caused by the bacterium *Vibrio cholerae* which, upon colonization of the small bowel, secretes a potent enterotoxin. This toxin, known as cholera toxin, or CT, is responsible for the clinical symptoms of the disease. These symptoms generally begin with the sudden onset of watery diarrhea, which in severe cases can result in the loss of several litres of fluid in a period of a few hours. This leads to severe dehydration, electrolyte imbalance and possible death without medical intervention. Although antibiotics have been shown to shorten the course of the disease, treatment must also include oral or intravenous rehydration, preferably with a solution containing both electrolytes (NaCl, KCl, $NaHCO_3$) and glucose [25].

A toxin secreted by enterotoxigenic *Escherichia coli* is structurally and functionally similar to CT and is known as heat-labile enterotoxin, or LT. The milder traveler's diarrhea is the clinical manifestation of an intestinal infection with this strain of *E. coli*. Many studies have been undertaken to investigate the similarities and differences between these two toxins [26].

1.3.1.1 Description of Toxin Structure

Both CT and LT are heterohexameric AB_5 proteins, wherein the doughnut shaped B_5 homopentamer is responsible for binding to cell-surface receptors on the intestinal epithelium and the monomeric A subunit is responsible for the catalytic activity of the toxin(Figure 1.6a). The A subunit is 27.2 kDa in size, while the B pentamer is composed of five identical, noncovalently linked 11.7 kDa units (Figure 1.6b) [27]. The two toxins are almost 80% homologous at both the nucleotide and amino acid levels [23].

Figure 1.6: AB_5 structure of holotoxin

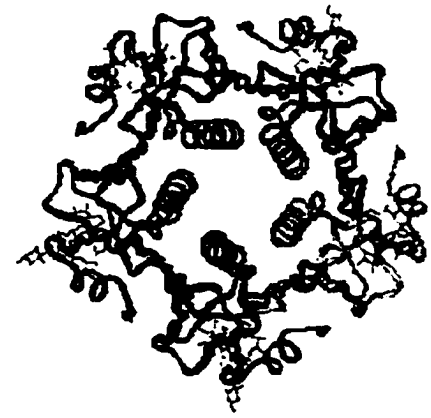


Figure 1.6b: Top view of B_5 subunit

The multimeric nature of the B subunit is a common structural motif found in the binding moieties of many bacterial toxins. Besides CT and LT, other examples of toxins with multiple binding sites include shiga toxin (*Shigella dysentariae*) and verotoxin (*E. coli* O157:H7) [28]. Since carbohydrate-protein interactions are generally low affinity, the

presence of multiple binding sites can result in very large increases in the functional affinity, or avidity, of the toxin. The binding of one unit to a cell-surface receptor brings the other units into close proximity to other possible receptors. While one weak interaction may be transient, multiple interactions behave in a cooperative manner, making it easier for the toxin to remain attached to the cell surface.

The A subunit is synthesized as a single polypeptide in both *V. cholerae* and *E. coli*. In *V. cholerae* A is proteolytically "nicked" during secretion from the bacterium, producing two polypeptides, A1 and A2, which remain covalently linked through a disulfide bond [29]. The A1 subunit contains the catalytic function, while A2 extends into the central pore formed by the B pentamer, maintaining the interaction between the B pentamer and A1. In *E. coli*, A does not undergo this proteolytic processing. It has been shown that this processing step is necessary to achieve catalytic activity of A1 in the epithelial cell [30].

It should be mentioned that while CT is actively secreted by *V. cholerae*, there exists no parallel mechanism for toxin secretion by *E. coli*, thus LT remains cell-associated. The contrast in severity between cholera and traveler's diarrhea has been attributed to both the absence of a "nicking" mechanism for the LT A subunit, and the subsequent inability of LT to exit the cell [31].

The first step in the pathogenesis of the toxins is adherence to the epithelium. It is well established that the B subunits bind to the membrane-bound ganglioside receptor GM_1

[32]. Ganglioside GM_1 is the only known natural receptor for CT and also acts as a functional receptor for LT, although additional receptors for LT have been identified as well [33]. Two theories exist regarding internalization of the toxin once binding to the cell-surface has been achieved [34]. The first proposal involves a conformational change in the B pentamer, allowing the A1 subunit to be presented to the surface of the cell with subsequent insertion into the membrane bilayer (Figure 1.7). Another proposal involves endocytosis of the intact holotoxin, but this would still require a mechanism for transporting the A subunit out of the endosome and into the cytosol.

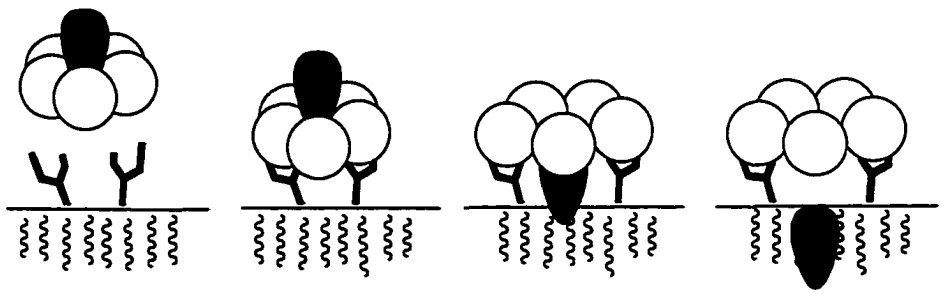


Figure 1.7: Presentation of the toxin to the cell surface

1.3.1.2 What Happens Inside the Cell?

Once the toxin has entered the cell, the A subunit must undergo another processing step before it is capable of enzymatic activity. The disulfide bond linking A1 and A2 is reduced, liberating the A1 fragment. The A1 fragment acts enzymatically, catalyzing the transfer of ADP-ribose from nicotinamide-adenine dinucleotide (NAD^+) to the G protein, G_s [35]. The G_s protein is a regulatory protein associated with the adenylate cyclase (AC) pathway. In a normally functioning AC system, G_s is active only when bound to

guanosine triphosphate (GTP) (Figure 1.8a). The G_s·GTP complex in turn forms a ternary complex with AC, thus activating it. This ternary complex then catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Over time, the bound GTP is hydrolyzed to GDP through the intrinsic GTPase activity of the G_s protein. The association between AC and the resulting G_s·GDP complex is weak, leading to the dissociation of the ternary complex and the subsequent inactivation of the AC protein. Reassociation of AC with the G_s protein occurs only after the bound GDP is replaced by another molecule of GTP.

The effect of ADP-ribosylation of the G_s protein by the A1 subunit is irreversible

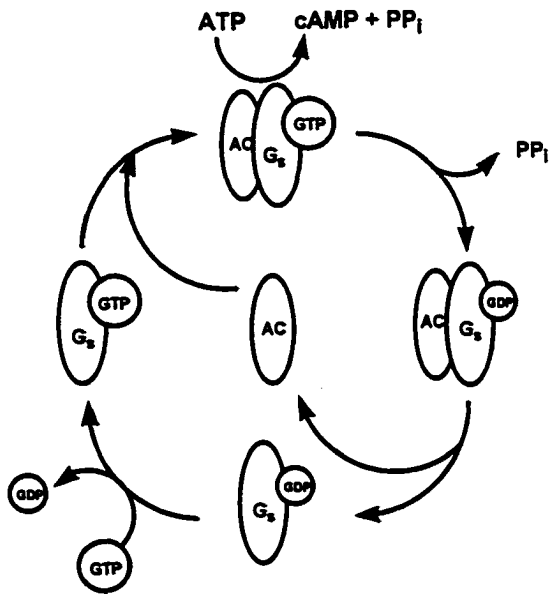


Figure 1.8a: Normal adenylate cyclase pathway

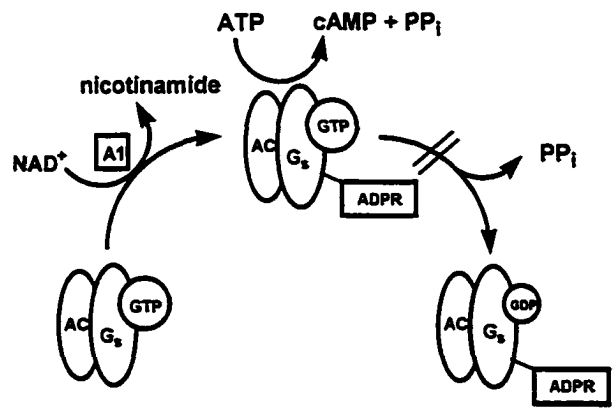


Figure 1.8b: Activation of adenylate cyclase by A1 subunit

activation of the adenylate cyclase (Figure 1.8b). This occurs because the intrinsic GTPase activity of the G_s protein is inhibited by the presence of the ADP-ribose moiety, resulting in the preservation of the active AC·G_s·GDP ternary complex. This leads to the

excessive production of cAMP, which in turn promotes the secretion of electrolytes from the cell followed by large amounts of water. This is the cause of the severe diarrheal symptoms experienced by individuals suffering from *V. cholera* infections.

1.3.1.3 Crystal Structure of GM_1 in Binding Site

The complex between GM_1 and cholera toxin is observed in a crystal structure refined to 2.2 Å resolution [23]. The hydrogen bonding map (Figure 1.9) has been deduced from the crystal structure by considering only donor-acceptor distances of less than 3.2 Å as possible hydrogen bonding interactions. It can be assumed that the toxin-GM_1 interactions observed in the CT binding site are the same in the LT binding site. This is due to the fact that the two sites are identical except for a single amino acid substitution at the periphery of the binding cleft in which a histidine residue in CT is replaced by arginine in LT.

1.3.1.3.1 Hydrogen Bonding Interactions

Since carbohydrates present an array of polar groups, i.e. hydroxyls and carboxylates, it is no surprise that hydrogen-bonding contacts are crucial to favourable binding. Cooperative hydrogen bonding is a common pattern observed in carbohydrate binding sites [36]. This occurs when a hydroxyl group on the sugar acts as both hydrogen bond donor and hydrogen bond acceptor. The sugar hydroxyl generally acts as a hydrogen bond donor to the carboxylate functionality of acidic amino acids, and accepts hydrogen

bonds from positively charged side chains, as well as from the amides of the main chain and side chains of asparagine and glutamine. The most prevalent cooperative binding scheme involving sugar hydroxyls is $(NH)_{1,2} \rightarrow OH \rightarrow O{=}C$.

It should also be noted that the amino acid functional groups commonly involved in hydrogen bonding are planar, with fixed bond geometry [22]. Sugar hydroxyls, on the other hand, have some torsional freedom, which permits optimization of the hydrogen bonding interactions. Another interesting feature of protein-sugar interactions is the rare use of protein hydroxyls for hydrogen bonding with sugar hydroxyls. This may possibly be attributed to the entropic penalty required for fixing the rotamers of both protein and sugar hydroxyl groups.

The acetamido functionality found on GlcNAc, GalNAc and NeuAc residues may also play a significant role in recognition. The planar amide is often seen acting as a donor to carbonyl or carboxylate groups.

Another functional group present on certain carbohydrates is the carboxylate. As mentioned before, one might expect the carboxylate to form salt bridges with positively charged side chains, but this is generally not the case [22]. Instead the carboxylate is frequently observed to interact with main chain amides or polar side chains in what are considered simple hydrogen bonding interactions.

The hydrogen bonding map obtained from the crystal structure of the GM_1:CT complex echoes many of the generally observed binding motifs cited above (Figure 1.9) [23]. The amino acid residues employed in hydrogen bonding with the sugar hydroxyls are indeed planar (asparagine, glutamine) or charged (lysine) as expected, with no interactions between protein hydroxyls and sugar hydroxyls observed. As well, the acetamido group in NeuAc does in fact donate a hydrogen bond to the main chain carbonyl of a glutamic acid. The acetamido group of GalNAc also acts as a hydrogen bond donor, but this time to the carboxylate of the NeuAc residue. This intrasaccharidic hydrogen bond may act to constrain the flexibility of the pentasaccharide moiety, even in the absence of toxin [23].

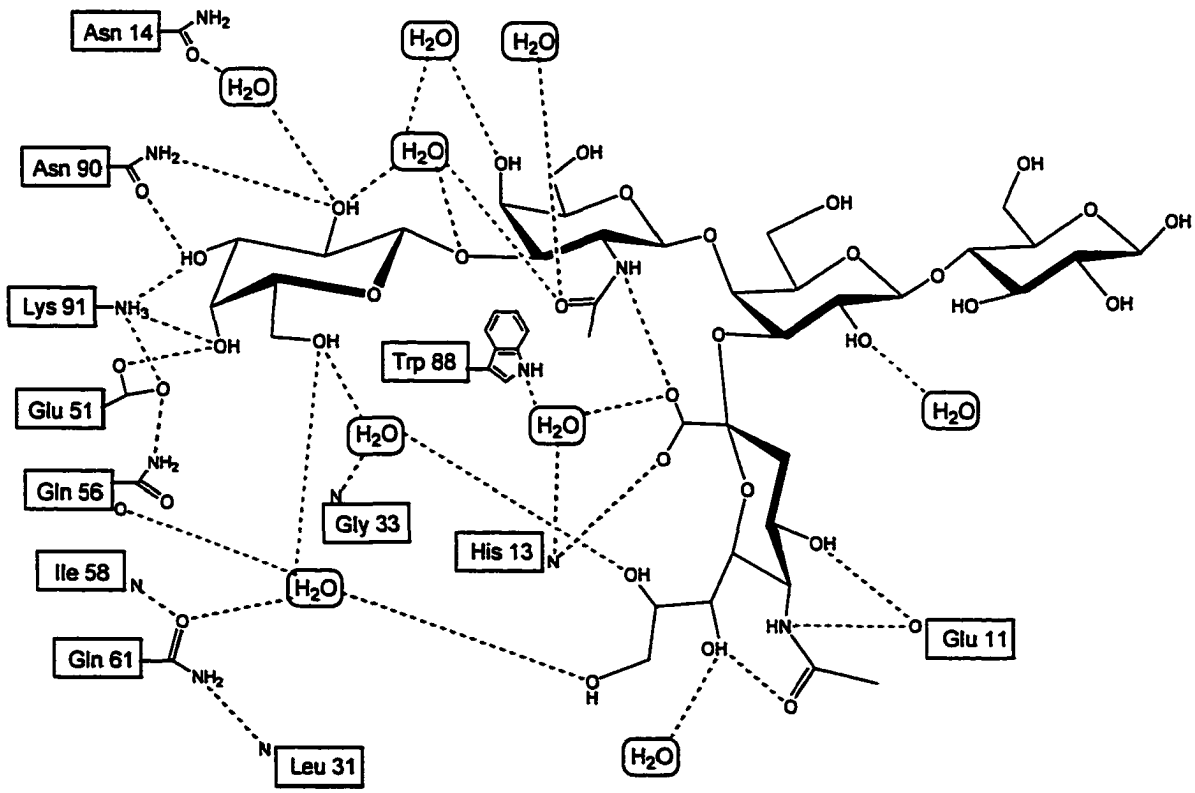


Figure 1.9: Hydrogen bonding interactions in GM_1 binding site of cholera toxin.

Water molecules mediate a large number of the hydrogen bonding interactions between the toxin and GM_1. This is not surprising, since the binding site is a shallow cleft, with GM_1 adopting what can be described as a "two fingered grip" on the toxin [23]. The terminal galactose, or "forefinger", is deeply embedded in the toxin, while the sialic acid, or "thumb", occupies a shallow depression on the surface of the protein. This means that the remaining sugar residues are quite solvent exposed, as is evidenced by the nine water molecules located in the crystal structure.

1.3.1.3.2 Nonpolar Interactions

In spite of the predominance of the polar groups found on sugar residues, there also exist significant nonpolar patches that play an important role in protein-carbohydrate recognition. These nonpolar regions are formed by aliphatic ring protons and can extend out to the exocyclic C-6 position for hexoses and the glycerol moiety (C-7 to C-9) of NeuAc. A common interaction between amino acid side chains and nonpolar patches on sugars involves the "stacking" of the sugar's hydrophobic face against an aromatic side chain. This is a binding motif found in every galactose binding site described in the literature, wherein the bottom face (or B face) of the galactose is found facing the aromatic region of tryptophan or phenylalanine. This stacking is generally not parallel; rather the angle between the two planes has been observed ranging from 17° to 52°, with an average angle of 32° [22].

The methyl group of an acetamido functionality can also provide a hydrophobic contact point. This is observed in the GM_1:CT complex where the acetamido methyl of NeuAc approaches a tyrosine phenyl ring.

A comment should be made on the infrequent observation of aliphatic side chains in nonpolar interactions with carbohydrates. It has been proposed that the ring protons carry a net positive charge due to the electronegativity of the hydroxyl substituents. The π-electron cloud of an aromatic ring can provide a stabilizing interaction, and not just the presence of a geometrically complimentary nonpolar surface [22].

1.4 Carbohydrate Synthesis

In order to define the roles that sugars play in biological systems, it is necessary to develop methodologies for the synthesis of natural and unnatural oligosaccharides. This is not a trivial task due to the complexity of carbohydrate structures, with the regio- and stereo-controlled synthesis of glycosidic linkages being a significant challenge. Not only is it important to be able to manipulate protecting groups to create appropriately blocked sugar acceptors with a free hydroxyl available for glycosylation, it is essential that the glycosylation be carried out with some control over the stereochemical outcome [37]. To this end, some general strategies have evolved which provide some control over the stereochemistry of glycosylation. As well, a number of different donors have been developed for the chemical synthesis of oligosaccharides. A brief review of some of the

tactics employed in synthetic carbohydrate chemistry will be undertaken, with an emphasis on approaches used in the present work.

1.4.1 Neighbouring Group Participation

The most frequently adopted method of creating a new glycosidic linkage is through the reaction of a glycosyl acceptor with the oxocarbonium intermediate generated upon the activation of a glycosyl donor (Figure1.10) [38, 39].

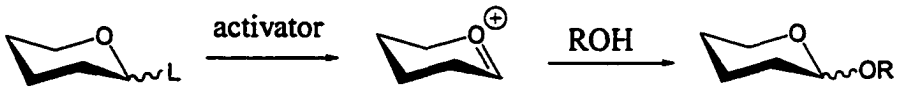


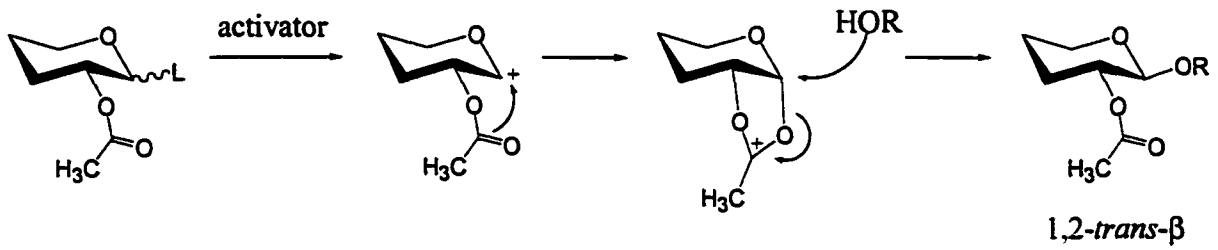
Figure 1.10: General glycosylation scheme

The two general outcomes to this reaction are designated 1,2-*trans* and 1,2-*cis*, which are further categorized according to the configuration at the anomeric centre (Figure 1.11). For the purposes of this discussion, the glycosidic bond will be described as α if it is axial and β if equatorial. While this does not follow the strict rules for α/β designation, it is a generalization that holds for most of the common mammalian hexoses, which are usually of D configuration. Achieving control over these outcomes provides the synthetic carbohydrate chemist with one of her biggest challenges.

1,2-*trans*-β 1,2-*trans*-α 1,2-*cis*-α 1,2-*cis*-β

Figure 1.11: Potential glycosylation outcomes

The 1,2-*trans* linkages are readily achieved through the use of protecting groups (acetates or benzoates, for example) at the 2-position that are capable of neighbouring group participation (Figure 1.12). The acetoxonium intermediate effectively blocks one face from nucleophilic attack by the acceptor, leading to the formation of the desired *trans* linkage as the major product.



1,2-*trans*-β

Figure 1.12: Neighbouring group participation during glycosylation

The formation of a 1,2-*cis*-α linkage is more challenging to obtain than either of the 1,2-*trans* linkages. Lemieux and co-workers have developed the "halide ion method" to reliably overcome this problem [40]. This method requires a non-participating group, typically a benzyl ether, at the 2-position. The proposed mechanism involves *in situ* anomerization of the α-bromide to the β-bromide, which in turn is the reactive species. The increased reactivity of the β-bromide toward the formation of α-glycosides can be

justified in two ways. The first rationale is the lower stability of the β-bromide relative to the α-bromide, which implies it is higher in energy and thus energetically closer to the transition state. The second rationale is described as the "kinetic anomeric effect," which invokes the antiperiplanar arrangement of the ring oxygen lone pair and that of the incoming oxygen on the acceptor. The favourable axial approach of the nucleophilic acceptor leads to formation of the α-anomer as the major product (Figure 1.13).

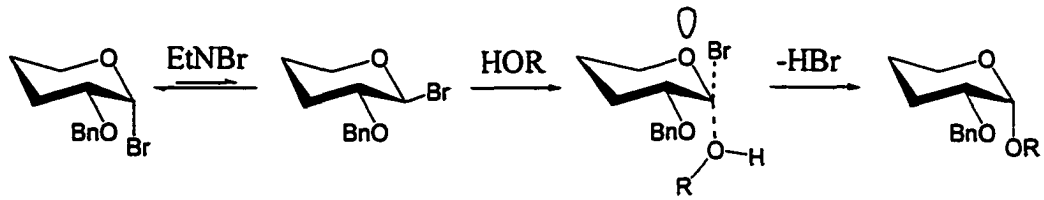


Figure 1.13: Halide ion method for the preparation of 1,2-*cis*-α products

1.4.2 Common Donors

1.4.2.1 Halides (Br, Cl)

One of the first methods for the formation of 1,2-*trans* linkages is the Koenigs-Knorr reaction, which was first introduced almost one hundred years ago [41]. It employs the use of an α-bromo or α-chloro sugar as donor, activated by an insoluble silver salt such as Ag_2CO_3 and Ag_2O. This method was often found to be low yielding, especially with hindered or otherwise unreactive acceptors. Modifications lead to the use of catalysts that are soluble in organic solvents, such as AgOTf or $Hg(CN)_2$ and $HgBr_2$ (the Helferich

modification) [42]. These reactions are presumed to proceed *via* the formation of the oxocarbonium intermediate as shown in Figure 1.14.

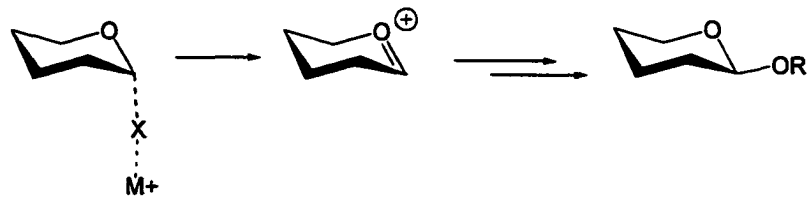


Figure 1.14: Proposed mechanism for activation of bromides by heavy metals

1.4.2.2 Thioglycosides

While bromide donors remain useful in many situations, problems with hydrolytic instability and facile elimination to yield glycals led to the inevitable development of alternatives. Significant advancement in this area came with the introduction of thioglycoside donors [43]. Thioalkyl and thioaryl groups at the anomeric position offer versatility to the carbohydrate chemist with their ability to act as both donor and temporary protecting group. The thioglycoside can be installed early in the synthetic procedure, remaining intact under many conditions that may be encountered in subsequent protecting group manipulations.

The synthesis of thioglycosides from anomeric acetates is typically undertaken by reaction with a thiol in the presence of a Lewis acid (Figure 1.15a) [44]. Another method of installing thioglycosides is through the reaction of a halide donor with a thiolate nucleophile (Figure 1.15b) [45].

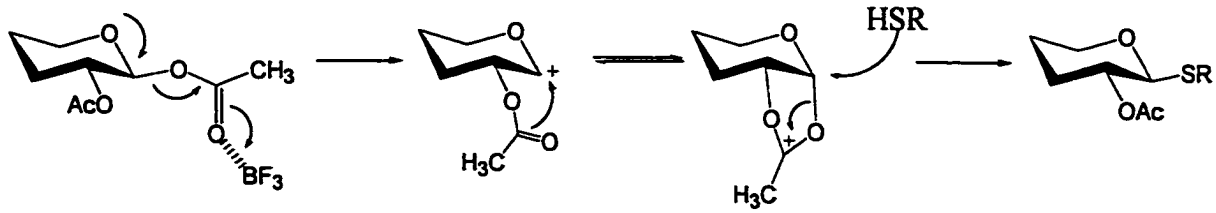


Figure 1.15a: Lewis acid-catalysed synthesis of thioglycoside donor

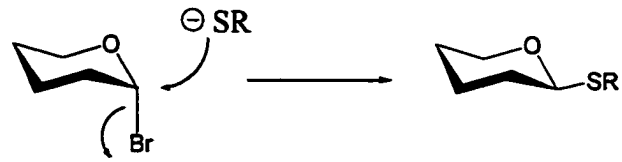


Figure 1.15b: Thiolate approach to synthesis of thioglycoside donors

Activation of thioglycosides can be achieved with a number of thiophilic promoters (Figure 1.16). Common activators include iodonium sources such as IBr, [46] or *N*-iodosuccinimide in combination with either trifluoromethanesulfonic acid [47] or silver trifluoromethanesulfonate [48]. Also popular are methylating agents such as methyl trifluoromethanesulfonate [49], or dimethyl(methylthio)sulfonium trifluoromethanesulfonate (DMTST) [50].

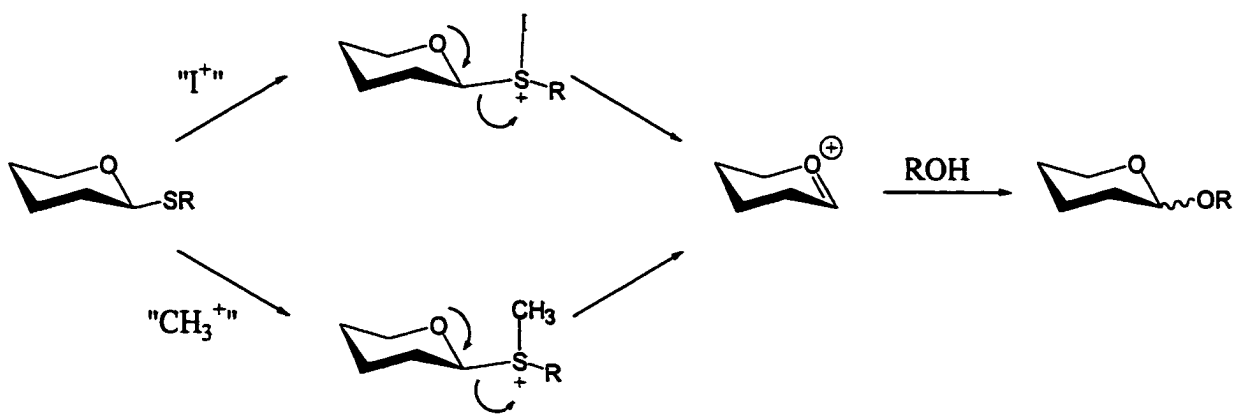


Figure 1.16: Activation of thioglycosides

1.4.2.3 Imidates

Another donor in common usage is the trichloroacetimidate. These are easily prepared from the reducing sugar under basic conditions through reaction of the anomeric alkoxide with trichloroacetonitrile (Figure 1.17) [51]. Trichloroacetimidates are activated under Lewis acidic conditions, with $BF_3{\cdot}OEt_2$ and TMSOTf commonly used activators [52, 53].

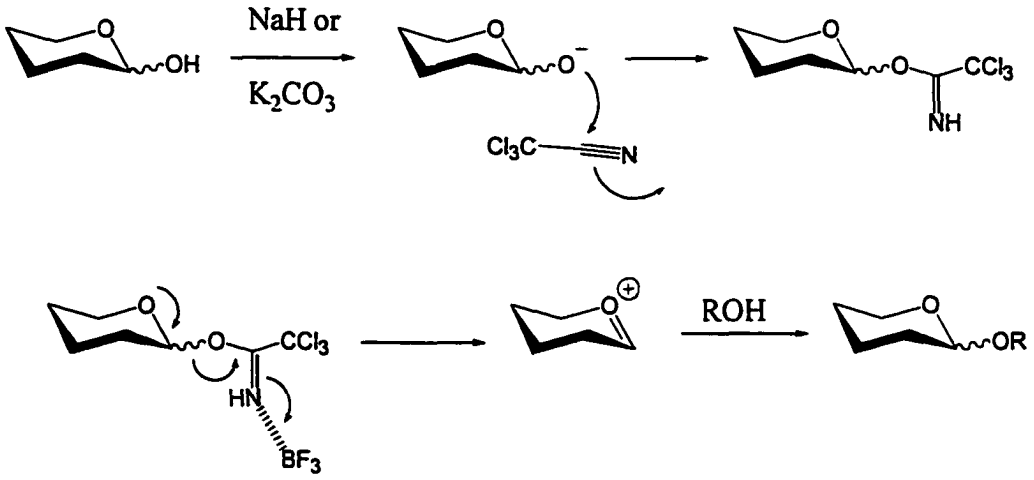
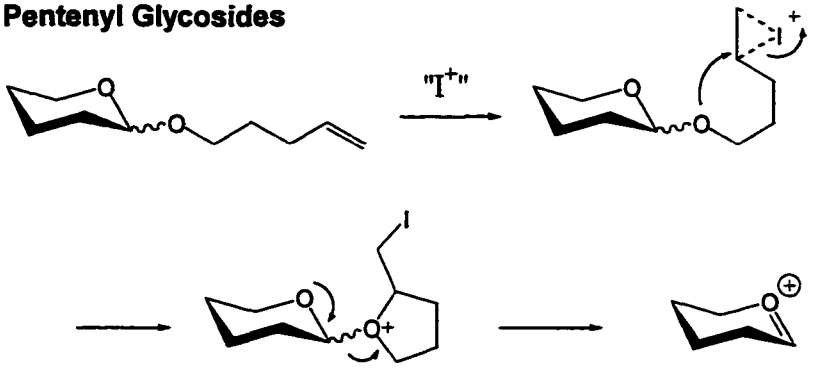


Figure 1.17: Synthesis and activation of trichloroacetimidate donors

1.4.2.4 Miscellaneous Donors (Pentenyl, Sulfoxide, Fluoro)

A number of alternative glycosyl donors have been developed that have not attained the same broad usage as bromides, thioglycosides and trichloroacetimidates. Among these choices are pentenyl glycosides [54], sulfoxides [55] and fluorides [56], to name only a few. Representative activation procedures for these donors are presented in Figure 1.18.

Pentenyl Glycosides



Sulfoxides

Fluorides

Figure 1.18: Activation of pentenyl glycoside, sulfoxide and fluoride donors

1.4.3 Synthesis of Oligosaccharides

The synthesis of oligosaccharides is certainly not as straightforward as that of disaccharides. Synthesis of a disaccharide requires simply that a suitably protected monosaccharide acceptor be glycosylated by a suitably protected monosaccharide donor.

Oligosaccharides, on the other hand, require much more planning, with two general approaches to consider for their construction [37, 57]. The first is the stepwise approach, which involves the synthesis of a disaccharide with a temporary protecting group that can be selectively removed to free a hydroxyl group for further glycosylation. This process can be repeated with the addition of another suitably protected monosaccharide, until the desired oligosaccharide has been built (Figure 1.19).

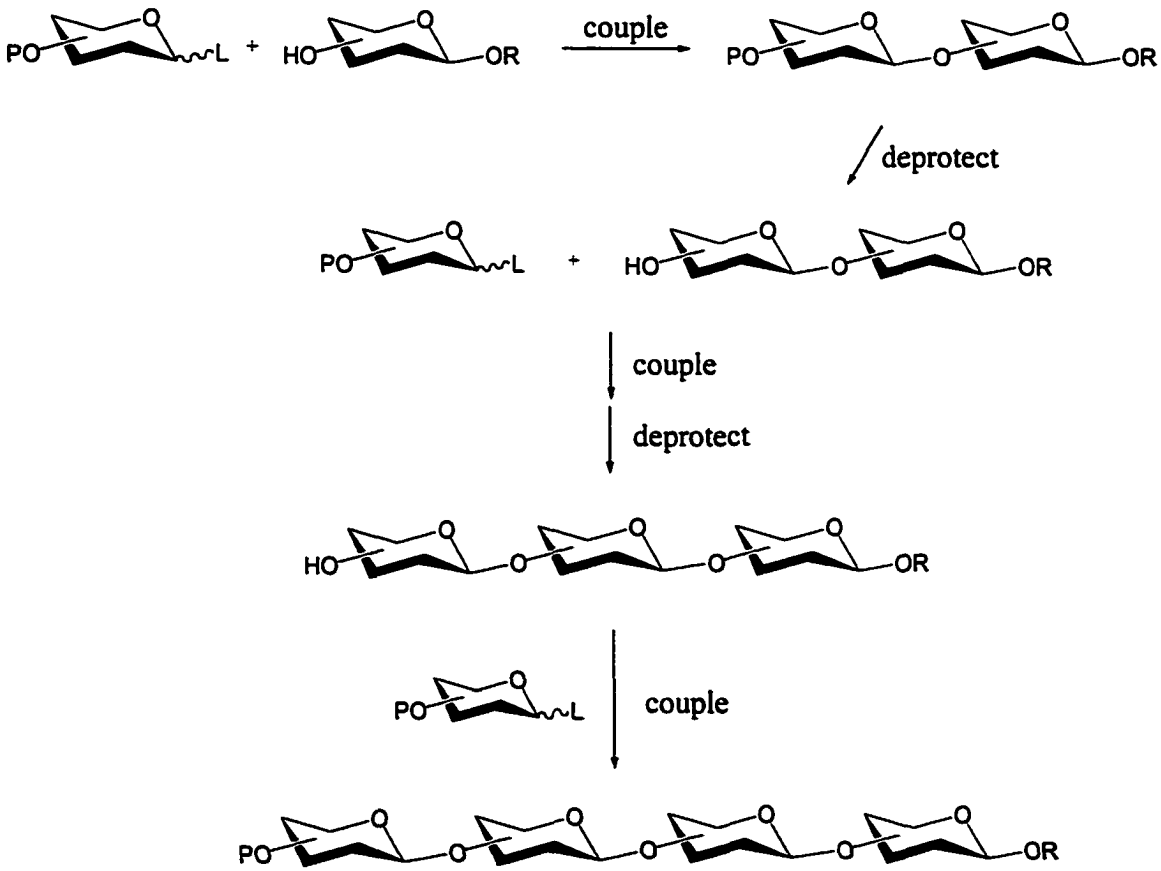


Figure 1.19: Stepwise approach to synthesis of oligosaccharides

The second method for oligosaccharide construction is *via* block synthesis. This sees the preparation of "blocks" of two or more sugar residues, which are subsequently coupled together to provide the chosen oligosaccharide. This approach requires that the disaccharide blocks be readily converted into a donor, or be capable of acting as one directly. A schematic description of the first approach is found in Figure 1.20. The

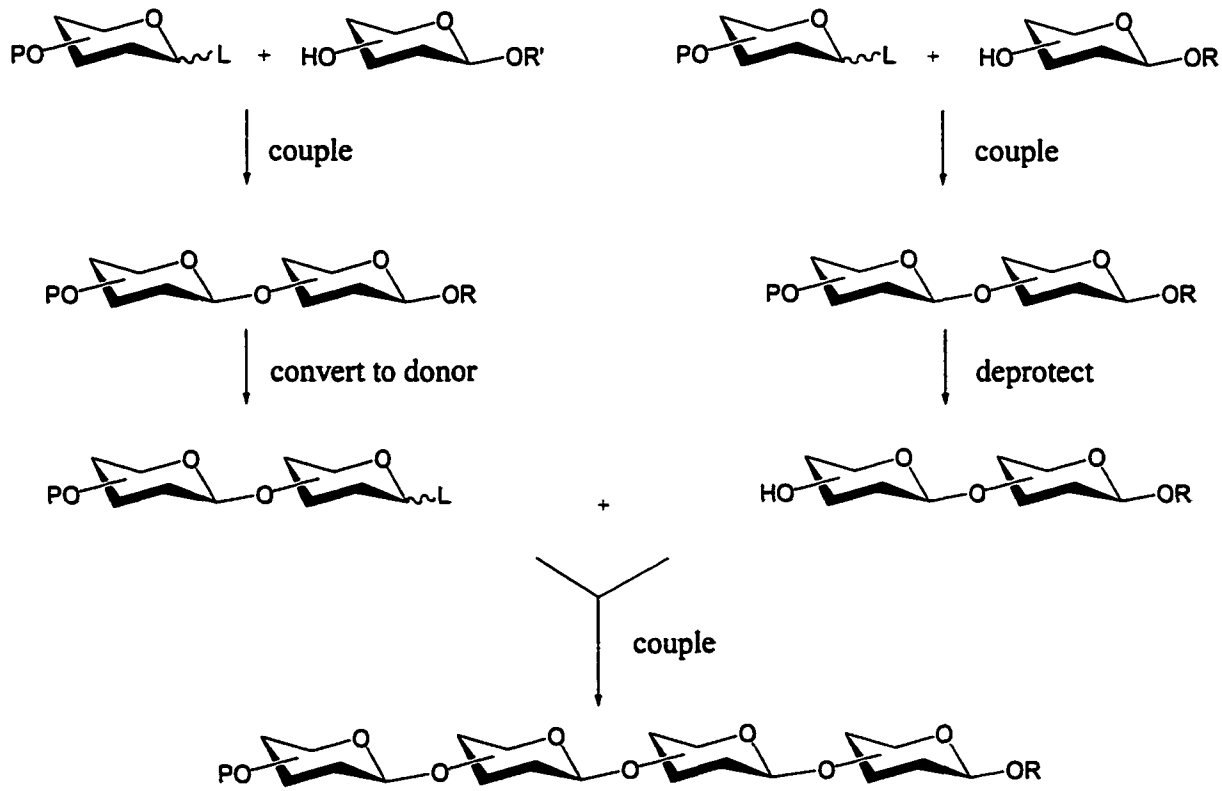


Figure 1.20: Example of block synthesis of oligosaccharide

acceptor monosaccharide contains an aglyconic moiety acting as a temporary protecting group. It may be selectively removed at a later stage in the synthetic scheme for conversion to a donor that is then used to glycosylate another sugar acceptor.

As previously mentioned, it is also possible to synthesize blocks of sugars that can be used directly as donors without an intermediate conversion step. Success with this method requires the selection of donor pairs with distinct activation conditions. Some useful donor combinations are thioglycosides and fluorides [58], thioglycosides and trichloroacetimidates [59], and thioglycosides and sulfoxides [60] (Figure 1.21).

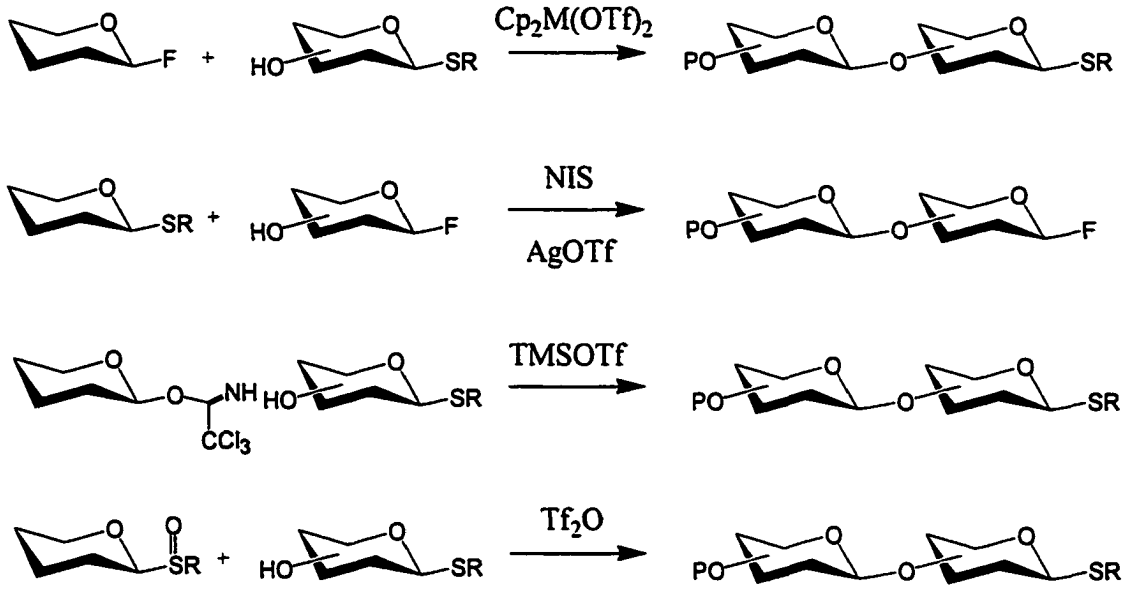


Figure 1.21: Different donor combinations useful for selective activation method

A particularly subtle approach to this problem is the use of "armed" and "disarmed" glycosyl donors. This strategy involves the use of the same type of anomeric group on both the donor and acceptor, but through the use of appropriate reaction conditions, only one of the anomeric groups is selectively activated. The utility of this approach has been demonstrated using pentenyl glycoside [61], thioglycosides [47] and phosphoramidate donors [62].

Installation of an electron-withdrawing group such as a benzoate or another ester at C-2 causes the formation of the positively charged oxocarbonium intermediate to be disfavoured. On the other hand, an ether substituent at C-2 does not have this destabilizing effect, which makes it possible to selectively activate one donor in the presence of another, as shown in Figure 1.22 [47].

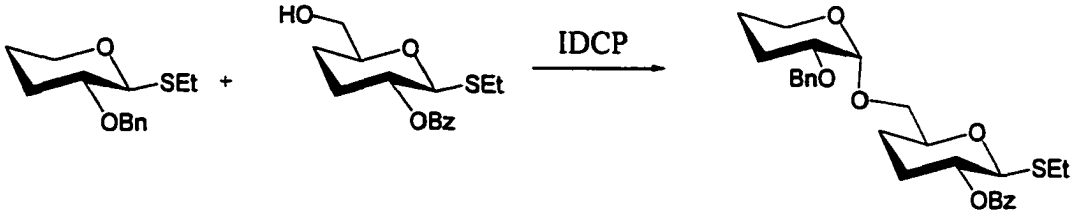


Figure 1.22: Example of armed/disarmed glycosylation strategy

This selectivity can also be induced with the use of cyclic acetal protecting groups [63]. The increased rigidity imposed on the sugar ring prevents the flattening required for the formation of the oxocarbonium intermediate (Figure 1.23).

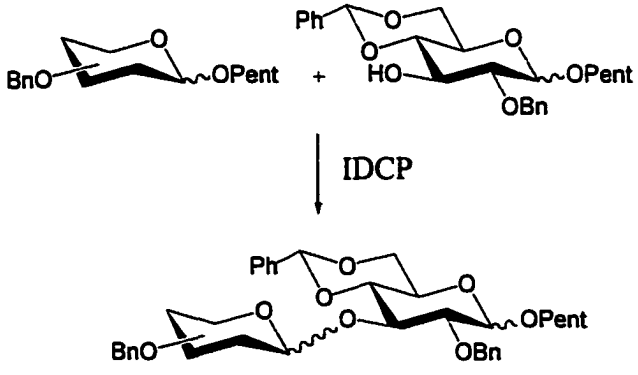


Figure 1.23: Deactivation of donor due to cyclic acetal

1.4.4 Synthesis of 2-Amino-2-Deoxy Glycosides

Much consideration has been given to the synthetic challenges presented by the incorporation of 2-amino-2-deoxy sugars in the synthesis of oligosaccharides. Due to the seemingly ubiquitous nature of 2-amino-2-deoxy glycosides in glycoconjugates, this problem can not easily be ignored. Instead, a variety of different approaches have been described in the literature, providing the carbohydrate chemist with a number of options in the design of synthetic schemes.

The nucleophilicity of the amino group presents a problem during the glycosylation step, in that undesired N-glycosylation may take place in the absence of adequate protection of the amine. A wide selection of amine protecting groups has been developed, and since the amine is located at the 2-position, the protecting group chosen can dictate the stereoselectivity of the glycosylation reaction. A brief review of some of the more frequently used amine protecting groups will be presented here.

The most common 2-amino-2-deoxy sugars found in nature are *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc). These are sugars in which the amine is already "protected" in the form of an acetamide, and glycosylation methods have been developed in which neighbouring group participation by the acetamido is invoked. The oxazoline can either be isolated and used as a donor itself [64], or it can be formed as a transient intermediate during the glycosylation reaction (Figure 1.24) [65]. Both methods

have limited utility with unreactive or hindered acceptors, leading frequently to isolation of the oxazoline as the major product. This has necessitated the development of other strategies in which the protecting groups used during glycosylation are temporary and can be removed and replaced by an acetamide at later stages in the synthetic scheme.

The most widely used temporary amine protecting group has been the phthalimido group (Figure 1.25) [66]. This has the advantage of offering neighbouring group assistance toward the synthesis of 1,2-*trans* glycosides. Unfortunately, the conditions necessary for its removal can be quite harsh, demanding the use of large excesses of an amine such as hydrazine or the less toxic ethylenediamine, often requiring temperatures as high as 80-100 °C for prolonged periods. Modifications through the use of the dichlorophthaloyl and tetrachlorophthaloyl derivatives lead to milder removal conditions, which include the use of a slight excess of ethylenediamine as well as lower reaction temperatures [67].

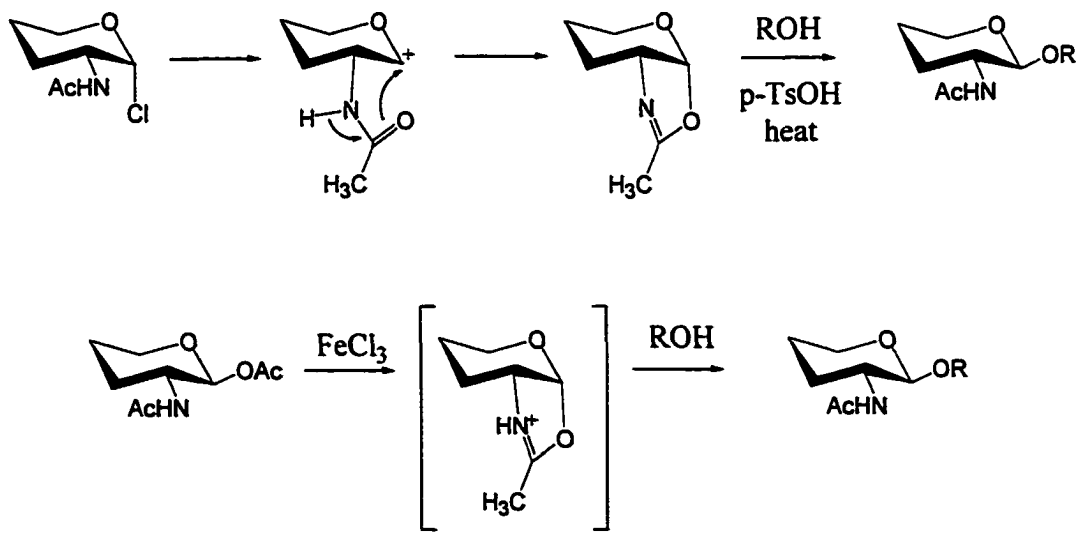


Figure 1.24: Oxazoline methods for synthesis of 1,2-*trans*-amino glycosides

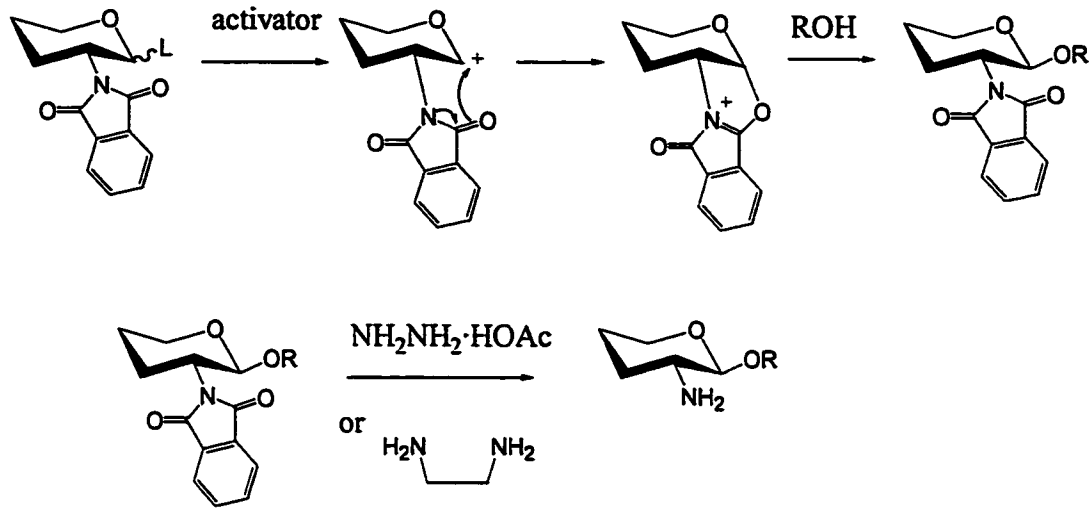


Figure 1.25: Use of phthalimido group and methods for its removal

Other amine protecting groups also in use are amides such as the mono-, di- and trichloroacetamides, as well as the *N*-pent-4-enamide [67]. Also in use are various carbamates, for example the trichloroethoxycarbonyl (Troc) [68], allyloxycarbonyl (Alloc) [69] and the *p*-nitrobenzyloxycarbonyl (PNZ) groups [70] (Figure 1.26). These groups are capable of neighbouring group participation and are thus useful for the synthesis of 1,2-trans glycosides.

The synthesis of 1,2-*cis*-α glycosides requires the use of a nonparticipating group at the 2-position. While groups that satisfy these requirements do exist, a more frequently adopted approach makes use of the azido group (Figure 1.26) [71]. This acts as a temporarily masked amine, which can be "unmasked" following the glycosylation step using various reductive methods.

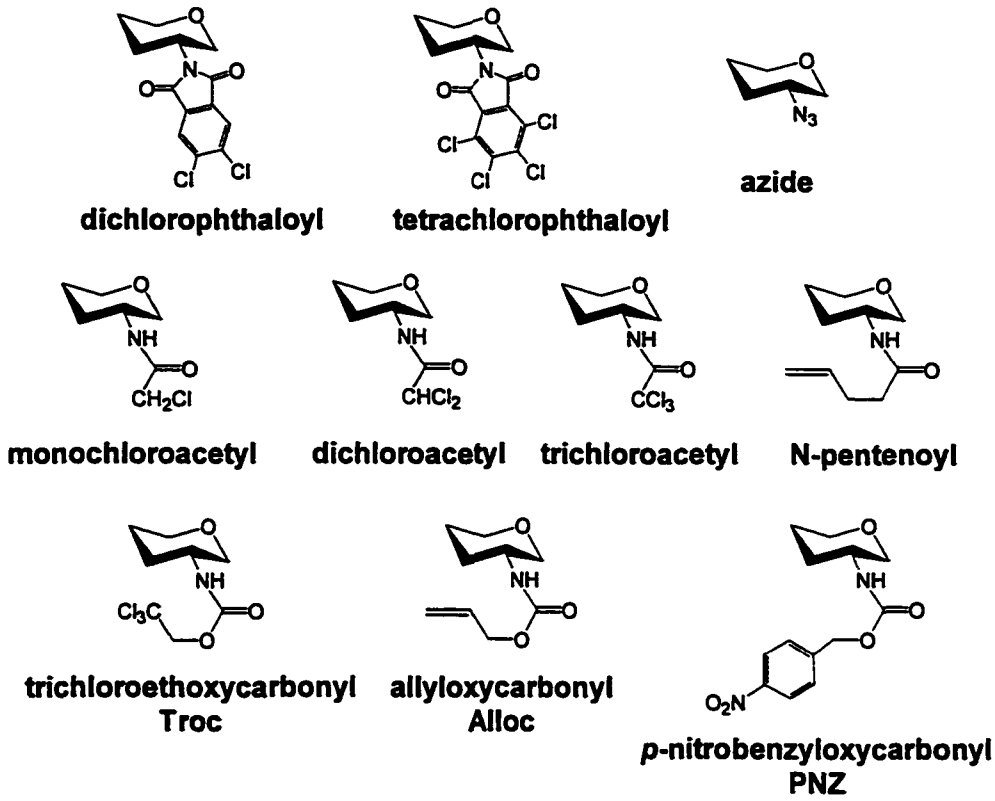


Figure 1.26: A selection of amino protecting groups

1.4.5 Synthesis of Sialyl Glycosides

1.4.5.1 Challenges of Sialic Acid Chemistry

Sialic acids are important constituents of many glycoproteins and glycolipids [72]. Their importance in recognition events at the cell surface is widely recognized, and the study of these roles is dependent upon the development of efficient methods for the synthesis of sialic acid conjugates. Sialosides in nature are invariably α-glycosides, which presents a real challenge to the carbohydrate chemist.

There are several reasons for the low yields and poor stereoselectivity observed in the synthesis of α-sialosides [73]. The first is steric hindrance at the anomeric centre (C-2) due to the adjacent carboxylic acid functionality. This carboxylate also disfavours the formation of a positive charge at C-2 upon activation of the glycosyl donor. Unfortunately, due to the anomeric effect, the thermodynamically favoured product is the β-sialoside, and the absence of a participating group at C-3 prevents the redirection away from the favoured stereochemical outcome (Figure 1.27). Another factor leading to low yielding glycosylation reaction is the competitive formation of the glycal elimination product.

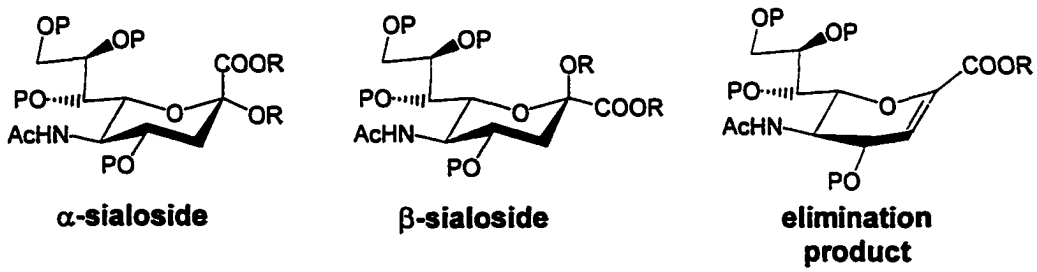


Figure 1.27: Possible outcomes of sialylation reaction

1.4.5.2 Commonly Used Sialyl Donors

The earliest attempts at the synthesis of sialosides used the classical Koenigs-Knorr methods with 2-halo donors [74]. These provided only disappointing coupling yields and poor stereoselectivity, so alternatives were sought (Figure 1.28). Thioglycoside donors were successfully used under similar activation conditions as those used for their hexose analogues [75]. Another method developed was the use of glycosyl phosphites, which

are activated under mild conditions with catalytic quantities of TMSOTf [76]. Xanthate derivatives comprise a third method that has been used with considerable success [77]. These are generally activated under electrophilic conditions with reagents such as DMTST and methylsulfenyl bromide (MSB).

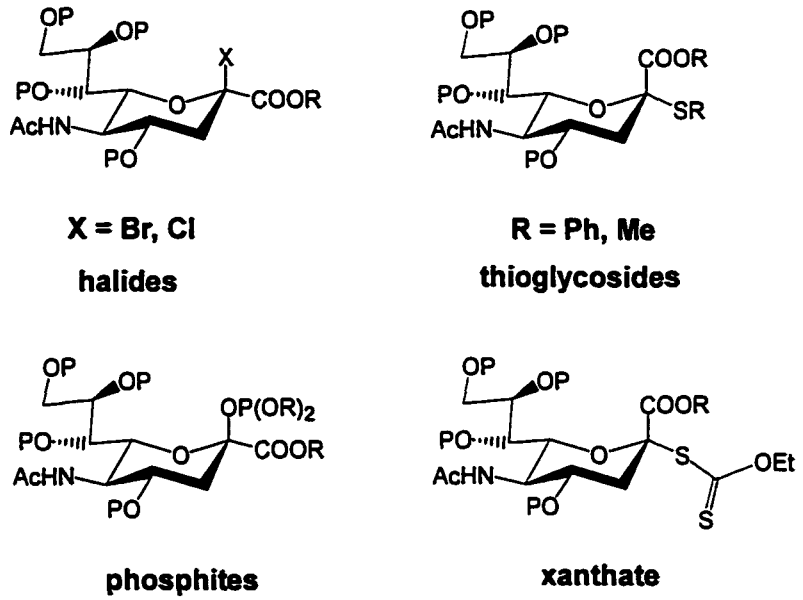


Figure 1.28: Glycosyl donors for the synthesis of α-sialosides

1.4.5.3 Solvent Participation

Each donor described above can be efficiently used to generate the oxocarbonium intermediate, but there still remains the problem of stereoselectivity. Fortunately, it has been observed that carrying out the reaction in acetonitrile as the solvent leads to the formation of predominantly α-sialoside product [78]. It has been proposed that the oxocarbonium, once formed, reacts with the solvent to form an acetonitrilium ion (Figure 1.29). The β-acetonitrilium ion then undergoes S_N2 attack by the acceptor nucleophile to

form the α-sialoside. In the absence of a nitrile solvent, the proportion of the oxocarbonium intermediate that is diverted toward the formation of the elimination product has been shown to increase [78].

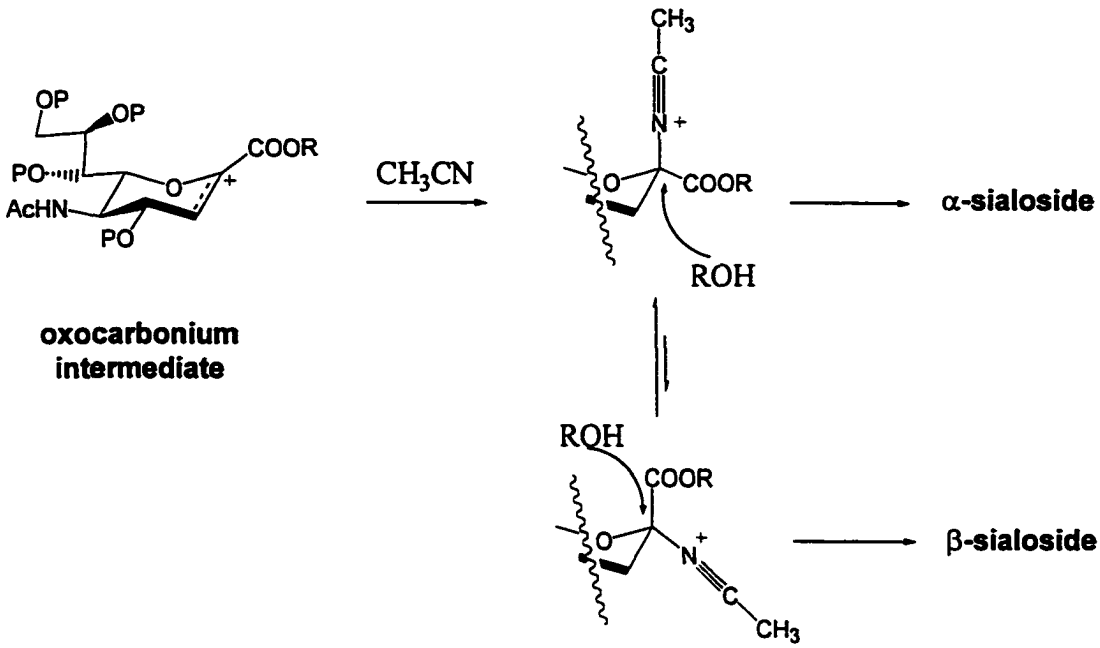


Figure 1.29: Nitrile effect in sialyl glycoside synthesis

1.4.5.4 2-Halo 3-thio or 3–seleno Auxiliaries

A novel approach to the stereoselective synthesis of α-sialoside employs donors in which the C-3 position has been substituted with a stereocontrolling auxiliary [79]. This auxiliary takes the form of a phenylthio or phenylseleno substituent (Figure 1.30a). The use of *N*-acetylneuraminyl halide donors in combination with the 3-thio or 3-seleno groups produces the α-anomer as the major glycosylation outcome. The proposed mechanism involves the formation of an episulfonium or episelenonium intermediate

(Figure 1.30b) [80]. This precludes axial attack at the anomeric centre and formation of the β-anomer. Instead, the desired α-anomer is the major product as a result of the neighbouring group participation. Two factors limit the general utility of this method, the first being numerous synthetic steps required to obtain the 3-substituted intermediate, and the second is the extra step required for the reductive removal of the auxiliary upon completion of the glycosylation.

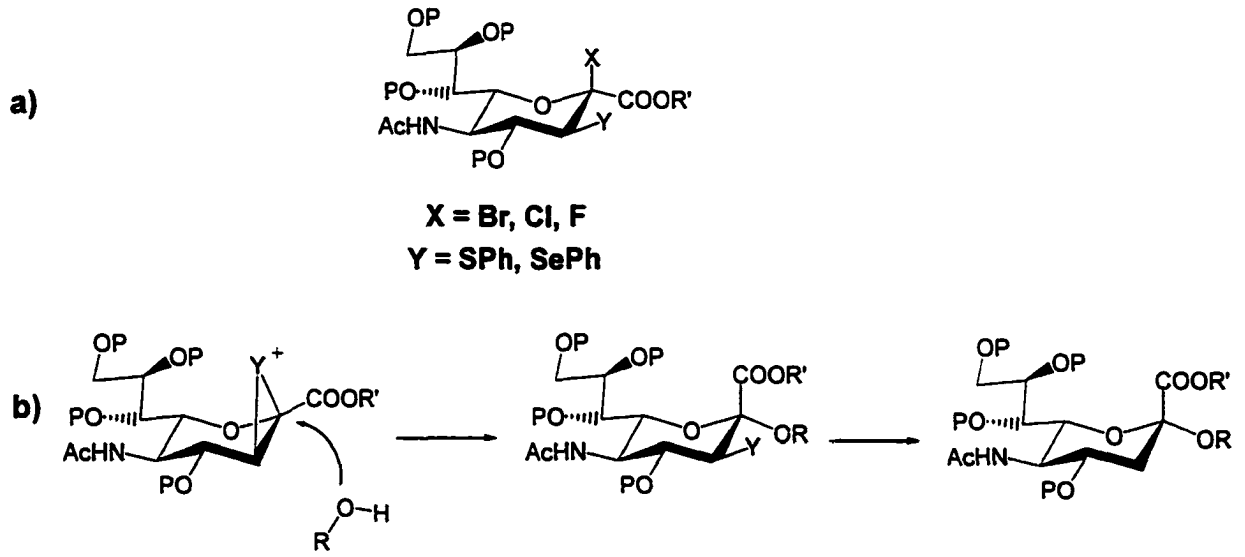


Figure 1.30: Stereocontrolling auxiliaries for the synthesis of α-sialosides

CHAPTER 2:

Design and Synthesis of GM_1 Analogues as Inhibitors of Cholera Toxin and Heat-Labile Toxin

2.1 Introduction

This chapter describes the design and synthesis of analogues of GM_1 for inhibition studies of cholera toxin (CT) and heat-labile enterotoxin (LT). Factors considered during the design process will be reviewed, including previously reported toxin binding studies of modified GM_1 substrates, as well as approaches taken by other groups toward the rational design of carbohydrate based inhibitors. The methods employed for the synthesis of the analogues will also be discussed.

2.1.1 Binding Studies on Modified GM_1 Substrates

Once it was recognized that ganglioside GM_1 was the receptor for CT and LT, studies were undertaken to establish specific GM_1 binding determinants. No crystal structure of either toxin with bound oligosaccharide existed in the literature until the publication of a lactose:LT complex in 1992 [81] and GM_1:CT complex in 1994 [23]. This meant that the specific interactions between the host toxin and its carbohydrate ligand could only be elucidated through the systematic manipulation of structural components of the native GM_1 ganglioside.

The importance of the terminal galactose for binding was first established in 1973 in a study by Cuatrecasas, which described the screening of a large number of mono-, di- and oligosaccharides for their ability to prevent binding of CT to liver cell membranes [82]. None of the smaller saccharides demonstrated any significant inhibitory effect at submillimolar concentrations. It was shown, however, that GM_1 was a potent inhibitor, and GM_2 was notably weaker. GM_2 is obtained by removing the terminal galactose of GM_1.

