University of Alberta

Investigation of Fluorescent Labels in Carbohydrate-Lectin Interactions and Discovery of Glycoside-Binding Boronic Acids

Meenakshi Dowlut ©

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 2006

Your file Votre référence
ISBN: 978-0-494-23018-3
Our file Notre référence
ISBN: 978-0-494-23018-3

Abstract

Carbohydrate-protein interactions are essential in a multitude of biological recognition events. Their importance can be seen in the rapid development of combinatorial chemistry to provide useful sources of synthetic carbohydrates as potential ligands for biological receptors. This thesis is divided into three main parts, but all are concerned with the development of receptors and sensors for carbohydrates.

In the first part, fluorescent dyes used in labeling carbohydrates were investigated. This was performed to improve the detection of positive hits arising from specific interactions between a carbohydrate and a protein. A study of labeled carbohydrates to lectins conjugated to a solid-support shows that succinimidyl 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoate (NBD-X) dye provides by far the lowest level of nonspecific interactions with immobilized proteins. This observation is in stark contrast with the commonly used labeling reagents constituted of charged and aromatic groups, for instance, FITC and TAMRA dyes. We encountered some failure with europium labeled carbohydrates.

The second part of the thesis is focused on the synthesis of small molecule models of a heparin sulfated carbohydrate that is used as an anticoagulant drug. Due to the ionic nature of the sulfated carbohydrates, there is still a lot to accomplish in the development of receptors for these charged biomolecules. Our aim was to screen a number of polyamines supported on polystyrene beads against dye-labeled sulfated carbohydrates.

We deemed it necessary to halt the project due to disappointing results in the synthetic route toward sulfated carbohydrates.

Limitations usually encountered in developing boronic acid-based receptors for carbohydrates are imposed by their poor solubility and their restricted complexation to carbohydrates that can isomerize to the furanose ring. Cell-surface carbohydrates are mostly present in the hexopyranose form. The third part of the thesis focused on identifying new monoboronic acids that bind strongly to carbohydrates under aqueous physiological conditions and eventually develop 'oligomers' of these boronic acids for improved binding potency and higher selectivity. Our qualitative and quantitative results show that 2-(hydroxymethylphenyl) boronic acids (benzoboroxoles) stand out as excellent binding agents for both monosaccharides and glycopyranosides. To explain the unusual binding selectivity, preliminary structural studies were carried out.

Acknowledgements

I would like to thank my two supervisors, Professor Dennis G. Hall and Professor Ole Hindsgaul for their never-ending guidance, mentorship and patience. A special thanks to Professor Hall for allowing me to step into a new field and enjoying the tough challenges of research. I would like to extend my thanks to past scientific colleagues from Professor Hindsgaul's Lab, especially Jignesh Bulsara who always seemed to find some humor at any time and Dr. Massoud Amanlou who taught me to keep smiling even during hard times. Of course, research would never have been so much fun without the support of my labmates, members of the Hall group. So, I thank all of them. To Eric Pelletier who always finds time to relieve my workload and meet my deadlines, I would like to extend my deepest thanks for doing such an excellent job in the purifications of my priceless boronic acid compounds. I am particularly grateful to Hugo Lachance, Yu Bai, Wei Shi (members of the Lowary's group), Sam Mothana, Tim Elford and Bilkiss Issack for proofreading the earlier versions of my thesis.

I am indebted to the University of Alberta, Department of Chemistry for providing excellent research support and expertise. I would like to express my gratitude toward Glen Bigam, Mark Miskolzie and Lai Kong in the NMR laboratory, Dr. Angie Morales-Izquierdo in the mass spectrometry laboratory as well as all the dedicated technicians at the Analytical and Instrumentation Laboratory. I would like to thank Rakesh Bhatnagar and Jack Scott in the Department of Biological Sciences for the fluorescence readings.

I would also like to thank Professor Bundle who kindly allowed me to use his microplate reader, Professor Rik Tykwinski for the use of the fluorimeter and Dr. Christie Mc. Dermott for the use of the autotitrator.

I am grateful to the undergraduate laboratory coordinators, Dr. Norman Gee and Dr. Lois Browne for the enjoyable, resourceful and invaluable teaching experience at the University of Alberta.

I was always fortunate to have good friends around me. My special thanks go to Soleiman Hisaindee and all the Mauritian friends I know and of course Bilkiss who always had something at home to nibble. My final thanks go to Monisha and Shridhar and my loving parents for their constant support and source of inspiration.

Table of Contents

Chapter 2 Investigation of Non-Specific Effects of Fluorescent Tags in the Screening of Labeled Carbohydrates Against Immobilized Proteins

List of Tables

List of Figures

List of Schemes

List of Abbreviations

Ac	Acetyl
Ac_2O	Acetic anhydride
εAhx	6-Aminohexanoic acid
Anal.	Analysis
aq	Aqueous
Ar	Aryl
Bn	Benzyl
BOC	*Tert*-butoxylcarbonyl
t-Bu	*Tert*-butyl
Bz	Benzoyl
c	Concentration
°C	Degrees Celsius
Calcd	Calculated
CD	Circular dichroism
Con A	Concanavalin A
CSA	10-Camphorsulfonic acid
d (in ^{1}H NMR)	Doublet
DBU	1,8-Diazabicyclo[5.4.0]unde-7-ene
DCM	Dichloromethane
dd	Doublet of doublets

ddd	Doublet of doublets of doublets
DEAM-PS	*N*,*N*-Diethanolaminomethyl polystyrene
DELFIA	Dissociation enhanced time resolved fluoroimmunoassay
DMSO	Dimethyl sulfoxide
DMAP	4-(*N*,*N*-Dimethylamino)pyridine
DMF	*N*,*N*-Dimethylformamide
dppf	1,1'-Bis(diphenylphosphino)ferrocene
dt	Doublet of triplets
DTPA	Diethylenetriaminepentaacetate
DTPAa	Diethylenetriaminepentaacetic acid dianhydride
EI	Electron impact
ELISA	Enzyme-linked immunoassay
equiv	Equivalent
ES	Electrospray
ε	Molar extinction coefficient
Fmoc	9-Fluorenylmethoxycarbonyl-*N*-hydroxy-succinimide
HBTU	*O*-Benzotriazoyl-1-yl-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate
^{1}H NMR	Proton nuclear magnetic resonance spectum
HMBC	Heteronuclear multiple bond coherence
HMPA	Hexamethylphosphoramide
HMQC	Heteronuclear multiple quantum coherence
HOBT	1-Hydroxybenzotriazole

HPLC	High performance liquid chromatography
HRES-MS	High resolution electrospray masss spectrometry
Hz	Hertz
J	Coupling constant value
K_a	Binding association constant
kDa	KiloDaltons
m	Multiplet
Me	Methyl
mg	Milligrams
min	Minutes
mL	Milliliters
MS	Mass spectrometry
NBD	Nitrobenzoxadiazole
NMR	Nuclear magnetic resonance
OBz	Benzoate
PBS	Phosphate buffered saline
Ph	Phenyl
PNA	Peanut agglutinin
^{i}Pr	Isopropyl
q	Quartet
θ_F	Fluorescence quantum yield
rt	Room temperature
s	Singlet

sLex	Sialyl Lewis X
t	Triplet
TBDMS	*Tert*-butyldimethylsilyl
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TR-FIA	Time-resolved fluoroimmunoassay
p-TsOH	*para*-Toluenesulfonic acid
UV	Ultraviolet
$\lambda_{emission}$	Emission wavelength
$\lambda_{excitation}$	Excitation wavelength

Chapter 1

Introduction

1.1 Carbohydrates

Carbohydrates exhibit a wide range of structural and functional diversity in nature. These features allow them to fulfill various biological functions such as energy sources, structural materials (cellulose), elements of molecular recognition, and also as factors determining the structure and role of protein in metabolic processes. Interactions between carbohydrates and proteins, both intra and extra cellular, play key roles in the above biological functions.

The three-dimensional structure of the carbohydrate is translated by a protein receptor into a signal, which determines the fate of a cell or glycoprotein. Such non-covalent interactions are present in a number of physiological processes[1,2] that comprise cell-cell communication,[3] fertilization, cell growth, immune response, cancer cell metastasis[4,5] and microbial infections. Studies centered on interactions between carbohydrates and proteins (enzymes,[6,7] lectins,[8-11] antibodies[12]) have revealed that one or two edges or faces of the carbohydrate ligands are bound by the protein and that the recognition element or epitope is often limited to a disaccharide or at most a trisaccharide unit. Thus, oligosaccharides are attractive targets as they are synthetically easily accessible and can therefore be used in carbohydrate-based therapeutics.

Examples of such therapeutics that have reached advanced stages of development are the inflammatory sialyl Lewis X tetrasaccharide,[13] and glycosphingolipid-protein conjugates used in cancer therapy,[14] antidiabetic α–glucosidase inhibitors,[15,16] anti-HIV agents[17] and heparin analogs.[18-20] Other classes of important carbohydrate-containing drugs on the market are bleomycin, gentamycin, amphothericin B, vancomycin and etoposide. Many more examples are in late or early stages of clinical development. In all of these cases, the removal or modification of the sugar moieties often alters or causes complete loss of activity of the drug. Because of the increasing popularity of carbohydrate-based drugs, intense research is focused on the development of combinatorial libraries of carbohydrates and glycoconjugates.

1.2 Fluorescence labeling

With the development of combinatorial chemistry, the need for efficient screening strategies for the discovery of biactive compounds as target drugs has become more important. For instance, interactions between carbohydrates and proteins can be studied by labeling the protein or carbohydrate (or glycoconjugates) ligands with radioisotopes or enzymes.

Fluorescent labels provide the following advantages: [21,22]

(a) They provide tremendous sensitivity due to their high quantum emission yield upon excitation. Certain fluorophores can absorb large amounts of energy at specific wavelengths and consequently, they provide excellent detection for sensitive measurements with tiny amounts of material. For instance, proteins, nucleic acids

and other biomolecules can be labeled with fluorescent probes to provide highly receptive reagents for *in vivo* assay procedures.

(b) Fluorophores emit at specific wavelengths. So, more that one fluorescent label can be used on the protein, nucleic acid or antibody at the same time for the detection of specific ligands.

(c) Fluorescent tags avoid the danger and expenses associated with handling radioactive substances.

1.2.1 Fluorescence spectroscopy

A fluorescent molecule absorbs energy at a particular wavelength and subsequently emits it at another wavelength. The process of absorbing energy is called excitation, during which, the quantum energy levels of the electrons of the fluorophore molecule increase with photon uptake. A range of energies are absorbed, giving rise to an absorption band with a maximum. The extinction coefficient (ε, expressed as M^{-1} cm^{-1}) at the absorption peak maximum is a unique characteristic of a fluorophore under a given set of experimental conditions.

The excited fluorophore will then release energy[22] as heat or as photons of light. The electrons of the fluorescent compound will eventually return to the lower, ground-state energy level (Figure 1-1). The emission rates of fluorescence are typically 10^8 s^{-1} so that typical fluorescence lifetime is near 10 ns (1×10^{-9} s). The Jabloñski diagram shown in Figure 1-1 depicts the fluorescence phenomenon. The singlet ground, first, and second electronic states are represented by S_0, S_1, and S_2 respectively. At each of these

electronic energy levels, the fluorophores can exist in a number of vibrational energy levels. The transitions between the states are depicted as solid vertical lines (Figure 1-1), to illustrate the instantaneous nature of light absorption. The Franck-Condon principle states that during the electronic transitions in a molecule, the nuclei can be assumed to be in the same place before and after the transition. Transitions occur in about 10^{-15} s, a time too short for relaxation through collisions.

When a fluorophore is irradiated with light energy, it is excited to some higher vibrational level, in either S_1 or S_2 (Figure 1-1). Molecules in the condensed phase rapidly relax to the lowest vibrational level. This process is called internal conversion and generally occurs in 10^{-12} s or less. Since fluorescence lifetimes are typically near 10^{-8} s, internal conversion is generally complete prior to emission. Hence, fluorescence

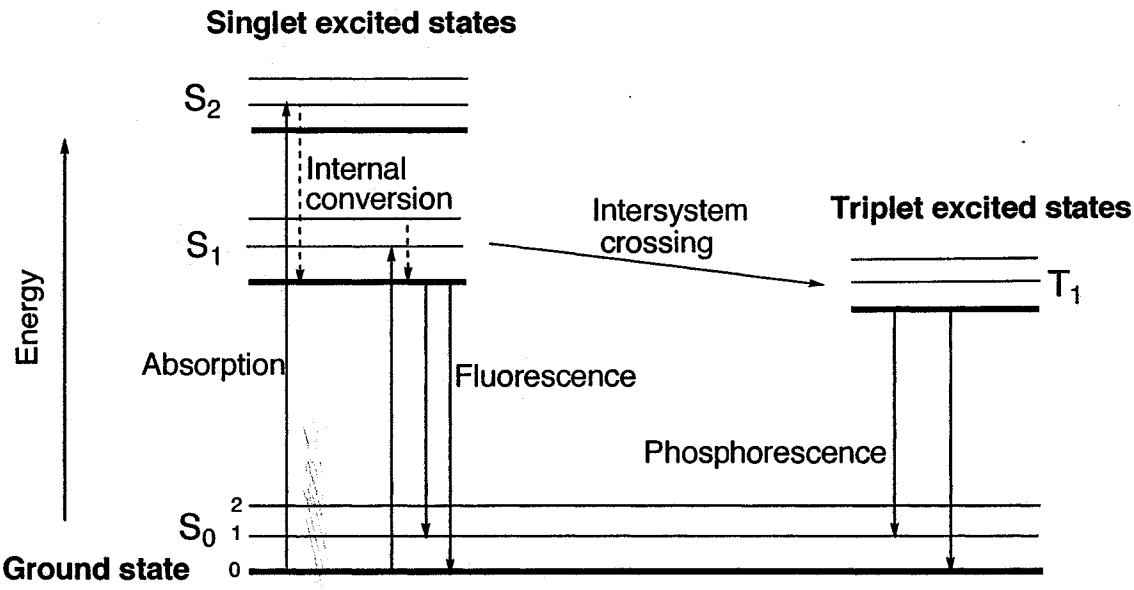


Figure 1-1. Electronic transition[22] energy-level diagram (reproduced from reference 22).

emission generally results from a thermally equilibrated state, that is the lowest vibrational state of S_1.

According to Stoke's law, the emission wavelength is always longer and thus of lower energy than the wavelength of excitation.[23] The ratio of total photon emission over the entire range of fluorescence to the total photon absorption is called quantum yield (Q). Quantum yields range from 0-1. A larger quantum yield means a larger emission or luminescence. Under fixed environmental conditions, a particular fluorophore has a constant extinction coefficient and quantum yield, characteristic of its photochemical behavior. For a fluorophore to be suitable for analytical studies, the quantum yield[21] must be high and its fluorescence spectrum must be sufficiently separated from its excitation spectrum to ensure good signal isolation. The separation between the maximal absorbance wavelength and the emission wavelength maxima is called Stoke's

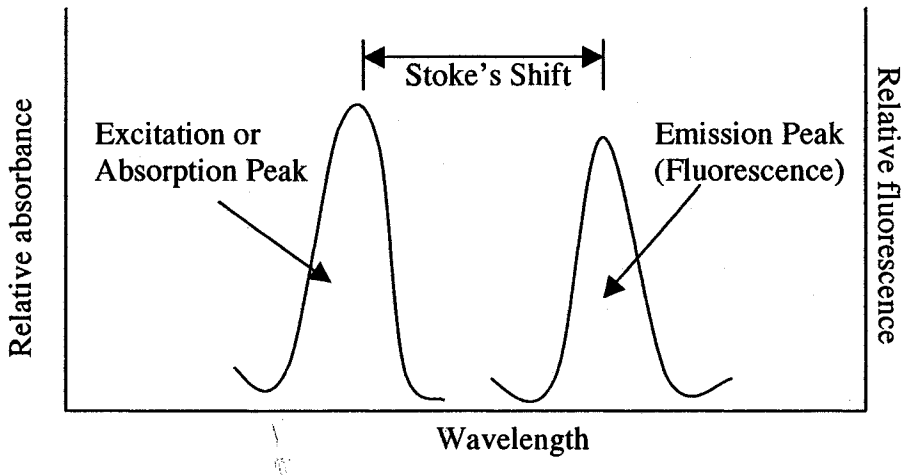


Figure 1-2. Typical spectral scan[21] (modified from reference 21) of a fluorescent compound showing its absorption peak and its emission peak at different wavelengths.

shift. The greater the Stoke's shift (>100 nm), the better the signal isolation and therefore there is less interference from Rayleigh-scattered excitation light.

1.2.2 Aromatic fluorescent compounds as labels for biomolecules

Fluorophores are divided onto two classes, intrinsic and extrinsic. Intrinsic fluorophores are those that occur naturally. Extrinsic fluorophores are those which are added to a sample that does not show any luminescent properties, also called fluorescent labels or tags. The structural motif of a compound that brings about fluorescence is the presence of a planar aromatic ring system. The fluorescence quantum yield (θ_F) of larger ring systems is typically greater than smaller ring systems.

Fluorophores are gaining popularity in labeling biomacromolecules[24,25] (nucleic acids, proteins, DNA). They are commercially available in several analogue forms, each with a different reactive group able to couple to specific functional groups or target molecules, for example the amines, sulfhydryl, or histidine side chains in proteins (Figure 1-3).

Conventional fluorescent tags are derivatives of fluorescein, rhodamine, coumarin, and Texas Red. Fluoresceins and rhodamines are widely used because of their favorably long absorption maxima at 480 nm and 560 nm, respectively and their large molar extinction coefficients (ε), near 80,000 M^{-1} cm^{-1} (Table[26] 1.1). The long wavelength of the emission photons minimizes the problems of background fluorescence from biological samples and eliminates the need for quartz optics.

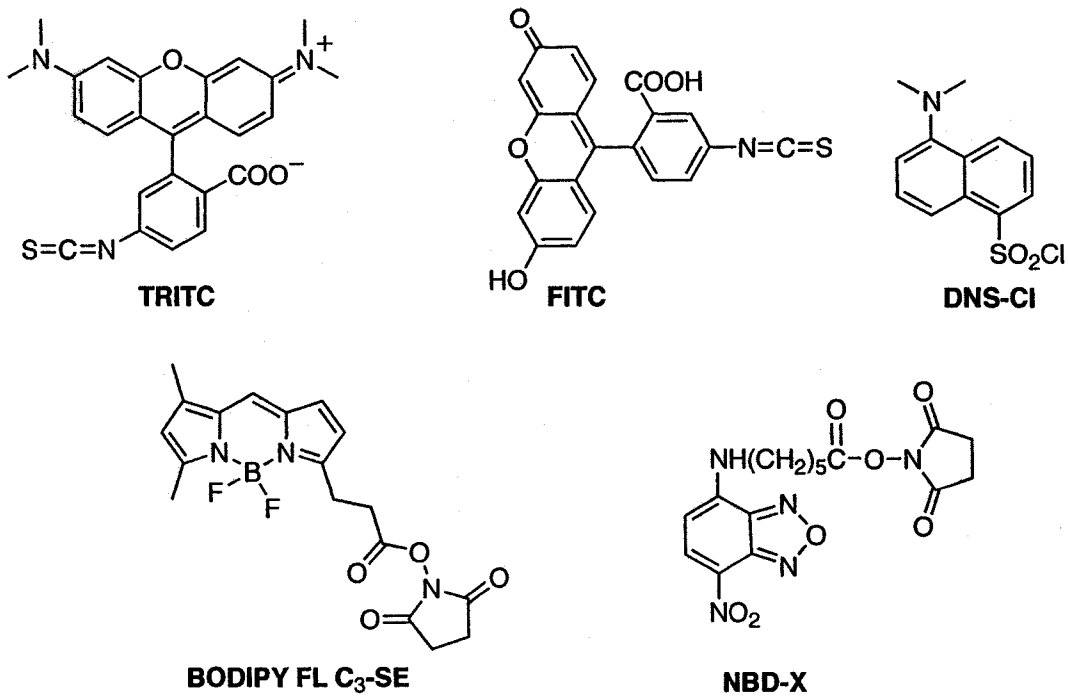


Figure 1-3. Reactive probes for conjugation with biomacromolecules. TRITC, Tetramethylrhodamine 5-(and 6-)isothiocyanate; FITC, fluorescein 5-isocyanate; DNS-Cl, Dansyl chloride; BODIPY FL C_3-SE, 4,4-Difloro-5,7-dimethyl-4-bora-3a,4a-diaza-sindacene-3-propionic acid, succinimidyl ester; NBD-X, succinimidyl 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) hexanoate.

Table 1.1 Fluorescence spectral properties[26] of commonly used extrinsic probes.

Fluorophore	Absorption (nm)	ε ($M^{-1}cm^{-1}$)	Emission (nm)	θ_F Quantum yield
DNS-Cl	340-350	4,300	510-560	0.1-0.3
BODIPY FL C_3-SE[21]	502	77,000	510	0.03
FITC	492	72,000	516-525	0.3-0.85
TRITC	535-545	107,000	570-580	0.3
NBD-X[27]	465-469	22,000	534-538	-

1.2.3 Lanthanide chelates

In recent years, time-resolved fluorometry[28-30] (TRF) involving carbohydrates and their derivatives, labeled with chelated lanthanides, has been gaining popularity as an easy and sensitive analytical method. Time-delayed fluorometry is a detection technology based on the unique abilities and the luminescence properties of the lanthanide chelates using pulsating excitation light. As shown in Figure 1-4, through a delay time of 200 μs, no luminescence measurement is taken. Background fluorescence is eliminated since its lifetime in the order of ns has decayed. Thus, a good signal to noise ratio is obtained.

The chelates are formed using lanthanides such as europium (Eu) and samarium (Sm).

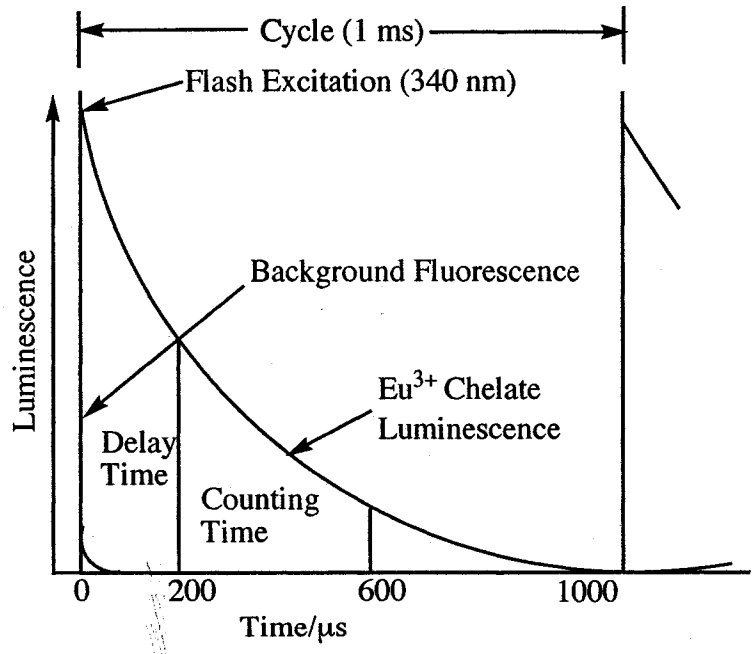


Figure 1-4. Time-delayed fluorometric measurement[31] of a europium label (reproduced from reference 31).

Lanthanide chelates are highly luminescent and have a very long luminescence decay time and large Stokes' shift. Because the lanthanide chelates can be measured as sensitively as radioisotopes[32,33] and are stable and easy to handle, they are widely used as tracers[31] in biological sciences.

For instance, Eu-labeled antibodies are used in time-resolved fluoroimmunoassays (TR-FIA).[29,30] Polycarboxylates[34] (Figure 1-5) such as DTPA anhydrate **1** and N_1(p-isothiocyanatobenzyl)-diethylenetriamine-N_1,N_2,N_3,N_3-tetraacetic acid **2** form eight-coordinate complexes with europium, which emit little luminescence. However, after immuno-reactions, the europium can be dissociated[29,30] from the complex using an enhancement solution (trioctylphosphine oxide, 2-naphthoyltrifluoroacetone, Triton X-100) to form a new and highly luminescent chelate **3** (Figure 1-5). This technology is referred to as dissociation enhanced lanthanide fluoroimmunoassay (DELFIA). Other types of ligands that form chelates with europium are beta-diketones[35,36] **4**, which emit high luminescence and they react directly with proteins. Aryl amines also form luminescent chelates, for example, Quantum-Dye (QD) **5**.

TR-FIA immunoassays have found numerous applications[37-40] in the field of glycobiology such as binding specificity studies of lectins. Activities[41] of glycosyltransferases and glycopeptidase have been measured using Eu-labeled lectins. Lee[42] and co-workers have reported binding and inhibition assays of carbohydrate-recognition domain of rat serum mannose-binding protein coated on microtiter plates

using QD-labeled neoglycoprotein, QD-Man-BSA. Their methodology has yielded results comparable to those obtained using radiolabeled I_{125}-Man-BSA.

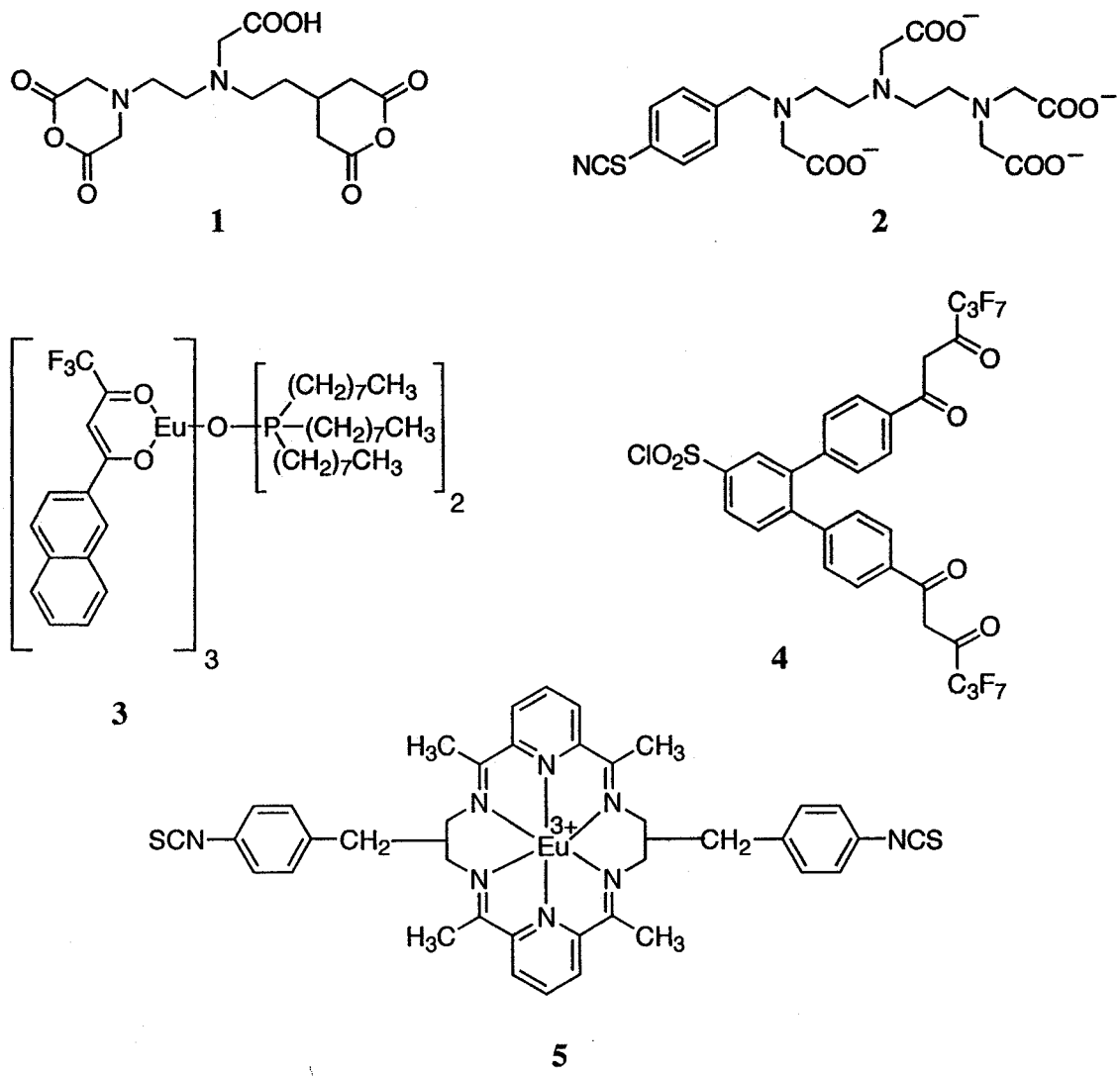


Figure 1-5. Common protein labeling reagents used for TR-FIA.[30,43]

1.3 Lectins: carbohydrate-binding proteins

Lectins[44-46] are carbohydrate-binding proteins with no catalytic ability. Furthermore, they are not products of the immune response but they mediate a variety of biological processes, such as cell-cell and host-pathogen interactions. They are usually oligomeric proteins composed of subunits, usually with one sugar binding site per unit. Lectins are found in plants, animals, microorganisms and viruses. Lectins are classified into five groups (mannose, galactose/N-acetylgalactosamine, N-acetylglucosamine, fucose and N-acetylneuraminic acids), according to the specificity of monosaccharide towards which they exhibit high affinity. Interest in lectins has increased since the 1960's due to their usefulness in detecting and studying carbohydrates in solution and cell surfaces. Lectins immobilized on agarose (a linear polysaccharide support) are typically used for fractionation and purification of glycoproteins.[47]

1.3.1 Concanavalin A (Con A)

Lectin Con A was the first lectin to be isolated[48] in 1919, from jack bean (*Canavalia ensiformis*), by Sumner who later reported the sugar specificity of lectins. Con A was also the first lectin to be sequenced,[49,50] and to have its three-dimensional structure[51,52] determined by X-ray crystallography. Since then, Con A has been the most extensively[53] studied lectin. In solution, Con A exists as a dimer[47,54] below pH 5.2, and as a tetramer above pH 7.0. Each identical subunit[47] binds one saccharide and two metal ions (Mn^{2+} and Ca^{2+}), both of which are necessary for carbohydrate binding. Each monomer consists of a sandwich[53,55] of two β sheets which are responsible for forming

dimers or tetramers of Con A through hydrogen-bonding between different amino acids found on the lectins.

Lectins in general bind reversibly to monosaccharides and oligosaccharides with typically modest binding constants in the range[9,47,56-58] of 10^{-3} to 10^{-6} M. In the 1980's, Brewer[59] and co-workers have identified the trisaccharide methyl-3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (**6**) as the tightest binding oligosaccharide, which fills up the entire site of the carbohydrate recognition domain of the Con A lectin. The binding was reported to be roughly 100 times tighter than for the monosaccharide, methyl α-D-mannopyranoside (**7**) shown in Figure 1-7. N-linked-glycoproteins[47,56,59] with a trimannose fragment display greater binding due to extended binding interactions.

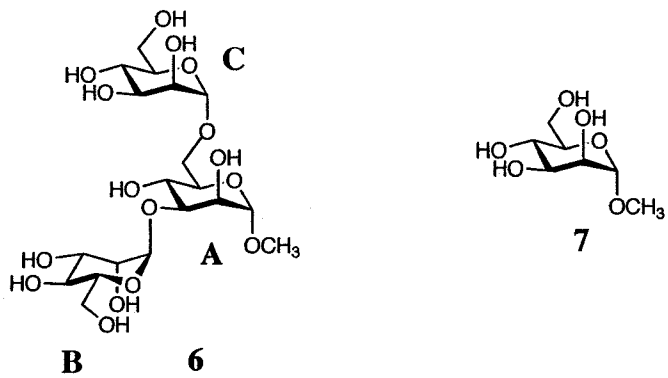


Figure 1-6. Methyl-3,6-di-O-(α-D-mannopyranosyl)-α-D-mannopyranoside (**6**) and methyl α-D-mannopyranoside (**7**), carbohydrate ligands for lectin Con A.

In the crystal structure of Con A bound to the trisaccharide **6**, ring **C** of (α–(1→6)–Man*p*) resides in a monosaccharide binding domain (Figure 1-7) of the

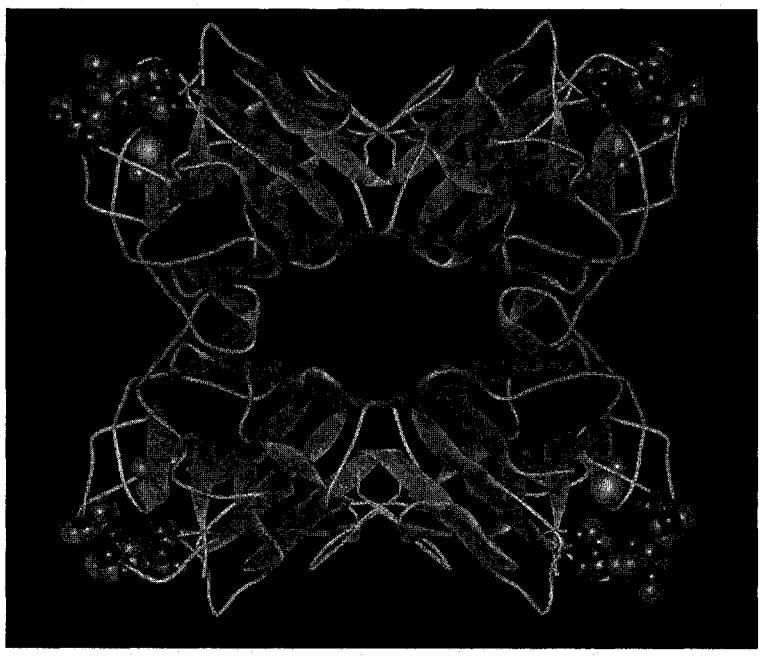


Figure 1-7. Crystal structure[60,61] of concanavalin A complexed with methyl-3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (**6**). (PDB code = IONA)

lectin Con A; 3, 4, and 6-hydroxyl groups have been found to be critical for tight binding.[53,61,62] The dissociation of the trisaccharide **6** ($K_d = 2.0 \times 10^{-7}$ M)[39] from the lectin would occur less easily when compared to the monosaccharide **7** ($K_d = 1.2 \times 10^{-4}$ M).[57,58,63]

1.3.2 Peanut Agglutinin (PNA)

Another lectin of the same legume family is PNA (peanut agglutinin), isolated[64] from peanuts (*Arachis hypogea*) and first[65] characterized by Sharon and co-workers. This 110-kDa lectin is a homotetramer[65-67] at neutral pH (Figure 1-8). It shows an unusual open quartenary structure of four sugar binding sites each containing one divalent cation of Ca^{2+} and Mg^{2+} per subunit. PNA is specific for D-galactopyranosyl[65] end groups and

its binding constants at room temperature, are in the range[68] of 10^{-3} M for monosaccharides such as methyl-galactopyranosides and 10^{-7} M for the T-antigen, which is a glycopeptide. Because PNA lectin has a strong affinity[65,68-71] for β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-galactopyranose (**8**) (Figure 1-9), which is also found in the tumor-associated T-antigenic disaccharide (Thomsen-Friendenreich antigen), the lectin has valuable clinical applications, for example, in the screening of cancerous malignancies. As depicted in Figure[72] 1-10, the ligand β-D-galactopyranosyl

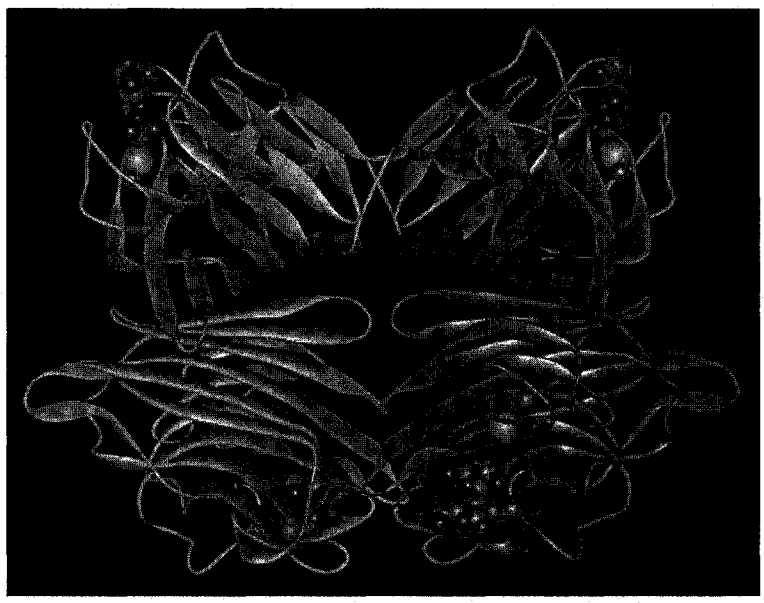
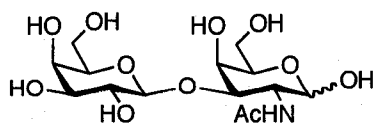


Figure 1-8. The crystal structure[60,63,72] of peanut agglutinin T-antigen complex. (PDB code = 2TEP)



8

Figure 1-9. β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-galactopyranose (**8**).

-(1→3)-2-acetamido-2-deoxy-galactopyranose (**8**) [Gal (1→3)GalNAc] is found with the non-reducing sugar unit,[65] ring E, in the carbohydrate recognition site, forming hydrogen bonds either directly or via water molecules[72] (**w**) with the amino acids of the lectin. Ring **D** is hydrogen bonded mainly to the extended site of PNA lectin.

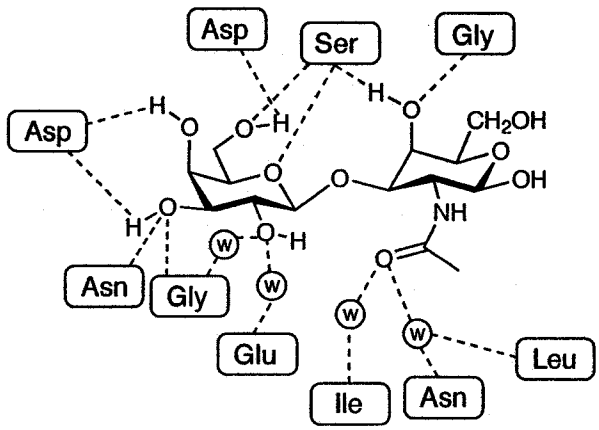


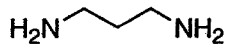
Figure 1-10.[72] Galβ(1→3)GalNAc in the combining site of peanut agglutinin (reproduced from reference 72).

1.4 Receptors for charged carbohydrates

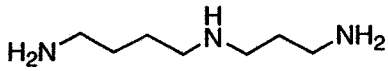
Charged complex carbohydrates fulfil many important biological functions. For example, heparin, the polysulfated oligosaccharide has powerful anti-coagulating properties. More research is being done to investigate the medicinal properties of sulfated and other polyanionic carbohydrates. The use of receptors and sensors will help in better understanding their roles, and thus may prove useful in medical therapy.

1.4.1 Polyamine sensors for negatively charged carbohydrates

Because of their cationic nature under physiological conditions, polyamines are known to bind strongly through ion-pairing to polyanionic biomolecules such as proteins, oligonucleic acids and sulfated oligosaccharides such as heparins.[73] The pK_a's of each amine on putrescine, and spermidine are given in Figure 1-11.[74]



Putrescine (pK_a : 10.8, 9.6)



Spermidine ((pK_a : 10.9, 9.8, 8.3)

Figure 1-11. Natural polyamines[74] present in animal and plant cells with pK_a values.[75,76]

Our group (Manku and Hall) optimized the synthesis of split-pool polyamine libraries[77] and screened them against sulfated dyes as targets for multivalent ion pairing. These dyes were serving as charge models for sulfated biomolecules such as polysulfated heparin oligosaccharide[78] and therefore might help in the development of ligands and sensors for these biologically important carbohydrates (Figure 1-12).[79]

An example of a polysulfated polysaccharide is heparin (Figure 1-12A). Heparin is made[80] pharmaceutically as an anticoagulant drug to prevent and treat cardiovascular diseases. The sulfated polysaccharide consists of a carbohydrate backbone[78,80] made of alternating 1,4-linked uronic acid (D-glucuronic acid or L-iduronic acid) and

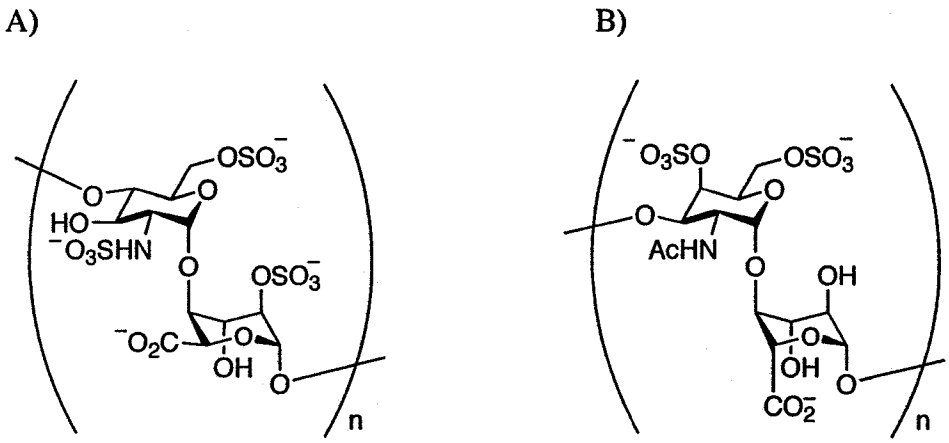


Figure 1-12. Repeating disaccharide unit[79] in (A) heparin and (B) chondroitin sulfate.

glucosamine residues.