Schengrund *et al.* modified the sialic acid segment of GM_1 to investigate the critical binding contacts between this part of the sugar and the protein binding site [83]. The sialic acid glycerol chain was either truncated at C-7 or replaced with an ethanolamine fragment with no observable effect on binding. The *N*-acetyl was replaced by an *N*-glycolyl, which also resulted in a negligible change in binding strength. Conversion of the carboxylate to the methyl ester lead to a slightly more noticeable decrease in binding

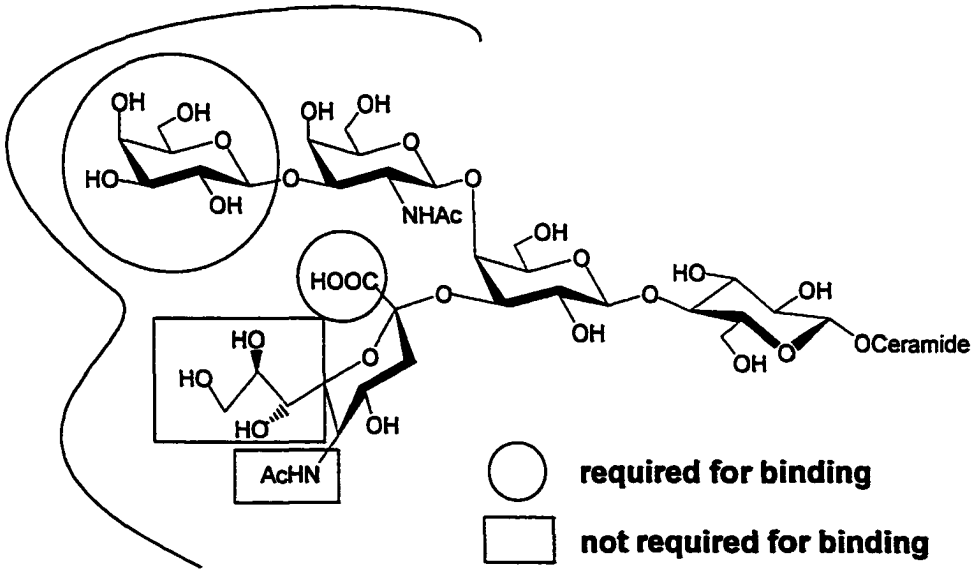


Figure 2.1: Results of Modified GM_1 Substrate Assays

by factors of 5.5 for CT and 2.5 for LT. It would seem that the functional groups on sialic acid can be manipulated quite extensively without significant penalty (Figure 2.1). With these observations in mind it is interesting to note that the complete removal of the sialic acid leads to abolition of binding altogether.

Further study of the role sialic acid plays in binding was undertaken by Lanne *et al.* through the synthesis of GM_1 derivatives in which its carboxylate functionality is chemically modified [84]. It was found that the amide and benzylamide of GM_1 retain their CT binding capabilities, although weaker by a factor of 14 and 81 respectively. Replacement of the carboxylate with a methyl, ethyl or propyl amide, on the other hand, leads to a significant drop in binding strength. Reduction of the carboxylate to an alcohol reduces binding by a factor of 33. The conclusion was drawn that the carboxylate is required for hydrogen bonding interactions and these interactions are sustained to some degree with the amide derivative. The weaker binding of the alcohol suggests the importance of the carbonyl in particular. The inability of the methyl, ethyl and propyl amides to bind well to the toxin may be a result of the hydrophobic substituents preventing access to the hydrogen bonding determinants. The anomalous result with the benzylamide may be due to a conformational change in the sugar as a result of the size of the amide substituent, or there may possibly be a favourable interaction with a hydrophobic patch on the periphery of the binding site.

The data gained from binding studies of modified GM_1 substrates and the GM_1:CT crystal structure provides some insight into the minimum carbohydrate structure required

for strong ligand binding. This information can be applied to efforts regarding the rational design of small molecules that maintain strong interactions with the toxin binding site.

2.1.2 Considerations in Carbohydrate-Based Drug Design

When considering the development of drug therapies for the treatment of cholera or traveler's diarrhea, a logical place to start is the design of small molecules that hinder the recognition step at the cell surface. If binding of the B pentamer to GM_1 on epithelial cells can be prevented, the toxin will be denied the opportunity to enter the cell and the development of the clinical symptoms of the disease will be averted. Since it has been amply demonstrated that the toxin binds to the carbohydrate receptor GM_1, this will be considered the starting point for the design of inhibitory small molecules.

The oligosaccharide portion of GM_1 would make an efficient and strong binding inhibitor if not for two problems. The first is the extremely high cost of synthesizing a pentasaccharide on a scale large enough for clinical trials, let alone global distribution in the form of a widely available medication. The second problem with using a molecule such as GM_1 oligosaccharide is the susceptibility of the glycosidic linkages to enzymatic degradation once administered to the patient [85]. These are problems inherent to the use of any carbohydrate as a potential drug, and some cues for avoiding these obstacles may be taken from another system currently under investigation.

The sialyl Lewis^x (SLe^x) tetrasaccharide epitope is found on membrane glycoproteins and glycolipids and is known to play a role in the adhesion and recruitment of leukocytes to sites of infection or injury [6]. The recognition step occurs when cells lining blood capillaries in the area of injured tissue are stimulated to produce a protein called E-selectin on their cell surface. E-selectin interacts with SLe^x on the surface of leukocytes, recruiting them to the injured tissue, leading to an inflammatory response, which in severe or chronic cases can lead to tissue damage. If the cell-adhesion step could be blocked, this inflammatory response could be checked and tissue damage prevented.

Considerable research has been undertaken to develop molecules that mimic the SLe^x tetrasaccharide. It is known that the intact tetrasaccharide effectively inhibits the adhesion process in vivo, but its cost and vulnerability to glycosidases make the design of mimics an attractive target for research.

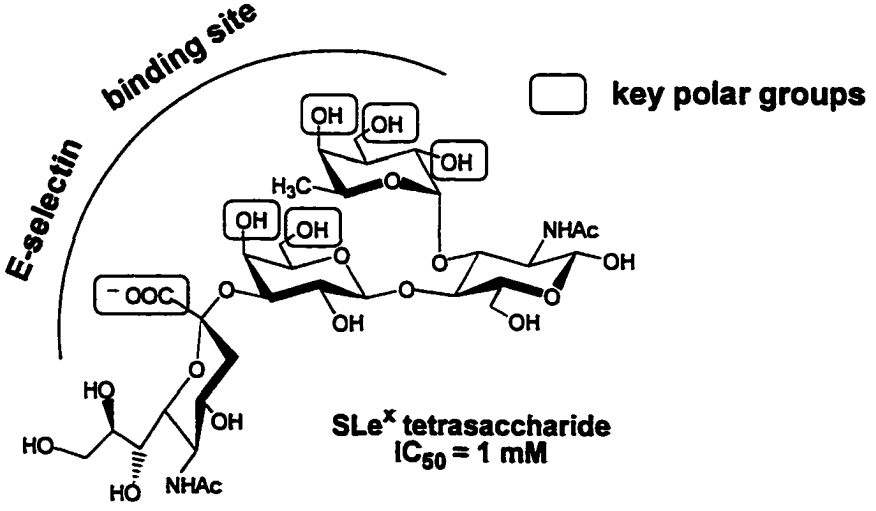


Figure 2.2: Important interactions between SLe^x and E-selectin

Since a crystal structure of E-selectin has been published [86], and key polar interactions between E-selectin and SLe^x have been elucidated [87] (Figure 2.2), a number of research groups have taken this information and rationally designed potential inhibitors [88], some of which are shown in Figure 2.3. These molecules contain features such as non-carbohydrate linkers (**A** and **D**) [89, 90], unnatural 1,1-glycosidic linkages (**C**) [91] and C-linkages (**B**) [92], acid fragments in place of a sialic acid residue (**A**, **C** and **D**) and the replacement of a sugar residue with an amino acid-based moiety (**B**). These target molecules incorporate decreased vulnerability to glycosidases while maintaining comparable or even improved binding to E-selectin.

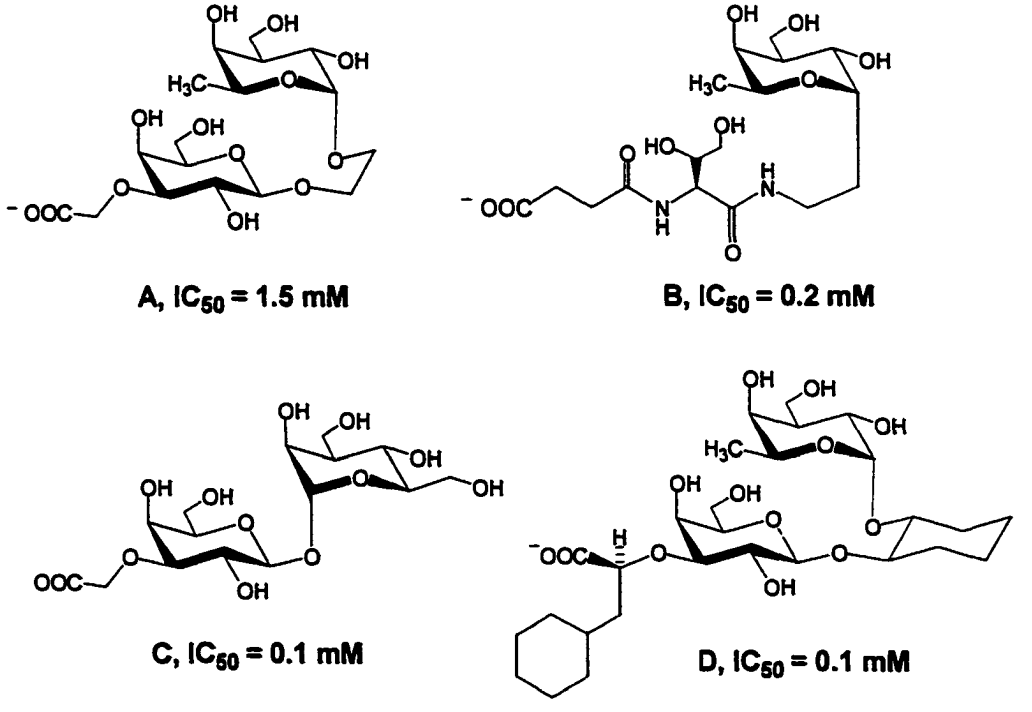


Figure 2.3: Rationally Designed SLe^x mimics

2.1.3 Description of Target Molecules

Once provided with data from binding studies of modified GM_1 substrates that demonstrate that the terminal galactose and the carboxylate of sialic acid are required for strong binding, strategies for designing potential inhibitors of CT and LT can be developed. It is evident from the SLe^x/E-selectin studies that a sialic acid residue can be replaced with acid fragments and non-carbohydrate linkers can be inserted in place of carbohydrate moieties without a significant penalty to recognition by the protein. The question posed in this thesis is whether these synthetic tools can be employed in the design of potent inhibitors of cholera toxin and heat-labile toxin.

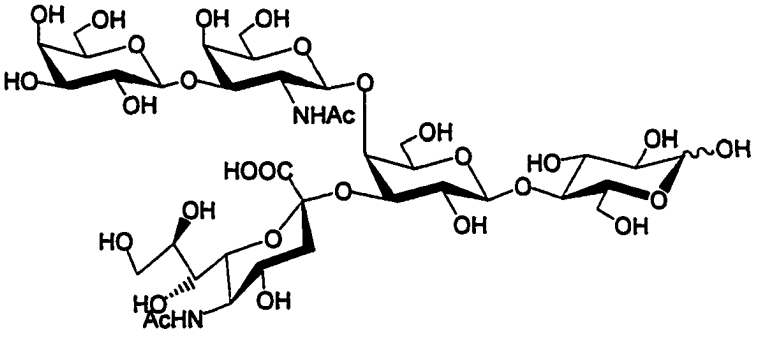


Figure 2.4: GM_1 oligosaccharide

To answer this question, a series of target molecules based on the GM_1 terminal oligosaccharide (Figure 2.4) were designed. The targets incorporated either a flexible two-carbon linker in the place of the branching galactose residue (**1**), a carboxymethyl fragment in place of the sialic acid residue (**2**), or both of these modifications (**3**) (Figure 2.5).

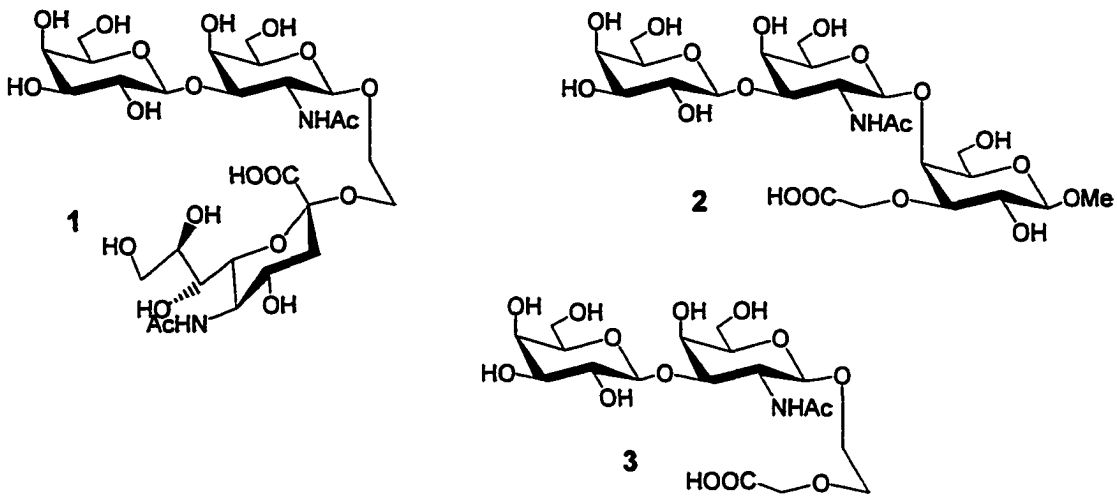


Figure 2.5: GM_1 analogues incorporating ethylene linker and/or acid fragment

Two other trisaccharide targets (**4** and **5**) were designed wherein the acetamido of the GalNAc residue was replaced by a hydroxyl in order to investigate the contribution of the acetamido toward favourable binding interactions (Figure 2.6).

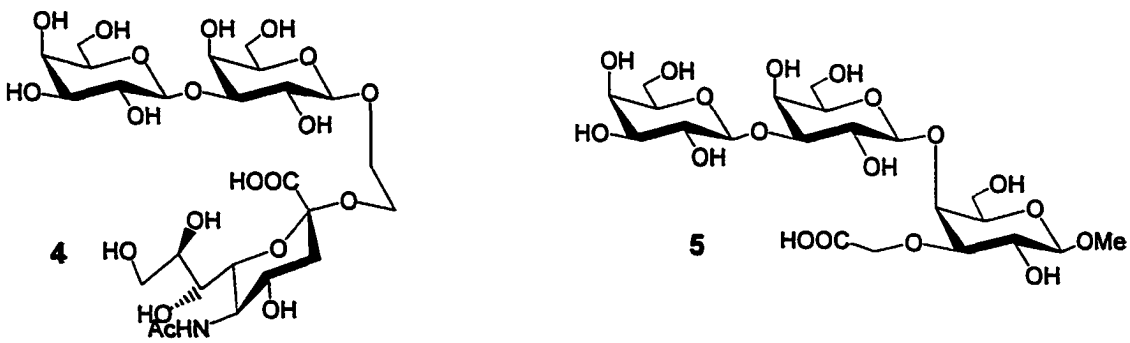


Figure 2.6: GM_1 analogues incorporating Gal in place of GalNAc

Since the chemistry toward the synthesis of the trisaccharide targets was first optimized on disaccharides lacking the terminal galactose, the products **6**, **7** and **8** were also included in the final collection of compounds for inhibition testing (Figure 2.7).

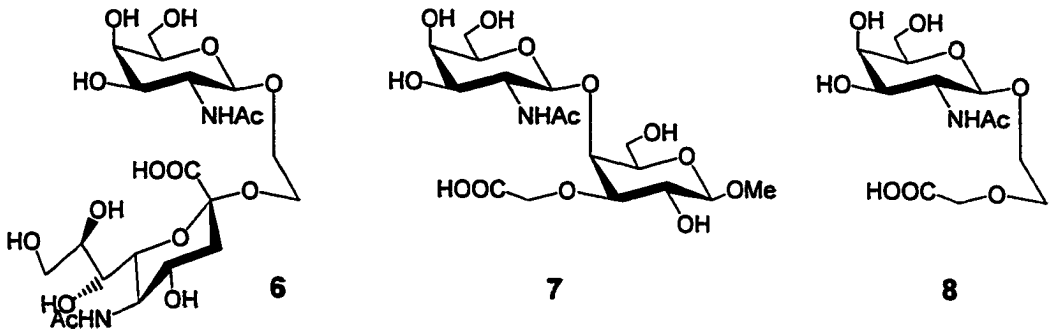


Figure 2.7: Disaccharide GM_1 analogues

2.2 Synthesis of Carbohydrate analogues

2.2.1 Gal-GalNAc Chemistry

2.2.1.1 GalNAc and Gal-GalNAc Primary Acceptors

The target molecules containing a flexible ethylene linker (**1**, **3**, **6** and **8**) were synthesized according to the retrosynthetic approach shown in Figure 2.8. The first goal was to make the Galβ(1→3)GalNAcβOCH$_2$CH$_2$OH (**16**) and GalNAcβOCH$_2$CH$_2$OH (**10**) primary alcohol acceptors available for sialylation and carboxymethylation.

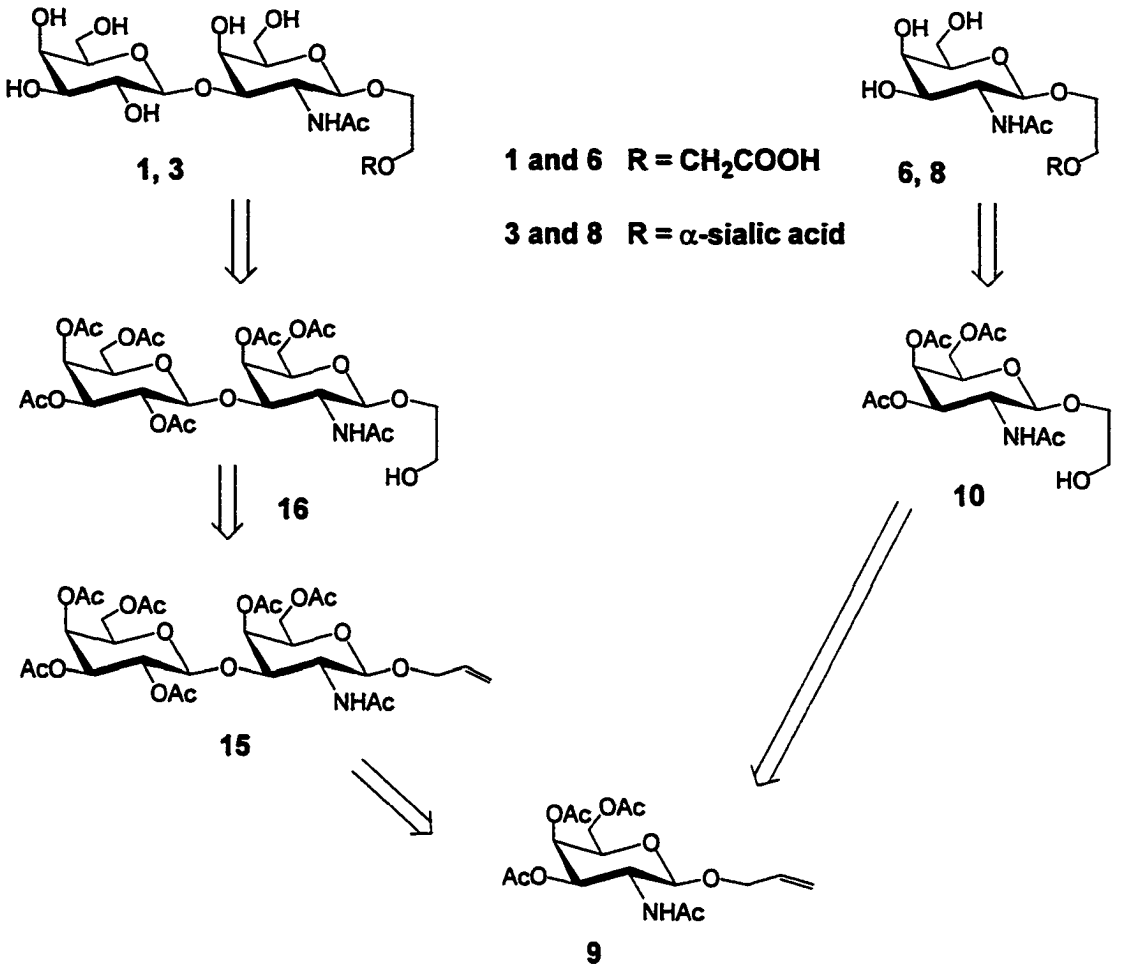
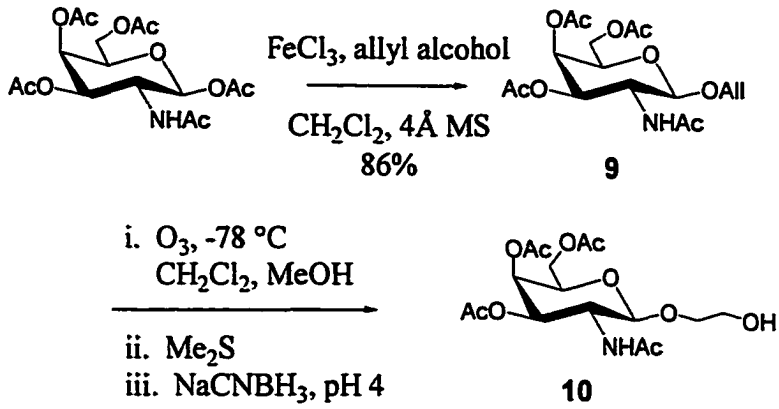


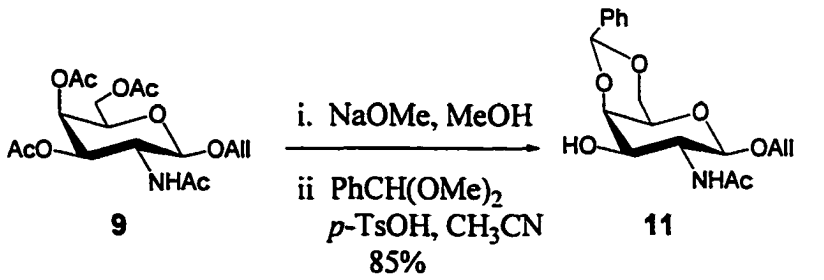
Figure 2.8: Retrosynthesis of flexible linker target compounds

The allyl glycoside was chosen for the ease with which it can be converted to the 2-hydroxyethylene moiety. The first step towards this goal was the transformation of the β-1-O-acetate of peracetylated galactosamine into the β-allyl glycoside **9** (Scheme 2.1). This was easily achieved in 86% yield under Lewis acid conditions in the presence of $FeCl_3$ [65]. Synthesis of the monosaccharide acceptor **10** began with ozonolysis of the allyl double bond followed by reductive work-up with dimethyl sulfide. Further reduction to the alcohol was accomplished using sodium cyanoborohydride under mildly acidic conditions, giving the product **10** in 95% overall yield (2 steps).



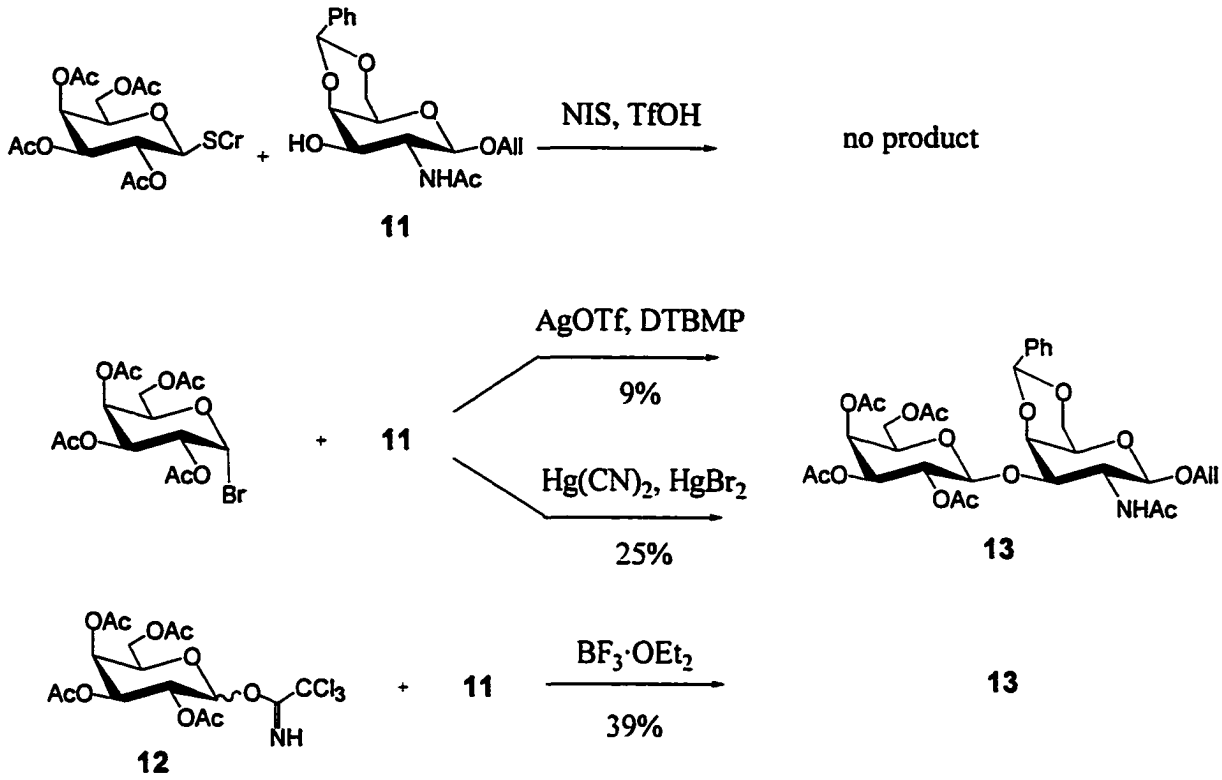
Scheme 2.1: Synthesis of primary alcohol acceptor **10**

The first step toward the construction of the disaccharide acceptor **16** was the synthesis of the 4,6-*O*-benzylidene protected GalNAc derivative **11**. Zemplèn deacetylation of **9**, followed by benzylidene acetal formation using benzaldehyde dimethyl acetal and *p*-toluenesulfonic acid gave **11** in 85% overall yield (Scheme 2.2).



Scheme 2.2: Synthesis of 3-*O* GalNAc acceptor **11**

Many attempts were made to glycosylate the acceptor **11**, as shown in Scheme 2.3. The use of acetobromogalactose [93] and thioglycoside donors [94] was explored, with marginal success. The imidate donor **12** [95] was ultimately selected (in spite of the fact that the optimized yield remained quite low) due to the reproducibility of the coupling



Scheme 2.3: Attempts at synthesis of Galβ(1→3)GalNAc disaccharide **13**

reaction in the author's hands. It should be noted that the use of larger amounts of donor relative to acceptor, i.e. greater than a 1:1 ratio, led to a decrease in coupling yield. For example, the use of 3-4 equivalents of donor relative to acceptor did not yield any disaccharide product at all, contrary to expectations. This may be attributed to the glycosylation of the oxygen of the acetamido group as shown in Figure 2.9.

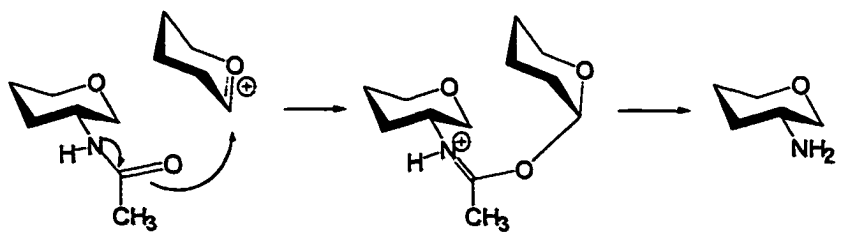
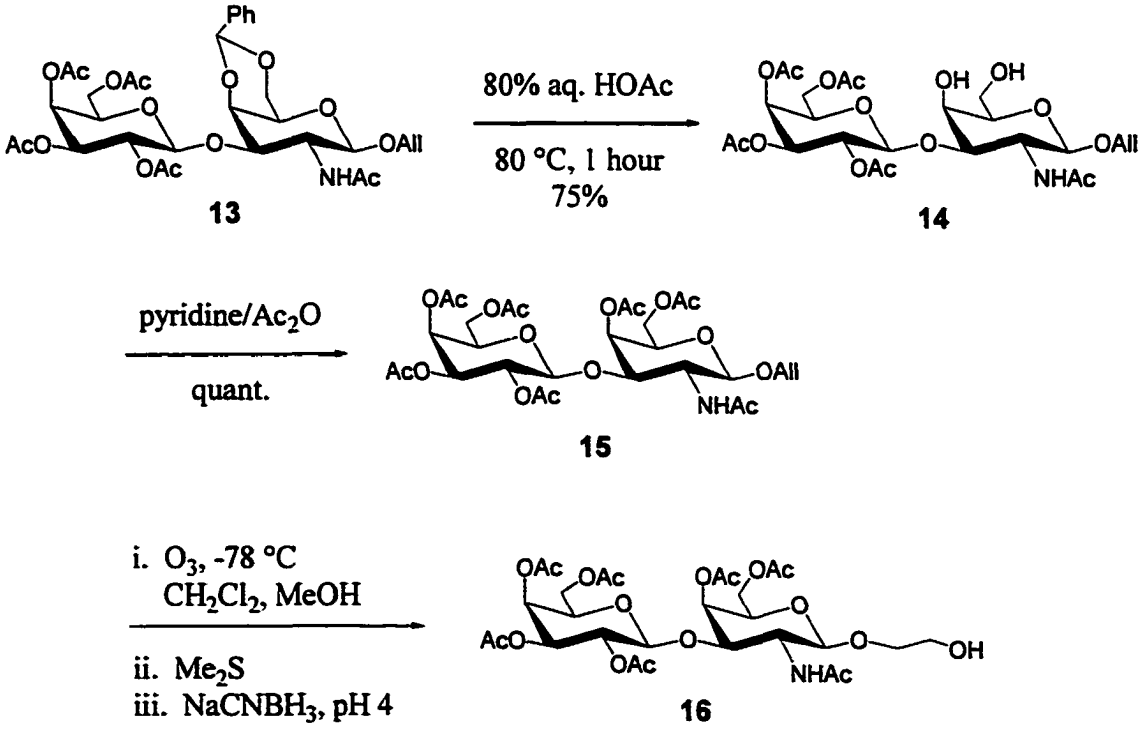


Figure 2.9: Possible explanation for low yield of glycosylation reaction

The formation of the imidate intermediate followed by aqueous work-up leads to hydrolysis to the free amine. The amino sugar is then effectively lost during chromatography.

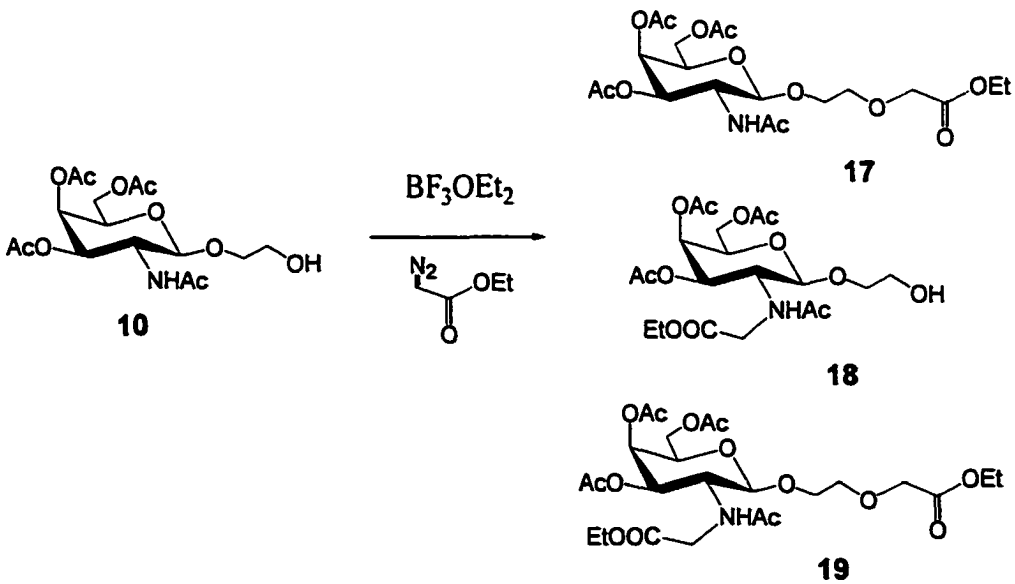
Once the disaccharide **13** was obtained, the benzylidene acetal was hydrolyzed in 80% acetic acid at 80°C, and the resulting diol **14** was acetylated with pyridine and acetic anhydride in 75% overall yield (2 steps) to give disaccharide **15**. This was then ozonolyzed with reductive work-up, followed by reduction to the primary alcohol using sodium cyanoborohydride (pH 4) as for compound **10**. The disaccharide acceptor **16** was thus obtained in 83% yield (Scheme 2.4).



Scheme 2.4: Synthesis of disaccharide primary alcohol acceptor **16**

2.2.1.2 GalNAc and Gal-GalNAc with Acid Fragments

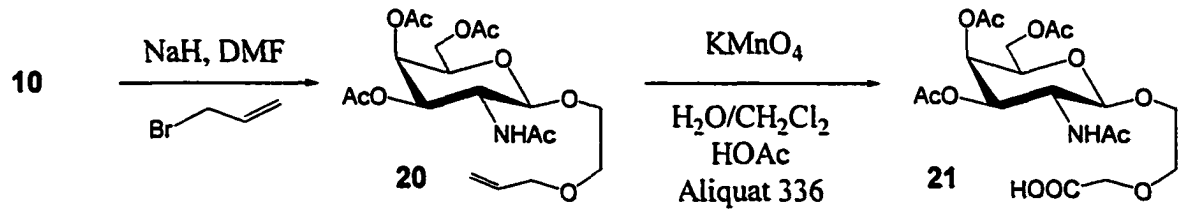
A number of different approaches were considered for the addition of the carboxymethyl functionality. Methods investigated included direct introduction of the carboxymethyl fragment through alkylation of the 2-hydroxyethyl group using either ethyl diazoacetate under acid-catalyzed conditions, or *tert*-butyl α-bromoacetate under basic conditions. Another more indirect method for obtaining the carboxymethyl group involved the allylation of compound **10** followed by the oxidation of the double bond to a carboxylic acid. All methods were first attempted on the monosaccharide, with none of the methods being very successful. A significant problem with the Lewis acid-catalyzed alkylation using the diazoester [96] was in the lack of discrimination between O-alkylation and N-alkylation (on the acetamido) as shown in Scheme 2.5. Addition of 2 equivalents of the diazoacetate led to the formation of a mixture of products **17**, **18**, and **19** in a 1:1:2 ratio.



Scheme 2.5: Ethyl diazoacetate used for installation of carboxymethyl group

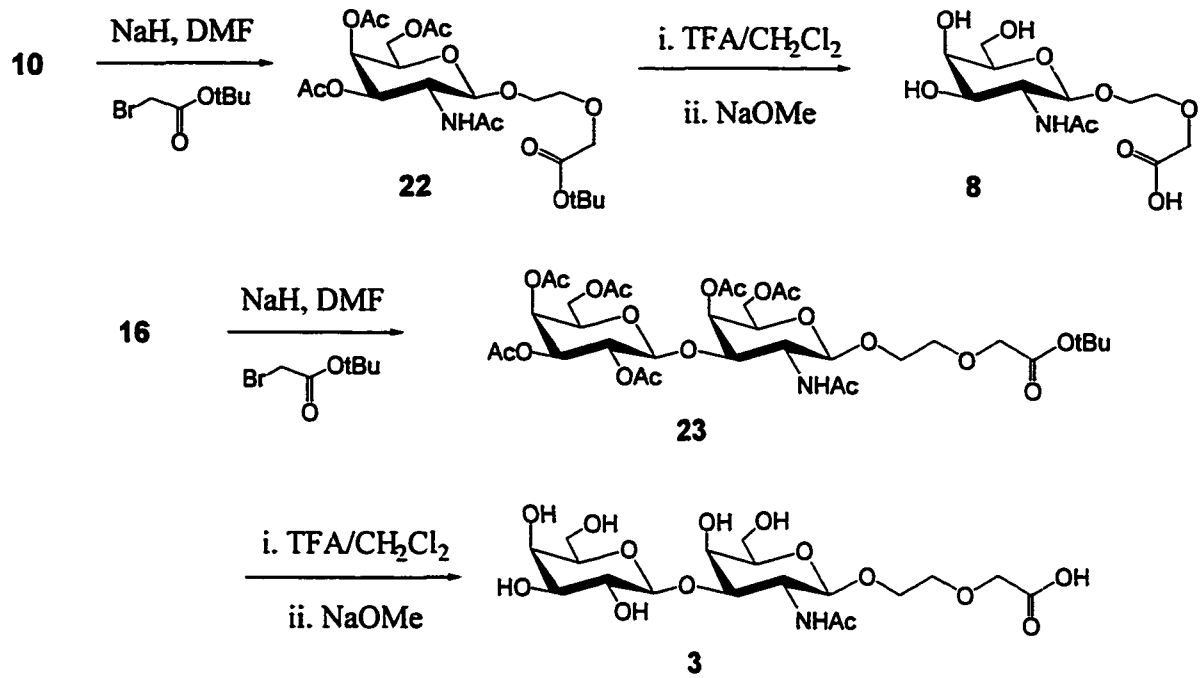
The proposed structures were confirmed using ^1H NMR and mass spectrometry. The addition of fewer equivalents of the diazoacetate led to similar mixtures, leading to the conclusion that this was not a useful process.

The indirect method of installing the acid fragment involved alkylation of **10** with allyl bromide followed by the transformation of the resulting alkene **20** to the carboxylic acid **21** using phase-transfer catalyzed $KMnO_4$ oxidation [97] (Scheme 2.6). The product was isolated in very low yield, which precluded further investigations using this procedure.



Scheme 2.6: Carboxymethyl fragment from oxidation of allyl group

Alkylation of the primary acceptors **10** and **16** employing *tert*-butyl α-bromoacetate and sodium hydride in DMF gave the products **22** and **23** in 33% and 27% yield respectively (Scheme 2.7). While these yields were disappointing, further optimization was not pursued since enough of each compound was already in hand for inhibition testing. The products were deprotected through acidolysis of the *tert*-butyl ester, followed by deacetylation to provide the final carboxymethyl product in 44% yield for the monosaccharide **8** and 54% yield for the disaccharide **3**.

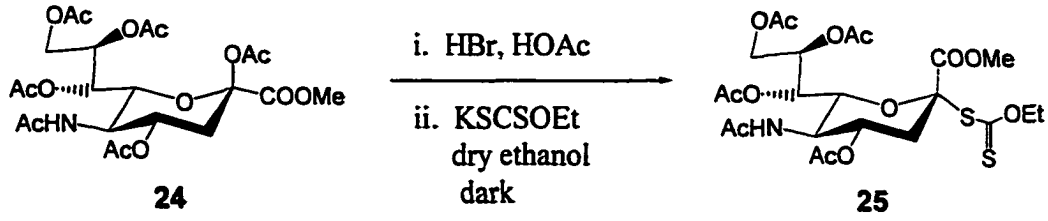


Scheme 2.7: *tert*-Butyl ester method for installation of carboxymethyl group

2.2.1.3 GalNAc and Gal-GalNAc with Sialic Acid

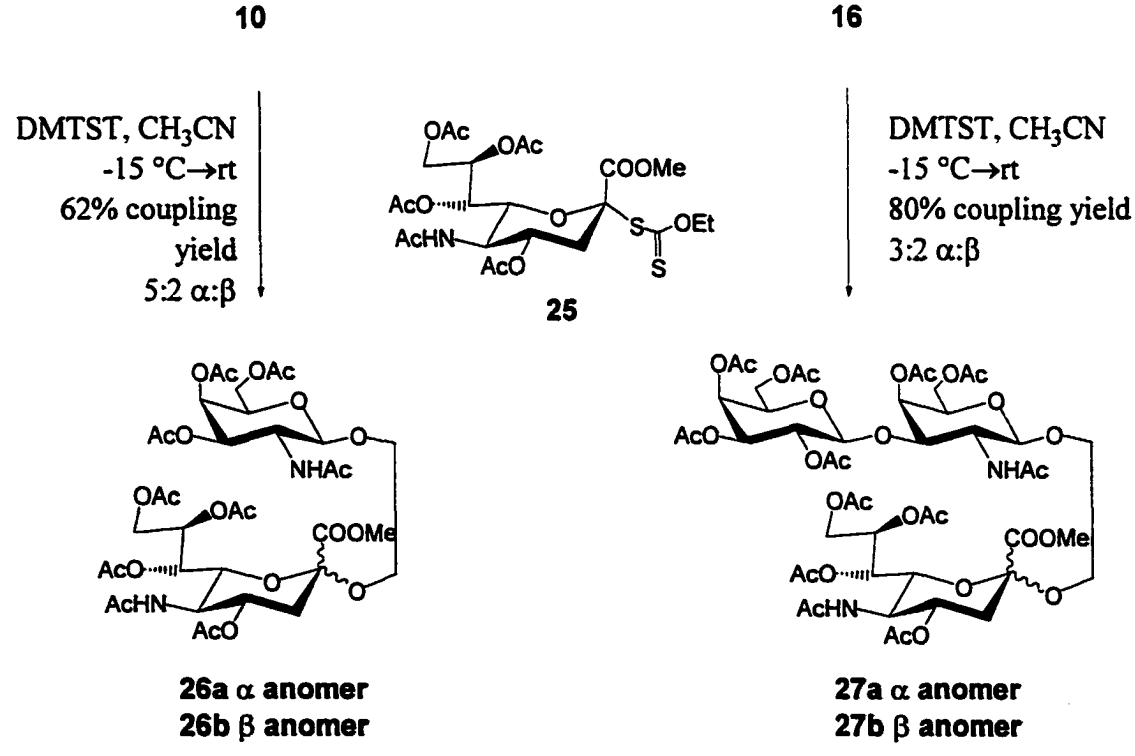
A number of different N-acetylneuraminic acid derivatives are available as donors for the introduction of a sialic acid residue to an hydroxyl acceptor, including thioglycosides, phosphites and xanthate derivatives. Synthesis of the sialylated inhibitors was carried out using the S-glycosyl xanthate derivative of N-acetylneuraminic acid **25** as donor. This donor was chosen for the ease with which it can be made, its availability in an easily handled crystalline form, as well as its stability to long term storage. The first step in the formation of this donor was conversion of the *per*-acetylated methyl ester **24** [98] to the β-bromo sugar in HBr (45% v/v in acetic acid). A solution of the bromo donor and

potassium ethyl xanthogenate in ethanol was then stirred overnight in the absence of light [77] (Scheme 2.8). Inversion at the anomeric centre provides the α-xanthate **25** in 62% overall yield, which is easily crystallized to give a yellow needle-like product.



Scheme 2.8: Synthesis of sialyl xanthate donor

The two primary alcohol acceptors **10** and **16** were sialylated with the donor **25** using the activator dimethyl(methylthio)sulfonium triflate (DMTST) in acetonitrile [99] (Scheme

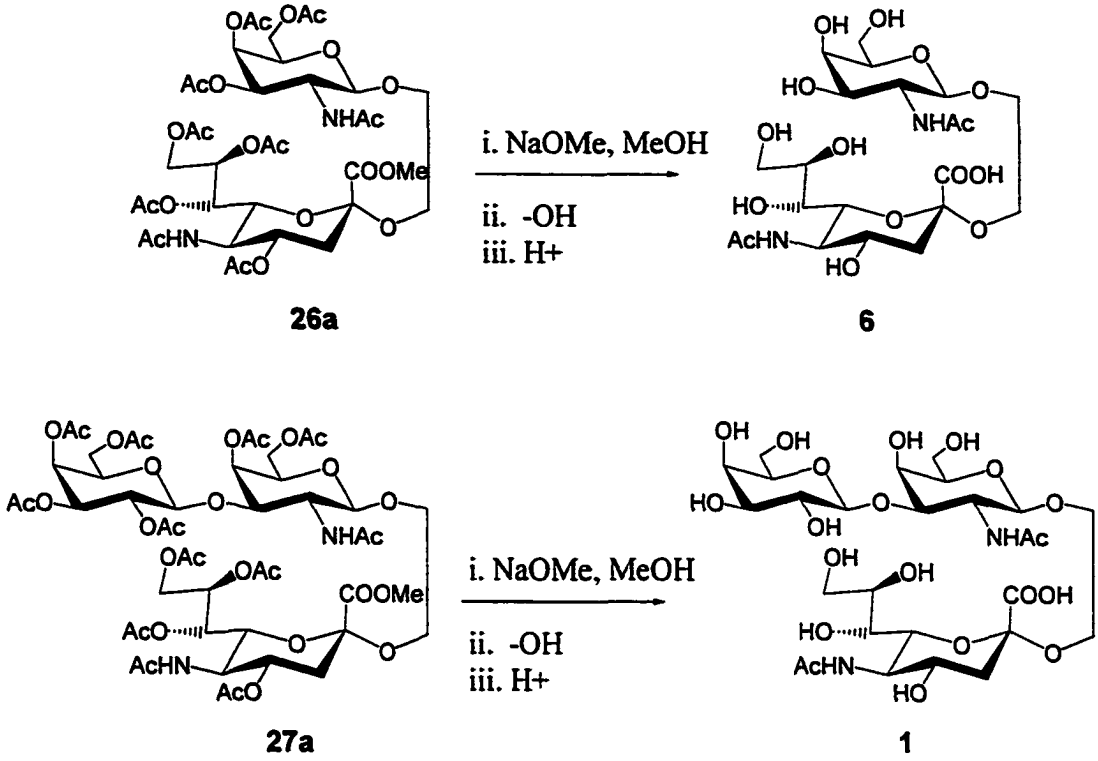


Scheme 2.9: Synthesis of sialosides

2.9). The overall coupling yields in both situations were acceptable, 62% relative to the monosaccharide acceptor (5:2 α:β) and 80% relative to the disaccharide acceptor (3:2 α:β ratio).

The anomeric configuration at the quaternary C-2 of the sialic acid was determined by NMR analysis. HMBC experiments are used to determine the three bond ($^3J_{^1H,^{13}C}$) coupling between the carbon of the carboxylate and the axial hydrogen at the 3-position. If the carboxylate is axial as well, the antiperiplanar nature of the two substituents leads to a correlation that can be detected using this technique, with a coupling constant on the order of approximately 6 Hz being observed. If the carboxylate is equatorial, the coupling between the carbon and hydrogen is weaker, and the correlation is difficult to detect.

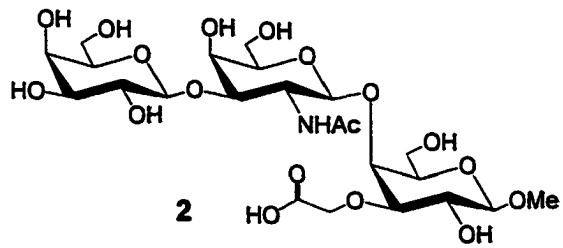
Once the α-sialosides (**26a** and **27a**) were each isolated, the deprotection was carried out without difficulty. The first step of the deprotection was deacetylation in methanolic sodium methoxide followed by the addition of water directly to the reaction mixture to effect saponification of the methyl ester (Scheme 2.10). Neutralization of the reaction with Amberlite IR-120 (H+) provided the final products **6** and **1** in quantitative yield.



Scheme 2.10: Deprotection of sialosides

2.2.1.4 Phthalimido Disaccharide Studies

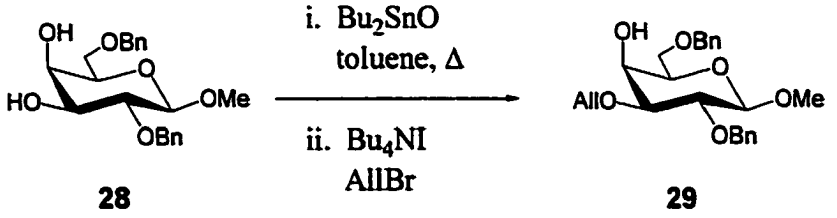
The next synthetic target was the trisaccharide **2** containing the acid fragment in place of the sialic acid residue. Model studies on the GalNAcβ(1→4)Gal disaccharide were



undertaken to establish the best approach to the synthesis of the corresponding

trisaccharide. Phthalimido protection on the galactosamine was chosen to provide temporary masking of the amino group until the acetamide could be installed. The phthalimido group is also expected to provide anchimeric assistance during the glycosylation step, leading to the β-anomer as the major product. As with the incorporation of the acid fragment described in Section 2.3.1.2, a few alternatives were considered when trying to decide how to install the carboxymethyl group at the 3-position of galactose. The two options explored included the acid-sensitive *tert*-butyl ester derivative and, alternatively, oxidation of the allyl group to provide the desired acid fragment. It was established experimentally that neither method held a significant advantage over the other in terms of overall yield. Only the allyl oxidation method will be described.

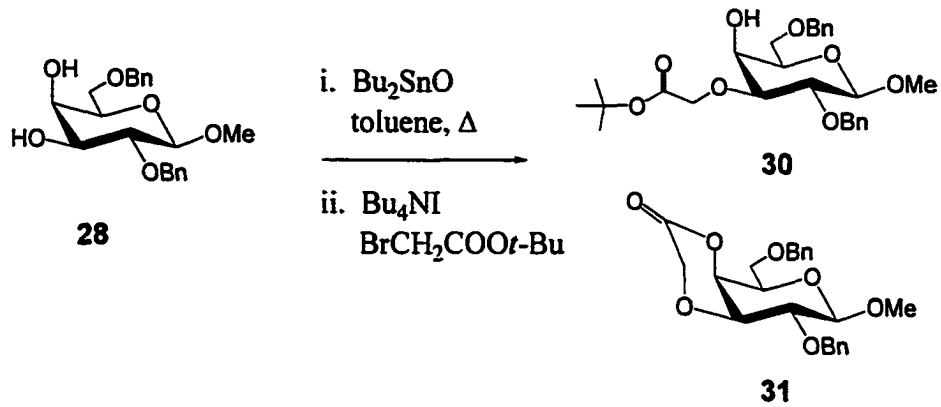
Synthesis of the galactose acceptor **29** [100] began with the stannylidene mediated regioselective alkylation at the 3-position of methyl 2,6-di-*O*-benzyl-β-D-galactoside **28** [101]. Alkylation with allyl bromide to give **29** proceeded in high yield (91%) (Scheme 2.11).



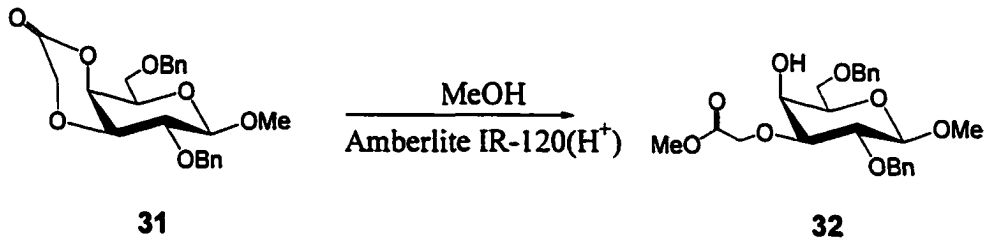
28 **29**

Scheme 2.11: Regioselective allylation of **28**

Regioselective alkylation with *tert*-butyl α-bromoacetate provided both the *3-O-(tert*-butoxycarbonyl)methyl derivative **30** and the corresponding lactone **31** (Scheme 2.12). Conveniently, the lactone **31** could be easily converted to the methyl ester **32** by stirring overnight in methanol with Amberlite IR-120(H+) (Scheme 2.13).



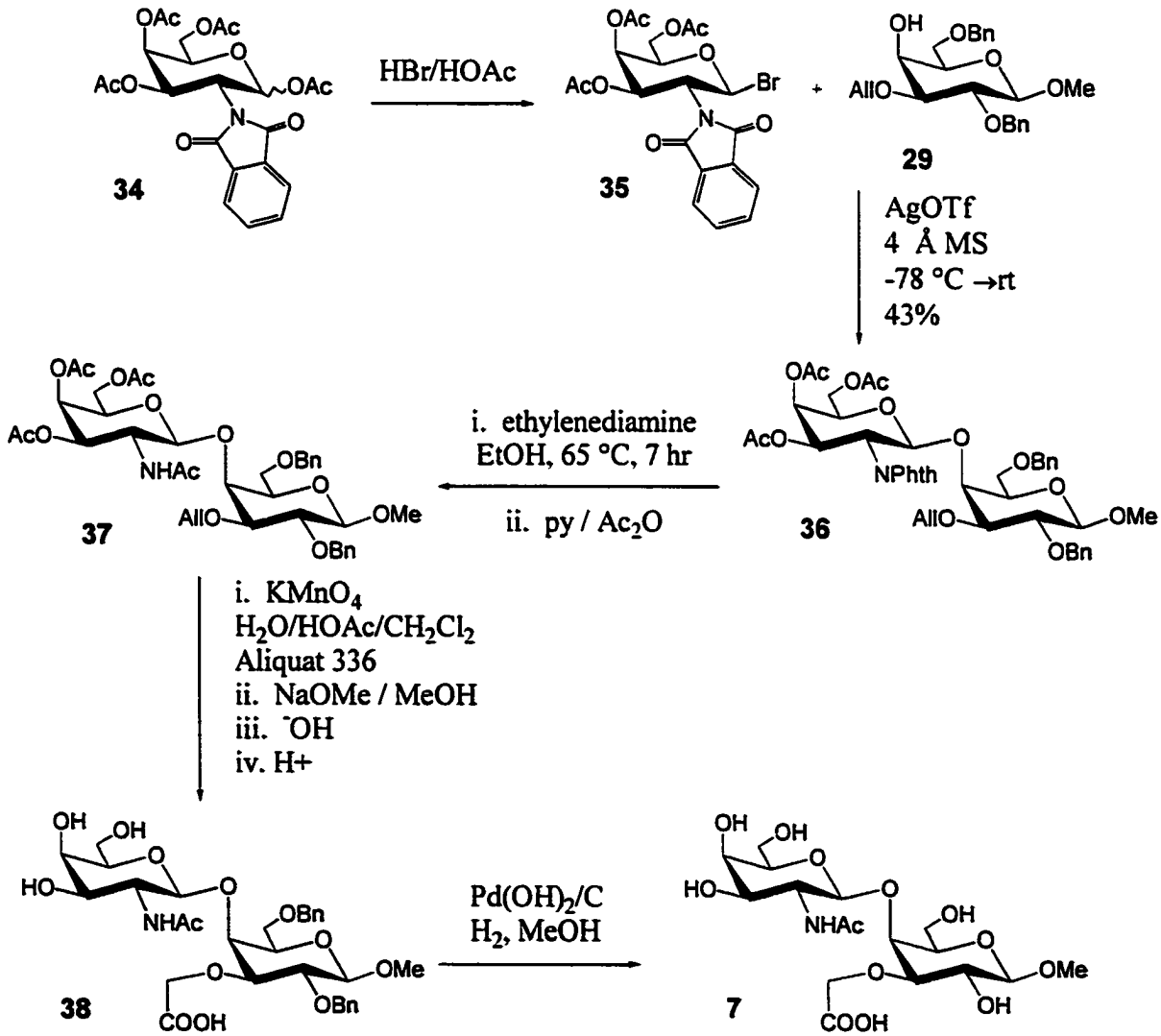
Scheme 2.12: Regioselective alkylation of **28** with *tert*-butyl α-bromoacetate



Scheme 2.13: Transforming lactone **31** to methyl ester **32**

In order to construct the 3-*O*-allyl disaccharide **36**, the bromo donor **35** [101] was chosen (Scheme 2.14). This was generated using the established method of converting the 1-*O*-acetate **34** to the bromide in HBr/HOAc (84% yield) [102]. The coupling reaction was carried out using silver trifluoromethanesulfonate as the activator, providing the disaccharide **36** in a disappointing 43% yield. This low yield is unsurprising, since a review of the literature reveals that this linkage is particularly problematic when employing the phthalimido as the neighbouring group [103]. The phthalimido group was

then removed with ethylenediamine in ethanol (65 °C, 7 hours) [104], followed by acetylation in pyridine/acetic anhydride to produce **37** in 93% yield. The allyl group was then transformed to the acid using phase-transfer catalysis $KMnO_4$ oxidation. The product was not directly isolated, instead the acetate protecting groups were removed via the Zemplèn method to provide **38** in 33% yield (2 steps). The benzyl groups were hydrogenolyzed in the presence of $Pd(OH)_2/C$ to give the deprotected disaccharide **7** in 95% yield.



Scheme 2.14: Synthesis of disaccharide **7**

2.2.1.5 Difficulty using Phthalimido Amine Protection for Synthesis of Galβ(1→3)GalNAc Disaccharide

The first step towards the construction of the Galβ(1→3)GalNAcβ(1→4)Gal trisaccharide **2** would be to build first the Galβ(1→3)GalNAc disaccharide. This would be followed by its conversion to a suitable donor to be available for subsequent coupling to a galactose residue containing the acid fragment already installed at the 3-position. This is described in the retrosynthetic approach shown in Figure 2.10.

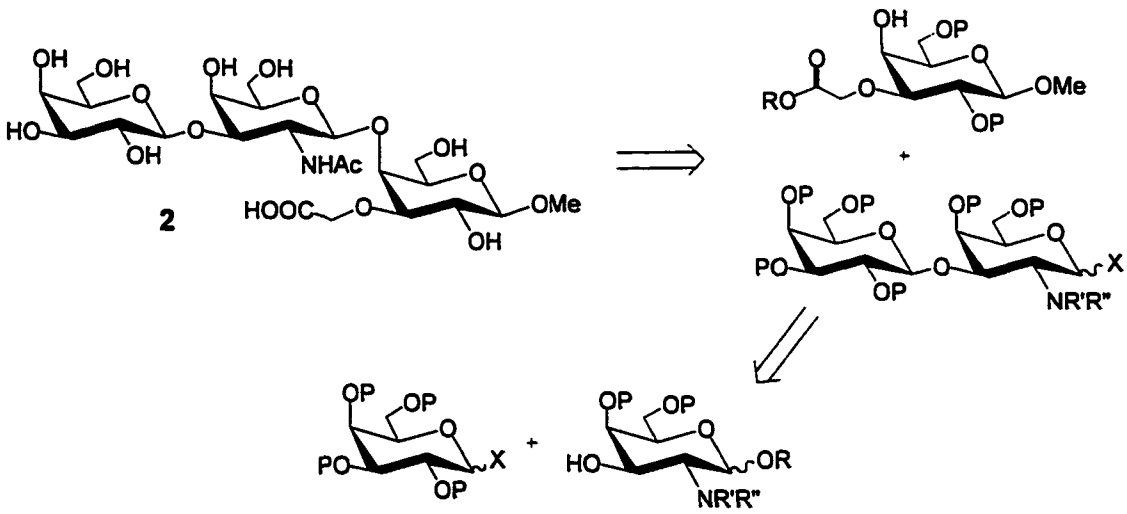
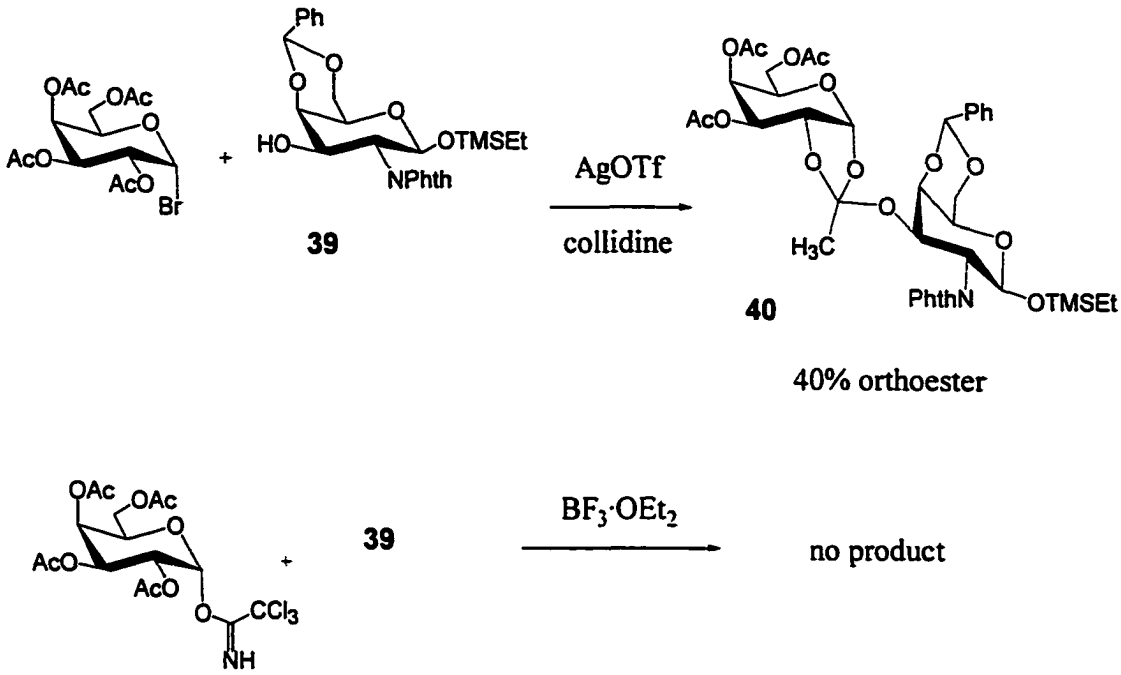


Figure 2.10: Retrosynthetic approach to trisaccharide **2**

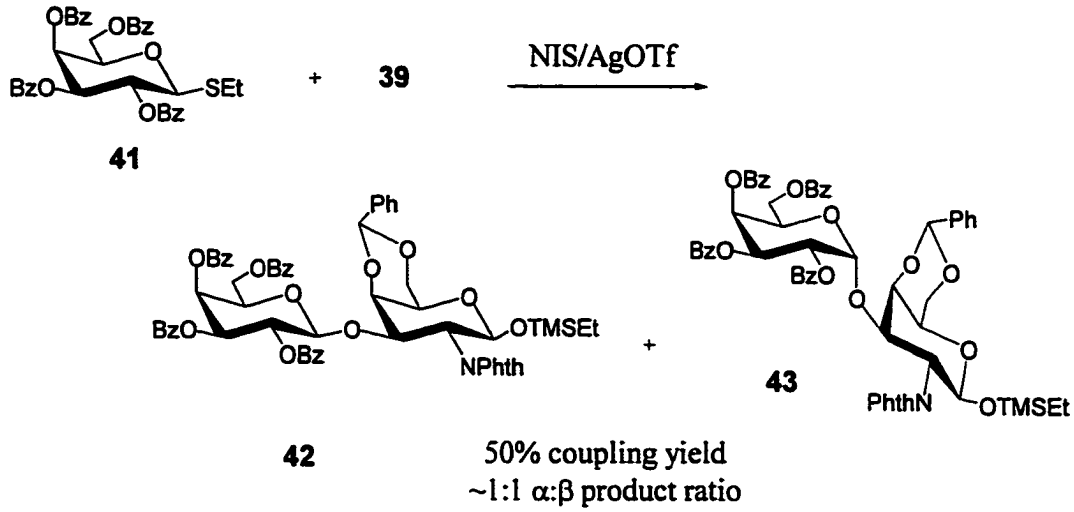
The first attempts made toward building the Galβ(1→3)GalNAc disaccharide employed phthalimido protection on the galactosamine nitrogen (Scheme 2.16). This approach turned out to be very problematic. Efforts using acetobromogalactose as the donor and the acceptor **39** (synthesized using methods found in ref. 105) resulted in the formation of

orthoester **40** as the major product (40% yield). The use of the acetylated imidate donor was no more successful, with no disaccharide product being isolated at all.



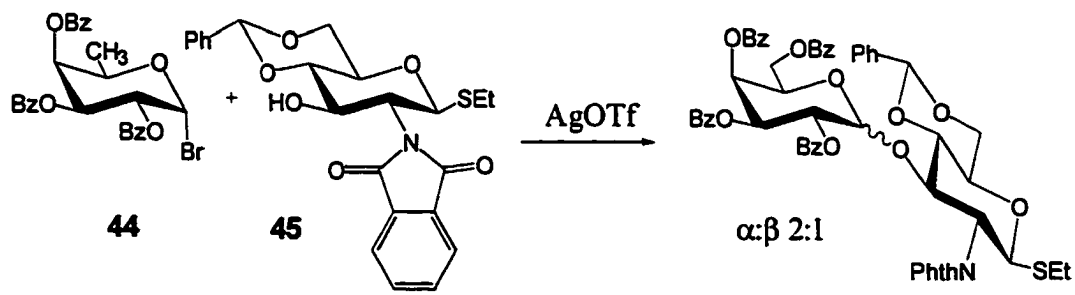
Scheme 2.16: First attempts at Galβ(1→3)GalNAc using phthalimido protection

In an attempt to eliminate the possibility of orthoester formation, benzoylated donors such as **41** [106] were selected. This was marginally more successful, with one experiment providing **42** in 32% yield. All efforts to repeat this relative success failed, and a new problem became apparent in the appearance of the α-anomer **43** as a major product (Scheme 2.17). This was unexpected, since the presence of a benzoate at the 2-position of the donor should lead to neighbouring group participation, which should in turn induce the formation of the β-anomer **42**.



Scheme 2.17: Unexpected α-anomer as major product in glycosylation reaction

Continued investigation led to the discovery of a paper describing a very similar problem [107]. This paper by van Boeckel described the outcome of the glycosylation reaction between benzoylated donor **44** and phthalimido-protected acceptor **45** (Scheme 2.18). The α-anomer was the major product in spite of the participating 2-benzoate.



Scheme 2.18: Previously reported unusual glycosylation outcome

The authors proposed that steric hindrance between the participating benzoate and the incoming phthalimido group prevented the formation of the expected β-anomer, leading to the observed α-anomer as the major product (Figure 2.11).

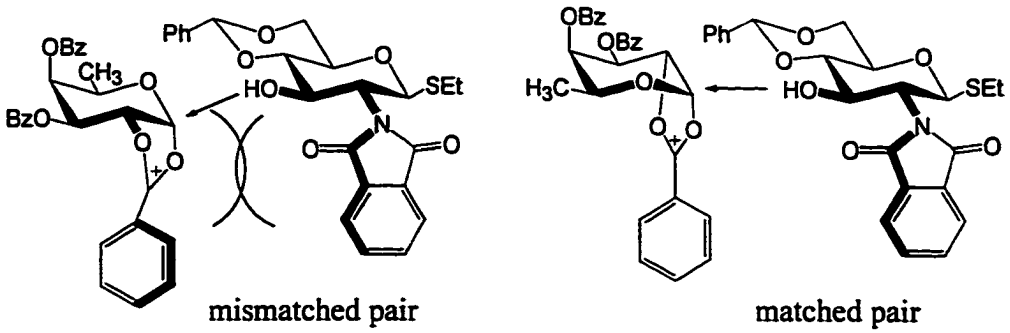


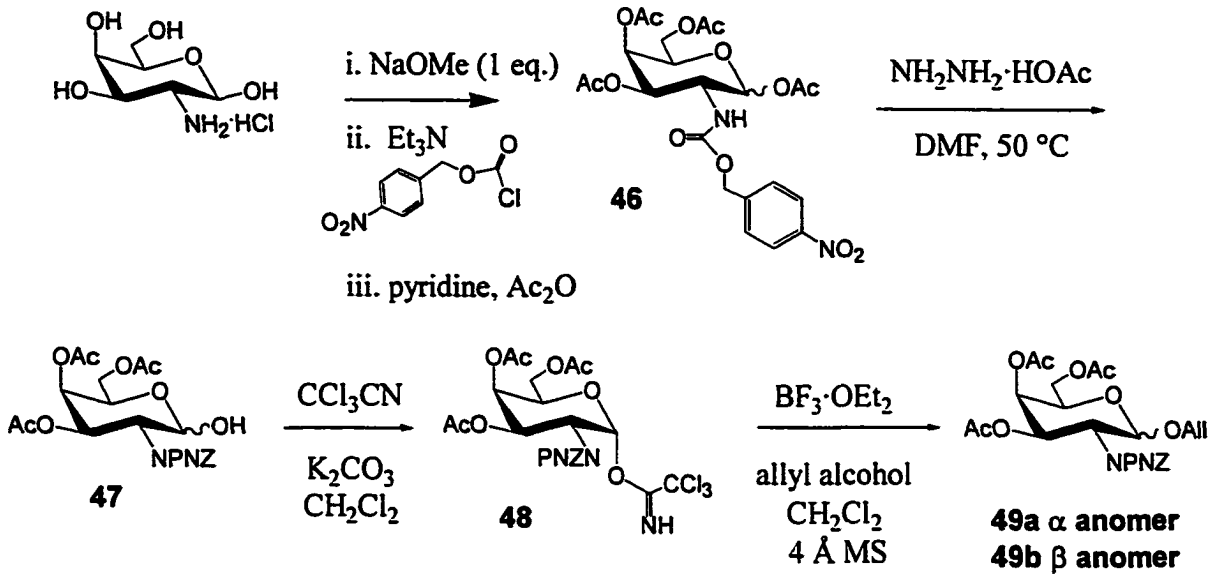
Figure 2.11: Mismatched vs. matched donor acceptor pairs

This hypothesis was tested with the use of the equivalent L-donor and, as anticipated in the absence of steric interference, the major product was indeed the β-anomer (8.4:1 β:α). The same "mismatched donor-acceptor pair" interactions can be envisioned with the analogous galactosamine acceptor, which would explain the unexpected results observed. Still the question remained: How can the Galβ(1→3)GalNAc disaccharide be constructed in satisfactory yield?

2.2.1.6 Use of the PNZ Group in the Synthesis of the Gal-GalNAc Disaccharide

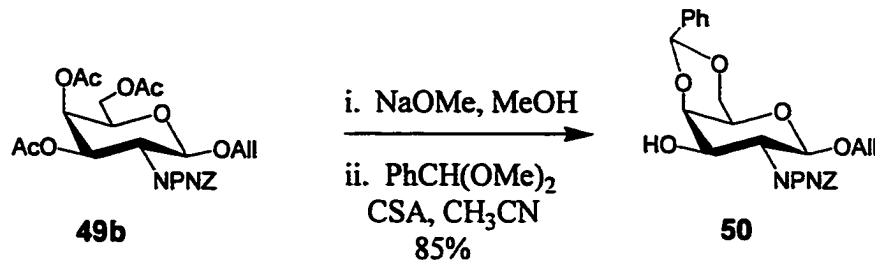
Fortunately, at the same time as a solution to this problem was being sought, Xiangping Qian in our group was exploring the use of *p*-nitrobenzyl carbamate (PNZ) as a protecting group for amino sugars and its utility as a neighboring group for the synthesis of β-glycosides [70]. His experiments were successful, and it was decided to investigate whether the PNZ group would be useful in the synthesis of the Galβ(1→3)GalNAc disaccharide. As it turned out, the coupling yield for this reaction was very high

compared to the yields obtained using any of the other methods to construct this disaccharide linkage attempted previously in this thesis.



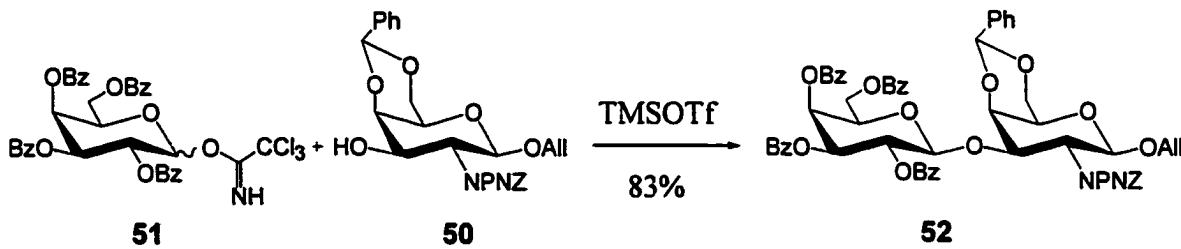
Scheme 2.19: Synthesis of PNZ protected allyl glycoside

The starting galactosamine derivative **46** was synthesized as reported by Qian [70]. The anomeric acetate was selectively removed using hydrazine acetate in DMF at 50°C to give **47** in 65% yield (Scheme 2.19). This was converted into the imidate donor **48** in 84% yield through the reaction of the reducing sugar with CCl_3CN in the presence of K_2CO_3. The allyl glycosides **49a** and **49b** were then obtained from **48** and allyl alcohol using $BF_3{\cdot}OEt_2$ activation to give a 3:1 β:α mixture in 95% overall yield. The β-anomer **49b** was deacetylated in NaOMe/MeOH and benzylidenated with benzaldehyde dimethyl acetal and CSA to provide **50** in 85% overall yield (2 steps) (Scheme 2.20).



Scheme 2.20: Synthesis of PNZ protected acceptor **50**

The PNZ protected disaccharide **52** was obtained in 83% yield through the TMSOTf-mediated coupling of the benzoylated imidate donor **51** [108] and the acceptor **50** (Scheme 2.21).

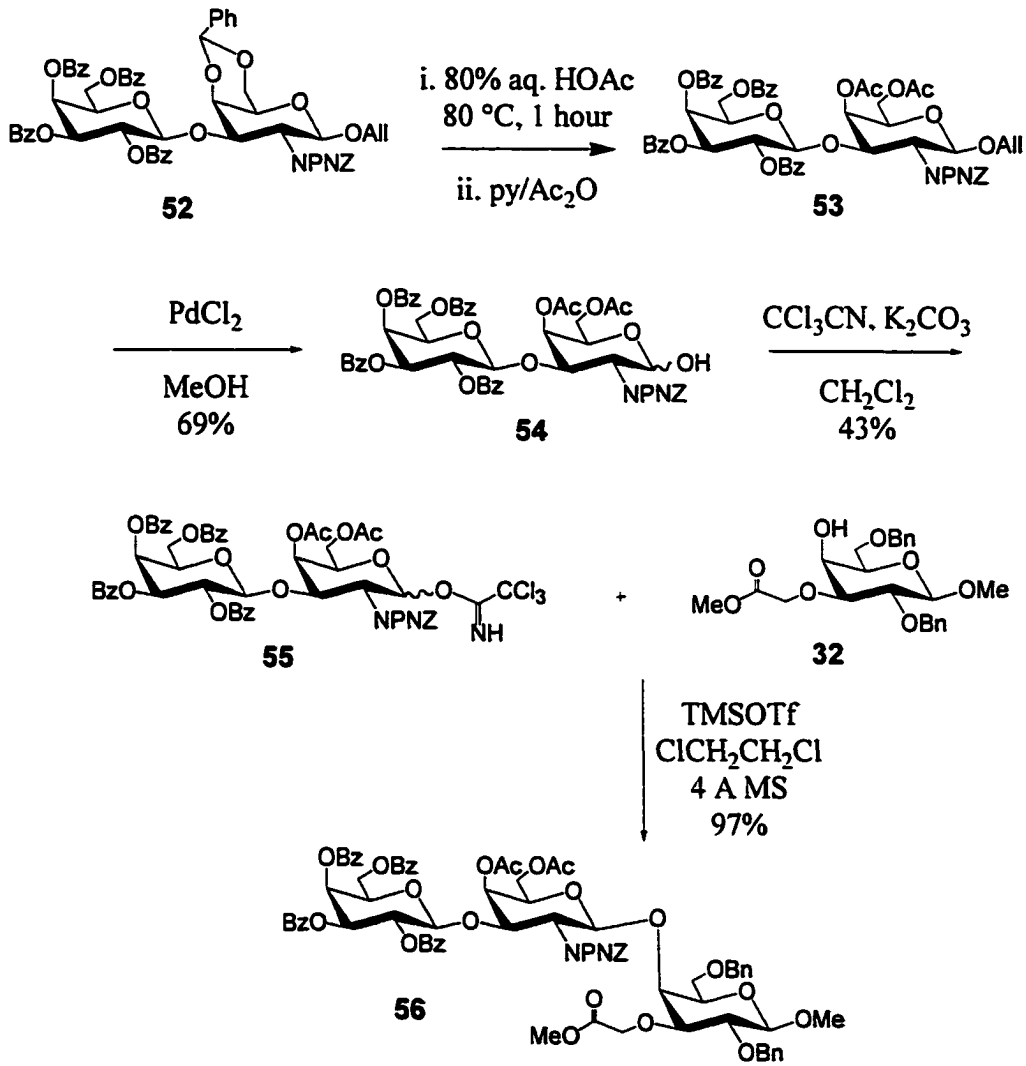


Scheme 2.21: Synthesis of PNZ protected disaccharide **52**

2.2.1.7 Synthesis of PNZ-acid Fragment Trisaccharide

The experiences with the allyl group (as shown in Section 2.2.1.2) and its apparent low yields in conversion to the carboxymethyl fragment were not encouraging. Instead, the methyl ester **32** was chosen due to the simplicity of saponification as a deprotection reaction, as well as the fact that there was no longer a concern about amide formation during removal of the phthalimido group with either hydrazine or ethylene diamine.

The first step in the conversion of **52** into the imidate donor **55** was removal of the benzylidene in 80% aqueous acetic acid (80°C, 1 hour) followed by acetylation in pyridine and acetic anhydride to provide **53** in 91% yield over two steps (Scheme 2.22).



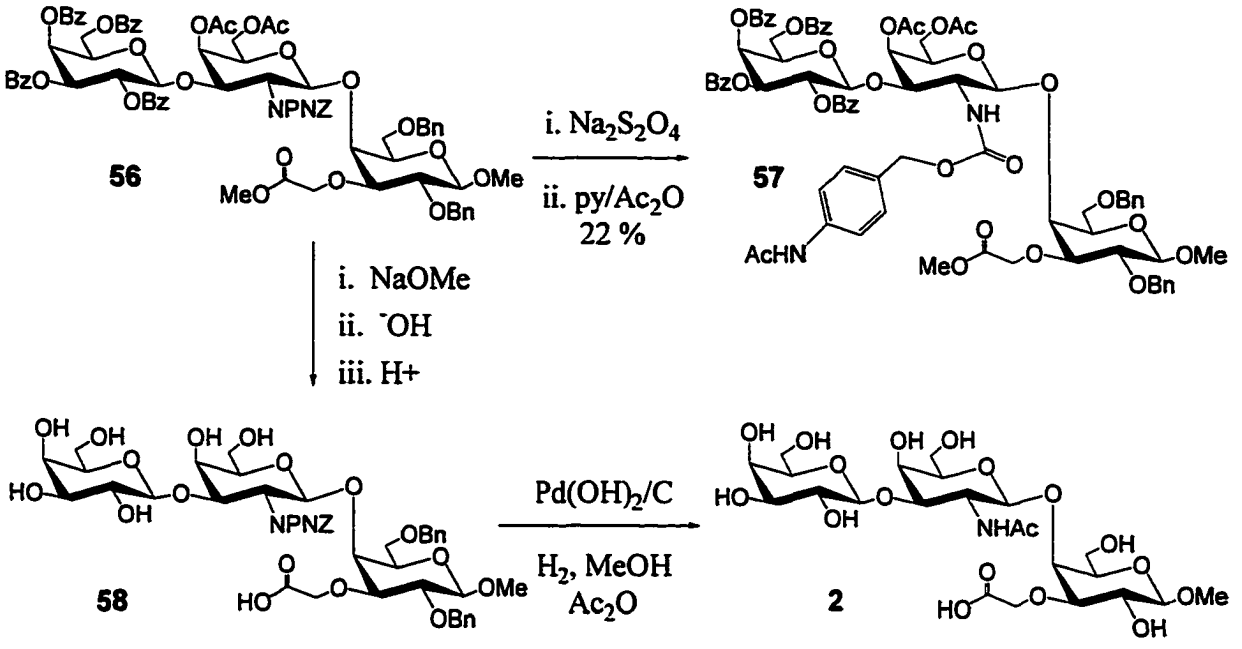
Scheme 2.22: Synthesis of PNZ trisaccharide **56**

The anomeric allyl group was then removed with palladium(II)chloride in methanol [109], providing the reducing sugar **54** in 69% yield. This was converted to the imidate

donor **55** in 43% yield using the same method used to make imidate **48**. This was then coupled with methyl ester **32** using TMSOTf activation providing the trisaccharide **56** in a very heartening 97% yield.

2.2.1.8 Deprotection of PNZ-acid Fragment Trisaccharide *56*

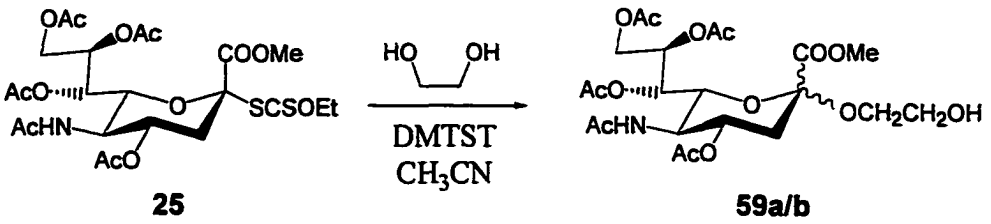
The initial step in the deprotection of the trisaccharide **56** was conversion of the PNZ to an acetamido group (Scheme 2.23). This was first undertaken through reaction of **56** with $Na_2S_2O_4$ followed by acetylation in pyridine and acetic anhydride. This led to a mixture of products, including the undesired acetamido derivative **57** in 22% yield, probably due to steric hindrance from the close proximity of the carboxymethyl substituent. As well, the mixture of products could be a result of the reaction of the intermediate amine with the nearby methyl ester to form an intramolecular amide. The easiest solution to the second problem would be to remove the methyl ester before the PNZ group. Thus, the trisaccharide was deacylated in methanolic sodium methoxide followed by saponification of the ester to give **58** in overall quantitative yield. The removal of the benzoates also allowed easy monitoring of the PNZ removal by TLC, since the hydrogenolysis product is no longer UV active in the absence of both benzoate and PNZ protection. The benzyl groups were removed and the PNZ group converted to the acetamido group through catalytic hydrogenation in the presence of $Pd(OH)_2/C$ in a methanol solution containing acetic anhydride. This provided the final trisaccharide **2** in 74% yield.



Scheme 2.23: Deprotection of Galβ(1→3)GalNAcβ(1→4)Gal trisaccharide **56**

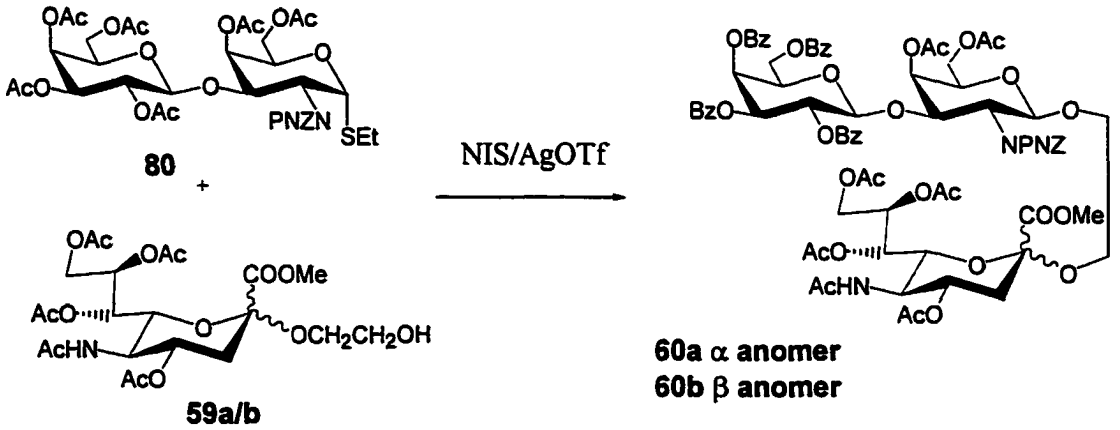
2.2.1.7 Alternative Synthesis of Sialoside *1*

In a further attempt to explore the utility of PNZ protection for the synthesis of the sialoside **1**, an alternative approach was investigated. This scheme was designed to use the thioglycoside donor **80**, whose synthesis is described in Chapter 5. The first step was the glycosylation of ethylene glycol with the xanthate donor **25** using DMTST activation (58% yield, ~1:1 α/β mixture) (Scheme 2.24). At this stage it was impossible to separate the two anomeric products by column chromatography. This purification step proved unnecessary, since the mixture could be used directly for the next step in the reaction sequence.



Scheme 2.24: Synthesis of the sialic acid acceptor **59**

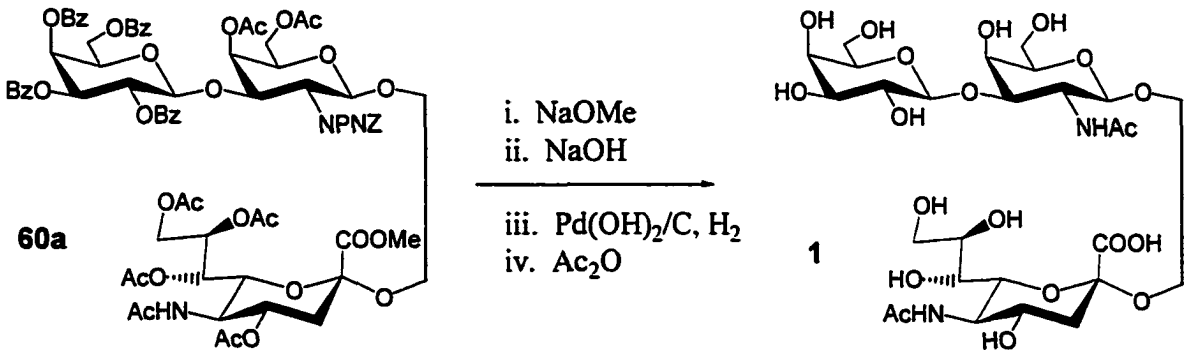
This next step involved glycosylation of the ethylene glycol sialoside mixture **59a/b** using thioglycoside **80** using NIS and AgOTf as activators, providing the trisaccharides **60a** and **60b** in 65% overall yield (Scheme 2.25). This glycosylation step was stereoselective, providing the β anomer as the major product. Separation of the α/β sialosides **60a** and **60b** proved to be straightforward.



Scheme 2.25: Synthesis of the trisaccharides **60a** and **60b**

Attempts to begin the deprotection of **60a** with the removal of the PNZ group led to the formation of multiple product spots by TLC. This may be a result of the amine reacting

with the methyl ester of the sialic acid to form an amide. It was determined that this problem could be avoided if the ester was saponified before the amine was liberated. To this end, the acyl protecting groups were removed first in methanolic sodium methoxide, followed by the addition of water to the reaction mixture to effect saponification (Scheme 2.26). Removal of the PNZ by hydrogenolysis and subsequent acetylation of the amine provided methyl ester, which was easily saponified to yield the final product **1**.



Scheme 2.26: Deprotection of the sialoside **60a**

2.2.2 Gal-Gal-Gal Chemistry

In an attempt to gain some insight into the importance of the acetamido group on the GalNAc to the recognition of potential inhibitors, two trisaccharide analogues were synthesized in which the NHAc was replaced by an OH group. The first trisaccharide **4** employed the same flexible ethylene linker found in analogue **1**, and the second target trisaccharide **5** was based on the carboxymethyl derivative **2**.

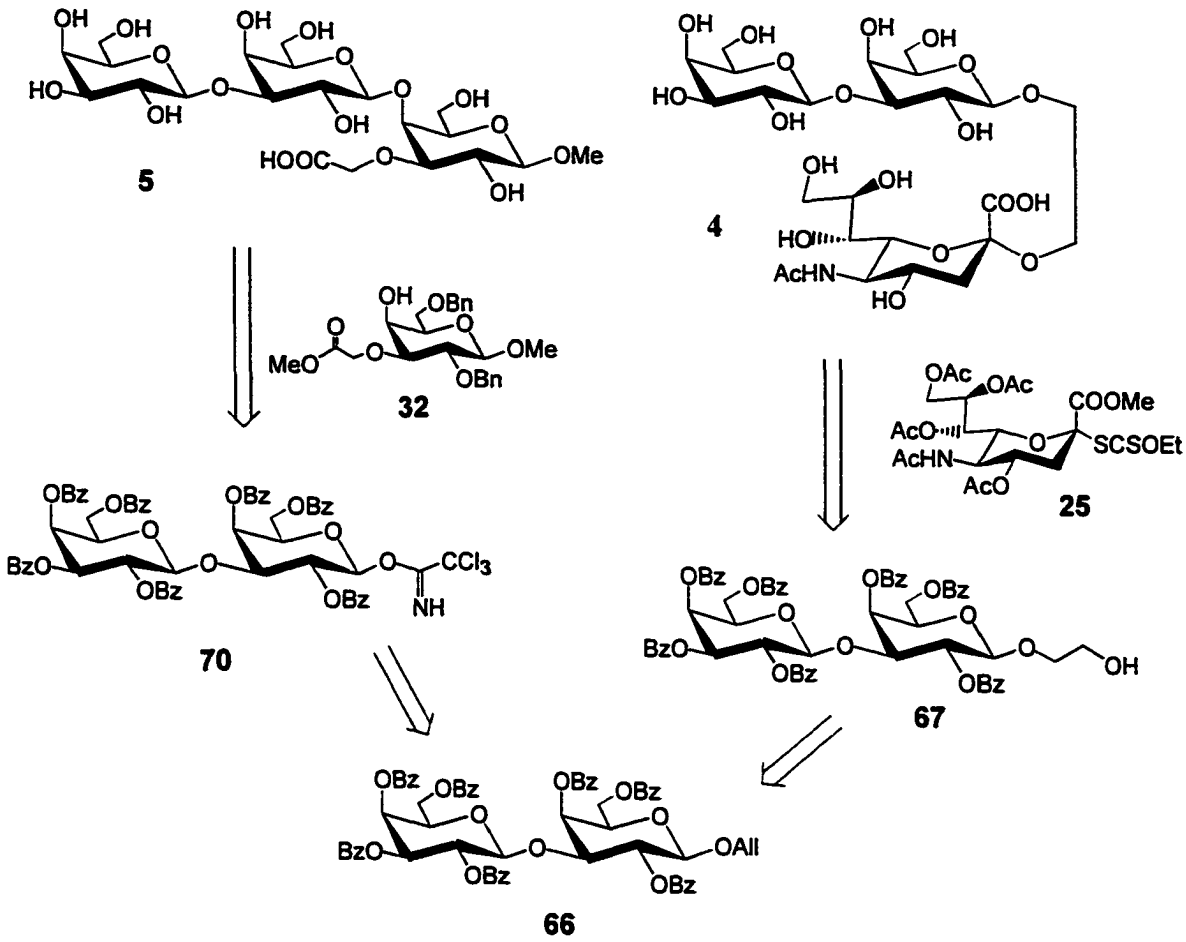
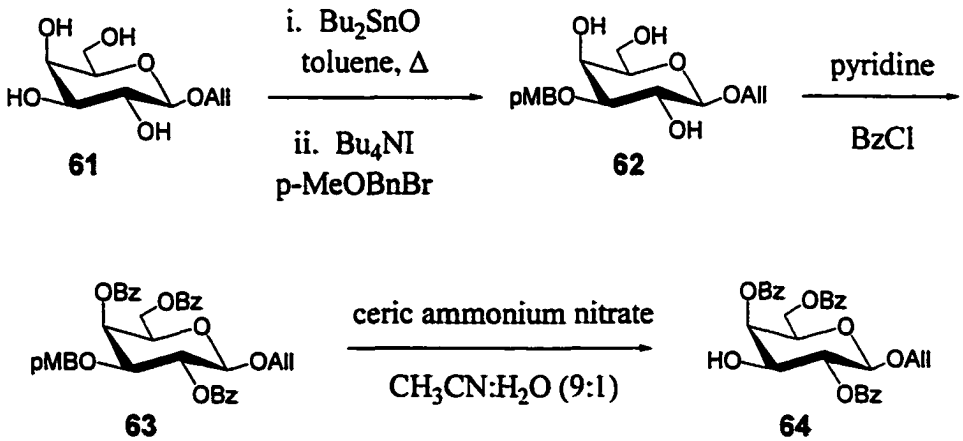


Figure 2.12: Retrosynthesis of GM_1 analogues **4** and **5**

The retrosynthetic approaches for **4** and **5** paralleled that for **1** and **2** (Figure 2.12). The β-allyl glycoside was again chosen for its easy conversion to the 2-hydroxyethyl group (target **4**), as well as the ease with which the allyl can be removed and the resulting reducing sugar transformed to the imidate donor (target **5**).

2.2.2.1 Gal-Gal-Sialic Acid *4*

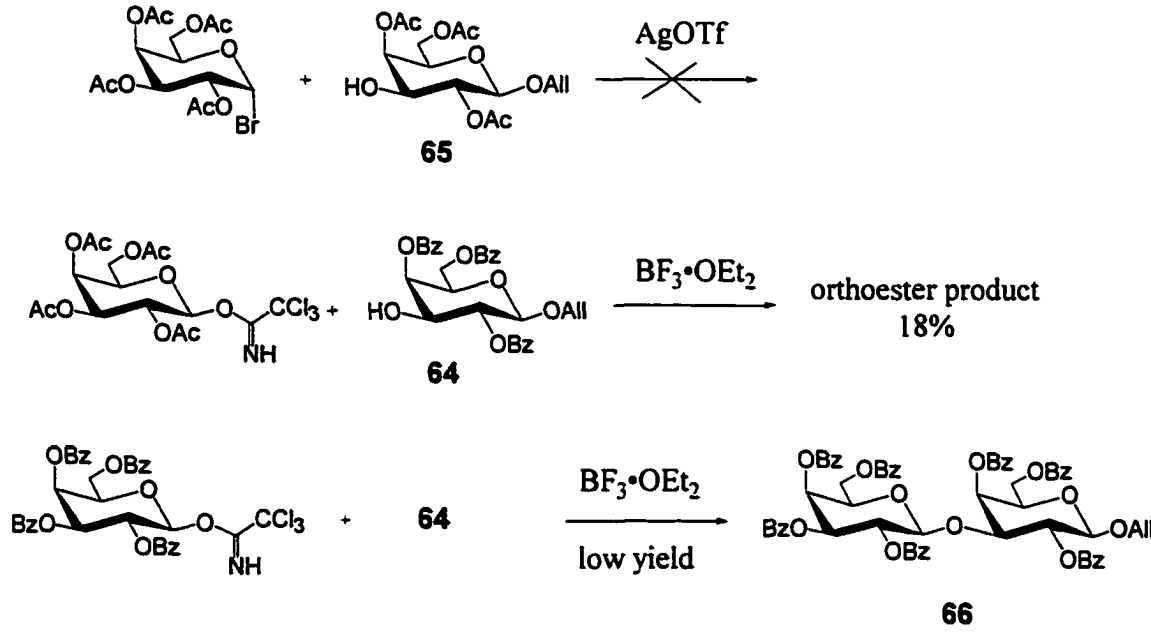
Both the acetylated and benzoylated acceptors were employed in attempts to construct the Galβ(1→3)Gal disaccharide. The synthesis of acceptor **64** began with the introduction of a *p*-methoxybenzyl group at the 3-position of allyl galactoside **61** [110] via a stannylidene intermediate providing **62** in 64% yield (Scheme 2.27). The product **62** was benzoylated in pyridine and benzoyl chloride to provide **63** in 91% yield. The benzoylated derivative **63** then underwent reaction with ceric ammonium nitrate to furnish the 3-OH acceptor **64** in quantitative yield. Synthesis of the acetylated acceptor **65** [111] was carried out using an analogous procedure to that used for **64**, with comparable yields.



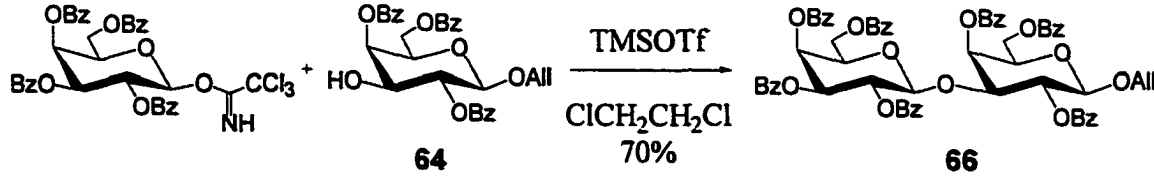
Scheme 2.27: Synthesis of 3-OH galactose acceptor **64**

Attempts to glycosylate the acetylated acceptor **65** with the donor acetobromogalactose failed to provide any disaccharide at all (Scheme 2.28). Fortunately, results with the benzoylated acceptor **64** were a little more promising. Although the $BF_3{\cdot}OEt_2$-activated

glycosylation of acceptor **64** with the acetylated imidate yielded the orthoester product in 18% yield and no other discernible disaccharide product, the analogous reaction using the benzoylated imidate did provide the desired disaccharide **66**, albeit in very low yield. When the activator was changed to TMSOTf, the reaction provided disaccharide **66** in a very satisfactory 70% yield (Scheme 2.29).

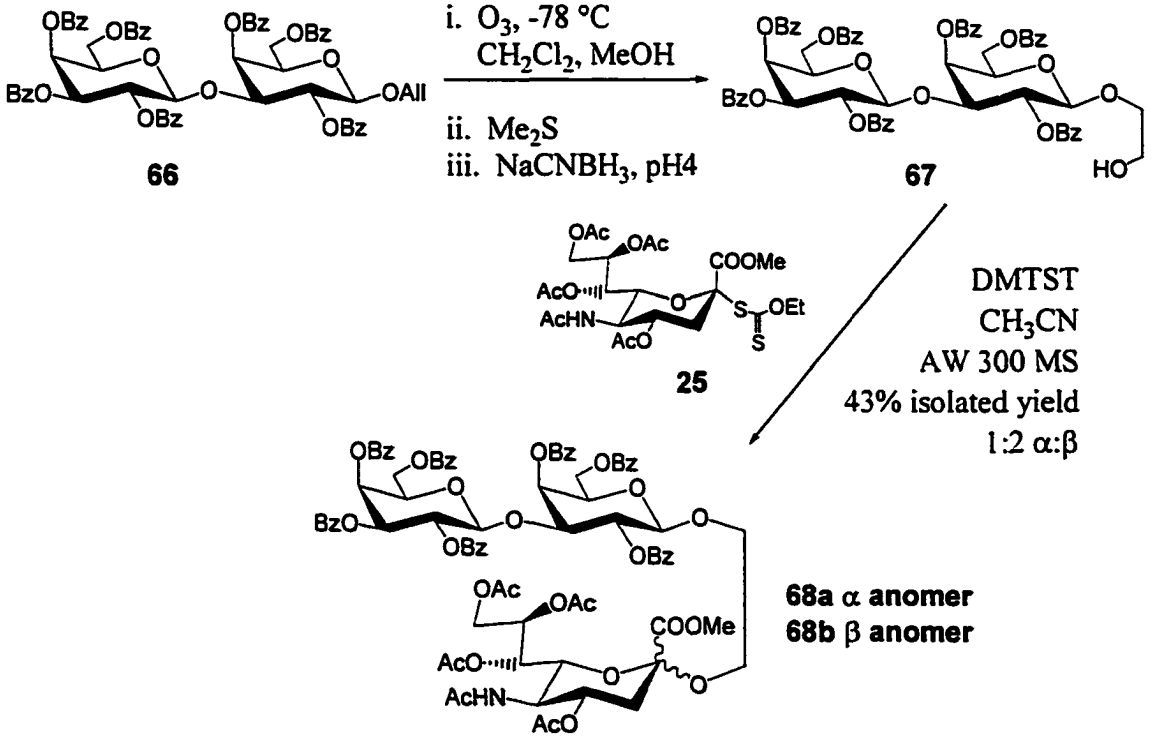


Scheme 2.28: Unsuccessful attempts at building Galβ(1→3)Gal disaccharide **66**

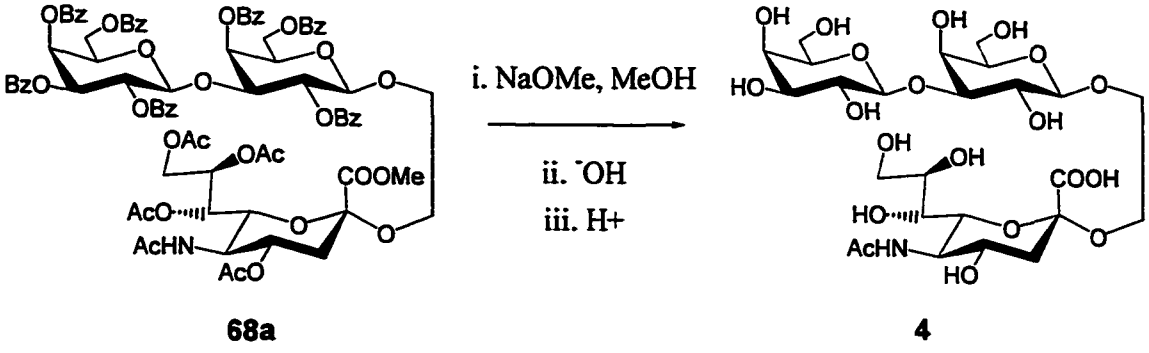


Scheme 2.29: Successful attempt at building Galβ(1→3)Gal disaccharide **66**

The benzoylated disaccharide **66** underwent ozonolysis with reductive workup, followed by further reduction of the resulting aldehyde with sodium cyanoborohydride to the alcohol **67** in 63% (Scheme 2.30). This was then coupled with the sialyl xanthate donor **25** with DMTST activation in 43% yield. Unexpectedly, the β-anomer **68b** was the major anomer isolated (2:1 β:α). The α-anomer **68a** was deacylated using sodium methoxide in methanol, followed by saponification to provide the product **4** in quantitative yield (Scheme 2.31).



Scheme 2.30: Synthesis of Galβ(1→3)Gal sialosides **68a** and **68b**

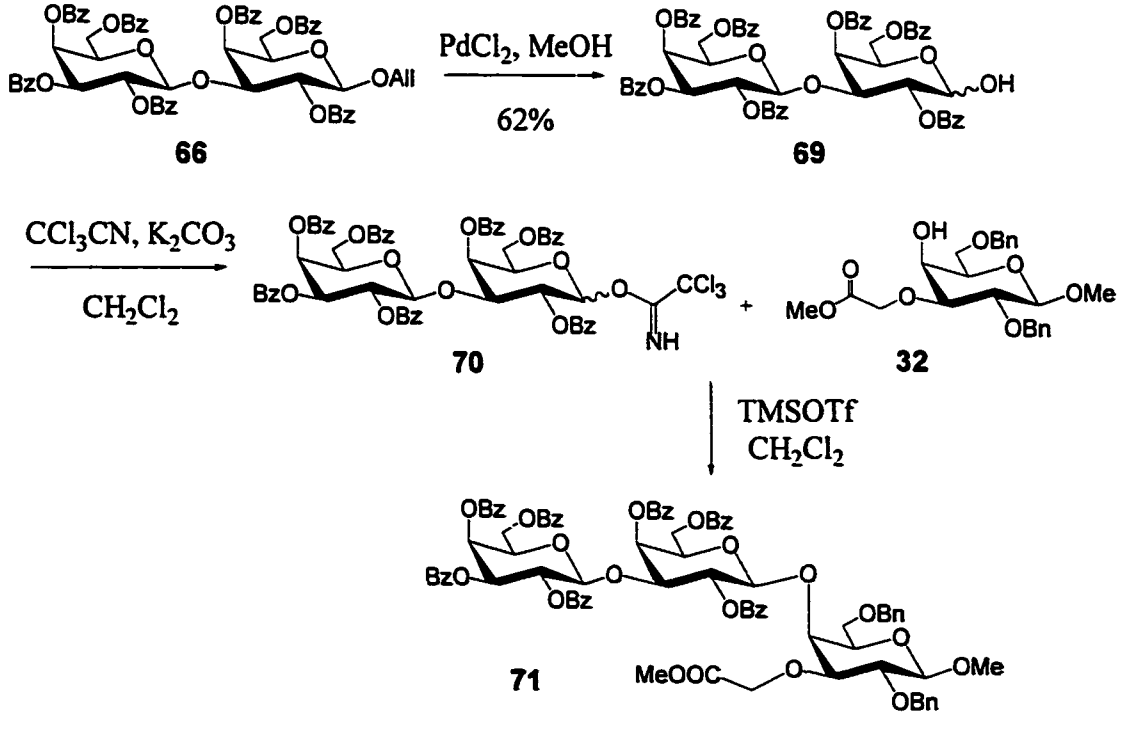


68a **4**

Scheme 2.31: Deprotection of Galβ(1→3)Gal sialoside **68a**

2.2.2.2 Gal-Gal-Gal Trisaccharide *5*

The β-allyl disaccharide **66** was also used as a building block in the synthesis of the trisaccharide **5** (Scheme 2.32). The allyl group was first removed using $PdCl_2$ in

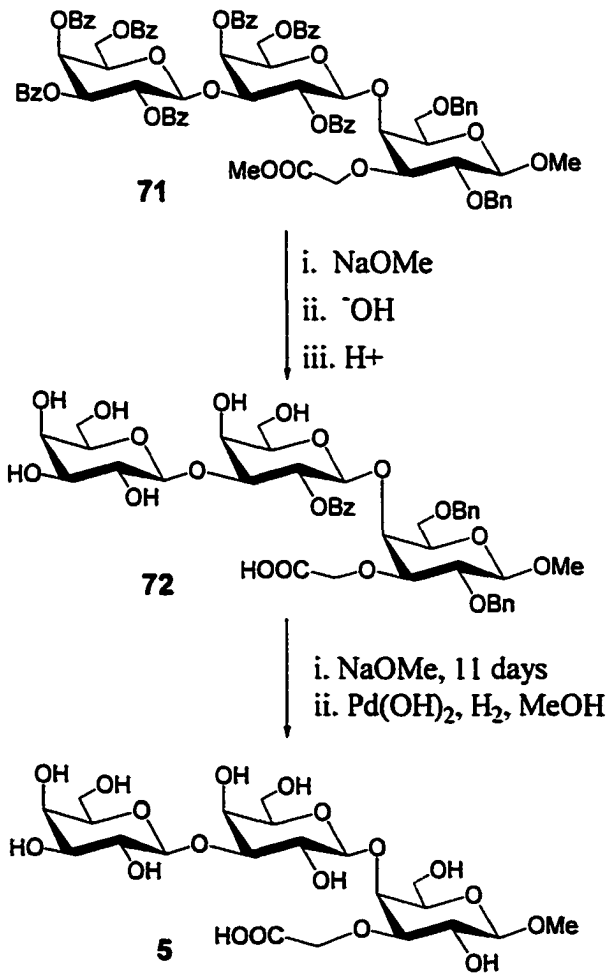


66 **69** **70** **32** **71**

Scheme 2.32: Synthesis of Galβ(1→3)Galβ(1→4)Gal trisaccharide **71**

methanol to provide the reducing sugar **69** in 62% yield. This was then transformed into the imidate donor **70** by reaction with trichloroacetonitrile in the presence of K_2CO_3. The imidate **70** was used without purification to glycosylate the methyl ester acceptor **32** providing the trisaccharide **71** in the dismal, while unoptimized, 9% yield.

Deprotection of trisaccharide **71** was not straightforward. Deacylation with NaOMe and subsequent saponification seemed to proceed as anticipated and a major product was isolated by chromatography. Unfortunately, ^{1}H NMR data and mass spectrometry of the



Scheme 2.33: Deprotection of Galβ(1→3)Galβ(1→4)Gal trisaccharide **71**

product **72** indicated the presence of a persistent benzoate at the 2-position of the central galactose residue (Scheme 2.33). This benzoate required prolonged stirring in methanolic sodium methoxide (11 days) for complete removal. This can probably be explained by increased steric hindrance at this position provided by the carboxymethyl group. In spite of the extra deprotection steps, the fully deprotected trisaccharide **5** was obtained in 84% overall yield.

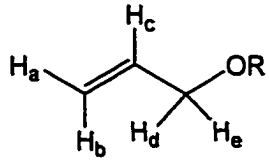
2.3 Experimental

General Methods

Analytical TLC was performed on Silica Gel 60-F_{254} (E. Merck, Darmstadt) with detection by quenching of fluorescence, by charring with 5% H_2SO_4 or with orcinol/ H_2SO_4, or with phosphomolybdic acid. Unless otherwise noted, column chromatography was performed on Silica Gel 60(E. Merck, 40-63 μm). Beaded silica gel (Iatrobeads) was from Iatron Laboratories, Inc. (Japan). Millex-GV (0.22 μm) filter units were from Millipore (Missisauga, ON). Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 22 ±2°C. All commercial reagents were used as supplied and all chromatography solvents were distilled prior to use. ^{1}H NMR spectra were recorded at 360 megahertz (MHz) (Bruker WM-360), 300 MHz (Bruker WM-300), 300 MHz (Varian i300) in a solution of $CDCl_3$ (proton chemical shifts referenced to residual proton signal of chloroform at δ 7.24), CD_3OD (residual proton signal of CHD_2OD at δ 3.30), or D_2O (DOH at δ 4.8). ^{13}C NMR were recorded on 75 MHz (Bruker AM-300) or 125

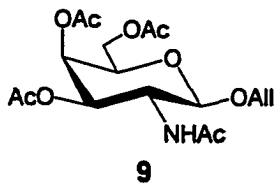
MHz(Varian UNITY 500) in $CDCl_3$ (δ 77.07), CD_3OD (δ 49.0), or D_2O (external acetone at δ 31.07). All coupling constants are reported as observed splitting of signals using first order analysis. Electrospray ionization mass spectra (ES-MS) were obtained from a Micromass ZabSpec Hybrid Sector-TOF instrument.

The protons of the allyl group are defined H_a, H_b, H_c, H_d and H_e as shown. These protons demonstrated similar coupling constants and multiplicity patterns in all the compounds presented in this thesis, only the chemical shifts changed. The observed couplings were as follows: H_a (dddd, $J_{a,c}$ 10.5 Hz, $J_{a,d} = J_{a,e} = J_{a,b}$ 1.5 ± 0.5 Hz), H_b (dddd, $J_{b,c}$ 17.0 Hz, $J_{b,d} = J_{b,e} = J_{a,b}$ 1.5 ± 0.5 Hz), H_c (dddd, $J_{b,c}$ 17.0 Hz, $J_{a,c}$ 10.5 Hz, $J_{c,d} = J_{c,e}$ 5.5 Hz), H_d (dddd, $J_{d,e}$ 13.5 Hz, $J_{c,d}$ 5.5 Hz, $J_{b,d} = J_{a,d}$ 1.5 ± 0.5 Hz), H_e (dddd, $J_{d,e}$ 13.5 Hz, $J_{c,e}$ 5.5 Hz, $J_{b,e} = J_{a,e}$ 1.5 ± 0.5 Hz).



The priorities of the monomers of the flexible linker sialosides are set so that the numbering begins at the sialic acid. The protons of the carbohydrate monomer at the other end of the ethylene linker (Gal or GalNAc) are then designated by a superscript prime, i.e. H-1'. The designations continue from reducing end to non-reducing end as is the accepted practice.

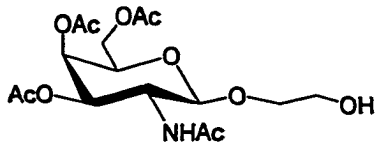
Allyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranoside (**9**).



9

A mixture of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D galactose (680 mg, 1.75 mmol) and allyl alcohol (1.2 ml, 17.6 mmol) in dry CH_2Cl_2 (25 ml) containing crushed 4 Å molecular sieves (1 g) was stirred for 1 hour. $FeCl_3$ (600 mg, 3.7 mmol) was added and stirring was continued overnight. The reaction was quenched with solid $NaHCO_3$, filtered through Celite, washed with H_2O and brine, and concentrated. Column chromatography of the residue (1:1 toluene:acetone) yielded **9** (582 mg, 86%) as a white solid. ^1H NMR ($CDCl_3$): δ 5.85 (1 H, H_c allyl), 5.72 (d, 1 H, NH), 5.33 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1 Hz, H-4), 5.28 (dd, 1 H, $J_{2,3}$ 11 Hz, $J_{3,4}$ 3.5 Hz, H-3), 5.25 (1 H, H_b allyl), 5.17 (1 H, H_a allyl), 4.72 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 4.33 (1 H, H_d allyl), 4.09-4.18 (m, 2 H, H-6a, H-6b), 4.08 (1 H, He allyl), 3.97 (ddd, 1H, $J_{2,3}$ 11 Hz, $J_{1,2}$ 8.5 Hz, $J_{2,NH}$ 9 Hz, H-2), 3.91 (dt, 1 H, $J_{4,5}$ 1 Hz, $J_{5,6a}$ =$J_{5,6b}$ 7 Hz, H-5), 2.12 (s, 3 H, NHAc), 1.92, 1.98 and 2.02 (3 s, 9 H, 3 x OAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.5, 170.5, 170.4, 170.3 ($COCH_3$), 133.6 (-CH=, allyl), 117.8 (=CH_2, allyl), 99.9 (C-1), 70.7, 69.9, 66.9 (C-3, C-4, C-5), 70.0, 61.5 (C-6, CH_2 allyl), 51.7 (C-2), 23.5, 20.7 ($COCH_3$). HR-ESMS calcd for $C_{17}H_{25}NO_9Na$ ($M+Na^+$) 410.1427, found 410.1425.

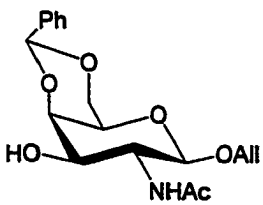
2-hydroxyethyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranoside (**10**).



10

A solution of **9** (396 mg, 1.02 mmol) in CH_2Cl_2 (5 ml) and MeOH (5ml) was cooled to –78 °C. Ozone was bubbled through the solution until blue colour persisted, then reaction was quenched with Me_2S. After removal of solvent *in vacuo*, the residue was redissolved in MeOH (10 ml), the solution was acidified to pH 4 with glacial acetic acid, $NaCNBH_3$ (200 mg, 3.18 mmol) was added and stirring was continued overnight. Upon evaporation of solvent, column chromatography (19:1→9:1 CH_2Cl_2:MeOH) yielded **10** (371 mg, 95%). ^{1}H NMR ($CDCl_3$): δ 5.37 (dd, 1 H, $J_{3,4}$ 3 Hz, $J_{4,5}$ 1 Hz, H-4), 5.10 (dd, 1 H, $J_{2,3}$ 11 Hz, $J_{3,4}$ 3 Hz, H-3), 4.63 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 4.12-4.26 (m, 2 H, H-6a, H-6b), 4.18 (dd, 1H, $J_{2,3}$ 11 Hz, $J_{1,2}$ 8.5 Hz, H-2), 4.02 (dt, 1 H, $J_{4,5}$ 1 Hz, $J_{5,6a}$=$J_{5,6b}$ 6.5 Hz, H-5), 3.86-3.97, 3.64-3.76 (m, 4 H, O*C*H_2*C*H_2O), 2.17 (s, 3 H, NHAc) 1.97, 1.99 and 2.07 (3 s, 9 H, 3 x OAc).

*Allyl 2-acetamido-4,6-di-*O*-benzylidene-2-deoxy-β-D-galactopyranoside* (**11**).

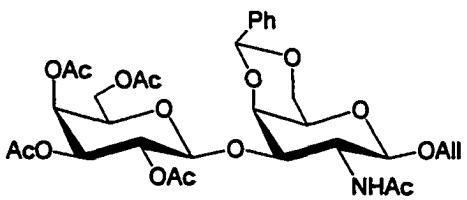


11

Allyl glycoside **9** (389 mg, 1.0 mmol) was treated with methanolic sodium methoxide (10 ml, 0.02M) for 16 hours, then neutralized with Amberlite IR-120 (H+) resin, filtered and concentrated. The crude residue was dissolved in dry CH_3CN (15 ml). Benzaldehyde dimethyl acetal (0.6 ml, 4.0 mmol) and a catalytic amount of *p*-toluene sulfonic acid were then added to this solution. Reaction mixture was stirred for 14 hours, quenched with Et_3N and concentrated. Column chromatography (19:1 CH_2Cl_2:MeOH) yielded **11** (297 mg, 85% overall yield) as a white solid. ^{1}H NMR (CD_3OD): δ 7.26-7.60 (m, 5 H, Ph),

5.88 (1 H, H_c allyl), 5.59 (s, 1 H, PhC*H*O_2), 5.25 (1 H, H_b allyl), 5.11 (1 H, H_a allyl), 4.49 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 4.32 (1 H, H_d allyl), 4.16 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1.5 Hz, H-4), 4.15 (dt, 1 H, $J_{6a,6b}$ 12.5 Hz, $J_{5,6a}$ 1.5 Hz, H-6a), 4.13 (dt, 1 H, $J_{6a,6b}$ 12.5 Hz, $J_{5,6a}$ 1.5 Hz, H-6b), 4.05 (1 H, H_e allyl), 4.00 (dd, 1H, $J_{2,3}$ 11 Hz, $J_{1,2}$ 8.5 Hz, H-2), 3.73 (dd, 1 H, $J_{2,3}$ 11 Hz, $J_{3,4}$ 3.5 Hz, H-3), 3.49 (broad m, 1 H, H-5), 1.91 (s, 3 H, NHAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 172.6 (*C*OCH_3), 137.5 (aromatic quat.), 133.8 (-CH=, allyl), 128.9, 127.8, 126.1 (aromatic CH), 117.0 (=CH_2, allyl), 101.2, 99.7 (PhCH, C-1), 75.3, 70.3, 66.4 (C-3, C-4, C-5), 69.4, 69.0 (C-6, CH_2 allyl),53.3 (C-2), 22.6 ($COCH_3$). HR-ESMS calcd for $C_{18}H_{23}NO_6Na$ ($M+Na^+$) 372.1423, found 372.1419.

Allyl 2-acetamido-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-4,6-di-O-benzylidene-2-deoxy-β-D-galactopyranoside (**13**).

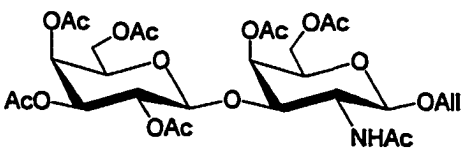


13

Imidate donor **12** (180 mg, 0.365 mmol) and acceptor **11** (144 mg, 0.412 mmol) in CH_2Cl_2 (5 ml) were cooled to –5 °C under an inert atmosphere. To this mixture a solution of BF_3OEt_2 in CH_2Cl_2 (0.1 M, 800 μl, 0.08 mmol) was added dropwise. The reaction was allowed to reach rt, and stirring continued for 16 hours. The reaction was diluted with CH_2Cl_2 washed with saturated $NaHCO_3$ and H_2O, dried over Na_2SO_4 and concentrated. Column chromatography (6:1→2:1 toluene:acetone) yielded product **13** (82 mg, 39% based on recovered acceptor). ^{1}H NMR ($CDCl_3$): δ 7.30-7.60 (m, 5 H, Ph), 5.88 (1 H, H_c allyl), 5.80 (d, 1 H, $J_{2,NH}$ 7 Hz, NH), 5.55 (s, 1 H, PhC*H*O_2), 5.36 (dd, 1 H, $J_{3',4'}$ 3.5 Hz,

$J_{4',5'}$ 1 Hz, H-4'), 5.25 (1 H, H_b allyl), 5.22 (dd, 1H, $J_{2',3'}$ 10 Hz, $J_{1',2'}$ 8.5 Hz, H-2'), 5.20 (1 H, H_a allyl), 5.17 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.98 (dd, 1 H, $J_{2',3'}$ 10 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 4.78 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1'), 4.75 (dd, 1 H, $J_{2,3}$ 11 Hz, $J_{3,4}$ 3.5 Hz, H-3), 4.37 (1 H, H_d allyl), 4.32 (dd, 1 H, $J_{5,6a}$ 1 Hz, $J_{6a,6b}$ 12 Hz, H-6a), 4.28 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1 Hz, H-4), 4.05-4.17 (m, 3 H, H-6a', H-6b', H-6b), 4.05 (1 H, H_e allyl), 3.88 (dt, 1 H, $J_{4',5'}$ 1 Hz, $J_{5',6a'} = J_{5',6b'}$ 7.5 Hz, H-5'), 3.48 (broad m, 1 H, H-5), 3.43 (ddd, 1H, $J_{2,3}$ 10.5 Hz, $J_{1,2}$ 8 Hz, $J_{2,NH}$ 7 Hz, H-2), 1.96, 1.97, and 2.02 (3 s, 12 H, 4 x OAc), 2.14 (s, 3 H, NHAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 171.0, 170.4, 170.1 (*C*OCH_3), 137.9 (aromatic quat.), 134.1 (-CH=, allyl), 128.9, 128.1, 126.3 (aromatic CH), 117.7 (=CH_2, allyl), 101.3, 100.8, 98.1 (Ph*C*H, C-1, C-1'), 76.0, 75.6, 71.1, 71.0, 69.2, 67.2, 66.5 (C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 70.2, 69.4, 61.8 (C-6, C-6', CH_2 allyl), 54.7 (C-2), 23.8, 20.9, 20.7, 20.6 (CO*C*H_3). HR-ESMS calcd for $C_{32}H_{41}NO_{15}Na$ ($M+Na^+$) 702.2374, found 702.2377.

*Allyl 2-acetamido-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-4,6-di-*O*-acetyl-2-deoxy-β-D-galactopyranoside* (**15**).

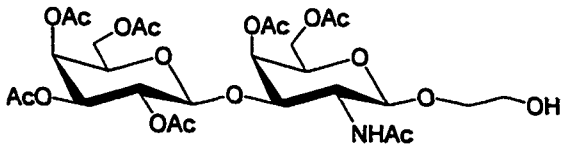


15

A solution of **13** (90 mg, 0.13 mmol) in 80% aqueous acetic acid (10 ml) was stirred at 80 °C for 1 hour, then concentrated and co-concentrated with toluene. The crude residue was stirred in pyridine (5ml) and acetic anhydride (3 ml) overnight, then concentrated and co-concentrated with toluene. Column chromatography (2:1 toluene:acetone) yielded **15** (65 mg, 75%). ^1H NMR ($CDCl_3$): δ 5.87 (1 H, H_c allyl), 5.68 (d, 1 H, $J_{2,NH}$ 7 Hz, NH), 5.42 (dd, 1 H, $J_{3',4'}$ 3.5 Hz, $J_{4',5'}$ 1 Hz, H-4'), 5.33 (dd, 1 H,

$J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1 Hz, H-4), 5.26 (1 H, H_b allyl), 5.20 (1 H, H_a allyl), 5.14 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 8 Hz, H-2'), 5.09 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 4.96 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 4.72 (dd, 1 H, $J_{2,3}$ 10.5 Hz, $J_{3,4}$ 3.5 Hz, H-3), 4.61 (d, 1 H, $J_{1',2'}$ 7 Hz, H-1'), 4.32 (1 H, H_d allyl), 4.02-4.19 (5 H, H-6a, H-6b, H-6a', H-6b', H_e allyl), 3.82-3.90 (2 H, H-5, H-5'), 3.30 (ddd, 1H, $J_{2,3}$ 10.5 Hz, $J_{1,2}$ 8.5 Hz, $J_{2,NH}$ 7 Hz, H-2), 1.96, 2.05, 2.06 and 2.09 (4 s, 18 H, 6 x OAc), 2.12 (s, 3 H, NHAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 171.0, 170.6, 170.4, 170.3, 170.1, 170.0, 169.4 (*C*OCH_3), 133.7 (-CH=, allyl), 118.2 (=CH_2, allyl), 100.0, 98.1 (C-1, C-1'), 77.3, 74.7, 71.3, 71.0, 70.9, 69.4, 68.0 (C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 70.5, 62.5, 61.0 (C-6, C-6', CH_2 allyl), 55.5 (C-2), 23.7, 20.9, 20.8, 20.7, 20.7, 20.6 (CO*C*H_3).). HR-ESMS calcd for $C_{29}H_{41}NO_{17}Na$ ($M+Na^+$) 698.2272, found 698.2266.

2-hydroxyethyl 2-acetamido-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-4,6-di-O-acetyl-2-deoxy-β-D-galactopyranoside (**16**).

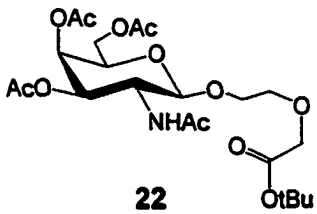


16

A solution of **15** (200 mg, 0.296 mmol) in CH_2Cl_2 (10 ml) and MeOH (10 ml) was cooled to –78 °C. Ozone was bubbled through this solution until a blue colour appeared, then dimethyl sulfide was added dropwise until colour disappeared. The mixture was evaporated under reduced pressure and the resulting residue was dissolved in methanol (5ml). The solution was acidified to pH 4 with glacial acetic acid, then $NaCNBH_3$ (20 mg, 0.32 mmol) was added and stirring continued overnight. Concentration, followed immediately by column chromatography

(1:1 toluene:acetone) yielded **16** (167 mg, 83%). ^{1}H NMR ($CDCl_3$): δ 6.21 (d, 1 H, NH), 5.40 and 5.36 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1 Hz, H-4), 5.13 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 8 Hz, H-2'), 4.99 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 4.97 (d, 1 H, $J_{1,2}$ 7 Hz, H-1), 4.67 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 4.49 (dd, 1 H, $J_{2,3}$ 10.5 Hz, $J_{3,4}$ 3.5 Hz, H-3), 4.05-4.20 (m, 4 H, H-6a, H-6b, H-6a', H-6b'), 3.89-3.96 (m, 2 H, OCH_2CH_2OH), 3.84 (2 H, H-5, H-5'), 3.65-3.79 (m, 2 H, OCH_2CH_2OH), 3.56 (broad dt, 1H, $J_{2,3}$ 10.5 Hz, $J_{1,2}$ 7 Hz, $J_{2,NH}$ 7 Hz, H-2), 1.97, 2.02, 2.07, 2.10, 2.14 and 2.16 (6 s, 21 H, 6 x OAc, NHAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 171.2, 170.5, 170.3, 170.1, 169.9, 169.8, 169.3 ($COCH_3$), 100.1, 99.8 (C-1, C-1'), 74.8, 71.4, 70.8, 69.1, 67.9, 66.7 (C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 72.9, 62.5, 62.0, 60.8 (C-6, C-6', OCH_2CH_2OH, OCH_2CH_2OH), 55.0 (C-2), 23.5, 20.7, 20.6, 20.5, 20.5, 20.4 ($COCH_3$). HR-ESMS calcd for $C_{28}H_{41}NO_{18}Na$ ($M+Na^+$) 702.2221, found 702.2212.

*2-(*tert*-butoxycarbonyl)methoxyethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranoside* (**22**).

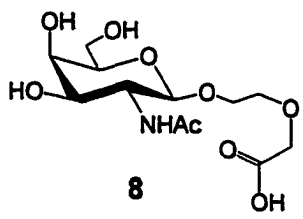


22

A solution of **10** (73 mg, 0.19 mmol) in DMF (1 ml) was cooled to 0 °C, *tert*-butyl α-bromoacetate was added (33 μl, 0.22 mmol), then NaH (24 mg, 60% dispersion in oil, 0.60 mmol) was added. After stirring at room temperature for 2 hours, TLC indicated the presence of product and unreacted starting material. The reaction was quenched with glacial acetic acid, concentrated and chromatographed (19:1 CH_2Cl_2:MeOH) to yield the alkylated product **22** (32 mg, 33%). ^{1}H NMR ($CDCl_3$): δ

6.32 (d, 1 H, NH), 5.34 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1 Hz, H-4), 5.08 (dd, 1 H, $J_{2,3}$ 11 Hz, $J_{3,4}$ 3.5 Hz, H-3), 4.92 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 4.09-4.20 (m, 2 H, H-6a, H-6b), 4.18 (ddd, 1H, $J_{2,3}$ 11 Hz, $J_{1,2}$ 8.5 Hz, $J_{2,NH}$ 9 Hz,H-2), 4.02 (dt, 1 H, $J_{4,5}$ 1 Hz, $J_{5,6a}$ =$J_{5,6b}$ 6.5 Hz, H-5), 4.06 (d, 1 H, J_{gem} 16.5, C*H*$_2$COOtBu), 3.85-3.93 (5 H, C*H*$_2$COOtBu, H-5, OCH$_2$C*H*$_2$O), 3.62-3.77 (m, 2 H, OC*H*$_2$CH$_2$O), 1.95, 2.01, 2.05, 2.16 (4 s, 12 H, 3 x OAc, NHAc), 1.49 (s, 9 H, O*t*Bu). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.7, 170.6, 170.5, 170.4, 170.0 (*C*OCH$_3$, CH$_2$*C*OOtBu), 101.3 (C-1), 82.3 (*C*H$_2$COOtBu), 71.7, 68.6, 67.8, 61.6 (C-6, O*C*H$_2$CH$_2$OR, OCH$_2$*C*H$_2$OR, O*C*(CH$_3$)), 71.2, 70.7, 66.8 (C-3, C-4, C-5), 50.6 (C-2), 28.1 (C(*C*H$_3$)), 23.2, 20.7, 20.7 (CO*C*H$_3$).

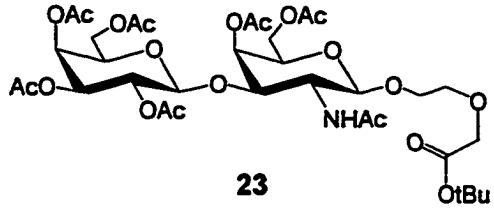
2-(carboxymethoxy)ethyl 2-acetamido -2-deoxy-β-D-galactopyranoside (**8**).



8

Ester **22** (33 mg, 0.065 mmol) was stirred in CH_2Cl_2 (0.5 ml) and trifluoroacetic acid (0.5 ml) for 45 minutes. The solvents were removed *in vacuo* and the residue was chromatographed (99:1→19:1 CH_2Cl_2:MeOH) to yield the acid (15 mg, 51%). This was then deacetylated in 91% yield in 0.02 M methanolic NaOMe (standard work-up) to yield the final product **8** (9.7 mg, 91%). ^{1}H NMR (CD_3OD): δ 4.41 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 4.07 (s, 2 H, C*H*$_2$COOH), 3.89 (dd, 1H, $J_{2,3}$ 11 Hz, $J_{1,2}$ 8.5 Hz, H-2), 3.77 (broad d, 1 H, $J_{3,4}$ 3.5 Hz, H-4), 3.57-3.75 (OC*H*$_2$C*H*$_2$O, H-6a, H-6b), 3.53 (dd, 1 H, $J_{2,3}$ 11 Hz, $J_{3,4}$ 3.5 Hz, H-3), 3.44 (m, 1 H, H-5), 1.94 (s, 3 H, NHAc).