Heparin inhibits[78,80] blood coagulation through activation of antithrombin III (ATIII), a glycoprotein that physiologically inhibits coagulation. Heparin binding sites, commonly observed on the external surfaces of proteins, correspond to shallow pockets of positive charges.

Another sulfated carbohydrate (part of the glycosaminoglycans- GAGs), important in cell-cell communication, is chondroitin sulfate. It is found localized[81] on cell surfaces and in the extracellular matrix. Polyamine sensors or reporters for these types of carbohydrates would help in better understanding their biological functions.

The screening of synthetic polyamines against heparins would also aid in the development of receptors and drugs that would suppress cancer-related events.[78,80,82] In a cell, polyamines assemble[82] through a biosynthetic pathway that can be inhibited by

α–difluoromethylornithine (DFMO). However, cell-surface heparan sulfate proteoglycans help in the uptake[83-86] of extracellular polyamines, thus canceling the cytostatic effect of DFMO. It was proposed by Belting and co-workers[82] that a combined inhibition of heparan sulfate assembly and polyamine synthesis may represent an additional strategy for cancer therapy.

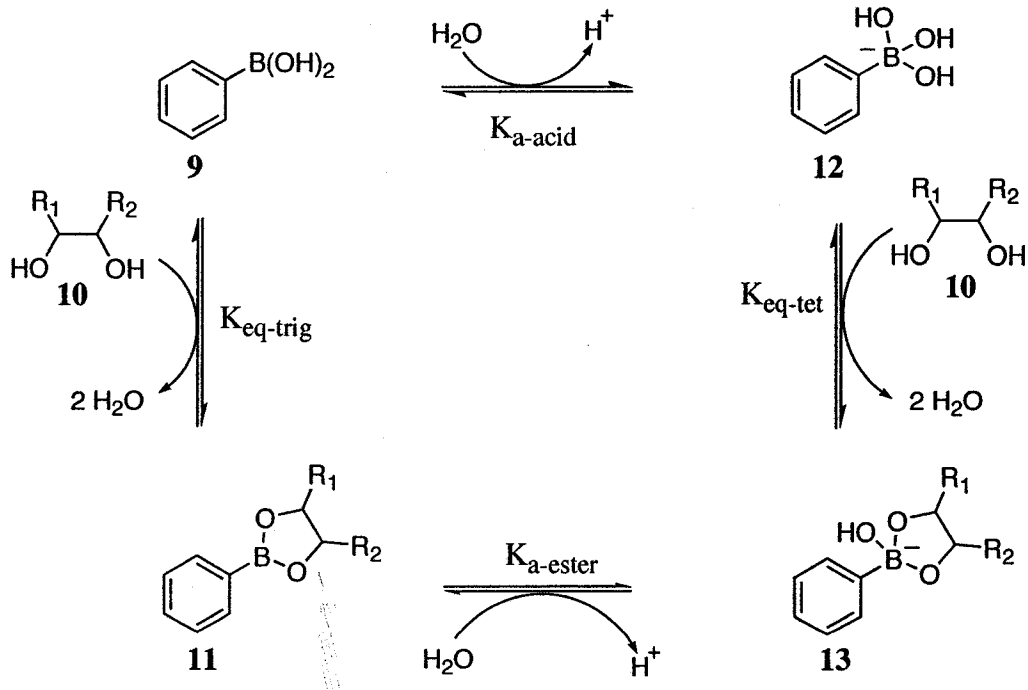
1.5 Boronic acid sensors for saccharides

1.5.1 Determination of binding constants

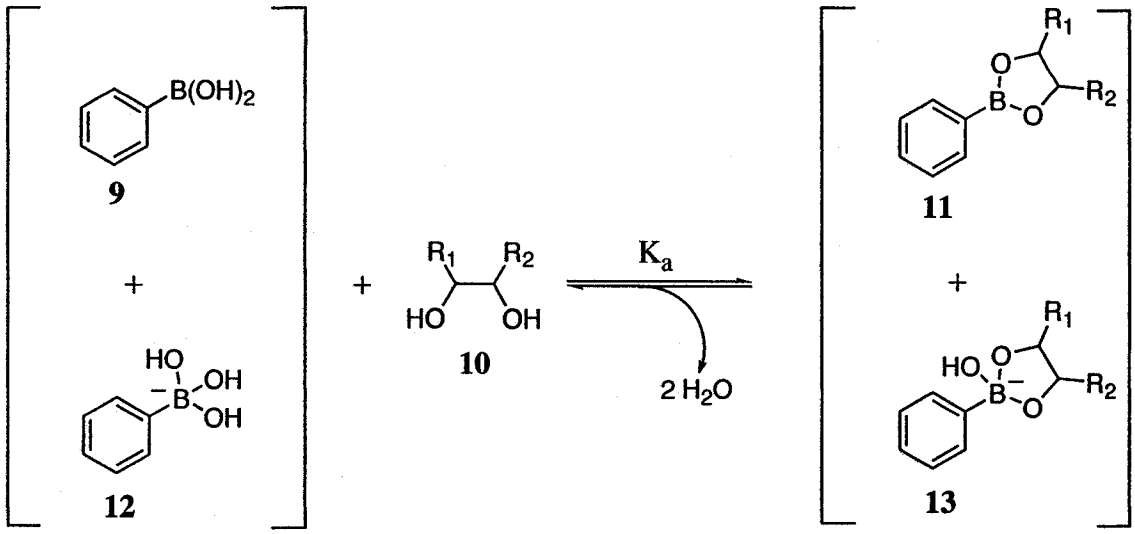
The need for small receptor molecules that bind with high selectivity and affinity with biomolecules is greatly felt in diverse areas of medicinal and clinical chemistry. In contrast to biopolymers such as polypeptides[87,88] (proteins and amino acids) and nucleic acids[89,90] (DNA and RNA), not much progress has been made in the development of receptors for oligosaccharides. This is due to the multiple stereocentres and ability of carbohydrates to exist in either furanose or pyranose forms, rendering molecular recognition and chemosensory detection[91-94] of carbohydrates more challenging.

Existing detection methods for saccharides, such as enzyme assays, suffer from numerous disadvantages[95,96] such as poor stability, heat and pH sensitiviy of the enzyme and consumption of substrate during the process. Although less sensitive than protein-based biosensors, synthetic chemosensors can be more reliable and stable under a wider range of conditions.

Complex formation between an aromatic boronic acid and different polyols was first mentioned in 1957 by Kuivila and co-workers.[97] Thence, boronic acids became an example of synthetic sensors[93] that could be used with carbohydrates. Boronic acids have already been used for the generation of selective transporters[98-101] of nucleosides, saccharides and nucleotides, and as inhibitors of proteases,[102-105] thus affirming their relative non-toxicity in the human body. There is a growing interest in developing sensitive detection methods involving boronic acids to bind selectively and with high affinity to saccharides. Boronic acid saccharide sensors can have numerous applications in medicinal therapy, for example in glucose[92] detection during the diagnosis of diseases such as diabetes. Boronic acid receptors can also be used in industry, for monitoring fermentation processes.



Scheme 1.1



Scheme 1.2

The main feature of boronic acids as sensors is that they are capable of binding reversibly in a covalent fashion to diol functionalities (Scheme 1.1) found in some carbohydrate molecules. Both the boronic acid **9** and its ester **11** are considered acids because of their Lewis acidic character imparted by the open shell of the boron atom, which can accept a lone pair of electrons.

In an aqueous solution, phenylboronic acid can exist in the neutral trigonal planar form **9** or the tetrahedral anionic form **12** (Scheme 1.2). The equilibrium would be determined by the pK_a of the acid. Similarly, the boronate ester can exist in the neutral trigonal form **11**, and the anionic tetrahedral form **13**. So, $K_{a\text{-}trig}$ represents the binding constant of the boronic acid **9** which forms an ester in its trigonal form, and $K_{a\text{-}tet}$ represents the binding constant of the boronic acid when in the tetrahedral form **12**. The

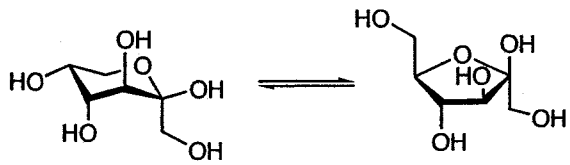
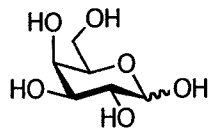
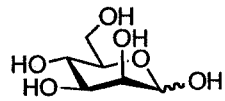
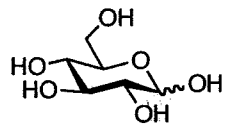
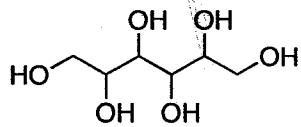
overall binding constant K_a (Scheme 1.2) is more representative of the binding constant of the phenylboronic acid to the diol as all the species are represented.

In 1959, Lorand and co-workers[106] did an extensive study on the binding constants between various diol-containing compounds and phenylboronic acid. Their study showed the lowering of the pH when a boronic acid binds to a diol compound in a boronic acid buffered solution. The decrease in pH with the increased addition of the diols was correlated with the binding constant of phenylboronic acid, termed as pH depression method.[106] The numbers listed in Table 1.2 reported by Lorand and co-workers[106] are very different from the binding constants of other monoboronic acids determined later using spectroscopy. The discrepancy was due to an unclear definition of the term "binding constants". The paper reported essentially $K_{a\text{-}tet}$ as it was assumed that no neutral boronate ester **11** existed (or was too negligible to be taken into account) because of the decreased pK_a of the boron upon ester formation. Thus, $K_{a\text{-}trig}$ was omitted in the mathematical derivations. Since, equilibrium between boronic acid **9** and boronic ester **12** was an acid-base equilibrium, the only unknown was $K_{a\text{-}tet}$, assumed to be the same as K_a. Experimental results by Wang and co-workers[107] proved this assumption to be wrong, and the author expected that under experimental conditions, the boronic ester **11** constituted about 20-30% of the complexed form.

Other conventional methods for determining binding constants of boronic acids involve ^{11}B NMR method[108-113] and the measurement of intrinsic fluorescence emitted or

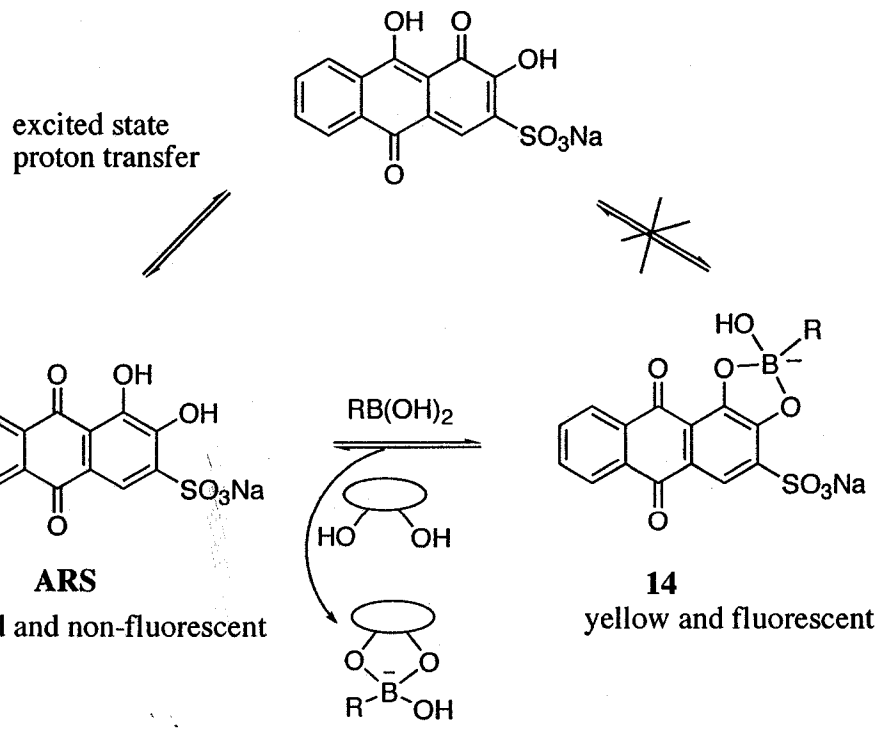
ultraviolet light absorbed by boronic acids when binding to diols. ^{11}B NMR is however based on the same concept that addition of a diol to a boronic acid solution would result

Table 1.2 Equilibrium constants for phenylboronic acid-polyol complexes.

Polyols	Association constants	
	K_a (M^{-1}) by pH[106] depression	K_a (M^{-1}) by ARS[107] spectroscopic method (pH 7.4)
 D-fructose	4370	160
 D-galactose	276	15
 D-mannose	172	13
 D-glucose	110	4.6
 D-mannitol	2275	120

in an increased portion of the boron being converted from the trigonal form to the anionic tetrahedral form. A significant shift difference can be seen for the neutral form of boronic acid at approximately 30 ppm and the anionic form of boronic acid at about 7 ppm. The mathematical derivation in the ^{11}B NMR method is the same as the pH depression method and gives the same $K_{a\text{-}tet}$. For example, the binding constants[109] of boric acid with glucose and galactose were determined using ^{11}B NMR method and were found to be in the range of 100 M^{-1}, similar to that obtained by pH-depression method. However, the ^{11}B NMR method suffers from low sensitivity, difficulties with peak resolution, and the requirement for high concentration of the boronic acid.

On the other hand, spectroscopic methods are generally more sensitive than NMR or pH determination. The binding event needs to trigger a change in the spectroscopic properties of the boronic acid component, for example, CD,[114-116] absorption[117-120] and

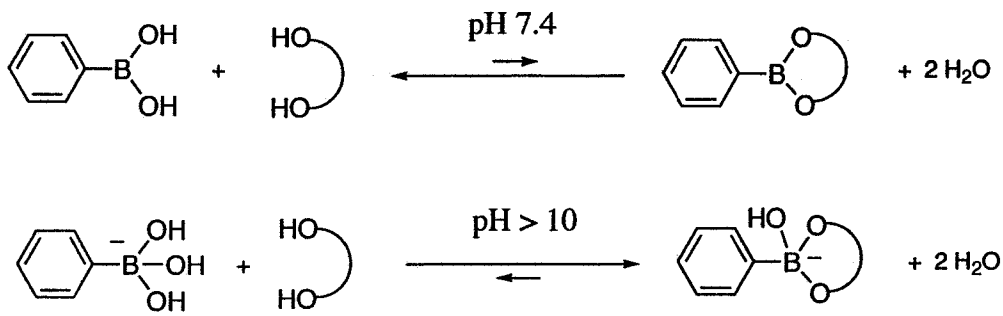


Scheme 1.3

fluorescence.[107,121-125] Therefore, this method is limited to boronic acids that change their spectroscopic properties sufficiently for their binding constants to be determined this way. To circumvent the problem, a competitive dye displacement assay called the ARS (Alizarin Red S) assay developed by Springsteen and Wang[107] allows the determination of binding constants for non-fluorescent boronic acids. In this system (Scheme 1.3), two equilibria are present. One of which involves the formation of yellow fluorescent complex between the boronic acid and the pink red dye Alizarin Red S. Free ARS is non-fluorescent because of the quenching effect of excited state proton transfer from the phenol hydroxyl group to the ketone oxygen of ARS (Scheme 1.3). The quenching mechanism does not operate under boronate ester formation with the ARS dye. On addition of a diol moiety in the form of a carbohydrate, the yellow colored fluorescent complex reverts back to the free dye as the boronic acid complexes with the added carbohydrate and is removed from the equilibrium. The resultant change in color can be observed and the emitted fluorescence or absorbed UV (ultraviolet light) can be measured spectrophotochemically. Treatment of the resulting data yields the corresponding binding constants between boronic acids and saccharides (Table 1.2), which are significantly different from those obtained by pH-depression method. The development of the ARS method allows for the first time the sensitive determination of K_a beween a boronic acid and a diol regardless of the spectroscopic nature of the boronic acid. The relationship between $K_{a\text{-}tet}$, $K_{a\text{-}ttrig}$ and K_a has also been described in a recent report by Springsteen and Wang.[107]

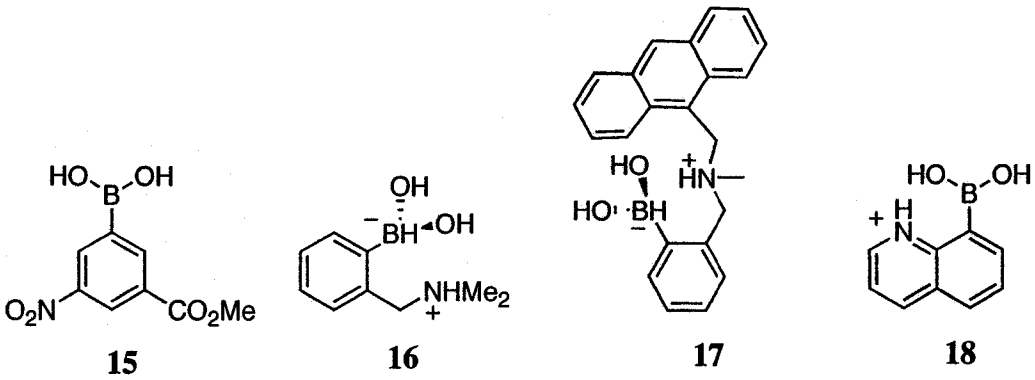
1.5.2 Factors affecting the operating pH of boronic acid based sensors

At physiological pH of 7.4, phenylboronic acid (**9**) ($pK_a = 8.8$)[93,106] exists mostly as the free acid. To favor the formation of boronate esters, the pH must be increased to at least 10 units as shown in Scheme 1.4. However, a neutral pH is required for glucose measurements in blood. Reducing the pK_a of the boronic acid can allow for the use of neutral conditions.



Scheme 1.4

This can be achieved by introducing electron-withdrawing groups on the arylboronic acid. The presence of electron-withdrawing groups in the aromatic ring result in better stabilization of the developing negative charge during boronate ester formation, for instance in 3-methoxycarbonyl-5-nitrophenylboronic acid[126] **15** ($pK_a = 6.9$) (Figure 1-13).



15 **16** **17** **18**

$pK_a = 7.9$ $pK_a = 5.2$ $pK_a = 2.9$[127] $pK_a = 4.0$

Figure 1-13. Aryl boronic acids with low pK_a values.

Another attractive modification of aromatic boronic acids is the incorporation of an *ortho*-dialkylaminomethyl substituent as examplified in boronic acids **16** (pK_1 = 5.2) and **17** (pK_1 = 2.9) (Figure 1-13). This modification was first reported by Wulff.[128] The very good fructose-selective fluorescent anthracene-based sensor **17** was designed by Shinkai and co-workers.[92,107,121] It was previously demonstrated by Wulff[128] that the putative B-N dative bond formation depicted in Figure 4-14 decreased the apparent pK_a of the boronic acid. It was presumed that upon formation of a boronate ester,

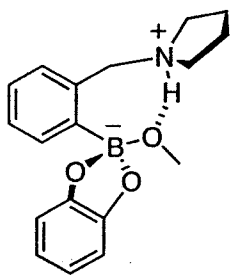
16 **16a**

Figure 1-14. Putative B-N dative bond in boronic acid **16**.

the Lewis acidity of the boron is increased, with a resultant increase in B-N dative bond strength, hence a shorter bond. Contradictory studies by Wang[129] and co-workers have shown that the B-N bond is longer for certain boronate esters than for the free boronic acid because of the steric strain involved.

The lone pair of electrons on the benzylic amine moiety was known to quench the anthracene fluorescence. The large change in fluorescence intensity associated with binding of boronic acid **17** with diols was attributed to the strength of the B-N bond, through the modulation of the excited state photoinduced electron transfer (PET). Wang and co-workers[121,129,130] showed increasing evidence (calculated B-N bond strength) of a hydrolysis mechanism versus the B-N mechanism whereby the lone pair of electrons is tied up by protonation, thus preventing fluorescence quenching through PET.

Moreover, Anslyn and co-workers[131] have recently reported that in the presence of methanol, a methoxy group coordinates to the boron atom while the nitrogen is protonated, giving the complex illustrated in Figure 1-15.



19

Figure 1-15. Representation of complex **19** between catechol and aromatic boronic acid (reproduced from reference 131).

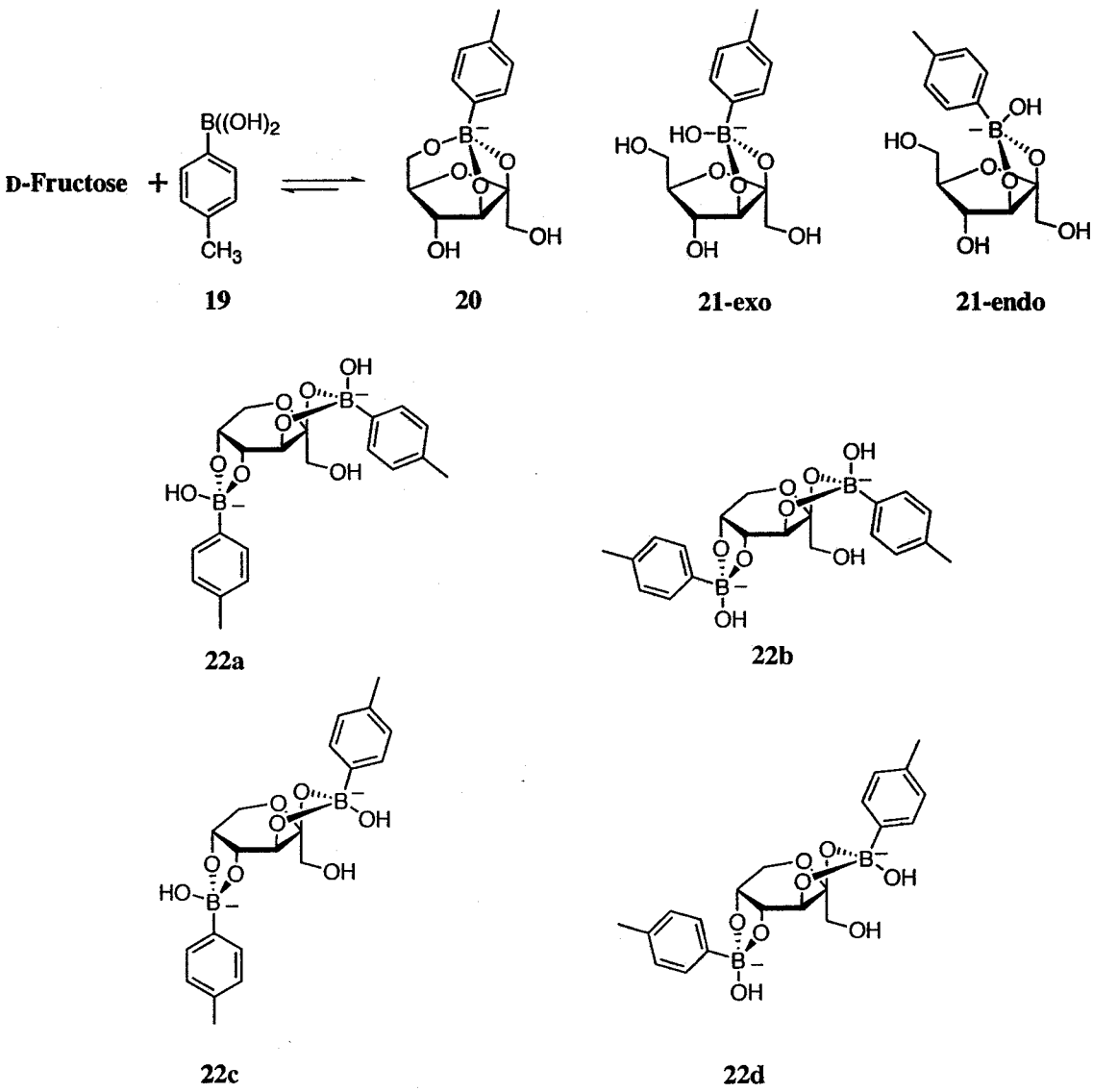
These findings thus weaken the argument of any B-N interaction in the binding of carbohydrates.Wang and co-workers[121] also discovered other classes of water-soluble sugar-binding boronic acids such as 8-quinolineboronic acid[132] **18** (pK_a of the boronic acid moiety = 4) shown in Figure 1-13. The carbohydrate complexes of the latter exist with the boron in the sp^3 hybridization state at pH 7.4. These types of very acidic boronic acids can give rise to attractive receptors for carbohydrates at the physiological pH of 7.4.

1.5.3 Geometry of saccharides bound to boronic acids

Until recently, there was a debate concerning the geometry of monosaccharides bound to the arylboronic acids as several reducing carbohydrates can exist in either the five-membered furanose form or the six-membered pyranose form.[92,133,134]

In 1995, Eggert and Norrild[134] studied the complex formation between *p*-tolylboronic acid and D-glucose by ^1H and ^{13}C NMR spectroscopy under aqueous and non-aqueous conditions and found that glucose binds mostly in its weakly populated furanose form (0.14 % in water). Later, in 1996, Norrild and Eggert[135] reported the presence of different complexes (Scheme 1.4) formed at high pD upon mixing *p*-tolylboronic acid with fructose. 2,3,6 Tridentate complex **20** was found to be the most abundant form in the equilibrating mixture of 1:1 boronic acid and fructose mixture (Scheme 1.5, 82 %). A small amount of 2,3 exo and endo isomers (**21-exo** and **21-endo**) were present and in all cases the fructose was found in the five-membered ring form. With a high ratio of *p*-

tolylboronic acid/fructose such as 2:1 and 4:1 (Scheme 1.5), endo/exo isomers of bisboronate complex **22** were formed with fructose in its pyranose form.

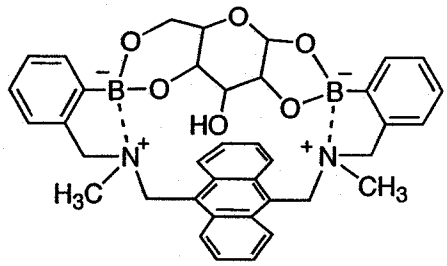


Scheme 1.5

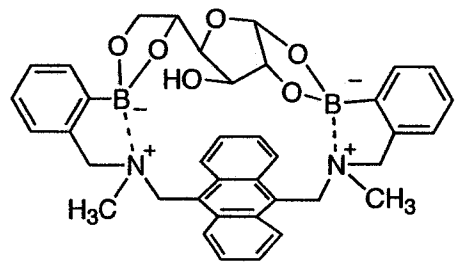
1.5.4 Development of sensors for glycoconjugates

Carbohydrate conjugates such as glycoproteins and glycolipids exist in nature mainly in the pyranose form as glycosylation often occurs at the 1- and 4-OH positions of the carbohydrate ring. Many of these carbohydrates are on the surface on mammalian cells and can serve as cell markers.[136] Over-expression of cell-surface carbohydrates such as sialyl Lewis X (sLex) is associated with the development of gastro-intestinal, pancreatic and breast cancer.[137,138] To increase the selectivity towards glycopyranosides, that is carbohydrates in the six-membered forms, one of the strategies would be to incorporate two boronic acid moieties in the same sensor molecule that would interact with two adjacent diol units on the saccharide.[139,140]

Following ^{1}H NMR studies in methanol, Shinkai and co-workers proposed the structure of the complex between the bis-boronic acid and glucose involving the monosaccharide in its pyranose form, giving complex **23a** (Figure 1-16).[127] Norrild and co-workers,[141] however, found that the six-membered cyclic form of glucose (in methanol) isomerized to the five-membered ring in the presence of water resulting in stronger binding to the receptor within the proposed complex **23b**. Syn 1,2-diol units of furanoses are strongly preferred to minimize angle strain in the resulting boronic ester When a coplanar boronate ester is formed with non-coplanar diols of a glycopyranoside, a highly unfavorable conformational change is induced in the puckered sugar ring.[97,142]



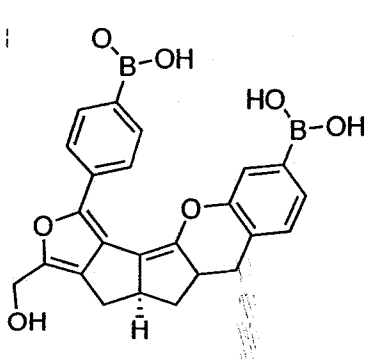
23a



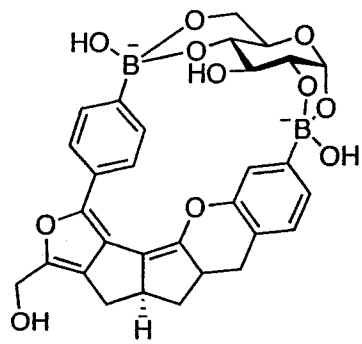
23b

Figure 1-16. Complex **23a** showing glucose binding to a bis-boronic receptor in its pyranose form and complex **23b** showing glucose bound in its furanose form. [141]

Recently, Drueckhammer and co-workers[143] synthesized a rigid bis-boronic acid (Figure 1-17) based on computational design of the best interspatial arrangement of two boronic acids for glucopyranose binding. The authors compared the coupling constants of the complex **24a** (Figure 1-17) with related reference compounds, the pyranose forms and the furanose forms of glucose. Fluorescence experiments were also carried out in 33% MeOH/aqueous buffer at pH 7.5 to evaluate the affinity of **24** (Figure 1-16) with different monosaccharides.



24



24a

Figure 1-17. Receptor **24** and complex **24a** when glucose is bound in the pyranose[143] form.

The selectivity and binding constant were found to be higher for glucose in the pyranose form. This selectivity was attributed to the tight bidendate glucose-diboronic acid complex **24a**.Despite these recent successes, there is still an urgent need for a boronic acid moiety that is capable of binding to glycosides or carbohydrates in the pyranose form, and which can form a carbohydrate complex under aqueous conditions at pH 7.4 for *in vivo* applications.

1.6 Thesis goals

Protein-carbohydrate interactions are responsible for a number of biological processes in nature, including cellular recognition by organisms such as viruses or bacteria. The search for carbohydrate-based therapeutics has motivated the development of carbohydrate and glycopeptide libraries. In order to screen carbohydrates as high-binding ligands to specific protein receptors, fluorescent labels are the ideal non-lethal tools in the detection of positive hits. The aromatic moieties of fluorescent dyes, however, tend to display non-specific interactions with the aromatic residues of proteins and the solid-support. The first part of the project will be centered on investigating a number of known dye molecules that could be covalently attached to the carbohydrate ligand. The non-specific effects of these dyes will be studied in a specific carbohydrate-protein interaction, in particular, carbohydrate-lectin interactions on a solid support. Semi-quantitative studies will be performed to compare the aromatic fluorescent dyes and a europium-based label covalently bound to the carbohydrate ligands.

The second part of the thesis will be focused on the screening of polyamine libraries as potential receptors for sulfated saccharides. High concentrations of natural polyamines[74,144] seem to be vital for the growth of cancer cells and their depletion represents a strategy for the treatment of cancer. Small-molecule models of sulfated carbohydrates such as heparin, an anticoagulant drug, will be synthesized and they will be conjugated with fluorescent labels. The libraries of polyamines supported on polystyrene beads will be screened against these fluorescently labeled carbohydrates.

The need for small receptor molecules that bind with high selectivity and affinity to biomolecules is greatly felt in diverse areas of medicinal and clinical chemistry. The objective of the final part of this thesis is on the identification of boronic acid receptors and sensors for reducing and non-reducing sugars. At present, the problems encountered with current boronic acids are their poor solubility in water and their inability to bind to the hexopyranoside units of complex carbohydrates found on cell surfaces. Our efforts will focus firstly on a preliminary qualitative screening of the best boronic acids that bind to non-reducing sugars, followed by a quantitative determination of their binding constants. Then, by performing structural studies, we can attempt to better understand their mode of binding and utilize the multivalency effects of two or more boronic acids in a sensor to enhance the binding with hexopyranosides of complex carbohydrates.

1.7 References

(1) Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683-720.

(2) Lis, H.; Sharon, N. *Chem. Rev.* **1998**, *98*, 637-674.

(3) Sharon, N.; Lis, H. *Glycobiology* **2004**, *14*, 53R-62R.

(4) Hakomori, S.; Zhang, Y. *Chem. Biol.* **1997**, *4*, 97-104.

(5) Alper, J. *Science* **2003**, *301*, 159-160.

(6) Lindth, I.; Hindsgaul, O. *J. Am. Chem. Soc.* **1991**, *113*, 216-223.

(7) Waston, K. A.; Mitchell, E. P.; Johnson, L. N.; Son, J. C.; Bichard, C. J. F.; Orchard, M. G.; Fleet, G. W. J.; Oikonomakos, N. G.; Leonidas, D. D.; Kontou, M.; Papageorgioui, A. *Biochemistry* **1994**, *33*, 5745-5758.

(8) Isbister, B. D.; St. Hilaire, P. M.; Toone, E. J. *J. Am. Chem. Soc.* **1995**, *117*, 12877-12878.

(9) Lemieux, R. U. *Acc. Chem. Res.* **1996**, *29*, 373-380.

(10) Izumi, M.; Tsuruta, O.; Hashimoto, H.; Yazawa, S. *Tetrahedron Lett.* **1996**, *37*, 1809-1812.

(11) Gohier, A.; Espinosa, J. F.; Jimenez-Barbero, J.; Carrupt, P. A.; Perez, S.; Imberty, A. *J. Mol. Graphics* **1996**, *14*, 322-327.

(12) Lemieux, R. U. *Chem. Soc. Rev.* **1989**, *18*, 347-374.

(13) Mulligan, M. S.; Paulson, J. C.; Defrees, S.; Zheng, Z. L.; Lowe, J. B.; Ward, P. *Nature* **1993**, *363*, 149-151.

(14) Livingston, P. O. *Immunol. Rev.* **1995**, *145*, 147-166.

(15) Balfour, J. A.; Mc Tavish, D. *Drugs* **1993**, *46*, 1025-1054.

(16) Johnston, P. S.; Coniff, R. F.; Hoogwerf, B. J.; Santiago, J. V.; Pi-Sunyer, F. X.; Krol, A. *Diabetes Care* **1994**, *17*, 20-29.

(17) Jacob, G. S. *Curr. Opin. Struct. Biol.* **1995**, *5*, 605-611.

(18) Avila, M. A.; Velasco, J. A.; Cho, C.; Lupu, R.; Wen, D. Z.; Notario, V. *Oncogene* **1995**, *10*, 963-971.

(19) Lian, R. H.; Kotwal, G. H.; Wellhausen, S. R.; Hunt, L. A.; Justus, D. E. *Immunology* **1996**, *15*, 39-46.

(20) Hoppensteadt, D. A.; Jeske, W.; Fareed, J.; Nicolaides, A. N. *Int. Angio.* **1996**, *15*, 39-46.

(21) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: California, 1995.

(22) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; 2 ed.; Klumer Academic/Plenum publishers: New York, 1999.

(23) Kawamura, A. J. *Fluorescent Antibody Techniques and their Applications*; University of Tokyo Press: Baltimore, Maryland, 1977.

(24) Brinkley, M. *Bioconjugate Chem.* **1992**, *3*, 3-12.

(25) Iyer, P. K.; Wang, S. *Tetrahedron Lett.* **2006**, *47*, 437-439.

(26) Hemmilä, I. A. *Applications of Fluorescence in Immunoassays*; John and Wiley & Sons: New York, 1991; 107-127.

(27) Website address: www.probes.com (4/1/06).

(28) Hemmilä, I. A. *Progress in Delayed Fluorescence Immunoassay*; Springer-Verlag, 1990; 259-266.

(29) Lee, Y. C. *J. Biochem.* **1997**, *121*, 818-825.

(30) Lee, Y. C. *Anal. Biochem.* **2001**, *297*, 123-127.

(31) Hemmilä, I. A.; Dakubu, S.; Mukkula, V.-M.; Siitari, H.; Lövgren, T. *Anal. Biochem.* **1984**, *137*, 335-343.

(32) Menjívar, M.; Ortiz, G.; Gárdenas, M.; Garza-Flores *J. Rev. Invest. Clin.* **1993**, *45*, 579-584.

(33) Schoket, B.; Doty, W. A.; Vincze, I.; Strickland, P. T.; Ferri, G. M.; Assennato, G.; Poirier, M. C. *Cancer Epidemiol. Biomarkers Prev.* **1993**, *2*, 349-353.

(34) Kawasaki, N.; Lee, Y. C. *Anal. Biochem.* **1997**, *250*, 260-262.

(35) Matsumoto, K.; Nojima, T.; Sano, H.; Majima, K. *Macromol. Symp.* **2002**, *186*, 117-121.

(36) Yuan, J.; Matsumoto, K.; Kimura, H. *Anal. Chem.* **1998**, *70*, 596-601.

(37) Taki, T.; Nishiwaki, S.; Handa, N.; Hattori, N.; Handa, S. *Anal. Biochem.* **1994**, *219*, 104-108.

(38) Yu, L. S.; Reed, S. A.; Golden, M. H. *J. Microb. Methods* **2002**, *49*, 63-68.

(39) Butcher, H.; Kennette, W.; Collins, O.; Demoor, J.; Koropatnick, J. *J. Immunol. Methods* **2003**, *272*, 247-256.

(40) Hemmilä, I.; Laitala, V. *J. Fluorescence* **2005**, *15*, 529-542.

(41) Deras, I. L.; Sano, M.; Kato, I.; Lee, Y. C. *Anal. Biochem.* **2000**, *278*, 213-220.

(42) Lee, Y. C.; Kawasaki, N.; Lee, R. T.; Suzuku, N. *Glycobiology* **1994**, *219*, 104-108.

(43) Website address: http://www.gak.co.jp/FCCA/glycoword/GT-C04/GT-C04E.html. (4/1/06)

(44) Goldstein, I. J.; Hughes, R. C.; Monsigny, M.; Osawa, T.; Sharon, N. *Nature* **1980**, *285*, 66-66.

(45) Lis, H.; Sharon, N. *Biology of Carbohydrates*; Ginsburg, V., Robbins, P. W. Eds.; John-Wiley & Sons: New York, 1984.

(46) Sharon, N.; Lis, H. *Lectins*; Chapman and Hall: New York, 1989.

(47) Lotan, R.; Beattie, G.; Hubbell, W.; Nicolson, G. L. *Biochemistry* **1977**, *16*, 1787-1794.

(48) Sumner, J. B. *J. Biol. Chem.* **1919**, *37*, 137-142.

(49) Wang, J. L.; Cunningham, B. A.; Waxdal, M. J.; Edelman, G. M. *J. Biol. Chem.* **1975**, *250*, 1490-1502.

(50) Min, W.; Dunn, A. J.; Jones, D. H. *EMBO J.* **1992**, *11*, 1303-1307.

(51) Hardman, K. D.; Ainsworth, C. F. *Biochemistry* **1972**, *11*, 4910-4919.

(52) Becker, J. W.; Reeke, G. N. J.; Wang, J. L.; Cunningham, B. A.; Edelman, G. M. *J. Biol. Chem.* **1975**, *250*, 1513-1524.

(53) Naismith, J. H.; Field, R. A. *J. Biol. Chem.* **1996**, *271*, 972-976.

(54) Kantardjieff, K. A.; Höchtl, P.; Segelke, B. W.; Tao, F.-M.; Rupp, B. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2002**, *58*, 735-743.

(55) Derewenda, Z.; Yariv, J.; Helliwell, J. R.; Kalb, A. J.; Dodson, E. J.; Papiz, M. Z.; Wan, T.; Campbell, J. *EMBO J.* **1989**, *8*, 2189-2193.

(56) Brewer, C. F.; Bhattacharyya, L. *J. Biol. Chem.* **1986**, *261*, 7306-7310.

(57) Mandal, D. K.; Kishore, N.; Brewer, C. F. *Biochemistry* **1994**, *33*, 1149-1156.

(58) Dam, T. K.; Roy, R.; Das, S. K.; Oscarson, S.; Brewer, C. F. *J. Biol. Chem.* **2000**, *275*, 14223-14230.

(59) Brewer, F.; Bhattacharyya, L.; Brown, R. D.; Koenig, S. H. J. *Biochem. Biophys. Res. Commun.* **1985**, *127*, 1066-1071.

(60) 3D Lectin Database Query Form on World Wide Web URL: http://www.cermav.cnrs.fr/cgibin/wdbi.cgi/lectines/lectine/form/monos/monos.cgi. (4/1/06)

(61) Loris, R.; Maes, D.; Poortmans, F.; Wyns, L.; Bouckaert, J. *J. Biol. Chem.* **1996**, *271*, 30614-30618.

(62) Gupta, D.; Dam, T. K.; Oscarson, S.; Brewer, C. F. *J. Biol. Chem.* **1997**, *272*, 6388-6392.

(63) Carver, J. P.; Mackenzie, A. E.; Hardman, K. D. *Biopolymers* **1985**, *24*, 49-63.

(64) Bird, G. W. G. *Vox Sang.* **1964**, *9*, 748-749.

(65) Lotan, R.; Skutelsky, E.; Danon, D.; Sharon, N. *J. Biol. Chem.* **1975**, *250.*

(66) Fish, W. W.; Hamlin, L. M.; Miller, R. L. *Arch. Biochem. Biophysics.* **1978**, *190*, 693-698.

(67) Bannerjee, R.; Mande, S. C.; Ganesh, V.; Das, K.; Dhanraj, V.; Mahanta, S. K.; Suguna, K.; Surolia, A.; Vijayan, M. *Proc. Natl. Acad. Sci.* **1994**, *91*, 227-231.

(68) Neurohr, K. J.; Young, N. M.; Mantsch, H. H. *J. Biol. Chem.* **1980**, *255*, 9205-9209.

(69) Irimura, T.; Kawaguchi, T.; Terao, T.; Osawa, T. *Carbohydr. Res.* **1975**, *39*, 317-327.

(70) Pereira, M. E. A.; Kabat, E. A.; Lotan, R.; Sharon, N. *Carbohydr. Res.* **1976**, *51*, 107-118.

(71) Vaith, P.; Uhlenbruck, G. Z. *Immunitaets Forsh* **1978**, *154*, 1-14.

(72) Ravishankar, R.; Ravindran, M.; Suguna, K.; Surolia, A.; Vijayan, M. *Curr. Sci.* **1997**, *72*, 855-861.

(73) Belting, M.; Havsmartk, B.; Jonsson, M.; Persson, S.; Fransson, L.-Å. *Glycobiology* **1996**, *6*, 121-129.

(74) Cohen, S. S. *Guide to Polyamines*; Oxford University Press: New York, 1998.

(75) Delfini, M.; Segre, A. L.; Conti, f.; Barbucci, R. *J. Chem. Soc. Perkin Trans. 2* **1980**, 900-903.

(76) Kanavorioti, A.; Baird, E. E.; Smith, P. J. *J. Org. Chem.* **1995**, *60*, 4873-4883.

(77) Manku, S.; Hall, D. G. *Org. Lett.* **2002**, *4*, 31-34.

(78) Petitou, M.; van Boeckel, C. A. A. *Angew. Chem. Int. Ed.* **2004**, *43*, 3118-3133.

(79) Marks, R. M.; Lu, H.; Sundaresan, R.; Toida, T.; Suzuki, A.; Imanari, T.; Hernáiz, M. J.; Linhardt, R. J. *J. Med. Chem.* **2001**, *44*, 2178-2187.

(80) Linhardt, R. J. *J. Med. Chem.* **2003**, *46*, 2551-2564.

(81) Maruyama, T.; Toida, T.; Imanari, T.; Yu, G.; Linhardt, R. J. *Carbohydr. Res.* **1998**, *306*, 35-43.

(82) Belting, M.; Borsig, L.; Fuster, M. M.; Brown, J. R.; Persson, L.; Fransson, L.-Å.; Esko, J. D. *Proc. Natl. Acad. Sci.* **2002**, *99*, 371-376.

(83) Tabor, C. W.; Tabor, H. *Annu. Rev. Biochem.* **1984**, *53*, 749-790.

(84) Pegg, A. E. *Cancer Res.* **1988**, *48*, 759-774.

(85) Heby, O.; Persson, L. *Trends Biochem. Sci.* **1990**, *15*, 153-158.

(86) Pegg, A. E. *Biochem. J.* **1986**, *234*, 249-262.

(87) Wess, G.; Urmann, B.; Sickenberger, B. *Angew. Chem. Int. Ed.* **2001**, *40*, 3341-3350.

(88) Schreiber, S. L. *Bioorg. Med. Chem. Lett.* **1998**, *6*, 1127-1152.

(89) Dervan, P. B.; Burli, R. W. *Curr. Opin. Chem. Biol.* **1999**, *3*, 688-693.

(90) Gallego, J.; Varani, G. *Acc. Chem. Res.* **2001**, *34*, 836-843.

(91) Davis, A. P.; Wareham, R. S. *Angew. Chem. Int. Ed.* **1996**, *35*, 2978-2996.

(92) James, T. D.; Sandanayake, K. R. A. S.; Shinkai, S. *Angew. Chem. Int. Ed.* **1996**, *35*, 1910-1922.

(93) Wang, W.; Gao, X.; Wang, B. *Curr. Org. Chem.* **2002**, *6*, 1285-1317.

(94) Cao, H.; Heagy, M. D. J. *Fluorescence* **2004**, *14*, 569-584.

(95) Heller, A. *Annu. Rev. Biomed. Eng.* **1999**, *1*, 153-175.

(96) Gough, D. A.; Armor, J. C. *Diabetes* **1995**, *44*, 1005-1009.

(97) Kuivila, H. G.; Keough, A. H.; Soboczenski, E. J. *J. Org. Chem.* **1954**, *8*, 780-783.

(98) Westmark, P. R.; Gardiner, S. J.; Smith, B. *J. Am. Chem. Soc.* **1996**, *118*, 11093-11100.

(99) Mohler, L. K.; Czarnik, A. W. *J. Am. Chem. Soc.* **1993**, *115*, 2998-2999.

(100) Riggs, J. A.; Hossler, K. A.; Smith, B. D.; Karpa, M. J.; Griffin, G.; Duggan, P. J. *Tetrahedron Lett.* **1996**, *37*, 6303-6306.

(101) Paugam, M.-F.; Bien, J. T.; Smith, B. D.; Chrisstoffels, L. A. J.; de Jong, F.; Reinhoudt, D. N. *J. Am. Chem. Soc.* **1996**, *118*, 9820-9825.

(102) Suenaga, H.; Yamamoto, H.; Shinkai, S. *Pure Appl. Chem.* **1996**, *68*, 2179-2186.

(103) Weston, G. S.; Blazquez, J.; Baquero, F.; Shoichet, B. K. *J. Med. Chem.* **1998**, *41*, 4577-4596.

(104) Teicher, B. A.; Ara, G.; Herbst, R.; Palombella, V. J.; Adams, J. *Clin. Cancer Res.* **1999**, *5*, 2638-2645.

(105) Myung, J.; Kim, K. B.; Crews, C. M. *Med. Res. Rev.* **2001**, *21*, 245-273.

(106) Lorand, J. P.; Edwards, J. O. *J. Org. Chem.* **1959**, *24*, 769-774.

(107) Springsteen, G.; Wang, B. *Tetrahedron* **2002**, *58*, 5291-5300.

(108) Oi, T.; Takeda, T.; Kakihana, H. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 1903-1909.

(109) Vandenberg, R.; Peters, J. A.; Vanbekkum, H. *Carbohydr. Res.* **1994**, *253*, 1-12.

(110) Vanduin, M.; Peters, J. A.; Kieboom, A. P. G.; Vanbekkum, H. *Tetrahedron* **1984**, *40*, 2901-2911.

(111) Vanduin, M.; Peters, J. A.; Vanbekkum, H. *Tetrahedron* **1985**, *41*, 3411-3421.

(112) van Haveren, J.; Peters, J. A.; Batelaan, J. G.; Kieboom, A. P. G.; van Bekkum, H. *Recl. Trav. Chim. Pays-Bas* **1989**, *108*, 179-184.

(113) van Haveren, J.; van den Burg, M. H. B.; Peters, J. A.; Batelaan, J. G.; Kieboom, A. P. G.; van Bekkum, H. *J. Chem. Soc. Perkin Trans. 2* **1991**, *3*, 321-327.

(114) Takeuchi, M.; Taguchi, M.; Shinomori, H.; Shinkai, S. *Bull. Chem. Soc. Jpn.* **1996**, *69*, 2613-2618.

(115) Mizuno, T.; Yamamoto, M.; Takeuchi, M.; Shinkai, S. *Tetrahedron* **2000**, *56*, 6193-6198.

(116) Yamamoto, H.; Takeuchi, M.; Shinkai, S. *Tetrahedron* **2002**, *58*, 7251-7258.

(117) Ni, W.; Fang, H.; Springsteen, G.; Wang, B. *J. Org. Chem.* **2004**, *69*, 1999-2007.

(118) Soundararajan, S.; Badawi, M.; Kohlrust, C. M.; Hageman, J. H. *Anal. Biochem.* **1989**, *178*, 125-134.

(119) Shinmori, H.; Takeuchi, M.; Shinkai, S. *J. Chem. Soc. Perkin Trans. 2* **1996**, 1-3.

(120) Ward, C. J.; Patel, P.; James, T. D. *Org. Lett.* **2002**, *4*, 477-479.

(121) Yan, J.; Fang, H.; Wang, B. *Med. Res. Rev.* **2005**, *25*, 490-520.

(122) Suenaga, H.; Mikami, M.; Sandanayake, K. R. A. S.; Shinkai, S. *Tetrahedron Lett.* **1995**, *36*, 4825-4828.

(123) Sandanayake, K. R. A. S.; James, T. D.; Shinkai, S. *Chem. Lett.* **1995**, *7*, 503-504.

(124) James, T. D.; Shinmori, H.; Shinkai, S. *Chem. Commun.* **1997**, 71-72.

(125) Yang, W.; He, H.; Drueckhammer, D. G. *Angew. Chem. Int. Ed.* **2001**, *40*, 1714-1718.

(126) Mulla, H. R.; Agard, N. J.; Basu, A. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 25-27.

(127) James, T. D.; Sandanayake, K. R. A. S.; Iguchi, R.; Shinkai, S. *J. Am. Chem. Soc.* **1995**, *117*, 8982-8987.

(128) Wulff, G. *Pure Appl. Chem.* **1982**, *54*, 2093-2102.

(129) Franzen, S.; Ni, W.; Wang, B. *J. Phys. Chem. B.* **2003**, *107*, 12942-12948.

(130) Ni, W.; Kaur, G.; Springsteen, G.; Wang, B.; Franzen, S. *Bioorg. Chem.* **2004**, *32*, 571-581.

(131) Zhu, L.; Shabbir, S. H.; Gray, M.; Lynch, V. M.; Sorey, S.; Anslyn, E. V. *J. Am. Chem. Soc.* **2006**, *128*, 1222-1232.

(132) Yang, W.; Yan, J.; Springsteen, G.; Deeter, S.; Wang, B. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1019-1022.

(133) James, T. D.; Sandanayake, K. R. A. S.; Shinkai, S. *Supramol. Chem.* **1995**, *6*, 141-157.

(134) Norrild, J. C.; Eggert, H. *J. Am. Chem. Soc.* **1995**, *117*, 1479-1484.

(135) Norrild, J. C.; Eggert, H. *J. Chem. Soc. Perkin Trans. 2* **1996**, 2583-2588.

(136) Fukuda, M. *Cell Surface Carbohydrates and Cell Development*: CRC: Boca Raton, 1992.

(137) Jorgensen, T.; Berner, A.; Kaalhus, O.; Tveter, K. J.; Danielsen, H. E.; Bryne, M. *Cancer Res.* **1995**, *55*, 1817-1819.

(138) Idikio, H. A. J. *Glycoconjugate J.* **1997**, *14*, 875-877.

(139) Yang, W.; Gao, S.; Gao, X.; Karnati, V. V. R.; Ni, W.; Wang, B.; Hooks, W. B.; Carson, J.; Weston, B. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2175-2177.

(140) Yang, W.; Fan, H.; Gao, X.; Gao, S.; Ni, W.; Hooks, W. B.; Carson, J.; Weston, B.; Wang, B. *Chemistry & Biology* **2004**, *11*, 439-448.

(141) Bielecki, M.; Eggert, H.; Norrild, J. C. *J. Chem. Soc. Perkin Trans. 2* **1999**, *449-455*.

(142) Sugihara, J. M.; Bowman, C. M. *J. Am. Chem. Soc.* **1958**, *80*, 2443-2446.

(143) Yang, W.; He, H.; Drueckhammer, D. G. *Angew. Chem., Int. Ed. Engl.* **2001**, *40*, 1714-1718.

(144) Karigiannis, G.; Papaioannou, D. *Eur. J. Org. Chem.* **2000**, 1841-1863.

Chapter 2

Investigation of Non-Specific Effects of Fluorescent Tags in the Screening of Labeled Carbohydrates Against Immobilized Proteins

2.1 Introduction

Protein binding to cell-surface carbohydrates plays an important role in many biological processes including immune response, cancer cell metastasis, inflammation and fertilization.[1-8] Many parasitic, bacterial and viral pathogens recognize and adhere to a host cell's surface through protein-carbohydrate interactions. Because of the roles of protein-carbohydrate interactions in human disease,[9-11] carbohydrate-based therapeutics is an intense area of research. Consequently, there has been growing interest in the development of carbohydrate and glycopeptide libraries.[12-16] The use of biphasic assays based on the binding of soluble, tagged ligands to an immobilized protein target is a popular approach. However, the problem of nonspecific interactions between the labeled ligand and the solid support or the receptor often makes the screening process troublesome, especially with fluorescently labeled substrates that have charged or hydrophobic functional groups that would induce false positive results. Yet, fluorescence labeling of carbohydrates is still an attractive recourse in the visualization of oligosaccharides[17,18] during purification[19-21] processes or the monitoring of a substrate for an enzyme.[22]

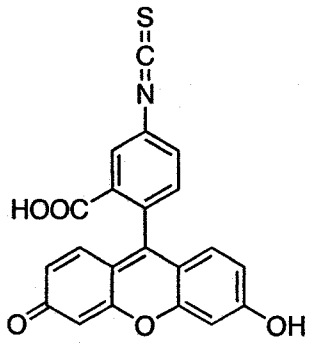
Fluorescent labels have proved to be useful tools as they have specific wavelengths of emission and they enable the monitoring of extremely low concentrations of the chemical species. This approach avoids the recourse to hazardous radioactive tags, another tool frequently used in biological screening studies.

Unfortunately, most organic dyes embed several aromatic rings that can induce weak 'non-specific' interactions with aromatic units of the protein receptors. Consequently, positive hits are hard to distinguish in a definite way from negative ones, especially at high concentrations of the fluorescent species.

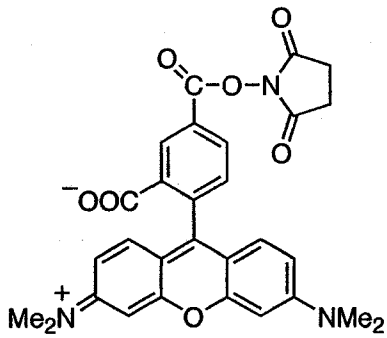
Therefore, we wanted to describe a semi-quantitative study comparing the non-specific interactions of carbohydrates conjugated with aromatic fluorophores[23-26] and carbohydrates labeled with a fluorescent non-aromatic europium chelate[27-29] (Figure 2-1) with solid-supported lectins. This model study, described conceptually in Figure 2-2, can be of use not only to the field of carbohydrate chemistry but also in the screening of other molecules.

Our biphasic screening model consisted of two different lectins immobilized on agarose beads, namely PNA[9] lectin from peanuts (*Arachis hypogaea*) and Con A lectin from jackbean (*Canavalia ensiformis*). These lectins were chosen because of their commercial availability, low cost, and strong binding affinities[10,11] (with a range of 10^7 -10^8 M^{-1}) to saccharides (Table 1). The solid support chosen for this study is beaded agarose, which is compatible with water, the ideal biologically relevant solvent for

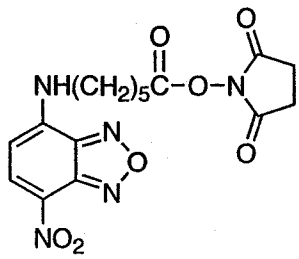
reactions involving deprotected carbohydrates. Thus, results with agarose can serve as a useful guide for other water-compatible supports such as Tentagel resins or aqueous biphasic assays such as ELISA.



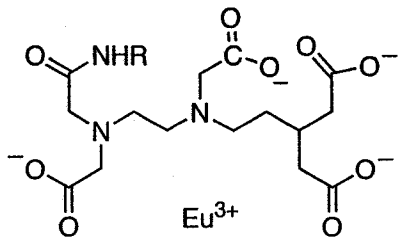
FITC



TAMRA



NBD



Europium chelate

Figure 2-1. Dye reagents used as labels for model carbohydrate molecules and the europium chelate that could be conjugated to a carbohydrate moiety.

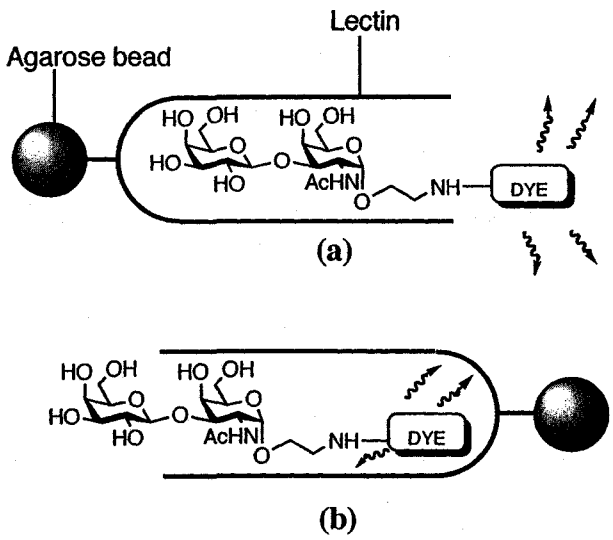


Figure 2-2. (a) Favorable interaction between an agarose-supported lectin and a labeled disaccharide. (b) Unfavorable 'nonspecific' interactions between an agarose-supported lectin and a labeled disaccharide.

Table 2.1 Natural ligands for agarose supported lectins.

Lectins	Substrates	Binding constants M^{-1}
Con A[30]	Glycopeptide containing 3,6-di-*O*-α-D-mannopyranosyl-α-D-mannopyranoside	2.38×10^7
PNA[31]	T-antigen	1×10^7

Therefore, based on the values of binding constants[30,31] found in the literature, the chosen ligand for Con A lectin was 3,6-di-*O*-α-D-mannopyranosyl-α-D-mannopyranoside, with a 2-aminoethyl group to conjugate the fluorescent label and 2-aminoethyl (β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside having the

core structure of the T-antigen (disaccharide β-Gal-(1→3)-α-D-Gal-NAc O-linked to serine or threonine) was the ligand of choice for PNA lectin.

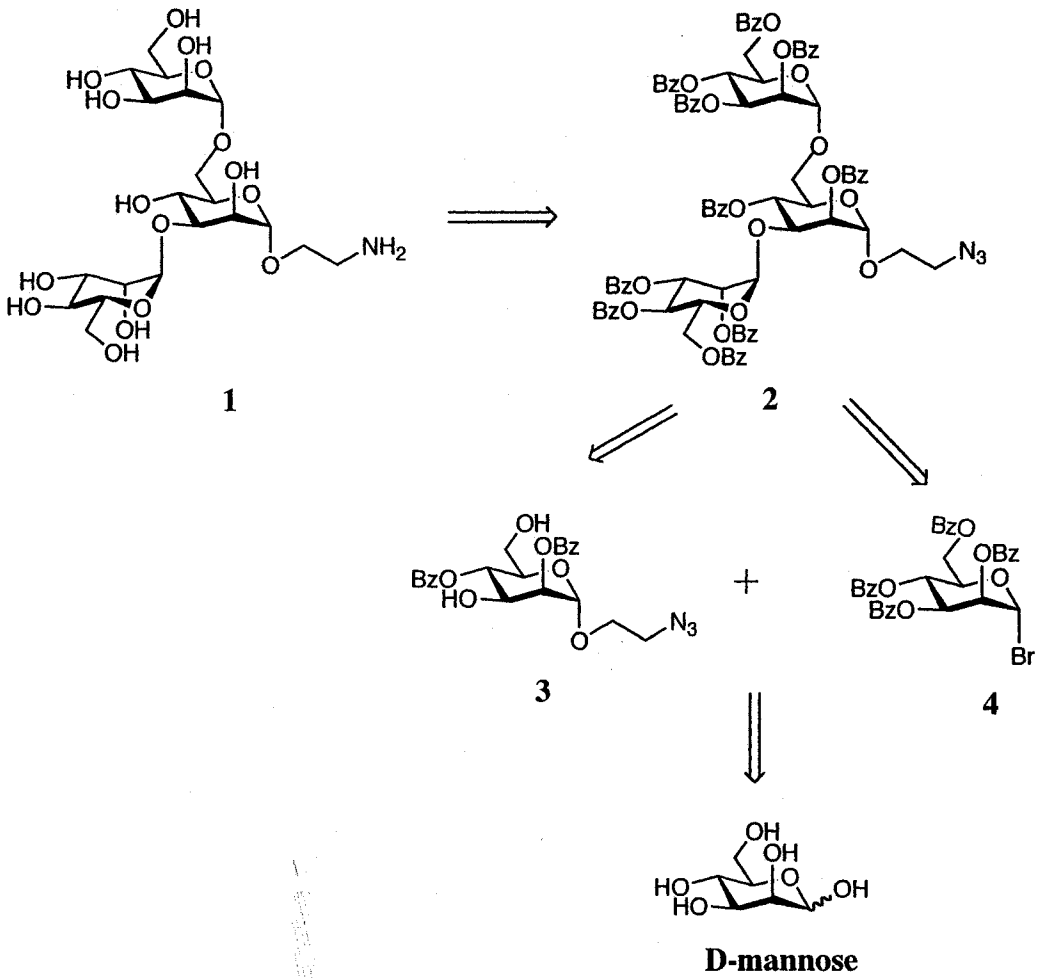
2.2 Synthesis of unlabeled ligand for Con A lectin

2.2.1 Retrosynthetic analysis of a ligand for Con A lectin

The ligand for Con A lectin would be 2-aminoethyl 3,6-di-*O*-α-D-mannopyranosyl-α-D-mannopyranoside **1.** The trisaccharide unit, being part of the high mannose type of N-linked glycoproteins, is of great biological importance and has been synthesized by a number of research groups. The groups of Furneaux,[32] Ogawa,[33] Roy[34] and Krepinsky[35] synthesized the trisaccharide core of **1** by consecutive addition of 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide **4** (Scheme 2.1) at the 3- and 6-positions of the glycosyl acceptor. Other synthetic approaches used would involve a 2,4-di-O-protection in the glycosyl acceptor. The most frequently used method involves tin activation followed by allylation to give the 3,6-di-*O*-allyl compound, which was benzylated. Subsequent removal of the allyl groups gave the required 2,4-di-*O*-benzyl derivative.[36,37]

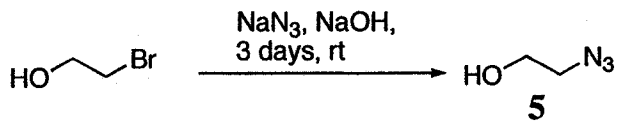
In our case, we chose to introduce two mannose rings at the same time within a 2,4 di-protected monosaccharide acceptor. The retrosynthetic analysis (Scheme 2.1) was based on the synthesis of the core 3,6-di-O-α-D-mannopyranosyl-α-D-mannopyranose developed by Oscarson and Tidén,[38] using a different aglycon in **3**. Oscarson's approach involved di-ortho esters, which would open regioselectively to give the required protection pattern, that is, 2,4-di-*O*-benzoyl derivative.

In the double glycosylation of the diprotected monosaccharide **3**, the 1,2 *trans* relationship between the 1-Br and 2-OBz in **4** will dictate the stereoselectivity at the anomeric centre. The axial orientation of the 2-OBz would allow the carbonyl oxygen to donate one of its lone pairs to stabilize the carbocation as the bromide is being abstracted.[35] This neighboring group participation also facilitates the stereoselective nucleophilic attack from the bottom face of the molecule, thus allowing access to the



Scheme 2.1

desired α-glycoside. Both building blocks would be derived from D-mannose. The aglycon containing a terminal amine was required to attach the fluorescent label to the carbohydrate molecule. The amine was formed by reduction of an azide group. The alternative aglycon would be one bearing a halogen group that could be displaced by nucleophilic azide anions.



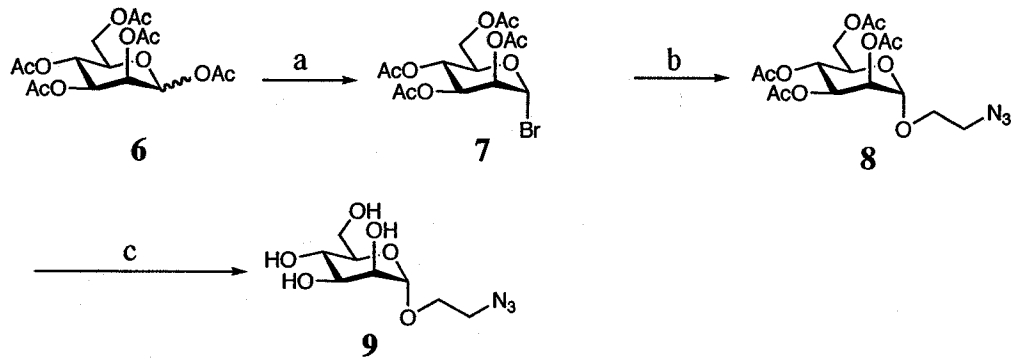
5

Scheme 2.2

The aglycon **5**[39-41] was obtained in 61% yield, by reaction of azide ions with the epoxide intermediate formed in situ. The azide compound **5** was used without any purification as the ^{1}H NMR analysis provided a clean spectrum[39] and it was reported that heating of low-molecular weight azides[41] during distillation may result in explosions.

2.2.2 Synthesis of 2-azidoethyl-α-D-mannopyranoside (3)

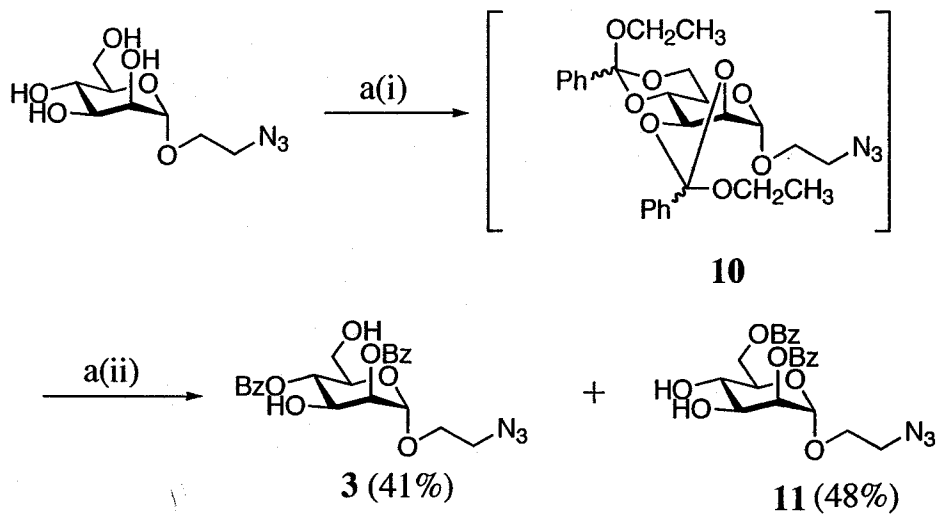
The first step in the synthesis of 2-azidoethyl α-D-mannopyranoside (Scheme 2.3) was the acetylation of D-mannose with acetic anhydride and pyridine, resulting in the per-acetylated mannose **6**[42,43] present as a 3:1 mixture of α and β anomers. The anomeric conformation of 1,2,3,4,6-penta-*O*-acetyl-α,β-D-mannopyranoside was assigned based on the C-H coupling constants of the anomeric carbon: 178 Hz for the α-anomer and



Scheme 2.3 Reagents and conditions: (a) 33% HBr in glacial acetic acid, quantitative yield ; (b) AgOTf, 2,4,6-collidine, $HOCH_2CH_2N_3$, dry DCM, -20 °C to rt , 84%; (c) 0.1 M NaOMe, MeOH, rt, quantitative yield.

164 Hz for the β-anomer. The anomer mixture **6** was then converted to a suitable glycosyl donor, the bromide **7**,[43] in quantitative yield using 33% HBr in acetic acid. 1,2,3,4,6-Penta-*O*-acetyl-α-D-mannopyranosyl bromide **7** would then be reacted with 2-azidoethanol (**5**), under Lewis acid conditions with silver triflate[44,45] and 2,4,6-collidine. It was found that silver triflate as a Lewis acid increased the yield of 2-azidoethyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside **8** to 84% compared to 42% as obtained by Chernyak and co-workers[39] under boron trifluoride etherate conditions at 0 °C (Scheme 2.3). De-acetylation of **8** with 0.1 M NaOMe afforded pure 2-azidoethyl α-D-mannopyranoside **9**. In the ^1H NMR spectrum of **9**, H-1 occurred at δ4.81 ppm. Moreover, from the coupled HMQC spectrum, the coupling constant of H-1 with the anomeric C-H was determined to be 169 Hz, indicative of an α-anomer in mannopyranoside **9**. With the aglycon installed, 2,4 protection of the hydroxyl groups in **9** was achieved by the formation of an intermediate, 2,3: 4,6-di-*O*-ethoxybenzylidene

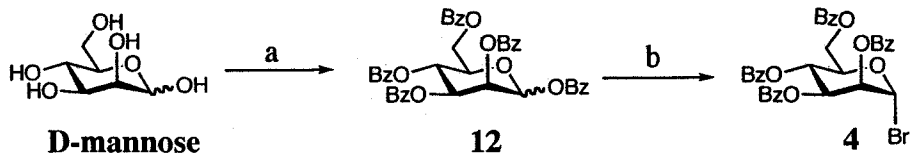
derivative of the 2-azidoethyl α-D-mannopyranoside using triethyl orthobenzoate and a camphor-sulfonic acid-trifluoroacetic acid mixture in dry acetonitrile. It was reported by Oscarson and Tidén[39] that addition of trifluoroacetic acid as a catalyst followed by evaporation forced the reaction to go to completion. These orthoesters (Scheme 2.4) were not isolated but they were made to open by treatment with aqueous trifluoroacetic acid. According to Lemieux and Driguez,[46] the 2,3-ortho ester should open up regiospecifically to the 2-*O*-acyl derivative, whereas the 4,6-ortho ester should give a mixture of the 2,4- and the 2,6-di-*O*-benzoyl derivatives, the relative amount being difficult to predict.[47] Opening of the di(ortho ester) compounds gave a mixture of 2,4- and 2,6-di-*O*-benzoyl derivatives. In our case, the 2,4 dibenzoyl derivative **3** was obtained with a yield of 41% and the 2,6 dibenzoyl derivative **11** was afforded with a yield of 48% (Scheme 2.4).



Scheme 2.4 Reagents and conditions: (i) $C_6H_5C(OCH_2CH_3)_3$, CSA, TFA, rt, 1 h, (ii) aqueous TFA, rt, 30 min.

2.2.3 Synthesis of 2-aminoethyl 3,6-di-*O*-α-D-mannopyranosyl-α-D-manno-pyranoside (1)

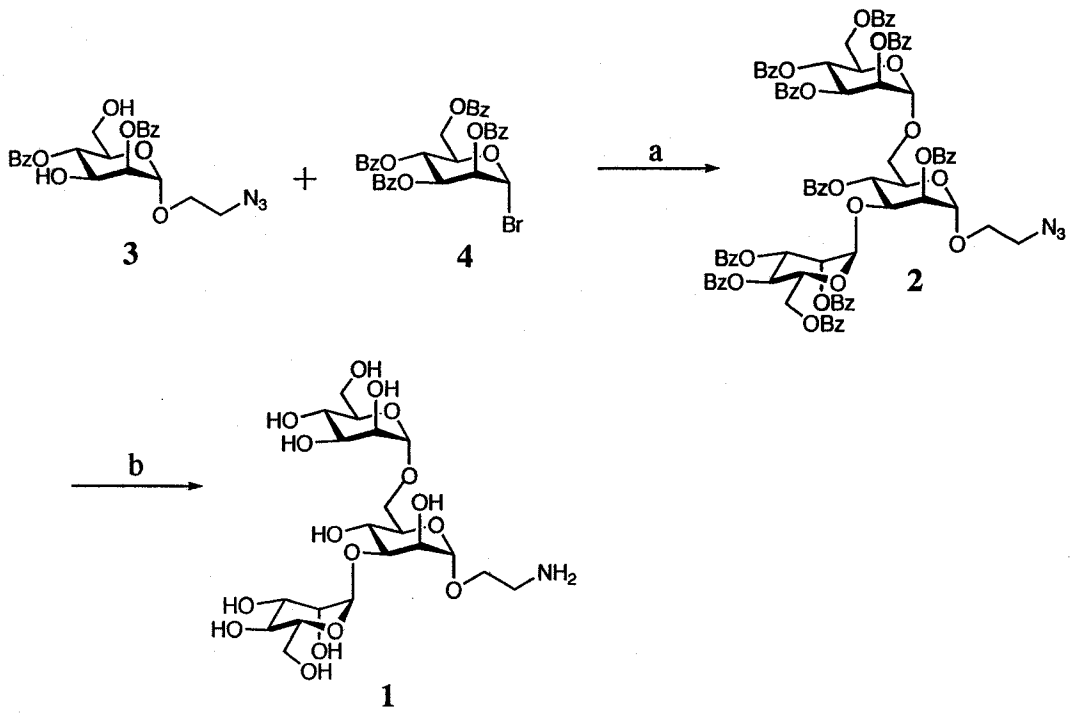
To synthesize the donor 2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl bromide **4**, the perbenzoylated mannose[48] was made to react with 33% HBr in acetic acid at a low temperature of 0 °C.



D-mannose **12** **4**

Scheme 2.5 Reagents and conditions: (a) BzCl, pyridine, DCM, 0°C, 2 h, quantitative yield; (b) HBr, DCM, 0 °C, 45 min, quantitative yield.

The protected trimannose trisaccharide was obtained[38] (Scheme 2.6) by silver-triflate-promoted couplings between 2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl bromide **4** and 2-azidoethyl 2,4-di-*O*-benzoyl-α-D-mannopyranoside **3** in dry dichloromethane at 0 °C. After purification, the trisaccharide **2** was obtained in 67% yield in one step. The values of C-H coupling (173 Hz) of the anomeric protons obtained from the HMQC-coupled spectrum proved that all the mannose rings were in the α-configuration. The α glycosidic linkages (1→3) and (1→6) relationship in the trisaccharide **2** were clearly seen in the HMBC spectrum. Mass spectrum also confirmed the molecular structure of the protected trisaccharide.



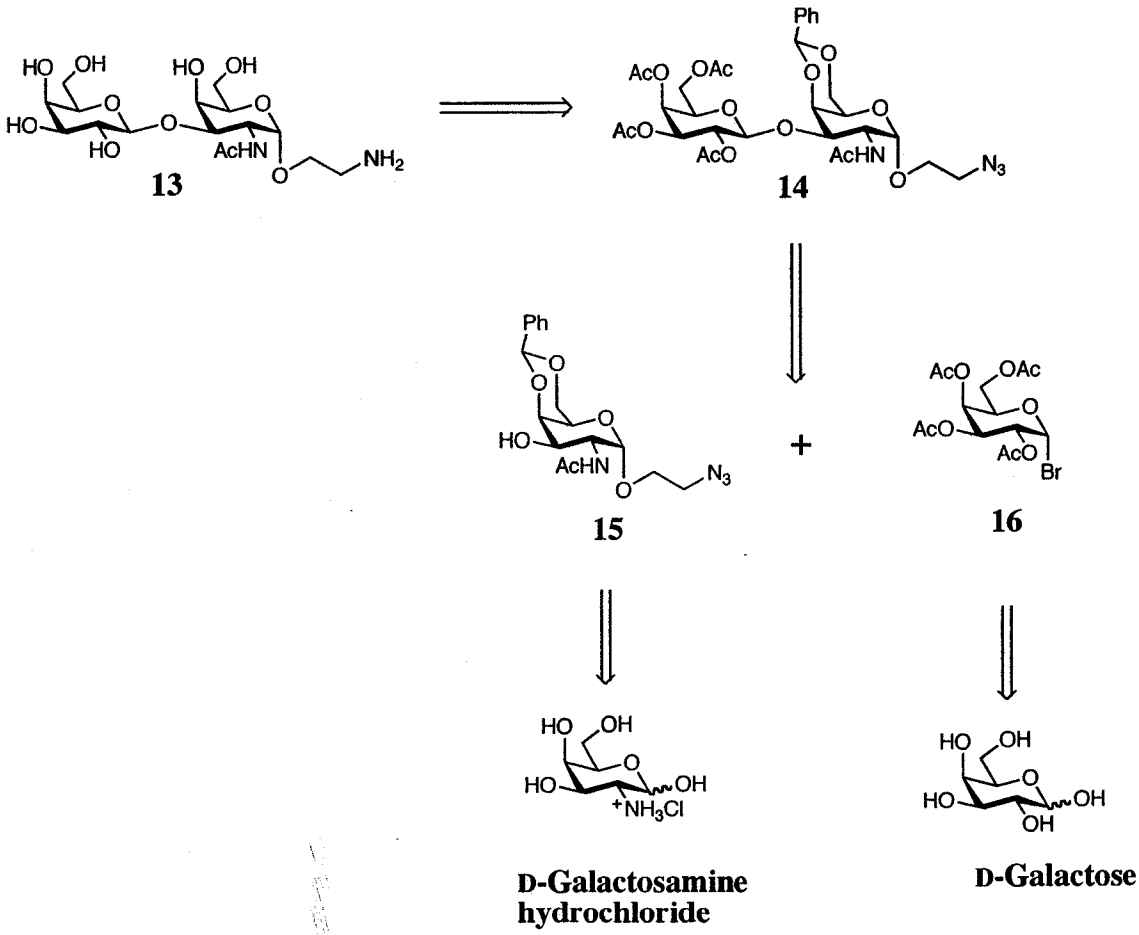
Scheme 2.6 Reagents and conditions: (a) AgOTf, DCM, 0°C, 1h, 67%; (b) (i) NaOMe, MeOH, rt, 6 h, (ii) H_2/Pd, rt, 24 h, 89%.

The final steps towards the synthesis of 2-aminoethyl 3,6-di-*O*-α-D-mannopyranosyl-α-D-mannopyranoside **1** consisted of the debenzoylation and hydrogenation of the trisaccharide **2** at room temperature, resulting in a yield of 89% after work-up. The ^{1}H and ^{13}C NMR spectra of the amine **1** were consistent with the structure of the unprotected trisaccharide, which was further confirmed by the high resolution mass spectrum.

2.3 Synthesis of unlabeled ligand for PNA lectin

2.3.1 Retrosynthetic analysis of a ligand for PNA lectin

The unlabeled ligand of choice for PNA lectin was 2-aminoethyl (β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside **13**. The monosaccharides from which the disaccharide would be synthesized were donor **15** and acceptor **16** (Scheme 2.7).

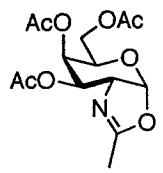


Scheme 2.7 Retrosynthetic analysis of the disaccharide ligand for lectin PNA.

The α-linkage[49] in **15** resulted from the steric effect of the benzylidene ring, which hindered the approach of 2-azidoethanol from the top face resulting in the α–glycoside being the major product. N-galactosamine hydrochloride and D-galactose were the starting reagents.

2.3.2 Synthesis of 2-azidoethyl 2-acetamido-2-deoxy-4,6-*O*-benzylidene-α-D-galactopyranoside (15)

The use of N-acetylgalactosamine for the synthesis of α-N-acetylgalactosaminides was made difficult due to the collapse of the donor during glycosylation reactions to the bicyclic oxazoline derivatives.[50]



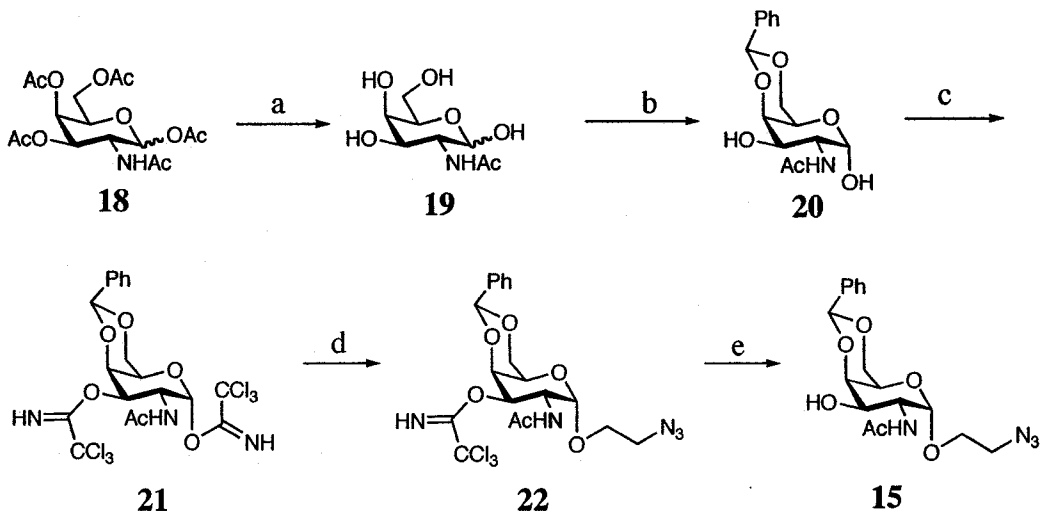
17

Figure 2-3 Oxazoline derivative **17**.