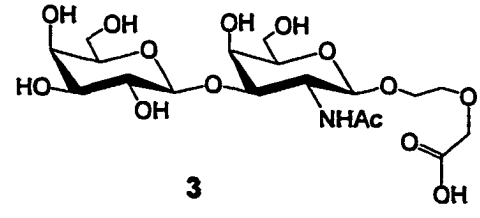
2-(tert-butoxycarbonyl)methoxyethyl 2-acetamido-3-O-(2,3,4,6-tetra-O-acetyl--β-D-galactopyranosyl)-4,6-di-O-acetyl-2-deoxy-β-D-galactopyranoside (**23**).



23

A solution of disaccharide **16** (25 mg, 0.037 mmol) and *tert*-butyl α-bromoacetate (10 μl, 0.068 mmol) in DMF was cooled to 0 °C. NaH (11 mg, 60% dispersion in oil, 0.28 mmol) was added and stirring was continued for 90 minutes. Glacial acetic acid was added to quench the reaction, followed by concentration and column chromatography (19:1 9:1 CH_2Cl_2:MeOH) to yield the ester **23** (8 mg, 27%). ^{1}H NMR ($CDCl_3$): δ 6.29 (d, 1 H, $J_{2,NH}$ 7.5 Hz, NH), 5.39 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 5.34 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1 Hz, H-4), 5.13 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 7.5 Hz, H-2'), 5.02 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 4.96 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 4.62 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1'), 4.57 (dd, 1 H, $J_{2,3}$ 11 Hz, $J_{3,4}$ 3.5 Hz, H-3), 4.20-3.92 (7 H, H-6a, H-6b, H-6a', H-6b', CH_2COOtBu, OCH_2CH_2O), 3.82-3.90 (2 H, H-5, H-5'), 3.73-3.82, 3.64-3.70 (3 H, OCH_2CH_2O), 3.56 (ddd, 1H, $J_{2,3}$ 10.5 Hz, $J_{1,2}$ 8.5 Hz, $J_{2,NH}$ 7.5 Hz, H-2), 1.96, 2.04, 2.06, 2.07, 2.11, 2.14 (6 s, 21 H, 6 x OAc, NHAc), 1.43 (s, 9 H, OtBu).

2-(carboxymethoxy)ethyl 2-acetamido-2-deoxy-3-O-β-D-galactopyranosyl-β-D-galactopyranoside (**3**).

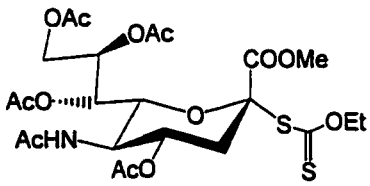


3

The ester **23** (8 mg, 0.01 mmol) was stirred in CH_2Cl_2:trifluoracetic acid (1:1, 1 ml) for 1.5 hours. The reaction was concentrated, followed

by chromatography (9:1 CH_2Cl_2:MeOH) to yield the acid (4 mg, 54%). This was deacetylated as for compound **8** to provide the final product **3** in quantitative yield (2.8 mg). ^{1}H NMR (D_2O): δ 4.64 (d, 1 H, $J_{1'2'}$ 8.5 Hz, H-1'), 4.48 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.22 (broad d, 1 H, $J_{3'4'}$ 3 Hz, H-4'), 4.01-4.08 (H-2', H-3', CH_2COOH), 3.66-3.96 (11 H, H-4, H-5, H-5', H-6a, H-6b, H-6a', H-6b', OCH_2CH_2O), 3.65 (dd, 1 H, $J_{2,3}$ 10 Hz, $J_{3,4}$ 3 Hz, H-3), 3.56 (dd, 1 H, $J_{2,3}$ 10 Hz, $J_{1,2}$ 7.5 Hz, H-2), 2.07 (s, 3 H, NHAc). LR-ESMS calcd for $C_{18}H_{31}NO_9Na$ ($M+Na^+$) 508.2, found 508.2.

O-*Ethyl* S-*[methyl (5-acetamido-4,7,8,9-tetra*-O-*acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosyl)onate] dithiocarbonate* (**25**).

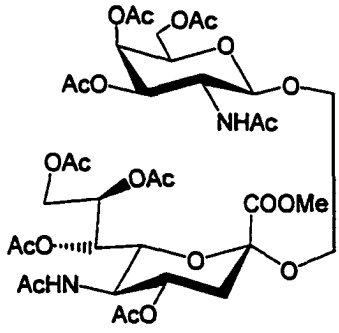


25

A solution of acetylated methyl ester **24** (243 mg, 0.438 mmol) in acetic anhydride (0.5 ml) was stirred at room temperature for 15 minutes, then hydrobromic acid (33% in acetic acid, 5 ml) was added. The reaction was stirred for 1 hour, then diluted with CH_2Cl_2, washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated to yield the crude bromo donor. The crude product and potassium ethyl xanthogenate (196 mg, 1.22 mmol) were stirred in anhydrous ethanol in the dark overnight. The reaction was diluted with CH_2Cl_2 and washed with H_2O and brine, dried over Na_2SO_4 and evaporated. Column chromatography (2:1 toluene:acetone) yielded xanthate donor **25** (162 mg, 62% overall yield). The donor was crystallized in benzene:pentane to provide a pale yellow needle-like product (145 mg). ^{1}H NMR ($CDCl_3$): δ 5.27-5.34 (2 H, H-7, H-8), 5.14 (1 H, $J_{5,N}$ 10.5 Hz, NH), 4.90 (ddd, 1 H, $J_{3a,4}$ 11.5 Hz, $J_{4,5}$ 10.5 Hz, $J_{3e,4}$ 4.5 Hz, H-4), 4.82 (m, 1 H, H-6), 4.56 (m, 2 H, SCH_2CH_3),

4.33 (dd, 1 H, $J_{8,9a}$ 2.5 Hz, $J_{9a,9b}$ 12 Hz, H-9a), 4.20 (dd, 1 H, $J_{8,9b}$ 5 Hz, $J_{9a,9b}$ 12 Hz, H-9b), 4.03 (ddd, 1 H, $J_{4,5} = J_{5,6}=J_{5,N}$ 10.5 Hz, H-5), 3.80 (s, 3 H, OMe), 2.64 (dd, 1 H, $J_{3e,4}$ 4.5 Hz, $J_{3e,3a}$ 12.5 Hz, H-3e), 1.90, 2.03, 2.04, 2.13 and 2.15 (5 s, 15 H, 4x OAc, NHAc), 1.19 (t, 3 H, SCH_2CH_3).

*2-[methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosyl)onate]oxyethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranoside* (**26a**).

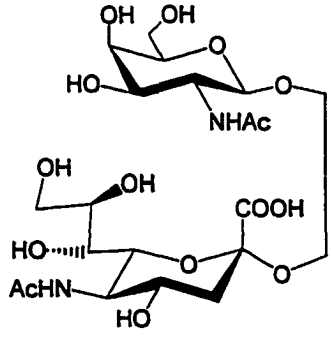


26a

Acceptor **10** (103 mg, 0.263 mmol) and DMTST (70 mg, 0.271 mmol) were stirred in dry CH_3CN (1 ml) under argon atmosphere at –15 °C. A solution of xanthate donor **25** (164 mg, 0.275 mmol) in dry CH_3CN (2 ml) was added dropwise over a period of 15 minutes. Stirring continued at –15 °C for 2 hours, after which Et_3N was added, the reaction mixture was diluted with CH_2Cl_2, filtered through Celite and concentrated. Column chromatography (2:1→1:1 toluene:acetone) yields both **26b** (β product, 39 mg, 17%) and **26a** (α product, 102 mg, 45%). Compound **26a**: ^{1}H NMR ($CDCl_3$): δ 6.22 (d, 1 H, $J_{2'NH}$ 9.5 Hz, NH), 5.46 (ddd, 1 H, $J_{7,8}$ 9 Hz, $J_{8,9a}$ 3 Hz, $J_{8,9b}$ 7.5 Hz, H-8), 5.36 (broad d, 1 H, $J_{3'4'}$ 3.5 Hz, H-4'), 5.27 (dd, 1 H, $J_{7,8}$ 9 Hz, $J_{6,7}$ 1.5 Hz, H-7), 5.12 (1 H, NH), 5.11 (dd, 1 H, $J_{2'3'}$ 11 Hz, $J_{3'4'}$ 3.5 Hz, H-3'), 4.84 (ddd, 1 H, $J_{3a,4}$ 12.5 Hz, $J_{4,5}$ 10 Hz, $J_{3e,4}$ 4.5 Hz, H-4), 4.64 (d, 1 H, $J_{1'2'}$ 8.5 Hz, H-1'), 4.34 (dd, 1 H, $J_{8,9a}$ 3 Hz, $J_{9a,9b}$ 12.5 Hz, H-9a), 4.20 (dd, 1 H, $J_{5'6a'}$ 6.5 Hz, $J_{6a'6b'}$ 11 Hz, H-6a'), 4.17 (1 H, H-2'), 4.13 (dd, 1 H, $J_{5'6b'}$ 7 Hz,

$J_{6a'6b'}$ 11 Hz, H-6b'), 3.91-4.12 (6 H, H-5, H-5', H-6, H-9b, OCH_2CH_2O), 3.71-3.85 (5 H, OMe, OCH_2CH_2O), 3.41 (1 H, OCH_2CH_2O), 2.56 (dd, 1 H, $J_{3e,4}$ 4.5 Hz, $J_{3e,3a}$ 12.5 Hz, H-3e), 1.95 (t, 1 H, $J_{3a,4}=J_{3e,3a}$ 12.5 Hz, H-3a), 1.88, 1.96, 1.98, 2.02, 2.05, 2.15 and 2.18 (8 s, 27 H, 7 x OAc, 2 x NHAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 171.0, 170.0, 170.9, 170.5, 170.4, 170.3, 170.2 ($COCH_3$), 168.1 (carbonyl), 101.7 (C-1'), 100.0 (C-2), 72.4, 70.9, 70.6, 68.9, 67.8, 67.6, 66.8 (C-3', C-4', C-5', C-4, C-6, C-7, C-8), 68.0, 63.1, 61.5 (C-6', OCH_2CH_2OH, OCH_2CH_2OH), 52.9 (OMe), 50.7, 49.4 (C-2', C-5), 37.7 (C-3), 23.3, 23.2, 21.3, 20.9, 20.9, 20.7, 19.6 ($COCH_3$), HR-ESMS calcd for $C_{36}H_{52}N_2O_{22}Na$ ($M+Na^+$) 887.2909, found 887.2920.

2-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic acid)oxyethyl 2-acetamido-2-deoxy-β-D-galactopyranoside (**6**).

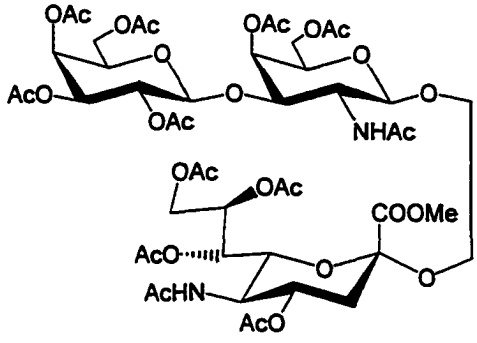


6

A solution of **26a** (6 mg, 0.007 mmol) in methanolic sodium methoxide (0.02 mM, 2 ml) was stirred for 3 hours, after which 0.5 ml of H_2O was added. Stirring continued overnight, followed by neutralization of the solution with Amberlite IR-120 (H+), filtration and concentration. The residue was dissolved in H_2O and lyophilized to yield **6** (3.8 mg, 94%). ^1H NMR (D_2O): δ 4.52 (d, 1 H, $J_{1'2'}$ 8.5 Hz, H-1'), 3.96 (ddd, 1 H, $J_{7,8}$ 2.5 Hz, $J_{8,9a}$ 5.5, $J_{8,9b}$ 8.5 Hz, H-8), 3.94 (broad d, 1 H, $J_{3'4'}$ 3.5 Hz, H-4'), 3.90 (dd, 1 H, $J_{2'3'}$ 11 Hz, $J_{1'2'}$ 8.5 Hz, H-2'), 3.78-3.89 (4 H, H-6, H-9a, H-9b, H-6b'), 3.76 (dd, 1 H, $J_{5'6a'}$ 4 Hz, $J_{6a'6b'}$ 12 Hz, H-6a'), 3.73 (dd, 1 H, $J_{2'3'}$ 11 Hz, $J_{3'4'}$ 3.5 Hz, H-3'), 3.62-3.71 (3 H, H-

4, H-7, H-5'), 3.58 (dd, 1 H, $J_{4,5}$ 1.5 Hz, $J_{5,6}$ 9 Hz, H-5), 2.74 (dd, 1 H, $J_{3e,4}$ 4.5 Hz, $J_{3e,3a}$ 12.5 Hz, H-3e), 2.04 and 2.07 (2 s, 2 x NHAc), 1.67 (t, 1 H, $J_{3a,4}$=$J_{3e,3a}$ 12.5 Hz, H-3a). HR-ESMS calcd for $C_{21}H_{36}N_2O_{15}Na$ ($M+Na^+$) 579.2013, found 579.2014.

2-[methyl (5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosyl)onate]oxyethyl 2-acetamido-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-4,6-di-O-acetyl-2-deoxy-β-D-galactopyranoside (**27a**).

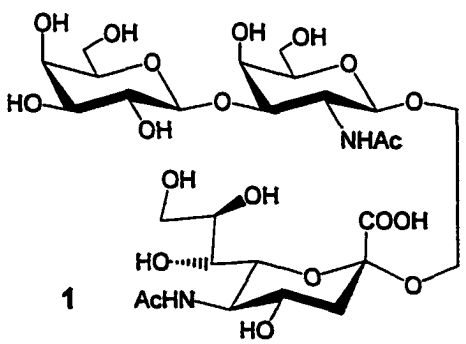


27a

A solution of acceptor **16** (71 mg, 0.10 mmol) and DMTST (47 mg, 0.18 mmol) was stirred in dry CH_3CN (1 ml) under argon atmosphere at –15 °C. A solution of xanthate donor **25** (100 mg, 0.168 mmol) in dry CH_3CN (2 ml) was added dropwise. Stirring continued at room temperature for 18 hours, after which Et_3N was added. The reaction mixture was then diluted with CH_2Cl_2, filtered through Celite and concentrated. Column chromatography (2:1→1:1→1:2 toluene:acetone) yielded the α anomer **27a** and a mixture of the α and β product. Repeated chromatography (7:5 toluene:acetone) of the mixture lead to an overall yield of α anomer **27a** (58 mg, 48%) and β anomer **27b** (38 mg, 32%). Compound **27a**: ^1H NMR ($CDCl_3/CD_3OD$): δ 5.18 (broad d, 1 H, $J_{3''4''}$ 3 Hz, H-4''), 5.17 (ddd, 1 H, $J_{7,8}$ 6.5 Hz, $J_{8,9a}$ 2.5 Hz, H-8), 5.14 (d, 1 H, $J_{3'4'}$ 3.5 Hz, $J_{4'5'}$ 1 Hz, H-4'), 5.11 (dd, 1 H, $J_{7,8}$ 6.5 Hz, $J_{6,7}$ 2 Hz, H-7), 4.87 (dd, 1 H, $J_{2''3''}$ 10.5 Hz, $J_{1''2''}$ 7.5 Hz, H-2''), 4.78 (dd, 1 H, $J_{2''3''}$ 10.5 Hz, $J_{3''4''}$ 3 Hz, H-3''), 4.59(ddd, 1 H, $J_{3a,4}$ 12.5 Hz, $J_{4,5}$ 10 Hz, $J_{3e,4}$ 4.5 Hz, H-4), 4.53 (d, 1 H,

$J_{1'2'}$ 8.5 Hz, H-1'), 4.45 (d, 1 H, $J_{1''2''}$ 7.5 Hz, H-1''), 4.18 (dd, 1 H, $J_{2'3'}$ 11 Hz, $J_{3'4'}$ 3.5 Hz, H-3'), 4.12 (dd, 1 H, $J_{8,9a}$ 2.5 Hz, $J_{9a,9b}$ 12.5 Hz, H-9a), 3.95 (dd, 1 H, $J_{5'6a'}$ 6.5 Hz, $J_{6a'6b'}$ 11.5 Hz, H-6a'), 3.64-3.95 (9 H, H-2', H-5', H-5'', H-6, H-6b', H-6a'', H-6b'', H-9b, OCH_2CH_2O), 3.63 (s, 3 H, OMe), 3.40-3.50 (m, 2 H, OCH_2CH_2O), 3.21-3.29 (m, 1 H, OCH_2CH_2O), 2.42 (dd, 1 H, $J_{3e,3a}$ 12.5 Hz, $J_{3e,4}$ 4.5 Hz, H-3e), 1.63-2.03 (10 s, 36 H, 10 x OAc, 2 x NHAc), 1.71 (1 H, H-3a). ^{13}C NMR (75 Hz, CD_3OD): δ 173.5, 173.4, 172.5, 172.4, 172.1, 172.0, 171.9, 171.8, 171.5, 171.5, 171.2 ($COCH_3$), 169.5 (carbonyl), 102.6, 102.5 (C-1', C-1''), 100.2 (C-2), 77.7, 73.2, 72.5, 72.3, 71.8, 70.8, 70.7, 70.3, 69.5, 68.7 (C-2'', C-3', C-3'', C-4, C-4, C-4'', C-5', C-5'', C-6, C-7, C-8), 65.1, 63.6, 63.5, 62.3 (C-6', C-6'', OCH_2CH_2OH, OCH_2CH_2OH), 53.4, 53.2 (C-2', C-5), 50.7 (OMe), 38.9 (C-3), 23.4, 22.7, 21.3, 20.9, 20.7, 20.5 ($COCH_3$). HR-ESMS calcd for $C_{48}H_{68}N_2O_{30}Na$ ($M+Na^+$) 1175.3755, found 1175.3743.

2-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic acid)oxyethyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-β-D-galactopyranoside (**1**).

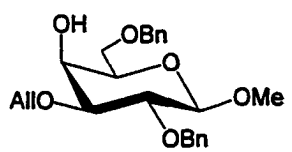


1

A solution of **27a** (4.2 mg, 0.0036 mmol) in methanolic sodium methoxide (0.02 mM, 3 ml) was stirred overnight. H_2O (1 ml) was added and stirring continued overnight, after which the solution was neutralized with Amberlite IR-120(H+), filtered and concentrated. The residue was dissolved in H_2O and lyophilized to yield a white fluffy product **1** (2 mg, 76 % yield). ^1H NMR (D_2O): δ 4.59 (d, 1 H, $J_{1'2'}$ 8.5

Hz, H-1'), 4.47 (d, 1 H, $J_{1''2''}$ 7.5 Hz, H-1''), 4.20 (broad d, 1 H, $J_{3'4'}$ 3 Hz, H-4'), 4.03 (dd, 1 H, $J_{2'3'}$ 11 Hz, $J_{1'2'}$ 8.5 Hz, H-2'), 4.01 (m, 1 H, H-8), 3.93 (broad d, 1 H, $J_{3''4''}$ 3 Hz, H-4''), 3.90 (dd, 1 H, $J_{2'3'}$ 11 Hz, $J_{3'4'}$ 3.5 Hz, H-3'), 3.65-3.90 (H-4, H-5, H-6, H-7, H-9a, H-9b, H-5', H-6a, H-6b, H-5'', H-6a'', H-6b''), 3.61 (dd, 1 H, $J_{2''3''}$ 11 Hz, $J_{3''4''}$ 3 Hz, H-3''), 3.55 (dd, 1 H, $J_{2''3''}$ 11 Hz, $J_{1''2''}$ 7.5 Hz, H-2''), 2.74 (dd, 1 H, $J_{3e,4}$ 4.5 Hz, $J_{3e,3a}$ 12.5 Hz, H-3e), 2.05 (s, 6 H, NHAc), 1.75 (t, 1 H, $J_{3a,4}$=$J_{3e,3a}$ 12.5 Hz, H-3a). ^{13}C NMR (75 Hz, D_2O): δ 175.9 (C=O), 105.7, 101.9 (C-1', C-1''), 80.8, 75.8, 75.6, 73.5, 73.3, 72.3, 71.4, 69.4, 69.1, 68.9, 68.8 (C-2'', C-3', C-3'', C-4, C-4, C-4'', C-5', C-5'', C-6, C-7, C-8), 64.0, 63.6, 61.8, 61.75 (C-6', C-6'', OCH_2CH_2OH, OCH_2CH_2OH), 52.6, 52.0 (C-2', C-5), 40.6 (C-3).). HR-ESMS calcd for $C_{27}H_{46}N_2O_{20}Na$ ($M+Na^+$) 741.2542, found 741.2546.

Methyl 3-O-allyl-2,6-di-O-benzyl-β-D-galactopyranoside (**29**).

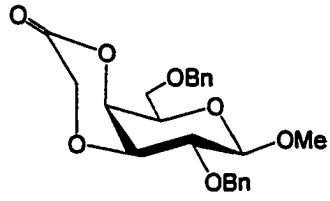


29

A solution of **28** (633 mg, 1.69 mmol) and dibutyltin oxide (4420 mg, 1.69 mmol) in toluene (50 ml) was refluxed in a Dean-Stark apparatus for 16 hours. The reaction was cooled to 65 °C, Bu_4NI (724 mg, 1.96 mmol) was added, then allyl bromide (750 µl, 8.67 mmol). Stirring continued for 20 hours at 65 °C. Removal of the solvent *in vacuo* and column chromatography (9:1 toluene:acetone) of the resulting residue yielded **29** (635 mg, 91%). ^{1}H NMR ($CDCl_3$): δ 7.1-7.3 (10 H, 2 x OBn), 5.84 (1 H, H_c allyl), 5.21 (1 H, H_b allyl), 5.10 (1 H, H_a allyl), 4.78 (d, 1 H, J_{gem} 11.5, PhCH_2), 4.62 (d, 1 H, J_{gem} 11.5, PhCH_2), 4.52 (s, 2 H, PhCH_2), 4.18 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.05-4.17 (2 H, H_d,H_e allyl), 3.95 (broad m, 1 H, H-4), 3.74 (dd, 1 H, $J_{6a,6b}$ 10 Hz, $J_{5,6a}$ 6 Hz, H-6a), 3.66 (dd, 1 H, $J_{6a,6b}$ 10 Hz, $J_{5,6b}$ 6 Hz, H-6b), 3.45-3.54 (5 H, H-2, H-5, OMe), 3.33 (dd, 1 H, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 3.5 Hz, H-3),

2.39 (broad m, 1 H, OH). ^{13}C NMR (75 Hz, $CDCl_3$): δ 138.7, 138.0 (aromatic quat.), 134.6 (-CH=, allyl), 128.5, 128.3, 128.0, 127.8, 127.8, 127.6 (aromatic CH), 117.4 (=CH_2, allyl), 104.7 (C-1), 80.4, 79.0, 71.5, 69.2 (C-2, C-3, C-4, C-5), 75.1, 73.8, 73.2, 67.0 (PhCH_2, CH_2 allyl, C-6), 57.0 (OMe). HR-ESMS calcd for $C_{24}H_{30}O_6Na$ ($M+Na^+$) 437.1940, found 437.1944.

Methyl 3,4-O-(2-carbonylethylene)-2,6-di-O-benzyl-β-D-galactopyranoside (**31**).

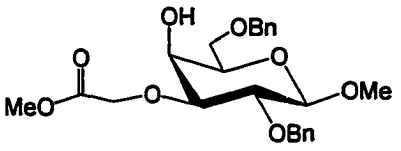


31

A solution of **28** (500 mg, 1.34 mmol) and dibutyltin oxide (370 mg, 1.49 mmol) in a toluene (50 ml) was refluxed at 120 °C with azeotropic removal of water for 22 hours. The reaction mixture was cooled to 80 °C, then Bu_4NI (540 mg, 1.46 mmol) and bromo ester (600 µl, 4.06 mmol) were added. Stirring continued at 65 °C for 28 hours. Solvent was removed under reduced pressure and residue was chromatographed on 19:1 toluene:acetone to yield **31** (225 mg, 41%). ^1H NMR ($CDCl_3$): δ 7.2-7.4 (10 H, 2 x OBn), 4.85, 4.71 (d, 1 H, J_{gem} 12 Hz, PhCH_2), 4.71 (broad d, 1 H, $J_{3,4}$ 4 Hz, H-4), 4.54, 4.56 (d, 1 H, J_{gem} 12 Hz, PhCH_2), 4.32 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.13 (d, 1 H, J_{gem} 18 Hz, CH_2 lactone), 3.87 (dd, 1 H, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 3.5 Hz, H-3), 3.80 (dd, 1 H, $J_{6a,6b}$ 7.5 Hz, $J_{5,6a}$ 5 Hz, H-6a), 3.74 (ddd, 1 H, $J_{4,5}$ 1 Hz, $J_{5,6a}$ 5 Hz, $J_{5,6b}$ 5.5 Hz, H-5), 3.70 (dd, 1 H, $J_{6a,6b}$ 7.5 Hz, $J_{5,6b}$ 5.5 Hz, H-6b), 3.57 (s, 3 H, OMe), 3.55 (d, 1 H, J_{gem} 18 Hz, CH_2 lactone), 3.53 (dd, 1H, $J_{2,3}$ 10 Hz, $J_{1,2}$ 7.5 Hz, H-2). ^{13}C NMR (75 Hz, $CDCl_3$): δ 166.6 (carbonyl), 137.7, 137.7 (aromatic quat.), 128.7, 128.5, 128.2, 128.0, 127.8 (aromatic CH), 105.0 (C-1), 74.4, 72.2, 72.0, 71.7 (C-2, C-3,

C-4, C-5), 74.0, 73.8 ($PhCH_2$), 67.1, 60.2 (C-6, CH_2 lactone), 57.2 ($COOCH_3$). HR-ESMS calcd for $C_{23}H_{26}O_7Na$ ($M+Na^+$) 437.1576, found 437.1571.

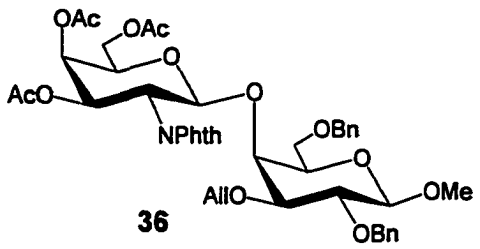
Methyl 2,6-di-O-benzyl-3-O-(methoxycarbonyl)methyl-β-D-galactopyranoside (**32**).



32

A solution of lactone **31** (220 mg, 0.531 mmol) in dry methanol (20 ml) was stirred in the presence of Amberlite IR-120 (H+) ion exchange resin overnight. Removal of the resin by filtration, followed by evaporation of the solvent and column chromatography of the residue (9:1 toluene:acetone) yielded the methyl ester **32** (210 mg, 89%). ^1H NMR ($CDCl_3$): δ 7.2-7.4 (10 H, 2 x OBn), 4.86 (d, 1 H, J_{gem} 11, $PhCH_2$), 4.66 (d, 1 H, J_{gem} 11, $PhCH_2$), 4.59 (d, 1 H, J_{gem} 12, $PhCH_2$), 4.57 (d, 1 H, J_{gem} 12, $PhCH_2$), 4.40 (d, 1 H, J_{gem} 17, CH_2COOMe), 4.24 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.18 (d, 1 H, J_{gem} 17, CH_2COOMe), 4.04 (dd, 1 H, $J_{4,5}$ 1 Hz, $J_{3,4}$ 3 Hz, H-4), 3.81 (dd, 1 H, $J_{6a,6b}$ 10 Hz, $J_{5,6a}$ 5.5 Hz, H-6a), 3.74 (dd, 1 H, $J_{6a,6b}$ 10 Hz, $J_{5,6b}$ 6 Hz, H-6b), 3.71 (s, 3 H, COOMe), 3.66 (dd, 1H, $J_{2,3}$ 9.5 Hz, $J_{1,2}$ 7.5 Hz, H-2), 3.57 (broad t, 1 H, $J_{5,6a}$ 5.5 Hz, $J_{5,6b}$ 6 Hz, $J_{4,5}$ 1 Hz, H-5), 3.54 (s, 3 H, OMe), 3.37 (dd, 1 H, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 3.5 Hz, H-3). ^{13}C NMR (75 Hz, $CDCl_3$): δ 172.0 (carbonyl), 138.7, 138.1 (aromatic quat.), 128.5, 128.3, 128.0, 127.8, 127.7, 127.6 (aromatic CH), 104.6 (C-1), 83.5, 79.1, 73.2, 67.3 (C-2, C-3, C-4, C-5), 75.1, 73.8, 69.4, 68.5 ($PhCH_2$, CH_2COOMe, C-6), 56.9 (OMe), 52.1 (Me ester). HR-ESMS calcd for $C_{24}H_{30}O_8Na$ ($M+Na^+$) 469.1838, found 469.1838.

Methyl 4-O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl)-3-O-allyl-2,6-di-O-benzyl-β-D-galactopyranoside (**36**).

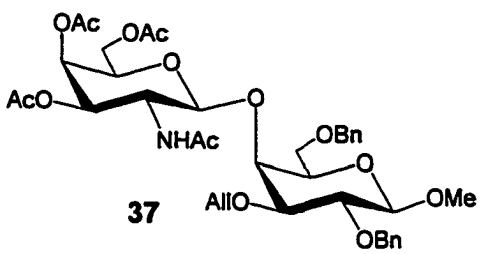


36

Acceptor **29** (44 mg, 0.106 mmol), AgOTf (68 mg, 0.265 mmol) and 4 Å molecular sieves were stirred in CH_2Cl_2 (6 ml) for 30 minutes at room temperature. A solution of the bromo donor **35** (101 mg, 0.203 mmol) in CH_2Cl_2 (1.5 ml) was added dropwise. After stirring for 13 hours, the reaction was quenched with solid $NaHCO_3$, diluted with CH_2Cl_2 and filtered through Celite. The filtrate was washed with saturated $NaHCO_3$, brine, dried over Na_2SO_4 and concentrated. Column chromatography (2: 1→1:1 pentane:EtOAc) provided disaccharide **36** in 59% isolated yield (52 mg). ^{1}H NMR ($CDCl_3$): δ 6.88-7.87 (14 H, ArH), 6.03 (dd, 1 H, $J_{2',3'}$ 11.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 5.69 (1 H, H_c allyl), 5.47 (dd, 1 H, $J_{4',5'}$ 1 Hz, $J_{3',4'}$ 3.5 Hz, H-4'), 5.30 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1'), 4.93-5.10 (2 H, H_a,H_b allyl), 4.61 (dd, 1 H, $J_{1',2'}$ 8.5 Hz, $J_{2',3'}$ 11.5 Hz, H-2'), 4.55 (s, 3 H, OMe), 4.28 (d, 1 H, J_{gem} 10.5, PhCH_2), 4.01-4.16 (4 H, H-1, H_d allyl, H-5', H-6a', H-6b'), 3.77-3.87 (3 H, H-4, H_e allyl, H-6a), 3.67 (dd, 1 H, $J_{6a,6b}$ 9.5 Hz, $J_{5,6b}$ 5.5 Hz, H-6b), 3.51 (d, 1 H, J_{gem} 10.5, PhCH_2), 3.46 (s, 3 H, OMe), 3.42 (broad t, 1 H, $J_{5,6b}$ 5.5 Hz, $J_{5,6a}$ 6 Hz, $J_{4,5}$ 1 Hz, H-5), 3.12 (dd, 1 H, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 3 Hz, H-3), 2.99 (dd, 1 H, $J_{1,2}$ 7.5 Hz, $J_{2,3}$ 9.5 Hz, H-2), 1.87, 2.03 and 2.19 (3 s, 9 H, 3 x OAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.4, 169.9, 168.4, 167.6 (carbonyl), 138.4, 138.3, 133.8, 132.5 (aromatic quat.), 134.9, 133.9, 131.8, 128.5, 128.2, 127.7, 123.4, 123.2 (-CH= allyl, aromatic CH), 118.0 (=CH_2 allyl), 104.3, 99.9 (C-1, C-1'), 79.8, 79.7, 76.9, 73.2, 70.2, 67.6, 66.5 (C-2, C-3, C-4, C-5, C-3', C-4', C-5'), 75.0,

73.6, 72.4, 69.6, 61.4 (PhCH$_2$, CH$_2$ allyl, C-6, C-6'), 56.8 (OMe), 51.3 (C-2'), 20.8, 20.7. 20.6 (COCH$_3$). HR-ESMS calcd for $C_{44}H_{49}NO_{15}Na$ ($M+Na^+$) 854.3000, found 854.3008.

Methyl 4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranosyl)-3-O-allyl-2,6-di-O-benzyl-β-D-galactopyranoside (**37**).

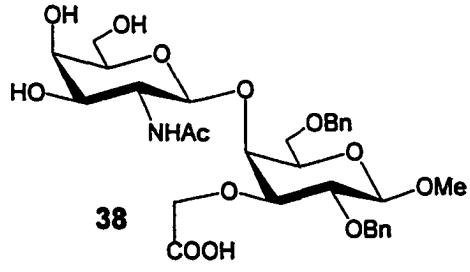


37

A solution of **36** (122 mg, 0.147 mmol) and ethylene diamine (5 ml, 7.5 mmol) in ethanol (20 ml) was stirred at 65 °C for 7 hours. The reaction was concentrated and co-concentrated with toluene. The residue was acetylated overnight in pyridine (6 ml) and acetic anhydride (4 ml). The reaction was concentrated and co-concentrated with toluene, followed by column chromatography on 3:1 toluene:acetone. This yielded an impure product which was rechromatographed (6:1→2:1 toluene:acetone) to yield **37** (102 mg, 93%). ^{1}H NMR ($CDCl_3$): δ 7.25-7.4 (10 H, 2 x OBn), 5.94 (1 H, H_c allyl), , 5.90 (d, J 8.5 Hz, NH), 5.32 (dd, 1 H, $J_{4',5'}$ 1 Hz, $J_{3',4'}$ 3.5 Hz, H-4'), 5.30 (1 H, H_a allyl), 5.27 (1 H, H_b allyl), 5.05 (dd, 1 H, $J_{2',3'}$ 11 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 4.91 (d, 1 H, J_{gem} 11, PhCH_2), 4.74 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1'), 4.59 (d, 1 H, J_{gem} 11, PhCH_2), 4.58 (d, 1 H, J_{gem} 12, PhCH_2), 4.55 (d, 1 H, J_{gem} 12, PhCH_2), 4.30 (1 H, H_d allyl), 4.26 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.19 (1 H, H_e allyl), 4.11 (dd, 1 H, $J_{6a,6b}$ 11 Hz, $J_{5,6a}$ 7.5 Hz, H-6a), 4.03 (dd, 1 H, $J_{6a,6b}$ 11 Hz, $J_{5,6b}$ 6 Hz, H-6b), 4.00 (dd, 1 H, $J_{4,5}$ 1 Hz, $J_{3,4}$ 3 Hz, H-4), 3.84 (broad t, 1 H, $J_{5',6b'}$ 6 Hz, $J_{5',6a'}$ 7.5 Hz, $J_{4',5'}$ 1 Hz, H-5'), 3.80 (dd, 1 H, $J_{6a',6b'}$ 10 Hz, $J_{5',6a'}$ 6 Hz, H-6a'), 3.68 (dd, 1 H, $J_{6a',6b'}$ 10 Hz, $J_{5',6b'}$ 6 Hz, H-6b'), 3.48-3.60 (5 H, H-2, H-5, OMe), 3.44 (dd, 1 H, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 3

Hz, H-3), 1.86, 1.99and 2.17 (3 s, 12 H, NHAc, 3 x OAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.6, 170.4, 170.3, 170.0 ($COCH_3$), 138.6, 138.2 (aromatic quat.), 134.2 (-CH=, allyl), 128.5, 128.4, 127.7, 127.7 (aromatic CH), 118.7 ($=CH_2$, allyl), 104.7, 102.4 (C-1, C-1'), 81.0, 79.9, 75.9, 73.3, 71.7, 70.8, 66.6 (C-2, C-3, C-3', C-4, C-4', C-5, C-5'), 75.0, 73.6, 73.1, 69.4, 61.3 (C-6, C-6', CH_2 allyl, $PhCH_2$), 56.9 (OCH_3), 51.3 (C-2'), 23.5, 20.8, 20.6 ($COCH_3$). HR-ESMS calcd for $C_{38}H_{49}NO_{14}Na$ ($M+Na^+$) 766.3051, found 766.3059.

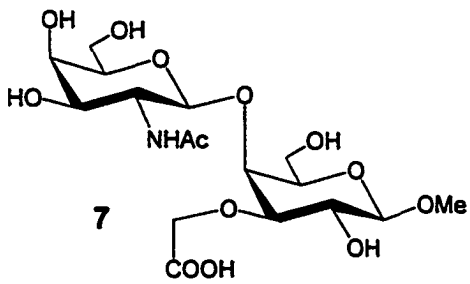
Methyl 4-O-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-2,6-di-O-benzyl-3-O-carboxymethyl-β-D-galactopyranoside (**38**).



To a solution of the allyl ether **37** (61 mg, 0.082 mmol) in CH_2Cl_2 (1.2 ml) was added aqueous acetic acid (17%, 1.5 ml), then Aliquat-336 (0.1 M in diethyl ether, 120 μl, 0.012 mmol). $KMnO_4$ (51 mg, 0.32 mmol) was added slowly to the vigorously stirring solution. Stirring continued for 12 hours, then reaction was quenched with sodium sulfite and acidified with 1 M HCl. The CH_2Cl_2 layer was washed with brine, dried over Na_2SO_4 and concentrated. Column chromatography (2:1 toluene:acetone→60:20:1 toluene:acetone:HOAc) yielded a 1:1 mixture of compounds (24 mg, one spot by TLC) which was directly deacetylated in 0.05 M methanolic sodium methoxide. After 1 hour, H_2O was added, stirred for 15 minutes, followed by neutralization with Amberlite IR-120(H+), filtration and evaporation of the solvents *in vacuo*. Column chromatography (4:1 CH_2Cl_2:MeOH) yields **38** (17 mg, 33% overall yield). ^1H NMR (CD_3OD): δ 7.13-

7.35 (10 H, 2 x OBn), 4.61 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 4.55 (d, 1 H, J_{gem} 11, PhCH_2), 4.52 (s, 2 H, PhCH_2), 4.31 (d, 1 H, J_{gem} 15.5, CH_2COOH), 4.21 (d, 1 H, $J_{1'2'}$ 7 Hz, H-1'), 4.18 (d, 1 H, J_{gem} 15.5, CH_2COOH), 4.09 (dd, 1 H, $J_{4,5}$ 1 Hz, $J_{3,4}$ 2.5 Hz, H-4), 3.95 (dd, 1 H, $J_{2,3}$ 10.5 Hz, $J_{1,2}$ 8.5 Hz, H-2), 3.81 (dd, 1 H, $J_{2'3'}$ 9.5 Hz, $J_{3'4'}$ 3 Hz, H-3'), 3.75 (dd, 1 H, $J_{4'5'}$ 1 Hz, $J_{3'4'}$ 3 Hz, H-4'), 3.55-3.72 (5 H, H-2', H-6a, H-6b, H-6a', H-6b'), 3.36-3.49 (6 H, OMe, H-3, H-5, H-5') , 1.93 (s, 3 H, NHAc).

Methyl 4-O-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-3-O-carboxymethyl-β-D-galactopyranoside (7).

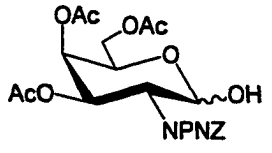


7

A solution of **38** (17 mg, 0.027 mmol) and $Pd(OH)_2$ (45 mg) in methanol (10 ml) was stirred overnight under flow of hydrogen. Filtration of the reaction mixture and removal of the solvent yields 7 (11.5 mg, 95% yield). ^{1}H NMR (D_2O): δ 4.62 (d, 1 H, $J_{1'2'}$ 8.5 Hz, H-1'), 4.41, 4.38 (d, 1 H, J_{gem} 17.5, CH_2COOH), 4.33 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.33 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4), 3.90 (broad d, 1 H, $J_{3'4'}$ 3 Hz, H-4'), 3.89 (dd, 1 H, $J_{2'3'}$ 10.5 Hz, $J_{1'2'}$ 8.5 Hz, H-2'), 3.75-3.85 (4 H, H-6a, H-6b, H-6a', H-6b'), 3.72 (dd, 1 H, $J_{2'3'}$ 10.5 Hz, $J_{3'4'}$ 3 Hz, H-3'), 3.62-3.69 (2 H, H-5, H-5'), 3.58 (dd, 1 H, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 3 Hz, H-3), 3.55 (s, 3 H, OMe), 3.45 (dd, 1 H, $J_{2,3}$ 9.5 Hz, $J_{1,2}$ 8 Hz, H-2), 2.06 (s, 3 H, NHAc). ^{13}C NMR (75 Hz, D_2O): δ 184.1 (*C*OOH), 175.0 (*C*OCH_3), 103.5, 102.4)C-1, C-1'), 81.7, 80.0, 74.1, 73.5, 71.2, 70.4, 67.9 (C-2, C-3, C-4, C-5, C-3', C-4', C-5'), 61.1, 60.6 (C-6, C-6'), 57.1 (OMe),

52.7 (C-2'), 20.9 ($COCH_3$). HR-ESMS calcd for $C_{17}H_{29}NO_{13}Na$ ($M+Na^+$) 478.1537, found 478.1541.

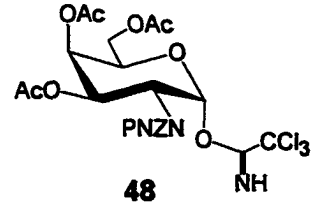
3,4,6-tri-O-acetyl-2-(p-nitrobenzyloxycarbonyl)amino-2-deoxy-β-D-galactopyranose (**47**).



47

A solution of **46** (994 mg, 1.90 mmol) and hydrazine acetate (230 mg, 2.50 mmol) in DMF (5 ml) was stirred at 50 °C for 30 minutes. The reaction mixture was diluted with ethyl acetate and washed with brine. The organic layer was dried over Na_2SO_4 and concentrated. Column chromatography (9:1toluene:acetone) yielded the reducing sugar **47** (596 mg, 65 %). ^1H NMR ($CDCl_3$): δ 8.20 (d, 2 H, J_{ortho} 9 Hz, ArC*H*), 7.49 (d, 2 H, J_{ortho} 9 Hz, ArC*H*), 5.40 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.35 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 5.12-5.32 (4 H, NH, H-3, OCH_2PhNO_2), 4.43 (broad t, 1 H, $J_{5,6a}$ 6 Hz, $J_{5,6b}$ 6.5 Hz, H-5), 4.26 (ddd, 1H, $J_{2,3}$ 10.5 Hz, $J_{1,2}$ 3.5 Hz, $J_{2,NH}$ 10 Hz, H-2), 4.03-4.15 (2 H, H-6a, H-6b), 1.95, 2.03 and 2.17 (3 s, 9 H, 3 x OAc).

O-[3,4,6-tri-O-acetyl-2-deoxy-2-N-(p-nitrobenzyloxycarbonyl)amino-α-D-galactopyranosyl] trichloroacetimidate (**48**).

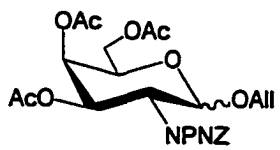


48

Reducing sugar **47** (590 mg, 1.22 mmol), K_2CO_3, (680 mg, 4.92 mmol) and trichloroacetonitrile (1.2 ml, 12.0 mmol) were stirred in dry CH_2Cl_2 for 24 hours. The reaction was diluted

with CH_2Cl_2, washed with H_2O and brine, and the organic layer was dried over Na_2SO_4 and concentrated. Column chromatography (19:1 toluene:acetone) yielded the α imidate donor **48** (645 mg, 84 %). ^{1}H NMR ($CDCl_3$): δ 8.78 (s, 1 H, $NH_{imidate}$), 8.20, 7.47 (d, 2 H, J_{ortho} 9 Hz, ArC*H*), 6.44 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 5.49 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.26 (dd, 1 H, $J_{2,3}$ 11.5 Hz, $J_{3,4}$ 3.5 Hz, H-3), 5.22 (d, 1 H, J_{gem} 14, OC*H*$_2$PhNO$_2$), 5.18 d, 1 H, J_{gem} 14, OC*H*$_2$PhNO$_2$), 4.92 (d, 1 H, , $J_{2,NH}$ 9.5 Hz, NH), 4.51 (ddd, 1H, $J_{2,3}$ 11.5 Hz, $J_{1,2}$ 3.5 Hz, $J_{2,NH}$ 9.5 Hz, H-2), 4.37 (dt, 1 H, $J_{5,6a}$ 6.5 Hz, $J_{5,6b}$ 6.5 Hz, H-5), 4.17 (dd, 1 H, $J_{6a,6b}$ 11 Hz, $J_{5,6a}$ 6.5 Hz, H-6a), 4.07 (dd, 1 H, $J_{6a,6b}$ 11 Hz, $J_{5,6b}$ 6.5 Hz, H-6b), 1.99, 2.02 and 2.18 (3 s, 9 H, 3 x OAc).

*Allyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-(*p*-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside* (**49b**) and *Allyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-(*p*-nitrobenzyloxycarbonyl)amino-α-D-galactopyranoside* (**49a**).

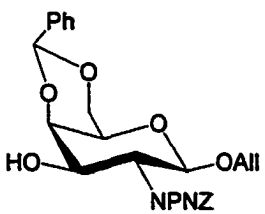


49a/b

The imidate donor **48** (642 mg, 1.02 mmol), allyl alcohol (350 μl, 5.25 mmol) and 4 Å molecular sieves (900 mg) were stirred in dry CH_2Cl_2 under argon for 15 minutes. A solution of $BF_3{\cdot}OEt_2$ in CH_2Cl_2 (0.1 M, 200 μl, 0.02 mmol) was added via syringe. Stirring continued overnight at room temperature. The reaction was filtered through Celite and concentrated. The residue was chromatographed (9:1 toluene:acetone) to yield the α-glycoside **49a** (123 mg, 23 %) and the β-glycoside **49b** (414 mg, 77 %). Compound **49a**: ^{1}H NMR ($CDCl_3$): δ 8.21, 7.50 (d, 2 H, J_{ortho} 9 Hz, ArC*H*), 5.88 (1 H, H_c allyl), 5.38 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4),, 5.29 (1 H, H_b allyl), 5.24

(1 H, H_a allyl), 5.22 (d, 1 H, J_{gem} 13.5 Hz, OCH_2PhNO_2), 5.16 (dd, 1 H, $J_{2,3}$ 11 Hz, $J_{3,4}$ 3.5 Hz, H-3), 5.15 (d, 1 H, J_{gem} 13.5 Hz, OCH_2PhNO_2), 5.04 (d, 1 H, , $J_{2,NH}$ 10.5 Hz, NH), 4.95 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.27 (ddd, 1H, $J_{2,3}$ 11 Hz, $J_{1,2}$ 3.5 Hz, $J_{2,NH}$ 10.5 Hz, H-2), 4.19 (1 H, H_d allyl), 4.05-4.19 (3 H, H-5, H-6a, H-6b), 4.01 (1 H, H_e allyl), 1.93, 2.07 and 2.15 (3 s, 9 H, 3 x OAc). Compound **49b**: ^{1}H NMR ($CDCl_3$): δ 8.21, 7.49 (d, 2 H, J_{ortho} 9 Hz, ArC*H*), 5.83 (1 H, H_c allyl), 5.37 (dd, 1 H, $J_{3,4}$ 3 Hz, $J_{4,5}$ 1 Hz, H-4), 5.26 (1 H, H_b allyl), 5.18 (1 H, H_a allyl), 4.37 (1 H, H_d allyl), 4.18 (dd, 1 H, $J_{6a,6b}$ 11 Hz, $J_{5,6a}$ 6.5 Hz, H-6a), 4.13 (dd, 1 H, $J_{6a,6b}$ 11 Hz, $J_{5,6b}$ 6.5 Hz, H-6b), 4.08 (1 H, H_e allyl), 3.89 (dt, 1 H, $J_{5,6a}$ = $J_{5,6b}$ 6.5 Hz, $J_{4,5}$ 1 Hz, H-5), 1.99, 2.06 and 2.16 (3 s, 9 H, 3 x OAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.4, 170.4, 170.2 (*C*OCH_3), 155.4 (carbonyl PNZ), 143.8 (aromatic quat.), 133.4 (-CH=, allyl), 128.1, 123.8 (aromatic CH), 118.0 (=CH_2, allyl), 70.8, 69.9, 66.8 (C-3, C-4, C-5), 70.3, 65.4, 61.4 (C-6, CH_2 allyl, CH_2 PNZ), 53.0 (C-2), 20.7 ($COCH_3$). HR-ESMS calcd for $C_{23}H_{28}N_2O_{12}Na$ ($M+Na^+$) 547.1540, found 547.1540.

Allyl 4,6-di-O-benzylidene-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside (**50**).

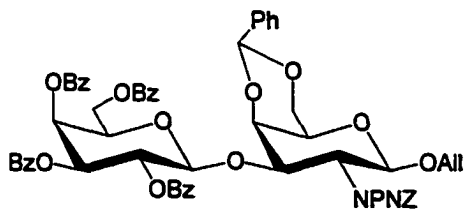


50

Allyl glycoside **49a** (400 mg, 0.763 mmol) was deacetylated in methanolic sodium methoxide. The reaction was neutralized with Amberlite IR-120(H+), filtered and the solvents were removed under reduced pressure. The resulting product was suspended in anhydrous CH_3CN (20 ml), a catalytic amount of camphorsulfonic acid was added, then benzaldehyde dimethyl acetal (130 µl, 0.866 mmol) was added via syringe. The reaction

mixture was stirred overnight. The reaction was quenched with solid $NaHCO_3$, concentrated, and chromatographed (6:1→3:1→2:1 toluene acetone) to yield **50** (267 mg, 92 % overall yield). ^1H NMR ($CDCl_3$): δ 7.18-8.15 (9 H, Ar*H*), 5.74 (1 H, H_c allyl), 5.49 (s, 1 H, PhC*H*O_2), 5.16 (1 H, H_b allyl), 5.04 (1 H, H_a allyl), 4.28 (1 H, H_d allyl), 4.21 (dd, 1 H, $J_{6a,6b}$ 12.5 Hz, $J_{5,6a}$ 1.5 Hz, H-6a), 4.10 (dd, 1 H, $J_{3,4}$ 3 Hz, $J_{4,5}$ 1 Hz, H-4), 4.01 (dd, 1 H, $J_{6a,6b}$ 12.5 Hz, $J_{5,6b}$ 1.5 Hz, H-6b), 3.97 (1 H, H_e allyl), 3.64-3.82 (H-5, OC*H*$_2$PhNO_2). ^{13}C NMR (75 Hz, CD_3OD): δ 156.8 (C=O, PNZ), 147.0, 144.2, 137.4 (aromatic quat.), 133.7 (-CH=, allyl), 128.6, 128.1, 127.7, 127.6, 127.3, 126.1, 126.0, 123.1 (aromatic CH), 116.2 (=CH_2, allyl), 100.9 (PhCH), 75.1, 74.5, 71.2, 69.8, 68.066.2 (C-3, C-4, C-5), 69.3, 68.7, 64.5, 60.8 (C-6, CH_2 allyl, CH_2 PNZ), 54.2 (C-2). HR-ESMS calcd for $C_{24}H_{26}N_2O_9Na$ ($M+Na^+$) 509.1536, found 509.1533.

Allyl 3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-4,6-di-O-benzylidene-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside (**52**).

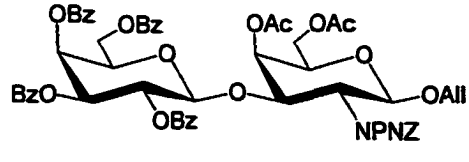


52

A flask containing donor **51** (385 mg, 0.520 mmol, 1.5 eq.), acceptor **50** (169 mg, 0.347 mmol) and 4 Å molecular sieves (650 mg) was purged with argon. 1,2-Dichloroethane (10 ml) was syringed in and reaction mixture was stirred for 15 minutes at room temperature. A solution of TMSOTf (0.4 M in toluene, 70 μl, 0.028 mmol) was added dropwise and stirring continued for 4 hours. The reaction was quenched with pyridine, and the reaction mixture was diluted with CH_2Cl_2, filtered through Celite and concentrated. Column

chromatography (20:1→10:1→6:1 toluene acetone) yielded the disaccharide **52** (306 mg, 83%). ^{1}H NMR ($CDCl_3$): δ 7.0-8.2 (29 H, Ar*H*), 5.88 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 5.74 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 7.5 Hz, H-2'), 5.64 (1 H, H_c allyl), 5.52 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 5.39 (s, 1 H, PhC*H*O_2), 5.08 (1 H, H_b allyl), 5.05 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 4.99 (d, 1 H, J_{gem} 13.5 Hz, OC*H*$_2$PhNO$_2$), 4.96 (1 H, H_a allyl), 4.64 (dd, 1 H, $J_{6a',6b'}$ 11.5 Hz, $J_{5',6a'}$ 7 Hz, H-6a'), 4.47 (d, 1 H, J_{gem} 13.5 Hz, OC*H*$_2$PhNO$_2$), 4.33 (dd, 1 H, $J_{6a',6b'}$ 11.5 Hz, $J_{5',6b'}$ 5 Hz, H-6b'), 4.27 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4), 4.22 (1 H, H_d allyl), 4.12 (broad d, 1 H, $J_{6a,6b}$ 12 Hz, H-6a), 3.88 (1 H, H_e allyl), 3.67 (broad d, 1 H, $J_{6a,6b}$ 12 Hz, H-6b), 3.18 (m, 1 H, H-5). ^{13}C NMR (75 Hz, $CDCl_3$): δ 166.1, 165.6, 165.5, 165.0 (*C*OPh), 155.2 (carbonyl PNZ), 147.5, 143.9 (aromatic quat. PNZ), 137.9 (-CH=, allyl), 134.5, 133.8, 133.6, 133.5, 133.4, 130.1, 129.8, 129.0, 128.7, 128.7, 128.6, 128.4, 128.3, 128.1, 127.6, 127.5, 126.2 (aromatic CH), 129.4, 129.3, 129.2, 129.0, 128.6 (aromatic quat.), 117.4 (=CH_2, allyl), 102.3, 102.1, 100.7 (C-1, C-1', Ph*C*H), 75.9, 73.8, 71.9, 71.4, 69.9, 68.2, 66.6 (C-3, C-4, C-5, C-2', C-3', C-4', C-5'), 70.0, 69.0, 64.7, 62.6, 62.3 (C-6, C-6', CH_2 allyl, CH_2 PNZ), 53.9 (C-2). HR-ESMS calcd for $C_{58}H_{52}N_2O_{18}Na$ ($M+Na^+$) 1087.3113, found 1087.3102.

Allyl 4,6-di-O-acetyl-3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside (**53**).

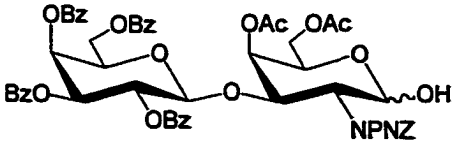


53

A solution of **52** (137 mg, 0.129 mmol) in 80% aqueous acetic acid was stirred at 80 °C for 1 hour. The reaction mixture was concentrated and

co-concentrated with toluene. The crude residue was stirred overnight in pyridine (10 ml) and acetic anhydride (8 ml). The reaction mixture was again concentrated and co-concentrated with toluene, followed by column chromatography (6:1 toluene:acetone) to yield **53** (125 mg, 91% overall yield). ^{1}H NMR ($CDCl_3$): δ 7.15-8.25 (29 H, Ar*H*), 5.92 (broad d, 1 H, $J_{3'4'}$ 3.5 Hz, H-4'), 5.72 (1 H, H_c allyl), 5.67 (dd, 1H, $J_{2'3'}$ 10.5 Hz, $J_{1'2'}$ 7.5 Hz, H-2'), 5.58 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.54 (dd, 1 H, $J_{2'3'}$ 10.5 Hz, $J_{3'4'}$ 3.5 Hz, H-3'), 5.17 (1 H, H_b allyl), 5.07 (1 H, H_a allyl), 4.98 (d, 1 H, J_{gem} 14 Hz, OC*H*$_2$PhNO$_2$), 4.92 (d, 1 H, $J_{1'2'}$ 7.5 Hz, H-1'), 4.67 (dd, 1 H, $J_{6a'6b'}$ 11.5 Hz, $J_{5'6a'}$ 6.5 Hz, H-6a'), 4.36 (dd, 1 H, $J_{6a'6b'}$ 11.5 Hz, $J_{5'6b'}$ 7 Hz, H-6b'), 4.21-4.30 (3 H, H_d allyl, H-5', OC*H*$_2$PhNO$_2$), 4.01-4.13 (2 H, H-6a, H-6b), 3.98 (1 H, H_e allyl), 3.77 (ddd, 1 H, $J_{5'6a'}$ 6.5 Hz, $J_{5'6b'}$ 7 Hz, H-5), 2.02 and 2.17 (2 s, 6 H, 2 x OAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.5, 169.7 (*C*OCH$_3$), 166.0, 165.6, 165.4, 165.0 (*C*OPh), 155.1 (carbonyl PNZ), 147.7, 143.8 (aromatic CH, PNZ), 133.6, 133.5, 133.3, 128.7, 128.5, 128.4, 128.3, 127.8, 123.7 (aromatic CH), 129.5, 129.4, 129.1, 128.7 (aromatic quat.), 117.8 (=CH$_2$, allyl), 101.7 (C-1'), 71.4, 71.3, 70.3, 69.1, 67.8 (C-3, C-4, C-5, C-2', C-3', C-4', C-5'), 70.2, 64.9, 62.5, 61.8 (C-6, C-6', CH$_2$ allyl, CH$_2$ PNZ), 54.9 (C-2), 20.8, 20.7 (COC*H*$_3$). HR-ESMS calcd for $C_{55}H_{52}N_2O_{20}Na$ (M+Na$^+$) 1083.3011, found 1083.3010.

*4,6-di-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl)-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranose* (**54**).

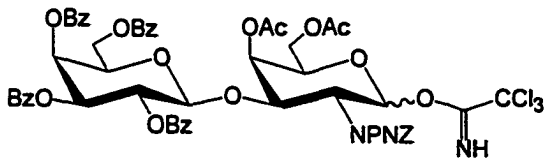


54

Disaccharide **53** (61.5 mg, 0.058 mmol) was dissolved in methanol (6 ml), then $PdCl_2$ (5.6 mg,

0.032 mmol) was added. Stirring continued for 3.5 hours, then solvent was removed in vacuo. The crude product was immediately charged on a silica gel column and chromatographed (3:1 toluene:acetone) to yield the reducing sugar **54** (41 mg, 69 % yield) which was carried directly on to the next step.

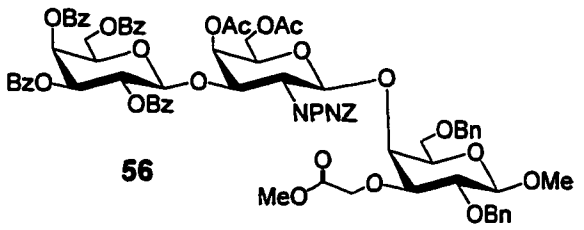
*O-[4,6-di-*O*-acetyl-3-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl)-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranosyl] trichloroacetimidate* (**55**).



55

A solution of **54** (35 mg, 0.035 mmol) and CCl_3CN (60 µl, 0.60 mmol) in dry CH_2Cl_2 with K_2CO_3 (57 mg, 0.41 mmol) was stirred at room temperature for 4 days. The reaction mixture was concentrated directly and the crude product was chromatographed (10:1→2:1 toluene:acetone) to yield the imidate donor **55** (17 mg, 43%). ^{1}H NMR ($CDCl_3$): δ 8.67 (NH imidate), 7.10-8.20 (24 H, ArH), 6.40 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 5.96 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 5.70-5.78 (2 H, H-2', H-4), 5.60 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 5.04 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1'), 4.91 (d, 1 H, J_{gem} 14 Hz, OCH_2PhNO_2), 4.78 (1 H, NH), 4.70 (dt, 1 H, H-2), 4.30-4.51 (H-5, H-6a, H-6b, OCH_2PhNO_2), 4.27 (broad t, H-5'), 4.20 (dd, 1 H, $J_{5',6a'}$ 6.5 Hz, $J_{6a',6b'}$ 11.5 Hz, H-6a'), 4.05 (dd, 1 H, $J_{3,4}$ 3 Hz, $J_{2,3}$11 Hz, H-3), 3.98 (dd, 1 H, $J_{5',6b'}$ 7 Hz, $J_{6a',6b'}$ 11.5 Hz, H-6b'), 2.20, 2.00 (2 s, 6 H, OAc).

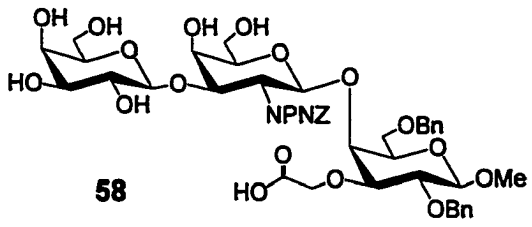
Methyl [4,6-di-O-acetyl-3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranosyl]-2,6-di-O-benzyl-3-O-(methoxycarbonyl)methyl-β-D-galactopyranoside (**56**).



56

A flask containing donor **55** (17 mg, 0.015 mmol), acceptor **32** (13 mg, 0.029 mmol) and 4 Å molecular sieves was purged with argon. 1,2-Dichloroethane (2 ml) was syringed in, the reaction mixture was stirred for 10 minutes, followed by the addition of TMSOTf (0.2M in toluene, 50 µl, 0.01 mmol). After 35 minutes the reaction was quenched with pyridine, and the reaction mixture was filtered through Celite and concentrated. Column chromatography (9:1→2:1 toluene:acetone) yielded the trisaccharide **56** (20.5 mg, 97%). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.6, 169.9 (*C*OCH_3), 166.0, 165.6, 165.5, 164.9 (*C*OPh), 133.6, 133.3, 133.3, 130.2, 129.8, 128.7, 128.5, 128.5, 128.3, 128.3, 128.0, 127.7, 127.6, 123.7 (aromatic CH), 138.3, 129.5, 129.1, 128.8 (aromatic quat.), 101.6 (C-1), 80.4, 73.5, 71.6, 71.4, 71.1, 70.1, 69.2, 67.8 (C-2, C-3, C-4, C-5, C-3', C-4', C-5', C-2", C-3", C-4", C-5"), 73.6, 69.6, 69.2, 62.3, 61.6 (C-6, C-6', C-6", *C*H_2COOMe, CH_2 PNZ), 57.0 (OMe), 54.4 (C-2'), 52.0 (COO*Me*), 20.9, 20.7 (CO*C*H_3). LR-ESMS calcd for $C_{76}H_{76}N_2O_{27}Na$ ($M+Na^+$) 1472.5, found 1472.5.

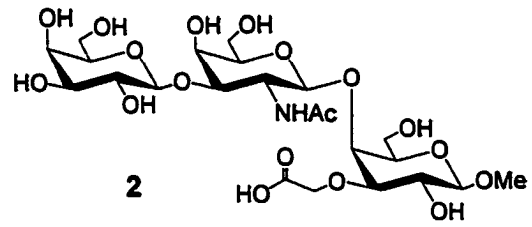
Methyl 2,6-di-O-benzyl-3-O-carboxymethyl-[2-deoxy-3-O-(β-D-galactopyranosyl)-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranosyl]-β-D-galactopyranoside (**58**).



58

Trisaccharide **56** (5.7 mg, 0.0039 mmol) was deacetylated in 0.4 M NaOMe in methanol for 24 hours, after which a few drops of H_2O were added and stirring was continued for another 24 hours. The reaction was neutralized with Amberlite IR-120(H+), filtered and evaporated. Column chromatography (65:34:1 $CHCl_3$:MeOH:H_2O, Iatrobeads) yielded **58** (3.5 mg, quant.). ^{1}H NMR (D_2O): δ 6.9-8.0 (14 H, ArH), 5.11 (2 H, CH_2 PNZ), 4.52 (s, 2 H, CH_2COOH), 4.13-4.38 (H-4, CH_2Ph), 4.03 (H-4), 3.81 (H-3), 3.76 (H-4), 3.44 (s, OMe). HR-ESMS calcd for $C_{43}H_{54}N_2O_{21}Na$ ($M+Na^+$) 957.3117, found 957.3110.

Methyl [2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-β-D-galactopyranosyl]-3-O-carboxymethyl-β-D-galactopyranoside (**2**).