Other research groups such as those of Paulsen[51] and Lemieux[52] performed glycosylation reactions using the 2-azido precursor, hence avoiding the interference of the 2-acetamido group with the anomeric carbon during the glycosidic bond formation. Koganty and Qiu[49] also found out that 4,6-*O*-benzylidenyl-*N*-acetylgalactosamine could be converted to a suitable acceptor. The latter would react with an alcohol, including serine and threonine, forming α–glycosides in moderate to high yields depending on the reactivity of the acceptor. We therefore based our synthesis on Koganty's

methodology,[49] which minimized the number of protecting steps while carrying out the glycosylation reaction with the acetamido group at the 2-position.

In Scheme 2.8, 2-acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-galactopyranoside **18**[53] was prepared in good yield (90%) from acetylating D-galactosamine with acetic anhydride and pyridine at room temperature. By performing a deacetylation on **18** with NaOMe, GalNac **19**[54,55] was obtained with the 4- and 6-hydroxyls free to form a



Scheme 2.8 Reagents and conditions: (a) NaOMe, MeOH, rt, 30 min, 99%; (b) $PhCH(OMe)_2$, CSA, CH_3CN, rt, 24h, 84%; (c) Cl_3CCN, DBU, DCM, -20 °C, 1 h, 90%; (d) $HOCH_2CH_2N_3$, $BF_3.OEt_2$, molecular sieves (3Å), -20 °C, 20 min, 61%; (e) 80% CH_3COOH in H_2O, rt, 45 min, 95%.

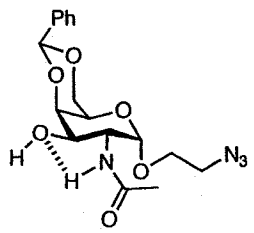
benzylidene ring with benzaldehyde dimethyl acetal in the presence of camphor sulfonic acid at room temperature. It was important to introduce a benzylidene ring in the monosaccharide so that the α-anomer was favored when the glycosidation was done

with 2-azidoethanol. [49] Two trichloroacetimidate groups were then introduced by reacting **20** with trichloroacetonitrile[49,56] in the presence of DBU as a base at –20 °C. The trichloroacetimidate group at the anomeric position served as a donor and the one at the 3-position fulfilled the role of a protecting group. This strategy served as a good way to generate (1→3) linkages[49] in linear oligosaccharides by reducing the number of protection/deprotection steps. In the ^{1}H NMR spectrum of **21**, peaks of the NH of the trichloroacetimidate groups were found at δ 8.73 ppm and 8.47 ppm and in the ^{13}C APT spectrum, quartenary carbons (C=N) at δ 163.21 and 160.30 ppm were present. The mass spectrum also confirmed the structure of **21**.

Glycosylation of **21** was carried under Lewis acid conditions (boron trifluoride etherate) with 2-azido ethanol at -20 °C. The reaction was carefully monitored by TLC as the second trichloroacetimidate group has a tendency to get cleaved if the mixture was left for a longer period of time. After purification of the residue, 2-azidoethyl 2-acetamido-2-deoxy-4,6-*O*-benzylidene-3-*O*-trichloroacetimidato-α-D-galactopyranoside **22** was obtained in a reasonable yield of 61%. In the ^{1}H NMR spectrum, the proton at the anomeric position appeared at δ 5.06 ppm with a coupling constant of 3.5 Hz with H-2. Moreover, since the coupling constant (C-H) of the anomeric carbon was 173 Hz, an α linkage was confirmed. To obtain the glycosyl donor **15**, the trichloroacetamidate was removed with 80% acetic acid to give the 2-azidoethyl 2-acetamido-2-deoxy-4,6-*O*-benzylidene-α-D-galactopyranoside with a yield of 95%. Absence of the trichloroacetimidate proton in the region 8-9 ppm confirmed the structure of **15**.

2.3.3 Synthesis of 2-azidoethyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-4,6-*O*-benzylidene-α-D-galactopyranoside (14)

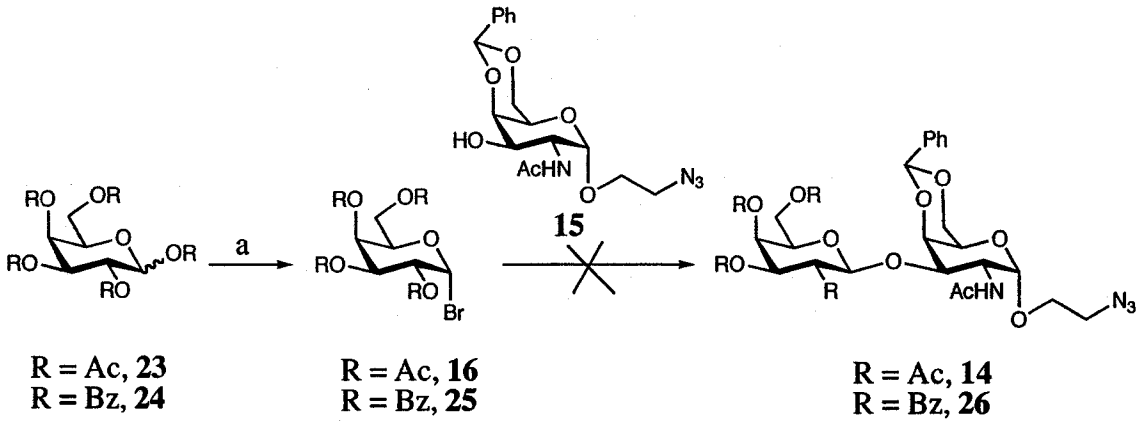
Conversion of **15** into an analogue of the T antigen required regioselective β-galactosylation of 3-hydroxyl. The formation of a β-galactosidic bond in derivatives of T-antigen is usually performed by employing derivatives of *N*-acetylgalactosamine as glycosyl acceptors, in which the acetamido group is masked as an azido group.[57] In comparison to the acetamido group, the azido group reduces steric hindrance for glycosylation at the adjacent 3-hydroxyl and increases the nucleophilicity of this hydroxyl group by avoiding an unfavorable hydrogen-bonding pattern (Figure 2-4).[58,59]



15

Figure 2-4. Unfavorable H-bond that reduces the nucleophilicity of the adjacent hydroxyl group.

In our case, since acceptor **15** was used, special glycosylation conditions were to be used. Different glycosyl donors were investigated with either a bromide or an imidate group as a leaving group. Tetra-*O*-acetyl galactopyranosyl bromide **23**,[60] synthesized from penta-*O*-acetyl-α,β-D-galactopyranoside, was reacted[61] with **15** at different temperatures (-25, -10, 0, 25 °C) using silver triflate as Lewis acid in different solvents such as dichloromethane and acetonitrile (Figure 2.9). Under these conditions, no



R = Ac, **23**
R = Bz, **24**

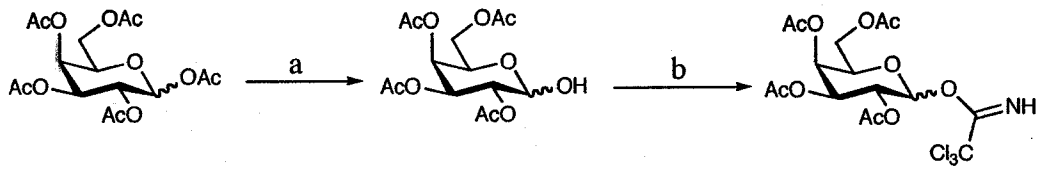
R = Ac, **16**
R = Bz, **25**

R = Ac, **14**
R = Bz, **26**

Scheme 2.9 Reagents and conditions: (a) 33% HBr/acetic acid, pyridine, DCM, 0 °C to rt, 1-2 h; (b) AgOTf, DCM or CH_3CN, -25 to 25 °C, 1 h-24 h.

product was formed; in some cases, the α-linked disaccharide was observed in the crude mixture. The latter consisted mostly of the hydrolysed donor and the starting material **15** after purification by column chromatography.

When the same reaction conditions were tried on the benzoyl bromide derivative **25**,[62] prepared from perbenzolylated galactopyranoside **24**,[63] no product **26** was observed (Scheme 2.9). Other glycosyl donors were then investigated, with the imidate group as the leaving group. The hemiacetal **27**[64] was prepared by removal of the anomeric acetate group from commercially available 1,2,3,4,6-penta-*O*-acetyl-α,β-D-galactopyranoside by reaction with hydrazine sulfate at 50 °C, resulting in a satisfactory yield of 92% (Scheme 2.10). The imidate group was then introduced[65] at the anomeric position by treating hemiacetal **27** with sodium and trichloroacetonitrile at room temperature.

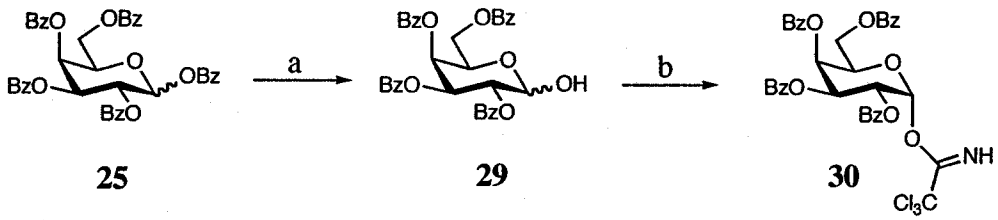


23 **27** **28**

Scheme 2.10 Reagents and conditions: (a) NH_2NH_2.HOAc, DMF, 50 °C, 20 min, 92%; (b) Na, DCM, Cl_3CCN, rt, 2 h, 73%.

O-(2,3,4,6-Tetra-*O*-acetyl-galactopyranosyl)trichloroacetimidate **28** was then obtained as the α–anomer with a yield of 32% and the β-anomer was obtained with a yield of 41%. However, both the α and β forms of **28** did not react with acceptor **15** under different Lewis acid conditions of silver triflate or boron trifluoride etherate in dry dichloromethane. Different temperature conditions ranging from -20 °C to room temperature were employed but no β-glycosidic bond was formed.

The acetate groups were then substituted by benzoate groups (Scheme 2.11) to give the alternate glycosyl acceptor **30**. 1,2,3,4,6-Penta-*O*-benzoyl-α-D-galactopyranoside **24** was converted into the bromide derivative which was then hydrolysed in the presence of silver carbonate at room temperature to afford the hemiacetal **30**[62] in 96% yield. The imidate derivative **30** was then formed, with a yield of 42%, by reacting compound **29**[62] with trichloroacetonitrile and 1,8-diazabicyclo[5.4.0]undec-7-ene at room temperature.



25 29 30

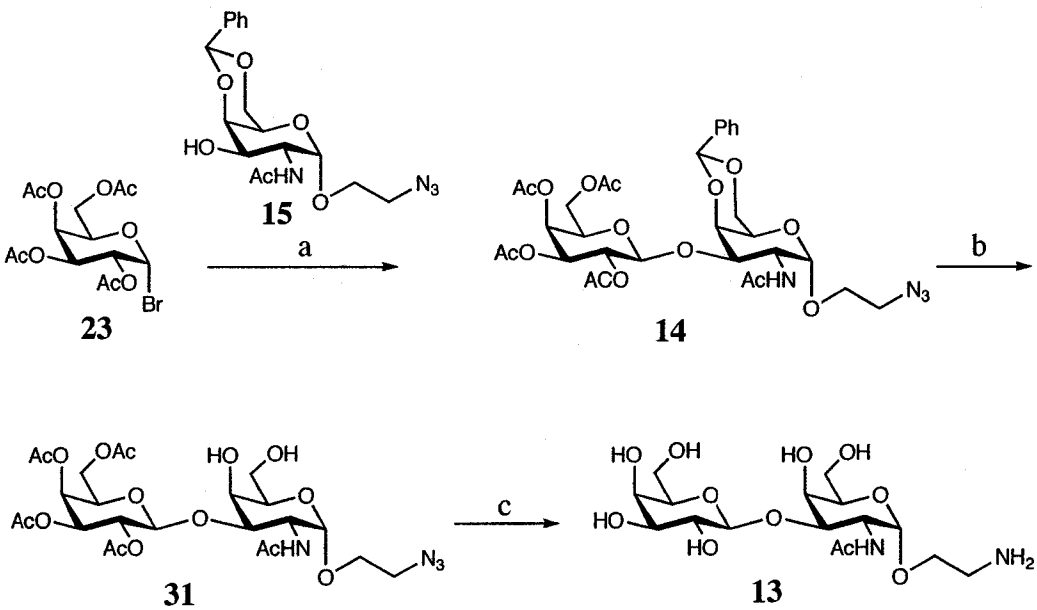
Scheme 2.11 Reagents and conditions: (a) (i) 33% HBr/acetic acid, 0 °C to rt, 2 h, 96%, (ii) $AgCO_3$, 4:1 acetone/water, rt, 30 min, quantitative; (b) CCl_3CN, DBU, CH_2Cl_2, 0 °C, 30 min, 47%.

When glycosylation was done with the protected monosaccharide **15** and the benzoyl imidate **30** under different temperature conditions (-25 °C and rt) in the presence of trimethyl silyl triflate as the Lewis acid,[66] no disaccharide was obtained. The reaction mixture gave rise to mostly unreacted starting material and hemiacetal **29**, the hydrolysis product of **30**.

It was therefore deduced that the low nucleophilicity of the 3-hydroxyl in the acceptor **15** and the steric hindrance caused by the acetamido group prevented the formation of the disaccharide at low temperatures and room temperature conditions. The different functional groups in the glycosyl donors and different leaving groups did not favor the glycosylation reaction. Alternative reaction conditions had to be found that would force the poorly nucleophilic glycosyl donor **15** to react.

2.3.4 Successful synthesis of 2-aminoethyl (β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (13)

Using modified glycosylation conditions from Lemieux and Tidén,[67] where toluene was used as the solvent and mercury (II) cyanide as the Lewis acid at a temperature of 70 °C, the disaccharide **14** was finally obtained in 92% yield (Scheme 2.12). Care had to be taken to ensure dry reaction conditions by using molecular sieves (4Å) or anhydrous calcium sulfate, otherwise the glycosyl donor was hydrolysed to the hemiacetal. In the protected disaccharide, the new glycosidic bond formed was the β linkage as seen by the chemical shift δ at 4.74 ppm as well as by the coupling constant of H-1' with H-2', which is of the magnitude 8.0 Hz typical of two axial protons. The β



Scheme 2.12 Reagents and conditions: (a) $Hg(CN)_2$, molecular sieves (4Å), CH_3NO_2:toluene (1:1), 70 °C, 4–6 h, 92%; (b) 80% AcOH, 50 °C, 2 h, 67%; (c) (i) NaOMe, MeOH, rt, 6 h, 96%, (ii) H_2/Pd, rt, 18 h, quantitative.

linkage was also confirmed by the C-H coupling constant of the anomeric carbon (161 Hz), in the coupled HMQC specrum. High resolution mass spectrum analysis also confirmed the formula of the disaccharide **14**.

The benzylidene ring was then removed by heating disaccharide **14** with 80% acetic acid[66] to give compound **31** with the 4,6-hydroxyls free. The HMBC spectrum showed distinct correlations between C-3 of the α-linked galactopyranoside and H-1' of the β-linked galactopyranoside, confirmed by the correlations between C-1' and H-3. A β (1→3) linkage was thus confirmed in the disaccharide **31**.

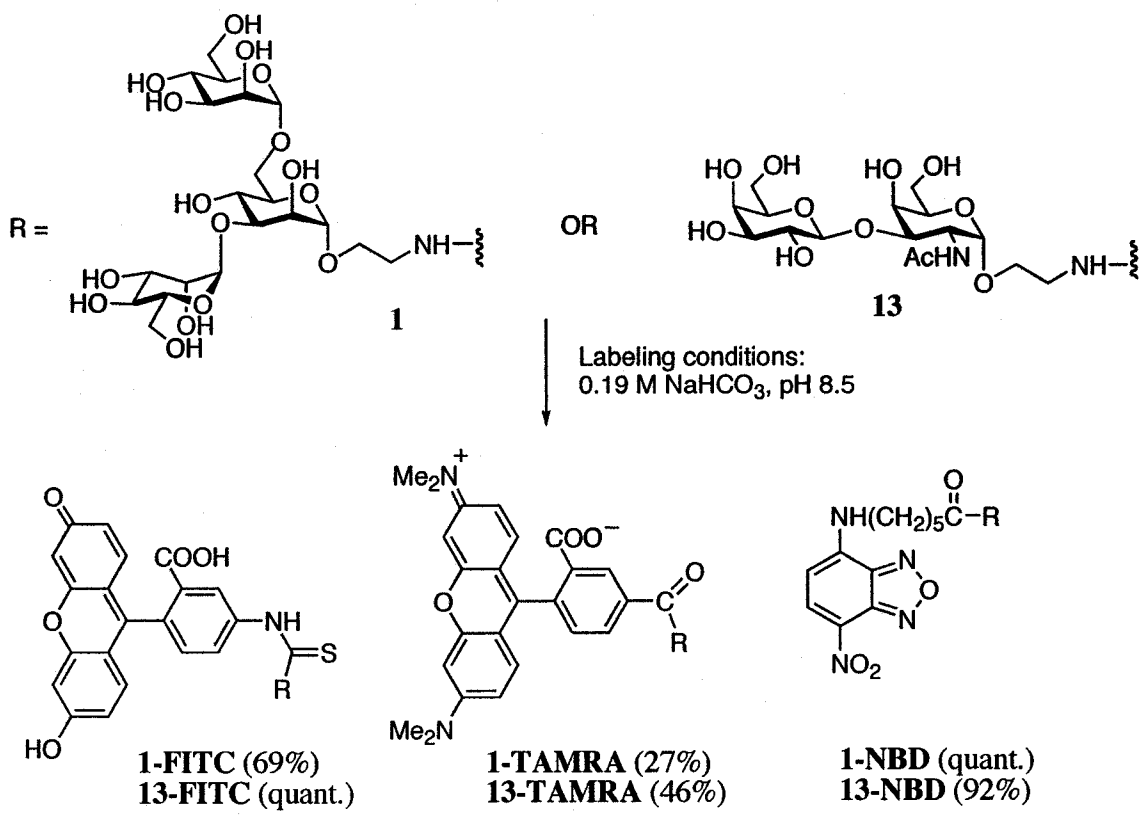
The ligand **13** for PNA lectin was obtained in 96% yield after two successive deprotection steps, that is, de-acetylation and catalytic hydrogenolysis of the intermediate on Pd/C in methanol at room temperature. Mass spectrum analysis again confirmed the formula of the disaccharide and the 1→3 linkage was also observed in the HMBC spectrum of the intermediate before the catalytic hydrogenolysis step.

2.4 Conjugation of saccharides with fluorescent labels

2.4.1 Conjugation of the saccharides with aromatic fluorescent dyes

Considerable progress had been made in finding ideal conditions[68] for the conjugation of fluorescent molecules, also called reporter molecules, with biopolymers such as proteins and DNA. These methods serve as an important tool in cell biology and immunology. Thus, we thought of adapting these conditions for conjugating[26] aromatic

fluorescent dye molecules to the ethyl amino group of the carbohydrates (**1** and **13**, Scheme 2.13). The labeled carbohydrates would serve as ligands for the agarose supported lectins.



Scheme 2.13

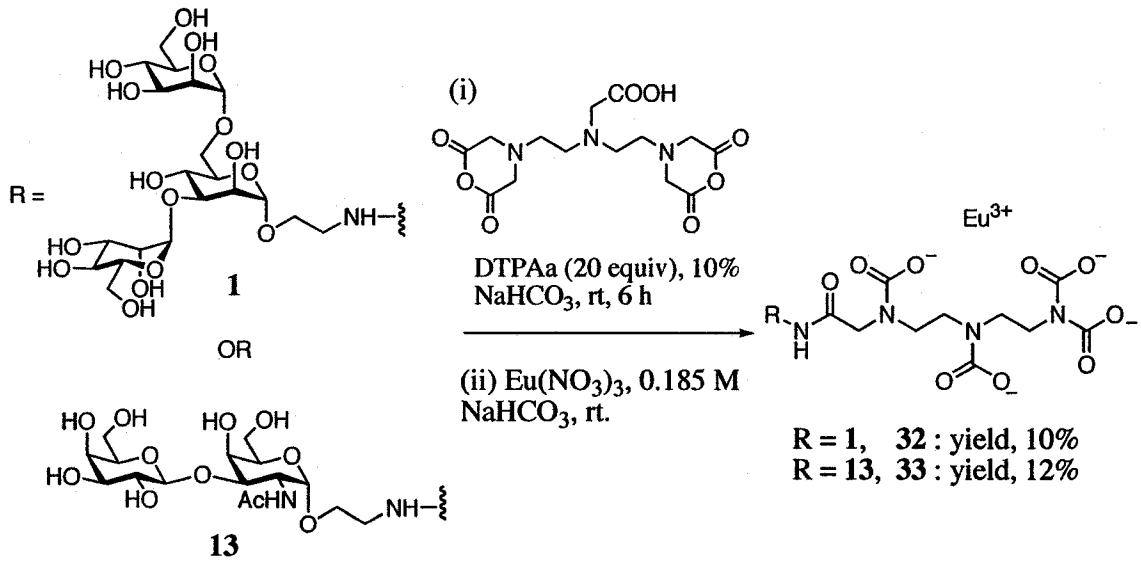
Under basic buffer conditions at pH 8.5 in water, the amine groups reacted through thiourea and amide bond formation to give the desired conjugates, which were purified on C-18 columns and lyophilized. The formulae of the conjugates were confirmed by the number of aromatic protons present in each conjugate and also by high resolution mass spectrum analysis.

2.4.2 Labeling of agarose-lectin ligands with europium chelates

Europium chelates are viable alternatives to radioisotopes,[69,70] especially, since the sensitivity of europium detection can be increased under conditions of dissociation enhancement.[71]

Commercially available europium labels[28] are generally hydrophobic because of the aromatic rings present. However, these are not suitable for labeling small hydrophilic compounds such as glycopeptides, often preferable for glycobiological studies. These reagents increase the hydrophobicity of glycopeptides and cause undesirable nonspecific binding to proteins, glassware, or plasticware.

Therefore, we wanted to synthesize a non-aromatic label, that is a europium chelate, by reacting the amine of the linker arm present in the carbohydrate ligands to the anhydride derivative of diethylenetriaminepentaacetic acid dianhydride[28] (DTPAa). The resulting carboxylate will then be made to react with a europium salt under aqueous conditions (Scheme 2.14). Purification of the europium-labeled carbohydrates was accomplished with Sephadex G-15 columns. The trisaccharide europium derivative **32** was obtained in 10% yield and the disaccharide europium salt **33** was obtained with a yield of 12%. The hydrolysis of the anhydride before the reaction with the amino group of **1** and **13** could account for the low yields of the amides.



Scheme 2.14

The diethylenetriaminepentaacetate (DTPA) derivatives of the carbohydrates are weakly fluorescent and therefore, a DELFIA (Dissocation Enhanced Lanthanide FluoroImmunoAssay) reagent[71] (trioctylphosphine oxide, 2-naphthoyltrifluoroacetone, Triton X-100) was used to dissociate the europium from the labeled compound and form a highly luminescent Eu^{3+} chelate (Figure 2-5). The aromatic chelates (Figure 2-5) can act as antenna molecules to absorb light and transfer excitation energy to lanthanide ions, which significantly increases the luminescence of lanthanides.[72]

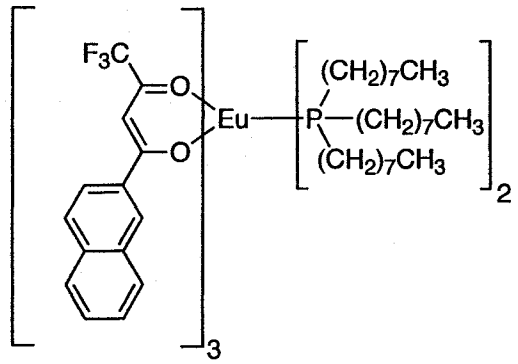


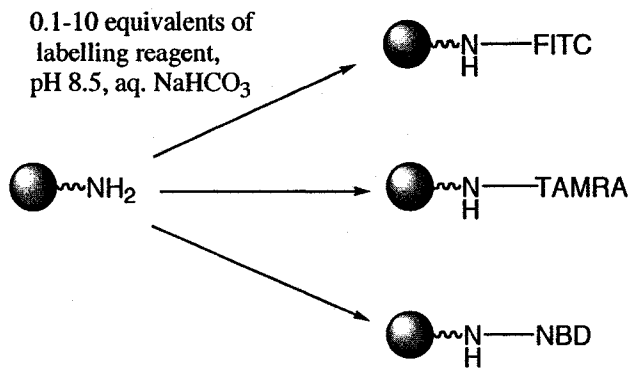
Figure 2-5. Highly luminescent chelate formed by the DELFIA technique.

2.5 Investigation of non-specificity of aromatic fluorescent labeling on solid phase

2.5.1 Study of agarose-supported lectins with labeled carbohydrates

In order to investigate non-covalent interactions between the labeled carbohydrates and the agarose-supported lectins, we first needed to effect covalent attachment of the aromatic dyes to ω-aminobutyl-agarose beads by amide or thiourea bond formation (Scheme 2.15). This control study was also performed to ascertain that fluorescent beads would be seen and that agarose would not quench any fluorescence under the aqueous medium used.

The results were satisfactory. Agarose beads were not fluorescent on their own and agarose beads were conjugated with the labeling dyes under basic conditions (Scheme 2.15). After a number of washing times in a phosphate buffered solution, the labeled beads were seen to be fluorescent under the epifluorescent microscope for a certain amount of time.



Scheme 2.15

Then, the existence of non-covalent interactions between the solid-supported lectins and fluorescent carbohydrates was investigated. Both negative and positive control experiments were planned. In the case of the PNA lectin, no binding should take place with the labeled trisaccharides of **1** (Con A ligand), hence the latter was the negative control. Whereas for Con A lectin, the conjugated disaccharides of **13** (PNA ligand) were used in the negative control assays.

To investigate the lowest concentration of the labeled carbohydrate for fluorescence to be detected in the confocal microscope, the lectins supported on agarose beads were incubated with different concentrations (1×10^{-7}-1×10^{-5} M) of the labeled saccharides in both the positive and negative controls. However, for a relative comparison between beads from different fluorescent dyes having different molar absorptivities, 10^{-5} M was the lowest concentration of dye used. After washing the beads with a fixed volume of buffer solution to remove any unbound conjugated carbohydrate, the average intensity of a lectin bead in the sample was taken from a plot of pixel intensity versus diameter of

the bead (Figure 2-6). This was repeated for three different beads of the same size (200-250 μm) in the sample, thus giving an average of the bead intensity in the sample (Tables 2.3 and 2.4).

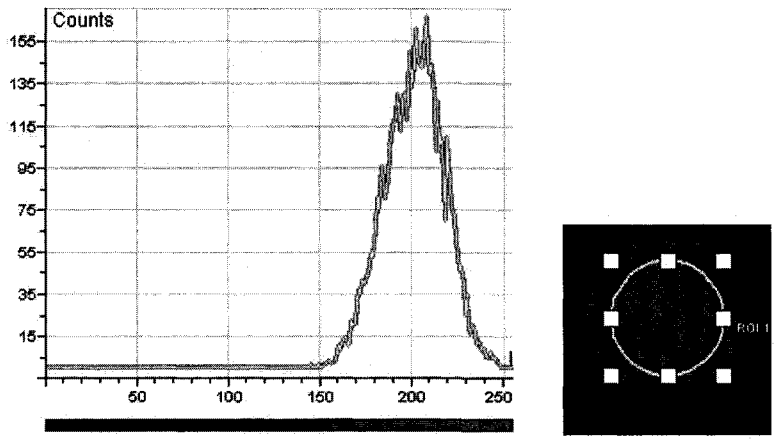


Figure 2-6 Average pixel intensity of 8-bit images (0-255) versus the distance in μM across the bead (the example shown is a Con A-agarose bead incubated with 10^{-4} M of the labeled trisaccharide (**1-TAMRA**).

Table 2.2 Parameters used in laser scanning confocal microscopy.

Dyes	Excitation wavelengths (nm)	Pinhole (μm)	
		Con A	PNA
FITC	500-535	70.82	75.26
TAMRA	553-596	107.00	56.70[a]
NBD	559-634	70.82[a]	70.82[a]

[a] a different beam had to be used, of higher intensity with six times the standard expansion.

Due to the different intensities of emission of the dyes and to avoid the problem of saturation of the pictures at higher fluorescence, different settings had to be chosen for the differently labeled glycosides bound to the agarose-supported lectins (Table 2.2). Agarose-supported PNA lectins were less fluorescent than agarose-supported Con A lectins. Morever, as discussed in Chapter 1 and indicated in Table 1.1. the large molar extinction coefficients of fluorescein in FITC and rhodamine in TAMRA dye require a lower beam intensity than NBD dye.

The tables (Tables 2.3 and 2.4) show the comparison of the positive and negative controls of the three dyes for both PNA and Con A lectins. As shown with its high level of background fluorescence in the negative controls, TAMRA displayed the greatest level of nonspecific interactions. However, compared to FITC and TAMRA dyes, NBD labeled carbohydrates showed by far the lowest level of nonspecific interactions as demonstrated by the absence of fluorescence in the negative controls for both lectins (Figure 2-7).

Table 2.3 Average intensities[a] of the agarose-supported Con A beads treated with different concentrations of labeled saccharides **1** and **13**.

Conc. of labeled saccharides (10^{-5} M)	Pixel intensity of beads (units)					
	FITC		TAMRA		NBD	
	+ control **1-FITC**	- control **13-FITC**	+ control **1-TAMRA**	- control **13-TAMRA**	+ control **1-NBD**	- control **13-NBD**
10	222	30	204	152	175	0
5	129	23	104	139	46	0
1	116	0	98	43	22	0

[a] Experimental errors are in the range of 5-20%.

Table 2.4 Average intensities[a] of the agarose-supported PNA beads treated with different concentrations of labeled saccharides **1** and **13**.

Conc. of labeled saccharides (10^{-5} M)	Pixel intensity of beads (units)					
	FITC		TAMRA		NBD	
	+ control **13-FITC**	- control **1-FITC**	+ control **13-TAMRA**	- control **1-TAMRA**	+ control **13-NBD**	- control **1-NBD**
10	250	45	236	81	106	0
5	48	0	105	79	13	0
1	10	0	57	75	8	0

[a] Experimental errors are in the range of 3-20%.

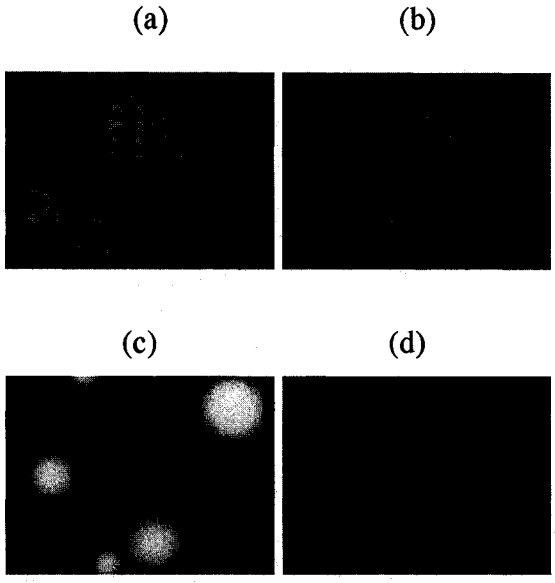


Figure 2-7. Fluorescence of the PNA-agarose beads treated with (a) + control in 1.0 × 10^{-5} M TAMRA-labeled disaccharide **13-TAMRA,** (b) – control in 1.0 × 10^{-5} M TAMRA-labeled trisaccharide **1-TAMRA,** (c) + control in 1.0 × 10^{-5} M NBD-labeled disaccharide **13-NBD,** (d) – control in 1.0 × 10^{-5} M NBD-labeled trisaccharide **1-NBD**.

2.5.2 Binding studies of agarose-lectins with NBD-labeled carbohydrates

To confirm the usefulness of the NBD dye for monitoring interactions between carbohydrates and proteins, the dye-labeled glycosides (1 × 10^{-4} M) were incubated with the agarose-supported lectins PNA and Con A. The beads became bright fluorescent yellow. The beads were then washed with equal volumes of buffer (PBS for PNA lectin and Tris buffer for Con A lectin) after equal intervals of times until the yellow color was completely washed out (Table 2.5). For the PNA lectin incubated with the NBD-labeled disaccharide, 165 seconds were required for complete washing compared to 405

seconds in the case of the Con A lectin incubated with the NBD-labeled trisaccharide. This result agrees with the above mentioned literature values of binding constants reported for the different lectins (c.f., Table 2.1).

Table 2.5 Qualitative study of binding strength of lectins towards NBD-labeled saccharides (**1-NBD** with ConA and **13-NBD** with PNA).

Time taken to wash beads (s)	Volume of buffer (mL)	Lectins (pixel intensity of bead)	
		PNA	ConA
15	0.25	163	209
45	0.5	55	169
105	1.0	22	96
165	1.0	0	47
225	1.0	0	24
285	1.0	0	14
345	1.0	0	5
405	1.0	0	0

Therefore, the NBD dye proved to be a labeling reagent that could be used in the screening of glycoconjugate libraries that would involve the minimum amount of non-specific interactions, and positive hits could be distinguished clearly from negative ones. The small size of the NBD dye compared to FITC and TAMRA surely contributes to this characteristic. The sensitive nature of NBD dye to its environment can also account for fluorescence quenching when NBD-labeled saccharides are surrounded by lectins. This could result in negligible fluorescence in negative controls.

2.6 Study of europium-labeled carbohydrates with agarose-supported lectins

2.6.1 Qualitative on-bead assays

The same experiments were performed with agarose-supported PNA lectin (20 μL) and europium-labeled disaccharide (0.1mM) in the positive control and europium labeled trisaccharide (0.1 mM) in the negative control (illustrated in Figure 2-8). After washing away the dye solutions with the appropriate buffer for the respective lectins, the enhancement solution DELFIA reagent, was added and after five minutes the agarose-supported PNA beads were observed under the epifluorescent microscope. Because the europium was dissociated from the carbohydrates immobilized non-covalently to the agarose-supported lectin and was forming a red fluorescent complex with the Delfia reagent, the red color was seen on the beads only for a short time and was diffusing away from the beads. It was thus difficult to localize the red spots on the beads as the solution was quickly becoming red.

Different solvents (propanol, tetrahydrofuran, isonitrile, dimethylsulfoxide, dimethylformamide, methanol, dichloromethane and monoglyme) were tested so as to precipitate the europium complex in the beads. Hence, localization and visualization of the red spots would become more definite. Unfortunately, no precipitation was seen.

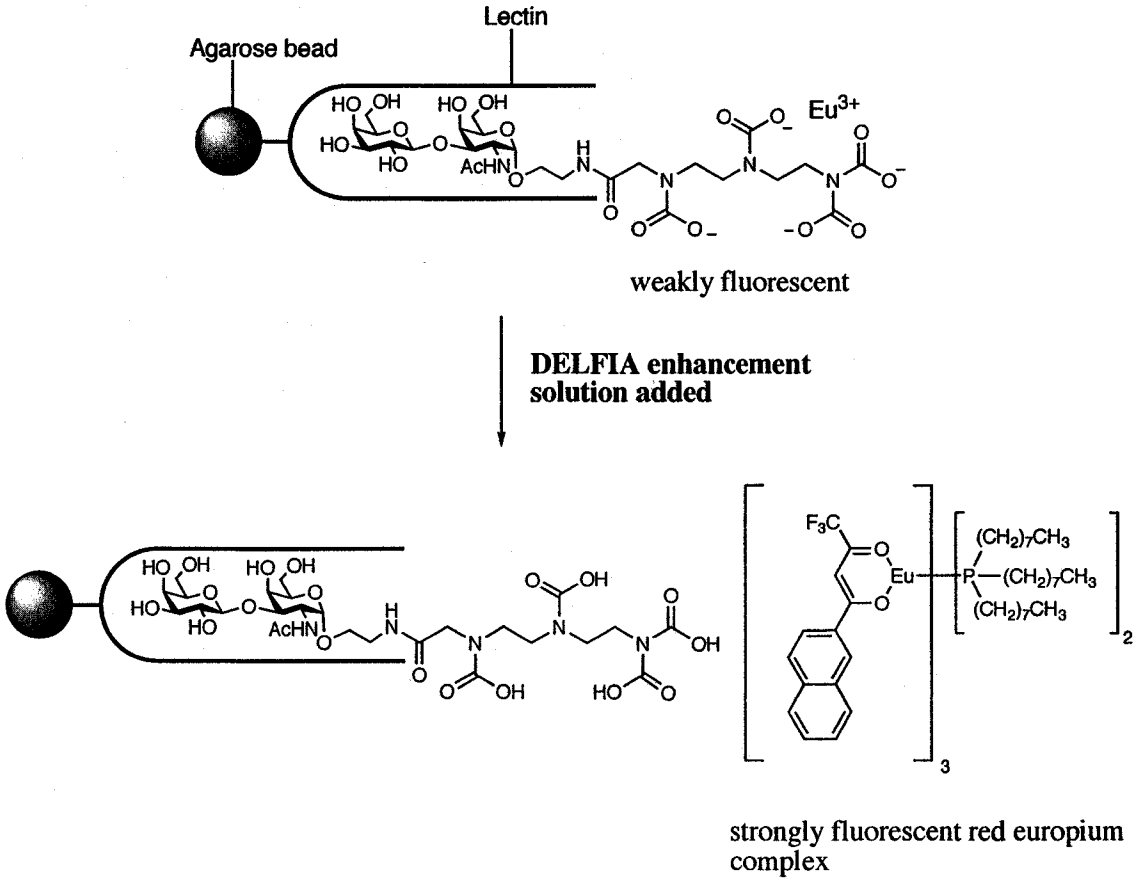


Figure 2-8. Fluorescence of europium label enhanced by the addition of DELFIA solution at pH 6.

The alternative option was then to immoblize the beads in a gel (more details are given in the supporting information)[73] that is usually employed in electrophoresis. We can then observe the red complex formed between the europium and the Delfia reagent while still embedded in the gel. However, when this was attempted with the agarose-supported PNA embedded in the gel, the beads could be seen, and on adding the DELFIA solution, the red spots again became diffused into the surrounding solution. From these gels, it was hard to confirm specific binding of europium-labeled carbohydrates to the agarose- supported lectins.

2.6.2 Quantitative determination of europium by fluorimetry

The amount of europium in the carbohydrates covalently attached to the agarose beads could not be determined visually. A way to solve the problem will be to quantify the amount of europium still bound to the agarose lectin bead by fluorimetry. After washing the lectin beads with the buffer solution to remove unbound europium conjugates, the DELFIA solution can be used to dissociate the residual europium in the carbohydrate conjugate that is still bound non-covalently to the lectin bead. Thus, fluorescence measurements can be used to quantify the europium in the DELFIA washings both in the positive controls and negative controls.

A volume of 20 μL of the agarose-supported lectin was incubated and diluted to 100 μL of buffer solution containing europium-tagged carbohydrates for 6 hours. The beads in the polypropylene tube were rinsed with the appropriate buffer solution (4 × 1 mL). After shaking the beads with 1 mL of the DELFIA solution for five minutes, the filtrate was collected in a quartz cuvette for readings to be taken. DELFIA washings (1 mL DELFIA reagent) were repeated three times and the fluorometric readings were taken with $\lambda_{excitation}$ = 315 nm, $\lambda_{emission}$ = 630 nm.

Table 2.6 Fluorometric measurements of DELFIA washings of the agarose-supported PNA lectin treated with different concentrations of labeled trisaccharide **32** and disaccharide **33.**

Cuvettes	Fluorescence readings[a] (units)					
	0.1 mM		1.0 mM		1.5 mM	
	+ control **33**	- control **32**	+ control **33**	- control **32**	+ control **33**	- control **32**
1	10	78	51	48	61	319
2	175	212	16	44	61	671
3	53	139	27	148	133	204

[a] Experimental error is ± 1 unit.

Table 2.7 Fluorometric measurements of DELFIA washings of the agarose-supported Con A lectin treated with 1.5 mM of labeled trisaccharide **32** and disaccharide **33.**

Cuvettes	Fluorescence measurements[a] (units)	
	1.5 mM	
	+ control **32**	- control **33**
1	35	47
2	82	37
3	168	33

[a] Experimental error is ± 1 unit.