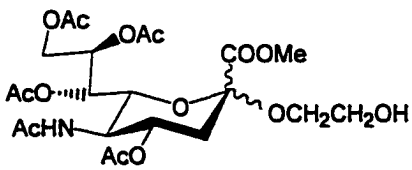


2

Trisaccharide **58** (3.5 mg, 0.0037 mmol) was dissolved in methanol (3 ml). $Pd(OH)_2$/C was added then Ac_2O (5 drops) and the reaction mixture was maintained under a flow of H_2 for 16 hours. The reaction was then filtered through a Millipore filter (0.22 μm) and evaporated. The residue was chromatographed (5:4:1 $CHCl_3$:MeOH:H_2O, Iatrobeads) to yield **2** (1.7 mg, 74%). ^{1}H NMR (D_2O): δ 4.63 (d, 1 H, $J_{1'2'}$ 8.5 Hz, H-1'), 4.48 (d, 1 H, $J_{1''2''}$ 7.5 Hz, H-1''), 4.33 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.31 (broad d, 1 H, $J_{3,4}$ 2.5

Hz, H-4), 4.15 (broad d, 1 H, $J_{3',4'}$ 3 Hz, H-4'), 4.14, 4.07 (d, 1 H, J_{gem} 15.5 Hz, OCH_2PhNO_2), 4.04 (dd, 1H, $J_{2',3'}$ 11 Hz, $J_{1',2'}$ 8.5 Hz, H-2'), 3.92 (broad d, 1 H, $J_{3'',4''}$ 3 Hz, H-4"), 3.86 (dd, 1 H, $J_{2',3'}$ 10 Hz, $J_{3',4'}$ 3 Hz, H-3'), 3.70-3.84 (H-6a, H-6b, H-6a', H-6a" , H-6b"), 3.64-3.74 (H-5, H-5', H-5"), 3.64 (dd, 1 H, $J_{2'',3''}$ 8 Hz, $J_{3'',4''}$ 3 Hz, H-3"), 3.56 (dd, H-6b'), 3.55 (s, OMe), 3.49-3.55 (2 H, H-2", H-3), 3.45 (dd, 1H, $J_{2,3}$ 10 Hz, $J_{1,2}$ 7.5 Hz, H-2), 2.05 (s, 3 H, NHAc). ^{13}C NMR (125 Hz, D_2O): δ 178.9 (C=O), 105.5, 104.3, 102.8 (C-1, C-1', C-1"), 82.0, 80.7, 75.6, 75.3, 74.9, 73.7, 73.2, 71.3, 70.8, 69.6, 69.3, 68.8, 61.7, 61.3 (C-2, C-3, C-4, C-5, C-6, C-3', C-4', C-5', C-6', C-2", C-3", C-4", C-5", C-6", CH_2COOH), 57.6 (OMe), 52.1 (C-2'), 23.1 ($COCH_3$). HR-ESMS calcd for $C_{23}H_{39}N_2O_{18}Na$ ($M+Na^+$) 640.2065, found 640.2068.

2-hydroxyethyl 2-O-[methyl (5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosyl)onate] (**59**).

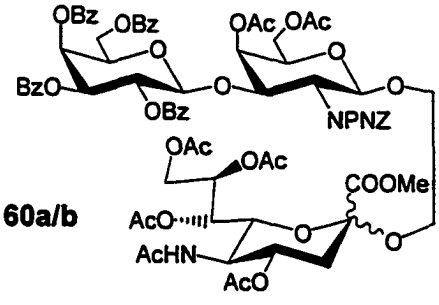


59a/b

A solution of xanthate donor **25**, (19 mg, 0.032 mmol) and anhydrous ethylene glycol (50 µl, 0.90 mmol) in dry CH_3CN (1 ml) was stirred over AW 300 molecular sieves (200 mg) under argon atmosphere. The reaction mixture was cooled to 0 °C, DMTST (20 mg, 0.077 mmol) was added and stirring was continued for 30 minutes. Triethylamine was added to quench the reaction, followed by filtration through Celite and concentration and co- concentration with toluene. Column chromatography (2:1 toluene:acetone) yielded the product **59a/b** (10

mg, 58%) as an approximately 1:1 mixture of α- and β-anomers. ^{1}H NMR ($CDCl_3$): δ 2.60 (H-3a, α-anomer), 2.47 (H-3a, β-anomer).

2-[methyl (5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosyl)onate]oxyethyl 3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-4,6-di-O-acetyl-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside (**60a**).

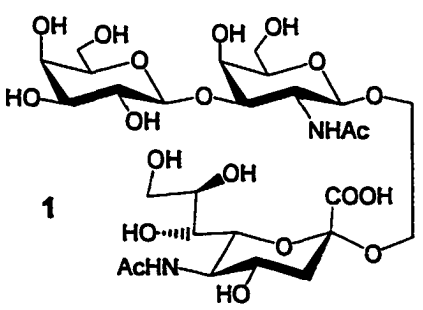


60a/b

A solution of the α/β mixture **59a/b** (50 mg, 0.093 mmol) and the thioglycoside donor **80** (110 mg, 0.103 mmol) in anhydrous CH_2Cl_2 (2 ml) was stirred in the presence of AW 300 molecular sieves (350 mg) under argon atmosphere. NIS (30 mg, 0.13 mmol) and AgOTf (5 mg, 0.02 mmol) in dry CH_3CN was added via syringe to this mixture, and stirring continued for 15 minutes, followed by dilution with CH_2Cl_2 and filtration through Celite. The organic layer was washed with saturated sodium thiosulfate solution and brine, then dried over Na_2SO_4 and concentrated. Column chromatography (3:1→2:1→1:1 toluene:acetone) yielded the α-sialoside **60a** (61 mg, 42%) and the β-sialoside **60b** (33 mg, 23%). Alpha anomer **60a**: ^{1}H NMR ($CDCl_3$): δ 7.1-8.3 (24 H, ArH), 5.91 (broad d, 1 H, $J_{3''4''}$ 3 Hz, H-4"), 5.67 (dd, 1 H, $J_{2''3''}$ 10.5 Hz, $J_{1''2''}$ 8 Hz, H-2"), 5.58 (broad d, 1 H, $J_{3'4'}$ 3.5 Hz, H-4'), 5.53 (dd, 1 H, $J_{2''3''}$ 10.5 Hz, $J_{3''4''}$ 3 Hz, H-3"), 5.42 (d, 1 H, $J_{1'2'}$ 8 Hz, H-1'), 5.34 (ddd, 1 H, $J_{7,8}$ 9 Hz, $J_{8,9a}$ 3 Hz, $J_{8,9b}$ 5.5 Hz, H-8), 5.30 (broad d, 1 H, $J_{7,8}$ 9 Hz, H-7), 5.14 (d, 1 H, $J_{2,NH}$ 10 Hz, NH), 4.95 (broad d, 1 H, $J_{1''2''}$ 8 Hz, H-1"), 4.81 (m, 1 H, H-4), 4.68 (dd, 1 H, $J_{5''6a''}$ 6 Hz, $J_{6a''6b''}$ 11 Hz, H-6a"), 4.33 (dd, 1

H, $J_{5''6b''}$ 7 Hz, $J_{6a''6b''}$ 11 Hz, H-6b''), 4.28 (dd, 1 H, $J_{8,9a}$ 3 Hz, $J_{9a,9b}$ 12.5 Hz, H-9a), 4.23 (broad t, 1 H, $J_{4''5''}$ 1 Hz, $J_{5''6a''}$ 6 Hz, $J_{5''6b''}$ 7 Hz, H-5''), 4.02-4.13 (4 H, H-5, H-6, H-6a', H-6b'), 4.00 (dd, 1 H, $J_{8,9b}$ 5.5 Hz, $J_{9a,9b}$ 12.5 Hz, H-9b), 3.90 (m, 1 H, OCH_2CH_2O), 3.80 (broad t, 1 H, $J_{5'6a'}$ 6 Hz, $J_{5'6b'}$ 7 Hz, H-5'), 3.72 (s, 3 H, OMe), 3.25-3.65 (broad, 3 H, OCH_2CH_2O), 2.50 (dd, 1 H, $J_{3e,4}$ 4.5 Hz, $J_{3e,3a}$ 12.5 Hz, H-3e), 1.75, 1.84, 1.99, 2.00, 2.05, 2.11 and 2.19 (7 s, 21 H, 6 x OAc, NHAc), 1.99 (t, 1 H, $J_{3a,4}=J_{3e,3a}$ 12.5 Hz, H-3a). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.9, 170.8, 170.5, 170.3, 170.1, 169.7, 168.1 ($COCH_3$), 166.0, 165.6, 165.4, 164.9 (COPh), 155.3 (carbonyl PNZ), 147.5, 144.2 (aromatic quat. PNZ), 133.6, 133.3, 133.1, 130.1, 129.8, 129.7, 128.7, 128.5, 128.4, 128.3, 127.4 (aromatic CH), 129.4, 129.1, 128.7 (aromatic quat.), 101.6 (C-1'), 98.8 (C-2), 72.6, 71.5, 71.2, 71.1, 70.2, 69.1, 68.9, 68.4, 67.8, 67.4 (C-2'', C-3', C-3'', C-4, C-4, C-4'', C-5', C-5'', C-6, C-7, C-8), 68.1, 64.8, 62.5, 62.4, 61.7 (C-6', C-6'', CH_2 PNZ, OCH_2CH_2OH, OCH_2CH_2OH), 54.5 (OMe), 52.9 , 49.4 (C-2', C-5), 37.7 (C-3), 23.2, 21.1, 20.8, 20.8 ($COCH_3$). HR-ESMS calcd for $C_{74}H_{79}N_3O_{33}Na$ ($M+Na^+$) 1560.4494, found 1560.4489.

2-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic acid)oxyethyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-β-D-galactopyranoside (**1**).

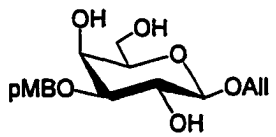


1

The α-sialoside **60a** (43 mg, 0.028 mmol) was stirred in 0.01 M sodium methoxide in methanol (10 ml) for 2 hours. At this point, H_2O (0.5 ml) was added, and stirring continued for 8 hours. The solution was neutralized with Amberlite IR-120(H+) ion exchange

resin, filtered, and concentrated. The crude product in ethanol (30 ml) was hydrogenolyzed over 20% $Pd(OH)_2/C$ overnight. The catalyst was removed by filtration through a Millipore filter (0.22 μm), solvents were removed under reduced pressure, and the residue was chromatographed on an Iatrobead column (65:34:1→5:4:1 CH_2Cl_2:MeOH:H_2O) to yield the methyl ester (11 mg, 52% yield). The ester was saponified in 0.1 M NaOH to yield the deprotected product **1**. HR-ESMS calcd for $C_{27}H_{46}N_2O_{20}Na$ ($M+Na^+$) 741.2542, found 741.2534.

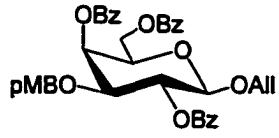
Allyl 3-O-(4-methoxybenzyl)-β-D-galactopyranoside (**62**).



62

Allyl galactoside **61** (165 mg, 0.754 mmol) and dibutyltin oxide (189 mg, 0.759 mmol) were refluxed in toluene (50 ml) with azeotropic removal of water. After 14 hours, the reaction was cooled to 60 °C, Bu_4NI (290 mg, 0.785 mmol) and 4-methoxybenzyl chloride (150 μl, 1.11 mmol) were added, and stirring continued at 80 °C for 24 hours. Upon removal of solvent under reduced pressure, column chromatography (2:1 toluene:acetone) yielded **62** (165 mg, 64%). ^1H NMR (CD_3OD): δ 7.31 (d, 2 H, J_{ortho}, 9 Hz, ArC*H*), 6.83 (d, 2 H, J_{ortho}, 9 Hz, ArC*H*), 5.95 (1 H, H_c allyl), 5.31 (1 H, H_b allyl), 5.14 (1 H, H_a allyl), 4.68, 4.58 (d, 1 H, J_{gem} 11.5, PhCH_2), 4.36 (1 H, H_d allyl), 4.26 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.13 (1 H, H_e allyl), 3.99 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1 Hz, H-4), 3.68-3.79 (m, 2 H, H-6a, H-6b), 3.66 (dd, 1H, $J_{2,3}$ 9.5 Hz, $J_{1,2}$ 8 Hz, H-2), 3.41 (dt, 1 H, $J_{4,5}$ 1 Hz, $J_{5,6a}$=$J_{5,6b}$ 6.5 Hz, H-5), 3.34 (dd, 1 H, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 3.5 Hz, H-3).

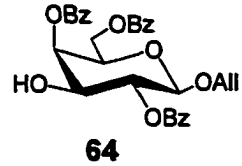
Allyl 2,4,6-tri-O-benzoyl-3-O-(4-methoxybenzyl)-β-D-galactopyranoside(**64**).



63

p-Methoxybenzyl ether **63** (79 mg, 0.232 mmol) was stirred in pyridine (5 ml), cooled to 0 °C, then benzoyl chloride (100 µl, 0.861 mmol) was added dropwise. Stirring continued at room temperature for 10 hours, then reaction quenched with the addition of methanol. Evaporation of the reaction mixture and column chromatography (19:1 toluene:acetone) yielded product **64** (138 mg, 91%). ^{1}H NMR ($CDCl_3$): δ 7.40-8.25 (15 H, 3 x OBz), 7.05 (d, 2 H, J_{ortho}, 9 Hz, ArC*H*), 6.60 (d, 2 H, J_{ortho}, 9 Hz, ArC*H*), 5.90 (broad m, 1 H, H-4), 5.78 (1 H, H_c allyl), 5.54 (ddd, 1 H, H-2), 5.18 (1 H, H_b allyl), 5.07 (1 H, H_a allyl), 4.65 (dd, 1 H, H-1), 4.63 (d, 1 H, J_{gem} 12.5, PhCH_2), 4.62 (ddd, 1 H, H-6a), 4.46 (ddd, 1 H, H-6b), 4.44 (d, 1 H, J_{gem} 12.5, PhCH_2), 4.35 (1 H, H_d allyl), 4.12 (1 H, H_e allyl), 4.06 (broad t, 1 H, H-5), 3.78 (ddd, 1 H, H-3), 3.71 (s, 3 H, OMe)

*Allyl 2,4,6-tri-*O*-benzoyl-β-D-galactopyranoside* (**64**).

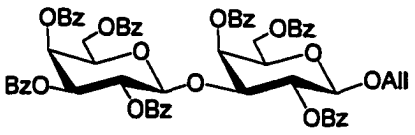


64

Compound **63** (135 mg, 0.207 mmol) and CAN (346 mg, 0.631 mmol) were stirred in CH_3CN:H_2O (9:1, 5 ml) for 30 minutes. The reaction mixture was diluted with CH_2Cl_2, washed with H_2O, brine and dried over Na_2SO_4. After evaporation of the reaction mixture, column chromatography (19:1 toluene:acetone) yielded **64** (107 mg, 97%). ^{1}H NMR ($CDCl_3$): δ 7.4-8.2 (15 H, 3 x OBz), 5.86 (1 H, H_c allyl), 5.77 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1 Hz, H-4), 5.40 (dd, 1H, $J_{2,3}$ 10 Hz, $J_{1,2}$ 8 Hz, H-2), 5.25 (1 H, H_b allyl), 5.15 (1 H, H_a allyl), 4.77 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.60 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, $J_{5,6a}$ 7 Hz, H-6a), 4.43 (dd, 1 H, $J_{6a,6b}$

11.5 Hz, $J_{5,6b}$ 6 Hz, H-6b), 4.39 (1 H, H_d allyl), 4.19 (1 H, H_e allyl), 4.12-4.17 (2 H, H-5, H-3).

Allyl 2,4,6-tri-O-benzoyl-3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-β-D-galactopyranoside (**66**).

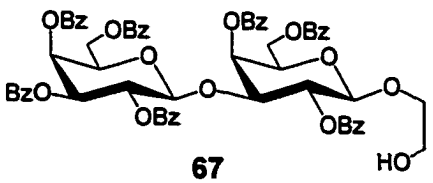


66

A solution of the benzoylated imidate donor (237 mg, 0.320 mmol) and allyl glycoside **64** (108 mg, 0.201 mmol) in anhydrous dichloroethane (5 ml) with 4 Å molecular sieves (550 mg) was stirred under argon atmosphere for 45 minutes. The reaction mixture was cooled to –20 °C, after which TMSOTf (0.25 M in toluene, 100 µl, 0.025 mmol) was added. Stirring continued for two hours, while reaction flask warmed to room temperature. TLC indicated unreacted acceptor, so more donor (97 mg, 0.131 mmol) in 2 ml dichloroethane was added via syringe. Stirring continued for 2 hours, then reaction quenched with a few drops of pyridine. Reaction mixture was diluted with CH_2Cl_2, filtered through Celite and solvent was removed *in vacuo*. Column chromatography (40:1→10:1 toluene:acetone) yielded disaccharide with some impurity, as well as unreacted acceptor. Product mixture was acetylated in pyridine/acetic anhydride and chromatographed again on 40:1 toluene:acetone to yield pure disaccharide **66** (113 mg, 70% coupling yield based on recovered acceptor). ^{1}H NMR ($CDCl_3$): δ 7.0-8.3 (35 H, 7 x OBz), 6.01 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1 Hz, H-4), 5.87 (dd, 1 H, $J_{3',4'}$ 3.5 Hz, $J_{4',5'}$ 1 Hz, H-4'), 5.68 (1 H, H_c allyl), 5.64 (dd, 1H, $J_{2,3}$ 10 Hz, $J_{1,2}$ 8 Hz, H-2), 5.58 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 7.5 Hz, H-2'), 5.38 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 5.11

(1 H, H_b allyl), 5.02 (1 H, H_a allyl), 5.00 (d, 1 H, $J_{1'2'}$ 7.5 Hz, H-1'), 4.70 (dd, 1 H, $J_{6a'6b'}$ 11 Hz, $J_{6a'5}$ 6 Hz, H-6a'), 4.64 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.47-4.58 (m, 2 H, H-6a, H-6b), 4.21-4.35 (4 H, H-6b', H-5', H-3, H_d allyl), 4.03-4.12 (2 H, H-5, H_e allyl). ^{13}C NMR (75 Hz, $CDCl_3$): δ 166.2, 166.0, 165.9, 165.5, 165.5, 164.6, 164.5 (C=O), 133.5, 133.5, 133.3, 133.2, 133.1, 132.6, 130.2, 130.1, 129.8, 129.8, 129.8, 129.5, 129.4, 128.6, 128.6, 128.5, 128.4, 128.4, 128.2, 128.0 (aromatic CH), 129.8, 129.4, 129.0, 129.0, 128.6 (aromatic quat.), 117.7 (=CH_2, allyl), 101.5, 100.0 (C-1, C-1'), 77.2, 71.9, 71.5, 71.3, 71.3, 70.3, 69.6, 67.7 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 69.8 (CH_2 allyl), 63.0, 61.8 (C-6, C-6'). HR-ESMS calcd for $C_{64}H_{54}O_{18}Na$ ($M+Na^+$) 1133.3208, found 1133.3208.

2-hydroxyethyl 2,4,6-tri-O-benzoyl-3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-β-D-galactopyranoside (**67**).

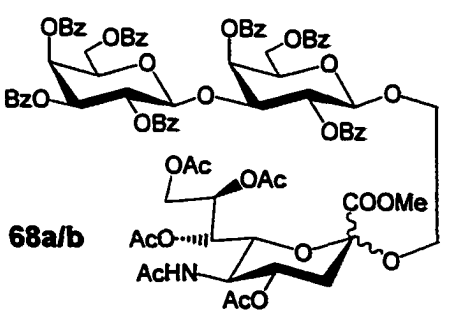


67

A solution of allyl glycoside **66** (60 mg, 0.054 mmol) in CH_2Cl_2 (15 ml) was cooled to –78 °C then ozone was bubbled through the solution until a blue colour persisted, then dimethyl sulfide (drops) were added to quench the reaction (colour disappeared). The solvent was removed in vacuo and the residue was dissolved in methanol (10 ml) and the reaction mixture was acidified with glacial acetic acid to pH 4. Sodium cyanoborohydride (25 mg, 0.239 mmol) was added and stirring continued overnight at room temperature. The reaction was concentrated and column chromatography of the residue (9:1 toluene:acetone) yielded **67** (38 mg, 63%). ^{1}H NMR ($CDCl_3$): δ 6.95-8.25 (35 H, 7 x OBz), 6.03 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1 Hz, H-4), 5.88 (dd,

1 H, $J_{3',4'}$ 3.5 Hz, $J_{4',5'}$ 1 Hz, H-4'), 5.63 (dd, 1H, $J_{2,3}$ 10 Hz, $J_{1,2}$ 8 Hz, H-2), 5.58 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 7.5 Hz, H-2'), 5.40 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 5.02 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1'), 4.72 (dd, 1 H, $J_{6a',6b'}$ 10.5 Hz, $J_{5',6a'}$ 6 Hz, H-6a'), 4.64 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.59 (dd, 1 H, $J_{6a,6b}$ 12 Hz, $J_{5,6a}$ 4.5 Hz, H-6a), 4.47 (dd, 1 H, $J_{6a,6b}$ 12 Hz, $J_{5,6b}$ 8.5 Hz, H-6b), 4.25-4.37 (3 H, H-3, H-5', H-6b'), 4.03 (ddd, 1 H, $J_{4,5}$ 1 Hz, $J_{5,6a}$ 4.5 Hz, $J_{5,6b}$ 8.5 Hz, H-5), 3.82 (dt, 1 H, J_{gem} 11.5 Hz, J_{vic} 4.5 Hz, OC*H*$_2$CH$_2$OH), 3.73 (dt, 1 H, J_{gem} 11.5 Hz, J_{vic} 4.5 Hz, OC*H*$_2$CH$_2$OH), 3.61 (t, 2 H, J_{vic} 4.5 Hz, OCH$_2$C*H*$_2$OH). ^{13}C NMR (75 Hz, $CDCl_3$): δ 166.2, 166.0, 165.8, 165.5, 165.4, 164.8, 164.5 (C=O), 133.4, 133.3, 133.2, 132.6, 130.2, 130.0, 129.8, 129.8, 129.7, 129.5, 129.4, 128.6, 129.6, 128.4, 128.2, 128.0 (aromatic CH), 129.7, 129.2, 128.9, 128.6 (aromatic quat.), 102.0, 101.6 (C-1, C-1'), 77.2, 72.2, 71.5, 71.4, 71.3, 70.3, 69.6 (C-2, C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 72.6, 63.2, 61.9, 61.8 (C-6, C-6', ROCH$_2$CH$_2$OH, ROCH$_2$CH$_2$OH). HR-ESMS calcd for $C_{63}H_{54}O_{19}Na$ ($M+Na^+$) 1137.3157, found 1137.3158.

2-[methyl (5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosyl)onate]oxyethyl 2,4,6-tri-O-benzoyl-3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-β-D-galactopyranoside (**68a**).



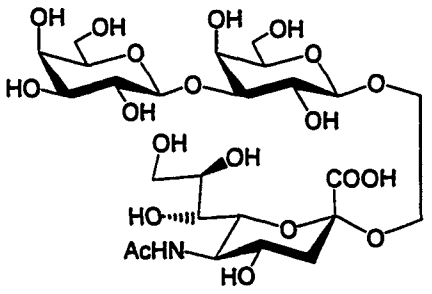
68a/b

Xanthate donor **25** (50 mg, 0.084 mmol), acceptor **67** (38 mg, 0.034 mmol) and AW 300 molecular sieves (250 mg) were placed in a flask. CH_3CN (2 ml) was added via syringe and mixture was stirred under argon for 15 minutes at room

temperature. DMTST (27 mg, 0.10 mmol) was added and stirring continued for 2.5 hours at room temperature. TLC of the reaction mixture indicated the presence of unreacted donor and acceptor, so a further 20 mg of DMTST (0.078 mmol) was added and stirring continued for 20 hours at room temperature. The reaction was quenched with Et_2NH, diluted with CH_2Cl_2, filtered through Celite and concentrated. Column chromatography of the residue (3:1→2:1 toluene:acetone) failed to completely purify the trisaccharide product. A second attempt at purification was made using 1:2 toluene:EtOAc. This yielded the β product **68b**, 15 mg (28%) and the α product **68a**, 8 mg (15%). Alpha sialoside **68a**: ^{1}H NMR ($CDCl_3$): δ 7.00-8.25 (35 H, Ar*H*), 6.00 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 5.84 (broad d, 1 H, $J_{3'',4''}$ 3.5 Hz, H-4''), 5.58 (dd, 1H, $J_{2',3'}$ 10 Hz, $J_{1',2'}$ 8 Hz, H-2'), 5.54 (dd, 1H, $J_{2'',3''}$ 10.5 Hz, $J_{1'',2''}$ 8 Hz, H-2''), 5.35 (dd, 1H, $J_{2'',3''}$ 10.5 Hz, $J_{3'',4''}$ 3.5 Hz, H-3''), 5.28 (ddd, 1 H, $J_{7,8}$ 8.5 Hz, $J_{8,9a}$ 2.5 Hz, $J_{8,9b}$ 5.5 Hz, H-8), 5.24 (dd, 1 H, $J_{7,8}$ 8.5 Hz, $J_{6,7}$ 1.5 Hz, H-7), 5.01 (1 H, $J_{5,NH}$ 9 Hz, NH), 4.97 (d, 1 H, $J_{1'',2''}$ 8 Hz, H-1''), 4.72 (m, 1 H, H-4), 4.69 (dd, 1 H, $J_{6a'',6b''}$ 11 Hz, $J_{6a'',5''}$ 6.5 Hz, H-6a''), 4.64 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 4.52 (dd, 1 H, $J_{6a',6b'}$ 11.5 Hz, $J_{5',6a'}$ 7 Hz, H-6a'), 4.44 (dd, 1 H, $J_{6a',6b'}$ 11.5 Hz, $J_{5',6b'}$ 5.5 Hz, H-6b'), 4.29 (dd, 1 H, $J_{6a'',6b''}$ 11 Hz, $J_{6b'',5''}$ 7 Hz, H-6b''), 4.19-4.28 (3 H, H-3', H-5'', H-9a), 4.07 (1 H, H-5'), 4.04 (dd, 1 H, $J_{8,9a}$ 5.5 Hz, $J_{9a,9b}$ 12.5 Hz, H-9b), 3.85-3.97 (H-5, H-6), 3.78 (m, 1 H, OCH_2CH_2O), 3.54-3.61 (5 H, OMe, OCH_2CH_2O), 3.29 (m, 1 H, OCH_2CH_2O), 2.22 (dd, 1 H, $J_{3e,4}$ 4.5 Hz, $J_{3e,3a}$ 12.5 Hz, H-3e), 2.08, 2.04, 1.98 and 1.84 (4s, 15 H, OAc, NHAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 171.6, 171.3, 170.8, 170.8, 170.6, 168.8, 166.6, 166.5, 166.2, 166.1, 165.1, 165.1 (C=O), 134.1, 134.0, 133.9, 133.8, 133.8, 133.5, 133.2 (aromatic quat.), 130.9, 130.7, 130.6, 130.5, 130.4, 130.3, 130.2, 130.1, 130.1, 129.7, 129.6, 129.3, 129.3, 129.2, 129.2,

129.2, 129.1, 129.0, 128.9, 128.6 (aromatic CH), 102.2, 102.0, 99.2 (C-2, C-1', C-1"), 73.1, 72.5, 72.2, 71.9, 71.8, 71.1, 70.3, 69.6, 69.1, 68.8, 68.3, 67.7, 64.3, 63.5, 63.0, 62.3 (C-2', C-2", C-3', C-3", C-4, C-4', C-4", C-5', C-5", C-6, C-6', C-6", C-7, C-8, CH_2CH_2), 53.3 (OMe), 50.1 (C-5), 38.1 (C-3), 23.9, 21.8, 21.7, 21.5, 21.5, 21.4 ($COCH_3$). LR-ESMS calcd for $C_{83}H_{81}O_{31}Na$ ($M+Na^+$) 1610.5, found 1610.5.

2-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic acid)oxyethyl 3-O-(β-D-galactopyranosyl)-β-D-galactopyranoside (**4**).

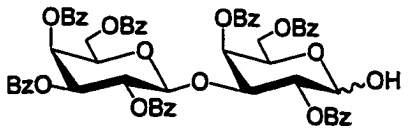


4

The trisaccharide **68a** was deprotected using the same method as for **27a**. The residue obtained upon removal of the solvents under reduced pressure was subjected to column chromatography (65:34:1→5:4:1 $CHCl_3$:MeOH: H_2O, Iatrobeads) to yield 1 mg of the deprotected trisaccharide. Electrospray mass analysis indicated the presence of the methyl ester. Residue was dissolved in H_2O/MeOH (1:1, 5 ml) and a few drops of 0.2 M NaOMe in MeOH were added and stirring was continued overnight. The reaction was neutralized using Amberlite IR-120(H+), filtered and evaporated to yield the acid **4** in quantitative yield. ^1H NMR (D_2O): δ 4.64, 4.52 (d, 1 H, H-1', H-1"), 4.22 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 4.08 (m, 1 H, H-8), 3.95 (broad d, 1 H, H-4"), 3.58-3.95 (H-4, H-5, H-6, H-7, H-9a, H-9b, H-5', H-6a, H-6b, H-5", H-6a", H-6b", OCH_2CH_2O), 2.78 (dd, 1 H, $J_{3e,4}$ 4.5 Hz, $J_{3e,3a}$ 12.5 Hz, H-3e), 2.06 (s, 3 H, NHAc), 1.71 (t, 1 H, $J_{3a,4}=J_{3e,3a}$ 12.5 Hz, H-3a). ^{13}C NMR (75 Hz, D_2O): δ 105.1, 103.4, 101.4 (C-

1', C-1", C-2), 83.0, 75.9, 75.6, 73.5, 72.5, 71.9, 70.7, 69.4, 69.2, 69.1, 69.0, 63.5, 61.7, 61.7, 58.2 (C-2, C-2', C-3, C-3', C-4, C-4', C-4", C-5', C-5", C-6, C-6', C-6", C-7, C-8, C-9, CH_2CH_2), 52.7 (C-5), 41.1 (C-3), 22.9 ($COCH_3$). HR-ESMS calcd for $C_{25}H_{43}NO_{20}Na$ ($M+Na^+$) 700.2276, found 700.2282.

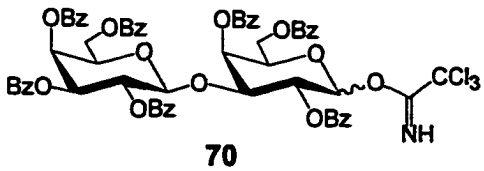
2,4,6-tri-O-*benzoyl-3*-O-*(2,3,4,6-tetra*-O-*benzoyl-β-D-galactopyranosyl)-D-galactopyranose* (**69**).



69

To a solution of allyl glycoside **66** (30 mg, 0.027 mmol) in MeOH (5 ml) was added $PdCl_2$ (5.5 mg, 0.031 mmol). Reaction mixture was stirred at room temperature for 1 hour. Very little product was observed by TLC, so more $PdCl_2$ (14 mg, 0.079 mmol) was added. After 1 hour reaction mixture was filtered through Celite and evaporated. Column chromatography (20:1 9:1 toluene:acetone) yielded the reducing sugar **69** (18 mg, 62%). ^1H NMR ($CDCl_3$): δ 6.8-8.2 (35 H, 7 x OBz), 6.09 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1 Hz, H-4), 5.93 (dd, 1 H, $J_{3',4'}$ 3.5 Hz, $J_{4',5'}$ 1 Hz, H-4'), 5.68 (d, 1 H, $J_{1,2}$ 4 Hz, H-1), 5.59 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 7.5 Hz, H-2'), 5.40-5.64 (2 H, H-2, H-3), 5.16 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1'), 4.72-4.88 (H-6a, H-6a'), 4.64 (H-5'), 4.25-4.55 (H-3, H-5, H-6b, H-6b').

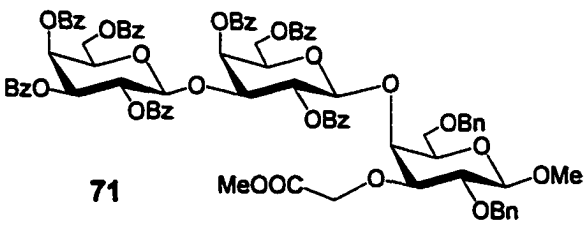
2,4,6-tri-O-*benzoyl-3*-O-*(2,3,4,6-tetra*-O-*benzoyl-β-D-galactopyranosyl)-β-D-galactopyranosyl trichloroacetimidate* (**70**).



70

A solution of the reducing sugar **69** (18 mg, 0.017 mmol), K_2CO_3, (38 mg, 0.28 mmol) and CCl_3CN (20 µl, 0.20 mmol) in CH_2Cl_2 (1 ml) was stirred at room temperature for 48 hours. Another 48 mg K_2CO_3 (0.35 mmol) and 50 µl CCl_3CN (0.50 mmol) were added. Stirring continued for 24 hours, then the reaction was filtered through Celite and evaporated. The crude product **70** (α/β mixture) was carried directly on to the next step.

Methyl 2,6-di-O-benzyl-4-O-[2,4,6-tri-O-benzoyl-3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-β-D-galactopyranosyl]-3-O-(methoxycarbonyl)methyl-β-D-galactopyranoside (**71**).

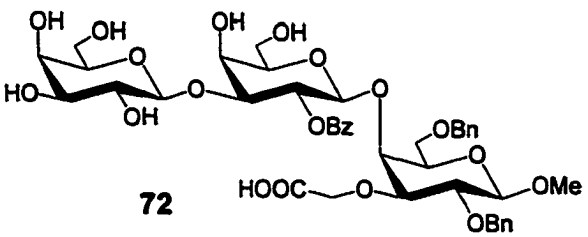


71

A flask containing the acceptor **32** (28 mg, 0.063 mmol) and 4 Å molecular sieves was flushed with argon. A solution of the crude imidate donor **70** in CH_2Cl_2 (1.5 ml) was added via syringe. After stirring at room temperature for 15 minutes, TMSOTf (20 ml, 0.1 M in toluene, 0.002 mmol) was added. After stirring at room temperature for 60 minutes, TLC shows presence of unreacted donor, so a further 70 µl of TMSOTf solution was added. After stirring for 20 minutes, the reaction was quenched with pyridine. The reaction mixture was then filtered through Celite and concentrated. The residue was immediately chromatographed (15:1 toluene acetone) to provide impure trisaccharide product. Further column chromatography (10:1

toluene:acetone) of the mixture yielded the pure trisaccharide **71** (8.2 mg, 8.7% isolated yield). ^{1}H NMR ($CDCl_3$): δ 6.95-8.25 (45 H, Ar*H*), 6.02 (dd, 1 H, $J_{3'4'}$ 3.5 Hz, $J_{4'5'}$ 1 Hz, H-4'), 5.90 (dd, 1 H, $J_{3''4''}$ 3.5 Hz, $J_{4''5''}$ 1 Hz, H-4''), 5.61 (dd, 1H, $J_{2''3''}$ 10.5 Hz, $J_{1''2''}$ 8 Hz, H-2''), 5.58 (dd, 1H, $J_{2'3'}$ 9.5 Hz, $J_{1'2'}$ 8 Hz, H-2'), 5.41 (dd, 1 H, $J_{2''3''}$ 10.5 Hz, $J_{3''4''}$ 3.5 Hz, H-3''), 5.24 (d, 1 H, $J_{1''2''}$ 8 Hz, H-1''), 5.06 (d, 1 H, $J_{1'2'}$ 8 Hz, H-1'), 4.76 (dd, 1 H, $J_{6a'6b'}$ 10.5 Hz, $J_{5'6a'}$ 6.5 Hz, H-6a'), 4.42-4.53 (H-3', H-6a'', H-6b'', PhCH_2 x 2), 4.39 (d, 1 H, J_{gem} 10.5, PhCH_2), 4.34 (dd, 1 H, $J_{6a''6b''}$ 11 Hz, $J_{6a''5''}$ 7 Hz, H-6b'') , 4.23-4.30 (2 H, H-4, H-5') , 4.08-4.14 (2 H, H-1, H-5''), 4.07 (d, 1 H, J_{gem} 17.5, CH_2COOMe), 3.98 (d, 1 H, J_{gem} 17.5, CH_2COOMe), 3.72-3.83 (2 H, H-6a, H-6b), 3.70 (s, 3 H, COOMe), 3.47-3.55 (5 H, OMe, PhCH_2, H-5).

Methyl 2,6-di-O-benzyl-4-O-[2-O-benzoyl-3-O-(β-D-galactopyranosyl)-β-D-galactopyranosyl]-3-O-carboxymethyl-β-D-galactopyranoside (**72**).

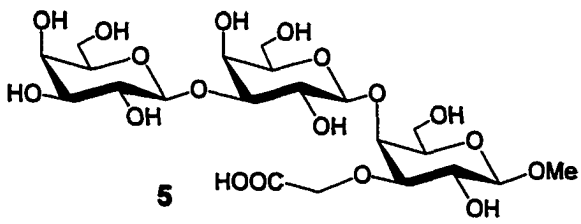


72

A solution of trisaccharide **71** (7.5 mg, 0.005 mmol) in 0.01 M sodium methoxide in methanol was stirred at room temperature for 2 days, after which a few drops of H_2O were added. Stirring was continued for another 24 hours, followed by neutralization using Amberlite IR-120(H+), filtration and evaporation of the solvents in vacuo. Column chromatography (65:34:1 $CHCl_3$:MeOH:H_2O, Iatro beads) yielded 3.6 mg of the monobenzoylated trisaccharide **72** in 84% yield. ^{1}H NMR (CD_3OD): δ 6.8-8.2 (15 H, OBz, 2 x OBn), 5.37 (dd, 1H, $J_{2'3'}$ 10 Hz, $J_{1'2'}$ 8 Hz, H-2'), 5.06 (d, 1 H, $J_{1'2'}$ 8 Hz,

H-1'), 4.57 (s, 2 H, PhCH_2), 4.31 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.25 (dd, 1 H, $J_{3,4}$ 2.5 Hz, $J_{4,5}$ 1 Hz, H-4), 4.20 (d, 1 H, J_{gem} 10.5, PhCH_2), 4.13 (dd, 1 H, $J_{3',4'}$ 3.5 Hz, $J_{4',5'}$ 1 Hz, H-4'), 4.02 (d, 1 H, $J_{1'',2''}$ 8 Hz, H-1''), 3.93-4.04 (3 H, H-3', CH_2COOH), 3.83 41 (dd, 1 H, $J_{2'',3''}$ 10.5 Hz, $J_{3'',4''}$ 3.5 Hz, H-3''), 3.52-3.76 (H2'', H-3, H-4'', H-5', H-5'', H-6a', H-6b', H-6a'', H-6b'', 3 x PhCH_2), 3.35-3.48 (2 H, H-2', H-5), 3.37 (s, 3 H, OMe), 3.28 (dd, 1 H, $J_{6a,6b}$ 10 Hz, $J_{5,6a}$ 3.5 Hz, H-6a), 3.21 (dd, 1 H, $J_{6a,6b}$ 10 Hz, $J_{5,6a}$ 3.5 Hz, H-6b), 3.13 (dd, 1H, $J_{2,3}$ 9.5 Hz, $J_{1,2}$ 8 Hz, H-2). ^{13}C NMR (75 Hz, CD_3OD): δ 167.6 (C=O), 142.6, 140.0, 132.1 (aromatic quat.), 134.1, 131.4, 129.4, 129.1, 128.9, 128.7, 128.6, 128.3 (aromatic CH), 106.3, 105.8, 103.3 (C-1, C-1', C-1''), 83.4, 81.6, 81.5, 77.1, 76.7, 75.9, 74.7, 74.5, 73.6, 72.4, 70.3, 70.2 (C-2, C-2', C-2'', C-3, C-3', C-3'', C-4, C-4', C-4'', C-5, C-5', C-5''), 75.6, 74.3, 71.3, 62.6, 62.2 (PhCH_2, CH_2COOH, C-6, C-6', C-6''), 57.0 (OMe). LR-ESMS calcd for $C_{56}H_{50}O_{18}Na$ ($M+Na^+$) 883.9, found 883.3.

Methyl 4-O-[3-O-(β-D-galactopyranosyl)-β-D-galactopyranosyl]-3-O-carboxymethyl-β-D-galactopyranoside (**5**).



5

The monobenzoylated trisaccharide **72** (2.5 mg, 0.003 mmol) was stirred in 0.04M methanolic sodium methoxide for 11 days. The reaction was worked up as for compound **72**. The residue obtained upon evaporation of the solvents was dissolved in methanol and stirred overnight under flow of H_2 in the presence of $Pd(OH)_2/C$. The reaction mixture was passed through a Millipore filter (0.22 μm) and evaporated.

Column chromatography (55:40:5 $CHCl_3$:MeOH:H_2O) yielded two products, the deprotected trisaccharide (0.7 mg) and the methyl ester (1.4 mg). The methyl ester product was saponified to yield the fully deprotected trisaccharide (1 mg). The two products were pooled to yield a total of 1.7 mg final product **5** (quantitative yield). ^{1}H NMR (D_2O): δ 4.70, 4.65 (d, 1 H, H-1', H-1"), 4.46 (broad d, 1 H, $J_{3,4}$ 2.5 Hz, H-4), 4.40 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.21 (d, 1 H, J_{gem} 17 Hz, CH_2COOH), 4.20 (broad d, 1 H, H-4"), 4.06 (d, 1 H, J_{gem} 17 Hz, CH_2COOH), 3.96 (broad d, 1 H, H-4'), 3.65-3.90 (H-2, H-2', H-2", H-3', H-3", H-5, H-5', H-5", H-6ab, H-6ab', H-6ab"), 3.55-3.65 (4 H, H-3, OMe). ^{13}C NMR (75 Hz, D_2O): δ 178.1 (C=O), 104.4, 103.9, 103.0 (C-1, C-1', C-1"), 82.2, 82.0, 75.1, 74.7, 74.4, 72.6, 71.8, 71.1, 70.6, 69.9, 68.7, 68.7, 68.6, 61.0, 60.7, 61.0 (C-2, C-2', C-2", C-3, C-3', C-3", C-4, C-4', C-4", C-5, C-5', C-5", C-6, C-6', C-6", CH_2COOH), 57.1 (OMe). HR-ESMS calcd for $C_{21}H_{36}O_{18}Na$ ($M+Na^+$) 599.1799, found 599.1811.

Chapter 3:

Binding Assays and Discussion of Assay Results

3.1 Introduction

Reproducible and reliable assays have been developed over the years in order to evaluate the ability of proteins to recognize and bind natural and unnatural ligands. One method of testing that has endured the test of time is the enzyme-linked immunosorbent assay, or ELISA [112]. This technique has been successfully implemented for the evaluation of inhibitors of cholera toxin (CT) and heat-labile enterotoxin (LT) [113]. Part A of this chapter will describe these enzyme-linked assays and their use in evaluating the inhibitory ability of mono-, di- and trisaccharide fragments of GM_1 synthesized in Chapter 2 of this thesis.

The Hindsgaul group has recently developed another procedure that can be applied to this problem, which employs frontal-affinity chromatography coupled with mass spectrometric detection (FAC-MS) [114]. This assay was employed to determine the dissociation constants of four trisaccharide GM_1 analogues. The assay and the results of the binding assays will be discussed in Part B of this chapter.

PART A:

3.2 Enzyme-Linked Assays

3.2.1 Introduction

The success of an enzyme-linked immunoassay relies on the ability of an antibody to recognize and bind to a specific antigen, and the harnessing of an enzyme's catalytic power to detect the antibody-antigen reaction. These two phenomena combine to provide a highly specific and sensitive method for determining the binding of a known receptor to a potential ligand.

The ELISA used in the present experiments measures the ability of a synthetic molecule to be recognized by the toxin, preventing subsequent toxin binding to a reference ligand. This is described as a competitive inhibition ELISA and can be used to provide information regarding the inhibitor's IC_{50} value, which is its concentration at 50% inhibition. The first step of the assay requires the preincubation of potential inhibitor with the toxin. This mixture is then added to microtiter wells containing immobilized GD_{1b} as the reference ligand. The ganglioside GD_{1b} was used instead of GM_1 because it is a weaker ligand than GM_1 by a factor of 11. This allows for the detection of more weakly binding ligands than a competitive inhibition assay using GM_1. The unattached toxin is washed away, and the amount of toxin remaining bound to the immobilized GD_{1b} is determined. For the LT assay, this determination is carried out indirectly through the

use of a primary antibody that recognizes the B-subunit of LT (anti-LTB) (Figure 3.1a). This step is followed by the addition of a horseradish peroxidase (HRP) conjugated secondary antibody, which had been raised against the first antibody [anti(anti-LTB)-HRP]. The HRP substrate *o*-phenylenediamine (OPD) is then added, furnishing a coloured product upon oxidation by HRP. The amount of colour produced can be correlated to the amount of toxin bound to the reference ligand, thus providing a measure of the inhibitory power of the test compounds, i.e. more colour = more toxin = poor inhibition. It should be noted here that the anti-CT primary antibody was employed in the LT assay since it is commercially available, and an anti-LT antibody is not.

For the CT assay, the quantitation of bound toxin is simplified by the use of the commercially available CT B-subunit which has been conjugated directly to horseradish-peroxidase (CTB-HRP) (Figure 3.1b). This obviates the need for intermediate antibodies, allowing the direct measurement of bound toxin with the OPD substrate. For this reason

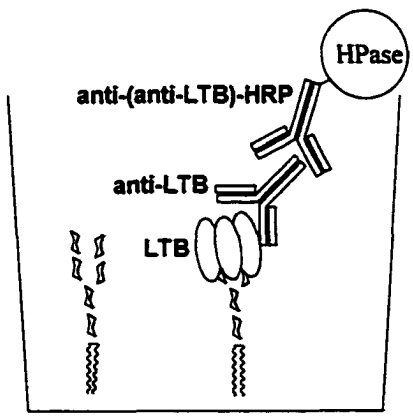


Figure 3.1a: Schematic diagram of LT ELISA

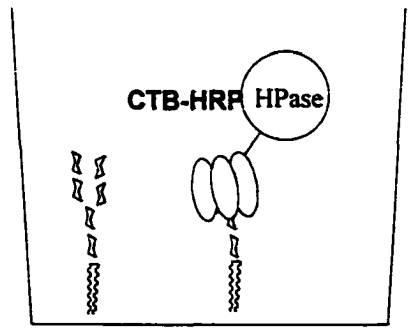


Figure 3.1b: Schematic diagram of CT DELA

the CT assay should not be referred to as an ELISA, and should instead be described as a direct enzyme-linked assay, or DELA.

3.2.2 Experimental for Enzyme-Linked Assays

Initial screening of inhibitors was undertaken in the labs of Dr Wim Hol at the University of Washington in Seattle, USA. The experimental procedure for these assays has been published [115]. Later testing of inhibitors was completed in the Hindsgaul labs at the University of Alberta and the modified experimental procedure used will be described in this chapter.

Commercially obtained materials included: C96 Maxisorp microtiter plates, Disialoganglioside GD_{1b} (Fluka), LT B-subunit (Sigma), rabbit anti-CT IgG (Sigma), CTB-HRP conjugate (Sigma), anti-rabbit IgG horseradish peroxidase conjugate (Sigma), bovine serum albumin (BSA, fraction V, 96%, Sigma). Phosphate-buffered saline (PBS, pH 7.2) contained 150 mM NaCl and 10 mM potassium phosphate. Optical densities were read at 450 nm on a Molecular Devices V-max ELISA microtiter plate reader. All error bars are reported as the standard deviation of the mean. Since the amounts of carbohydrate ligand used to make up the inhibitor solutions were quite small and errors in weighing such small masses are quite high, concentrations of the carbohydrate test solutions were quantified using a phenol-sulfuric acid spectrophotometric assay [116].

3.2.2.1 LT-ELISA

The samples to be tested for their inhibition are preincubated with 20 ng/ml LTB in 0.1% BSA in PBS at room temperature for 2 hours. As a reference, a concentration curve of toxin without inhibitor was also prepared using 0, 10, 20 and 30 ng/ml solutions of LTB. Microtiter plates were prepared for the assay by incubating each well with 100 µl of 2 µg/ml ganglioside GD_{1b} for 16 hours at 37 °C. Unattached ganglioside was removed by washing the wells twice with 200 µl PBS. Additional binding sites on the plastic surface were blocked with 200 µl of 1% BSA in PBS for 30 minutes at 37 °C, followed by washing 3 times with 200 µl PBS. The inhibitor solutions were added to the wells (100 µl each in triplicate or quadruplicate) and incubated at room temperature for 30 minutes, followed by washing 3 times with 200 µl PBS. The amount of LT-B remaining bound to GD_{1b} was established with the following sequence of steps: (a) addition of 100 µl anti-CT (1:10,000 dilution in 0.1% BSA in PBS) to each well, followed by incubation at room temperature for 1 hour, (b) washing 3 times with 200 µl 0.05% Tween-20 in PBS, (c) addition of 100 µl anti-rabbit IgG-HRP conjugate (1:20,000 dilution in 0.1% BSA and 0.05% Tween-20 in PBS), followed by incubation at 37 °C for 1 hour (d) washing 3 times with 200 µl 0.05% Tween-20 in PBS, (e) addition of a freshly made solution of 10 mg *o*-phenylenediamine in 5 ml 0.1 M sodium citrate, 5 ml citric acid and 4 µl 30% H_2O_2, followed by incubation at room temperature for 30 minutes, (f) measurement of the optical density (OD_{450}).

3.2.2.2 CT-DELA

The samples to be tested for their inhibition are preincubated with 20 ng/ml CTB-HRP in 0.1% BSA and 0.05% Tween-20 in PBS at room temperature for 2 hours. As a reference, a concentration curve of toxin without inhibitor was also prepared using 0, 10, 20 and 30 ng/ml solutions of CTB-HRP. Microtiter plates were prepared for the assay by incubating each well with 100 μl of 2 μg/ml ganglioside GD_{1b} for 16 hours at 37 °C. Unattached ganglioside was removed by washing the wells twice with 200 μl PBS. Additional binding sites on the plastic surface were blocked with 200 μl of 1% BSA in PBS for 30 minutes at 37 °C, followed by washing 3 times with 200 μl 0.05% Tween-20 in PBS. The inhibitor solutions were added to the wells (100 μl each in triplicate or quadruplicate) and incubated at room temperature for 30 minutes, followed by washing 3 times with 200 μl 0.05% Tween-20 in PBS. The amount of CTB-HRP bound to GD_{1b} was determined using the OPD method described in the LT-ELISA protocol.

3.2.3 Results of Enzyme-Linked Assays

Preliminary testing of GM_1 analogues was completed using the competitive ELISA assays. At this stage, only mono- and disaccharide fragments had been synthesized, with the exception of the flexible linker trisaccharide **1** and its β analogue (Figure 3.2).

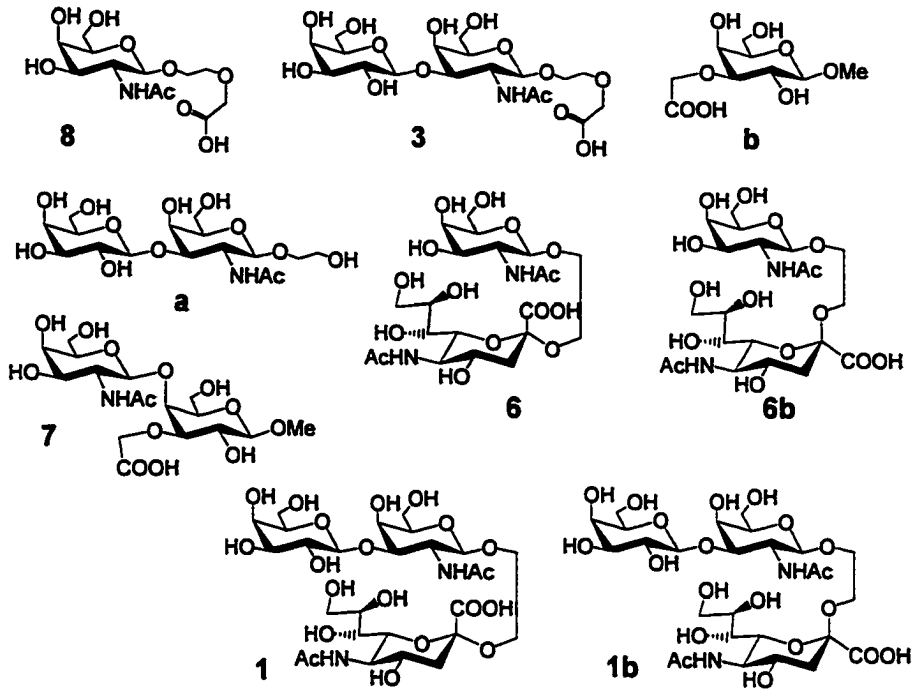


Figure 3.2: Molecules initially screened using LT-ELISA or CT-DELA

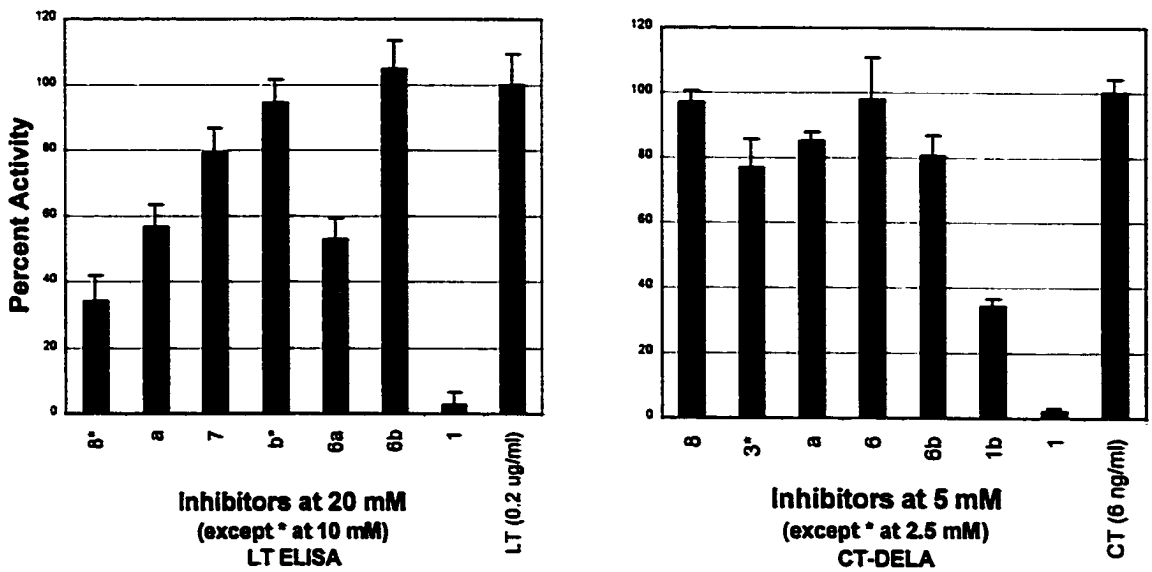


Figure 3.3: Summary of initial screening assays against LT and CT

Initial screening indicated that the mono- and disaccharides were at best only weak inhibitors, with IC_{50} values in the millimolar range (Figure 3.3). These values are comparable to the IC_{50} of galactose, which is approximately 30-40 mM.

The trisaccharide 1 was the only molecule that showed significant inhibition of both CT and LT at the millimolar concentrations used in the initial screening experiments. Further analysis of this molecule led to the determination of IC_{50} values in the micromolar range against both LT (Figure 3.4a) and CT (Figure 3.4b).

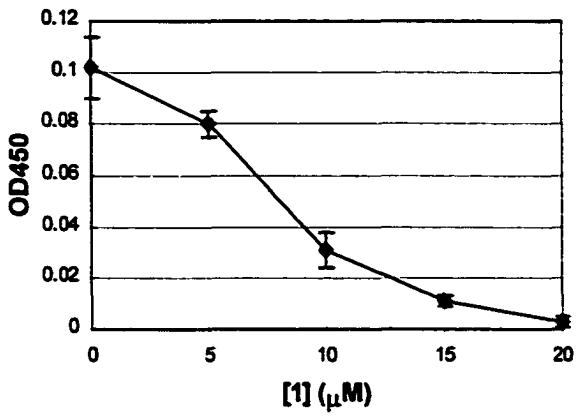


Figure 3.4a: Determination of IC_{50} of 1 against LT.

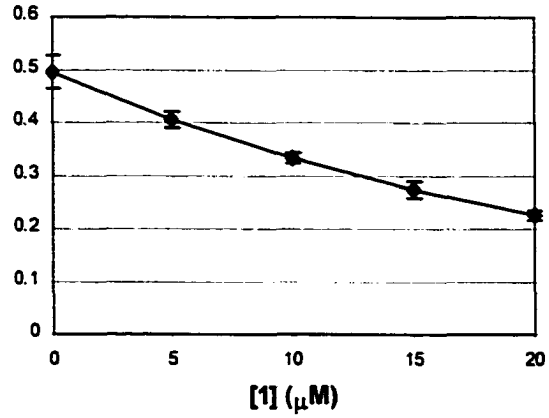


Figure 3.4b: Determination of IC_{50} of 1 against CT.

Another three GM_1 analogue trisaccharides were synthesized and tested in the Hindsgaul labs using the modified LT-ELISA and CT-DELA. These compounds included **4** (the galactose analogue of compound **1**), the carboxymethyl trisaccharide **2** and its galactose analogue **5** (Figure 3.5).

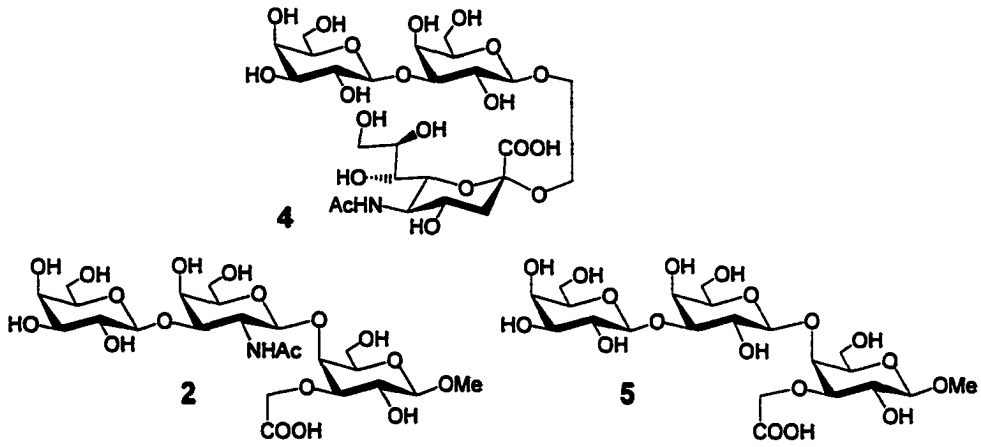


Figure 3.5: Trisaccharide GM_1 analogues

The competitive LT-ELISA was performed for all four trisaccharides **1**, **2**, **4** and **5** (Figure 3.6). Unfortunately, there were not enough of the weaker inhibitors **4** and **5** to perform an exact evaluation of the IC_{50} values, but general trends are observed and estimated IC_{50} values in the low millimolar range can be inferred. The data does suggest, however, that the acid fragment trisaccharides **4** and **5** are not much stronger binders than Galβ(1→3)GalNAcβOCH_2CH_2OH, which has an IC_{50} value of approximately 20 mM (Figure 3.3). Compound **4**, on the other hand, does prove to be a slightly stronger inhibitor than the acid fragment compounds, albeit the IC_{50} value appears to be around

275 μM, which is still significantly weaker than compound **1**. Results for **1** confirm the IC_{50} value obtained in the earlier assays (Figure 3.4a), with a determined value of approximately 8 μM.

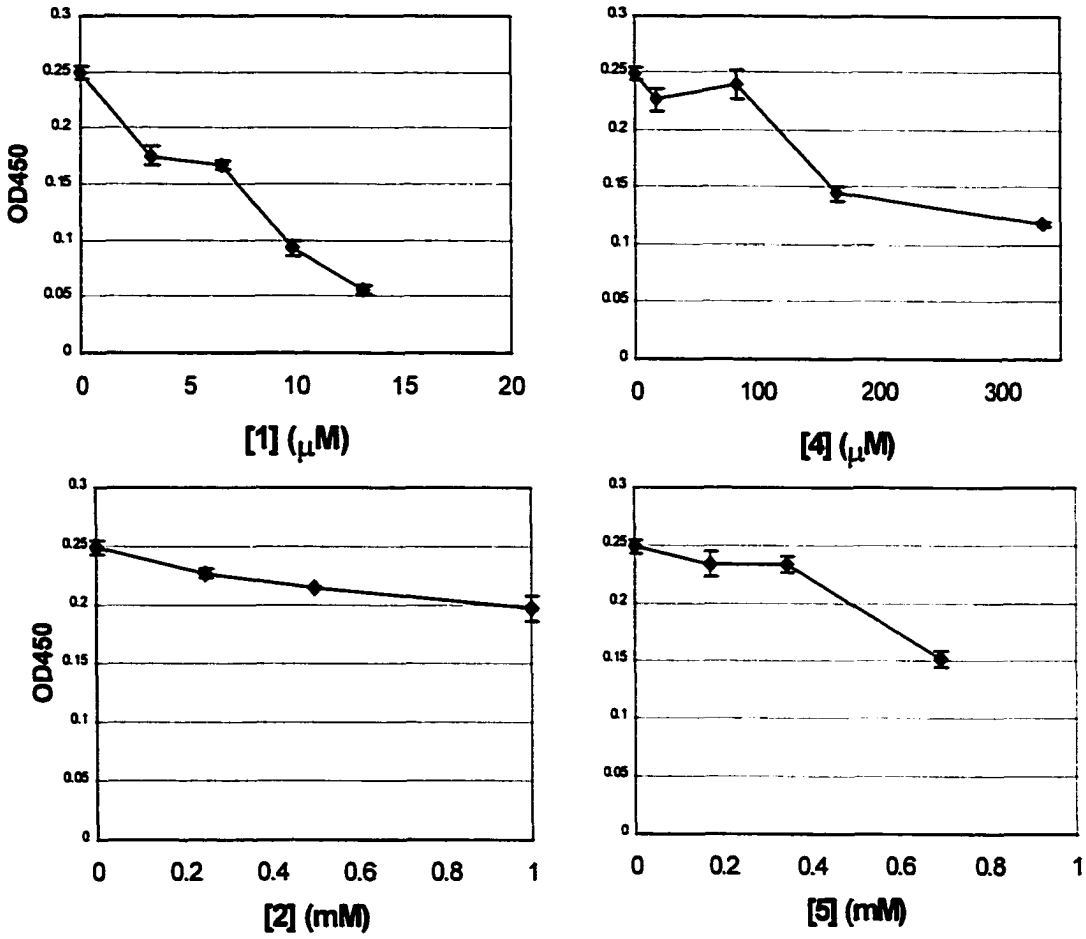


Figure 3.6: Results of LT-ELISA of trisaccharides **1**, **2**, **4** and **5**

The CT-DELA was carried out on only compounds **1**, **4** and **5**, since there was not enough compound **2** to complete the evaluation (Figure 3.7). Visual extrapolation of the

curve obtained for compound **1** would suggest an IC_{50} value around 20 μM, comparable to the results obtained from the earlier CT-DELA (Figure 3.4b). Again, results for the galactose-sialic acid trisaccharide **4** suggest an IC_{50} value in the high micromolar or low millimolar range. As well, carboxymethyl trisaccharide **5** appears to be a low millimolar inhibitor as well.

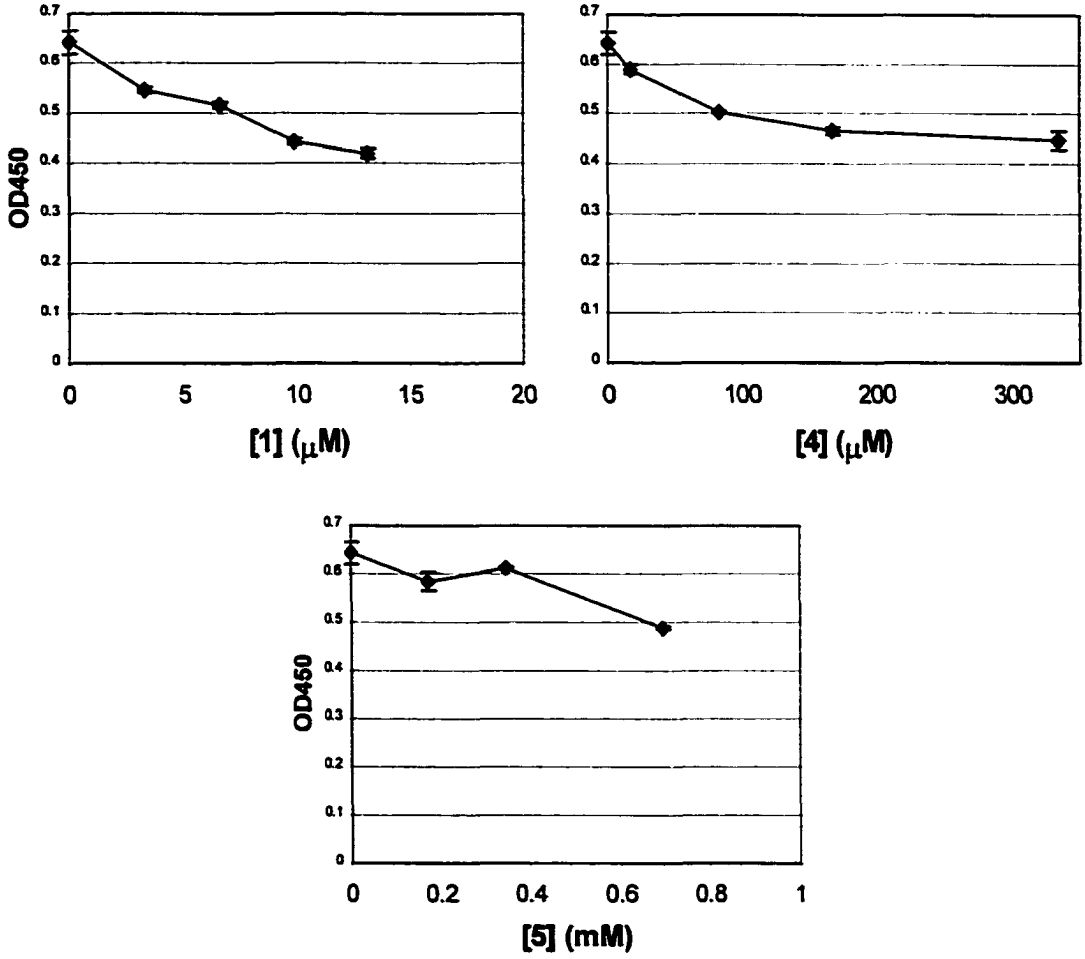


Figure 3.7: Results of CT-DELA assay of trisaccharides **1**, **4** and **5**.

PART B:

3.3 Frontal Affinity Chromatography with Mass Spectrometric Detection

3.3.1 Introduction to FAC-MS Assay

While the enzyme-linked assay has amply demonstrated its utility for screening potential inhibitors, a few shortcomings are inherent to this method. For example, the number of compounds that can be screened at one time is limited by the number of wells available on a microtiter plate. This may not seem significant until one realizes that a typical ELISA takes up to six hours to complete. Screening of a library of compounds becomes a very labour-intensive exercise that can take many days, with further evaluation of "hits" to determine IC_{50} values requiring yet even more work. To carry out these analyses, milligram quantities of inhibitors are sometimes required, which can sometimes present a major obstacle to the synthetic chemist.

A novel approach to the evaluation of libraries of compounds was developed by the Hindsgaul group [114]. This method employs frontal affinity chromatography coupled with electrospray mass spectrometric detection. This technique was applied to the problem of evaluating the binding strength of large numbers of potential CT and LT ligands and allowed the determination of the dissociation constant $\mathbf{K_d}$ of each compound.

The assay first requires the immobilization of toxin on a support material which has been packed into a microscale column. Molecules that bind to the toxin are identified by continuously infusing this column with a solution containing potential ligands while constantly analyzing the effluent by electrospray mass spectrometry. The use of an electrospray mass spectrometer as a detector allows the monitoring of specific molecules according to their mass/charge ratio (m/z). Many compounds can be assayed at the same time providing they have distinct m/z ratios. The miniaturization of the FAC columns significantly reduces the amounts of both the receptor and potential ligands required for analysis.

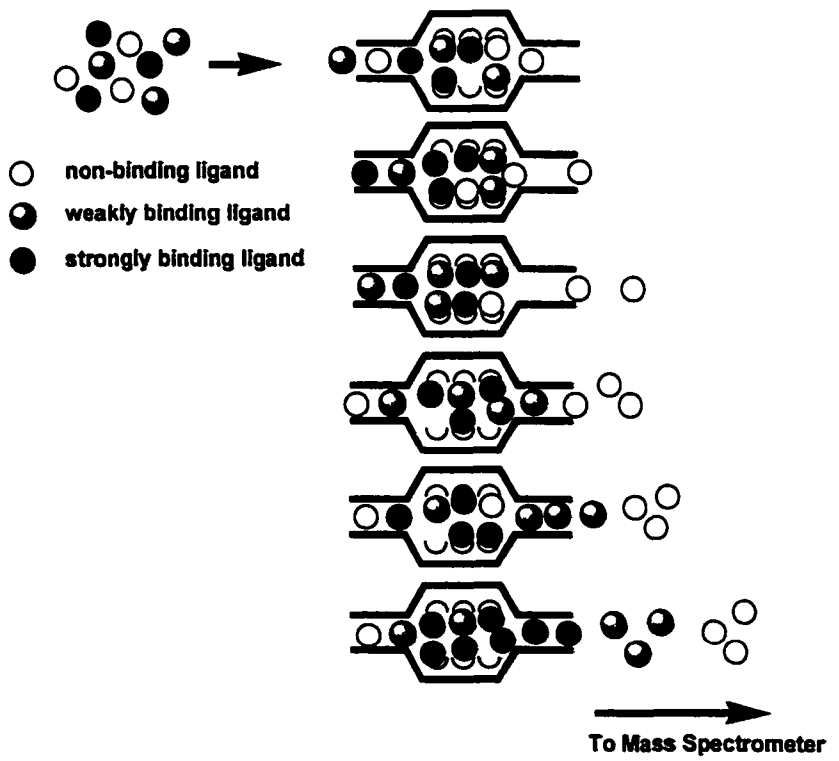


Figure 3.8: Schematic representation of FAC-MS assay

Theory suggests that, with continuous infusion of a mixture of compounds, nonbinding species will break through with the void volume (V_o), while active ones will be delayed [117] (Figure 3.8). These active compounds bind to the receptor, eventually exceeding the capacity of the column, leading to the observed delay in breakthrough [117]. This delay can be correlated to the dissociation constant K_d, if the number of receptor binding sites contained in the column, or column capacity B_t, is known. The equation describing this relationship is

$$V_x - V_o = \frac{B_t}{[X]_o + (K_d)_x},$$

where $[X]_o$ is the concentration of the ligand, and V_x is the volume at which the compound broke through [117].

The value for B_t can be calculated by measuring the V_x-V_o values for a known active

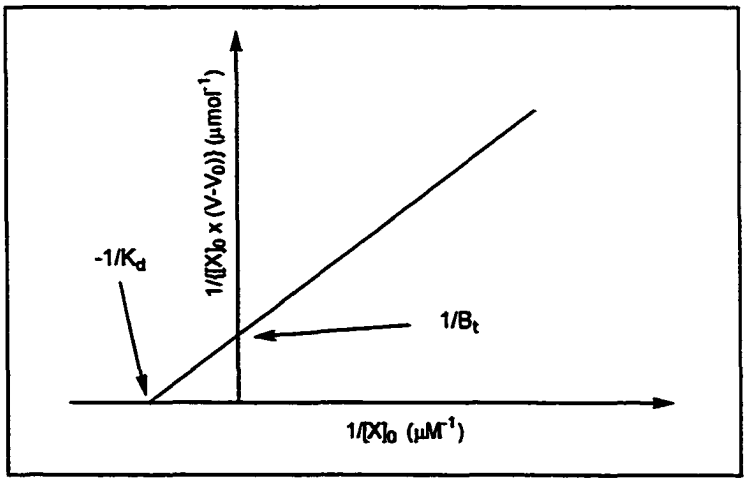


Figure 3.9: Sample plot of $1/([X]_o(V-V_o))$ versus $1/[X]_o$

compound X over a range of concentrations $[X]_o$. A plot of $([X]_o(V-V_o))^{-1}$ versus $[X]_o^{-1}$ provides the B_t value as the reciprocal of the y-intercept. The K_d value can also be extracted from this plot as the negative reciprocal of the x-intercept (Figure 3.9).

3.3.2 FAC-MS Experimental

The FAC-MS assay employs poly(ether ether ketone) (PEEK) microcolumns (500 μM i.d by 6 cm) containing controlled porous glass (CPG) beads covalently coupled to streptavidin as the packing material. These CPG-SA beads can later be derivatized with biotin-labeled protein. The B-subunits of both cholera toxin (CT-B) and heat-labile enterotoxin (LT-B) are commercially available from Sigma. Biotinylation of the toxins was achieved with the reagent NHS-LC-biotin (Pierce), which reacts with the amine side chains of basic amino acid residues on the protein. The biotinylation reaction takes place in sodium bicarbonate buffer containing 50 mM galactose, 5 μM toxin and 50 μM NHS-LC-Biotin. The degree of labeling is monitored by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Unreacted NHS-LC-biotin is consumed with the addition of Tris buffer. The buffer salts and excess biotin are then removed from the protein by dialysis across a 10 kDa cutoff membrane.

The CPG-SA beads are suspended in a buffer containing PBS:glycerol (6:4 ratio) for slurry-packing into the column. After packing is complete, a solution containing the biotin-labeled toxin is passed through the column at 8 μl/minute. Before the column is ready for testing compounds, it is washed with 2 mM NH_4OAc buffer (pH 6.7) to remove

any glycerol and phosphate salts. Unoccupied streptavidin binding sites are blocked by passing a 10 μM solution of biotin through the column until biotin is apparent in the effluent as monitored by electrospray mass spectrometry ($M\text{-}H^+$ 243).

To determine the column capacity B_t, a series of runs containing the known binding trisaccharide **1** at 1 μM, 4 μM, 10 μM and 20 μM was carried out. A sample plot of $([X]_o(V_x\text{-}V_o))^{-1}$ versus $[X]_o^{-1}$ for an LT column is shown in Figure 3.10. The calculated B_t for this plot is 632 ± 13 pmol and K_d for **1** is 8.1 ± 0.1 μM. The best-fit line was calculated using the graphing program Cricket Graph (version 1.3, Cricket Software, Malvern, PA) excluding the point which departs from linearity. The errors reported are the standard deviations determined using linear regression analysis.

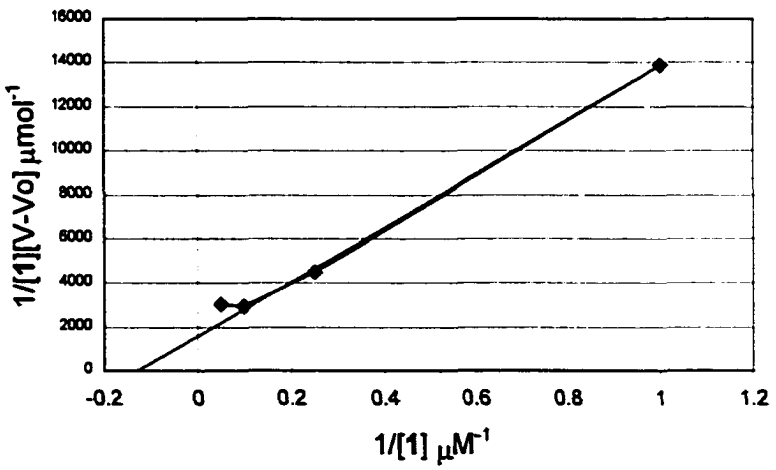


Figure 3.10: Plot of $1/([I](V\text{-}V_o))$ versus $1/[I]$

Prior to screening for activity against a toxin, each new compound is passed directly through the electrospray mass spectrometer in both positive and negative scan mode to establish the optimal m/z signal to be used during the assay. Once these m/z values have

been established, they are then monitored in selected ion mode (SIM) during the assay. All compounds are initially screened for activity at a concentration of 4 μM. Each run also contains a non-binding void volume marker (TriMan-OGr) at 4 μM and the reference compound **1** at 4 μM. The samples are diluted in 2 mM NH_4OAc buffer (pH 6.7) and are

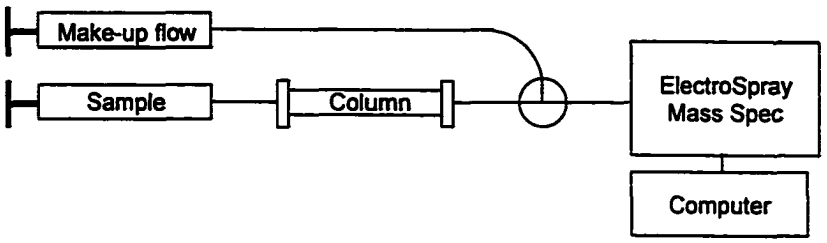


Figure 3.11: Schematic diagram of the FAC-MS assay

infused through the column at 8 μl/minute using a multi-syringe pump. The column eluent is combined at a tee joint with a make-up flow of acetonitrile before being introduced to the electrospray mass spectrometer for analysis (Figure 3.11).

3.3.3 Results of FAC-MS Assay

Since the activities of the smaller molecules had already been established using the ELISA method, they were not tested by FAC-MS. Instead, it was decided that the only molecules to be investigated using this new method were the four trisaccharides shown in Figure 3.12. Since it was already known that **1** was a relatively strong inhibitor of both toxins, it was used as the reference molecule. In other words, the activity of the other trisaccharides was evaluated relative to that of compound **1**. As expected, **1** remained the strongest binder of the four. The B_t calculated for this LT column was 632 ± 13 pmol

and K_d for **1** is 8.1 ± 0.1 μM. Binding constants for the four compounds are summarized in Table 3.1.

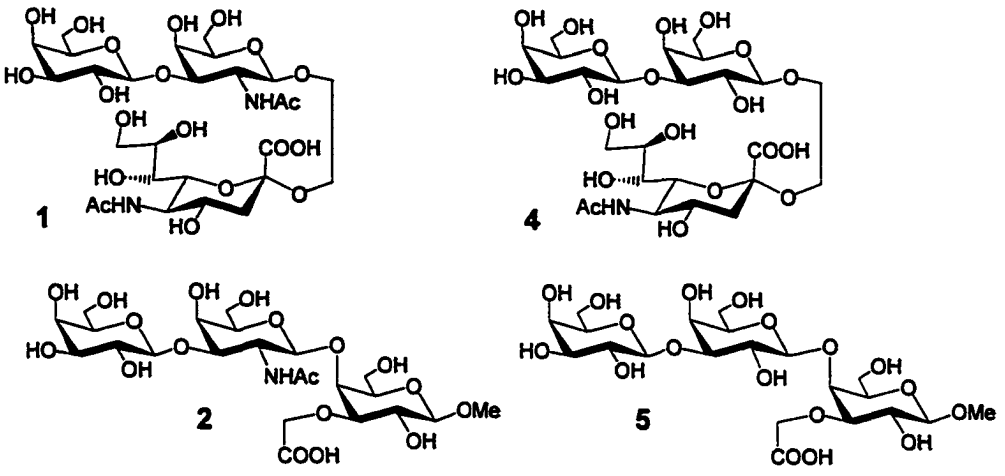


Figure 3.12: Four trisaccharide GM_1 analogues

Compound	Enzyme-Linked assays		FAC-MS	
	LT	CT	LT	CT
	IC_{50} (μM)	IC_{50} (μM)	K_d (μM)[a]	K_d (μM)[b]
1	8.2 ± 0.2[c]	19 ± 1	8.1 ± 0.1	20 ± 12
2	> 1000	--	710 ± 80	+
4	285 ± 17	> 350	55 ± 1	43 ± 34
5	> 1000	> 1000	+	+

\+ too high to evaluate using FAC-MS
-- not tested

[a] LT column B_t is 632 ± 13 pmol
[b] CT column B_t is 116 ± 58 pmol
[c] all errors reported as standard deviation

Table 3.1: Summary of ELISA ands FAC-MS assays of four trisaccharides

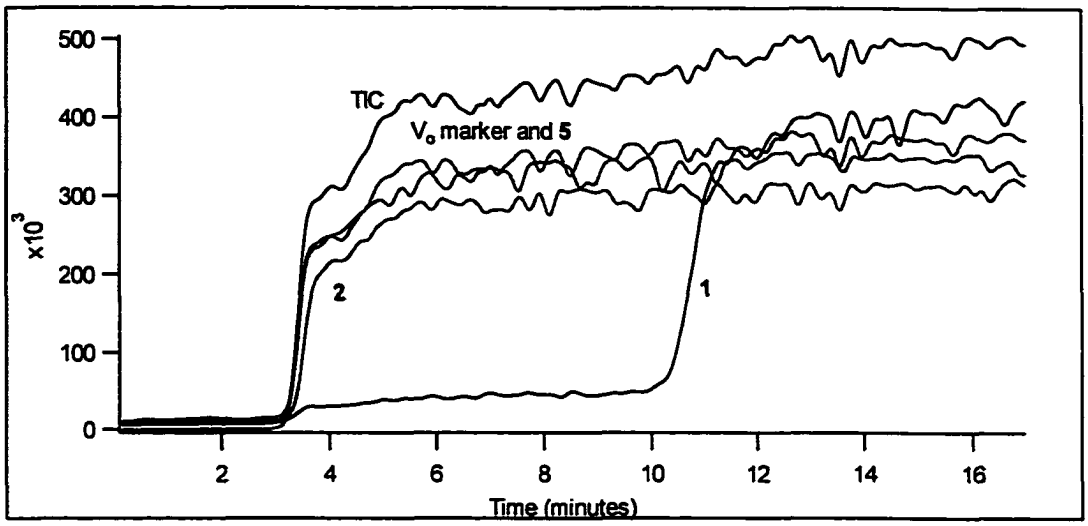


Figure 3.13: FAC-MS assay of trisaccharides **1**, **2** and **5** on LT column

It is difficult to deduce from Figure 3.13, but compound **2** appears to elute from the LT column slightly later than the void volume marker. Further investigation of this compound did indeed suggest a noticeable interaction between **2** and LT, leading to an

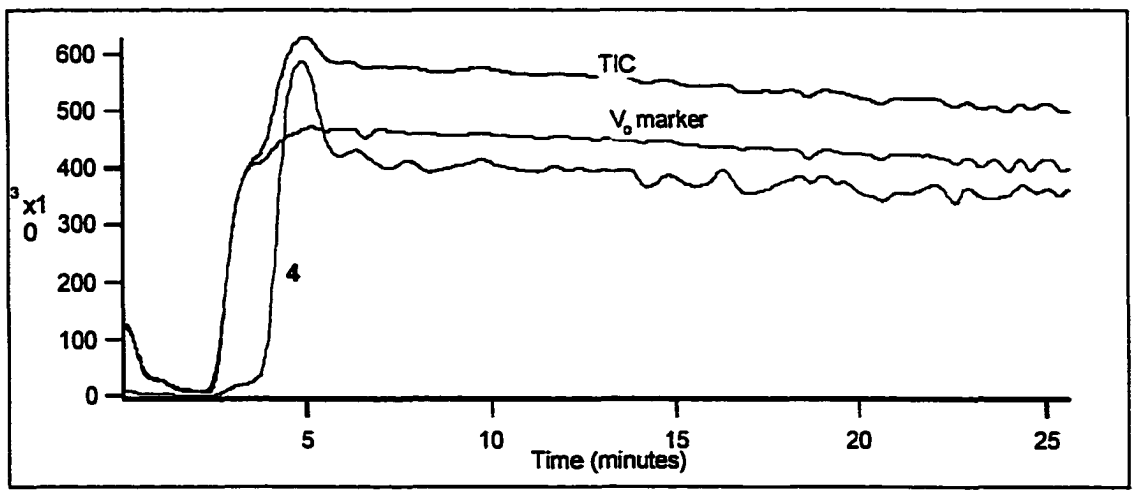


Figure 3.14: FAC-MS assay of trisaccharide **5** against LT.

estimated K_d value of 710 ± 80 μM. Compound **4** was evaluated individually, and a K_d value of 55 ± 1 μM was determined (Figure 3.14).

The column capacity B_t of the CT column was determined as for the LT column, providing a value of 116 ± 58 pmol. This method also produced a K_d value of 20 ±12 μM for **1** against CT. The smaller B_t of the CT column leads to a decreased ability to detect weakly binding compounds. This would explain the observation that only the two strongest binding ligands, **1** and **4** (43 ± 34 μM), were noticeably active, and the weaker disaccharides **2** and **5** were not retarded at all (Figure 3.15). Unfortunately, the smaller B_t increased the difficulty with which it could be accurately determined, leading to the large error.

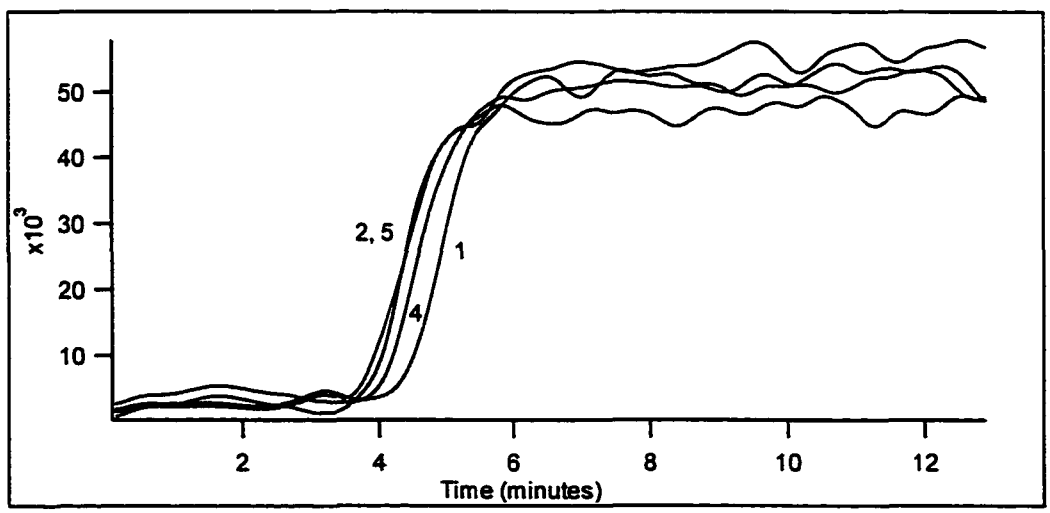


Figure 3.15: FAC-MS assay of trisaccharides **1**, **2**, **4** and **5** against CT

The discrepancy between the calculated column capacity B_t for the LT and CT columns (632 pmol for LT *vs.* 116 pmol for CT) can be due to a number of factors. The first may

be that there simply are not as many amino groups available as sites for biotinylation on the surface of CT as there are on LT. A lower degree of biotinylation may lead to a lower substitution level when derivatizing the packing material. But, since an excess of biotinylated toxin is passed through the microcolumn during the loading step, it would appear that this should not be the cause of such a drastic difference in the column capacity. Perhaps the reason for lower column capacity lay in the location of the amino groups. If biotinylation occurs near the opening of the binding site, it could effectively block access to the site for potential ligands. In fact, biotinylation anywhere on the face of the toxin that contains the binding sites would also effectively cause blocking of the binding site. This would be the result of situating the toxin so that its binding sites face in toward the streptavidin-labelled packing material, instead of out to where the potential ligands would have unhindered access to the binding cleft.

3.4 Discussion

As one would have predicted, the molecules lacking the terminal galactose (**6**, **7**, **8**) demonstrated very little inhibition of both cholera toxin and heat-labile enterotoxin when tested using the enzyme-linked assays. This is not surprising, since it has been well established from the crystal structure of GM_1 in the binding site of CT that the terminal galactose is engaged in a large number of hydrogen-bonding interactions with the protein [23]. Depriving a potential inhibitor of the cumulative strength of these interactions leaves it at a significant disadvantage, resulting in a weakly binding ligand.

Another conclusion that can be drawn from the assay results is that the carboxymethyl substituent is not an adequate replacement for the sialic acid residue. Both the trisaccharides (**2** and **5**) containing this acid fragment might be assumed to have affinities comparable to the Galβ(1→3)GalNAcβOCH_2CH_2OH disaccharide (low millimolar).

The molecule that exhibited the strongest binding to both LT and CT is compound **1**. This is as expected, since it is structurally the most similar to the native GM_1. When discussing the relative dissociation constants of **1** and GM_1, it would be more reasonable to compare it to GM_1-oligosaccharide (GM_1-OS) rather than the intact GM_1 ganglioside. This is due to the fact that the ganglioside has a much higher affinity for the toxins than GM_1-OS due to micellar effects. The ceramide of the ganglioside can be expected to induce the formation of micelles, which effectively lead to higher local concentrations of the carbohydrate ligand [118]. The critical micellar concentration of GM_1 has been estimated to be 10^{-5} to 10^{-4} M, but the ganglioside can probably be expected to aggregate to some extent at lower concentrations than the CMC [119]. This can result in a higher apparent affinity constant for GM_1 ganglioside relative to the GM_1-OS. An experiment evaluating the ability of intact GM_1 and GM_1-OS to prevent binding of the CT B-subunit to human fibroblast cells determined that GM_1-OS has a K_i of 600 nM in comparison to 25 nM for intact GM_1, which means the oligosaccharide ligand is 24 times weaker than GM_1 [118].

The dissociation constant for compound **1** against **CT** has been determined to be 20 μM using the FAC-MS method in the work presented here. This value can be compared to

that of GM_1-OS, which has a calorimetrically determined dissociation constant of 950 nM [120]. This would suggest that compound **1** is weaker by only a factor of 20 for binding to this toxin.

Before discussing the reasons for the weaker binding of **1** to toxin, some of the thermodynamic parameters pertinent to carbohydrate-protein interactions should be briefly described. It is well known that a relationship exists between the free energy of binding (ΔG) and a binding constant (K) such that

$$\Delta G = -RT\ln K.$$

This free energy can also be described in enthalpic (ΔH) and entropic (ΔS) terms according to:

$$\Delta G = \Delta H - T\Delta S.$$

The enthalpic term ΔH can be further partitioned into ΔH_{conf}, which describes the strain energy in the carbohydrate-protein complex, and ΔH_{bind}, which describes the sum of the van der Waals, electrostatic and hydrogen bonding interactions in this complex. The entropic term ΔS can also be partitioned into its component terms. These terms include $\Delta S_{rot+trans}$ (the change in entropy due to the loss of rotational and translational freedom upon binding), ΔS_{flex} (the change in entropy upon freezing of the torsional angles) and ΔS_{solv} (the change in entropy due to displacement of solvent molecules from the surface of the protein and ligand upon formation of the complex) [121, 122].

$$\Delta G = \Delta H_{conf} + \Delta H_{bind} - T(\Delta S_{rot+trans} + \Delta S_{flex} + \Delta S_{solv}).$$

The entropic terms deserve further discussion. The second law of thermodynamics states that all systems tend toward an increase in entropy, which can also be stated as a tendency toward a decrease in order. A loss in entropy through the restriction of a ligand in a protein binding site is therefore thermodynamically disfavoured. By restricting otherwise flexible molecules (both protein and ligand) into fixed conformations, a large entropic penalty must be paid. This penalty is balanced by the entropic gain associated with the expulsion of ordered solvent molecules from the surface of the protein and carbohydrate into the bulk solvent [122, 123].

It should be apparent from the preceding discussion that a flexible molecule will pay a larger price entropically upon binding than a conformationally restricted molecule. This would, of course, place the ethylene linker ligand **1** at an obvious disadvantage when compared to the relatively fixed conformation of the GM_1-OS [124]. The presence of the flexible linker requires significant preorganization of the carbohydrate in the toxin binding site. This fact is reinforced when compound **1** is compared to the structurally similar pseudotetrasaccharide synthesized by Bernardi *et al.* [125] (Figure 3.16). It is apparent that, although the sugars presented to the protein are identical, the rigidity of the cyclohexyl linker allows the sialic acid residue to assume a conformation similar to that of the native GM_1. Because of the smaller entropic penalty required, the molecule is a much stronger ligand for the toxin. A competitive ELISA assay demonstrated an inhibition profile identical to that of GM_1 oligosaccharide, suggesting that the

pseudotetrasaccharide is indeed an efficient mimic of and does not pay an entropic penalty relative to the native ligand [126].

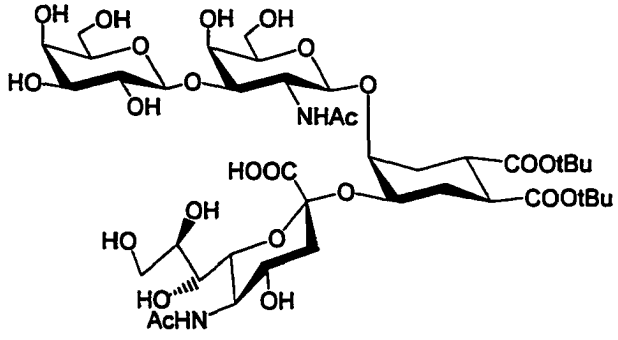


Figure 3.16: Pseudotetrasaccharide mimic of GM_1

A crystal structure of compound **1** in the binding site of cholera toxin has been obtained and kindly provided by the labs of Dr. W.G.J. Hol at the University of Washington in Seattle, USA. Overlap of the crystallographically obtained conformations of compound **1** and GM_1 in the toxin binding site allows a comparison of their conformations (Figure 3.17). Inspection of the overlap shows that the terminal Galβ(1→3)GalNAc disaccharide of **1** is in an identical position to that found in the GM_1:CT crystal structure. This is not surprising due to the large number of hydrogen bonding interactions between the protein and the terminal galactose (and to a lesser extent the *N*-acetylgalactosamine), which would serve to anchor the ligand in place.

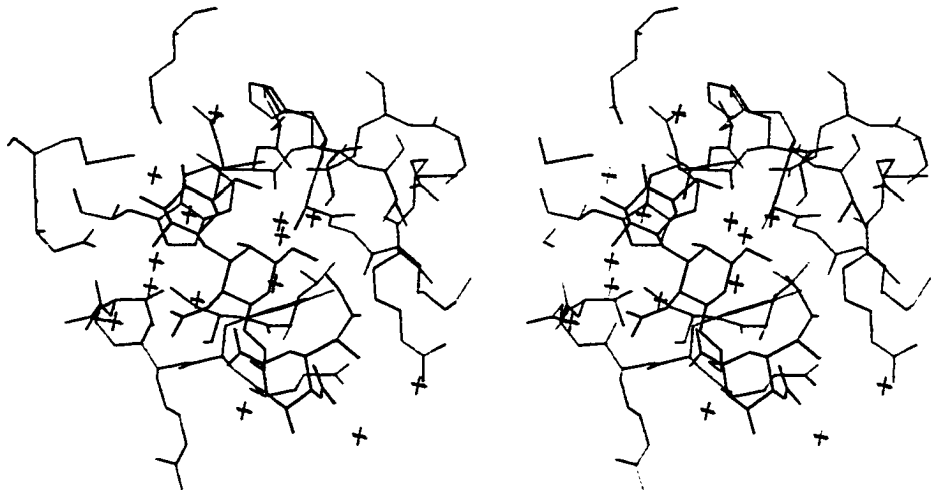


Figure 3.17: Stereogram of crystal structure of compound **1** in CT binding site

More interesting is the position of the sialic acid residue. One would expect that the flexibility of the linker would allow the sialic acid to assume any position in the binding site. Instead it is found in a position similar to, but not exactly, that of GM_1. While the polyhydroxy tail and the acetamido group are involved in a number of hydrogen bonding interactions, many of them are water mediated and thus may be not quite as strict as those directly to protein amino acids residues. This does imply that the interactions between the sialic acid and the protein are significant, but not as strong as those between the terminal galactose and protein, which may explain the variation in conformation.

Perhaps more significant is the position of the carboxylate of the sialic acid of compound **1**, which overlaps almost perfectly with the carboxylate of GM_1 (Figure 3.18). This would suggest that the hypothesis regarding the hydrogen bond between the COOH and

NHAc of the GalNAc residue may be valid [23]. The interatomic distance between the carboxylate oxygen and acetamido nitrogen is 2.9 Å, which falls within the range of known hydrogen bonding distances. This may be partly responsible for anchoring the sialic acid in position, in spite of the flexible linker and limited strength of the sialic acid-protein hydrogen bonding interactions.

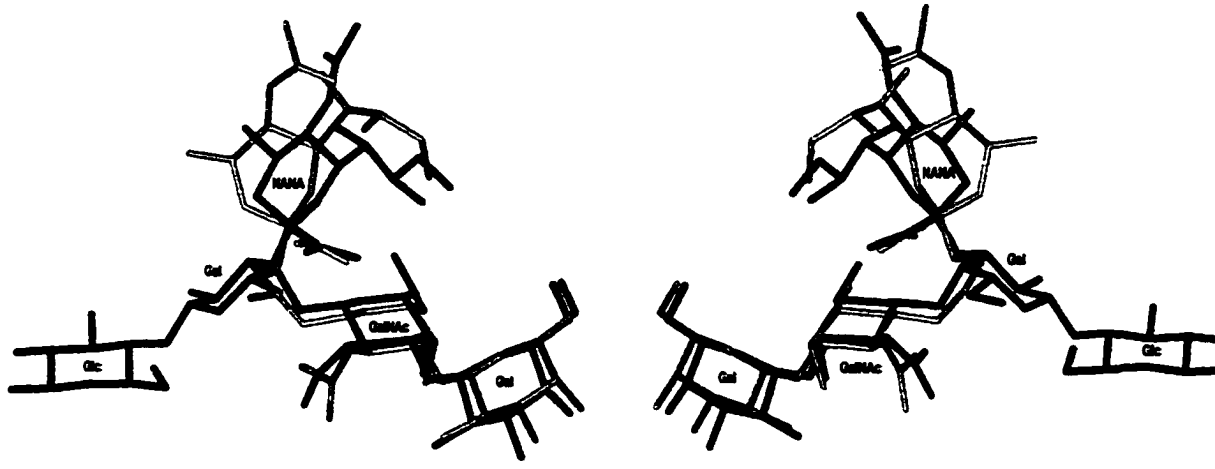


Figure 3.18: Overlap of GM_1 and compound **1** from two different views (180° rotation)

What about the difference between the dissociation constants of compound **1** and its galactose analogue **4**? The galactose analogue **4** (K_d 55 ± 1 μM) is obviously a weaker ligand than **1** (K_d 8.1 ± 0.1 μM). Establishing what role the acetamido plays that the hydroxyl cannot can only be discussed in speculative terms, since there is no crystal structure of **4** in the toxin binding site or calorimetric study comparing the two ligands.

The first proposed explanation would be that the supposed hydrogen bonding interaction between the COOH of the sialic acid and the NHAc of GalNAc would be attenuated by

torsional freedom than a planar acetamido, which may require some preorganization to orient itself into the correct conformer for hydrogen bonding to the carboxylate. Another factor to consider is the empirical observation that a hydroxyl group acting as a hydrogen bond donor must in turn act as a hydrogen bond acceptor [36]. This cooperativity requires that another functional group be in the vicinity to assume this role, which may not be the case. Assuming that this hydrogen bonding interaction is as important as proposed and that it has indeed been weakened, the sialic acid residue would not be fixed as closely into position, leading to a weaker overall interaction of the trisaccharide with the toxin.

Another possible explanation for the lower affinity of **4** may be due to the space left behind upon the replacement of the functional groups. A hydroxyl group takes up much less space than the native acetamido, which means that the region left open must be occupied by something. This could allow the sequestering of another water molecule in the binding site, which would be entropically disfavoured. The penalty paid for restricting a water molecule in this manner could also contribute to the lower affinity of compound **4** for the toxins relative to compound **1**.

When looking at the assay results for compound **1**, it appears that there is a significant correlation between the IC_{50} values obtained using the enzyme-linked assays and the K_d values obtained using the FAC-MS assay for both CT and LT. This correlation diminishes for the weaker ligand **4**. What does this suggest? Is this a cause for concern regarding the validity of the test results? The answer is no, since one should not expect to

find any real relationship between an IC_{50} value and a K_d value. The IC_{50} is determined using a competitive inhibition assay. The success of this type of assay depends on many factors, of which one of the most important is the development of assay parameters that allow the identification of moderately binding ligands in the presence of a strongly binding reference ligand. Because manipulation of these assay parameters can lead to variations in the calculated IC_{50} values, it is important to realize that values obtained using different protocols should not be compared. The determination of the dissociation constant K_d, on the other hand, provides information regarding a thermodynamic parameter for a given ligand. With this in mind, it should be stressed that much more weight should be placed on the significance of a K_d evaluation than that of an IC_{50} determination and the strong correlation between the experimentally determined binding constants should be viewed as little more than a coincidence.

It is apparent from this study that, unlike with the SLe^x/E-selectin model, the sialic acid residue cannot be replaced with a carboxymethyl moiety. This is unfortunate, since the synthesis of toxin inhibitors would be much easier if molecules containing sialic acid could be avoided, due to the complicated nature of the chemistry. It appears that this is not likely, and other strategies toward the synthesis of toxin inhibitors will have to be explored.

It also appears that the use of the flexible linker carries with it inherent shortcomings as a result of the entropic penalty required for preorganization of the sugar before binding. As well, the replacement of the acetamido of the GalNAc residue with a hydroxyl group

results in a decrease in affinity. Further molecular modeling or calorimetric studies would have to be undertaken to investigate why.

Part II

Solid Phase Synthesis of Small Molecule Libraries as Inhibitors of Cholera Toxin and Heat-Labile Enterotoxin

Chapter 4:

Solid Phase Methods for the Synthesis of a Disaccharide Library

4.1 Introduction to Small Library Synthesis and the Use of Solid Supports

Historically drug development has been a rather methodical venture, typically involving the laborious task of identifying a lead compound, followed by its synthesis and subsequent biological evaluation, which generally in turn necessitates further optimization through the design and synthesis of analogues. This method of drug development is tedious and often times inefficient, not to mention expensive. Typical timelines for the development of a single drug are often measured in decades and the monetary investments required are on the order of hundreds of millions of dollars [127]. A relatively recent innovation in drug discovery has been the use of combinatorial chemistry for the synthesis of large libraries of structurally related compounds [128]. These techniques are capable of greatly accelerating the discovery of lead compounds as well as their subsequent optimization.

The combinatorial approach can be used to generate large mixtures of compounds, or to provide arrays of individual compounds, both of which can be screened for biological activity. To illustrate the potential of combinatorial chemistry for the synthesis of large numbers of compounds, one need only consider the outcome of a serial synthesis of a tripeptide containing only three different amino acid substituents. There are 27 different combinations available using the three peptides (Figure 4.1). Using only the 20 common

amino acids, one can construct a tripeptide library containing 8000 different combinations, and a hexapeptide library containing 64 million combinations. It is clear from these examples that a combinatorial approach provides the chemist with a very powerful tool in the development of drugs.

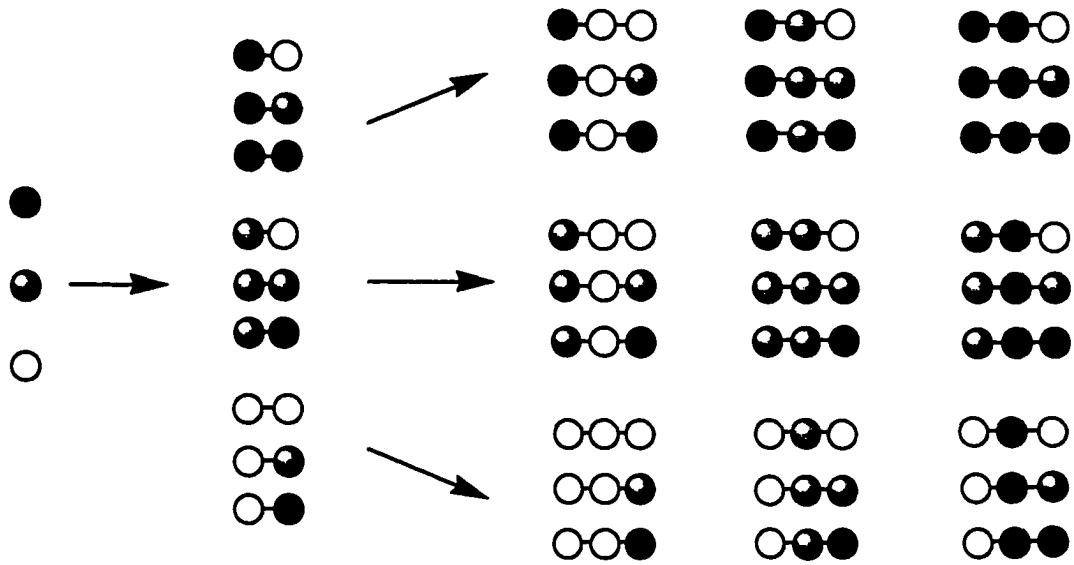


Figure 4.1: Tripeptide library constructed from 3 different amino acids

The combinatorial approach to drug development has been assisted by the accompanying evolution of solid phase synthetic techniques, which provide a number of advantages over the use of solution phase chemistry [129]. One obvious shortcoming of solution phase chemistry is the necessity of extensive purification steps for the isolation of the desired product from excess reagents and byproducts. This can be a significant problem when synthesizing large numbers of compounds for screening or carrying out a sequence of reactions on the same molecule. Solid phase techniques allow the molecule being manipulated to remain attached to a solid phase support throughout all the reaction steps. This permits the use of high molar excesses of reagent to achieve high yields, with any

unreacted reagent removed by a simple washing step. Providing the individual reactions are high yielding, a series of reactions can be carried out on this tethered molecule, and the only real purification required is the washing step. This allows the product to be obtained in a relatively pure state upon cleavage from the solid phase.

4.2 Disaccharide-Linker Library

4.2.1 Description of Library Approach

In the quest for small molecule inhibitors of cholera toxin (CT) and heat-labile enterotoxin (LT), it was decided that there was limited potential for a rational approach to the design of tight binding ligands. In an attempt to create a library of compounds for testing against the toxins, solid phase methods were chosen for the relative ease with which a large number of ligands could be synthesized. In an attempt to mimic the terminal tetrasaccharide of GM_1, a template in which a disaccharide is conjugated to a series of rigid 1,2-*cis* amino alcohol linkers was conceived (Figure 4.2). These

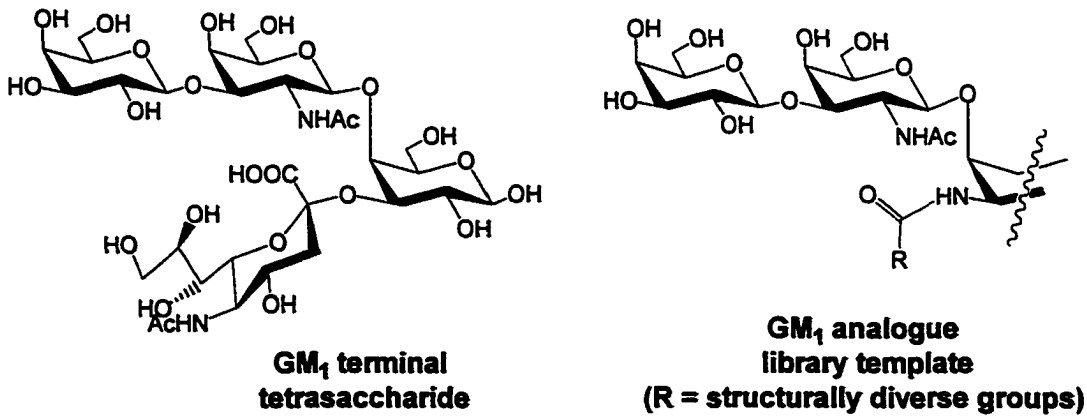


Figure 4.2: Analogue library in comparison to GM_1 terminal tetrasaccharide

disaccharide–linker conjugates could derive their structural diversity from the large number of acylating or alkylating agents which would be used to derivatize the amino functionality. The 1,2-*cis* amino alcohol linker could be envisioned to take the place of the galactose at the branching position, and the substituents on the amine would occupy the space normally occupied by the sialic acid residue.

4.2.2 Synthesis of Disaccharide-Linker Library

4.2.2.1 Synthesis of Disaccharide Thioglycoside Donor

Construction of the Galβ(1→3)GalNAcβLinker framework for the creation of the rigid linker library was carried out according to the retrosynthetic approach described in Figure 4.3. The GalNAc moiety contains a thioether function at the anomeric position, which acts as a temporary protecting group until the disaccharide is assembled. It then allows the disaccharide to be used directly as a donor without further chemical modification. The thioglycoside was then activated for glycosylation of the oxygen of the 1,2-*cis* amino alcohol linkers, followed by protecting group manipulation to provide the Fmoc protected disaccharide for loading onto the resin. Once the sugar was coupled to the resin, the Fmoc group would be removed to free the amine for reaction with a variety of acylating agents.

The thiocresyl derivative was selected for the construction of the disaccharide donor for reasons of practicality, since the excess thiocresol is easily eliminated from the reaction mixture (and its smell neutralized) with an aqueous NaOH workup [130]. This provides

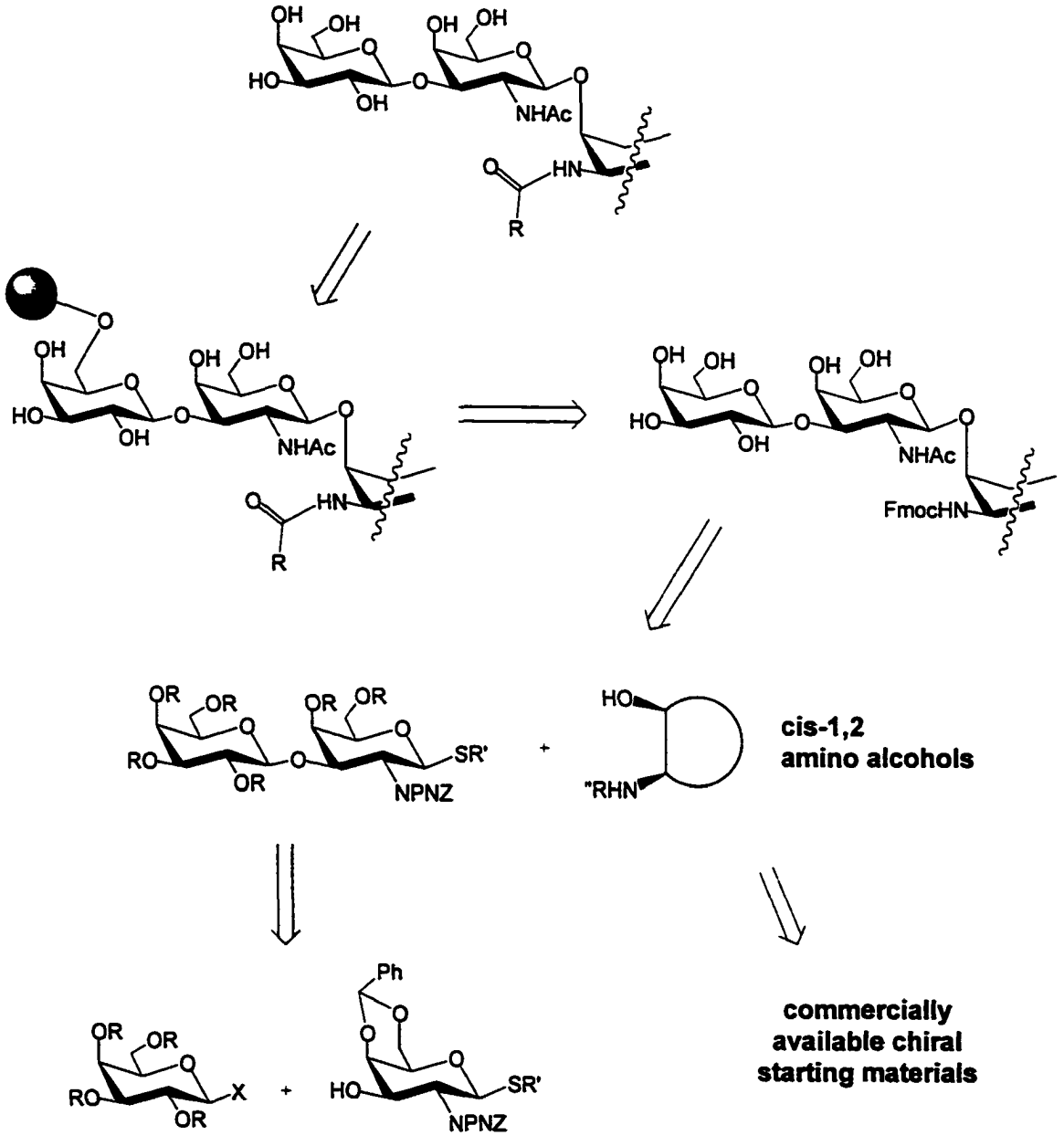
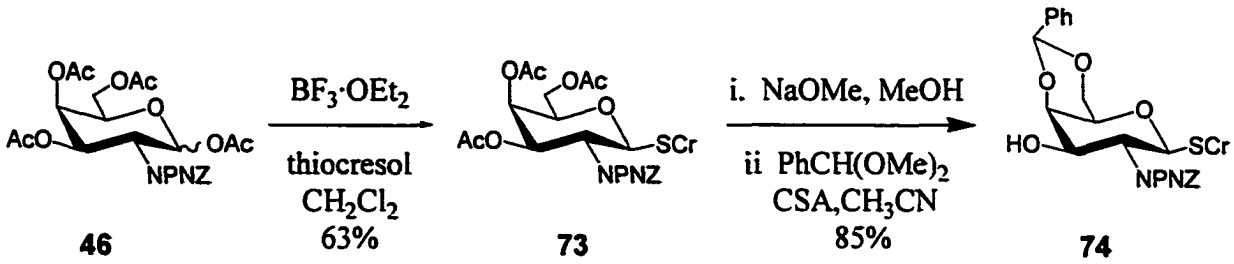


Figure 4.3: Retrosynthetic approach to the synthesis of rigid linker libraries

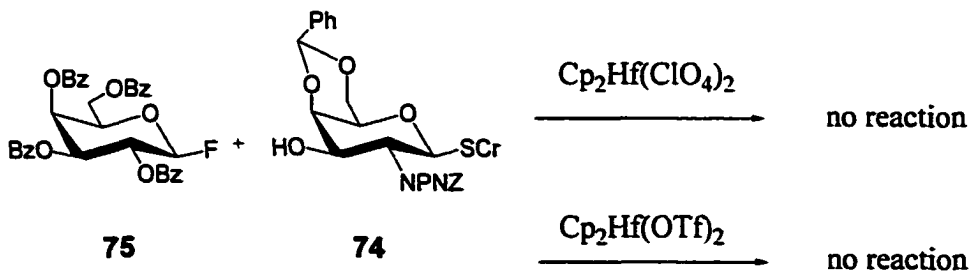
a significant advantage over the more volatile thioethyl and thiomethyl alternatives, since it allows the maintenance of a good working relationship with coworkers. Synthesis of the β-thioglycoside **73** proceeded with a reasonable 65% yield (Scheme 4.1). The

acetates were removed in methanolic sodium methoxide, and a 4,6-di-*O*-benzylidene group was installed using benzaldehyde dimethyl acetal and CSA in acetonitrile to provide **74** in 88% yield over two steps.



Scheme 4.1: Synthesis of thiocresyl acceptor **74**

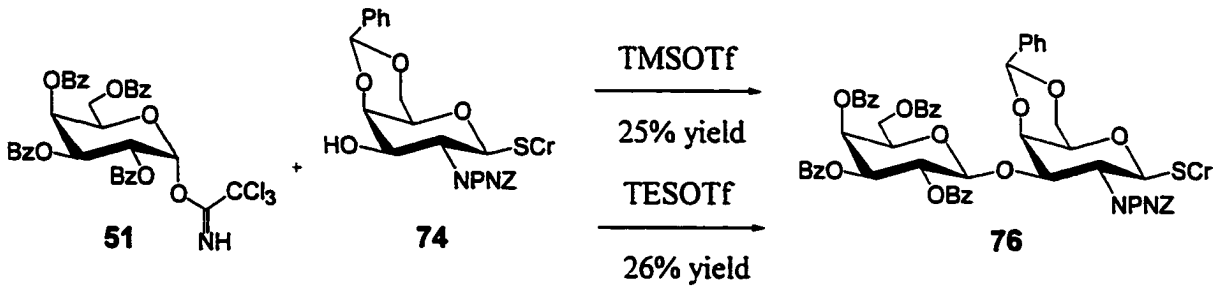
Synthesis of the Galβ(1→3)GalNAcβSCr disaccharide **76** turned out to be more problematic than anticipated. Unsuccessful early attempts employed the benzoylated fluoro donor **75** [131] with activation by the hafnocene derived promoters $Cp_2Hf(ClO_4)_2$ and $Cp_2Hf(OTf)_2$ (Scheme 4.2) [132]. Isolated product of both reactions included significant amounts of unreacted donor and acceptor. The presence of benzoate protecting groups may serve to deactivate the fluoro donor, preventing its ready activation under mild conditions. Instead of pursuing the questionable utility of the



Scheme 4.2: Unsuccessful glycosylation reactions with fluoro donor

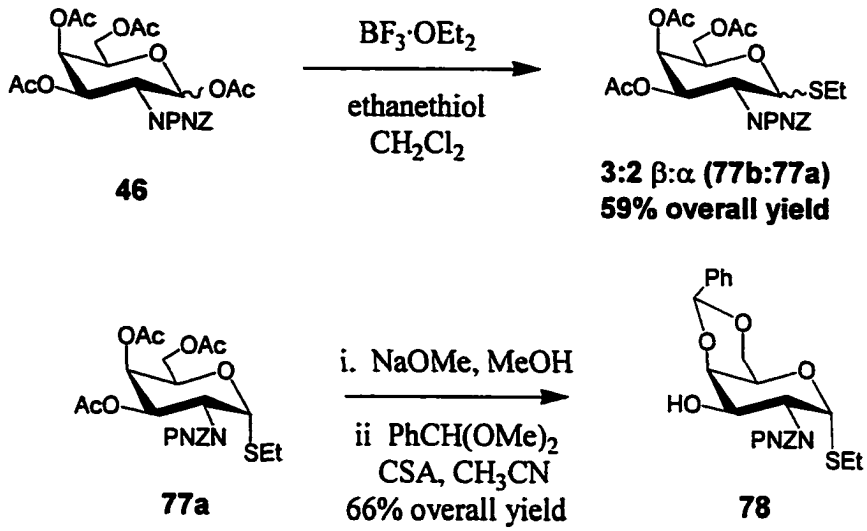
fluoro donor, the more familiar imidate donor was investigated.

The benzoylated imidate donor **51** gave poor results with $BF_3{\cdot}OEt_2$, TMSOTf or TESOTf as activators (Scheme 4.3). The highest coupling yield achieved with this donor-acceptor pair was 26%, obtained with TESOTf. Since a low yielding reaction so early in a multistep synthesis can be problematic, an alternative approach was sought with the use of the more orthodox thioethyl group.



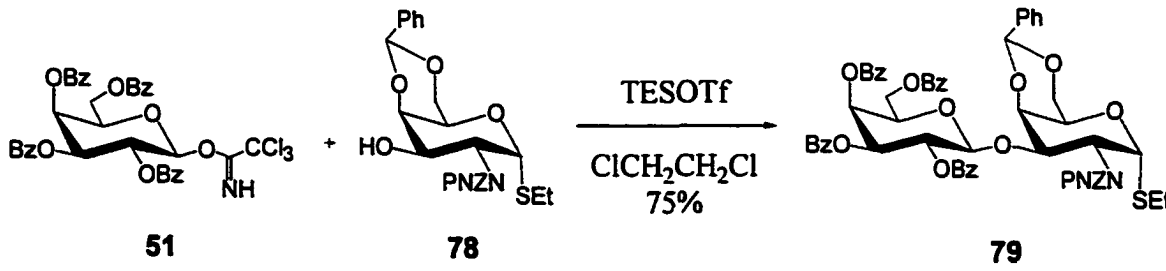
Scheme 4.3: Attempts at the synthesis of the thiocresyl disaccharide **76**

Synthesis of the ethyl thioglycoside yielded both the α- and β-anomers in a 3:2 ratio (59% overall yield) which were separated by column chromatography. The α-anomer **77a** was deacetylated and benzylidenated as for **74**, to provide **78** in 66% yield (Scheme 4.4). The same reaction sequence with the β-anomer **77b** was more troublesome due to the poor solubility of the benzylidene product, which lead to difficulties during the purification step. In any case, the β-anomer was not very useful as an acceptor, as will be indicated shortly.



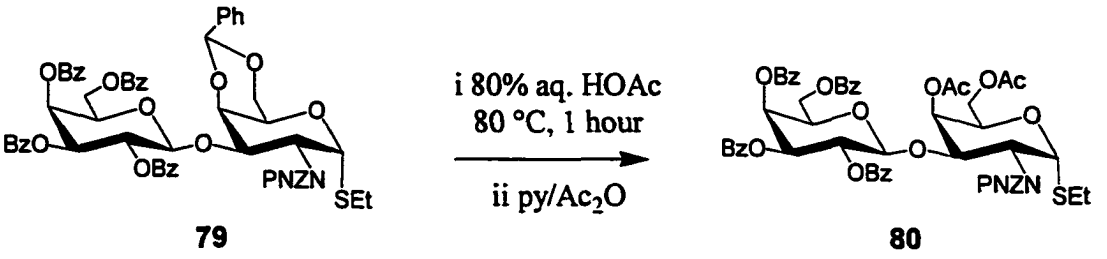
Scheme 4.4: Synthesis of the α-thioethyl acceptor **78**

The coupling reaction between the benzoylated donor **51** and the acceptor **78** proceeded in a satisfying 75% yield to provide the disaccharide **79** (Scheme 4.5). Attempts to reproduce this success with the β-anomer afforded the corresponding disaccharide in a very low yield.



Scheme 4.5: Synthesis of the α-thioethyl disaccharide **79**

The benzylidene protection was removed from disaccharide **79** in 80% aqueous acetic acid at 80°C for one hour, followed by acetylation in pyridine and acetic anhydride to provide the disaccharide **80** in 91% yield over two steps (Scheme 4.6). This thioglycoside was used as the donor for coupling to the 1,2-*cis* amino alcohol linkers.

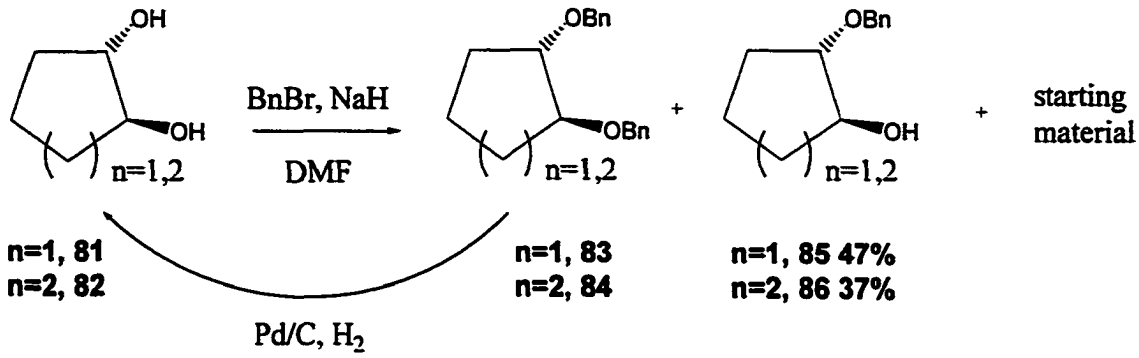


79 **80**

Scheme 4.6: Preparation of the α-thioethyl **80** disaccharide for coupling to linkers

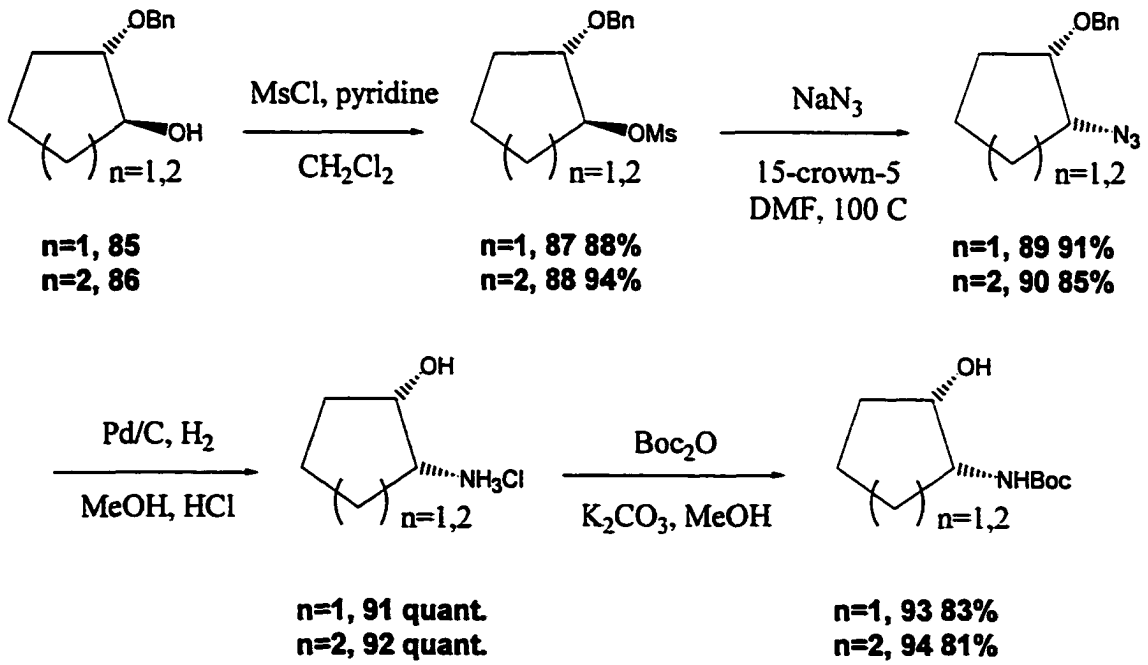
4.2.2.2 Synthesis of 1,2-*cis* Amino Alcohol Linkers

The synthesis of the 1,2-*cis* amino alcohol linkers began with commercially available chiral molecules. The cyclopentane and cyclohexane linkers **93** and **94** were both made using the 1*S*,2*S* diols **81** and **82** as the starting material (Scheme 4.7). The first step in the synthetic pathway was monobenzylation of the diols **81** and **82**. This was done with the addition of approximately one equivalent each of sodium hydride and benzyl bromide in DMF. No selectivity was observed, producing a mixture of the mono-, di- and non-benzylated products. The benzyl cyclopentyl ether **85** was obtained in 47% yield, and the cyclohexane analogue **86** in 37% yield. The dibenzylated products **83** and **84** were easily recycled by hydrogenolysis over palladium catalyst to yield the diol starting material.



Scheme 4.7: Synthesis of benzyl ethers **85** and **86**

The monobenzyl ethers **85** and **86** were mesylated with methanesulfonyl chloride and pyridine in CH_2Cl_2 in 94% yield for both cyclopentyl (**87**) and cyclohexyl (**88**) derivatives (Scheme 4.8). Nucleophilic displacement of the mesylate by sodium azide in



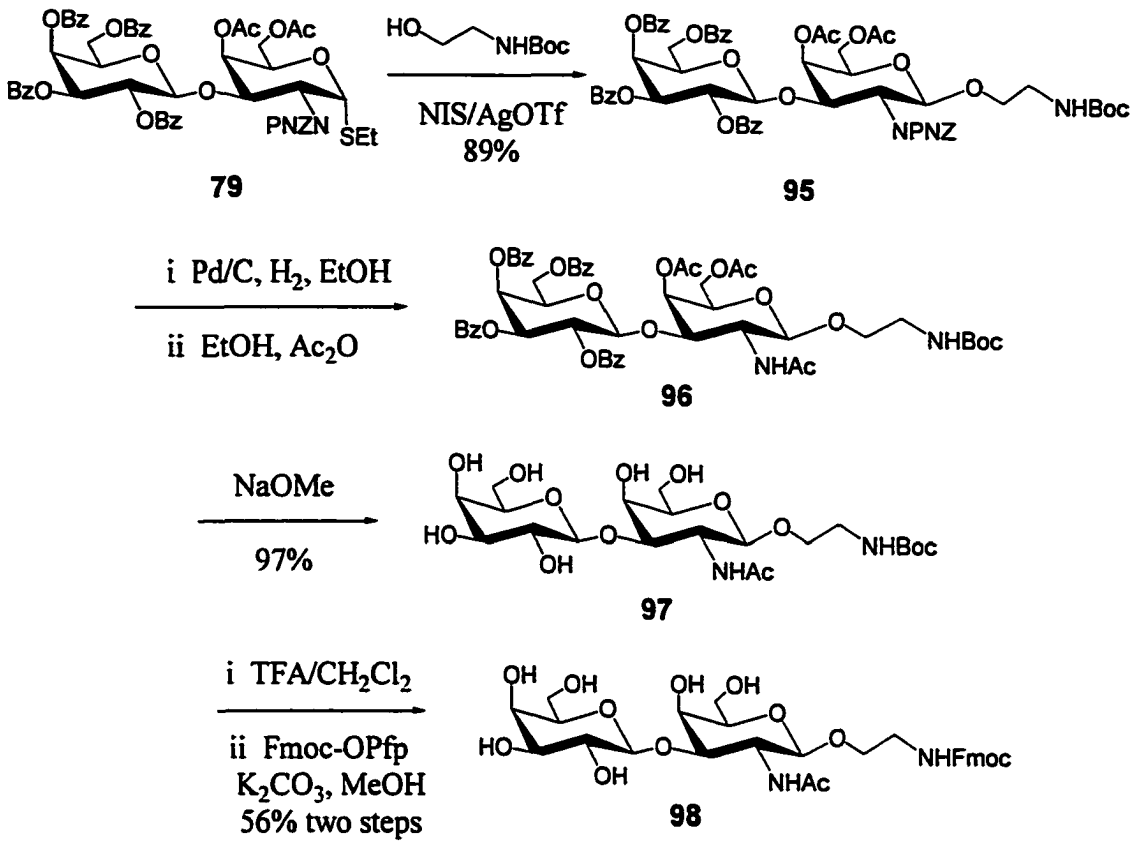
Scheme 4.8: Synthesis of the Boc-protected amino alcohol linkers **93** and **94**

DMF took place in good yield (91% for **89**, 85% for **90**). Reduction of the azide and removal of the benzyl group was achieved in quantitative yield with hydrogenolysis over $Pd(OH)_2/C$ in methanol and HCl. The Boc protective group was then installed by stirring the hydrochloride salts **91** and **92** in methanol with K_2CO_3 and Boc_2O, providing **93** in 83% yield and **94** in 80% yield.