In both cases, the fluorescence readings for agarose-supported PNA lectin (Table 2.6) and agarose-Con A lectin (Table 2.7) were high in case of the negative controls. It seemed that the washing protocol was not efficient and it was possible that the europium was also bound to the agarose bead, making the studies of non-specificity more complicated as both lectins were immobilized on the agarose beads. This possibility was confirmed with the following experiment: after washing agarose-supported PNA beads incubated with 1 mM of saccharide sixteen times with buffer solution (1 mL), the DELFIA washings gave fluorescence readings 953 in the positive control and 113 in the negative control. The agarose solid support was thus replaced by microtiter plates coated with the lectins.

2.6.3 Quantitative determination of europium by time-resolved fluorometry (TRF) using microtiter plates

This methodology made use of microtitration plates which would be coated with the proteins, lectins. It was based on a modified version of the ELISA assay (enzyme-linked immunosorbent assay), which is a biphasic immunoassay that requires only small amounts of proteins and relatively little sugar as the binding ligand.

Enzyme immunoassay methods (ELISA) were first developed for antibody receptors. ELISA is based on primary interactions, meaning it measures the direct binding of the protein to its antigen. This type of assay has several aims, including the quantitative measurement and specificity of antibodies, the amount of antigen or inhibitor activity.

ELISA methods, being safe and environmental friendly, have found numerous applications in immunology, biology and medicine.[74]

In our projected DELFIA assay, the unlabeled component (lectin) is adsorbed to a polystyrene surface, such as the wells of a microtiter plate. The adsorption of the unlabeled component to the polymeric support is due to intermolecular attractions (Van der Waals forces). Any remaining reactive sites on the ELISA plate are blocked by addition of protein (BSA or casein from milk). Labeled ligands, in our case europium-labeled carbohydrates would then be allowed to react with the immobilized lectin and the unbound labeled carbohydrates were removed by multiple washing steps. Since one of the reactants in the DELFIA assay became bound to the plate's surface, the separation of bound and free reagents was easily made by such washing procedures. The europium of the carbohydrates would then be dissociated and form a strongly fluorescent europium complex by addition of the DELFIA enhancement medium, which would then be measured by TRF in the microplate reader (Figure 2-9).

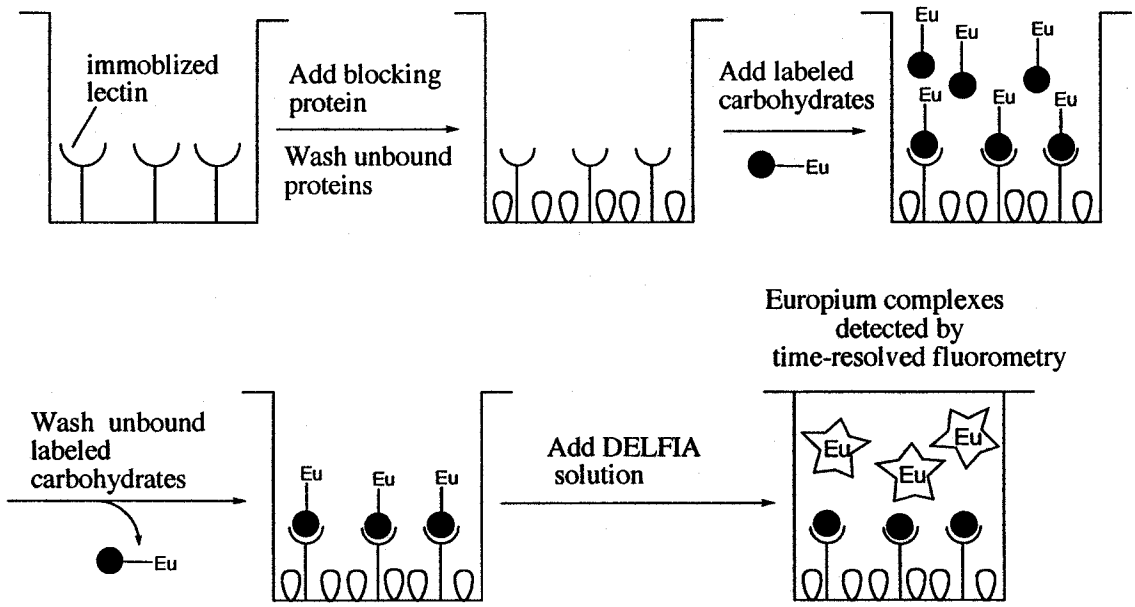


Figure 2-9. DELFIA assays performed on black-opaque microplates and europium fluorescence detected (time-resolved fluorometry) by the microplate reader.

DELFIA assays were performed in triplicate and the experimental sequence was modified from Bundle[75] and co-workers's assay as shown below:

1) Attachment of affininity purified lectin to the wells of a microtiter plate (96-well format).
2) Washing with the buffer to remove unadsorbed lectin.
3) Addition of 2.5% casein as a blocking protein.
4) Washing with buffer to remove excess casein.
5) Labeled carbohydrates were added to the well and incubation with the immobilized lectins was done at different temperatures from –4 °C to room temperature.
6) Washings were done to remove the unbound carbohydrates

7) DELFIA solution was added to dissociate europium from the bound carbohydrates and fluorescence readings were taken via time-resolved fluorometry.

2.6.3.1 Results for DELFIA assays for immobilized Con A lectin

Different conditions were tried to immobilize the Con A lectins to the black microplate as there was no prior procedure for this assay. Immobilized Con A lectin was first incubated with labeled carbohydrates for 6 hours at room temperature. The positive control consisted of the trisaccharide with the europium label, that is, **32**, and the negative control was the disaccharide with the europium chelate **33**. An average of three readings rounded to two significant figures were taken for each control ($\lambda_{excitation}$ = 332 nm, $\lambda_{emission}$ = 616 nm). As seen in Table 2.8, no conclusion could be made from the fluorescence readings (they were rounded to two significant figures), as a result of the rather high values of fluorescence in the negative controls and the reference control with the trisaccharide chelate without europium.

Table 2.8 DELFIA assay for immobilized Con A with labeled and non-labeled saccharides (with three buffer washings to remove unbound carbohydrates).

Concentration (M)	Fluorescence readings[a] (units)		
	+ control trisaccharide **32**	- control disaccharide **33**	trisaccharide chelate with no europium
10^{-7}	5,700	6,200	5,800
10^{-6}	7,600	7,300	6,200
10^{-5}	15,000	25,000	6,300
10^{-3}	saturation	saturation	saturation

[a]The experimental errors are in the range of 2-10%.

Another attempt (Table 2.9) was made to investigate the non-specificity of non-aromatic europium chelate with microplate wells filled with a solution of Con A for a longer period of time at room temperature for 42 hours as compared to 6 hours for Table 2.8. Our expectation of lower fluorescence readings for the negative and reference controls as compared to the positive control was not observed.

Table 2.9 DELFIA assay for immobilized Con A with labeled and non-labeled saccharides (with two buffer washings to remove unbound carbohydrates).

Concentration (M)	Fluorescence readings[a] (units)		
	+ control trisaccharide **32**	- control disaccharide **33**	Trisaccharide chelate with no europium
10^{-7}	5,800	6,200	5,500
10^{-6}	6,400	8,800	5,300
10^{-5}	6,300	130,000	5,900
10^{-4}	5,800	140,000	5,200

[a]The experimental errors are in the range of 2-10%.

Obviously, the assay was not working as projected. Background fluorescence was too high to ascertain specific binding of labeled trisaccharide **32** to the Con A lectin. We wanted to investigate if the number of washings performed to remove unbound labeled carbohydrates were responsible for the lower fluorescence values in the positive controls. From Table 2.10, we still did not see any significant increase in the order of 100,000 for fluorescence readings in the positive controls.

Table 2.10 DELFIA assay for immobilized Con A with labeled and non-labeled saccharides (with two buffer washings to remove unbound carbohydrates).

Washings	Concentration (M)	Fluorescence readings[a] (units)		
		+ control trisaccharide **32**	- control disaccharide **33**	trisaccharide chelate with no europium
1	10^{-5}	2,100	6,200	2,700
1	10^{-4}	23,000	8,800	2,700
2	10^{-5}	2,000	2,100	3,300
2	10^{-4}	17,000	15,000	1,900

[a]The experimental errors are in the range of 2-10%.

These experiments were also repeated for the PNA lectin (Table 2.11), which was immobilized on black microplates at -4 °C for 24 hours. Again, we could see no significant increase in fluorescence readings for the positive controls.

Table 2.11 DELFIA assay for immobilized PNA with labeled and non-labeled saccharides (with two buffer washings to remove unbound carbohydrates).

Concentration (M)	Fluorescence readings (units)		
	+ control disaccharide **33**	- control trisaccharide **32**	disaccharide chelate with no europium
10^{-7}	7, 300	5,400	none taken
10^{-6}	11, 000	8,500	none taken
10^{-5}	53, 000	37,000	none taken
10^{-4}	510, 000	160,000	4,800
10^{-3}	saturation	saturation	none taken

[a]The experimental errors are in the range of 2-10%.

Three other attempts were made at finding conditions for binding the PNA lectin to the mircroplate and with a different number of buffer washings.

Table 2.12 DELFIA assay for immobilized PNA with labeled and non-labeled saccharides (with three buffer washings to remove unbound carbohydrates).

Concentration (M)	Fluorescence readings[a] (units)		
	+ control disaccharide **33**	- control trisaccharide **32**	disaccharide chelate with no europium
10^{-7}	5,800	5,200	5,600
10^{-6}	9,200	5,200	6,300
10^{-5}	200,000	5,500	5,300
10^{-4}	290,000	4,900	5,800

[a]The experimental errors are in the range of 2-10%.

Table 2.13 DELFIA assay for immobilized PNA with labeled and non-labeled saccharides (with three buffer washings to remove unbound carbohydrates).

Concentration (M)	Fluorescence readings[a] (units)		
	+ control disaccharide **33**	- control trisaccharide **32**	disaccharide chelate with no europium
10^{-7}	4,800	6,800	6,900
10^{-6}	4,700	6,400	10,000
10^{-5}	5,100	7,100	5,800
10^{-4}	100,000	81,000	12,000

[a]The experimental errors are in the range of 2-10%.

Table 2.14 DELFIA assay for immobilized PNA with labeled and non-labeled saccharides (with one buffer washing to remove unbound carbohydrates).

Concentration (M)	Fluorescence readings[a] (units)		
	+ control disaccharide **33**	- control trisaccharide **32**	disaccharide chelate with no europium
10^{-7}	12,000	14,000	11,000
10^{-6}	12,000	12,000	9,600
10^{-5}	16,000	20,000	14,000
10^{-4}	400,000	200,000	13,000

[a]The experimental errors are in the range of 2-10%.

The results for both the immobilized Con A and PNA showed no conclusive evidence of specific binding to the carbohydrate of high affinity, hence no information could be obtained for the amount of non-specificity seen in the use of the non-aromatic dye.

Possible reasons for this failure include:

The lectins were not truly coated on the plates, which were Nunc plates. It is known that different lectins prefer certain types of plates for strong binding, and since these were black microplates for specific measurement of low fluorescence, the experimental conditions differed from the transparent microplates.

Background fluorescence was high in the case of negative controls. The ideal background fluorescence should be around 2,000. Special plates, or even the buffer solutions, might contain interfering contaminants that affected the fluorescence readings. The number of factors affecting these readings were innumerable, hence it was tough to pinpoint the exact problem with the assay. Because of time constraints, it was decided to abandon this part of the project dealing with the investigation of non-specificity of the europium label as compared to polyaromatic dyes.

2.7 Conclusions

Hydrophobic and other interactions often exist between lectins and dye molecules on labeled glycosides, which make specific interactions hard to ascertain. The predominantly large and polyaromatic labeling dyes traditionally used are rendered impractical because of the difficulty in distinguishing positive hits from negative hits especially at high concentrations of the labeled ligands. This study confirmed that

NBD-X dye was an ideal labeling tool in the screening or monitoring of the binding interactions between promising ligands and immobilized protein receptors. Our studies suggest that NBD-labeled libraries of synthetic carbohydrates and other small molecules could be screened in a reliable fashion against bead and surface-immobilized proteins with minimal interference from nonspecific dye interactions. The low molar extinction coefficient of the NBD[76] dye (ε = 22,000 $M^{-1}cm^{-1}$) compared to the FITC (ε = 72,000 $M^{-1}cm^{-1}$) and TAMRA[76] dyes (ε = 65,000 $M^{-1}cm^{-1}$) may limit its use in certain cases. The use of higher powered beams may be necessary to perform the visualization. The different concentrations of the carbohydrate conjugates used in the positive and negative controls, however, show that NBD dye exhibits the least amount of non-specificity compared to the largely aromatic dyes.

In the case of europium chelates, no conclusion could be derived as to the non-specificity of the label. DELFIA solution was used to dissociate the europium complex from the carbohydrate conjugate bound to the agarose bead. The diffusion of the red color made it hard to ascertain nonspecific binding. Some europium may have been attached to the agarose beads themselves as agarose is a linear polysaccharide (average molecular mass about 12,000) through chelating effects from the hydroxyl groups.

Disappointing results were also obtained with the assays performed on the microplates. The fluorescence readings were unreliable and non-reproducible. The protocol for attaching the lectin to the plates was not working well and hence, there was not much

difference between positive and negative controls when europium conjugates were incubated with the lectins.

2.8 Experimental

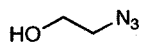
2.8.1 General

All chemical reagents were of analytical grade, used as supplied without further purification unless otherwise indicated. Solvents used in reactions were distilled under an inert atmosphere. Molecular sieves that were stored in an oven (> 200 °C) and cooled in vacuo were used. The acidic ion-exchange resin used was Amberlite (H^+ form). Analytical thin layer chromatography (TLC) was conducted on silica gel 60-F_{254}. Plates were visualized under UV light and/or treatment with 5% ethanolic sulfuric acid followed by heating.

The succinimidyl esters of NBD, and TAMRA and the isocyanate ester of FITC were commercially available. The lectin gels, Con A-agarose and PNA-agarose were commercially available from EY laboratories. Stock buffer solutions for Con A-agarose were composed of 0.05 M Tris, 0.15 M HCl and 0. 004 M $CaCl_2$ (pH 7.0) and for PNA-agarose, the buffer solutions were made of 0.01M phosphate, 0.15 M NaCl, and 0.005 M $CaCl_2$ (pH 7.2). The one-bead fluorescence assays were visualized with Leica TLS-SP2 multiphoton confocal laser scanning microscope (TLS-MP) located in the Biological Sciences Building. For the reading of microtiter plates, the SPECTRAmax GEMINI microplate spectrofluorometer was used.

^{1}H and ^{13}C NMR spectra were recorded on Varian INOVA 400, 500 or 600 MHz spectrometers. Chemical shifts were reported in d (ppm) units using ^{13}C and residual ^{1}H signals from deuterated solvents. ^{1}H and ^{13}C NMR spectra were assigned with the assistance of COSY, HMQC and HMBC and TOCSY spectra where necessary. ^{13}C NMR spectra of dye-saccharide conjugates tend to show low signal-to-noise ratio due to limited quantities of these complex compounds. The two protons of the C-6 of the carbohydrates are denoted by H_a and H_b respectively. A Micromass Zabspec TOF mass spectrometer provided the electrospray ionization mass spectra. Optical rotations were measured using a 1 mL cell with a 1 dm length on a Perkin-Elmer model 241 polarimeter at 22 °C.

2.8.2 Experimental procedures

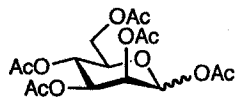


2-Azidoethanol (5) is known and its preparation was modified from a known litterature[39] method using 2-bromoethanol rather than 2-chloroethanol.

2-Bromoethanol (10 mL 14.1 mmol) was added to a stirred solution of sodium azide (11.3 g, 17.3 mmol, 1.23 equiv) and sodium hydroxide (0.564 g, 1.41 mmol) in water. The mixture was stirred for 3 days at room temperature until complete disappearance of the starting material. Anhydrous sodium sulfate (13.5 g) was added and the mixture was extracted with dichloromethane (3 × 45 mL) and the combined extracts were dried over anhydrous sodium sulfate. Filtration and evaporation afforded 2-azidoethanol (7.46 g, 8.60 mmol, 61%) which was found to be sufficiently pure by ^{1}H NMR to be used for subsequent glycosylation reactions. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.80-3.77

(m, 2H, O$\underline{CH_2}CH_2N_3$), 3.45 (t, 2H, J = 2.0, 5.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 61.5 (O$\underline{CH_2}CH_2N_3$), 53.5 (OCH$_2\underline{CH_2}N_3$). HR-ESMS calcd for $C_2H_5N_3O$: 87.0483. Found: 87.0435.

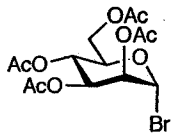
1,2,3,4,6 Penta-*O*-acetyl-α,β-D-mannopyranoside (6)



D-Mannose (15 g, 0.083 mol) was dissolved in acetic anhydride (219 mL) containing a few crystals of DMAP. Pyridine (294 mL) was added to the solution at 0 °C. Stirring was continued at rt for 24 h. The mixture became clear and the solvents were coevaporated with toluene (3 × 40 mL). The residue was taken up in ethyl acetate (200 mL), washed with 2 N HCl (3 × 50 mL), water (2 × 40 mL), saturated sodium bicarbonate (3 × 50 mL) and brine (2 × 40 mL). After drying the organic phase over anhydrous Na_2SO_4, the filtered solvent was evaporated to give a yellow white solid which was purified by silica gel column chromatography using ethyl acetate:hexanes (1:8→1:1) to give the per-acetylated mannopyranoside as a white solid (32.5 g, 0.083 mol, quantitative yield) as a 3:1 mixture of α and β anomers: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.06 (d, 1H, H-1α, $J_{1\alpha,2\alpha}$ = 1.5 Hz, $J_{C\text{-}H}$ = 178 Hz), 5.84 (d, 1H, H-1β, $J_{1\beta,2\beta}$ = 1.0 Hz, $J_{C\text{-}H}$ = 164 Hz), 5.46 (dd, 1H, H-2β, $J_{2\beta,1\beta}$ = 1.0 Hz, $J_{2\beta,3\beta}$ = 3.0 Hz), 5.33-5.30 (m, 2H, H-3α, H-4α), 5.30- 5.21 (m, 2H, H-4β, H-2α), 5.11 (dd, 1H, H-3β, $J_{3\beta,2\beta}$ = 3.0 Hz, $J_{3\beta,4\beta}$ = 10.0 Hz), 4.30 - 4.22 (m, 2H, H-6aβ, H-6aα), 4.12 (dd, 1H, H-6bβ, $J_{6b\beta,5\beta}$ = 2.4 Hz, $J_{6b\beta,6a\beta}$ = 12.0 Hz), 4.08 (dd, 1H, H-6bα, $J_{6b\alpha,5\alpha}$ = 2.5 Hz, $J_{6b\alpha,6a\alpha}$ = 12.0 Hz), 4.05-3.99 (m, 1H, H-5α), 3.79 (ddd, 1H, H-5β, $J_{5\beta,6b\beta}$ = 2.4 Hz, $J_{5\beta,6a\beta}$ = 5.5 Hz, $J_{5\beta,4\beta}$ = 8.0

Hz), 2.19, 2.15, 2.14, 2.08, 2.07, 2.03, 2.02, 1.98, 1.977 (s, 3H, $COCH_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.32, 170.29, 169.9, 169.7, 169.5, 169.36, 169.4, 169.3, 168.1, 167.84 (C=O), 90.4 (C-1α), 90.2 (C-1β), 72.9 (C-5β), 70.42 70.40 (C-5α), 68.6 (C-3α), 68.2, 68.1 (C-2α, C-2β), 65.35, 65.32 (C-4α, C-4β), 61.89 (C-6α, C-6β), 20.6, 20.48, 20.49, 20.47, 20.45, 20.43, 20.41, 20.39, 20.35, 20.27 ($CO\underline{CH_3}$); HR-ESMS calcd for $C_{16}H_{22}O_{11}Na$: 413.1060. Found: 413.1063.

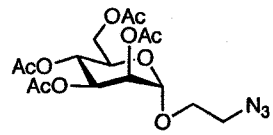
2,3,4,6 Tetra-*O*-acetyl-α-D-mannopyranosyl bromide (7)[43]



To a stirred solution of the per-acetylated mannose **6** (3.45 g, 8.84 mmol) in dry dichloromethane (10 mL) at 0 °C was added 33% HBr (in glacial acetic acid). Stirring was continued at rt for 3 h. After removal of solvents under reduced pressure, the residue was dissolved in dichloromethane (80 mL) and added to ice water. The organic layer was separated. The aqueous layer was extracted with dichloromethane (2 x 50 mL). The combined organic extracts were washed with saturated solution bicarbonate solution (2 x 80 mL) and brine (2 x 70 mL). The organic portion was dried over anhydrous Na_2SO_4, filtered and evaporated to give the bromide **7** (3.63 g, 8.83 mmol, quantitative yield) as a solid. : ^1H NMR (500 MHz, $CDCl_3$) δ 6.26 (d, 1H, H-1, $J_{1,2}$ = 1.5 Hz), 5.41 (dd, 1H, H-3, $J_{3,2}$ = 3.5 Hz, $J_{3,4}$ = 10.5 Hz), 5.41 (dd, 1H, H-2, $J_{2,1}$ = 1.5 Hz, $J_{2,3}$ = 3.5 Hz), 5.33 (dd, 1H, H-4, $J_{4,3}$ = $J_{4,5}$ = 10.3 Hz), 4.30 (dd, 1H, H-6a, $J_{6a,5}$ = 5.0 Hz, $J_{6a,6b}$ = 12.5 Hz), 4.18 (ddd, 1H, H-5, $J_{5,6b}$ = 2.5 Hz, $J_{5,6a}$ = 5.0 Hz, $J_{5,4}$ = 10.3 Hz), 4.10 (dd, 1H, H-6b, $J_{6b,5}$ = 2.5 Hz, $J_{6b,6a}$ = 13.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ

169.6, 169.5, 169.5 (C=O), 83.1 (C-1), 72.8 (C-5), 72.1 (C-2), 67.9 (C-3), 65.3 (C-4), 61.4 (C-6).

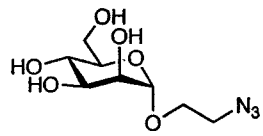
2-Azidoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (8)[43]



A mixture of silver triflate (3.14 g, 13.2 mmol), 2,4,6-collidine (0.094 mL, 0.71 mmol,) and 2-azidoethanol (1.59 mL, 26.5 mmol) in dry dichloromethane (6 mL) with molecular sieves 4Å was stirred for 30 min at -20 °C. 2,3,4,6 Tetra-O-acetyl-α-D-mannopyranosyl bromide **7** (3.63 g, 8.83 mmol) dissolved in dry dichloromethane (4 mL) was added to the mixture, which was left stirring for 9 h with the temperature rising to rt. Triethylamine (0.3 mL) was added to neutralize the reaction mixture, which was diluted with dichloromethane (50 mL), then filtered through celite. The filtrate was then washed with water (2 × 40 mL), 1 N HCl (2 × 40 mL), saturated aqueous sodium bicarbonate (2 × 40 mL) and brine (2 × 20 mL). After drying over anhydrous Na_2SO_4 and filtering the mixture, the solvent was evaporated to give a crude product which was purified by silica gel chromatography using ethyl acetate/hexane (1:4 →1:1) to give **8** (3.11 g, 7.45 mmol, 84%) as a white solid. $[\alpha]_D^{22}$ + 39.02 (*c*, 0.62, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 5.34 (dd, 1H, H-3, $J_{3,2}$ = 3.0 Hz, $J_{3,4}$ = 10.0 Hz), 5.30 (t, 1H, H-4, $J_{4,3} = J_{4,5}$ = 10.2 Hz), 5.26 (dd, 1H, H-2, $J_{2,1}$ = 1.8 Hz, $J_{2,3}$ = 3.40 Hz), 4.85 (d, 1H, H-1, $J_{1,2}$ = 2.0 Hz), 4.27 (dd, 1H, H-6a, $J_{6a,5}$ = 5.0 Hz, $J_{6a,6b}$ = 12.4 Hz), 4.11 (dd, 1H, H-6b, $J_{6b,5}$ = 2.6 Hz, $J_{6b,6a}$ = 12.2 Hz), 4.03 (ddd, 1H, H-5, $J_{5,6b}$ = 2.6 Hz, $J_{5,6a}$ = 5.4 Hz, $J_{5,4}$ = 9.6 Hz), 3.85 (ddd, 1H, O$\underline{CH_2}CH_2N_3$, *J* = 3.8, 6.8, 10.6 Hz), 3.65 (ddd, 1H,

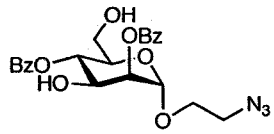
$O\underline{CH_2}CH_2N_3$, *J*= 3.7, 6.1, 10.7 Hz), 3.51-3.39 (m, 2H, $OCH_2\underline{CH_2}N_3$), 2.14, 2.09, 2.03, 1.98 ($CO\underline{CH_3}$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.46, 169.4, 169.3, 168.1, 167.84 (C=O), 97.7 (C-1α), 69.4 (C-2), 68.84, 68.81 (C-3, C-4), 67.0 ($O\underline{CH_2}CH_2N_3$), 66.0 (C-5), 62.4 (C-6), 50.3 ($OCH_2\underline{CH_2}N_3$), 20.8, 20.69, 20.66, 20.61 ($CO\underline{CH_3}$). HR-ESMS calcd for $C_{16}H_{23}N_3O_{10}Na$: 440.1281. Found: 440.1280.

2-Azidoethyl α-D-mannopyranoside (9)

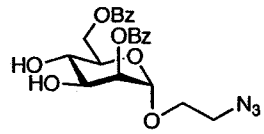


2-Azidoethyl 2,3,4,6 tetra-*O*-acetyl-α-D-mannopyranoside **8** (1.62 g, 3.88 mmol) was dissolved in dry methanol (9 mL) and 0.1 N sodium methoxide (0.76 mL, 0.076 mmol, 0.02 equiv) was added to the solution. Stirring was continued at room temperature for 30 min, after which Amberlite-H^+ was added to neutralize the solution. The resin was filtered off and the solvent was evaporated to afford **9** quantitatively as a white solid (0.97 g, 3.88 mmol). $[\alpha]_D^{22}$ + 54.84 (*c*, 0.91, CH_3OH); ^1H NMR (500 MHz, CD_3OD) δ 4.81 (dd, 1H, H-1, $J_{1,2}$ = 1.8 Hz, $J_{C\text{-}H}$ = 169 Hz), 3.91 (ddd, 1H, $O\underline{CH_2}CH_2N_3$, *J* = 4.5, 5.1, 10.6 Hz), 3.85-3.81 (m, 2H, H-6a, H-2), 3.74-3.68 (m, 2H, H-3, H-6b), 3.64-3.54 (m, $O\underline{CH_2}CH_2N_3$, H-4, H-5), 3.40 (t, 2H, $OCH_2\underline{CH_2}N_3$, *J* = 5.0 Hz); ^{13}C NMR (125 MHz, CD_3OD, δ) 101.8 (C-1), 75.0 (C-4), 72.5 (C-3), 72.1 (C-2), 68.6 (C-2), 67.8 ($O\underline{CH_2}CH_2N_3$), 63.0 (C-6), 51.8 ($OCH_2\underline{CH_2}N_3$). HR-ESMS calcd for $C_8H_{15}N_3O_6Na$: 272.0859. Found: 272.0856.

2-Azidoethyl 2,4- and 2.6 -di-*O*-benzoyl-α-D-mannopyranoside (3) and (11)



3



11

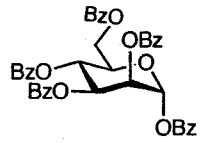
The preparation of compound **3** was adapted from the procedure of Oscarson and Tidén[38] with a different aglycon at the anomeric position. Compound **9** (0.066 g, 0.27 mmol) was made into a solution with dry acetonitrile (4 mL). Triethyl orthobenzoate (0.16 mL, 0.69 mmol) was added to it, followed by camphorsulfuric acid (40 mg, 0.016 mmol) and 90% TFA (1.6 mL, 0.022 mmol). The stirred suspension became clear after 1 h. Triethylamine (0.5 mL) was added and the solvent removed under reduced pressure to give diorthoesters. The latter was dissolved in dry acetonitrile (3.5 mL) and 90% TFA (in water) was added. The solution was stirred for 30 min. The solvents were removed by evaporation and the crude was chromatographed using ethyl acetate:hexane (1:9→1:3) as eluant to give three fractions. The first fraction was 2-azidoethyl 2,4-*O*-benzoyl-α-D-mannopyranoside **3** (0.051 g, 0.11 mmol, 41%). Then came a 1:1 mixture of 2-azidoethyl 2,4 and 2,6 -di-*O*-benzoyl-α-D-mannopyranoside (0.014 g, 0.030 mmol, 11%) and a pure fraction of 2-azidoethyl 2,6-di-*O*-benzoyl-α-D-mannopyranoside **11** (0.061 g, 0.13 mmol, 48%).

2-Azidoethyl 2,4-di-*O*-benzoyl-α-D-mannopyranoside (3): $[\alpha]_D^{22}$ –50.69 (*c*, 0.37, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 8.08-8.03 (m, 4H, Ar), 7.59-7.55 (m, 2H, Ar), 7.46-7.42 (m, 4H, Ar), 5.50 (t, 1H, H-4, $J_{4,3} = J_{4,5} = 10.0$ Hz), 5.41 (dd, 1H, H-2, $J_{2,1} = 1.5$ Hz, $J_{2,3} = 3.5$ Hz), 5.07 (d, 1H, H-1, $J_{1,2} = 1.0$ Hz, $J_{C\text{-}H} = 172$ Hz), 4.41 (dd, 1H, H-3,

$J_{3,2}$ = 3.5 Hz, $J_{3,4}$ = 9.5 Hz), 3.97 (ddd, 1H, H-5, $J_{5,6a}$ = 2.5 Hz, $J_{5,6b}$ = 4.0 Hz, $J_{5,4}$ = 6.5 Hz), 3.92 (ddd, 1H, $O\underline{CH_2}CH_2N_3$, *J*= 3.0, 7.0, 10.5 Hz), 3.79 (dd, 1H, H-6a, $J_{6a,5}$ = 2.5 Hz, $J_{6a,6b}$ = 13.0 Hz), 3.75-3.65 (m, 2H, H-6b, $O\underline{CH_2}CH_2N_3$), 3.51-3.35 (m, 2H, $OCH_2\underline{CH_2}N_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.2, 166.0 (C=O), 133.70, 133.60, 129.93, 129.90 (Ar CH), 129.2, 129.0 (Ar C), 128.59, 128.53 (Ar CH), 97.7 (C-1), 72.7 (C-3), 70.9 (C-2), 70.1 (C-4), 68.4 (C-5), 67.1 ($O\underline{C}H_2CH_2N_3$), 61.4 (C-6), 50.5 ($OCH_2\underline{C}H_2N_3$). HR-ESMS calcd for HR-ESMS for $C_{22}H_{23}N_3O_8Na$: 480.1383. Found: 480.1384.

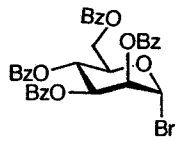
2-Azidoethyl 2,6-di-*O*-benzoyl-α-D-mannopyranoside (11): ^1H NMR (500 MHz, $CDCl_3$) δ 8.07-8.05 (m, 2H, Ar), 7.90-7.88 (m, 2H, Ar), 7.58-7.55 (m, 1H, Ar), 7.49-7.45 (m, 1H, Ar), 7.41-7.38 (m, 2H, Ar), 7.24-7.20 (m, 2H, Ar), 5.38 (dd, 1H, H-2, $J_{2,1}$ = 2.0 Hz, $J_{2,3}$ = 3.5 Hz), 4.97 (dd, 1H, H-1, $J_{1,2}$ = 1.0 Hz), 4.81 (br d, 1H, H-6a, $J_{6a,6b}$ = 12.0 Hz), 4.53 (dd, 1H, H-6b, $J_{6b,5}$ = 1.5 Hz, $J_{6a,6b}$ = 12.0 Hz), 4.22 - 4.15 (m, 1H, H-3), 3.97-3.93 (m, 2H, H-5, H-4), 3.89 (ddd, 1H, $O\underline{CH_2}CH_2N_3$, *J*= 4.0, 7.0, 11 Hz), 3.64 (ddd, 1H, $O\underline{CH_2}CH_2N_3$, *J*= 3.5, 6.0, 9.5 Hz), 3.46-3.35 (m, 3H, $OCH_2\underline{CH_2}N_3$ and OH), 3.01 (br s, 1H, OH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.1, 166.0 (C=O), 133.32, 133.26, 129.8, 129.73 (Ar CH), 129.7, 129.4 (Ar C), 128.5, 128.4 (Ar CH), 97.9 (C-1), 72.1 (C-2), 71.2 (C-4), 70.1 (C-3), 67.7 (C-5), 66.9 ($O\underline{C}H_2CH_2N_3$), 63.5 (C-6), 50.4 ($OCH_2\underline{C}H_2N_3$). HR-ESMS calcd for $C_{22}H_{23}N_3O_8Na$: 480.1383. Found: 480.1384.

1,2,3,4,6-Penta-*O*-benzoyl-α-D-mannopyranoside (12)



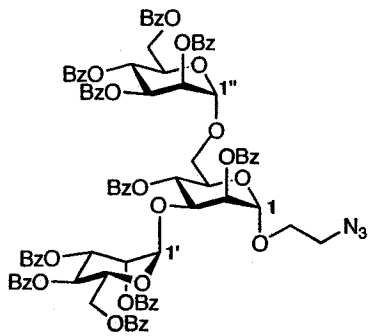
was prepared according to a literature procedure.[48] To a solution of dry α-D-mannose (200 g, 0.011 mol) in dry dichloromethane (7mL) and pyridine (6.8 mL) was added benzoyl chloride (16.1 mL,0.14 mol) at 0 °C. Stirring was continued for 2 h and on completion of reaction, the solvent was removed by evaporation. The residue was taken in dichloromethane (70 mL), washed with 2 M HCl (2 x 40 mL), water (2 x 40 mL) and brine (2 x 40 mL). After drying over Na_2SO_4 anf filtering the solution, the solvent was removed under reduced pressure, leaving a crude that was purified by flash column chromatography with ethyl acetate/hexane (1:4) as eluant to give **12** (7.78 g, 0.011 mol, quantitative yield) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$, δ) 8.20-8.19 (m, 2H, Ar), 8.09-8.07 (m, 4H, Ar), 7.96-7.94 (m, 2H, Ar), 7.86-7.84 (m, 2H, Ar), 7.67 (br t, 1H, Ar, J = 7.5 Hz), 7.61 (br t, 1H, Ar, J = 7.5 Hz), 7.57-7.53 (m, 3H, Ar), 7.49 (t, 1H, Ar, J = 7.5 Hz), 7.45-7.34 (m, 7H, Ar), 7.27 (t, 2H, Ar, J = 8.0 Hz), 6.63 (d, 1H, H-1, $J_{1,2}$ = 2.3 Hz), 6.28 (t, 1H, H-4, $J_{4,3}$= $J_{4,5}$= 10.4 Hz), 6.07 (dd, 1H, H-3, $J_{3,2}$ = 3.5 Hz, $J_{3,4}$ = 10.4 Hz), 5.91 (dd, 1H, H-2, $J_{2,1}$= 2.3 Hz, $J_{2,3}$ = 3.5 Hz), 4.70 (dd, 1H, H-6a, $J_{6a,5}$ = 2.5 Hz, $J_{6a,6b}$ = 12.5 Hz), 4.58 (ddd, 1H, H-5, $J_{5,6a}$ = 2.5 Hz, $J_{5,6b}$ = 3.8 Hz, $J_{5,4}$ = 10.4 Hz), 4.50 (dd, 1H, H-6b, $J_{6a,5}$ = 3.8 Hz, $J_{6a,6b}$= 12.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$, δ) 165.9,165.32, 165.25, 165.1, 165.8 (C=O), 129.79 (Ar CH), 129.78 (Ar C), 129.76 (Ar CH), 129.0 (Ar C), 128.8 (Ar CH), 128.71 (Ar C), 128.65 (Ar C), 128.5, 128.41, 128.38 (Ar CH), 91.4 (C-1), 71.2 (C-5), 70.0 (C-3), 69.5 (C-2), 66.2 (C-4), 62.4 (C-6). HR-ESMS calcd for $C_{41}H_{32}O_{11}Na$: 723.1842. Found: 723.1844.

2,3,4,6-Tetra-*O*-benzoyl-α-D-mannopyranosyl bromide (4)[48]



A solution of the perbenzoylated mannose **12** (0.30 g, 0.43 mol) in dry DCM (2 mL) was stirred at 0 °C. A solution of 33% HBr (glacial acetic acid, 3 mL) was added to the solution, which was stirred for 45 min. The solvents were removed by co-evaporation with toluene (2 × 50 mL) and the residue was dissolved in ice-cold DCM (50 mL) and neutralized with ice-cold saturated aqueous $NaHCO_3$ (20 mL). The organic phase was separated and washed with ice-cold water (3 × 40 mL), saturated aqueous $NaHCO_3$ (3 × 20 mL) and brine (2 × 15 mL). After drying over anhydrous Na_2SO_4 and filtration, followed by evaporation of solvent, the bromide **4** (0.28 g, 0.43 mmol, quantitative yield) was obtained as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12-7.92 (m, 6H, Ar), 7.84-7.80 (m, 2H, Ar), 7.61-7.20 (m, 12H, Ar), 6.57 (br s, 1H, H-1), 6.27 (dd, 1H, H-3, $J_{3,2}$ = 3.3 Hz, $J_{3,4}$ = 10.3 Hz), 6.22 (t, 1H, H-4, $J_{4,3} = J_{4,5}$ = 10.3 Hz), 5.89 (dd, 1H, H-2, $J_{2,1}$ = 1.5 Hz, $J_{2,3}$ = 3.3 Hz), 4.72 (dd, 1H, H-6a, $J_{6a,5}$ = 1.8 Hz, $J_{6a,6b}$ = 12.5 Hz), 4.63 (m, 1H, H-5), 4.50 (dd, 1H, H-6b, $J_{6b,5}$ = 3.8 Hz, $J_{6b,6a}$ = 12.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.9, 165.3, 165.25, 164.9 (C=O), 83.1 (C-1), 73.10 (C-5), 72.9 (C-2), 65.0 (C-3), 65.9 (C-4), 61.8 (C-6). HR-ESMS calcd for $C_{34}H_{27}O_9NaBr$: 681.0736. Found: 681.0735.

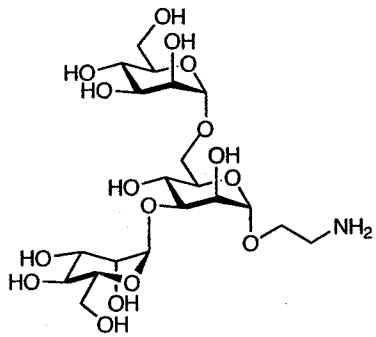
2-Azidoethyl 2,4-di-*O*-benzoyl-3,6-di-*O*-(2,3,4,6-tetra-*O*-benzoyl-α-mannopyranosyl-α-D-mannopyranoside (2)



To a mixture of the dibenzoate **3** (1.97 g, 4.31 mmol) and 2,3,4,6-tetra-O-benzoyl-α-D-mannopyranosyl bromide (**4**) (6.08 g, 9.22 mmol) in dry DCM (27 mL) and 4Å molecular sieves, a solution of silver triflate (2.61 g, 10.2 mmol) in dry toluene (10 mL) was added at –20 °C. The mixture was allowed to stir for 4 h at a temperature rising to 0 °C, then to rt. After complete disappearance of the donor by TLC, triethylamine (0.5 mL) was added to neutralize the mixture, which was then filtered over celite and concentrated. The resulting crude was chromatographed using ethyl acetate:hexane (6:1→2:1) as eluant to afford **2** (4.69 g, 2.90 mmol, 67%) as a white solid. $[\alpha]_D^{22}$ -59.99 (*c*, 0.59, $CHCl_3$); ^1H NMR (600 MHz, $CDCl_3$) δ 8.30-8.28 (m, 2H, ArH), 8.11-7.99 (m, 12H, ArH), 7.85-7.82 (m, 5H, ArH), 7.76-7.74 (m, 2H, ArH), 7.71-7.69 (m, 2H, ArH), 7.61-7.16 (m, 24H, ArH), 7.20-7.16 (m, 3H, ArH), 6.10 (t, 1H, H-4", $J_{4",3"} = J_{4",5"} = 10.2$ Hz), 6.02 (t, 1H, H-4', $J_{4',3'} = J_{4',5'} = 9.9$ Hz), 5.98 (dd, 1H, H-3", $J_{3",2"} = 3.6$ Hz, $J_{3",4"} = 10.2$ Hz), 5.88 (t, 1H, H-4, $J_{4,3} = J_{4,5} = 9.9$ Hz), 5.74 (dd, 1H, H-2, $J_{2,1} = 1.8$ Hz, $J_{2,3} = 3.6$ Hz), 5.73 (dd, 1H, H-2", $J_{2",1"} = 1.8$ Hz, $J_{2",3"} = 3.6$ Hz), 5.71-5.68 (m, 1H, H-3'), 5.37-5.35 (m, 2H, H-1', H-2'), 5.19 (d, 1H, H-1, $J_{1,2} = 1.8$ Hz), 5.15 (d, 1H, H-1", $J_{1",2"} = 1.8$ Hz), 4.67 (dd, 1H, H-3, $J_{3,2} = 3.6$ Hz, $J_{3,4} = 9.9$ Hz), 4.62-4.56 (m, 2H, H-6'', H-6'),

4.53-4.46 (m, 2H, H-5'', H-5'), 4.40-4.32 (m, 3H, H-6", H-6', H-5), 4.18-4.32 (m, 2H, H-6, $O\underline{CH_2}CH_2N_3$), 3.81-3.77 (m, 2H, H-6, $O\underline{CH_2}CH_2N_3$), 3.52-3.55 (m, 2H, $OCH_2\underline{CH_2}N_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.2, 166.07, 166.05, 165.7, 165.5, 165.28, 165.27, 165.2, 164.7, 164.6 (C=O), 133.6, 133.43, 133.40, 133.38, 133.22, 133.20, 133.1, 133.0, 132.9, 132.8, 130.2 (ArCH), 130.01 (ArC), 130.0, 129.82, 129.78, 129.70, 129.69, 129.67, 129.6 (Ar CH), 129.3, 129.20, 129.19, 129.17, 129.07, 128.96 (ArC), 128.9 (ArCH), 128.8 (ArCH), 99.7 (C-1', $J_{C\text{-}H}$ = 173 Hz), 97.7 (C-1, $J_{C\text{-}H}$ = 173 Hz), 97.4 (C-1", $J_{C\text{-}H}$ = 173 Hz), 76.2 (C-3), 71.8 (C-2), 70.4 (C-2"), 70.2 (C-2'), 69.98, 69.96 (C-3", C-5), 69.7 (C-5'), 69.4 (C-3'), 69.0 (C-5"), 68.5 (C-4), 67.2 ($O\underline{CH_2}CH_2N_3$), 66.8 (C-6), 66.7 (C-4"), 66.5 (C-4'), 62.7 (C-6'), 62.4 (C-6"), 50.5 ($OCH_2\underline{CH_2}N_3$). ESMS found for $C_{90}H_{75}N_3O_{26}Na$: 1637.4.