4.2.2.3 Synthesis of the Disaccharide-Linker Adducts

Since derivatization of the linker-disaccharide adduct was to take place on the acid-labile trityl resin, the temporary Boc protection on the linker amine would have to be replaced with an acid-stable protecting group before loading. The Fmoc was selected since it is easily removed under mild basic conditions and is well known to be compatible with solid phase synthetic methods. Unfortunately, the Fmoc cannot be installed until the acetate protecting groups have been removed, since it is not stable to the basic deprotection conditions.

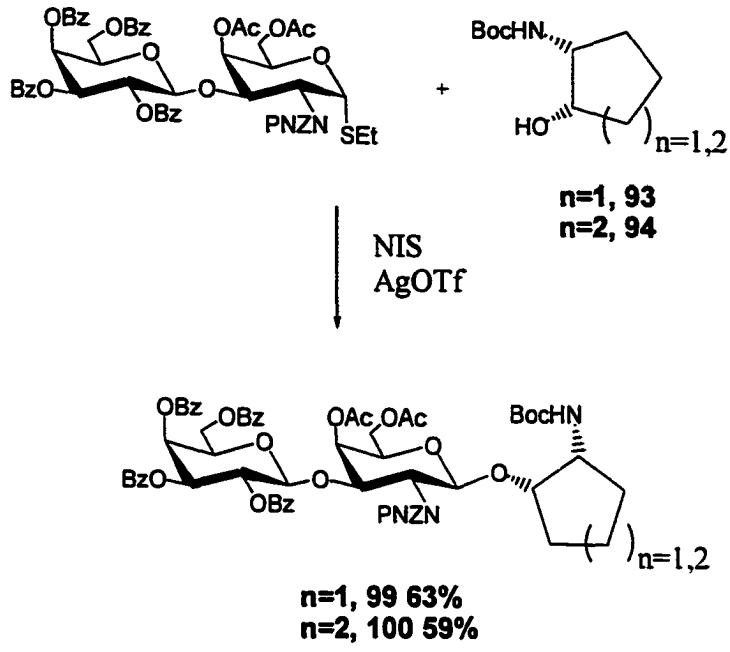
Evaluation of this synthetic plan for its utility was undertaken with Boc-ethanolamine linker as a model (Scheme 4.9). Coupling of the disaccharide thioglycoside **79** with Boc-ethanolamine was carried out under N-iodosuccinimide/silver trifluoromethane sulfonate activating conditions providing **95** in 89% yield. The PNZ group was hydrogenolyzed over Pd/C in ethanol, followed by acetylation of the amine with the addition of acetic anhydride to form **96**. The *O*-acetates were then removed in methanolic sodium methoxide affording **97** in quantitative yield. The Boc group was removed in 5%



Scheme 4.9: Preparation of the ethanolamine linker disaccharide **98**

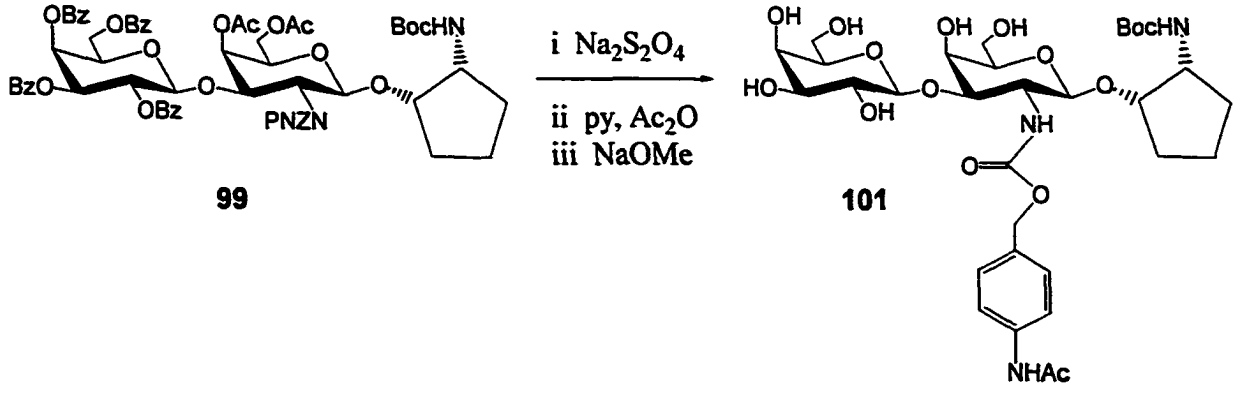
trifluoroacetic acid in dry CH_2Cl_2, to subsequently be replaced with Fmoc as shown in Scheme 4.9. While the yield of **98** for the Boc-Fmoc exchange was only 56%, it demonstrated that the scheme as outlined was feasible.

The cyclopentyl linker-disaccharide **99** and cyclohexyl linker-disaccharide **100** analogues were synthesized as for the ethanolamine, in 63% and 59% yield, respectively (Scheme 4.10).



Scheme 4.10 Synthesis of rigid linker disaccharides **99** and **100**

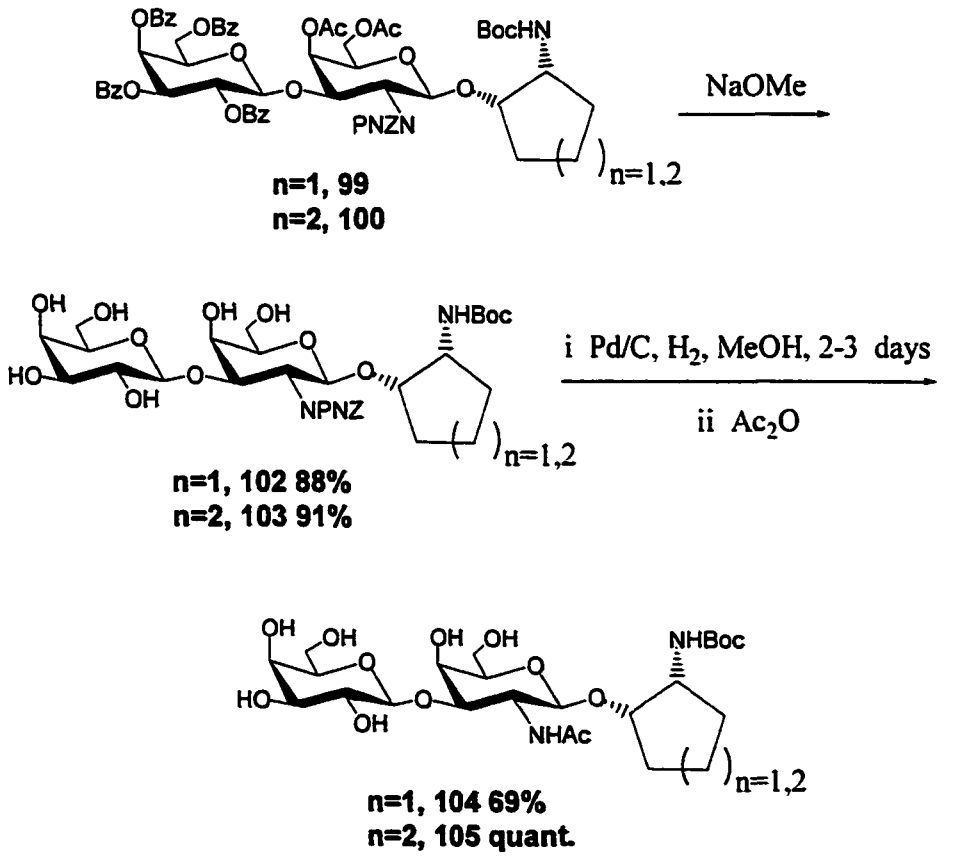
Unfortunately, attempts to remove the PNZ group from **99** under reductive conditions led to a mixture of products, with the major product being the *p*-acetamidobenzyl carbamate **101** (Scheme 4.11). The *p*-aminobenzyl intermediate was not eliminating as fast as it had with the flexible ethanolamine linker and was getting trapped during the acetylation step,



99 **101**

Scheme 4.11: Problems with the removal of the PNZ group

so another strategy was chosen. Since the presence of the *p*-aminobenzyl carbamate intermediate could be easily monitored by TLC with UV lamp detection, it was decided that the UV active benzoates, along with the acetates, would be removed before removing the PNZ group. This was carried out with little difficulty with both the cyclopentyl and cyclohexyl derivatives (Scheme 4.12). As suspected, hydrogenolysis of the PNZ group of both **102** and **103** required a few days to complete as monitored by TLC, being considerably more sluggish than the ethanolamine analogue, which reaches completion in a matter of hours.



Scheme 4.12: Removal of the PNZ and acetylation of the galactosamine

As soon as the acetamido group was in place (**104** and **105**), the plan would be to remove the Boc group and replace it with the Fmoc group. Unfortunately, problems were encountered at this stage. Unable to reproduce the relative success of the corresponding reaction with the flexible ethanolamine linker, all attempts at installing the Fmoc group on the cyclopentyl derivative failed.

A possible explanation would be the rigidity of the linker coupled with the proximity of the linker amine to the GalNAc acetamido group (Figure 4.4). The tetrahedral intermediate required for the Fmoc installation may be sterically precluded, preventing the successful derivatization of the linker amine. This is not only a severe problem for preparing Fmoc protected linker-disaccharides for loading onto a resin, but it also makes the synthesis of a small library with this approach quite challenging as well.

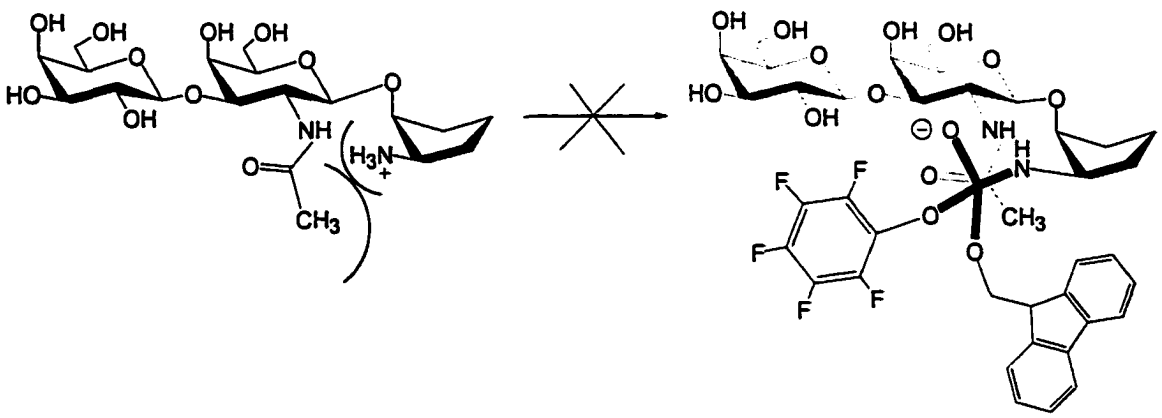


Figure 4.4: Possible explanation for difficulty in Fmoc installation

It was decided that although the rigid linker approach to a solid phase library seemed destined to fail, perhaps the flexible ethanolamine linker analogue could be used instead. The plan was then changed to use this flexible molecule to create a library using the solid

phase methodology as originally envisioned. If a member of the ethanolamine based library was found to be active, its rigid analogue would be synthesized directly from the Boc protected disaccharides using solution chemistry. The resulting product could then be assayed for activity.

Unfortunately, when the ethanolamine disaccharide was synthesized on a scale large enough to provide adequate product for loading onto the resin for synthesis of a small library, it became apparent that the insolubility of the Fmoc protected molecule was going to be a problem. This compound demonstrated no appreciable solubility in acetone, THF, water, DMF, or combinations of these solvents. Worse yet was its insolubility in pyridine, the solvent used during the resin loading step. All attempts to find suitable loading conditions failed. It seemed that the design of this approach to the solid phase-assisted synthesis of the disaccharide libraries was fundamentally doomed.

4.2.3 Discussion

"Hindsight is 20/20" is an adage that could easily be applied to the difficulties encountered in this project. While it is important to meet the challenges of predicting what may go wrong with an experimental plan, it is usually much easier to rationalize the shortcomings of these plans well after they have failed. This is indeed the case with the failures described in the preceding sections regarding the rigid linker disaccharides.

Some insight may be gained into the apparent inability to install the Fmoc group on the amino group of the cyclopentyl linker after considering the difficulty encountered during the removal of the PNZ from the GalNAc residue. This reaction, while normally quite facile, became very sluggish with the Boc group on the rigid linker in close proximity. This problem disappeared when the flexible ethanolamine analogue was subjected to the same reaction conditions. One explanation may be that the region between the GalNAc and the Boc group may be congested just enough to force the PNZ into a conformation unfavourable for ready elimination. Once the acetamido group is finally in place and the Boc group is removed, this congestion would be exacerbated when trying to establish Fmoc protection on the linker amine. As mentioned earlier, a bulky tetrahedral intermediate is required for Fmoc installation using the Fmoc pentafluorophenyl ester. The size of this intermediate is apparently not accommodated by the limited space available.

Similar congestion in this region was encountered in Part I of this thesis. If the cyclopentyl linker can really be considered to take the place of the galactose at the branching position, then perhaps it is valid to draw conclusions from observations made during the synthesis of the trisaccharide analogues. During the synthesis of the Galβ(1→3)GalNAcβ(1→4)Gal trisaccharide **2** containing the carboxymethyl substituent (Section 2.2.1.8), problems were observed during the removal of the PNZ that foreshadowed the problems encountered with the linker analogues. Again, longer reaction times were required for the removal step than for corresponding unhindered molecules, possibly due to steric interference from the proximal carboxymethyl moiety.

Another indication of the crowding at this position came from the persistence of the 2'-*O*-benzoate during deprotection of the Galβ(1→3)Galβ(1→4)Gal trisaccharide **5** (Section 2.2.2.2). Stirring in methanolic sodium methoxide for many days was necessary for complete removal of this hindered protecting group, presumably also as a result of the adjacent carboxymethyl group.

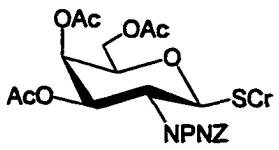
If these cues had been heeded, the futility of using this approach to synthesize these disaccharide libraries may have been foreseen. Unfortunately, the shortcomings of the plan were not realized until the Fmoc installation failed. At that point, the larger picture became quite clear, and the powers of hindsight kicked into gear.

Attempts to salvage this project using the flexible ethanolamine linker were thwarted by solubility problems of the final Fmoc protected ethanolamine disaccharide. Obstacles of this sort are not as easy to predict, but are nonetheless sometimes very difficult to overcome, as was the case in this instance.

4.2.3 Experimental

General methods used were as described in Chapter 2.

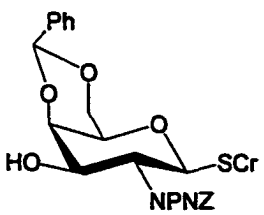
*Cresyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-(*p*-nitrobenzyloxycarbonyl)amino-1-thio-β-D-galactopyranoside* (**73**).



73

A solution of **46** (4.82 g, 9.15 mmol) and thiocresol (3.5 g, 28.2mmol) in anhydrous CH_2Cl_2 (50 ml) was cooled to 0°C. $BF_3{\cdot}OEt_2$ (4.5 ml, 37 mmol) was added and the reaction mixture was allowed to reach room temperature. Stirring continued for 20 hours, at which point the reaction was diluted with CH_2Cl_2, washed with 5% NaOH (twice) and brine, dried over Na_2SO_4 and concentrated. The β–product **73** was obtained by crystallizing the resulting syrup in 95% ethanol (3.5 g, 65 % yield). ^{1}H NMR ($CDCl_3$): δ 8.19, 7.49 (d, 2 H, J_{ortho} 9 Hz, Ar*H*), 7.37, 7.08 (d, 2 H, J_{ortho} 8.5 Hz, Ar*H*), 5.35 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.20 (broad s, 2 H, OCH_2PhNO_2), 4.15 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, $J_{6a,5}$ 7 Hz, H-6a), 4.08 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, $J_{6b,5}$ 6 Hz, H-6b), 2.32 (s, 3 H, $ArCH_3$), 2.10, 2.01 and 1.93 (3 s, 9 H, 3 x OAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.4, 170.3, 170.2 ($COCH_3$), 155.2 (C=O, PNZ), 147.7, 143.8, 138.5, 128.7 (aromatic quat.), 133.0, 129.7, 128.0, 123.8 (aromatic CH), 87.6 (C-1), 74.5, 71.0, 67.0 (C-3, C-4, C-5), 65.5, 61.7 (C-6, CH_2 PNZ), 51.4 (C-2), 21.2, 20.7 (CH_3, $COCH_3$). HR-ESMS calcd for $C_{27}H_{30}N_2O_{11}SNa$ ($M+Na^+$) 613.1468, found 613.1470.

Cresyl 4,6-di-O-benzylidene-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino–1–thio–β-D-galactopyranoside (**74**).

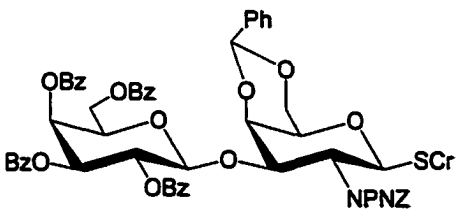


74

A suspension of the thioglycoside **73** (3.5 g, 5.9 mmol) in methanolic sodium methoxide (0.02 M, 100 ml) was stirred for 6 hours. The reaction was then neutralized using Amberlite IR-120(H+), filtered and evaporated to yield the deprotected

product in quantitative yield (2.75 g). This product was then suspended in dry CH_3CN, $PhCH(OMe)_2$ (2.2 ml, 14.5 mmol) was added then a catalytic amount of camphorsulfonic acid. This mixture was stirred overnight, then quenched with solid $NaHCO_3$. The reaction was concentrated and co-concentrated with toluene. The residue was chromatographed on silica gel (6:1→2:1 toluene:acetone) to yield benzylidene **74** (2.9 g, 88%). ^1H NMR ($CDCl_3$): δ 8.19 (2 H, J_{ortho} 9 Hz, Ar*H*), 7.3-7.6 (9 H, Ar*H*), 7.07 (d, 2 H, J_{ortho} 8.5 Hz, Ar*H*), 5.52 (s, 1 H, PhC*H*O_2), 5.18 (broad s, 2 H, OC*H*$_2$PhNO_2), 4.36 (dd, 1 H, $J_{6a,6b}$ 12.5 Hz, $J_{6a,5}$ 1.5 Hz, H-6a), 4.20 (dd, 1 H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1 Hz, H-4), 4.01 (dd, 1 H, $J_{6a,6b}$ 12.5 Hz, $J_{6b,5}$ 1.5 Hz, H-6b), 3.54 (1 H, $J_{5,6a}$1.5 Hz, $J_{5,6b}$ 1.5 Hz, $J_{4,5}$ 1 Hz, H-5), 2.31 (broad s, 3 H, ArC*H*$_3$). HR-ESMS calcd for $C_{28}H_{28}N_2O_8SNa$ ($M+Na^+$) 575.1464, found 575.1469.

Cresyl 3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-4,6-di-O-benzylidene-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-1-thio-β-D-galactopyranoside (**76**).

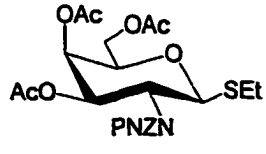


76

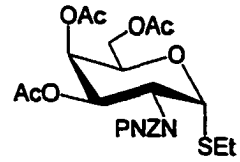
Acceptor **74** (21 mg, 0.038 mmol), donor Y (30 mg, 0.040 mmol) and AW 300 molecular sieves (150 mg) were stirred in anhydrous 1,2-dichloroethane under argon for 10 minutes. A solution of TESOTf (0.2 M in toluene, 20 µl, 0.004 mmol) was added via syringe. Stirring continued at room temperature for 30 minutes, then the reaction was quenched with pyridine, diluted with CH_2Cl_2, filtered through Celite and concentrated. Column chromatography (100:1→10:1→2:1

toluene:acetone) yielded the disaccharide **76** (11 mg, 26%). ^{1}H NMR ($CDCl_3$): δ 6.95-8.23 (33 H, Ar*H*), 5.95 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4'), 5.77 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 8 Hz, H-2'), 5.56 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3 Hz, H-3'), 5.43 (s, 1 H, PhC*H*O_2), 5.27 (d, 1-H, $J_{2,NH}$ 10 Hz, NH), 5.08 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 5.07 (d, 1 H, J_{gem} 14 Hz, OC*H*$_2$PhNO_2), 4.71 (dd, 1 H, $J_{6a',6b'}$ 11.5 Hz, $J_{6a',5'}$ 7.5 Hz, H-6a'), 4.44 (d, 1 H, J_{gem} 14 Hz, OC*H*$_2$PhNO_2), 4.28-4.50 (3 H, H-4, H-6b', H-5'), 4.22 (broad d, 1-H, $J_{6a,6b}$ 12 Hz, H-6a), 3.66 (broad d, 1-H, $J_{6a,6b}$ 12 Hz, H-6b), 3.46 (m, 1-H, H-2), 3.33 (broad s, 1 H, H-5), 2.29 (broad s, 3 H, ArC*H*$_3$). ^{13}C NMR (75 Hz, $CDCl_3$): δ 165.9, 165.5, 165.5, 167.9 (*C*OPh), 154.9 (C=O, PNZ), 143.9, 138.15, 138.0, 129.4, 128.9 (aromatic quat.), 133.6, 133.5, 133.3, 133.2, 130.0, 129.5, 129.7, 128.6, 128.6, 128.5, 128.3, 128.1, 127.5, 123.7 (aromatic CH), 102.2, 100.7 (C-1', Ph*C*H), 83.9 (C-1), 75.8, 71.8, 71.6, 70.0, 69.9, 68.2 (C-2', C-3, C-3', C-4, C-4, C-5, C-5'), 45.9, 41.5, 39.4 (C-6, C-6', CH_2 PNZ), 20.9 (CH_3, CO*C*H$_3$). HR-ESMS calcd for $C_{62}H_{54}N_2O_{17}SNa$ ($M+Na^+$) 1153.3041, found 1153.3029.

*Ethyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-(*p*-nitrobenzyloxycarbonyl)amino-1-thio-α-D-galactopyranoside* (**77a**) and *ethyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-(*p*-nitrobenzyloxycarbonyl)amino-1-thio-β-D-galactopyranoside* (**77b**).



77b

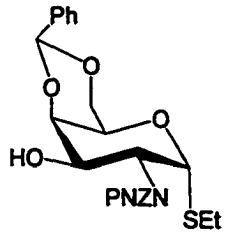


77a

A solution of **46** (7.7 g, 14.6 mmol) and ethanethiol (3.2 ml, 44 mmol) in anhydrous CH_2Cl_2 (100 ml) was cooled to 0°C, then $BF_3{\cdot}OEt_2$ (7.2 ml, 59 mmol) was slowly added via syringe. The resulting solution was stirred at room temperature for 23 hours.

The reaction was washed with saturated $NaHCO_3$, brine, dried over Na_2SO_4, followed by concentration and co-concentration with toluene. Column chromatography (50:1→15:1 toluene:acetone) yielded the α anomer **77a** in 22% yield (1.73 g) and the β anomer **77b** in 37% yield (2.86 g). Compound **77a**: ^{1}H NMR ($CDCl_3$): δ 8.21, 7.49 (2 H, J_{ortho} 9 Hz, Ar*H*), 5.47 (d, 1 H, $J_{1,2}$ 5.5 Hz, H-1), 5.36 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.17 (broad s, 2 H, O*C*H_2PhNO$_2$), 4.94-5.03 (2 H, N*H*, H-3), 4.55 (broad t, 1 H, H-5), 4.49 (ddd, 1H, $J_{2,3}$ 11.5 Hz, $J_{1,2}$ 5.5 Hz, $J_{2,NH}$ 9.5 Hz, H-2), 4.10 (2 H, H-6a, H-6b), 2. (dq, 2 H, J_{vic} 7.5 Hz, J_{gem} 7.5 Hz, CH_2CH$_3$), 1.93, 2.02 and 2.13 (3 s, 9 H, 3 x OAc), 1.28 (t, 3 H, J_{vic} 7.5 Hz, CH$_2$CH_3). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.6, 170.4, 170.2 (*C*OCH$_3$), 155.3 (C=O, PNZ), 143.6 (aromatic quat.), 128.0, 123.8 (aromatic CH), 85.2 (C-1), 68.8, 67.4 (C-3, C-4, C-5), 65.5, 61.8 (C-6, CH_2 PNZ), 50.0 (C-2), 25.5 (CH_2, ethyl), 20.7 (CO*C*H$_3$), 15.1 (CH_3, ethyl). HR-ESMS calcd for $C_{22}H_{28}N_2O_{11}SNa$ ($M+Na^+$) 551.1312, found 551.1310. Compound **77b**: ^{1}H NMR ($CDCl_3$): δ 8.18, 7.47 (2 H, J_{ortho} 9 Hz, Ar*H*), 5.37 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.18 (broad s, 2 H, O*C*H_2PhNO$_2$), 4.14 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, $J_{6a,5}$ 7 Hz, H-6a), 4.07 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, $J_{6b,5}$ 6.5 Hz, H-6b), 3.80-3.97, 2.71 (dq, 2 H, J_{vic} 7.5 Hz, J_{gem} 7.5 Hz, CH_2CH$_3$), 1.95, 2.02 and 2.13 (3 s, 9 H, 3 x OAc), 1.27 (t, 3 H, J_{vic} 7.5 Hz, CH$_2$CH_3). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.4, 170.2 (*C*OCH$_3$), 155.3 (C=O, PNZ), 147.7, 143.8 (aromatic quat.), 128.0, 123.8 (aromatic CH), 85.1 (C-1), 74.5, 71.2, 67.0 (C-3, C-4, C-5), 65.5, 61.6 (C-6, CH_2 PNZ), 51.6 (C-2), 24.6 (CH_2, ethyl), 20.7, 20.7 (CO*C*H$_3$), 14.9 (CH_3, ethyl).

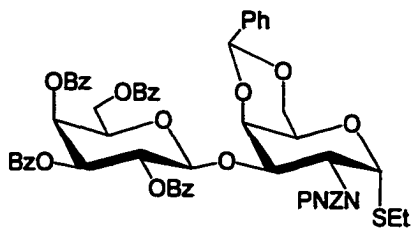
***Ethyl 4,6-di-O-benzylidene-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-1-thio-α-D-galactopyranoside* (78).**



78

Thioglycoside **77a** was deacetylated in methanolic NaOMe (0.005 M, 55 ml). After stirring at room temperature for two hours, the reaction was neutralized with Amberlite IR-120(H+), filtered and evaporated. The white solid obtained was suspended in dry CH_3CN (75 ml), $PhCH(OMe)_2$ (1.0 ml, 6.7 mmol) and a catalytic amount of camphorsulfonic acid were added. After stirring overnight, the reaction was quenched with solid $NaHCO_3$ and the solvents were removed under reduced pressure. Column chromatography (10:1→2:1 toluene:acetone) yielded the product **78** (0.81g, 66% overall yield). ^1H NMR ($CDCl_3$): δ 8.23 (2 H, J_{ortho} 9 Hz, Ar*H*), 7.3-7.6 (7 H, Ar*H*), 5.60 (s, 1 H, PhC*H*O_2), 5.56 (d, 1 H, $J_{1,2}$ 5 Hz, H-1), 5.21 (broad s, 2 H, OC*H*$_2$PhNO$_2$), 5.12 (d, 1 H, $J_{2,NH}$ 8.5 Hz, N*H*), 4.44 (ddd, 1H, $J_{2,3}$ 11 Hz, $J_{1,2}$ 5 Hz, $J_{2,NH}$ 8.5 Hz, H-2), 4.28 (dd, 1 H, $J_{6a,6b}$ 11 Hz, $J_{6a,5}$ 2 Hz, H-6a), 4.27 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4), 4.13 (dd, 1 H, $J_{6a,6b}$ 11 Hz, $J_{6b,5}$ 2 Hz, H-6b), 4.12 (1 H, $J_{5,6a}$ 2 Hz, $J_{5,6b}$ 2 Hz, $J_{4,5}$ 1 Hz, H-5), 3.71 (dd, 1 H, $J_{2,3}$ 11 Hz, $J_{3,4}$ 3.5 Hz, H-3), 2.66 (dq, 2 H, J_{vic} 7.5 Hz, J_{gem} 7.5 Hz, C*H*$_2$CH$_3$), 1.29 (t, 3 H, J_{vic} 7.5 Hz, CH$_2$C*H*$_3$). ^{13}C NMR (75 Hz, $CDCl_3$): δ 156.0 (C=O, PNZ), 147.7, 143.8, 137.4 (aromatic quat.), 129.4, 128.4, 128.1, 126.4, 123.8 (aromatic CH), 101.5 (PhCH), 85.6 (C-1), 75.4, 69.4, 63.6 (C-3, C-4, C-5), 69.5, 65.5 (C-6, CH_2 PNZ), 52.5 (C-2), 25.8 (CH_2, ethyl), 15.2 (CH_3, ethyl). HR-ESMS calcd for $C_{23}H_{26}N_2O_8SNa$ ($M+Na^+$) 513.1308, found 513.1312.

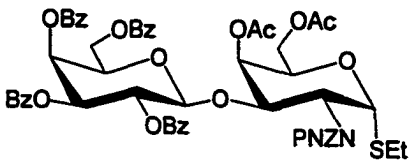
Ethyl 3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-4,6-di-O-benzylidene-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-1-thio-α-D-galactopyranoside (**79**).



79

A solution of acceptor **78** (199 mg, 0.406 mmol) and donor **51** (370 mg, 0.499 mmol) in anhydrous 1,2-dichloroethane (5 ml) was stirred at room temperature under argon atmosphere in the presence of AW 300 molecular sieves (0.6 g). A solution of TESOTf in toluene (0.2 M, 100 μl, 0.02 mmol) was added dropwise via syringe. Stirring continued for 15 minutes, then the reaction was quenched with the addition of pyridine. The mixture was diluted with CH_2Cl_2, filtered through Celite, concentrated, and immediately charged on a silica gel column. Chromatography (50:1→10:1 toluene:acetone) yielded the product **79** (316 mg, 75%). ^{1}H NMR ($CDCl_3$): δ 7.1-8.2 (29 H, Ar*H*), 6.00 (broad s, 1 H, H-4), 5.77-5.88 (2 H, H-2', H-4'), 5.57 (dd, 1 H, $J_{2',3'}$ 10 Hz, $J_{3',4'}$ 2.5 Hz, H-3'), 5.27-5.39 (2 H, H-1', PhC*H*O_2), 5.23 (d, 1 H, $J_{2,NH}$ 8 Hz, N*H*), 4.96 (d, 1 H, J_{gem} 14 Hz, OC*H*$_2$PhNO_2), 4.64-4.77 (2 H, H-5', OC*H*$_2$PhNO_2), 4.54 (m, 1 H, H-2), 4.10-4.22 (2 H, H-6a, H-6a'), 3.98 (broad s, 1 H, H-5), 3.80-3.92 (2 H, H-6b, H-6b'), 2.60 (dq, 2 H, J_{vic} 7.5 Hz, J_{gem} 7.5 Hz, C*H*$_2$CH_3), 1.25 (t, 3 H, J_{vic} 7.5 Hz, CH_2C*H*$_3$). ^{13}C NMR (75 Hz, $CDCl_3$): δ 166.0, 165.6, 165.5, 165.4 (*C*OPh), 155.3 (C=O, PNZ), 147.4, 144.2, 137.5 (aromatic quat.), 133.8, 133.5, 130.1, 129.8, 128.9, 128.8, 128.6, 128.5, 128.4, 128.1, 127.6, 126.2, 123.6, 129.3, 129.0, 128.7 (aromatic CH), 101.1, 100.7 (C-1', Ph*C*H), 85.2 (C-1), 74.2, 72.0, 69.5, 68.1, 63.5 (C-2', C-3, C-3', C-4, C-4, C-5, C-5'), 69.3, 64.8, 62.6 (C-6, C-6', CH_2 PNZ), 50.1 (C-2), 25.2 (CH_2, ethyl), 15.2 (CH_3, ethyl). HR-ESMS calcd for $C_{57}H_{52}N_2O_{17}$SNa (M+Na^+) 1091.2884, found 1091.2886.

Ethyl 4,6-di-O-acetyl-3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-1-thio-α-D-galactopyranoside (**80**).

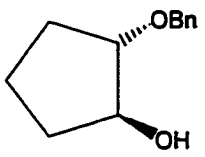


80

Disaccharide **79** (300 mg, 0.280 mmol) was stirred at 80°C in 80% aqueous acetic acid for 1 hour. The reaction was diluted with toluene and the solvents were removed under reduced pressure. The resulting crude product was stirred overnight in pyridine (15 ml) and acetic anhydride (10 ml). The reaction was concentrated and co-concentrated with toluene. Column chromatography of the residue (10:1 toluene:acetone) yielded the product **80** (271 mg, 91%). ^{1}H NMR ($CDCl_3$): δ 7.1-8.2 (24 H, Ar*H*), 5.97 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.77 (dd, 1H, $J_{2',3'}$ 10 Hz, $J_{1',2'}$ 8 Hz, H-2'), 5.54-5.63 (2 H, H-1, H-4'), 5.15 (d, 1 H, $J_{2,NH}$ 8.5 Hz, N*H*), 5.04 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 4.97 (d, 1 H, J_{gem} 13.5 Hz, OCH_2PhNO$_2$), 4.68 (dd, 1H, $J_{2,3}$ 11 Hz, $J_{1,2}$ 6 Hz, H-2), 4.60 (d, 1 H, J_{gem} 13.5 Hz, OCH_2PhNO$_2$), 4.32-4.52 (4 H, H-5, H-5', H-6a', H-6b'), 4.16 (dd, 1 H, $J_{6a,6b}$ 12 Hz, $J_{6a,5}$ 5 Hz, H-6a), 4.06 (dd, 1 H, $J_{6a,6b}$ 12 Hz, $J_{6b,5}$ 7.5 Hz, H-6b), 4.01 (dd, 1 H, $J_{2,3}$ 11 Hz, $J_{3,4}$ 3 Hz, H-3), 2.57 (dq, 2 H, J_{vic} 7.5 Hz, J_{gem} 7.5 Hz, CH_2CH$_3$), 2.15, 2.03 (2 s, 6 H, 2 x OAc), 1.21 (t, 3 H, J_{vic} 7.5 Hz, CH$_2$CH_3). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.3, 169.6 (*C*OCH$_3$), 165.9, 165.5, 165.3, 165.2 (*C*OPh), 155.0 (C=O, PNZ), 147.4, 143.8, 137.8 (aromatic quat.), 133.6, 133.3, 133.2, 130.1, 129.7, 129.7, 129.0, 128.6, 128.4, 128.3, 128.2, 128.2, 127.7, 125.2, 123.6 (aromatic CH), 100.5 (C-1'), 84.4 (C-1), 74.3, 71.7, 71.5, 69.6, 68.5, 68.0, 67.7 (C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 65.0, 62.4, 61.8 (C-6, C-6', CH$_2$ PNZ), 50.7 (C-2), 25.0

(CH_2, ethyl), 20.6 ($COCH_3$), 15.0 (CH_3, ethyl). HR-ESMS calcd for $C_{54}H_{52}N_2O_{19}SNa$ ($M+Na^+$) 1087.2783, found 1087.2782.

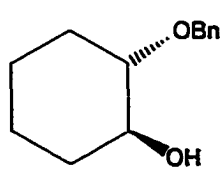
(1S,2S)-trans-*1*-O-*benzyl-2-cyclopentanediol* (**85**).



85

(1*S*,2*S*)-*trans*-1,2-cyclopentanediol **81** (485 mg, 4.75 mmol) was dissolved in dry DMF (50 ml) and the resulting solution was cooled to 0°C. Sodium hydride (217 mg, 60% dispersion in oil, 5.43 mmol, 1.14 equiv.) was added and the reaction mixture was stirred at room temperature under flow of argon for two hours. Benzyl bromide (720 ul, 6.05 mmol, 1.27 equiv.) was then added dropwise, and stirring was allowed to continue overnight. The reaction was quenched with glacial acetic acid, diluted with toluene and evaporated under reduced pressure. Column chromatography (50:1→10:1→1:1 toluene:acetone) yielded the benzyl ether **85** (340 mg, 37%). ^{1}H NMR ($CDCl_3$): δ 7.2-7.4 (5 H, Ar*H*), 4.52, 4.56 (d, 1 H, J_{gem} 11.5 Hz, $PhCH_2$), 4.14 (m, 1 H, C*H*OH), 3.76 (m, 1 H, C*H*OR), 2.47 (1 H, CHO*H*), 1.80-2.05, 1.45-1.75 (6 H, CH_2). HR-ESMS calcd for $C_{12}H_{16}O_2Na$ ($M+Na^+$) 215.1048, found 215.1044. $[\alpha]^{22}_D$ + 35.8 (*c* 0.01, $CHCl_3$).

(1S,2S)-trans-*1*-O-*benzyl-2-cyclohexanediol* (**86**).

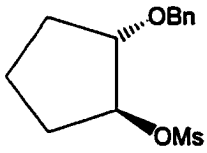


86

A solution of (1*S*,2*S*)-*trans*-1,2-cyclohexanediol **82** (680 mg, 5.85 mmol) in anhydrous DMF (60 ml) was cooled to 0°C. Sodium hydride (275 mg, 60% dispersion in oil, 6.88 mmol, 1.18 equiv.) was

added and the mixture was stirred at 0°C under a flow of argon for one hour. Benzyl bromide (850 μl, 7.15 mmol, 1.22 equiv.) was then added via syringe and the reaction mixture was stirred at room temperature for one hour. The reaction was quenched with glacial acetic acid and the solvents were removed under reduced pressure. Column chromatography (50:1→10:1→1:1 toluene:acetone) yielded the benzyl ether **86** (440 mg, 47%) and the starting diol **82** (130 mg, 19%). ^{1}H NMR ($CDCl_3$): δ 7.2-7.4 (5 H, Ar*H*), 4.68, 4.47 (d, 1 H, J_{gem} 11.5 Hz, Ph*C*H_2), 3.49 (m, 1 H, C*H*OH), 3.17 (m, 1 H, C*H*OR), 2.64 (1 H, CHO*H*), 2.13, 2.01, 1.62-1.80, 1.10-1.35 (8 H, CH_2). ^{13}C NMR (75 Hz, $CDCl_3$): δ 138.7 (aromatic quat.), 128.5, 127.8, 127.8 (aromatic CH), 83.6, 73.9 (*C*HOBn, *C*HOH), 70.8 (*C*H_2, OBn), 32.1, 29.2, 24.3, 24.0 (*C*H_2). HR-ESMS calcd for $C_{13}H_{18}O_2Na$ ($M+Na^+$) 229.1204, found 229.1205. $[\alpha]^{22}_D$ + 79.0 (*c* 0.01, $CHCl_3$).

(1S,2S)-trans-*2*-O-*benzyl-1*-O-*methanesulfonyl cyclopentanediol* (**87**).

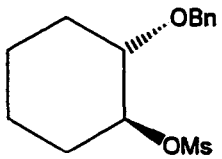


87

A solution of the cyclopentyl benzyl ether **85** (243 mg, 1.26 mmol), dry pyridine (1.0 ml, 12 mmol) and methanesulfonyl chloride (0.50 ml, 6.5 mmol) in anhydrous CH_2Cl_2 (10 ml) was stirred at room temperature overnight. The reaction was quenched with methanol, followed by dilution with toluene and concentration under reduced pressure. Column chromatography (100:1 toluene:acetone) yielded the mesylate **87** (300 mg, 88%). ^{1}H NMR ($CDCl_3$): δ 7.2-7.4 (5 H, Ar*H*), 5.00 (m, 1 H, C*H*OMs), 4.57, 4.59 (d, 1 H, J_{gem} 11.5 Hz, Ph*C*H_2), 4.08 (m, 1 H, C*H*OBn), 2.98 (s, 3 H, *C*H_3), 2.15, 2.02, 1.57, 1.65-1.92 (6 H, C*H*$_2$). ^{13}C NMR (75 Hz,

$CDCl_3$): δ 138.0 (aromatic quat.), 128.5, 127.8, 127.7 (aromatic CH), 86.4 (*C*HOMs), 83.8 (*C*HOBn), 71.6 (CH_2, OBn), 38.4 (CH_3), 30.6, 29.6, 21.0 (CH_2).

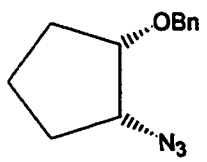
(1S,2S)-trans-*2*-O-*benzyl-1*-O-*methanesulfonyl cyclohexanediol* (**88**).



88

A solution of the benzyl ether **86** (320 mg, 1.55 mmol), dry pyridine (1.25 ml, 15.5 mmol) and methanesulfonyl chloride (600 ul, 7.75 mmol) in dry CH_2Cl_2 (15 ml) was stirred overnight. The work-up procedure was the same as for compound **87**. Column chromatography (100:1 toluene:acetone) yielded the mesylate **88** (414 mg, 94%). ^{1}H NMR ($CDCl_3$): δ 7.2-7.4 (5 H, Ar*H*), 4.64, 4.50 (d, 1 H, J_{gem} 11.5 Hz, Ph*C*H_2), 4.42 (ddd, 1 H, J = 4.5 Hz, 8.5 Hz, 10.5 Hz, C*H*OMs), 3.43 (ddd, 1 H, J = 4.5 Hz, 8.5 Hz, 10 Hz, C*H*OBn), 2.87 (s, 3 H, CH_3), 2.17, 1.71, 1.57, 1.14-1.38 (8 H, CH_2). HR-ESMS calcd for $C_{14}H_{20}O_4SNa$ ($M+Na^+$) 307.0980, found 307.0983.

(1S,2R)-cis-*1*-O-*benzyl-2-azido cyclopentanol* (**89**).

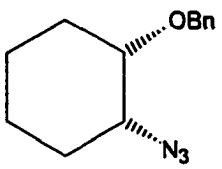


89

A solution of **87** (335 mg, 1.24 mmol), sodium azide (415 mg, 6.38 mmol) and 15-crown-5 (0.30 ml, 1.5 mmol) in anhydrous DMF (5 ml) was stirred overnight at 100°C. The reaction was then diluted with toluene and the solvents were removed in vacuo. Column chromatography (200:1 toluene:acetone) yielded the azide product **89** (228 mg, 85 %). ^{1}H NMR ($CDCl_3$): δ 7.2-7.5 (5 H, Ar*H*), 4.64 (dd, 2 H, , J_{gem} 12 Hz, Ph*C*H_2), 3.92 (1 H, C*H*OBn), 3.72 (1 H, C*H*N_3), 1.5-1.7, 1.8-2.0 (6 H, CH_2). ^{13}C NMR (125 Hz, $CDCl_3$):

δ 138.3 (aromatic quat.), 129.9, 128.4, 127.6 (aromatic CH), 81.5 (*C*HOBn), 71.7 (CH_2, OBn), 63.1 (CHN_3), 28.4, 27.8, 19.6 (CH_2). HR-ESMS calcd for $C_{12}H_{15}N_3ONa$ ($M+Na^+$) 240.1113, found 240.1112. $[\alpha]^{22}_D$ - 36.8 (*c* 0.01, $CHCl_3$).

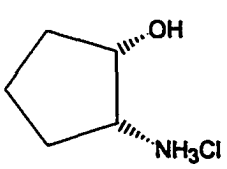
(1S,2R)-cis-*1*-O-*benzyl-2-azido cyclohexanol* (**90**).



90

Mesylate **88** (30 mg, 0.11 mmol), sodium azide (48 mg, 0.74 mmol), and 15-crown-5 (0.25 ml, 1.3 mmol) in dry DMF (1 ml) were stirred for 20 hours at 100°C. The reaction mixture was concentrated and co-concentrated with toluene, followed by chromatography (4:1→2:1 pentane:EtOAc) yielded the azide **90** (22 mg, 91%). ^1H NMR ($CDCl_3$): δ 7.2-7.4 (5 H, Ar*H*), 4.64 (dd, 2 H, , J_{gem} 12 Hz, PhCH_2), 3.56-3.67 (2 H, C*H*N_3, C*H*OBn), 1.85-2.02, 1.54-1.77, 1.26-1.45 (8 H, CH_2). ^{13}C NMR (75 Hz, $CDCl_3$): δ 138.6 (aromatic quat.), 128.4, 127.5 (aromatic CH), 77.5 (*C*HOBn), 61.0 (CHN_3), 70.6 (CH_2, OBn), 27.5, 27.5, 22.1, 21.7 (CH_2). HR-ESMS calcd for $C_{13}H_{17}N_3O_2Na$ ($M+Na^+$) 254.1269, found 254.1267. $[\alpha]^{22}_D$ – 24.9 (*c* 0.01, $CHCl_3$).

(1*S*,2*R*)-*cis*-cyclopentan-1-ol ammonium chloride (**91**).

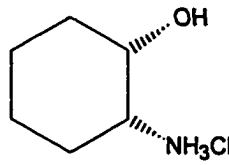


91

A solution of cyclopentyl azide **89** (228 mg, 1.04 mmol) in 95% ethanol (10 ml) and 1 M HCl (2.5 ml) was hydrogenated under flow of hydrogen on 10% Pd/C in 95% ethanol (5 ml) and 1 M HCl (0.5 ml). Filtration through a Millipore filter and evaporation of the

solvents, followed by column chromatography on an Iatrobead column (20:8:1 CH_2Cl_2:MeOH:NH_4OH) yielded the hydrochloride salt **91** in quantitative yield (141 mg). ^1H NMR (CD_3OD): δ 4.14 (1 H, C*H*OH), 3.34 (1 H, C*H*NH_3), 1.95-2.05 (6 H, CH_2).

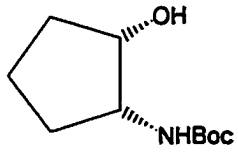
(1*S*,2*R*)-*cis*-cyclohexan-1-ol ammonium chloride (**92**).



92

Azide **90** (53 mg, 0.23 mmol) was hydrogenated under the same conditions used for compound **91**. The reaction mixture was filtered through a Millipore filter (0.22 μm), the filtrate was concentrated and the residue was chromatographed on an Iatrobead column (20:8:1 CH_2Cl_2:MeOH:NH_4OH) to yield the hydrochloride salt **92** in quantitative yield (35 mg). ^1H NMR (CD_3OD): δ 3.96 (1 H, C*H*OH), 3.16 (1 H, C*H*NH_3), 1.25-1.90 (8 H, CH_2).

(1S,2R)-cis-*2*-N-*Boc-cyclopentanol amine* (**93**).

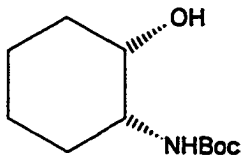


93

A solution of **91** (141 mg, 1.02 mmol), K_2CO_3 (480 mg, 3.47 mmol) and Boc_2O (590 mg, 2.70 mmol) in methanol (10 ml) was stirred overnight. The reaction mixture was concentrated and chromatography (10:1 toluene:acetone) yielded the Boc protected product **93** (167 mg, 81%). ^1H NMR ($CDCl_3$): δ 4.94 (1 H, N*H*Boc), 4.10 (1 H, C*H*NBoc), 3.75 (1 H, C*H*OH), 2.29(1 H, CHO*H*), 1.2-2.0 (6 H, CH_2), 1.43 (s, 9 H, tBu). ^{13}C NMR (75 Hz, $CDCl_3$): δ 156.0 (C=O), 79.4 (*C*(CH_3)), 72.6 (1 H, *C*HOH), 55.4 (1 H,

*C*HNBoc), 32.4, 29.1, 20.1 (CH_2), 28.4 (C(*C*H_3)). HR-ESMS calcd for $C_{10}H_{19}NO_3Na$ ($M+Na^+$) 224.1263, found 224.1262.

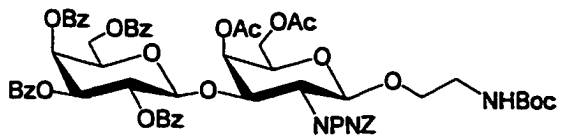
(1S,2R)-cis-2-N-Boc-cyclohexanol amine (**94**).



94

A solution of **92** (149 mg, 0.985 mmol), K_2CO_3 (383 mg, 2.77 mmol) and Boc_2O (248 mg, 1.14 mmol) in methanol (10 ml) was stirred overnight. The reaction mixture was concentrated and chromatography (10:1 toluene:acetone) yielded the Boc protected product **94** (178 mg, 83%). ^1H NMR ($CDCl_3$): δ 4.89 (1 H, N*H*Boc), 3.92 (1 H, C*H*NBoc), 3.59 (1 H, C*H*OH), 2.10 (1 H, CHO*H*), 1.2-1.8 (8 H, CH_2), 1.44 (s, 9 H, tBu). ^{13}C NMR (75 Hz, $CDCl_3$): δ 155.9 (C=O), 79.5 (*C*(CH_3)), 69.4 (1 H, *C*HOH), 52.1 (1 H, *C*HNBoc), 31.6, 27.5, 23.7, 20.0 (CH_2), 28.4 (C(*C*H_3)). HR-ESMS calcd for $C_{11}H_{21}N_1O_3Na$ ($M+Na^+$) 238.1419, found 238.1417.

1-(N-Boc-2-amino)ethyl 4,6-di-O-acetyl-3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside (**95**).



95

A solution of thioglycoside **79** (111 mg, 0.103 mmol) and Boc-ethanolamine (49 mg, 0.30 mmol) in anhydrous CH_2Cl_2 (2 ml) was stirred in the presence of AW

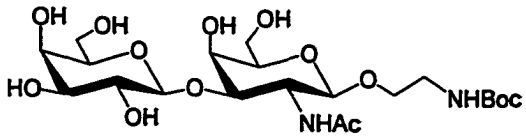
300 molecular sieves (250 mg) under argon atmosphere at room temperature for 10 minutes. A solution of silver trifluoromethane sulfonate (10 mg, 0.039 mmol) and N-iodosuccinimide (39 mg, 0.17 mmol) in 1 ml dry CH_3CN was added via syringe, and stirring was continued for another 15 minutes, at which point the reaction was quenched with triethylamine. The reaction mixture was diluted with CH_2Cl_2, filtered through Celite, washed successively with a saturated solution of sodium thiosulfate and brine. The organic layer was then dried over Na_2SO_4 and concentrated. Column chromatography (10:1→6:1 toluene:acetone) yielded the product **95** (108 mg, 89% yield). ^{1}H NMR ($CDCl_3$): δ 7.1-8.3 (24 H, ArH), 5.91 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 5.66 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 7.5 Hz, H-2'), 5.57 (broad d, 1 H, $J_{3,4}$ 3.5 Hz, H-4), 5.53 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 4.91 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.67 (dd, 1 H, $J_{6a',6b'}$ 11 Hz, $J_{5',6a'}$ 6.5 Hz, H-6a'), 4.35 (dd, 1 H, $J_{6a',6b'}$ 11 Hz, $J_{5',6b'}$ 7 Hz, H-6b'), 4.24 (broad t, 1 H, $J_{5',6a'}$ 6.5 Hz, $J_{5',6b'}$ 7 Hz, H-5'), 4.09 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, $J_{5,6a}$ 5.5 Hz, H-6a), 4.03 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, $J_{5,6b}$ 7.5 Hz, H-6b), 3.78 (broad t, H-5), 3.76, 3.59 (broad, CH_2NBoc), 3.22, 3.36 (OCH_2CH_2), 2.02 and 2.18 (2 s, 6 H, OAc), 1.39 (s, 9 H, tBu). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.5, 169.6 (*C*OCH_3), 166.0, 165.5, 165.4, 165.0 (*C*OPh), 155.9, 155.2 (C=O carbamate), 147.7, 143.6 (aromatic quat. PNZ), 133.7, 133.4, 133.3, 130.1, 129.8, 129.7, 129.1, 128.7, 128.5, 128.4, 128.3, 128.3, 127.8, 125.3, 123.8 (aromatic CH), 101.6 (C-1), 129.5, 129.3, 129.1, 128.7 (aromatic quat.), 75.2, 71.4, 71.3, 70.3, 69.0, 67.8 (C-3, C-4, C-5, C-2', C-3', C-4', C-5'), 69.6, 65.1, 62.5, 61.8 (C-6, C-6', *C*H_2OR, CH_2 PNZ), 54.8 (C-2), 40.5 (*C*NBoc), 29.6 (*C*(CH_3)), 28.4 (C(*C*H_3)), 20.8, 20.7 (CO*C*H_3). HR-ESMS calcd for $C_{59}H_{61}N_3O_{22}Na$ ($M+Na^+$) 1186.3644, found 1186.3638.

1-(N-Boc-2-amino)ethyl 2-acetamido-4,6-di-O-acetyl-3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-2-deoxy-β-D-galactopyranoside (**96**).

96

A solution of the PNZ protected sugar **95** (65 mg, 0.056 mmol) in 10 ml absolute ethanol was hydrogenolyzed over palladium on charcoal under flow of hydrogen gas overnight. Acetic anhydride (1 ml) was added and the reaction mixture was stirred at room temperature for another 4 hours. This mixture was filtered through a Millipore filter (0.22 μm), concentrated and chromatographed on 6:1 toluene:acetone to yield the acetamido product **96** (32 mg, 56% yield). ^{1}H NMR ($CDCl_3$): δ 7.1-8.3 (24 H, ArH), 5.94 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 5.69 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 7.5 Hz, H-2'), 5.60 (broad d, 1 H, $J_{3,4}$ 3.5 Hz, H-4), 5.58 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 5.37 (d, 1 H, NH), 5.04 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1'), 4.94 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.75 (dd, 1 H, $J_{2',3'}$ 11 Hz, $J_{3',4'}$ 3.5 Hz, H-3), 4.67 (dd, 1 H, $J_{6a',6b'}$ 11 Hz, $J_{5',6a'}$ 6.5 Hz, H-6a'), 4.38 (dd, 1 H, $J_{6a',6b'}$ 11 Hz, $J_{5',6b'}$ 7 Hz, H-6b'), 4.29 (broad t, 1 H, $J_{5',6a'}$ 6.5 Hz, $J_{5',6b'}$ 7 Hz, H-5'), 3.97-4.11 (2 H, H-6a, H-6b), 3.82 (broad t, H-5), 3.78, 3.62 (broad, CH_2NBoc), 3.14-3.35 (OCH_2CH_2), 2.03 and 2.20 (2 s, 6 H, OAc), 1.41 (s, 9 H, tBu). LR-ESMS calcd for $C_{53}H_{58}N_2O_{19}Na$ ($M+Na^+$) 1049.4, found 1049.4.

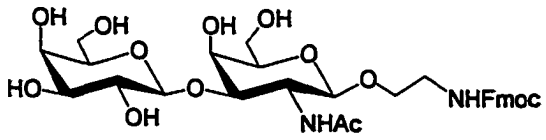
1-(N-Boc-2-amino)ethyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-β-D-galactopyranosyl (**97**).



97

A solution of the protected disaccharide **96** (30 mg, 0.029 mmol) in 0.01 M sodium methoxide in methanol was stirred for 1 hour. The reaction was then neutralized with Amberlite IR-120 (H+), filtered, and concentrated. Column chromatography (9:1→4:1 CH_2Cl_2:MeOH, Iatrobeads) yielded the deprotected product **97** in quantitative yield (14 mg). ^{1}H NMR (CD_3OD): δ 4.46 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1'), 4.32 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.09 (broad d, $J_{3,4}$ 3 Hz, H-4), 4.00 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 8.5 Hz, H-2'), 3.81 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 3.65-3.86 (6 H, H-3', H-5', H-6a, H-6b, H-6a', H-6b'), 3.46-3.62 (H-2, H-5, C*H*$_2$NBoc), 3.44 (dd, 1 H, $J_{2,3}$ 10 Hz, $J_{3,4}$ 3 Hz, H-3), 3.22 (broad t, 2 H, C*H*$_2$OR), 1.97 (s, 3 H, NHAc), 1.42 (9 H, tBu). HR-ESMS calcd for $C_{21}H_{38}N_2O_{13}Na$ ($M+Na^+$) 549.2272, found 549.2274.

1-(N-Fmoc-2-amino)ethyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-β-D-galactopyranosyl (**98**).

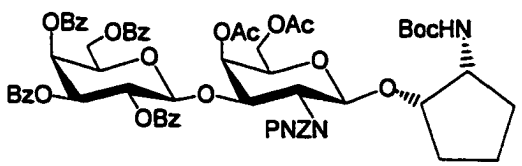


98

Boc-protected disaccharide **96** (13 mg, 0.025 mmol) was stirred in 4 ml of a 5% solution of trifluoroacetic acid in CH_2Cl_2 for 60 minutes, after which the reaction mixture was diluted with 10 ml dry toluene and concentrated. The crude residue was dissolved in methanol (5 ml), and Fmoc-OPfp (17 mg 0.042 mmol) and K_2CO_3 (17 mg, 0.12 mmol) were added. After stirring at room temperature for 30 minutes, the solvents were removed under reduced pressure, and the

crude product was purified by column chromatography (10:1→4:1 CH_2Cl_2:MeOH, Iatrobeads) to yield the Fmoc protected **97** (9 mg, 56% yield). ^{1}H NMR (CD_3OD): δ 7.79, 7.65, 7.38, 7.31 (Ar*H*), 4.46 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1'), 4.35 (2 H, OCH_2 (Fmoc)), 4.31 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.20 (1 H, CH (Fmoc)), 4.09 (broad d, H-4), 3.99 (dd, 1H, H-2'), 3.80 (broad d, 1 H, H-4'), 3.65-3.80 (5 H, H-5', H-6a, H-6b, H-6a', H-6b'), 3.45-3.63 (H-2, H-3', H-5, C*H*$_2$NBoc), 3.43 (dd, 1 H, $J_{2,3}$ 10 Hz, $J_{3,4}$ 3 Hz, H-3), 1.91 (s, 3 H, NHAc). HR-ESMS calcd for $C_{31}H_{40}N_2O_{13}Na$ ($M+Na^+$) 671.2428, found 671.2424.

*1-(1*S*,2*R*)-*cis*-N-*Boc-2-aminocyclopentyl 4,6-di*-O-*acetyl-3*-O-*(2,3,4,6-tetra*-O-*benzoyl-β-*D*-galactopyranosyl)-2-deoxy-2-(*p*-nitrobenzyloxycarbonyl)amino-β-*D*-galactopyranoside* (**99**).

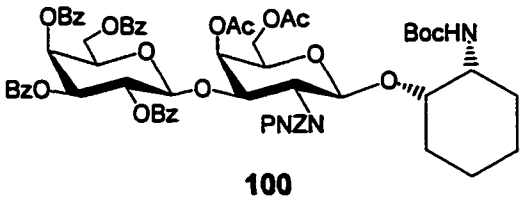


99

Disaccharide donor **79** (780 mg, 0.732 mmol) and Boc-cyclopentanolamine acceptor **93** (129 mg, 0.641 mmol) were stirred in 10 ml anhydrous CH_2Cl_2 under argon in the presence of 1.7 g AW 300 molecular sieves. A solution of AgOTf (46 mg, 0.18 mmol) and NIS (250 mg, 1.11 mmol) in 2 ml anhydrous CH_3CN was added via syringe. After 10 minutes, the reaction was quenched and underwent a work-up procedure as described for compound **95**. Column chromatography (10:1→6:1 toluene:acetone) yielded disaccharide **99** (484 mg, 63% yield). ^{1}H NMR ($CDCl_3$): δ 7.1-8.3 (24 H, ArH), 5.91 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 5.66 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 7.5 Hz, H-2'), 5.55 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.53 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 4.92 (d, 1 H,

$J_{1,2}$ 8 Hz, H-1) , 4.67 (dd, 1 H, $J_{6a'6b'}$ 11 Hz, $J_{6a'5'}$ 6.5 Hz, H-6a'), 4.35 (dd, 1 H, $J_{6a'6b'}$ 11 Hz, $J_{6b'5'}$ 7 Hz, H-6b'), 4.24 (dt, 1 H, $J_{6a'5'}$ 6.5 Hz, $J_{6b'5'}$ 7 Hz, H-5'), 4.00-4.12 (2 H, H-6a, H-6b), 3.89 (broad, C*H*NBoc), 3.73 (broad t, H-5), 3.45 (broad, C*H*OR), 2.02 and 2.18 (2 s, 6 H, OAc), 1.63-1.95, 1.25-1.57 (15 H, tBu, CH_2 cyclopentane). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.5, 169.7 (*C*OCH_3), 166.0, 165.6, 165.4, 165.0 (*C*OPh), 155.3 (carbonyl PNZ), 147.7, 143.7 (aromatic quat. PNZ), 133.7, 133.4, 133.3, 130.1, 129.8, 129.7, 128.7, 128.6, 128.4, 128.3, 127.9, 123.7 (aromatic CH), 129.5, 129.4, 129.1, 128.7 (aromatic quat.), 101.6 (C-1), 71.4, 70.2, 68.9, 67.8 (C-3, C-4, C-5, C-2', C-3', C-4', C-5', *C*HOR), 65.2, 62.4, 61.8 (C-6, C-6', CH_2 PNZ), 54.9 (C-2), 28.5 (tBu), 20.8, 20.7 (CO*C*H_3). HR-ESMS calcd for $C_{62}H_{65}N_3O_{22}Na$ ($M+Na^+$) 1226.3957, found 1226.3959.

*1-(1*S*,2*R*)-*cis*-N-Boc-2-aminocyclohexyl 4,6-di-O-acetyl-3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside* (**100**).

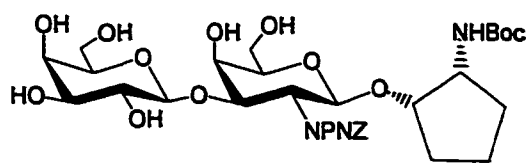


100

Thioglycoside donor **79** (739 mg, 0.694 mmol), Boc-cyclohexanolamine acceptor **94** (133 mg, 0.618 mmol) and AW 300 molecular sieves (1.35 g) were stirred in 10 ml dry CH_2Cl_2 for 10 minutes under argon atmosphere. A solution of NIS (238 mg, 1.06 mmol) and AgOTf (40 mg, 0.16 mmol) in dry CH_3CN (2 ml) was added via syringe. Stirring continued for 15 minutes, then the reaction was worked up according to the method used for compound **95**. Column chromatography (10:1 toluene:acetone) yields a mixture of

product and starting acceptor which is acetylated in pyridine (10 ml) and acetic anhydride (8 ml). Concentration and co-concentration with toluene, followed by column chromatography on 20:1→10:1→6:1 toluene:acetone yields the final cyclohexylamine product **100** (444 mg, 59% yield). ^{1}H NMR ($CDCl_3$): δ 7.1-8.3 (24 H, ArH), 5.91 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 5.66 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 7.5 Hz, H-2'), 5.54 (broad d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.53 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 4.92 (2 H, H-1, NH), 4.67 (dd, 1 H, $J_{6a',6b'}$ 11 Hz, $J_{6a',5'}$ 6.5 Hz, H-6a'), 4.35 (dd, 1 H, $J_{6a',6b'}$ 11 Hz, $J_{6b',5'}$ 7 Hz, H-6b'), 4.24 (dt, 1 H, $J_{6a',5'}$ 6.5 Hz, $J_{6b',5'}$ 7 Hz, H-5'), 4.09 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, $J_{6a,5}$ 7.5 Hz, H-6a), 4.01 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, $J_{6b,5}$ 5.5 Hz, H-6b), 3.67-3.76 (H-5, C*H*NBoc), 3.49 (broad, C*H*OR), 1.99 and 2.17 (2 s, 6 H, OAc), 1.1-1.7 (17 H, tBu, CH_2 cyclohexane). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.5, 169.8 (*C*OCH_3), 166.0, 165.6, 165.4, 165.0 (*C*OPh), 155.4 (carbonyl PNZ), 147.7, 143.7 (aromatic quat. PNZ), 133.7, 133.4, 133.3, 130.1, 129.8, 129.7, 128.7, 128.6, 128.4, 128.3, 128.0, 127.8, 123.8 (aromatic CH), 101.7 (C-1), 1287, 128.6, 128.4, 128.3 (aromatic quat.), 79.0, 77.3, 74.6, 71.4, 71.3, 70.3, 69.0, 67.8 (C-3, C-4, C-5, C-2', C-3', C-4', C-5', *C*HOR), 65.2, 62.4, 61.8 (C-6, C-6', CH_2 PNZ), 55.0 (C-2), 28.5 (tBu), 20.9, 20.7 (CO*C*H_3). HR-ESMS calcd for $C_{63}H_{67}N_3O_{22}Na$ ($M+Na^+$) 1240.4114, found 1240.4100.

*1-(1*S*,2*R*)-*cis*-N-Boc-2-aminocyclopentyl 2-deoxy-3-O-(β-D-galactopyranosyl)-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside* (**102**).