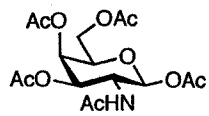
2-Aminoethyl 3,6-di-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (1)



A solution of **2** (4.12 g, 2.55 mmol) was made in dry MeOH (60 mL) and 0.1 N NaOMe (0.50 mL, 0.050 mmol) was added to it. Stirring was continued for 6 h at rt. After evaporation of the solvent, the crude was dissolved in EtOH and 10% Pd-C (0.15 g) was added to it. Hydrogenation was carried out for 16 h. The carbon was removed by filtration over celite, which was thoroughly washed with MeOH (15 mL). After

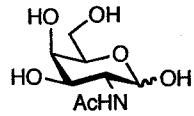
evaporation of the solvent, the residue was washed with chloroform (3 × 15 mL) to remove any methyl benzoate. $[\alpha]_D^{22}$ + 74.43 (*c*, 0.77, H_2O); ^1H NMR (500 MHz, D_2O) δ 5.11 (d, 1H, H-1, $J_{1,2}$ = 1.5 Hz), 4.90 (d, 1H, H-1, $J_{1,2}$ = 1.5 Hz), 4.86 (d, 1H, H-1, $J_{1,2}$ = 1.5 Hz), 4.14 (brd, 1H, H-2), 4.07-4.06 (m, 1H, H-2), 4.04 -3.97 (m, 2H), 3.97-3.61 (m, 18H); ^{13}C NMR (125 MHz, D_2O) δ 103.2, 100.9, 100.2, 79.3, 74.2, 73.5, 72.0, 71.4, 71.2, 70.8, 70.7, 70.4, 67.6, 67.5, 66.4, 66.0, 61.9, 61.8; HR-ESMS calcd for $C_{20}H_{35}N_3O_{16}Na$: 596.1915. Found: 596.1914.

2-Acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (18)



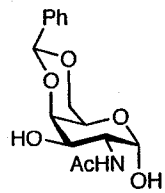
To a stirring solution of galactosamine (8.19 g, 21.0 mmol) in acetic anhydride (30 mL) was added pyridine (130 mL) at 0°C. Stirring was carried out for 12 h at rt. The solvents were removed under reduced pressure by co-evaporation with toluene (3 × 20 mL). The crude residue was recrystallised from methanol to give the title compound as a white solid (13.3 g, 34.2 mmol, 90%). ^1H NMR (400 MHz, $CDCl_3$) δ 5.68 (d, 1H, H-1, $J_{1,2}$ = 8.8 Hz), 5.36 - 5.32 (m, 2H, H-4,NH), 5.06 (dd, 1H, H-3, $J_{3,4}$ = 3.2 Hz, $J_{3,2}$ = 11.2 Hz), 4.43 (dt, 1H, H-2, $J_{2,1} = J_{2,NH}$ = 9.2 Hz, $J_{2,3}$ = 11.2 Hz), 4.20-4.06 (m, 2H, H-6a, H-6b), 4.00 (dt, 1H, H-5, $J_{5,4}$ = 1.0 Hz, $J_{5,6a} = J_{5,6b}$ = 6.6 Hz), 2.15, 2.11, 2.03, 2.00, 1.92 ($CO\underline{CH_3}$), 1.54 ($NHCO\underline{CH_3}$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.6, 170.2, 170.0, 169.4 (C=O), 93.1 (C-1), 71.9 (C-5), 70.3 (C-3), 66.4 (C-4), 61.3 (C-6), 49.9 (C-2), 23.4, 20.97, 20.74, 20.71 ($CO\underline{CH_3}$). HR-ESMS calcd for $C_{16}H_{23}NO_{10}Na$: 412.1220. Found: 412.1224.

2-Acetamido-2-deoxy-α,β-D-galactopyranose (19)



A freshly prepared solution of sodium methoxide (0.5N, 6 mL) was added to a stirring solution of 2-acetamido-2-deoxy-3,4,6-tetra-O-acetyl-α-D-galactopyranoside (**18**) (13.2 g, 33.9 mmol) in dry methanol (25 mL) at rt. When TLC analysis showed completion of reaction, after 30 min, Amberlite-H^+ was added to neutralize the solution mixture. After filtration, the solvent was removed under reduced pressure to yield 2-acetamido-2-deoxy-α,β-D-galactopyranoside **19** (α:β = 4:1) as a white solid (7.46 g, 33.7 mmol, 99%). ^{1}H NMR (500 MHz, CD_3OD) δ (α anomer) 5.32 (d, NHCOCH$_3$, J = 9.6 Hz), 5.12 (d, 1H, H-1, $J_{1,2}$ = 3.5 Hz), 4.20 (dd, 1H, H-2, $J_{2,1}$ = 3.5 Hz, $J_{2,3}$ = 11.0 Hz), 4.04-4.00 (m, H-5), 3.89 (dd, 1H, H-4, $J_{4,5}$ = 1.0 Hz, $J_{4,3}$ = 3.0 Hz), 3.80 (dd, 1H, H-3, $J_{3,4}$ = 3.0 Hz, $J_{3,2}$ = 11.0 Hz), 3.71-3.68 (m, 2H, H-6a, H-6b), 1.98 (s, 3H, NHCOCH$_3$); ^{13}C NMR (125 MHz, CD_3OD) δ 92.87 (C-1), 71.7 (C-5), 70.5 (C-4), 69.7 (C-3), 62.8 (C-6), 52.1 (C-2), 22.7 (COCH$_3$). HR-ESMS calcd for $C_8H_{15}NO_6Na$: 244.0797. Found: 244.0756.

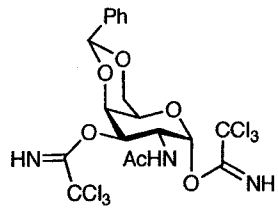
2-Acetamido-2-deoxy-4,6-*O*-benzylidene-α-D-galactopyranose (20)



The following experiment was adapted from a known procedure.[49] 2-Acetamido-2-deoxy-α-D-galactopyranose (**19**) (1.40 g, 6.33 mmol) was dissolved in dry acetonitrile

(15 mL) and camphor sulfonic acid (0.088 g, 38 mmol) was added to the stirred solution, followed by benzaldehyde dimethyl acetal (285 mL, 19 mmol). The mixture was stirred for 2 h and neutralized with triethylamine. The solvent was removed under reduced pressure and the residue recrystallised from 90% ethanol to afford **20** (1.65 g, 5.33 mmol, 84%) as a white solid (α anomer). ^{1}H NMR (500 MHz, $CDCl_3$ + 5% CD_3OD) δ 7.55-7.49 (m, 2H, Ar), 7.41-7.33 (m, 3H, Ar), 5.53 (s, 1H, PhCH), 5.19 (d, 1H, H-1, $J_{1,2}$ = 3.0 Hz), 4.27 (dd, 1H, H-2, $J_{2,1}$ = 3.0 Hz, $J_{2,3}$ = 10.5 Hz), 4.20-4.00 (m, 3H, H-5, H-6a, H-4), 3.90-3.75 (m, 2H, H-6b, H-3), 1.97 (s, 3H, NHCO$\underline{CH_3}$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.2 (C=O), 137.7 (Ar C), 128.8, 127.9, 126.3 (Ar CH), 100.9 ($C_6H_5\underline{C}H$), 92.1 (C-1), 75.8 (C-5), 69.6 (C-6), 68.3, 62.3 (C-4, C-3), 51.0 (C-2), 23.4 (CO$\underline{CH_3}$). HR-ESMS calcd for $C_{15}H_{19}NO_6Na$: 332.1110. Found: 332.1110.

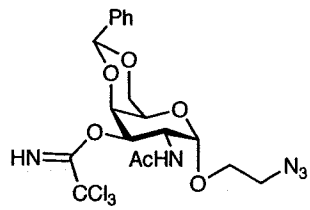
2-Acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-trichloroacetimidato-α-D-galactopyranosyl trichloroacetimidate (21)



The following experiment was adapted from a known procedure.[49] Compound **20** (5.55 g, 17.9 mmol) was dissolved in dry dichloromethane (30 mL) under argon at -20 °C, and in the presence of 4Å molecular sieves. To this stirred solution was added trichloroacetonitrile (43 mL, 0.43 mol, 24 equiv) followed by DBU (1.5 mL, 0.010 mol, 0.54 equiv). When the solution became clear after 1 h, the solvents were removed under reduced pressure. The resulting residue was purified by gradient silica gel

chromatography using ethyl acetate:hexanes (0:1→1:1, v/v with 1% Et_3N) to give the title compound as a white solid (9.74 g, 16.3 mmol, 90%). ^1H NMR (400 MHz, $CDCl_3$) δ 8.73 (s, 1H, NHCCl$_3$), 8.47 (s, 1H, NHCCl$_3$), 7.52-7.49 (m, 2H, Ar), 7.37-7.32 (m, 3H, Ar), 6.54 (d, 1H, H-1, $J_{1,2}$ = 3.2 Hz), 5.69 (d, NHCOCH$_3$, J = 8.4 Hz), 5.59 (s, 1H, PhCH), 5.38 (dd, 1H, H-3, $J_{3,4}$ = 3.2 Hz, $J_{3,2}$ = 11.6 Hz), 5.12 (ddd, 1H, H-2, $J_{2,1}$ = 3.2 Hz, $J_{2,NH}$ = 8.8 Hz, $J_{2,3}$ = 11.6 Hz), 4.67 (dd, 1H, H-4, $J_{4,5}$ = 0.5 Hz, $J_{4,3}$ = 3.2 Hz), 4.35 (dd, 1H, H-6a, $J_{6a,5}$ = 1.4 Hz, $J_{6a,6b}$ = 12.6 Hz), 4.07 (dd, 1H, H-6b, $J_{6b,5}$ = 1.8 Hz, $J_{6b,6a}$ = 12.6 Hz), 3.94 (m, 1H, H-5), 1.90 (s, 3H, NHCOCH$_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.6 (C=O), 163.2, 160.3 (C=N), 137.2 (Ar C), 128.9, 128.1, 126.0 (5 Ar CH), 100.5 (C_6H_5CH), 96.6 (C-1, J_{CH} = 181), 91.2, 90.8 (CCl_3), 73.4 (C-3), 72.0 (C-4), 68.9 (C-6), 64.9 (C-5), 47.6 (C-2), 23.2 (COCH$_3$). HR-ESMS calcd for $C_{19}H_{19}N_3O_6NaCl_6$: 617.9303. Found: 617.9309.

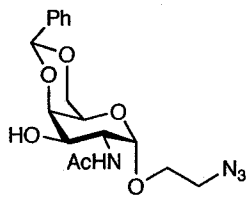
2-Azidoethyl 2-acetamido-2-deoxy-4,6-*O*-benzylidene-3-*O*-trichloroacetimidato-α-D-galactopyranoside (22)



To a homogeneous solution of **21** (0.23 g, 0.38 mmol) in dry THF (1 mL), at –20 °C, under argon, was added 2-azidoethanol (0.045 mL, 0.76 mol, 2 equiv), followed by BF_3OEt_2 (15 mL, 0.038 mmol, 0.1 equiv). Stirring was continued for 15 min and then on disappearance of the donor, triethylamine was added to neutralize the mixture. After evaporation, the residue was purified by silica gel column chromatography using as

eluant, ethyl acetate:hexanes (1:1, v/v with 1% Et_3N), to afford the title compound (0.12 g, 0.23 mmol, 61%) as white crystals. ^1H NMR (500 MHz, $CDCl_3$) δ 8.41 (s, 1H, C=NH), 7.50-7.49 (m, 2H, Ar), 7.33-7.31 (m, 3H, Ar), 5.78 (d, 1H, NHCOCH$_3$, $J_{NH,2}$ = 9.0 Hz), 5.57 (s, 1H, C_6H_5CH), 5.25 (dd, 1H, H-3, $J_{3,4}$ = 3.5 Hz, $J_{3,2}$ = 11.5 Hz), 5.06 (d, 1H, H-1, $J_{1,2}$ = 3.5 Hz), 4.95 (ddd, 1H, H-2, $J_{2,1}$ = 3.5 Hz, $J_{2,NH}$ = 9.0 Hz, $J_{2,3}$ = 11.5 Hz), 4.60 (br d, 1H, H-4, $J_{4,3}$ = 3.5 Hz), 4.28 (dd, 1H, H-6a, $J_{6a,5}$ = 1.5 Hz, $J_{6a,6b}$ = 12.5 Hz), 4.10-4.07 (m, 1H, H-6b), 3.92 (ddd, 1H, $OCH_2CH_2N_3$, J = 3, 6, 9 Hz), 3.78 (br s, 1H, H-5), 3.67 (ddd, 1H, $OCH_2CH_2N_3$, J = 3, 7.5, 10.5 Hz), 3.46 (ddd, 1H, $OCH_2CH_2N_3$, J = 3, 7.5, 10.5 Hz), 3.36 (ddd, 1H, $OCH_2CH_2N_3$, J = 3, 6.5, 9.5 Hz), 1.92 (s, 3H, NHCOCH$_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.8 (C=O), 163.0 (C=N), 137.4 (Ar C), 128.8, 128.1, 126.0 (Ar CH), 100.4 (C_6H_5CH), 98.7 (C-1, J_{CH} = 173 Hz), 91.0 (CCl_3), 73.7 (C-3), 72.2 (C-4), 69.2 (C-6), 67.4 ($OCH_2CH_2N_3$), 62.9 (C-5), 50.5 ($OCH_2CH_2N_3$), 47.5 (C-2), 23.3 ($COCH_3$). HR-ESMS calcd for $C_{19}H_{22}N_5O_6NaCl_3$: 544.0534. Found: 544.0509.

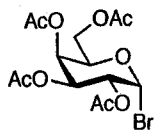
2-Azidoethyl 2-acetamido-2-deoxy- 4,6-*O*-benzylidene-α-D-galactopyranoside (15)



2-Azidoethyl 2-acetamido-2-deoxy-4,6-*O*-benzylidene-3-*O*-trichloroacetimidate-α-D-galactopyranoside (**22**) (0.11 g, 0.21 mmol) was dissolved in 80% acetic acid (2 mL) and stirred at rt for 45 min. On completion of reaction, the solvent was removed by co-evaporation with toluene (2 × 10 mL). Purification by silica gel column chromatography (dichloromethane:acetone, 8:1 → 6:1, v/v) then yielded **15** (0.076 g, 0.20 mmol, 95%)

as a white solid. $[\alpha]_D^{22}$ + 164.8 (*c*, 0.43, CH_3CN); ^1H NMR (500 MHz, $CDCl_3$) δ 7.50-7.48 (m, 2H, Ar), 7.38-7.33 (m, 3H, Ar), 5.80 (d, $\underline{NH}COCH_3$, $J_{NH,2}$ = 8.5 Hz), 5.56 (s, 1H, PhCH), 5.00 (d, 1H, H-1, $J_{1,2}$ = 3.5 Hz), 4.46 (ddd, 1H, H-2, $J_{2,1}$ = 3.5 Hz, $J_{2,NH}$ = 9.0 Hz, $J_{2,3}$ = 11.0 Hz), 4.25 (dd, 1H, H-6a, $J_{6a,5}$ = 1.5 Hz, $J_{6a,6b}$= 12.5 Hz), 4.23 (br d, 1H, H-4, $J_{4,3}$ = 3.0 Hz), 4.06 (dd, 1H, H-6b, $J_{6b,5}$ = 1.5 Hz, $J_{6b,6a}$= 12.5 Hz), 3.92 (ddd, 1H, $O\underline{CH_2}CH_2N_3$, *J* = 3.0, 7.5, 10.0 Hz), 3.85 (dd, 1H, H-3, $J_{3,4}$ = 3.0 Hz, $J_{3,2}$ = 11.0 Hz), 3.70 (m, 1H, H-5), 3.65 (ddd, 1H, $O\underline{CH_2}CH_2N_3$, *J* = 3.0, 7.5, 10.5 Hz), 3.44 (ddd, 1H, $OCH_2\underline{CH_2}N_3$, *J* = 3, 7.0, 10.0 Hz), 3.36 (ddd, 1H, $OCH_2\underline{CH_2}N_3$, *J* = 2.5, 6.0, 9.0 Hz), 2.01 (s, 3H, $CO\underline{CH_3}$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.2 (C=O), 137.3 (Ar C), 129.1, 128.2, 126.3 (Ar CH), 101.3 ($C_6H_5\underline{CH}$), 98.7 (C-1, J_{CH} = 174 Hz), 75.4 (C-4), 69.3 (C-6), 68.7 (C-3), 63.3 (C-5), 67.4 ($O\underline{CH_2}CH_2N_3$), 50.6 ($OCH_2\underline{CH_2}N_3$), 50.3 (C-2), 23.4 ($CO\underline{CH_3}$). HR-ESMS calcd for $C_{17}H_{22}N_4O_6Na$: 401.1437. Found: 401.1439.

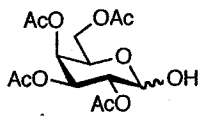
2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide (16)



It was made from a literature procedure[60] and was used without further purification. HBr (18 mL, 33% in glacial acetic acid) was added to a cooled solution of 1,2,3,4,6-penta-*O*-acetyl-galactopyranoside **23** (3.31 g, 8.48 mmol) in dry dichloromethane (6 mL) at 0 °C. Stirring was continued for 4 h and the acid removed by co-evaporation with toluene (2 x 20 mL). The residue was dissolved in dry DCM (30 mL) and added dropwise to ice water (20 mL). The organic layer was separated and the aqueous portion was extracted with dichloromethane (4 x 30 mL). The combined organic extracts were

washed with saturated sodium bicarbonate (3 x 100 mL) and brine (2 x 80 mL) and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure yielded **3** as a white solid (3.47 g, 8.44 mmol, 99.5%) which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.61 (d, 1H, H-1, $J_{1,2}$ = 4.0 Hz), 5.42 (dd, 1H, H-4, $J_{4,5}$ = 1.2 Hz, $J_{4,3}$ = 3.3 Hz), 5.30 (dd, 1H, H-3, $J_{3,4}$ = 3.3 Hz, $J_{3,2}$ = 10.4 Hz), 4.95 (dd, 1H, H-2, $J_{2,1}$ = 4.0 Hz, $J_{2,3}$ = 10.4 Hz), 4.40 (apparent t, 1H, H-5, $J_{5,6}$ = 6.8 Hz), 4.10 (dd, 1H, H-6a, $J_{6a,5}$ = 6.4 Hz, $J_{6a,6b}$= 11.6 Hz), 4.01 (dd, 1H, H-6b, $J_{6b,5}$ = 6.8 Hz, $J_{6b,6a}$= 11.6 Hz), 2.1, 2.01, 1.96, 1.91 (s, 3H, CO$\underline{CH_3}$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2, 169.9, 169.8, 169.6 (C=O), 88.1 (C-1, $J_{C\text{-}H}$ = 187 Hz), 71.0 (C-5), 67.9 (C-3), 67.7 (C-2), 66.9 (C-4), 60.8 (C-6), 20.6, 20.53, 20.47, 20.45 (CO$\underline{CH_3}$). HR-ESMS calcd for $C_{14}H_{19}O_9NaBr$: 433.0110. Found: 433.0110.

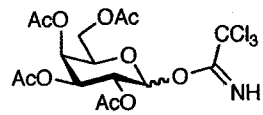
2,3,4,6-Tetra-*O*-acetyl-α,β-D-galactopyranose (27)



To a solution of 2,3,4,6 penta-*O*-acetyl-galactopyranoside (**23**) (0.25 g, 0.64 mmol) in DMF (1mL), was added hydrazine acetate (0.071 g, 0.77 mmol). The mixture was stirred for 20 mins at 55 °C until TLC indicated the disappearance of the starting material. The mixture was then cooled to room temperature and diluted with ethyl acetate (30 mL), which was then washed with brine (2 × 15 mL). The organic layer was then dried over anhydrous sodium sulfate and evaporated to give the title product as a mixture of anomers (α/β, 2/1) as a white solid (0.21 g, 0.60 mmol, 94%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.47 (d, 1H, H-1, $J_{1\alpha,2\alpha}$ = 3.5 Hz), 5.45-5.41 (d, 1H, H-4α, $J_{4\alpha,3\alpha}$ = 3.0

Hz), 5.40-5.32 (m, 2H, H-3α, H-4β), 5.10 (dd, 1H, H-2α, $J_{2\alpha,1\alpha}$ = 3.5 Hz, $J_{2,3}$ = 10.5 Hz), 5.08-5.00 (m, 2H, H-3β, H-2β), 4.68 (d, 1H, H-1β, $J_{1\beta,2\beta}$ = 7.0 Hz), 4.44 (t, 1H, H-5α, $J_{5\alpha,6\alpha}$ = 6.5 Hz), 4.14-4.02 (m, 4H, H-6α, H-6β), 3.94 (t, 1H, H-5β, $J_{5\beta,6\beta}$ = 6.5 Hz), 2.13, 2.11, 2.06, 2.02, 2.01, 1.96 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8, 170.62, 170.58, 170.5, 170.3, 170.11, 170.07 (C=O), 95.8 (C-1β), 90.50 (C-1α), 70.9 70.8 (C-5β, C-3α), 70.5 (C-3β), 68.4 (C-2α), 68.2 (C-4α), 67.3 (C-4β), 67.1 (C-2β), 66.0 (C-5α), 61.7 (C-6β), 61.4 (C-6α), 20.7, 20.61, 20.58, 20.56, 20.53, 20.47 ($COCH_3$). HR-ESMS calcd for $C_{14}H_{20}O_{10}Na$: 371.0949. Found: 371.0954.

2,3,4,6-Tetra-*O*-acetyl-α- and-β-D-galactopyranosyl trichloroacetimidate (28)



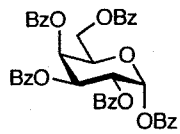
A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranose (**27**) (0.33 g, 0.95 mmol) in dry dichloromethane was stirred for 2 h at rt in the presence of trichloroacetonitrile (0.9 mL) and sodium (0.035 g, 1.52 mmol). After completion of reaction, the mixture was filtered over celite and concentrated. The residue was eluted from a column of silica gel using ethyl acetate:hexanes (0:1→1:1) to give two fractions.

The first fraction, a white solid, was the α-anomer (0.15 g, 0.30 mmol, 32%). ^1H NMR (500 MHz, $CDCl_3$) δ 8.64 (s, 1H, NH), 6.58 (d, 1H, H-1, $J_{1,2}$ = 3.5 Hz), 5.54-5.53 (m, 1H, H-4), 5.40 (dd, 1H, H-3, $J_{3,4}$ = 3.0 Hz, $J_{3,2}$ = 11.0 Hz), 5.34 (dd, 1H, H-2, $J_{2,1}$ = 3.5 Hz, $J_{2,3}$ = 11.0 Hz), 4.41 (t, 1H, H-5, $J_{5,6a}$ = $J_{5,6b}$ = 6.5 Hz), 4.14 (dd, 1H, H-6a, $J_{6a,5}$ = 6.5 Hz, $J_{6a,6b}$= 11.5 Hz), 5.06 (dd, 1H, H-6b, $J_{6b,5}$ = 6.5 Hz, $J_{6b,6a}$= 11.5 Hz), 2.16 , 2.02,

2.00, 1.99 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2, 170.02, 170.0, 169.9 (C=O), 160.9 (C=N), 93.5 (C-1), 90.8 (CCl_3), 68.9 (C-5), 67.5 (C-3), 67.4 (C-4), 66.9 (C-2), 61.2 (C-6), 20.60, 20.57, 20.54, 20.48 (CH_3). HR-ESMS calcd for $C_{16}H_{20}NO_{10}NaCl_3$: 514.0045. Found: 514.0050.

The second fraction was the β-anomer which was a white solid (0.19 g, 0.39 mmol, 41%). ^1H NMR (500 MHz, $CDCl_3$) δ 8.69 (s, 1H, NH), 5.80 (d, 1H, H-1, $J_{1,2}$ = 3.5 Hz), 5.46-5.41 (m, 2H, H-2, H-4), 5.09 (dd, 1H, H-3, $J_{3,4}$ = 3.5 Hz, $J_{3,2}$ = 10.5 Hz), 4.16-4.14 (m, 2H, H-6a, H-6b), 4.09-4.06 (m, 1H, H-5), 2.14 , 2.00, 1.98, 1.96 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2, 170.1, 169.92, 169.0 (C=O), 161.0 (C=N), 96.0 (C-1), 90.8 (CCl_3), 71.7 (C-5), 70.7 (C-3), 67.7, 66.7 (C-4, C-2), 60.8 (C-6), 20.60, 20.58, 20.5 (CH_3). HR-ESMS calcd for $C_{16}H_{20}NO_{10}NaCl_3$: 514.0045. Found: 514.0050.

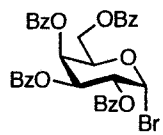
1,2,3,4,6-Penta-*O*-benzoyl-α-D-galactopyranoside (24)



To a solution of D-galactose (0.57 g, 3.15 mmol) in dry dichloromethane was added pyridine (1.9 mL), followed by benzoyl chloride (4.60 mL, 39.6 mmmol) at 0 °C. The mixture was allowed to stir at rt for 4 h. The mixture was co-evaporated thrice with toluene and the crude was diluted with dichloromethane (60 mL). The organic layer was washed with 2% aqueous hydrochloric acid (3 × 25 mL), water (2 × 20 mL), saturated sodium hydrogencarbonate ((3 × 25 mL) and brine (2 × 20 mL). After drying over anhydrous sodium sulphate, filtration and evaporation of the solvents, the crude

was purified by colum chromatography (eluant, EtOAc:hexane, 1:4) to give the title compound as a white solid (2.14 g. 3.05 mmol, 97%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.20-7.10 (m, 25H, Ar), 6.95 (d, 1H, H-1, $J_{1,2}$= 3.5 Hz), 6.19 (dd, 1H, H-4, $J_{4,5}$= 1.0, $J_{4,3}$= 3.5 Hz), 6.12 (dd, 1H, H-3, $J_{3,4}$= 3.5 Hz, $J_{3,2}$= 10.5 Hz), 6.03 (dd, 1H, H-2, $J_{2,1}$ = 4.0 Hz, $J_{2,3}$ = 11.0 Hz), 4.83 (br t, 1H, H-5, $J_{5,6a}$= $J_{5,6b}$ = 6.5 Hz), 4.63 (dd, 1H, H-6a, $J_{6b,5}$ = 6.5 Hz, $J_{6a,6b}$= 11.5 Hz), 4.42 (dd, 1H, H-6b, $J_{6b,5}$ = 6.5 Hz, $J_{6a,6b}$= 11.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.9, 165.7, 165. 53, 165.46, 164.50 (C=O), 133.9, 133.7, 133.44, 133.36, 133.2 (Ar CH), 130.0, 129.9, 129.7 (Ar CH), 129.3, 129.0, 128.97, 128.84 (Ar C), 128.76, 128.7, 128.44, 128.38, 128.3 (Ar CH), 90.7 (C-1), 69.5 (C-5), 68.54, 68.48 (C-3, C-4), 67.7 (C-2), 61.8 (C-6). HR-ESMS calcd for $C_{41}H_{32}O_{11}Na$: 723.1842. Found: 723.1838.

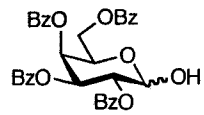
2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide (25)



To a solution of 1,2,3,4,6-penta-*O*-benzoyl-α-D-galactopyranoside (**24**) (3.02 g, 4.31 mmol) in dry dichloromethane (3 mL) and acetic amhydride (0.2 mL) was added 33% HBr in glacial acetic at 0°C. The solution mixture was allowed to stir for 2 h at rt until completion. The mixture was cooled and added to an ice-cold solution of saturated sodium bicarbonate (30 mL) in dichloromethane (60 mL) which was stirred for 30 min. The mixture was separated into two layers, and the organic layer was washed with ice-cold water (40 mL), saturated sodium bicarbonate (2 × 30 mL) and brine (30 mL). After drying the organic layer over anhydrous sodium sulfate and filtering the mixture,

the solvent was removed under vacuum to give the bromide as white crystals (2.73 g, 4.14 mmol, 96%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07-7.96 (m, 6H, Ar), 7.79-7.76 (m, 2H, Ar), 7.64-7.59 (m, 2H, Ar), 7.56-7.36 (m, 10H, Ar), 7.47-7.20 (m, 1H, Ar), 6.96 (d, 1H, H-1, $J_{1,2}$= 4.0 Hz), 6.10 (br d, 1H, H-4, $J_{4,3}$= 3.5 Hz), 6.04 (dd, 1H, H-3, $J_{3,4}$ = 3.5 Hz, $J_{3,2}$ = 10.5 Hz), 5.65 (dd, 1H, H-2, $J_{2,1}$ = 4.0 Hz, $J_{2,3}$ = 10.5 Hz), 4.91 (dd, 1H, H-5, $J_{5,6a}$ = $J_{5,6b}$ = 6.8 Hz), 4.62 (dd, 1H, H-6a, $J_{6b,5}$ = 6.5 Hz, $J_{6a,6b}$ = 11.5 Hz), 4.45 (dd, 1H, H-6b, $J_{6b,5}$ = 6.0 Hz, $J_{6a,6b}$= 11.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.9, 165.5, 165.31, 165.27 (C=O), 133.7, 133.33, 133.28, 130.0, 129.9, 129.8, 129.7 (Ar CH), 129.2, 128.8 (Ar C), 128.7 (Ar CH), 128.54 (Ar C), 128.52 (Ar CH), 128.4, 128.3 (Ar CH), 88.3 (C-1), 71.8 (C-5), 68.9, 68.6 (C-2, C-3), 68.1 (C-4), 61.6 (C-6). HR-ESMS calcd for $C_{34}H_{27}O_9NaBr$: 681.0736. Found: 681.0741.

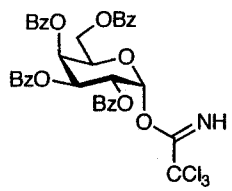
2,3,4,6-Tetra-*O*-benzoyl-α,β-D-galactopyranose (29)



To a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**25**) (0.44 g, 0.66 mmol) in 4:1 acetone-water (10 mL), was added silver carbonate (0.55 g, 1.99 mmol). The solution mixture was stirred for 30 min until completion of reaction. The suspension was then filtered through celite, and the filtrate was concentrated to give the hemiacetal **29** as a white amorphous solid (0.40 g, 0.66 mmol, quantitative yield) which was a mixture of the α/β (1: 3.5) anomers. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.10-8.05 (m, 3 H, Ar), 8.03-7.92 (m, 5 H, Ar), 7.80-7.74(m, 3 H, Ar), 7.60 (t, 1H, Ar, J = 7.5 Hz), 7.56-7.32 (m, 13 H, Ar), 7.36-7.18 (m, 1H, Ar), 6.08-6.04 (m, 2H, H-3α, H-4α),

5.99 (dd, 1H, H-4β, $J_{4\beta,\,5\beta}$ = 0.8 Hz, $J_{4\beta,\,3\beta}$ = 3.5 Hz), 5.84 (t, 1H, H-1α, $J_{1\alpha,2\alpha} = J_{1\alpha,OH}$ = 3 Hz), 5.72 (dd, 1H, H-3α, $J_{3\alpha,\,4\alpha}$ = 3.5 Hz, $J_{3\alpha,\,2\alpha}$ = 10.5 Hz), 5.69-5.67 (m, 1H, H-2α), 5.62 (dd, 1H, H-2β, $J_{2\beta,\,1\beta}$ = 8.2 Hz, $J_{2\beta,\,3\beta}$ = 10.5 Hz), 5.05 (t, 1H, H-1β, $J_{1\beta,2\beta} = J_{1\beta,OH}$ = 8.2 Hz), 4.85 (t, 1H, H-5α, $J_{5\alpha,6a\alpha} = J_{5\alpha,6b\alpha}$ = 6.5 Hz), 4.66 (dd, 1H, H-6aβ, $J_{6a\beta,\,5\beta}$ = 6.8 Hz, $J_{6a\beta,\,6b\beta}$ = 11.5 Hz), 4.60 (dd, 1H, H-6aα, $J_{6a\alpha,\,5\alpha}$ = 6.5 Hz, $J_{6a\alpha,\,6b\alpha}$ = 11.0 Hz), 4.43 (dd, 1H, H-6bβ, $J_{6b\beta,\,5\beta}$ = 6.5 Hz, $J_{6b\beta,\,6a\beta}$ = 11.0 Hz), 4.40-4.34 (H-6α, H-5β), 4.08 (br d, 1H, $J_{1,OH}$ = 8.2 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.0, 166.11, 166.08, 166.04, 165.58, 165.56, 165.49, 165.47, 133.64, 133.62, 133.5, 133.4, 133.33, 133.28, 133.2, 133.1, 129.96, 129.91, 129.9, 129.82, 129.80, 129.77, 129.73, 129.7 (Ar CH), 129.4, 129.3, 129.2, 129.13, 129.11, 129.0, 128.8, 128.7 (Ar C), 128.64, 128.61, 128.45, 128.42, 128.38, 128.3, 128.2 (Ar CH), 96.3 (C-1β), 91.1 (C-1α), 72.4 (C-2β), 71.6 (C-5β), 71.0 (C-3β), 69.5 (C-2α), 69.3 (C-3α), 68.2, 68.0 (C-4β, C-4α), 66.9 (C-5α), 62.4 (C-6α), 62.1 (C-6β). HR-ESMS calcd for $C_{34}H_{28}O_{10}Na$: 619.1580. Found: 619.1584.

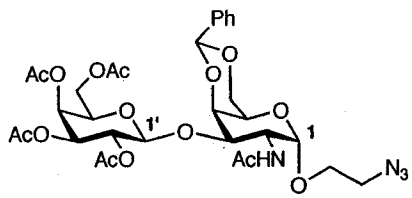
2,3,4,6-Tetra-*O*-benzoyl-α-D-galactopyranosyl trichloroacetimidate (30)



To a stirred solution of 2,3,4,6-Tetra-*O*-benzoyl-α,β-D-galactopyranose (**29**) (0.29 g, 0.49 mmol) in dry dichloromethane (3.5 mL) was added trichlotoacetonitrile (1.75 mL, 17 mmol) followed by DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) (0.073 mL, 0.49 mmol) at 0°C . The mixture was stirred for 30 min and evaporated. The residue was purified by column chromatography using ethyl acetate:hexanes (19:1) and 0.1% Et_3N.

Compound **30** was obtained as white solid (0.17 g, 0.23 mmol, 47%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.61 (s, 1H, C=NH), 8.07 (dd, 2H, Ar, J = 0.8, 8.0 Hz), 7.98-7.92 (m, 4H, Ar), 7.80-7.76 (m, 2H, Ar), 7.59-7.64 (m, 1H, Ar), 7.32-7.55 (m, 10H, Ar), 7.22-7.27 (m, 1H, Ar), 6.90 (d, 1H, H-1, $J_{1,2}$ = 3.5 Hz), 6.14 (dd, 1H, H-4, $J_{4,5}$ = 1.0 Hz, $J_{3,5}$ = 3.5 Hz), 6.06 (dd, 1H, H-3, $J_{3,4}$ = 3.5 Hz, $J_{3,2}$ = 11.0 Hz), 5.94 (dd, 1H, H-2, $J_{2,1}$ = 3.5 Hz, $J_{2,3}$ = 11.0 Hz), 4.85 (dd, 1H, H-5, $J_{5,6a}$ = 7.0 Hz, $J_{5,6b}$ = 6.0 Hz), 4.59 (dd, 1H, H-6a, $J_{6a,5}$ = 7.0 Hz, $J_{6a,6b}$ = 11.5 Hz), 4.42 (dd, 1H, H-6b, $J_{6b,5}$ = 6.0 Hz, $J_{6a,6b}$ = 11.5 Hz; ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.9, 165.6, 165.5, 165.4 (C=O), 160.6 (C=NH), 133.7, 133.5, 133.3, 133.2, 130.01, 129.99, 129.93, 129.9, 129.8, 129.73, 129.69, 129.67 (Ar CH), 129.4 (Ar C), 128.93, 128.86 (Ar CH), 128.69 (Ar CH), 128.43, 128.40, 128.36, 128.3 (Ar CH), 93.8 (C-1, $J_{C\text{-}H}$ = 181 Hz), 90.8 ($\underline{C}Cl_3$), 69.7 (C-5), 68.5 (C-4), 68.4 (C-3), 67.9 (C-2), 62.2 (C-6). HR-ESMS calcd for $C_{36}H_{28}NO_{10}NaCl_3$: 762.0676. Found: 762.0672.

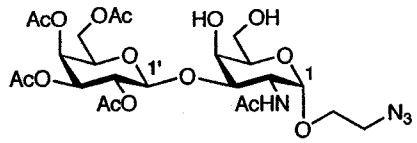
2-Azidoethyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-4,6-O-benzylidene-α-D-galactopyranoside (14)



The preparation of **14** was a modified procedure from Lemieux *et. al.*[67] A solution of tetra-*O*-acetyl-galatopyranosyl bromide (**16**) (2.27 g, 5.52 mmol) in dry toluene (15 mL) was added to a stirred mixture of the donor **15** (1.71 g, 4.52 mmol), mercuric cyanide (1.30 g, 5.51 mmol) and molecular sieves 4Å in a 1:1 (v/v) toluene:nitromethane

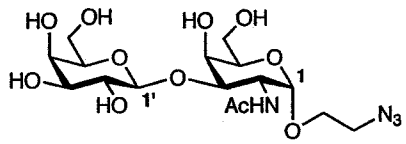
solution mixture (98 mL). The mixture was heated at 70 °C for 5 h. Then, more tetra-*O*-acetyl-galactopyranosyl bromide (**16**) (0.80 g, 2.0 mmol) was added and heating was carried out for 8 h. On disappearance of the donor by TLC analysis, the reaction mixture was cooled to rt and filtered with the residue being washed with dichloromethane (50 mL). The combined washings and organic layer were washed with water (2 × 150 mL), 2M potassium iodide solution (3 × 150 mL), brine (2 × 100 mL), and dried over anhydrous sodium sulfate. After filtration and evaporation, the residue was purified by silica gel column chromatography (eluant, EtOAc:MeOH, 1:0→1:1) to give the disaccharide as a white compound (2.95 g, 4.16 mmol, 92%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53-7.51 (s, 2H, Ar), 7.38-7.30 (m, 3H, Ar), 5.63 (d, 1H, NHCOCH$_3$, $J_{NH,2}$ = 8.5 Hz), 5.54 (s, 1H, PhCH), 5.37 (dd, 1H, H-4', $J_{4',5'}$ = 1.0 Hz, $J_{4',3'}$ = 3.5 Hz), 5.19 (dd, 1H, H-2', $J_{2',1'}$ = 8.0 Hz, $J_{2',3'}$ = 10.5 Hz), 5.11 (d, 1H, H-1, $J_{1,2}$ = 3.5 Hz), 4.97 (dd, 1H, H-3', $J_{3',4'}$ = 3.5 Hz, $J_{3',2'}$ = 10.5 Hz), 4.74 (d, 1H, H-1', $J_{1',2'}$ = 8.0 Hz), 4.66 (ddd, 1H, H-2, $J_{2,1}$ = 3.5 Hz, $J_{2,NH}$ = 8.5 Hz, $J_{2,3}$ = 11.5 Hz), 4.30 (br d, 1H, H-4, $J_{4,3}$ = 3.5 Hz), 4.25 (dd, 1H, H-6a, $J_{6a,5}$ = 2.0 Hz, $J_{6a,6b}$ = 12.5 Hz), 4.14 (m, 2H, H-6'a, H-6'b), 4.06 (dd, 1H, H-6b, $J_{6b,5}$ = 2.0 Hz, $J_{6b,6a}$ = 12.5 Hz), 4.00 (dd, 1H, H-3, $J_{3,4}$ = 3.5 Hz, $J_{3,2}$ = 11.5 Hz), 3.96-3.91 (m, 2H, OCH$_2$CH$_2$N$_3$), 3.89 (m, 1H, H-5'), 3.70-3.65 (m, 1H, H-5), 3.41-3.38 (m, 2H, OCH$_2$CH$_2$N$_3$), 2.14, 2.02, 1.98, 1.96 (s, 3H, COCH$_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.3, 170.2, 170.1, 169.7, 169.5 (C=O), 137.6 (Ar C), 128.9, 128.2, 126.2, 126.0 (Ar CH), 101.0 (PhCH), 100.8 (C-1'), 98.5 (C-1), 75.3 (C-4), 73.6 (C-3), 71.1 (C-5'), 70.9 (C-3'), 69.2 (C-6), 68.8 (C-2'), 67.4 (OCH$_2$CH$_2$N$_3$), 66.9 (C-4'), 63.4 (C-5), 61.4 (C-6'), 50.6 (OCH$_2$CH$_2$N$_3$), 48.2 (C-2), 23.4, 20.68, 20.66, 20.62, 20.52 (COCH$_3$). HR-ESMS calcd for $C_{31}H_{40}N_4O_{15}Na$: 731.2388. Found: 731.2397.

2-Azidoethyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (31)



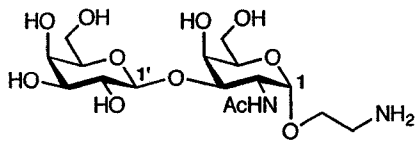
To a solution of 2-azidoethyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-4,6-*O*-benzylidene-α-D-galactopyranoside **14** (2.81 g, 3.97 mmol) in dichloromethane (44 mL), was added 80% acetic acid (15 mL) and the mixture was heated at 50 °C for 2 h. The mixture was then cooled, co-evaporated with toluene (3 × 15 mL). The residue was chromatographed using ethyl acetate:methanol (100:1) to afford the title compound (1.65 g, 2.66 mmol, 67%) as a white solid. $[\alpha]_D^{22}$ + 67.59 (*c*, 0.45, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.60 (d, 1H, N<u>H</u>COCH$_3$, $J_{NH,1}$ = 9.5 Hz), 5.35 (d, 1H, H-4', $J_{4',3'}$ = 3.5 Hz), 5.17 (dd, 1H, H-2', $J_{2',1'}$ = 8.0 Hz, $J_{2',3'}$ = 10.5 Hz), 4.97 (dd, 1H, H-3', $J_{3',4'}$ = 3.5 Hz, $J_{3',2'}$ = 10.5 Hz), 4.92 (d, 1H, H-1, $J_{1,2}$ = 3.5 Hz), 4.62-4.57 (m, 2H, H-1', H-2), 4.16-4.05 (m, 3H, H-6a', H-4, H-6b'), 3.95-3.89 (m, 3H, H-6a, O<u>CH$_2$</u>CH$_2$N$_3$, H-5'), 3.82-3.75 (m, 3H, H-5, H-6b, H-3), 3.64 (ddd, O<u>CH$_2$</u>CH$_2$N$_3$, *J* = 2.5, 8.0, 10.5 Hz), 3.47 (ddd, 1H, OCH$_2$<u>CH$_2$</u>N$_3$, *J* = 2.5, 7.5, 10.0 Hz), 3.32 (ddd, 1H, OCH$_2$<u>CH$_2$</u>N$_3$, *J* = 2.5, 6.0, 10.0 Hz), 2.14 (s, 3H, NHCOCH$_3$), 2.03, 2.026, 1.97, 1.95 (s, 3H, COCH$_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.4, 170.1, 170.1, 169.9 (C=O), 169.4 (NH<u>C</u>=O), 101.6 (C-1'), 98.1 (C-1), 77.8 (C-3), 71.0 (C-5'), 70.6 (C-3'), 69.9 (C-5), 69.2 (C-4), 68.6 (C-2'), 67.2 (O<u>C</u>H$_2$CH$_2$N$_3$), 66.9 (C-4'), 62.7 (C-6), 61.3 (C-6'), 50.5 (OCH$_2$<u>C</u>H$_2$N$_3$), 47.6 (C-2), 23.3 (NHCO<u>C</u>H$_3$), 20.61, 20.58, 20.57, 20.5 (CO<u>C</u>H$_3$). HR-ESMS calcd for $C_{24}H_{36}N_4O_{15}Na$: 643.2075. Found: 643.2072.

2-Azidoethyl (β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside



Sodium methoxide (0.2 N, 0.076 mmol, 0.38 mL) was added to a solution of 2-azidoethyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (1.19 g, 1.92 mmol) in dry methanol, which was stirred for 6 h for complete de-acetylation. Amberlite-H^+ resin was added to neutralize the solution, which was then filtered. After evaporation of the solvent, a white solid (0.83 g, 1.83 mmol, 96%) was obtained. $[\alpha]_D^{22}$ + 78.29 (*c*, 0.26, CH_3OH); ^1H NMR (600 MHz, CD_3OD) δ 4.91 (d, 1H, H-1, $J_{1,2}$ = 3.6 Hz), 4.43 (dd, 1H, H-2, $J_{2,1}$ = 3.6 Hz, $J_{2,3}$ = 11.4 Hz), 4.40 (d, 1H, H-1', $J_{1',2'}$ = 7.8 Hz), 4.18 (dd, 1H, H-4, $J_{4,5}$ = 1.8 Hz, $J_{4,3}$ = 3.0 Hz), 3.92 (dd, 1H, H-3, $J_{3,4}$ = 3.0 Hz, $J_{3,2}$ = 11.4 Hz), 3.90-3.86 (m, 2H, O$\underline{CH_2}CH_2N_3$, H-5), 3.81 (dd, 1H, H-4', $J_{4',5'}$ = 1.2 Hz, $J_{4',3'}$ = 3.6 Hz), 3.76-3.68 (m, 4H, H-6a, H-6b, H-6a', H-6b'), 3.62 (ddd, 1H, O$\underline{CH_2}CH_2N_3$, *J* = 3.6, 6.0, 7.8 Hz), 3.54 (dd, 1H, H-2', $J_{2',1'}$ = 7.8 Hz, $J_{2',3'}$ = 10.2 Hz), 3.52-3.50 (m, 1H, H-5'), 3.47-3.43 (m, 3H, $OCH_2\underline{CH_2}N_3$, H-3'), 1.97 (s, 3H, $NHCOCH_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.2 (CO), 106.4 (C-1', $J_{C\text{-}H}$ =160 Hz), 99.1 (C-1, $J_{C\text{-}H}$ = 171 Hz), 78.9 (C-3), 76.8 (C-5'), 74.8 (C-3'), 72.6 (C-2'), 72.4 (C-5), 70.3 (C-4'), 70.0 (C-4), 67.8 (O$\underline{CH_2}CH_2N_3$), 62.9, 62.5 (C-6, C-6'), 77.8 (C-2), 51.7 ($OCH_2\underline{CH_2}N_3$), 50.2 (C-2), 22.9 ($NHCO\underline{CH_3}$). HR-ESMS calcd for $C_{16}H_{28}N_4O_{11}Na$: 475.1652. Found: 475.1658.

2-Aminoethyl (β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (13)

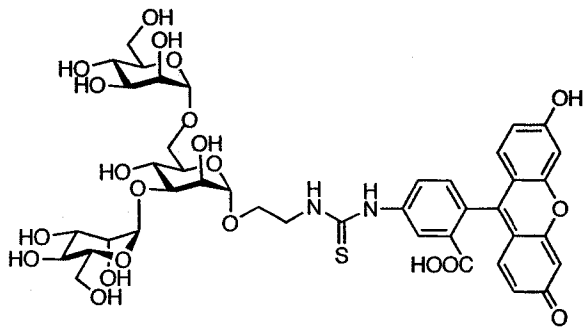


To a solution of 2-azidoethyl (β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (0.81g, 1.79 mmol) in a mixture of MeOH (20 mL) and water (2 mL) was added Pd-C (10% , 0.1 g) and hydrogenation was carried out with vigorous stirring overnight for 18 h. The palladium was removed by filtering the mixture through celite, which was subsequently washed with methanol (10 mL). After evaporating off the solvents under reduced pressure, the disaccharide **13** was produced in quantitative yield as a white solid (0.76 g, 1.79 mmol). $[\alpha]_D^{22}$ + 108.58 (*c*, 0.50, CH_3OH); ^1H NMR (600 MHz, CD_3OD) δ 4.81 (d, 1H, H-1, $J_{1,2}$ = 3.6 Hz), 4.46 (dd, 1H, H-2, $J_{2,1}$ = 3.6 Hz, $J_{2,3}$ = 9.5 Hz), 4.38 (d, 1H, H-1', $J_{1',2'}$ = 7.8 Hz), 4.16-4.15 (m, 1H, H-4), 3.91 (dd, 1H, H-3, $J_{3,4}$ = 3.0 Hz, $J_{3,2}$ = 9.5 Hz), 3.86-3.84 (m, 1H, H-5), 3.82-3.76 (m, 2H, O$\underline{CH_2}CH_2N_3$, H-4'), 3.76-3.72 (m, 2H, H-6'), 3.72-3.68 (m, 2H, H-6), 3.53 (dd, 1H, H-2', $J_{2',1'}$ = 7.8 Hz, $J_{2',3'}$ = 9.6 Hz), 3.50 (ddd, 1H, H-5', $J_{5',4'}$ = 1.2 Hz, $J_{5',6'a}$ = 5.4 Hz, $J_{5',6'b}$ = 6.0 Hz), 3.46-3.43 (m, 2H, H-3', O$\underline{CH_2}CH_2N_3$), 2.88-2.85 (m, 2H, OCH$_2\underline{CH_2}N_3$), 1.97 (s, 3H, CO$\underline{CH_3}$); ^{13}C NMR (125 MHz, CD_3OD) δ 174.1(CO), 106.5 (C-1'), 99.3 (C-1), 78.9 (C-3), 76.8 (C-5'), 74.7 (C-3'), 72.5 (C-2'), 72.3 (C-5), 70.3 (C-4'), 70.2 (C-4), 70.0 (O$\underline{CH_2}CH_2N_3$), 62.95, 62.64 (C-6, C-6'), 50.2 (C-2), 50.2 (C-2), 42.0 (OCH$_2\underline{CH_2}N_3$), 22.9 (NHCO$\underline{CH_3}$). HR-ESMS calcd for $C_{16}H_{31}N_2O_{11}$: 427.1928. Found: 427.1928.

General procedure for conjugating the dye molecules to the saccharide:

To a stirring solution (pH = 8.2) of the saccharide in 0.185 M $NaHCO_3$ (0.5 mL), the labeling agent in anhydrous DMF (0.5 mL) was added. Stirring was continued for 4–6 h at rt. The solvent was evaporated and the crude purified using reverse-phase chromatography on a C-18 column. Lyophilisation of the required fractions gave the dye-labeled saccharides as fluffy solids.

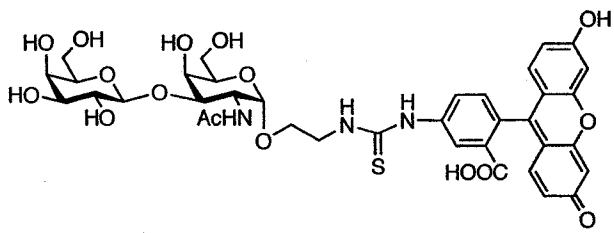
2-(6-Hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-{3-[2-(3,6-di-*O*-α-D-mannopyranosyl-α-D-mannopyranosyloxy)-ethyl]-thioureido}-benzoic acid (1-FITC)



With the trisaccharide **1** (30 mg, 0.55 mmol) and fluorescein isothiocyanate isomer I (5.0 mg, 0.013 mmol), the conjugated trisaccharide (7.9 mg, 0.009 mmol, 69%) was obtained in as a yellow fluffy solid and as isomers: ^{1}H NMR (500 MHz, D_2O) δ 7.75-7.70 (m, 1H, Ar), 7.71 (br s, 1H, ArH), 7.54 (d, 1H, Ar, J = 11.5 Hz), 7.36-7.17 (m, 3H, ArH), 6.70-6.56 (m, 3H, ArH), 5.10 (br s, 1H, H-1), 5.08 (br s, 1H, H-1), 4.89 (br s, 1H, H-1), 4.87 (br s, 1H, H-1), 4.85 (br s, 1H, H-1), 4.20-3.50 (m, 44H); ^{13}C NMR (125 MHz, D_2O) δ 184.2, 180.3, 174.2, 158.9, 158.7, 141.35, 138.46, 132.0, 131.9, 131.6, 130.7, 129.8, 126.0, 122.8, 122,5, 113.8, 113.5, 103.8, 102.7, 100.2, 99.7, 78.9, 73.6,

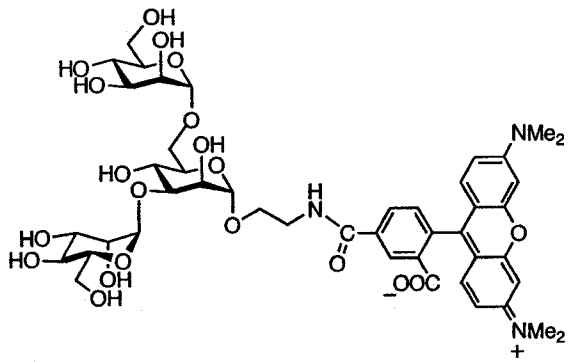
72.9, 71.6, 70.9, 70.7, 70.3, 70.2, 70.0, 67.0, 66.3, 66.1, 65.9, 65.7, 61.2, 44.6, 39.3. HR-ESMS calcd for $[M-H]^-$,$C_{41}H_{47}N_2O_{21}S$: 935.2387. Found: 935.2388.

2-(6-Hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-{3-[2-((β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranosyloxy)-ethyl]-thioureido}-benzoic acid (13-FITC)



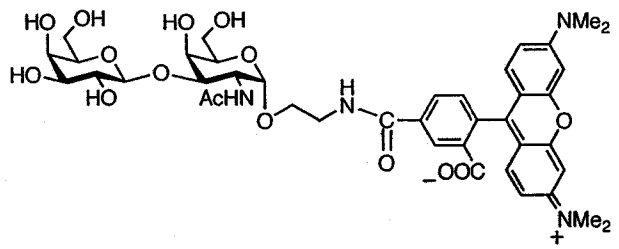
With the disaccharide **13** (20 mg, 0.47 mmol) and fluorescein isothiocyanate isomer I (4.2 mg, 0.01 mmol), the conjugated disaccharide (8.80 mg, 0.01 mmol) was obtained in quantitative yield as a yellow fluffy solid: (500 MHz, D_2O) δ 7.71 (br s, 1H, Ar), 7.55 (br d, 1H, Ar, J = 6Hz), 7.38 (d, 1 H, Ar, J = 8.5 Hz), 7.30-7.24 (m, 2H, ArH,), 6.97 (d, 1H, Ar, J = 2 Hz), 6.70-6.64 (m, Ar, 3H), 4.96 (d, 1H, H-1, $J_{1,2}$ = 3.0 Hz), 4.40-4.30 (m, 2H), 4.23 (br s, 1H), 4.04-3.30 (m, 14H), 2.30 (s, 3H, $CO\underline{CH_3}$); ^{13}C NMR (125 MHz, D_2O) δ 180.9, 174.9, 174.2, 167.5, 160.6, 159.2, 131.8, 130.3, 126.0, 125.2, 123.3, 112.8, 105.2, 103.9, 97.7, 78.1, 75.2, 72.8, 71.1, 70.8, 69.8, 69.0, 68.8, 66.6, 61.1, 48.8, 44.9, 22.5. HR-ESMS calcd for $[M-H]^-$, $C_{37}H_{40}N_3O_{16}S$: 814.2135. Found: 814.2138.

***N*-(9-{2-Carboxy-4-[2-(3,6-di-*O*-α-D-mannopyranosyl-α-D-mannopyranosyloxy)-ethylcarbamoyl]-phenyl}-6-dimethylamino-xanthen-3-ylidene)-dimethyl-ammonium (1-TAMRA)**



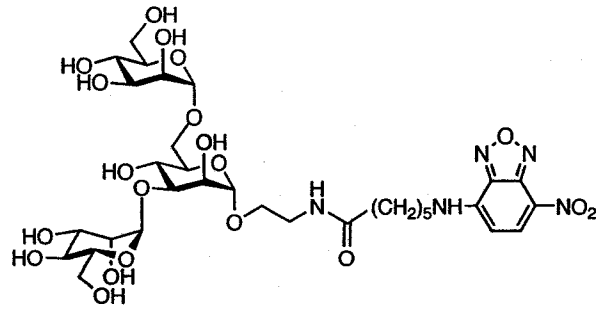
With the deprotected trisaccharide **1** (32 mg, 0.58 mmol) and 5-carboxytetramethylrhodamine, succinimidyl ester (5.0 mg, 0.0095 mmol), the conjugated trisaccharide (3.6 mg, 0.0044 mmol, 46%) was formed as a pink solid: ^{1}H NMR (500 MHz, D_2O) δ 8.31 (d, 1H, Ar, J = 2Hz), 8.26 (d, 1H, Ar, J = 2Hz), 8.18 (dd, 1H, J = 1.8 Hz, J = 8.0 Hz), 8.07 (dd, 1H, J = 1.5 Hz, J = 8.0 Hz), 7.60-7.54 (m, 2H, Ar), 7.24-7.16 (m, 4H, Ar), 6.87-6.80 (m, 4H, Ar), 6.46-6.36 (m, 4H, Ar), overlapping doublet at 5.13 (d, J = 1.5 Hz, H-1) and a broad singlet at 5.11 (H-1), overlapping doublets at 4.92 and 4.91 (d, J = 1.5 Hz, H-1), 4.85 (br s, H-1) and 4.81 (d, J = 2 Hz, H-1), 4.20-3.50 (m, 4H), 3.17 (s, 12H), 3.10 (s, 12H) ; ^{13}C NMR (125 MHz, D_2O) δ 202.0, 175.1, 174.2, 170.3, 159.7, 158.7, 157.8, 157.74, 157.66, 141.6, 141.0, 140.8, 139.0, 136.0, 135.1, 134.0, 131.9, 131.8, 131.2, 130.7, 130.6, 129.7, 128.9, 128.1, 115.5, 114.75, 114.71, 114.6, 113.7, 113.6, 103.3, 100.5, 100.3, 97.02, 97.00, 96.92, 96.91, 79.4, 74.2, 73.6, 73.5, 71.50, 73.49, 71.0, 70.92, 70.85, 70.81, 67.7, 67.65, 67.61, 67.58, 66.6, 66.4, 61.91, 61.84, 61.83, 61.80, 61.79, 49.76, 40.9, 40.80, 40.6. HR-ESMS calcd for $[M-H]^+$,$C_{45}H_{58}N_3O_{20}$: 960.3608. Found: 960.3603.

***N*-(9-{2-Carboxy-4-[2-((β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranosyloxy)-ethylcarbamoyl]-phenyl}-6-dimethylamino-xanthen-3-ylidene)-dimethyl-ammonium (13-TAMRA)**



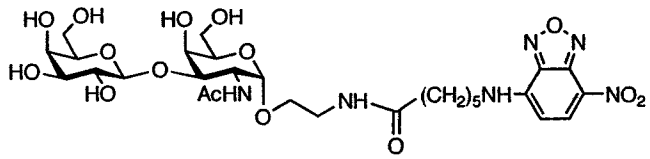
With the disaccharide **13** (20 mg, 0.47 mmol) and 5-carboxytetramethylrhodamine, succinimidyl ester (**13-TAMRA**) (8.0 mg, 0.015 mmol), the conjugated disaccharide (3.7 mg, 0.0041, 27% mmol) was formed as a pink fluffy solid: (500 MHz, D_2O) δ 8.28-8.27 (d, 1H, Ar, J = 1.5 Hz), 8.06 (dd, 1H, Ar, J = 1.8, 7.5 Hz), 7.58 (d, 1H, Ar, J = 8Hz), 7.18 (t, 2H, Ar, J = 9.3 Hz), 6.88 (dt, 2H, Ar, J = 2.5, 5.0 Hz), 6.51 (br s, 2H, Ar), 4.91 (d, 1H, H-1, J = 4.0 Hz), 4.48 (d, 1H, H-1', J = 8.0 Hz), 4.36 (1H, dd, H-2, $J_{2,1}$ = 4.0 Hz, $J_{2,3}$ = 11.0 Hz), 4.21 (d, 1H, H-4, $J_{4,3}$ = 3.0 Hz), 4.04 (1H, dd, H-3, $J_{3,4}$ = 3.0 Hz, $J_{3,2}$ = 11.0 Hz), 4.02-3.94 (m, 2H), 3.89 (d, 1H, H-4', $J_{4',3'}$ = 3.5 Hz), 3.88-3.82 (m, 1H), 3.79-3.64 (m, 7H), 3.62 (dd, 1H, $J_{3',4'}$ = 3.5 Hz, $J_{3',2}$ = 10.5 Hz), 3.51 (dd, 1H, H-2', $J_{2',1'}$ = 8.0 Hz, $J_{2',3'}$ = 10.5 Hz), 3.14 (s, 12H, CH_3), 1.92 (s, 3H, $NHCOCH_3$); ^{13}C NMR (100 MHz, D_2O) δ 174.7, 165.9 (C=O), 156.5, 151.5, 149.1, 147.5, 147.3, 145.2, 140.2, 138.3,137.6, 137.1, 128.0, 112.7, 105.0 (C-1), 97.9 (C-1'), 77.3, 75.3, 74.6, 72.9, 71.3, 70.9, 69.1, 68.9, 61.6, 61.4, 40.4, 39.8, 22.5. HR-ESMS calcd for $[M-H]^+$,$C_{41}H_{50}N_4O_{15}Na$: 861.3165. Found: 861.3157.

_N_-(2-(3,6-di-_O_-α-D-mannopyranosyl-α-D-mannopyranosyloxy)-ethyl)-6-(7-nitrobenzo[_c_][1,2,5]oxadiazol-4-ylamino)hexanamide (1-NBD)



The trisaccharide **1** (30 mg, 0.055 mmol) reacted with succinimidyl 6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl amino) hexanoate (NBD-X) (9.9 mg, 0.025 mmol) to give the conjugated trisaccharide (21 mg, 0.025 mmol) in quantitative yield as a yellow fluffy solid. ^{1}H NMR (500 MHz, D_2O) δ 8.35 (br s, 1H, Ar), 6.26 (d, 1H, Ar, J = 9.5 Hz), 5.09 (d, 1H, H-1, J = 1.5 Hz), 4.86 (d, 1H, H-1, J = 2.0 Hz), 4.80 (d, 1H, H-1, J = 1.5 Hz), 4.06-4.10 (m, 1H), 4.07 (dd, 1H, J = 1.8 Hz, 3.5 Hz), 3.98-3.61 (m, 20H), 3.60-3.46 (m, 3H), 3.37-3.42 (m, 1H), 2.30 (t, 2H, J = 7.5 Hz), 1.81-1.74 (m, 2H), 1.72-1.64 (m, 2H), 1.50-1.42 (m, 2H); ^{13}C NMR (125 MHz, D_2O) δ 177.7 (C=O), 147.5, 145.2, 144.9, 139.6, 120.4, 103.3 (C-1), 101.1, 100.7 (C-1), 100.3 (C-1), 79.5, 74.2, 73.5, 72.1, 71.5, 71.3, 70.9, 70.8, 70.5, 67.62, 67.58, 67.0, 66.4, 66.2, 61.9, 61.8, 49.8, 39.7, 36.5, 28.1, 26.6, 25.9. HR-ESMS calcd for $[M-H]^+$,$C_{32}H_{49}N_5O_{20}Na$: 846.2863. Found: 846.2865.

***N*-(2-((β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranosyloxy)-ethyl)-6-(7-nitrobenzo[*c*][1,2,5]oxadiazol-4-ylamino)hexanamide (13-NBD)**



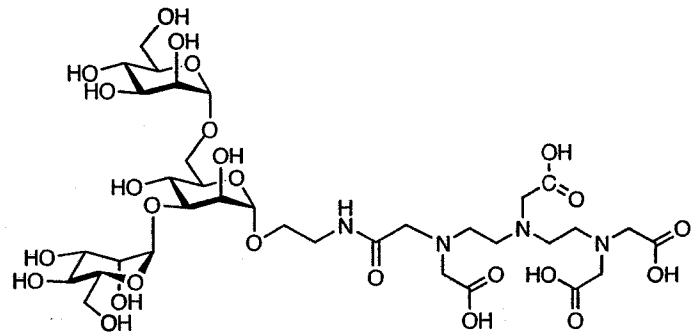
The disaccharide **13** (19 mg, 0.045 mmol) reacted with succinimidyl 6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl amino) hexanoate (NBD-X) (5.1 mg, 0.013 mmol) to give the conjugated disaccharide (8.5 mg, 0.012 mmol, 92%) as a yellow fluffy solid : ^{1}H NMR (500 MHz, D_2O) δ 8.06 (br s, 1H, ArH), 6.37 (d, 1H, ArH, J = 9.5 Hz), 4.80 (d, 1H, H-1, $J_{1,2}$ = 3.5 Hz), 4.43 (d, 1H, H-1', $J_{1',2'}$ = 8.0 Hz), 4.29 (dd, 1H, H-2, $J_{2,1}$ = 3.5 Hz, $J_{2,3}$ = 11.0 Hz), 4.20 (d, 1H, H-4, $J_{4,3}$ = 3.5 Hz), 3.95 (dd, 1H, H-3, $J_{3,4}$ = 3.5 Hz, $J_{3,2}$ =11.0 Hz), 3.92-3.88 (m, 2H), 3.80-3.44 (m, 12H), 3.34-3.27 (m, 1H), 2.29 (t, J = 7.3 Hz, 4.0 Hz), 2.01 (s, 3H, $COCH_3$), 1.84-1.75 (m, 2H), 1.71-1.62 (m, 2H), 1.48-1.40 (m, 2H); ^{13}C NMR (125 MHz, D_2O) δ 177.2, 174.7, 144.9, 144.5, 140.7, 139.2, 139.0, 116.3, 105.1, 100.6, 97.7, 77.6, 75.2, 72.8, 71.0, 70.9, 69.0, 68.8, 66.8, 61.4, 61.2, 49.1, 48.7, 39.2, 36.0, 27.5, 26.0, 25.4, 22.3. HR-ESMS calcd for $[M-H]^+$, $C_{28}H_{42}N_6O_{15}Na$: 725.2600. Found: 725.2596.

General procedure for preparing DTPA conjugates

The carbohydrate (0.037 mmol) was dissolved in 0.185M sodium hydrogen carbonate solution (0.7 mL) and solid diethylenetriaminepentaacetic acid dianhydride (0.147 mmol) was added. The mixture was gently stirred for 6 h at rt, until ninhydrin test indicated absence of amines on TLC. The mixture was then applied on a Sephadex G-

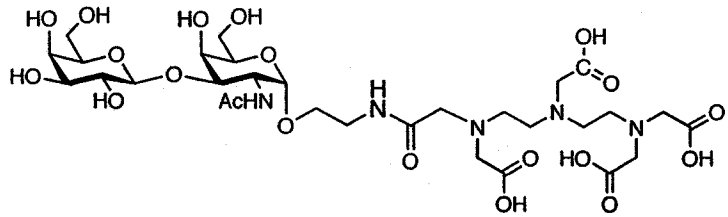
15 column and eluted with water. The different fractions collected were evaporated and analysed by ^{1}H NMR to identify the purified product. Water was removed by evaporation and lyophilization yielded the carbohydrate conjugate as a white fluffy solid.

((2*S*,3*S*,4*R*,5*R*)-2-(1-carboxy-2,5,8-tris(carboxymethyl)-10-oxo-2,5,8,11-tetraazatridecan-13-yloxy)-3,6-di-*O*-α-D-mannopyranosyl-α-D-mannopyranoside



Yield, 36%; ^{1}H NMR (500 MHz, D_2O) δ 5.09 (d, 1H, J = 1.2 Hz), 4.88 (d, 1H, J = 1.6 Hz), 4.83 (d, 1H, J = 1.6 Hz), 4.07-4.10 (m, 1H), 4.05 (dd, 1H, J = 1.6 Hz, 3.2 Hz), 3.98-3.63 (m, 30H), 3.54-3.34 (m, 5H), 3.33-3.18 (m, 3H); ^{13}C NMR (125 MHz, D_2O) δ 173.6, 172.8, 172.1, 171.2, 168.6 (C=O), 103.2, 100.6, 100.2 (C-1), 72.0, 71.4, 71.2, 70.9, 70.8, 70.4, 74.1, 73.5, 74.1, 73.5, 67.6, 67.5 (CH), 66.59, 66.4, 66.1, 61.81, 61.77, 57.6, 57.3, 57.2, 55.1, 53.0, 51.4, 50.8, 39.9, 39.8. HR-ESMS calcd for $[M-H]^-$,$C_{34}H_{57}N_4O_{25}$: 921.3318. Found: 921.3308.

((2*S*,3*S*,4*R*,5*R*)-2-(1-carboxy-2,5,8-tris(carboxymethyl)-10-oxo-2,5,8,11-tetraaza tridecan-13-yloxy)-(β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside



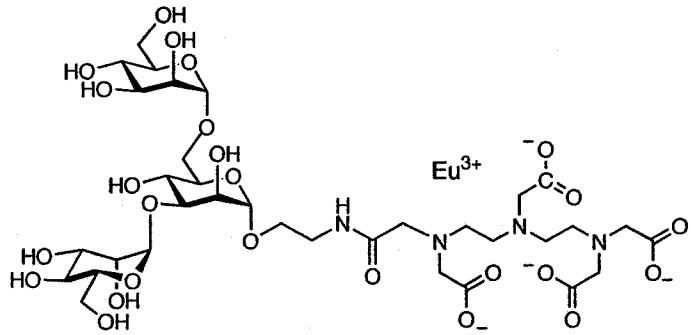
Yield, 40%; ^{1}H NMR (500 MHz, D_2O) δ 4.88 (d, 1H, H-1, $J_{1,2}$ = 4.0 Hz), 4.49 (d, 1H, H-1', $J_{1',2'}$ = 8.0 Hz), 4.33 (dd, 1H, H-2, $J_{2,1}$ = 4.0, $J_{2,3}$ = 11.5 Hz), 4.22 (d, 1H, H-4, $J_{4,3}$ = 3.0 Hz), 3.98 (dd, 1H, H-3, $J_{3,4}$ = 3.0 Hz, $J_{3,2}$ = 11.5 Hz), 3.96-3.42 (m, 25H), 3.40-3.30 (m, 4H), 3.18-3.10 (m, 2H), 2.03 (s, 3H, CH_3); ^{13}C NMR (125 MHz, D_2O) δ 175.4, 175.3, 173.7, 172.5, 171.2, 168.9 (C=O), 105.5 (C-1), 98.3 (C-1'), 78.03, 75.8, 73.3, 71.7, 71.4, 69.5, 69.4 (CH), 67.2, 62.0, 61.8, 58.0, 57.5, 57.44, 57.38, 55.23, 52.4, 51.6, 50.6 (CH_2), 49.3 (CH), 40.1, 40.01. HR-ESMS calcd for $[M-H]^-$, $C_{30}H_{50}N_5O_{20}$: 800.3044. Found: 800.3051.

General procedure for preparing europium complexes from the DTPA conjugates of carbohydrates

To a solution of DTPA conjugate (0.031 mmol) in water (0.7 mL) was added europium nitrate (0.034 mmol) and the mixture was allowed to stir at rt for 3 h. The mixture was then purified by sephadex G-15 and the fractions containing the product were analysed by ^{1}H NMR. The aqueous mixture was evaporated under vacuum and lyophilized to give a white fluffy solid as product. Because of the diamagnetic nature of europium, the

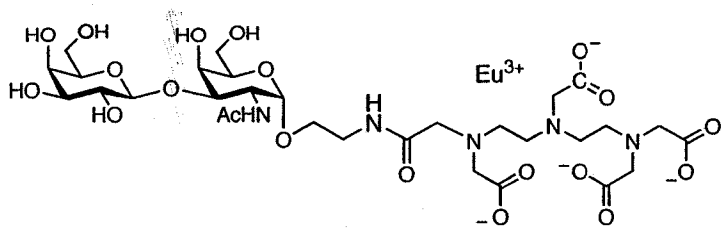
proton peaks were considerably broadened and would give higher values for their integration than expected. ^{13}C spectra were therefore not taken as sharp peaks were not obtained in the proton NMR spectra.

Europium (III) ((2*S*,3*S*,4*R*,5*R*)-2-(1-carboxylato-2,5,8-tris(carboxylatomethyl)-10-oxo-2,5,8,11-tetraazatridecan-13-yloxy)-3,6-di-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (32)



Yield, 28%; ^1H NMR (500 MHz, D_2O) δ 5.11 (d, 1H, J = 1.5 Hz), 4.90 (d, 1H, J = 2.0 Hz), 4.87 (d, 1H, J = 1.5 Hz), 4.20-3.24 (m, 40 H). HR-ESMS calcd for $[M]^-$, $C_{34}H_{54}N_4O_{25}Eu$: 1071.2295. Found: 1071.2291.

Europium (III) ((2*S*,3*S*,4*R*,5*R*)-2-(1-carboxylato-2,5,8-tris(carboxylatomethyl)-10-oxo-2,5,8,11-tetraazatridecan-13-yloxy)-(β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (33)



Yield, 30%; ^{1}H NMR (500 MHz, D_2O) δ 4.96 (br s, 1H), 4.70 (br s, 1H), 4.48-3.20 (m, 42H), 2.05-1.80 (m, 3H). HR-ESMS calcd for $[M]^-$, $C_{30}H_{47}N_5O_{20}Eu$: 950.2021. Found: 950.2018.

2.8.3 Derivatization of agarose beads with dye molecules

Buffer solution for just the agarose beads consisted of 0.01 M sodium hydrogen-phosphate, 0.15 M sodium chloride at pH 7.40. The agarose beads were ω-aminobutyl-agarose immobilized on cross-linked 4% beaded agarose, 95-165 μmoles. After washing 20 μL of gel suspension (1×10^{-7} moles of amine groups) with phosphate buffer solution, the beads were made into a solution with 0.5 mL of 0.19 M $NaHCO_3$ (pH 8.3) in a polypropylene vessel and treated with different equivalents (0.1, 0.5, 1, 2, 4, 6, 8, 10 equiv with respect to the amino groups) of dye solution in DMF (0. 1 g in 0.1 mL DMF). After 12 h of shaking in an automatic shaker, the beads were washed with 0.19 M $NaHCO_3$ (2 × 1 mL), methanol (1.5 mL), water (2 × 1 mL) and buffer solutions (1 mL). A volume of 1 mL of buffer solution was then added to the beads and 6 μL of the gel solution was placed on a glass slide to be observed under the epi-fluorescent microscope. Different filters were used for the dye-labeled beads and an average count was made as to the number of bright beads of the same size seen in the solution.

2.8.4 General procedure for preparing agarose-lectin assays with labeled carbohydrates

A solution of the gel solution (20 μL) was placed in a polypropylene vessel, followed by the saccharide solution (10×10^{-5} M, 5×10^{-5} M, 1×10^{5} M) made in the required buffer. The mixture was vortexed for 4 h at rt. Then, the resin was filtered and rinsed with the buffer solution (1mL) for 30 s. Then a volume (250 μL) of the buffer solution was added to the polypropylene vessel containing the lectin-agarose beads which was vortexed for 15 s. The gel solution (10 μL) was then placed on the glass slide for viewing under the microscope.

2.8.5 Visualization of beads on a gel matrix

1. The gel solution was of the same composition as the gel for electrophoresis. It was prepared by mixing 3.3 mL of 30% acrylamide mix, 50 μL of 10% ammonium persulfate 4 μL of TEMED (*N,N,N',N'*-Tetramethylethylenediamine) and 1.65 mL of buffer solution.

2. Agarose gel (20 μL) was placed onto one glass plate of a gel caster, left to dry and covered with another glass plate.

3. The gel solution was run down the slit between the plates and allowed to solidify for thirty minutes.

4. The plates were then separated from each other and the transparent gel was removed gently from the glass plate. A piece of the polymeric gel was broken off and 10 μL of

DELFIA solution was added. The gel was then observed under the epifluorescent microscope.

2.8.6 Solid-phase binding assay of lectins on microtiter plates

Buffers used for

(a) Con A lectin

Tris: 0.05 M Tris, 0.15 M NaCl, 0.004 M $CaCl_2$.

Tris-Tween: Tris + 0.05% Tween-20

(b) PNA lectin

PBS: 0.01 M Na_3PO_4, 0.15 M Na Cl, 0.5 mM $CaCl_2$.

PBST: PBS + 0.05% Tween-20.

A solution of lectin (PNA) dissolved in PBS buffer solution (2.5 μg in 1 mL) was added to 96-well plate (black Nunc plate) which was left overnight at - 4 °C (Con A, 2-3 h, rt). The microtiter plate was washed five times with PBST (for Con A lectin, Tris-tween). Blocking of wells was carried out at rt by incubating the lectin-coated plate with 2.5% casein. The plate was washed twice with PBST. A solution of the europium-labeled carbohydrate was added to the plate which was left overnight in the refrigerator at -4 °C for PNA lectin or at 3-4 h or at rt for Con A lectin. The plate cells were then washed three times with PBST (for PNA lectin) or Tris-tween (for Con A lectin). 100 μL of DELFIA reagent was added to each cell and left to react for thirty minutes as recommended for DELFIA assays and time-fluorescence readings were taken after 5 s of automatic shaking in the microplate reader. The wavelengths for excitation and emission were 332 nm and 616 nm respectively.

2.9 References

(1) Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683-720.

(2) Fukuda, M.; Hindsgaul, O. *Molecular Glycobiology*; Oxford University Press: New York, 1994.

(3) Hakomori, S.; Zhang, Y. *Chem. Biol.* **1997**, *4*, 97-104.

(4) Lis, H.; Sharon, N. *Chem. Rev.* **1998**, *98*, 637-674.

(5) Rudd, P. M.; Elliott, T.; Cresswell, P.; Wilson, I. A.; Dwek, R. A. *Science* **2001**, *291*, 2370-2376.

(6) Alper, J. *Science* **2003**, *310*, 159-160.

(7) Sharon, N.; Lis, H. *Gycobiology* **2004**, *14*, 53R-62R.

(8) Cobb, B. A.; Kasper, D. L. *Eur. J. Immunol.* **2005**, *35*, 352-356.

(9) Liener, I. E.; Sharon, N.; Goldstein, I. J. *The lectins: Properties, Functions and Applications in Biology and Medicine*; Academic Press: New York, 1996.

(10) Itzkowitz, S. H.; Bloom, E. J.; Kokal, W. A.; Modin, G.; Hakomori, S.; Kim, Y. S. *Cancer* **1990**, *66*, 1960-1966.

(11) Hiraizumi, S.; Takasaki, S.; Ohuchi, N.; Harada, Y.; Nose, M.; Mori, S.; Kobata, A. *Jpn. J. Cancer Res.* **1992**, *83*, 1063-1072.

(12) Haase, W. C.; Seeberger, P. H. *Curr. Org. Chem.* **2000**, *4*, 481-511.

(13) Ramström, O.; Lehn, J. M. *Chembiochem* **2000**, *1*, 41-48.

(14) Seeberger, P. H.; Haase, W. C. *Chem. Rev.* **2000**, *100*, 4349-4393.

(15) Arya, P.; Barkley, A.; Randell, K. D. *J. Comb. Chem.* **2002**, *4*, 193-198.

(16) Lockhoff, O.; Frappa, I. *Comb. Chem. High Throughput Screening* **2002**, *5*, 361-372.

(17) Hase, S.; Ikenaka, T.; Matsushima, Y. J. *J. Biochem.* **1981**, *90*, 1275-1279.

(18) Hu, G. F. *J. Chromatogr. A* **1995**, *705*, 89-103.

(19) Jackson, P. *Methods Enzymol.* **1994**, *230*, 250-265.

(20) Rudd, P. M.; Dwek, R. A. *Curr. Opin. Biotechnol.* **1997**, *8*, 488-497.

(21) Anumula, K. R.; Du, P. *Anal. Biochem.* **1999**, *275*, 236-242.