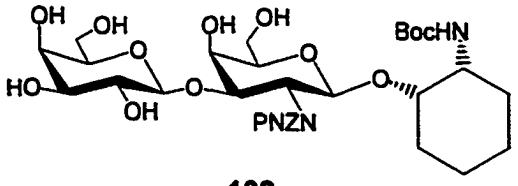


102

Compound **99** (310 mg, 0.257 mmol) was deacylated in 0.02 M methanolic sodium

methoxide. The reaction was neutralized and the reaction workup was carried out as for compound **97**. Column chromatography of the crude product (9:1→4:1 CH_2Cl_2:MeOH, Iatrobeads) yielded the PNZ protected disaccharide **102** (148 mg, 88% yield). ^{1}H NMR (CD_3OD): δ 8.23, 7.62 (d, 2 H, J_{ortho} 9 Hz, ArC*H*), 5.78 (1 H, N*H*Boc), 5.27 (m, 2 H, OCH_2PhNO_2), 4.48 (broad, H-1'), 4.39 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.10 (broad s, H-4), 4.03 (m, 1 H, C*H*NBoc), 3.81 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 3.63-3.79 (7 H, H-2', H-3', H-6a, H-6b, H-6a', H-6b', C*H*OR), 3.56 (dd, 1H, $J_{2,3}$ 10 Hz, $J_{1,2}$ 8 Hz, H-2), 3.45-3.53 (H-5, H-5'), 3.44 (dd, 1 H, $J_{2,3}$ 10 Hz, $J_{3,4}$ 3.5 Hz, H-3), 1.70-2.15, 1.25-1.60 (15 H, tBu, CH_2 cyclopentane). ^{13}C NMR (75 Hz, $CDCl_3$): δ 158.8, 158.0 (C=O carbamate), 148.9, 146.2, 129.1, 124.6, 122.3 (aromatic), 106.3, 104.0 (C-1, C-1'), 83.4, 80.9, 80.4, 76.8, 76.2, 74.7, 72.6, 70.3, 69.3, 66.3, 62.6, 62.3 (C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-6, C-6', *C*HOR, CH_2 PNZ), 56.0, 55.1 (C-2, *C*NBoc), 31.7, 30.5, 20.9 (CH_2 cyclopentane), 28.8 (tBu). HR-ESMS calcd for $C_{30}H_{45}N_3O_{16}Na$ ($M+Na^+$) 726.2698, found 726.2688.

*1-(1S,2R)-*cis*-N-Boc-2-aminocyclohexyl 2-deoxy-3-O-(β-D-galactopyranosyl)-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside* (**103**).

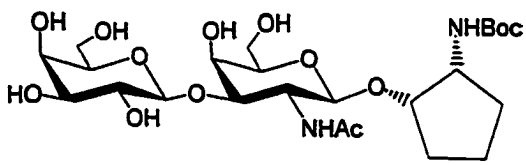


103

Compound **103** (430 mg, 0.352 mmol) was deacylated using the same procedure used for compound **97**. Column chromatography (9:1→4:1 CH_2Cl_2:MeOH, Iatrobeads) yielded the PNZ protected disaccharide **103** (229 mg, 91% yield). ^{1}H NMR (CD_3OD): δ 8.22 (d, 2 H, J_{ortho} 9 Hz, ArC*H*), 7.63 (d, 2 H, J_{ortho} 9 Hz, ArC*H*), 5.66 (1 H, N*H*Boc),

5.26 (m, 2 H, OCH_2PhNO$_2$), 4.52 (broad, H-1'), 4.38 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.11 (broad s, H-4), 3.81 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 3.65-3.87 (7 H, H-2', H-3', H-6a, H-6b, H-6a', H-6b', C*H*OR, C*H*NBoc), 3.57 (dd, 1H, $J_{2,3}$ 10 Hz, $J_{1,2}$ 8 Hz, H-2), 3.49, 3.48 (broad t, H-5, H-5'), 3.45 (dd, 1 H, $J_{2,3}$ 10 Hz, $J_{3,4}$ 3.5 Hz, H-3), 1.15-1.75, 2.00-2.15 (17 H, tBu, CH_2 cyclohexane). ^{13}C NMR (125 Hz, $CDCl_3$): δ 158.8, 157.5 (C=O carbamate), 148.9, 146.2, 129.1, 124.6, 122.3 (aromatic), 106.4, 103.8 (C-1, C-1'), 81.0, 80.4, 79.7, 76.8, 76.2, 74.7, 72.6, 70.3, 69.3, 66.3, 62.6, 62.3 (C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-6, C-6', *C*HOR, CH_2 PNZ), 55.1, 53.4 (C-2, *C*NBoc), 32.0, 29.0, 25.4, 20.8 (CH_2 cyclohexane), 28.8 (tBu). HR-ESMS calcd for $C_{31}H_{47}N_3O_{16}Na$ (M+Na$^+$) 740.2854, found 740.2842.

*1-(1S,2R)-*cis-N-*Boc-2-aminocyclopentyl 2-acetamido-2-deoxy-3-*O-*(β-D-galactopyranosyl)-β-D-galactopyranoside* (**104**).

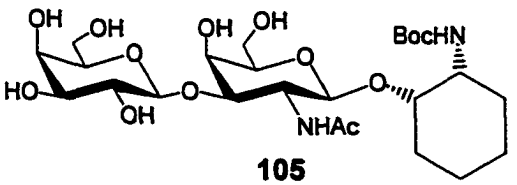


104

The PNZ group on compound **102** was hydrogenolyzed by stirring a solution of **102** (148 mg, 0.210 mmol) in methanol (10 ml) under a static atmosphere of hydrogen gas over Pd/C for two days, until TLC no longer indicated the presence of a UV active intermediate. Acetic anhydride was added directly to the reaction mixture, and stirring continued overnight. The reaction mixture was filtered and concentrated as for compound **96**. Column chromatography (4:1 CH_2Cl_2:MeOH) yielded the product **104** in 69% yield (82 mg). ^{1}H NMR (CD_3OD): δ 5.66 (1 H, N*H*Boc), 4.47 (d, 1 H, $J_{1',2'}$ 8.5 Hz,

H-1'), 4.33 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.10 (broad d, $J_{3,4}$ 3 Hz, H-4), 4.01 (dd, 1 H, $J_{1',2'}$ 8.5 Hz, $J_{2',3'}$ 10.5 Hz, H-2'), 3.97 (m, 1 H, C*H*NBoc), 3.81 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 3.65-3.82 (6 H, H-3', H-6a, H-6b, H-6a', H-6b', C*H*OR), 3.54 (dd, 1H, $J_{2,3}$ 9.5 Hz, $J_{1,2}$ 7.5 Hz, H-2), 3.46-3.53 (H-5, H-5'), 3.44 (dd, 1 H, $J_{2,3}$ 10.5 Hz, $J_{3,4}$ 3.5 Hz, H-3), 2.03 (s, 3 H, NHAc), 1.24-1.65, 1.70-2.00, 2.06-2.21 (15 H, tBu, CH_2 cyclopentane). ^{13}C NMR (75 Hz, $CDCl_3$): δ 174.5 (*C*OCH_3), 157.7 (C=O carbamate), 106.6, 104.4 (C-1, C-1'), 84.2, 81.4, 80.5, 76.8, 76.2, 74.7, 72.5, 70.3, 69.3, 66.3, 62.6, 62.3 (C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-6, C-6', *C*HOR, CH_2 PNZ), 55.9, 53.4 (C-2, *C*NBoc), 32.0, 30.6, 21.0 (CH_2 cyclopentane), 28.8 (tBu), 23.6 (CO*C*H_3). HR-ESMS calcd for $C_{24}H_{42}N_2O_{13}Na$ ($M+Na^+$) 589.2585, found 589.2580.

*1-(1S,2R)-*cis-N*-Boc-2-aminocyclohexyl 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-β-D-galactopyranoside* (**105**).



105

PNZ disaccharide **103** (184 mg, 0.256 mmol) was hydrogenolyzed as for compound **104**. After 3 days, there was no longer any UV active intermediate apparent by TLC. Workup conditions were also those used for compound **104**. Column chromatography (19:1→4:1 CH_2Cl_2:MeOH, Iatrobeads) yielded the product **105** in quantitative yield (147 mg). ^{1}H NMR (CD_3OD): δ 5.58 (1 H, N*H*Boc), 4.48 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1'), 4.34 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.11 (broad d, $J_{3,4}$ 3 Hz, H-4), 3.82 (broad d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 3.65-3.80 (7 H, H-2', H-3', H-6a, H-6b, H-6a', H-6b', C*H*OR, C*H*NBoc), 3.55 (dd, 1H, $J_{2,3}$ 10 Hz, $J_{1,2}$ 8 Hz, H-2), 3.40-3.53 (H-5, H-5'),

3.44 (dd, 1 H, $J_{2,3}$ 10 Hz, $J_{3,4}$ 3 Hz, H-3), 2.03 (s, 3 H, NHAc), 1.10-1.65, 2.05-2.15 (17 H, tBu, CH_2 cyclohexane). ^{13}C NMR (75 Hz, $CDCl_3$): δ 174.5 ($COCH_3$), 157.3 (C=O carbamate), 106.6, 104.1 (C-1, C-1'), 81.4, 80.7, 76.7, 76.1, 74.7, 72.4, 70.2, 69.2 (C-3, C-4, C-5, C-2', C-3', C-4', C-5', *C*HOR), 80.5, 62.6, 62.3 (C-6, C-6', CH_2 PNZ), 53.5, 53.2 (C-2, *C*NBoc), 31.8, 29.0, 25.4, 20.7 (CH_2 cyclohexane), 28.8 (tBu), 23.6 ($CO\mathit{C}H_3$). HR-ESMS calcd for $C_{25}H_{44}N_2O_{13}Na$ ($M+Na^+$) 603.2741, found 603.2736.

Chapter 5:

Synthesis of Galβ(1→3)Gal(2-*N*-acyl) Inhibitor Library

5.1 Introduction to Galβ(1→3)Gal(2-*N*-acyl) library

Since attempts to create a library of potential inhibitors using the linker method were unsuccessful (Chapter 4), another approach to constructing a library was explored. As mentioned earlier in this thesis, the terminal galactose is required for CT binding, and replacement of the acetamido group on the GalNAc residue with a hydroxyl group leads to a noticeable decrease in binding. With these facts in mind, a small disaccharide library of potential inhibitors was conceived in which the terminal disaccharide of GM_1, Galβ(1→3)GalNβOMe, acts as the scaffold. Derivatization of the galactosamine nitrogen with different acylating agents provides the structural diversity, maintaining the amide carbonyl of the native acetamido functionality, but replacing the acetamido methyl with a wide variety of groups (Figure 5.1). These will include both hydrophobic aromatic and alkyl substituents, as well as polar functionalities capable of acting as hydrogen bond donors and acceptors. The broad range of groups presented by the carbohydrate derivatives can probe the binding site in an attempt to discover possible favourable interactions.

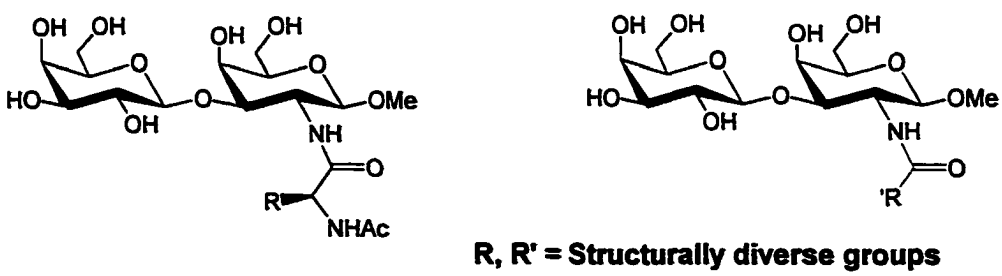


Figure 5.1: Galβ(1→3)Gal(2-*N*-acyl) inhibitor library

It was decided that the most efficient method for the synthesis of the small library would be using solid phase methods. This provided some useful experience with solid phase methods as well as the opportunity to investigate the utility of the *p*-nitrobenzyloxycarbonyl (PNZ) substituent as a temporary protecting group for the amine and its compatibility with solid phase manipulations.

5.2 Synthesis of Galβ(1→3)GalNPNZβOMe disaccharide

The *N*-acylated disaccharide library was created using the retrosynthetic approach outlined in Figure 5.2. The PNZ protected disaccharide was first assembled, then the

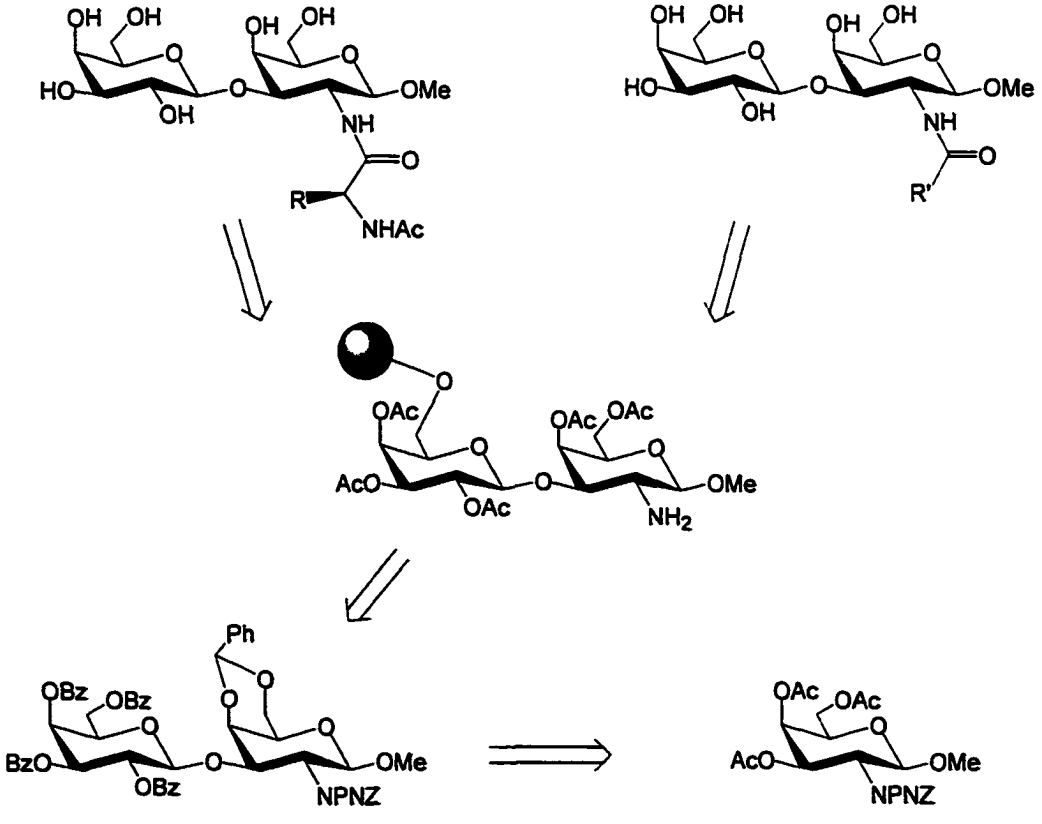
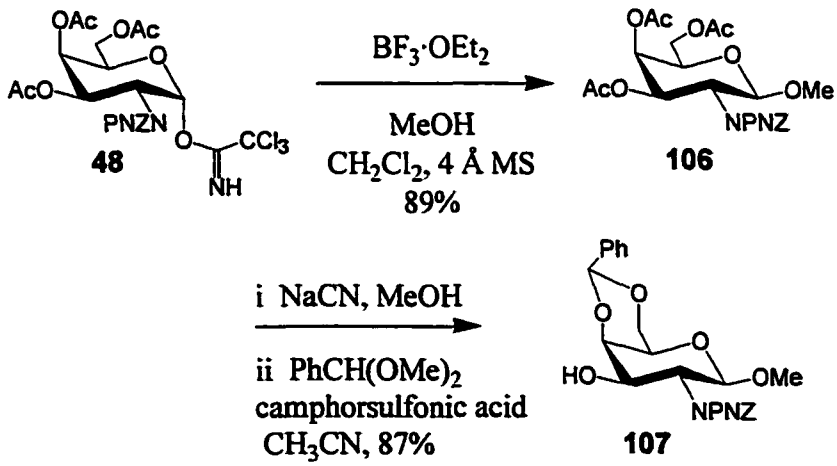


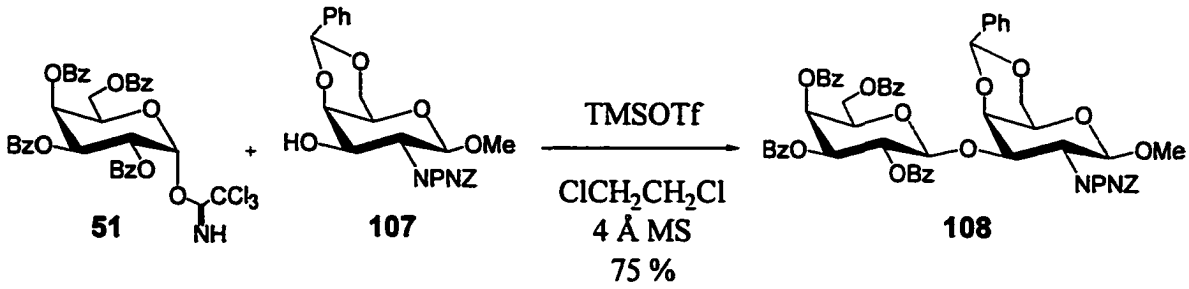
Figure 5.2: Retrosynthesis of N-acylation libraries

hydroxyl protecting groups were removed. This provided the deprotected disaccharide for loading onto the trityl resin. Once the resin was derivatized, the carbohydrate substituent was *O*-acetylated to prevent inadvertent *O*-acylation during any subsequent *N* -acylation steps. The PNZ protection was then removed and the resulting amine was reacted with a number of acylating agents, including activated ester amino acid derivatives, acyl chloride and anhydride reagents.

Previous experience in building the Galβ(1→3)GalNAcβOAll disaccharide (Section 2.3.1.6) suggested that the Galβ(1→3)GalNPNZβOMe **110** could be easily made using an analogous approach. The β-methyl glycoside **106** was synthesized in good yield from the imidate donor **48** and methanol using $BF_3{\cdot}OEt_2$ activation (89% yield) (Scheme 5.1). The product **106** was deacetylated with sodium cyanide in methanol and benzylidenated with benzaldehyde dimethylacetal to provide **107** in 87% yield over two steps. Acceptor **107** was then glycosylated using the benzoylated imidate donor **51** and TMSOTf activation to provide the disaccharide **108** in coupling yield of 75% (Scheme 5.2).

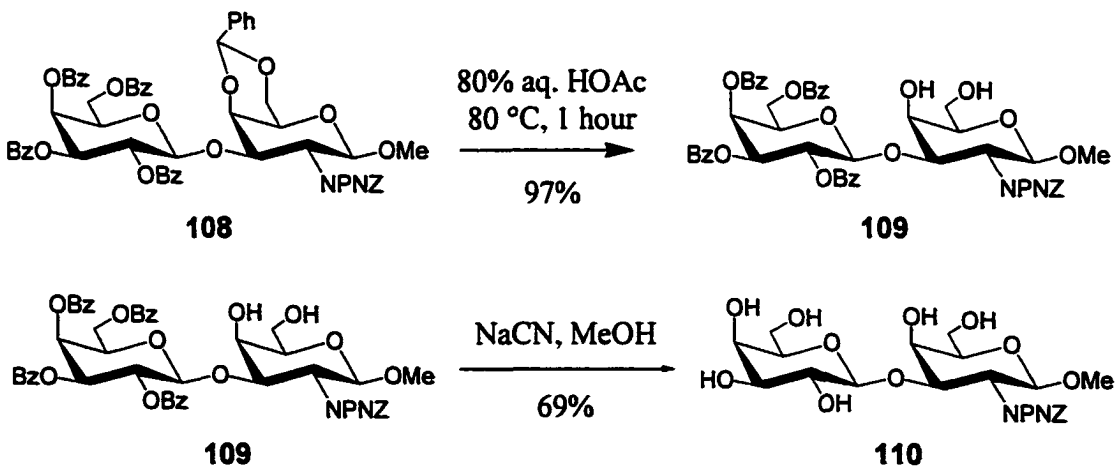


Scheme 5.1: Synthesis of PNZ protected methyl glycoside acceptor **107**



Scheme 5.2: Synthesis of disaccharide **108**

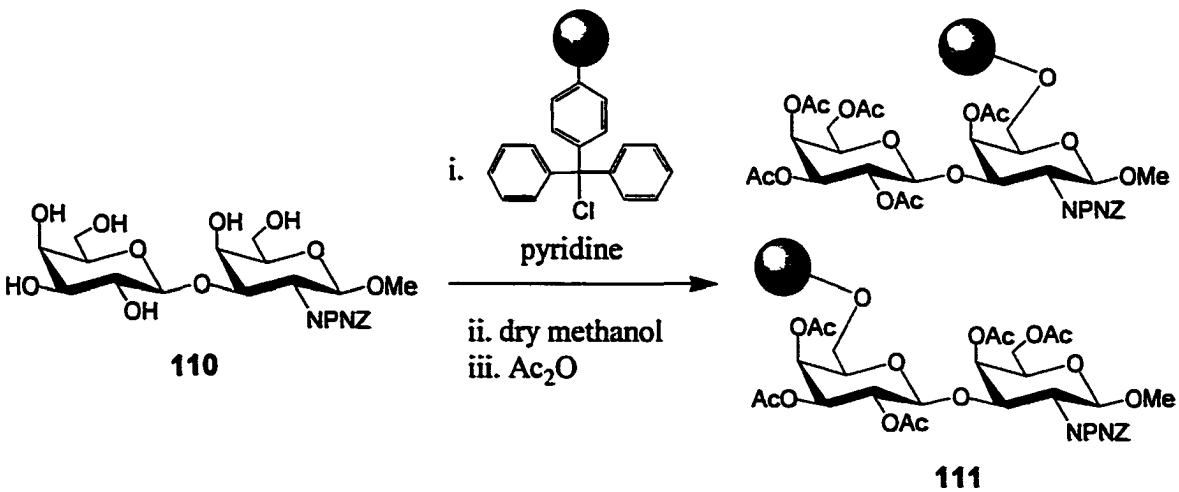
Deprotection of the disaccharide **108** began with removal of the benzylidene in 97% yield by stirring in 80% aqueous acetic acid at 80°C for one hour (Scheme 5.3). The resulting diol **109** was debenzoylated with sodium cyanide in methanol to provide **110** in 69% yield. The relatively low yield can be attributed to difficulties experienced during chromatography as a result of the strange solubility characteristics of the final product **110**, which was not soluble in water or acetone, and was very sparingly soluble in methanol.



Scheme 5.3: Deprotection of PNZ protected disaccharide **108**

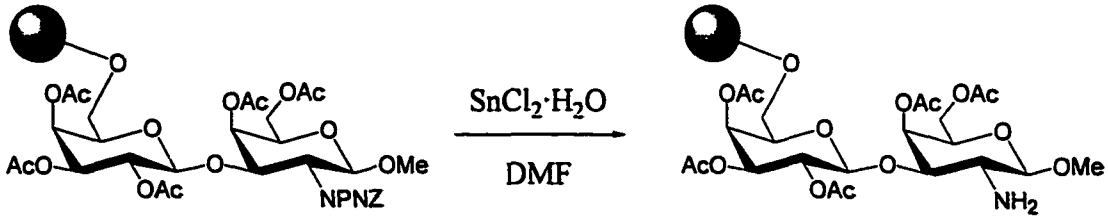
5.3 Loading onto resin and derivation

Reacting trityl chloride resin with the PNZ disaccharide **110** in a minimum volume of dry pyridine (60°C, 2 days) with minimal agitation provided the carbohydrate derivatized resin (Scheme 5.4). Approximately 1.5 equivalents of carbohydrate (relative to theoretical resin loading) were used in the loading step. Any unreacted trityl chloride sites were consumed with the addition of dry methanol. This step was followed by acetylation of the carbohydrate by the introduction of acetic anhydride directly to the pyridine-resin mixture. After being allowed to stand overnight at room temperature, the resin was filtered and washed. The filtrate was retained and concentrated, and the remaining residue was purified and weighed to provide an estimate of the loading levels. Using this method it was estimated that loading levels obtained in different experiments ranged in value from 21-38% of theoretical loading (0.43-0.47 mmol carbohydrate per gram resin). The Galβ(1→3)GalNPNZβOMe disaccharide tended to load less efficiently than its corresponding GalNPNZβOMe monosaccharide analogue. Loading levels of the monosaccharide were determined to be as high as 1.49 mmol/gram (67% theoretical).



Scheme 5.4: Loading and preparation of resin

Once assembly of the disaccharide-resin system was completed, the PNZ group was removed to make the amino group available for reaction with the various acylating agents (Scheme 5.5). This was done by reaction with $SnCl_2$ in DMF overnight. The resulting amine **112** was carried directly onto the next step.

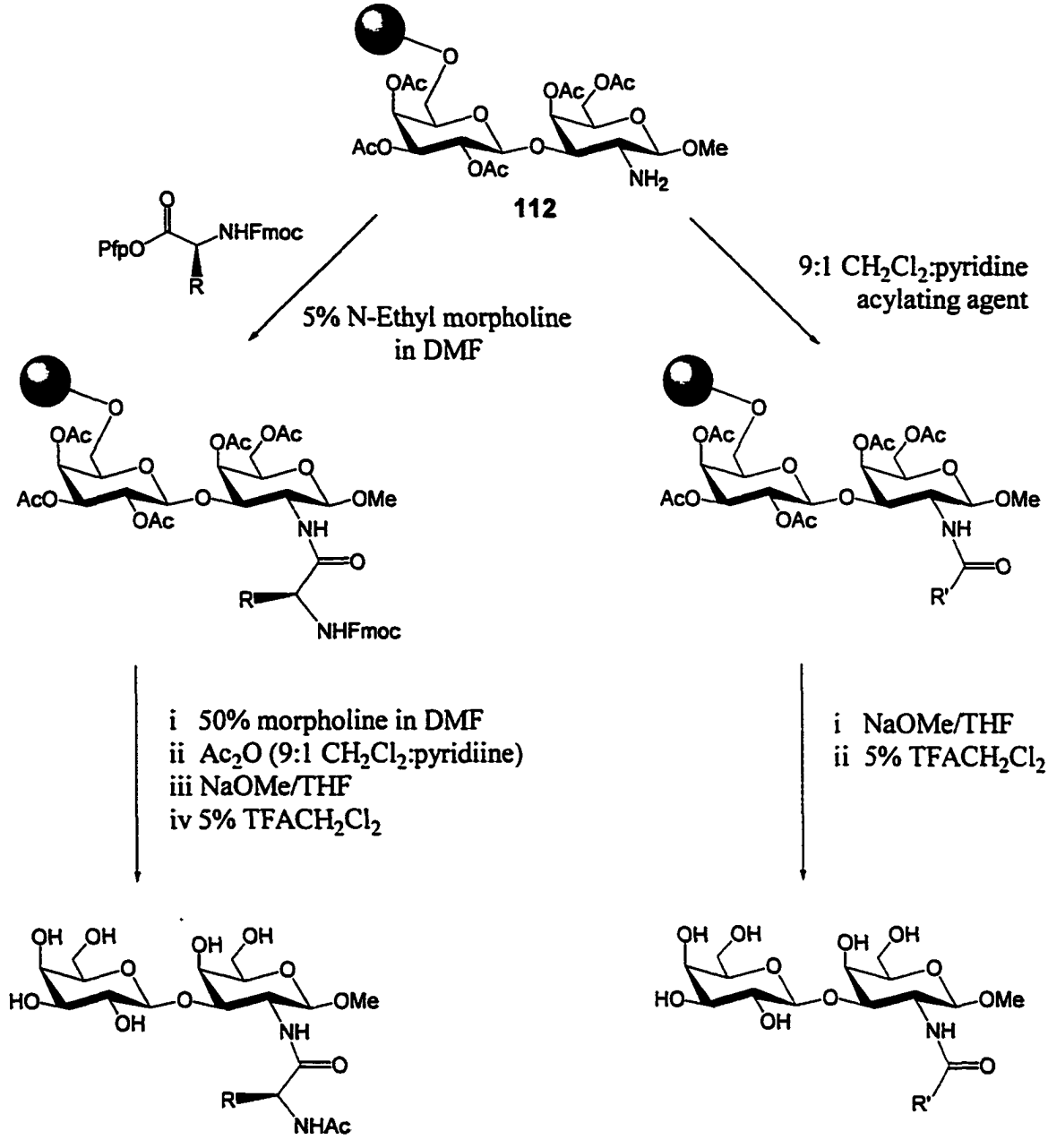


111 **112**

Scheme 5.5: Removal of PNZ protection from the resin-bound disaccharide **111**

To acylate with amino acids, the reagent used was the pentafluorophenyl ester derivative in DMF containing 5% N-ethylmorpholine (Scheme 5.6). The Fmoc protection of the α-amino group was removed in 50% morpholine in DMF, followed by acetylation with acetic anhydride in 9:1 CH_2Cl_2:pyridine. The *O*-acetates were then removed with 0.02 M NaOMe in THF. The resulting product was cleaved from the resin in 5% trifluoroacetic acid in CH_2Cl_2.

Acylation of the amino sugar **112** with anhydride or acyl chloride reagents began by swelling the resin in 9:1 CH_2Cl_2:pyridine, followed by the addition of 10 equivalents of the acylating agent (Scheme 5.6). After 24 hours, the *O*-acetates were removed with 0.02 M NaOMe in THF. The product was then cleaved from the resin with 5% TFA in CH_2Cl_2.

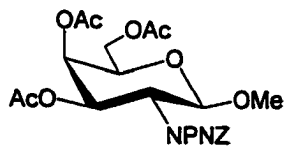


Scheme 5.6: Synthesis of *N*-acylated libraries

5.4 Experimental

General methods used were as described in Chapter 2. The trityl chloride polystyrene solid phase resins (1% DVB, 100-200 mesh) were obtained from either Advanced Chemtech or RAPP Polymere and were used directly as delivered. The solid phase reactions were carried out in sealed polypropylene columns (Biorad) and stirring was carried out by slow rotation of reaction vessels on a Labquake rotator.

Methyl 3,4,6-tri-O-acetyl-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside (**106**).

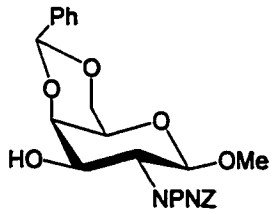


106

Imidate **48** (730 mg, 1.16 mmol) and 4Å molecular sieves (2 g) were stirred, under argon atmosphere, in dry CH_2Cl_2 (30 ml). Anhydrous methanol (1.0 ml, 25 mmol) was added via syringe, followed by $BF_3{\cdot}OEt_2$ (30 µl, 0.24 mmol). After stirring at room temperature for 1.5 hours, the reaction was quenched with solid $NaHCO_3$, filtered through Celite and evaporated under reduced pressure. Column chromatography (6:1 toluene:acetone) yielded the methyl glycoside **106** in 89% yield (517 mg). ^{1}H NMR ($CDCl_3$): δ 8.19, 7.47 (d, 2 H, J_{ortho} 8.5 Hz, ArC*H*), 5.34 (dd, 1 H, $J_{3,4}$ 3 Hz, $J_{4,5}$ 1 Hz, H-4), 4.18 dd, 1 H, $J_{6a,6b}$ 11.5 Hz, $J_{6a,5}$ 6.5 Hz, H-6a), 4.12 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, $J_{6b,5}$ 6.5 Hz, H-6b), 3.90 (dt, 1 H, $J_{5,6a} = J_{5,6b}$ 6.5 Hz, $J_{4,5}$ 1 Hz, H-5), 3.51 (s, 3 H, OMe), 2.13, 2.04, 1.96 (3 s, 9 H, 3 x OAc). ^{13}C NMR (75 Hz, $CDCl_3$): δ 170.4, 170.4, 170.2 (*C*OCH_3), 155.5 (C=O, PNZ), 147.6, 143.8 (aromatic quat.), 127.9, 123.7 (aromatic CH),

70.7, 70.0, 66.8 (C-3, C-4, C-5), 65.4, 61.4 (C-6, CH_2 PNZ), 57.1 (C-2), 52.9 (OMe), 20.7, 20.6 ($COCH_3$). HR-ESMS calcd for $C_{21}H_{26}N_2O_{12}Na$ ($M+Na^+$) 521.1383, found 521.1391.

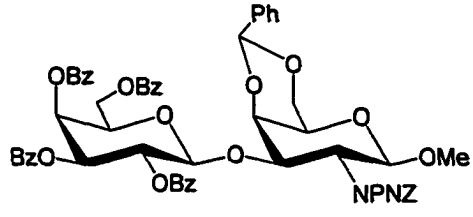
Methyl 4,6-di-O-benzylidene-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside (**107**).



107

A solution of methyl galactoside **106** (510 mg, 1.02 mmol) and sodium cyanide (43 mg, 0.66 mol) in anhydrous methanol (10 ml) was stirred at room temperature for 45 minutes. The reaction was then neutralized with Amberlite IR-120(H+), filtered and evaporated. The crude product was then dissolved in dry CH_3CN. A catalytic amount of camphorsulfonic acid was added, followed by dimethoxy toluene (350 μl, 2.33 mmol). The reaction mixture was stirred overnight, after which solid $NaHCO_3$ was added. The solvents were removed in vacuo and the residue was chromatographed (99:1→9:1 CH_2Cl_2:MeOH) to yield **107** (410 mg, 87% over two steps). ^1H NMR ($CDCl_3$): δ 8.53 (d, 2 H, J_{ortho} 9 Hz, ArC*H*), 7.63-7.93 (7 H, ArC*H*), 5.94 (s, 1 H, PhC*H*O_2), 5.47-5.62 (m, 2 H, OCH_2PhNO_2), 4.63 (dd, 1 H, $J_{6a,6b}$ 12.5 Hz, $J_{6a,5}$ 1.5 Hz, H-6a), 4.54 (broad d, $J_{3,4}$ 2.5 Hz, H-4), 4.46 (dd, 1 H, $J_{6a,6b}$ 12.5 Hz, $J_{6b,5}$ 1.5 Hz, H-6b), 3.85 (s, 3 H, OMe). ^{13}C NMR (75 Hz, $CDCl_3/CD_3OD$): δ 165.8 (C=O, PNZ), 147.0, 144.1, 137.4 (aromatic quat.), 128.5, 127.5, 127.2, 125.9, 123.0 (aromatic CH), 100.8 (PhCH), 75.1, 70.0, 66.1 (C-3, C-4, C-5), 68.6, 64.6 (C-6, CH_2 PNZ), 42.9 (C-2). HR-ESMS calcd for $C_{22}H_{24}N_2O_9Na$ ($M+Na^+$) 483.1380, found 483.1378.

Methyl 3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-4,6-di-O-benzylidene-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside (**108**).

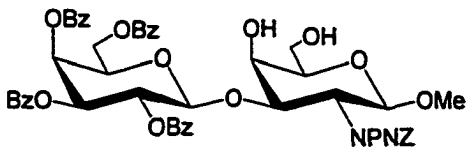


108

Imidate donor **51** (159 mg, 0.215 mmol), acceptor **107** (73 mg, 0.16 mmol) and 4 Å molecular sieves (600 mg) were stirred under argon atmosphere in anhydrous 1,2-dichloroethane for 30 minutes at room temperature. TMSOTf (0.2 M in toluene, 100 µl, 0.02 mmol) was added via syringe and stirring was continued for 30 minutes at room temperature. The reaction was quenched with pyridine, filtered through Celite and evaporated. Column chromatography (6:1 toluene:acetone) yielded the disaccharide **108** (124 mg, 75%). ^{1}H NMR ($CDCl_3$): δ 7.12-8.23 (29 H, Aromatic H), 5.94 (broad d, $J_{3',4'}$ 3.5 Hz, H-4'), 5.80 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 8 Hz, H-2'), 5.57 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 5.45 (s, 1 H, PhC*H*O_2), 5.12 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 5.05 (d, 1 H, J_{gem} 13.5, OC*H*$_2$PhNO_2), 4.70 (dd, 1 H, $J_{6a',6b'}$ 10.5 Hz, $J_{6a',5'}$ 7.5 Hz, H-6a'), 4.59 (d, 1 H, J_{gem} 13.5, OC*H*$_2$PhNO_2), 4.40 (dd, 1 H, $J_{6a',6b'}$ 10.5 Hz, $J_{6b',5'}$ 5.5 Hz, H-6b'), 4.29-4.37 (2 H, H-4, H-5'), 4.20 (dd, 1 H, $J_{6a,6b}$ 12.5 Hz, $J_{6a,5}$ 1 Hz, H-6a), 3.75 (dd, 1 H, $J_{6a,6b}$ 12.5 Hz, $J_{6b,5}$ 1 Hz, H-6b), 3.42 (s, 3 H, OMe). ^{13}C NMR (125 Hz, $CDCl_3$): δ 166.0, 165.6, 165.6, 165.0 (*C*OPh), 155.2 (C=O, PNZ), 147.5, 144.0, 137.8 (aromatic quat.), 133.7, 133.5, 133.4, 133.2 (aromatic quat., OBz), 130.1, 130.0, 129.8, 129.7, 129.6, 129.4, 129.4, 129.0, 128.9, 128.8, 128.7, 128.7, 128.6, 128.4, 128.2, 127.5, 127.2, 126.2, 123.8, 123.7 (aromatic CH), 102.2, 100.8, 100.4 (C-1, C-1', Ph*C*H), 76.5, 76.0, 71.7, 71.6, 70.0, 69.1,

68.3, 66.6, 64.8, 62.6 (C-2', C-3, C-3', C-4, C-4', C-5, C-5', C-6, C-6', CH_2 PNZ), 56.9 (OMe), 53.8 (C-2). HR-ESMS calcd for $C_{56}H_{50}N_2O_{18}Na$ ($M+Na^+$) 1061.2956, found 1061.2952.

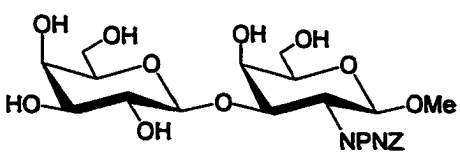
Methyl 3-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside (**109**).



109

The disaccharide **108** (91 mg, 0.088 mmol) was stirred in 80% aqueous acetic acid (20 ml) at 80 °C for 1 hour. The reaction was concentrated and co-concentrated with toluene, followed by column chromatography (2:1 toluene:acetone) which yielded the diol **109** (81 mg, 97%). ^1H NMR ($CD_3OD/CDCl_3$): δ 5.94 (broad d, $J_{3',4'}$ 3.5 Hz, H-4'), 5.74 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{1',2'}$ 8 Hz, H-2'), 5.61 (dd, 1 H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 5.04 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 4.62 (dd, 1 H, $J_{6a',6b'}$ 11 Hz, $J_{6a',5'}$ 7 Hz, H-6a'), 4.45 (dd, 1 H, $J_{6a',6b'}$ 11 Hz, $J_{6b',5'}$ 5.5 Hz, H-6b'), 4.40 (broad t, 1 H, $J_{6a',5'}$ 7 Hz, $J_{6b',5'}$ 5.5 Hz, H-5'), 3.77 (dd, 1 H, $J_{6a,6b}$ 11.5 Hz, $J_{6a,5}$ 7 Hz, H-6a), 3.40 (s, 3 H, OMe). HR-ESMS calcd for $C_{49}H_{46}N_2O_{18}Na$ ($M+Na^+$) 973.2643, found 973.2640.

Methyl 3-O-(β-D-galactopyranosyl)-2-deoxy-2-(p-nitrobenzyloxycarbonyl)amino-β-D-galactopyranoside (**110**).



110

Compound **109** (80 mg, 0.084 mmol) was dissolved in methanol, and NaCN (catalytic amount) was

added. Stirring was continued until no starting material remained (3 hours), then the reaction was neutralized with Amberlite IR-120(H+), filtered and evaporated. Column chromatography (9:1→4:1 CH_2Cl_2:MeOH) yielded the deprotected disaccharide **110** (31 mg, 69%). ^{1}H NMR (D_2O): δ 8.28 (d, 2 H, J_{ortho} 9 Hz, ArC*H*), 7.63 (d, 2 H, J_{ortho} 9 Hz, ArC*H*), 5.28 (d, 1 H, J_{gem} 13.5, OC*H*$_2$PhNO$_2$), 5.22 (d, 1 H, J_{gem} 13.5, OC*H*$_2$PhNO$_2$), 4.46 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.44 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 4.18 (d, 1 H, $J_{3,4}$ 2.5 Hz, H-4), 3.91 (broad d, $J_{3',4'}$ 3.5 Hz, H-4'), 3.90 (dd, 1 H, $J_{2',3'}$ 11.5 Hz, $J_{3',4'}$ 3.5 Hz, H-3'), 3.82 (dd, 1H, $J_{2',3'}$ 11.5 Hz, $J_{1',2'}$ 8 Hz, H-2'), 3.58-3.82 (6 H, H-5, H-5', H-6a, H-6b, H-6a', H-6b'), 3.60 (dd, 1 H, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 3.5 Hz, H-3), 3.53 (dd, 1H, $J_{2,3}$ 9.5 Hz, $J_{1,2}$ 7.5 Hz, H-2), 3.52 (s, 3 H, OMe). ^{13}C NMR (75 Hz, D_2O): δ 128.7, 124.7 (aromatic quat.), 105.4, 103.3 (C-1, C-1'), 80.2, 75.9, 75.6, 73.4, 71.5, 69.4, 69.0 (C-2', C-3, C-3', C-4, C-4', C-5, C-5'), 66.6, 61.8 (C-6, C-6', CH_2 PNZ), 58.0 (OMe), 53.9 (C-2). HR-ESMS calcd for $C_{21}H_{30}N_2O_{14}Na$ ($M+Na^+$) 557.1595, found 557.1598.

5.4.1 General procedures for Synthesis of *N*-acyl Library on the Solid Phase

Loading of Disaccharide onto Trityl Resin

A flask containing the disaccharide **110** (1.5 equivalents relative to theoretical loading) and trityl chloride resin in a minimum volume of dry pyridine (approximately 2 ml per 100 mg resin) was gently agitated for two days at 60°C. The unreacted trityl chloride sites were consumed with the addition of dry methanol (approximately 100 μl per 100 mg resin). Gentle agitation at 60°C continued for 24 hours, after which acetic anhydride (1 ml) was added. This mixture was allowed to stand at room temperature for 24 hours.

The resin was filtered and washed with 2x 1 ml CH_2Cl_2, 2x 1 ml MeOH, repeat cycle. The filtrate was retained, concentrated and purified by column chromatography (6:1 toluene:acetone). The amount of acetylated disaccharide obtained in this manner was used to estimate the loading of carbohydrate on the resin. The loading was approximated using the following method.

$$\text{moles}_{\text{initial CHO}} - \text{moles}_{\text{recovered CHO}} = \text{moles}_{\text{reacted CHO}}$$

$$(\text{moles}_{\text{reacted CHO}} \times \text{MW}_{\text{bound CHO}}) + \text{mass}_{\text{resin initial}} = \text{mass}_{\text{resin+CHO}}$$

$$\text{loading level} = \text{moles}_{\text{reacted CHO}} / \text{mass}_{\text{resin+CHO}}$$

Removal of PNZ group

The resin was swelled in DMF (5 ml). $SnCl_2 \cdot 2H_2O$ (10 equivalents) was added, and the mixture was rotated overnight. The resin was filtered and washed with 2x 1 ml DMF, 2x 1 ml THF, 2x 1 ml CH_2Cl_2, repeat cycle (except DMF step). The resin was then dried under vacuum.

Acylation Step: Amino acid derivatives

The deprotected resin was divided into 30 mg portions for the succeeding steps. The resin was swollen in DMF containing 5% N-ethylmorpholine (5 ml), followed by the addition of 10 equivalents of amino acid pentafluorophenyl ester. After rotating for 24 hours, the resin is filtered and washed with 2x 1 ml DMF, 2x 1 ml THF, 2x 1 ml MeOH, 2x 1 ml CH_2Cl_2, repeat cycle (except DMF step). Fmoc protection of the α-amino group was removed in 50% morpholine in DMF (5 ml). After rotating for 4 hours, the resin was filtered and washed with 2x 1 ml DMF, 2x 1 ml THF, 2x 1 ml MeOH, 2x 1 ml

CH_2Cl_2, repeat cycle (except DMF step). The resin is acetylated with acetic anhydride in 9:1 CH_2Cl_2:pyridine (5 ml) overnight, followed by filtration and washing with 2x 1 ml CH_2Cl_2, 2x 1 ml MeOH, 2x 1 ml THF, repeat cycle.

Acylation Step: Miscellaneous acylating reagents

The deprotected resin was divided into 38 mg portions for the succeeding steps. Acylation of the amino sugar X with anhydride or acyl chloride reagents began by swelling the resin in 9:1 CH_2Cl_2:pyridine (5 ml), followed by the addition of 10 equivalents of the acylating agent. After rotating for 24 hours, the resin was filtered and washed with 2x 1 ml CH_2Cl_2, 2x 1 ml MeOH, 2x 1 ml THF, repeat cycle.

Removal of *O*-acetates

The *O*-acetates were removed by rotating in 4.5 ml THF and 0.5 ml NaOMe in MeOH (0.2 M) for 5 hours, after which the resin is filtered and washed with 2x 1 ml THF, 2x 1 ml MeOH, 2x 1 ml CH_2Cl_2, repeat cycle.

Cleavage from Resin and Isolation of *N*-Acylated Product

The product was then cleaved from the resin by reaction in 5% TFA in CH_2Cl_2 for 30 seconds, followed by filtration, then 5% TFA in CH_2Cl_2 was again added to the resin, this time for 3-5 minutes. It was discovered that the short TFA step was required to neutralize any residual basicity from the deacetylation step, and the second step was responsible for the majority of the cleavage. The filtrate was collected and the resin was washed with 2x 1 ml CH_2Cl_2, 2x 1 ml THF, repeat cycle. The collected filtrate and

washings were diluted to twice the volume with toluene. The solvents were removed *in vacuo* and the residue was dissolved in H_2O, decolorizing charcoal was added, and the resulting mixture was filtered through a Millipore filter (0.22 μm) and the filtrate was lyophilized to yield the acylated product. Cleavage efficiency was typically in the range of 60-80% of ideal.

5.5 Results and Discussion

Commercially available acylating agents were selected to provide a monodimensional library of compounds which would present a variety of different functional groups at the nitrogen of galactosamine. Both aromatic and alkyl substituents were represented during the selection process, as well as groups that were capable of acting as hydrogen bond donors and acceptors. Anhydrides selected included those that would provide a carboxylate group, possibly in a position to mimic the carboxylate of the sialic acid residue.

The amino acid derivatives used for this experiment were Fmoc protected at the α-amino position. These derivatives were selected so that the Fmoc group could be removed under basic conditions while the disaccharide analogue was still attached to the resin, allowing acetylation or further derivation at this position. The basic side chains were protected with the acid labile Boc protection, the acidic side chains were protected by *tert*-butyl esters and the alcohols and phenols were protected with *tert*-butyl ethers. The *tert*-butyl-based protection would be expected to come off during the acidic cleavage step.

Some of the acylation reactions were carried out quite efficiently, while others were not. Among the successful acylating agents employed were benzoyl chloride (a), phthalic anhydride (b), 2,3-pyrazinedicarboxylic anhydride (c), glutaric anhydride (d) and diphenic anhydride (e) (Figure 5.3). Acylations were unsuccessful with diglycolic anhydride (f), 1,8-naphthalic anhydride (g) and nicotinoyl chloride hydrochloride (h) (Figure 5.4).

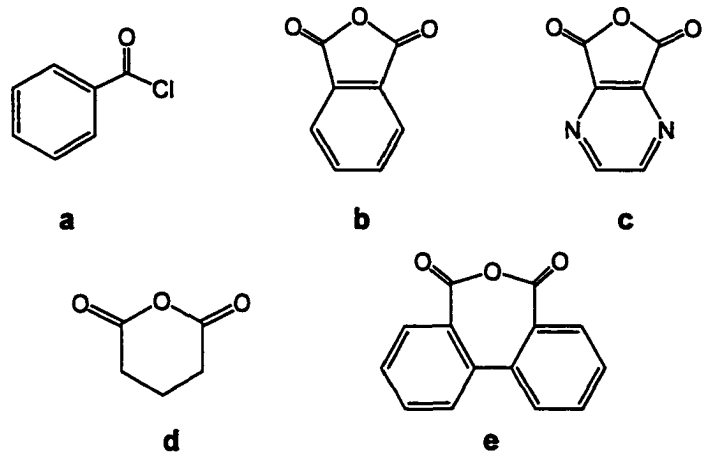


Figure 5.3: Successfully employed acylating agents

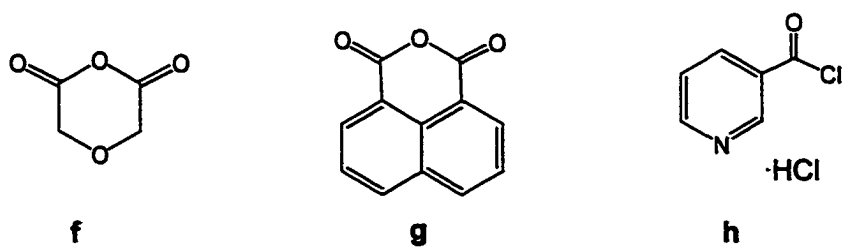


Figure 5.4: Unsuccessfully employed acylating agents

A selection of amino acids representing the different classes of side chains were chosen such that acidic (aspartic acid) and basic (lysine) groups, as well as alkyl (alanine, methionine) and aromatic (phenylalanine and tyrosine) groups were all represented (Figure 5.5). Most of the amino acid analogues were isolated in high yield, with the exception of the lysine derivative. This could be a result of the *N*-Boc protection on the basic side chain. Removal of this group may require more than 3-5 minutes in 5% TFA in CH_2Cl_2. If so, the persistent Boc group may interfere with the solubility of the analogue in water, resulting in its loss during the aqueous filtration step. It should also be

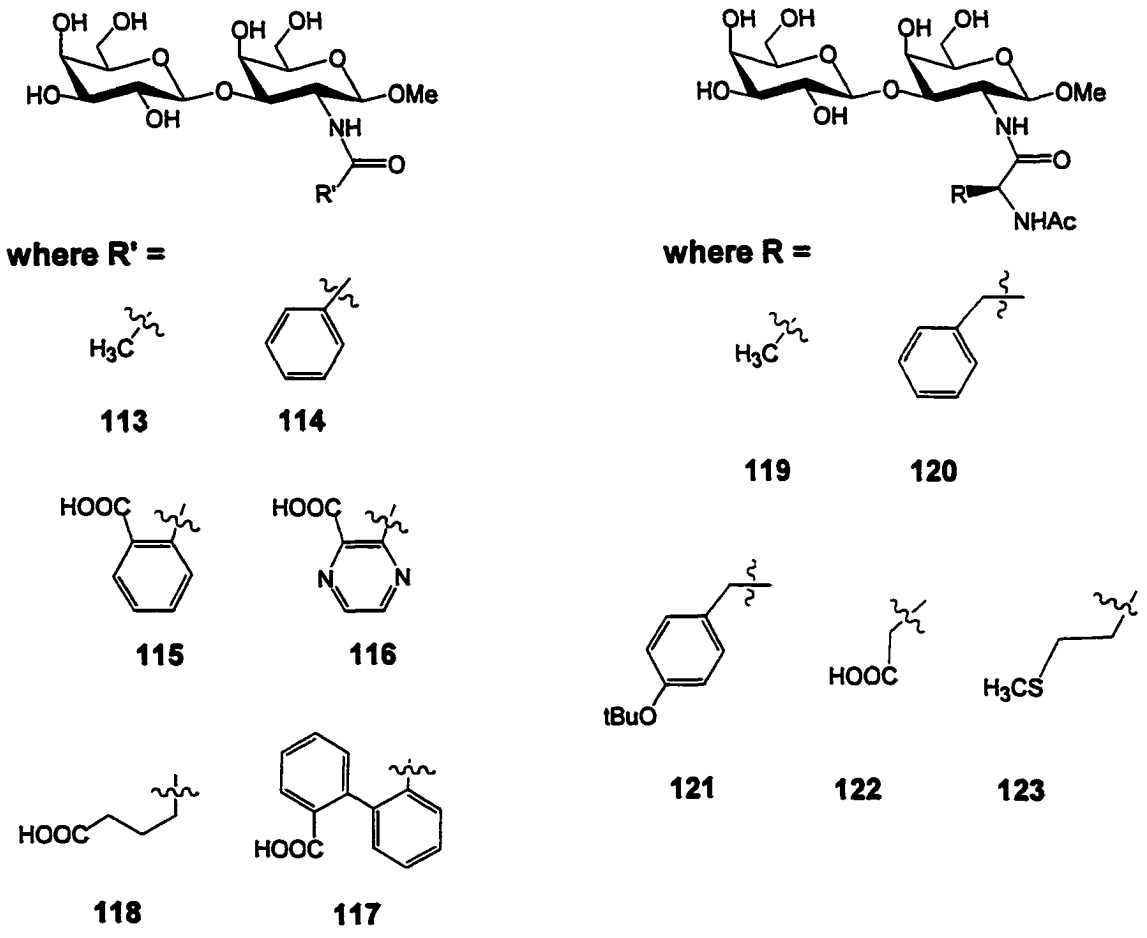


Figure 5.5: Galβ(1→3)Gal(2-*N*-acyl) inhibitor library members

mentioned that the tyrosine analogue was isolated (and assayed) with its *tert*-butyl ether protecting group intact. Acetylation of the α-amino was carried out primarily to prevent partial acetylation during the removal of the *O*-acetates. The *N*-acylated disaccharide analogues that were successfully synthesized are compiled with their *m/z* values in Table 5.1.

analogue	m/z (M-H)
113	396
114	458
115	502
116	504
117	511
118	578
Ala **119**	467
Phe **120**	543
Tyr-OtBu **121**	616
Asp **122**	511
Met **123**	543

Table 5.1: Compilation of the *m/z* values of the library members

The libraries were assayed against both cholera toxin and heat-labile enterotoxin using the FAC-MS method, with disappointing results. None of the compounds tested were identified to be a "hit". In other words, no library compound was bound by either CT or LT with a micromolar affinity. The FAC-MS assay is only capable of identifying moderately strong affinities, so if the acylated disaccharide members of this library were

recognized with affinities similar to that of the native Galβ(1→3)GalNAc disaccharide, which is in the millimolar range, they would not be detected using this method.

Examples of chromatograms obtained by screening eight library compounds against heat-labile enterotoxin and cholera toxin are shown in Figure 5.6 and Figure 5.7.

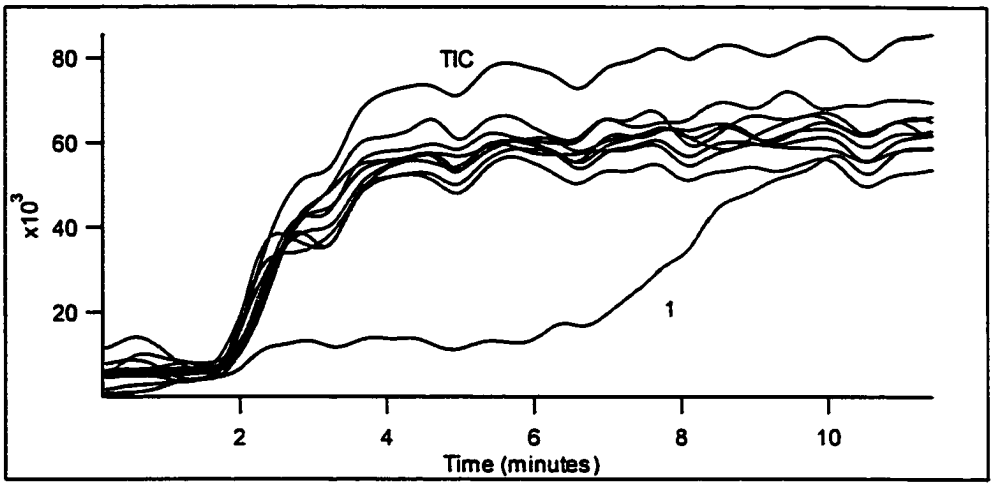


Figure 5.6: Results of FAC-MS assay of *N*-acyl library against heat-labile toxin

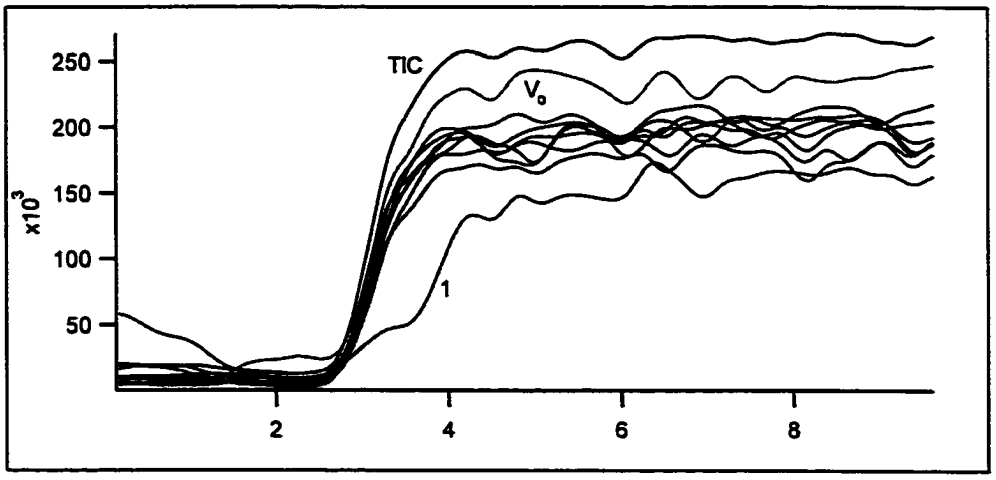


Figure 5.7: Results of FAC-MS assay of *N*-acyl library against cholera toxin

While the assay results of the library were disappointing, it should be noted that while the library represented a variety of functional groups, it was still quite small. In order for a more potent inhibitor to be found using this approach, a much larger library will probably be required.

This project was primarily designed to investigate the use of PNZ protection in a solid phase methodology toward the synthesis of disaccharide libraries. It has been successfully demonstrated that the PNZ carbamate may be used as a temporary amino protecting group on the solid phase.

Chapter 6

Future Work

While the binding assays of the GM_1 analogues synthesized in Part I of this thesis answered some questions about the minimum carbohydrate structure required for binding, the results also raised many more. For example, what is responsible for the decrease in binding strength of the compound **1** relative to GM_1? If it is indeed due to the entropic penalty paid as a result of the flexibility of the linker, can a conformational constraint alleviate this problem? In other words, if the sialic acid can be fixed into a position similar to that of the native GM_1, then perhaps a high affinity ligand would result. The feasibility of this approach was demonstrated by the design of the pseudotetrasaccharide, by Bernardi *et al.* (described in Section 3.4), which provided a molecule that was identical in activity to GM_1 in binding studies to cholera toxin.

Another method of restricting the sialic acid residue in a position favourable to binding would be through the use of an intramolecular tether. This approach has been attempted in other systems with mixed success [133]. Perhaps the proposed linker could be used to mimic the hydrogen bonding interaction between the COOH of the sialic acid and the NHAc of the galactosamine. One can envision the carboxylate acting as one end of the tether and the nitrogen of the galactosamine as the other (Figure 6.1). Molecular modeling could be employed to determine the optimum length of the tethering arm.

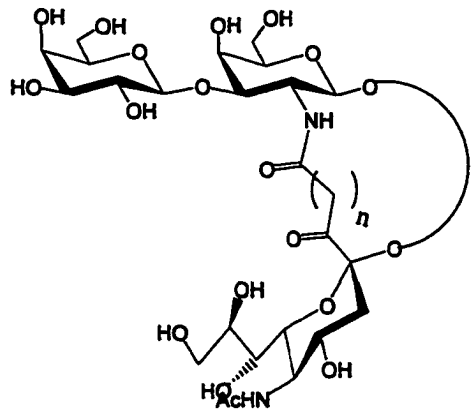


Figure 6.1: Intramolecular tether approach

Another approach to the design of high affinity ligands that has demonstrated remarkable success is the use of multivalent ligands. When multiple copies of a carbohydrate ligand are spatially organized to correlate to the binding sites of a multimeric protein, one may expect enhanced affinity between ligand and protein. Multivalency has been shown to transform weakly binding ligands into very highly potent inhibitors. This is due to the fact that the recognition of one arm of a multivalent molecule by the protein means that the other arms are in close proximity to the remaining binding sites. This preorganization reduces the entropically disfavoured preorganization of multiple ligands.

A striking example of multivalency in action is found in the STARFISH molecule synthesized by Kitov *et al.* (Figure 6.3) [134]. In this experiment, a P^k trisaccharide dimer presented in a pentavalent manner acts as a potent inhibitor of the Shiga-like toxin (SLT-I). The dimeric ligand itself is a 40-fold stronger binder than the univalent P^k trisaccharide. When the dimer is tethered to a pentavalent core, the resulting molecule is a 1-10 million times stronger inhibitor of SLT-I than the dimer. The tethering arms are

connected to a central glucose residue, and the pentavalent nature of the ligand is achieved by using the five hydroxyl groups as points of attachment.

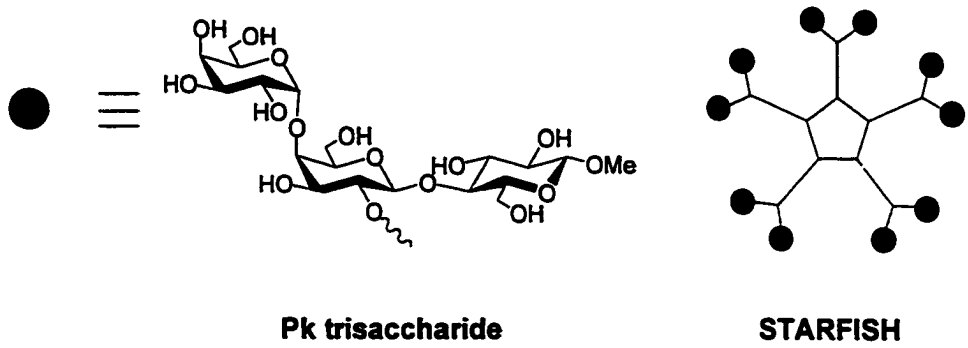


Figure 6.3: STARFISH inhibitor of SLT-I

Since cholera toxin and heat-labile enterotoxin are both homopentameric, they provide the perfect system for this type of investigation. This is made apparent through the results obtained by Fan *et al.* [135] with their pentavalent galactose molecule (Figure 6.2). Cholera toxin binds the monosaccharide galactose very weakly, but when galactose is presented to the toxin as a pentavalent molecule, its affinity increases 10^5-fold. While the improvement in affinity is not as dramatic as that of the STARFISH molecule, this example shows that the STARFISH approach can be extended with some success to monosaccharides.

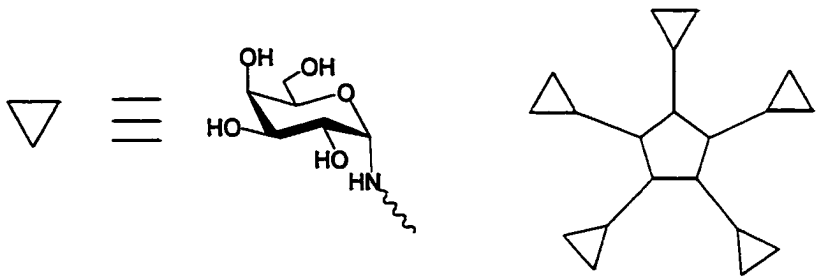


Figure 6.2: Pentavalent galactose ligand for inhibition of LT

These examples demonstrate that, with careful design, it is indeed possible to generate high affinity carbohydrate ligands. As indicated by the work of Fan *et al.*, the pentameric nature of the CT and LT toxins, provides a perfect model for the multivalent approach to high affinity ligand design. A pentavalent presentation of compound **1** at the end of carefully designed tethering arms would likely result in a molecule with a significant increase in affinity for the toxin.

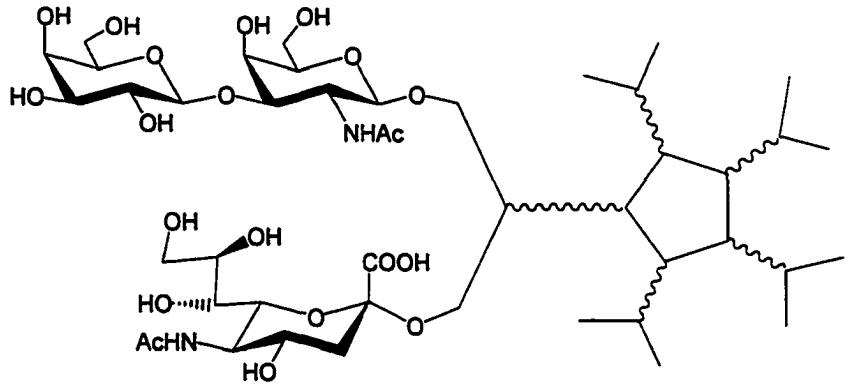


Figure 6.4: Multivalent approach to the design of high-affinity ligands for CT and LT

As indicated, there are still many areas to be explored toward the design of inhibitors for CT and LT.

Chapter 7

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