(22) Shibaev, V. N.; Veselovsky, V. V.; Lozanova, A. V.; Maltsev, S. D.; Danilov, L. L.; Forsee, W. T.; Xing, J.; Cheong, H. C.; Jedrzejas, M. J. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 189-192.

(23) Stoll, M. S.; Feizi, T.; Loveless, R. W.; Chai, W.; Lawson, A. M.; Yuen, C. T. *Eur. J. Biochem.* **2000**, *267*, 1795-1804.

(24) Drummond, K. J.; Yates, E. A.; Turnbull, J. E. *Proteomics* **2001**, *1*, 304-310.

(25) Arnosti, C. *J. Chromatogr. B* **2003**, *793*, 181-191.

(26) Zhang, Y.; Le, X.; Dovichi, N. J.; Compston, C. A.; Palcic, M. M.; Diedrich, P.; Hindsgaul, O. *Anal. Biochem.* **1995**, *227*, 368-376.

(27) Deras, I. L.; Sano, M.; Kato, I.; Lee, Y. C. *Anal. Biochem.* **2000**, *278*, 213-220.

(28) Kawasaki, N.; Lee, Y. C. *Anal. Biochem.* **1997**, *250*, 260-262.

(29) Saito, K.; Lee, R. T.; Lee, Y. C. *Anal. Biochem.* **1998**, *258*, 311-314.

(30) Baenziger, J. U.; Fiete, D. *J. Biol. Chem.* **1979**, *254*, 2400-2407.

(31) Neurohr, K. J.; Young, N. M.; Mantsch, H. H. *J. Biol. Chem.* **1980**, *255*, 9205-9209.

(32) Becker, B.; Furneaux, R. H.; Reck, F.; Zubkov, O. *Carbohydr. Res.* **1999**, *315*, 148-158.

(33) Ogawa, T.; Sasajima, K. *Carbohydr. Res.* **1981**, *93*, 231-240.

(34) Roy, R.; Page, D.; Perez, S. F.; Bencomo, V. V. *Glycoconjugate J.* **1998**, *15*, 251-263.

(35) Winnik, F. M.; Brisson, J.-R.; Carver, J. P.; Krepinsky, J. *J. Carbohydr. Res.* **1982**, *103*, 15-28.

(36) Arnarp, J.; Lönngren, J. *Acta Chem. Scand., Ser. B.* **1978**, *32*, 696-697.

(37) Ogawa, T.; Matsui, M. *Carbohydr. Res.* **1978**, *62*, C1-C4.

(38) Oscarson, S.; Tidén, A.-K. *Carbohydr. Res.* **1993**, *247*, 323-328.

(39) Chernyak, A. Y.; Sharma, G. V. M.; Kononov, L. O.; Krishna, P. R.; Levinsky, A. B.; Kochetkov, N. K.; Rama Rao, A. V. *Carbohydr. Res.* **1992**, *223*, 303-309.

(40) Forster, M. O.; Fierz, H. E. *J. Chem. Soc.* **1908**, *93*, 1174-1179.

(41) Smith, J. R. H.; Mehl, A. F.; Shantz, J. D. J.; Chmurny, G. N.; Michejda, C. J. *J. Org. Chem.* **1988**, *53*, 1467-1471.

(42) Gelas, T.; Horton, D. *Carbohydr. Res.* **1978**, *67*, 371-387.

(43) Nobréga, C.; Vázquez, J. T. *Tetrahedron: Assymmetry* **2003**, *14*, 2793-2801.

(44) Hanessian, S.; Banoub, J. *Carbohydr. Res.* **1977**, *53*, C13-C16.

(45) Garegg, P. J.; Norberg, T. *Acta Chem. Scand., Ser. B.* **1979**, *33*, 116-118.

(46) Lemieux, R. U.; Driguez, H. *J. Am. Chem. Soc.* **1975**, *97*, 4069-4075.

(47) Oscarson, S.; Szönyi, M. *J. Carbohydr. Chem.* **1989**, *1989*, 663-338.

(48) Ness, R. K.; Fletcher, J. H. G.; Hudson, C. S. *J. Am. Chem. Soc.* **1950**, *72*, 2200-2205.

(49) Qiu, D.; Koganty, R. R. *Tetrahedron Lett.* **1997**, *38*, 961-964.

(50) Jacquinet, J. C.; Zurabayan, S. E. *Carbohydr. Res.* **1973**, *32*, 137-143.

(51) Paulsen, H.; Kolar, C.; Stenzel, W. *Chem. Ber.* **1978**, *111*, 2358-2369.

(52) Lemieux, R. U.; Ratcliffe, R. M. *Can. J. Chem.* **1979**, *57*, 1244-1251.

(53) Goux, W. J.; Weber, D. S. *Carbohydr. Res.* **1992**, *233*, 65-80.

(54) Lipkind, G. M.; Shashkov, A. S.; Knirel, Y. A.; Vinogradov, E. V.; Kochetkov, N. K. *Carbohydr. Res.* **1988**, *175*, 59-75.

(55) Chaplin, D.; Crout, D. H. G.; Bornemann, S.; Hutchinson, D. W.; Khan, R. *J. Chem. Soc., Perkin Trans. 1* **1992**, 235-238.

(56) Yule, J. E.; Wong, T. C.; Gandhi, S. S.; Qiu, D.; Riopel, M. A.; Koganty, R. R. *Tetrahedron Lett.* **1995**, *36*, 6839-6842.

(57) Nakahara, Y.; Iijima, H.; Ogawa, T. *Stereocontrolled Approaches to O-Glycopeptide Synthesis.* In *ACS Symposium Series*; Kovác, P. Ed.; American Chemical Society: Washington DC, 1994; pp 249-266.

(58) Schmidt, R. R.; Zimmermann, P. *Angew. Chem. Int. Ed.* **1986**, *25*, 725-726.

(59) Polt, R.; Szabó, L.; Treiberg, J.; Li, Y.; Hruby, V. J. *J. Am. Chem. Soc.* **1992**, *114*, 10249-10258.

(60) Bukowski, R.; Morris, L. M.; Woods, R. J.; Weimar, T. *Eur. J. Org. Chem.* **2001**, *14*, 2697-2705.

(61) George, S. K.; Holm, B.; Reis, C. A.; Schwientek, T.; Clausen, H.; Kihlberg, J. *J. Chem. Soc., Perkin Trans.1* **2001**, 880-885.

(62) Ivanova, I. A.; Ross, A. J.; Ferguson, M. A. J.; Nikolaev, A. *J. Chem. Soc., Perkin Trans. 1* **1999**, 1743-1753.

(63) Kovác, P.; Taylor, R. B.; Glaudemans, C. P. J. *J. Org. Chem.* **1985**, *50*, 5323-5333.

(64) Excoffier, G.; Gagnaire, D.; Utile, J.-P. *Carbohydr. Res.* **1975**, *39*, 368-373.

(65) Amvam-Zollo, P.-H.; Sinaÿ, P. *Carbohydr. Res.* **1986**, *150*, 199-212.

(66) Paulsen, H.; Peters, S.; Bielfeldt, T.; Meldal, M.; Bock, K. *Carbohydr. Res.* **1995**, *268*, 17-34.

(67) Ratcliffe, R. M.; Baker, D. A.; Lemieux, R. U. *Carbohydr. Res.* **1981**, *93*, 35-41.

(68) Brinkley, M. *Bioconjugate Chem.* **1992**, *3*, 2-13.

(69) Menjívar, M.; Ortiz, G.; Gárdenas, M.; Garza-Flores, J. *Rev. Invest. Clin.* **1993**, *45*, 579-584.

(70) Elliott, C. T.; Francis, K. S.; McCaughey, W. J. *Analyst* **1994**, *119*, 2565-2569.

(71) Hemmilä, I. A.; Dakubu, S.; Mukkala, V.-M.; Siitari, H.; Lövgren, T. *Anal. Biochem.* **1984**, *137*, 335-343.

(72) Ge, P.; Selvin, P. R. *Bioconjugate Chem.* **2004**, *15*, 1088-1094.

(73) Harris, R. F.; Nation, A. J.; Copeland, G. T.; Miller, S. J. *J. Am. Chem. Soc.* **2000**, *122*, 11270-11271.

(74) Gervay, J.; McReynolds, K. D. *Curr. Med. Chem.* **1999**, *6*, 129-153.

(75) Kitov, P. I.; Sadowska, J. M.; Mulvey, G.; Armstrong, G. D.; Ling, H.; Pannu, N. S.; Read, R. J.; Bundle, D. R. *Nature* **2000**, *403*, 669-672.

(76) Website: www.probes.com. (4/1/06)

Chapter 3

Screening of Polyamine Libraries in the Search for Receptors for Polyanionic Sulfated Carbohydrates

3.1 Introduction

As discussed in Chapter 1, natural sulfated carbohydrates exist on the cell surfaces of proteoglycans[1] (PG), which are protein cores involved in a number of diseases. One effective way to treating cancer is to inhibit both polyamine synthesis and heparan sulfate assembly[2-4] (responsible for the replenishment of amines for cancerous cell growth). To date, no research has been done in screening combinatorial polyamine libraries for the discovery of unnatural ligands that bind strongly to sulfated carbohydrates[5] such as heparins or heparan sulfate.

Strong binding by multivalent ion pairing to sulfated carbohydrates depends on the separation between amine groups in the polyamine molecule as shown in Figure 3-1. Screening of combinatorial libraries provides a quick and easy way to find ligands for these sulfated targets in an aqueous medium, a mimic of the physiological conditions existing in the human body. Our aim was to synthesize fluorescently labeled water-soluble sulfated carbohydrate models of the polymer heparan sulfate. These will then be screened against a library of polyamines loaded on polystyrene-based supports, which were developed by Manku and Hall[6] and tested with sulfated aromatic water-soluble dyes.

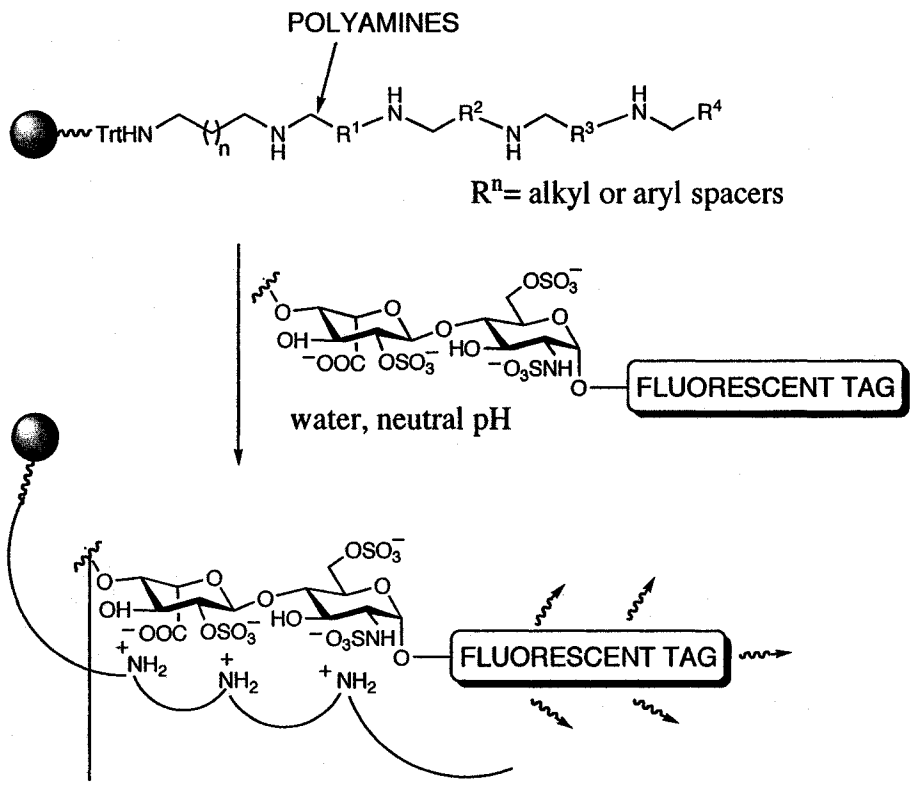


Figure 3-1. Screening of polyamine libraries against fluorescently tagged sulfated sugars.

The models of fluorescently labeled monosaccharide and disaccharide unit of heparan sulfate, the polysulfated version of heparin, are shown in Figure 3-2. Succinimidyl 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoate (NBD-X) dye would be the aromatic dye of choice used to label the sulfated carbohydrates so that the solubility of the carbohydrate molecules is not drastically affected by structure of the fluorescent tag used. The model saccharides would be monosacccharides and disaccharides that are units of heparin which have a linking arm to attach the reporter molecule. Before making more complicated versions of sulfated carbohydrates, the screening would be tested with simple NBD-labeled polyanionic carbohydrates **1** and **2**.

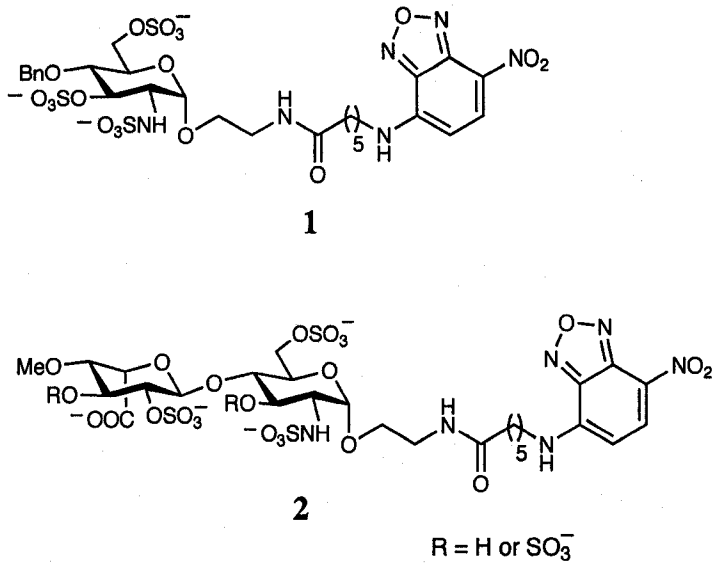


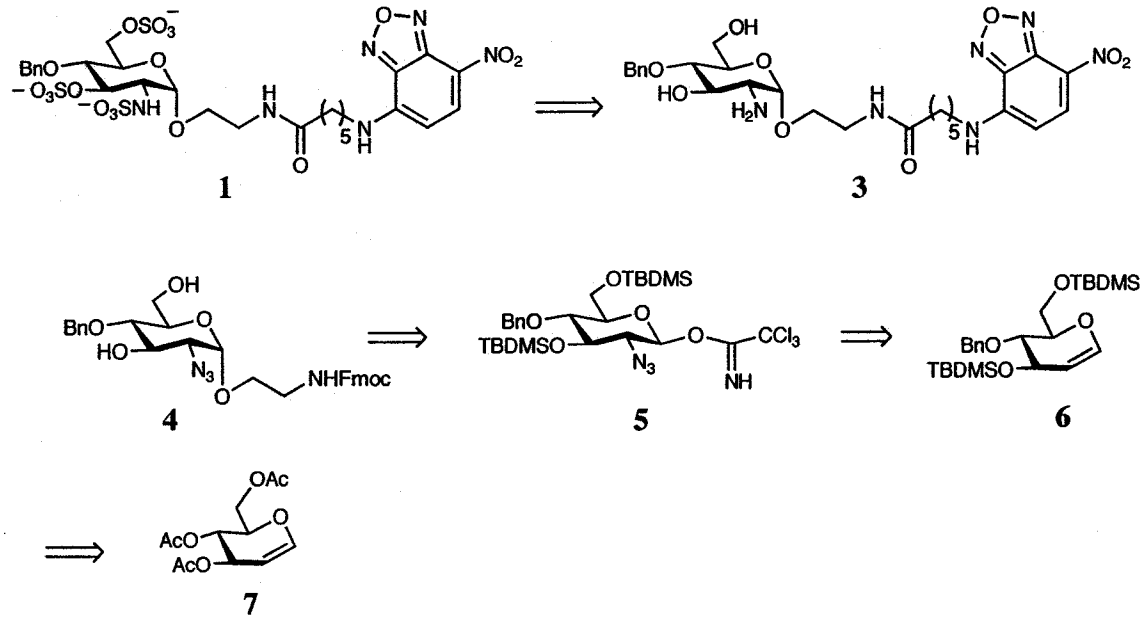
Figure 3-2. Model fluorescently tagged sulfated saccharides.

3.2 Retrosynthesis of sulfated compound 1

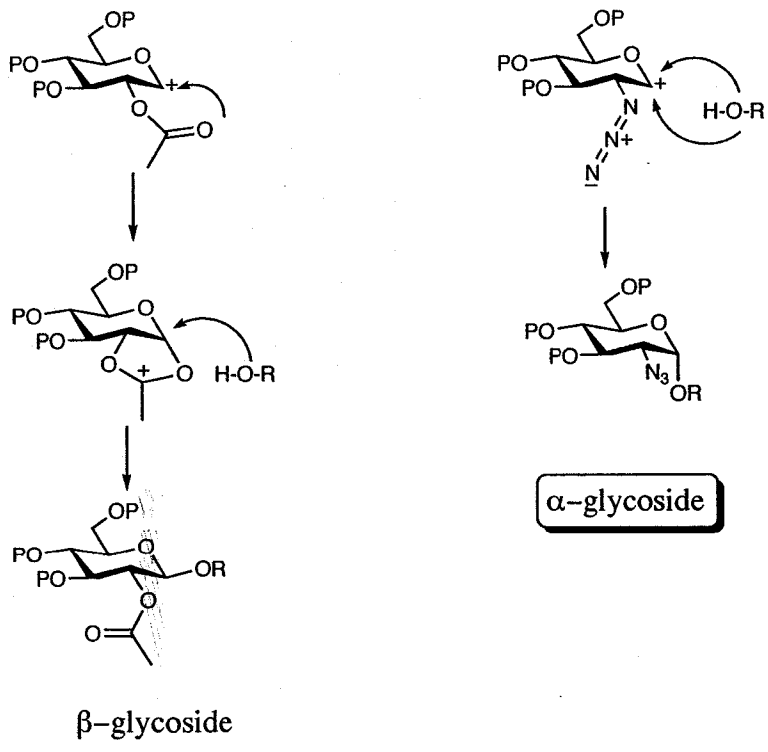
In the synthesis of the trisulfated carbohydrate **1** (Scheme 3.1), the sulfation process would be done in the last synthetic steps for the following reasons: (1) sulfated carbohydrates need ion-exchange columns for purification purposes; (2) certain conditions (harsh basic conditions) can favor elimination of sulfate ions.

Because it was found in the literature[7] that the amide NH was not sensitive to sulfation conditions, analogue **3,** a conjugate of succinimidyl 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoate (NBD-X) dye, would be one of the synthons as depicted in Scheme 3.1.

In the formation of an α-glycosidic bond in 2-amino glycoside, a non-participating neighboring group at the C-2 position is required during the activation of the anomeric position (Scheme 3.2), for example, an azido group.[8-10] Other neighboring groups that



Scheme 3.1



Scheme 3.2

favor the formation of α-glycosides are *O*-benzyl-, *O*-methoxybenzyl- or thioethers in the carbohydrate ring.[11] These functional groups are very bulky and cannot participate in the stabilization of the anomeric centre by donation of the lone pair of electrons on the oxygen (Scheme 3.2).

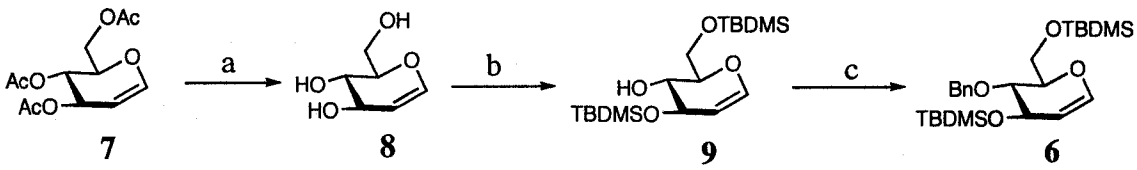
2-Amino sugar derivatives could be derived from 2-azido carbohydrates that are obtained by various methods.[12,13] The yields and selectivities of 2-azido derivatives are also high. We employed the azidonitration reaction,[12] which yielded the corresponding 2-azido carbohydrates from glycals (Scheme 3.1). Selective reduction of the azide to the amine would be carried out either under Staudinger conditions[14] (triphenyl phosphine) or with hydrogen sulfide. The corresponding amine would then be subjected to sulfation.[15]

To mimic the disaccharide unit in heparin, the hydroxyl group at C-4 was protected, so that similar protecting groups were needed for the 3- and 6-hydroxyl groups. For instance, *tert*-butyl silyl groups are known to regioselectively react with hydroxyl groups of glycals.[16] Thus, commercially available acetylated glycal **7** would be the starting material in this synthesis.

3.3 Synthesis of 4-*O*-benzyl-3,6-di-*O*-*tert*-butyldimethylsilyl-D-glucal (6)

In Scheme 3.3, removal of the acetyl groups from 3,4,6 tri-*O*-acetyl glucal **7** would yield D-glucal **8**. The hydroxyl groups at the 3 and 6 positions were protected selectively[16-18] with *tert*-butyl dimethylsilyl chloride to give the di-protected

carbohydrate **9** in 80% yield. Benzylation was then done at the 4-position with benzyl bromide and sodium hydride conditions. 4-*O*-Benzyl-3,6-di-*O*-*tert*-butyldimethylsilyl-D-glucal (**6**) was thus obtained in 99% yield.



7 **8** **9** **6**

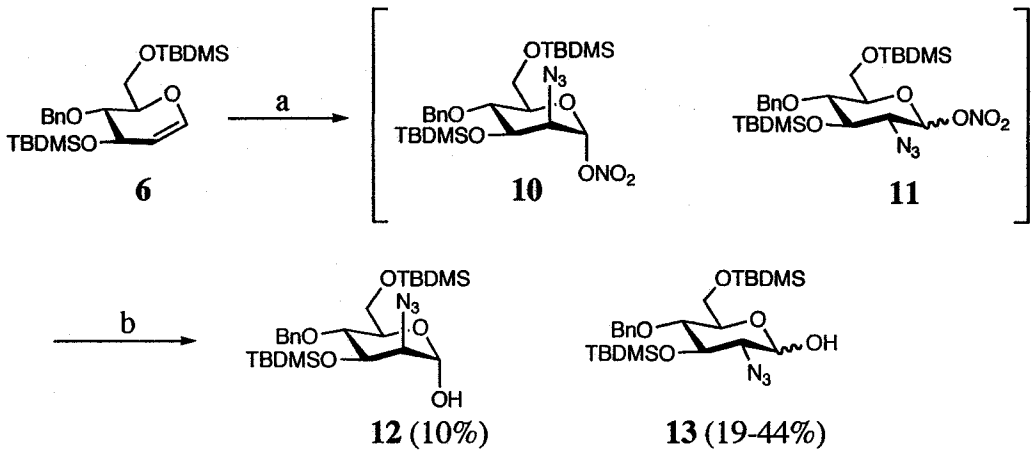
Scheme 3.3 Reagents and conditions: (a) NaOMe, MeOH, rt, 30 min, quantitative yield; (b) TBDMSCl, imidazole, DMF, 0 °C, 2 h, 80%; (c) BnBr, NaH, 0°C to rt, 1 h, 99%.

3.4 Synthesis of 2-*N*-(9-fluorenylmethyloxycarbonyl)-ethyl-2-azido-4-*O*-benzyl-2-deoxy-3,6-di-*O*-*tert*-butyldimethylsilyl-α-D-glucopyranoside (14)

With the protecting groups in place, the next step would be the introduction of the azido group at the C-2 position in **6** by azidonitration[12,18] with cerium ammonium nitrate and sodium azide as shown in Scheme 3.4. The work-up had to be modified from the literature conditions as the silyl groups were observed to cleave under acidic conditions, giving many side products.

The azidonitration reaction furnished the labile nitrate derivatives **10** and **11** which were hydrolysed to the compounds **12** (10% yield) and **13** (19-44% yield) respectively with thiophenol and diisopropylethylamine.[19] When column chromatography was directly attempted on the nitrates, low yields of impure compounds were obtained due to the

silyl groups cleaving. In addition, the isomers were difficult to separate as they were eluting very close to each other. As the hemiacetal derivative **13** was the desired product, it was deemed safe and more practical to perform the nitrate hydrolysis step first before attempting any purification process.

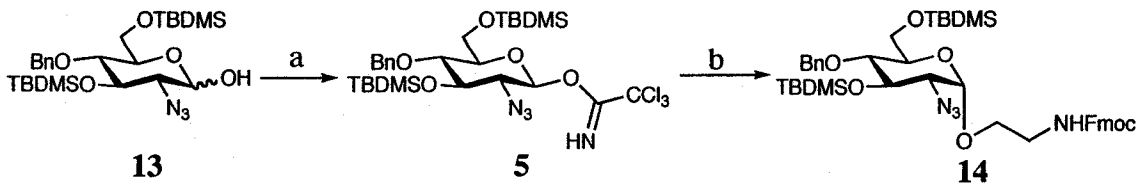


6 **10** **11**

12 (10%) **13** (19-44%)

Scheme 3.4 Reagents and conditions: (a) CAN, NaN_3, CH_3CN, -20 °C to -10 °C, 2 h, (b) thiophenol, $(^iPr)_2EtN$, CH_3CN, rt, 1 h.

To construct an α linkage in 2-azido-4-*O*-benzyl-2-deoxy-3,6-di-*O*-*tert*-butyldimethyl silyl-β-D-glucopyranose **13**, the hydroxyl group at the anomeric position was to be transformed to a good leaving group such as the β-trichloroacetimidate, by reaction with potassium carbonate and trichloroacetonitrile[20] (Scheme 3.4) under kinetic conditions. The glycosidic bond in **5** was of β configuration. ^1H NMR indicated a large coupling constant with H-1 at δ 5.65 (d, J = 8.0 Hz). With the acceptor ready for the next reaction, a linker with a protected amine group was introduced at the anomeric position.



13 5 14

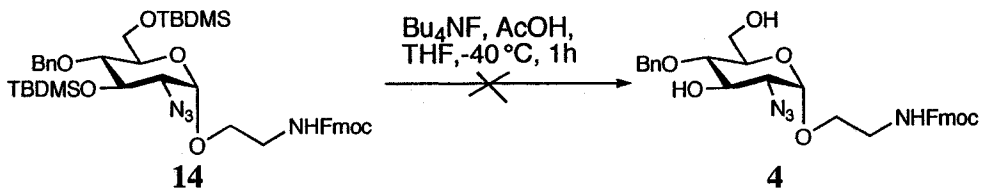
Scheme 3.5 Reagents and conditions: (a) K_2CO_3, CCl_3CN, DCM, rt, 8 h, 71%; (b) $BF_3.OEt_2$, THF, $HOCH_2CH_2NHFmoc$, 0 °C, 1 h, 34%.

To introduce another amine protecting group at the linker arm, the aglycon was chosen to be 2-*N*-(9-fluorenylmethoxycarbonylamino)-ethanol. Thus, ethanolamine was reacted with 9-fluorenylmethoxycarbonyl-*N*-hydroxy-succinimide[21] to give the Fmoc-protected aglycon in 97% yield under basic conditions with 10% sodium bicarbonate solution in tetrahydrofuran at 0 °C.

Glycosyl trichloroacetimidates with non-participating groups at C-2 undergo inversion of configuration at the anomeric centre with Lewis acids at low temperatures.[8] When glycosylation was carried out in dry dichloromethane with trimethylsilyl triflate[22] in the presence of molecular sieves at 0 °C, the desired product **14** was obtained in 17% yield. Some starting material was also recovered (22%). When the reaction was repeated using a different Lewis acid, boron trifluoride etherate at –20 °C, in dry dichloromethane, compound **14** was generated with a low yield of 16%. Boron trifluoride etherate as activating agent in dry tetrahydrofuran gave the best yield (37%) at 0 °C. Unreacted starting material was also recovered in 15%.

3.5 Synthesis of 2-*N*-(9-Fluorenylmethoxycarbonylamino)-ethyl-2-azido-4-*O*-benzyl-2-deoxy-α-D-glucopyranoside (4)

With the glycoside **14** ready, the next step was the removal of the silyl protecting groups. A mixture of tetrabutylammonium fluoride and acetic acid was used to maintain neutral conditions at – 40 °C. Disappointingly, no product **4** was obtained. Instead, a decomposition product was formed, whereby the azido group was lost.



Scheme 3.6

3.6 Conclusion to synthesis of NBD-labeled sulfated carbohydrates

The project was abandoned because of the low productivity of many reactions steps. We could see that silyl groups were not the best protecting groups to be used in the azidonitration reaction even if they could effect a 3,6 di-protection in the glucal derivative. Many side-products were obtained that made purification a lengthy and difficult process. The glycosidation step was also limited by the Lewis acid-sensitivity of the silyl groups. The azido group was cleaved on attempted removal of the silyl protecting groups.

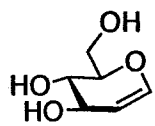
3.7 Experimental

3.7.1 General

The methods described in Section 2.8.1 also apply here.

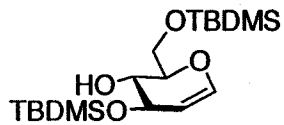
3.7.2 Experimental procedures

D-Glucal (8)



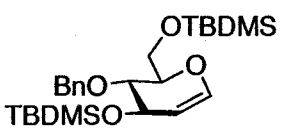
To a solution of 3,4,6 tri-*O*-acetyl glucal (4.40 g, 16.2 mmol) in dry methanol (18 mL), 0.1 M solution of sodium methoxide was added. The solution was stirred for 1 h at room temperature. Amberlite-H^+ was then added to neutralize the solution for 30 min. The solution mixture was filtered and the methanol was removed by evaporation to give a pale brown solid (2.37 g, 16.2 mmol) in quantitative yield. The spectral data matched those of the commercial compound. ^{1}H NMR (500 MHz, CD_3OD) δ 4.94 (d, 1H, H-1, $J_{1,3}$ = 2.0 Hz, $J_{1,2}$ = 6.0 Hz), 3.34 (dd, 1H, H-2, $J_{2,3}$ = 2.0 Hz, $J_{2,1}$ = 6.0 Hz), 2.76 (ddd, 1H, H-3, $J_{3,1} = J_{3,2}$ = 2.0 Hz, $J_{3,4}$ = 7.0 Hz), 2.46-2.36 (m, 3H, H-5, H-6a, H-6b), 2.21 (dd, 1H, H-4, $J_{4,3}$ = 7.0 Hz, $J_{4,5}$ = 9.0 Hz); ^{13}C NMR (125 MHz, CD_3OD) δ 143.8 (C-1), 102.9 (C-2), 78.2, 68.9, 67.4, 60.1. HR-ESMS calcd for $C_6H_{10}O_4Na$: 169.0471. Found: 169.0471.

3,6-Di-*O*-*tert*-butyldimethylsilyl-D-glucal (9)



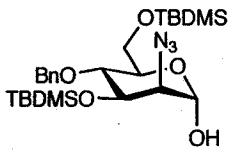
To a cooled solution of D-glucal (**8**) (2.37 g, 16.2 mmol) in dimethylformamide (60 mL) at 0 °C, imidazole (5.44 g, 79.9 mmol) and *tert*-butyldimethylsilyl chloride (5.07 g, 33.6 mmol) were added consecutively. The mixture was allowed to stir for 2 h. The solvent was evaporated and the crude was dissolved in dichloromethane (60 mL) and washed with water (2 × 40 mL). After drying over anhydrous Na_2SO_4, filtration and removal of solvent, the crude was obtained. Purification by silica gel column chromatography using hexane/ethyl acetate (1:10) as eluent afforded a white solid (4.82 g, 12.9 mmol) which was obtained in 80% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.28 (dd, 1H, H-1, $J_{1,3}$ = 2.0 Hz, $J_{1,2}$ = 6.0 Hz), 4.62 (dd, 1H, H-2, $J_{2,3}$ = 2.0 Hz, $J_{2,1}$ = 6.0 Hz), 4.24 (ddd, 1H, H-3, $J_{3,1} = J_{3,2}$ = 2.0 Hz, $J_{3,4}$ = 6.5 Hz), 3.98 (dd, 1H, H-6a, $J_{6a,5}$ = 4.5 Hz, $J_{6a,6b}$ = 11.0 Hz), 3.89 (dd, 1H, H-6b, $J_{6b,5}$ = 3.5 Hz, $J_{6b,6a}$ = 11.0 Hz), 3.85-3.80 (m, H-5), 3.76 (ddd, 1H, H-4, $J_{4,OH}$ = 4.0 Hz, $J_{4,3}$ = 6.5 Hz, $J_{4,5}$ = 9.0 Hz), 2.51 (d, 1H, OH, $J_{OH,4}$ = 4.0 Hz), 0.92, 0.91 (s, 9H, CH_3), 0.13, 0.12, 0.09, 0.09 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 143.3 (C-1), 103.4 (C-2), 77.9 (C-5), 71.1 (C-4), 69.9 (C-3), 63.2 (C-6), 25.9, 25.8 (CH_3), 18.4, 18.1 ($\underline{C}(CH_3)_3$), -4.45, -4.49 ($Si\underline{C}H_3$). HR-ESMS calcd for $C_{18}H_{38}O_4Si_2Na$: 397.2201. Found: 397.2199.

4-*O*-Benzyl-3,6-di-*O*-*tert*-butyldimethylsilyl-D-glucal (6)

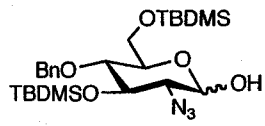


Sodium hydride (0.365 g, 15.2 mmol) was added to a solution of **9** (1.80 g, 4.81 mmol) in dry dimethylformamide (15 mL) at 0 °C, followed by benzyl bromide (1.15 mL, 9.63 mmol). The ice bath was removed and the reaction was allowed to warm to room temperature. After 1 h, on completion of reaction, methanol (2 mL) was added and the mixture was concentrated. Dichloromethane (60 mL) was added and the organic solution was washed with aqueous $NaHCO_3$ (2 × 40 mL) and brine (1 × 40 mL). After drying the organic layer over anhydrous Na_2SO_4 and filtration, the solvent was removed by evaporation to afford the crude. Purification was carried out using the eluent system hexane/ethyl acetate (100:1) on the silica gel column. The title compound was obtained as a colorless oil in 99% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42-7.26 (m, 5H. Ar), 6.32 (dd, 1H, H-1, $J_{1,3}$ = 1.5 Hz, $J_{1,2}$ = 6.0 Hz), 4.83 (d, 1H, PhC$\underline{H}_2$, J = 11.0 Hz), 4.74 (d, 1H, PhC$\underline{H}_2$, J = 11.0 Hz), 4.65 (dd, 1H, H-2, $J_{2,3}$ = 2.5 Hz, $J_{2,1}$ = 6.0 Hz), 4.36 (ddd, 1H, H-3, $J_{3,1}$ = 1.5 Hz, $J_{3,2}$ = 2.5 Hz, $J_{3,4}$ = 6.0 Hz), 3.97(dd, 1H, H-6a, $J_{6a,5}$ = 4.5 Hz, $J_{6a,6b}$ = 11.5 Hz), 3.94-3.90 (m, 1H, H-5), 3.86 (dd, 1H, H-6b, $J_{6b,5}$ = 2.0 Hz, $J_{6b,6a}$ = 11.5 Hz), 3.66 (dd, 1H, H-4, $J_{4,3}$ = 6.0 Hz, $J_{4,5}$ = 8.0 Hz), 0.93, 0.92 (s, 9H, 3 × CH_3), 0.12, 0.11 (s, 3H, CH_3), 0.08 (s, 6H, 2 × CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 128.4, 127.9, 127.8 (CH, Ar), 143.4 (C-1), 138.5 (C, Ar), 103.3 (C-2), 78.1(C-5), 76.6 (C-4), 74.0 (Ph$\underline{C}H_2$), 69.0 (C-3), 62.0 (C-6), 26.0, 25.9 (CH_3), 18.4, 18.1 ($\underline{C}(CH_3)_3$), -4.4, -4.6, -5.1, -5.3 (Si$\underline{C}H_3$). HR-ESMS calcd for $C_{25}H_{44}O_4Si_2Na$: 487.2670. Found: 487.2671.

2-Azido-4-*O*-benzyl-2-deoxy-3,6-di-*O*-*tert*-butyldimethylsilyl-α-D-galactopyranose (12) and 2-azido-4-*O*-benzyl-2-deoxy-3,6-di-*O*-*tert*-butyldimethylsilyl-α/β-D-glucopyranose (13)



12



13

Compound **6** (1.52 g, 3.26 mmol) was dissolved in dry acetonitrile (25 mL) and the solution was cooled to –20 °C. Cerium ammonium nitrate (6.61 g, 12.1 mmol) was added to the stirring mixture, followed by sodium azide (0.39 g, 6.06 mmol). The yellow solution was allowed to warm to –10 °C. After 2 h, when TLC showed no starting material present, a mixture of ethyl acetate (50 mL) and aqueous $NaHCO_3$ (25 mL) was added slowly. The yellow precipitate was filtered over celite and the organic layer was separated. It was washed with sodium bicarbonate solution (2 × 40 mL), water (1 × 40 mL) and brine (1 × 40 mL). The organic extract was then dried over anhydrous Na_2SO_4, filtered and the solvent was evaporated to afford the azidonitrate mixture.

For denitration, a solution of the crude in acetonitrile (20 mL) and thiophenol (0.7 mL, 10.1 mmol) was treated with *N*, *N*-diisopropylethylamine (0.50 mL, 3.59 mmol). After 1 h, the mixture was concentrated and the residue was subjected to column chromatography with hexane/ethyl acetate (100:1→1:1). Two main fractions were eluted with the first fraction being the glucose derivative **13** (0.75 g, 1.43 mmol, 44%

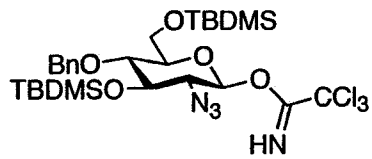
yield) as a mixture of α/β (1:1). Further elution afforded the mannose derivative **12** (0.17 g, 0.32 mmol, 10% yield) as the α-anomer only.

Mannose derivative **12**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42-7.26 (m, 5H. Ar), 5.73 (d, 1H, H-1, $J_{1,2}$ = 1.8 Hz), 4.86 (d, 1H, PhC$\underline{H}_2$, J = 11.4 Hz), 4.61 (d, 1H, PhC$\underline{H}_2$, J = 11.4 Hz), 4.30 (dd, 1H, H-3, $J_{3,2}$ = 3.6 Hz, $J_{3,4}$ = 9.0 Hz), 3.84-3.70 (m, 3H, H-2, H-6a, H-6b, H-5), 3.65 (t, 1H, H-4, $J_{4,3}$ =, $J_{4,5}$ = 9.0 Hz), 0.96, 0.89 (s, 9H, 3 × CH_3), 0.15, 0.13, 0.05, 0.03 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.4, 128.3, 127.7, 127.6, 127.5, 92.8, 75.7, 75.0, 73.3, 72.5, 65.2, 62.9, 45.9, 25.92, 25.86, 18.4, 18.0 ($\underline{C}(CH_3)_3$), -4.4, -4.7, -5.2, -5.4 (Si$\underline{C}H_3$). HR-ESMS calcd for $C_{25}H_{45}N_3O_5Si_2Na$: 546.2790. Found: 546.2792.

Glucose derivative **13** (α/β: 1/1 mixture): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36-7.26 (m, 10H. Ar), 5.33 (d, 1H, H-1α, $J_{1\alpha,2\alpha}$ = 3.0 Hz), 4.84 (d, 1H, PhC$\underline{H}_2$, J = 11.5 Hz), 4.83 (d, 1H, PhC$\underline{H}_2$, J = 11.5 Hz), 4.69 (d, 1H, PhC$\underline{H}_2$, J = 11.5 Hz), 4.66 (d, 1H, PhC$\underline{H}_2$, J = 11.5 Hz), 4.58 (d, 1H, H-1β, $J_{1\beta,2\beta}$ = 8.5 Hz), 4.03 (dd, 1H, H-3α, $J_{3\alpha,2\alpha} = J_{3\alpha,4\alpha}$ = 9.0 Hz) 3.90-3.74 (m, 5H, H-6aα, H-6bα, H-6aβ, H-6bβ, H-5β), 3.40-3.52 (m, H-4β, H-4α, H-3β), 3.28 (ddd, 1H, H-5α, J = 2.0, 4.0, 6.0 Hz), 3.23 (dd, 1H, H-2α, $J_{2\alpha,1\alpha}$ = 3.0 Hz, $J_{2,3}$ = 9.0 Hz), 3.15 (dd, 1H, H-2β, $J_{2\beta,1\beta}$ = 8.5 Hz, $J_{2\beta,3\beta}$ = 9.0 Hz), 0.96, 0.95, 0.90, 0.90 (s, 9H, 3 × CH_3), 0.22, 0.19, 0.09, 0.08, 0.07, 0.06, 0.05, 0.04 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.5, 138.3 (C, Ar), 128.33, 128.28, 127.6, 127.43, 127.40, 127.2 (CH, Ar), 96.3, 92.2, 78.9, 78.4, 76.2, 75.4, 74.7, 74.6, 72.5, 72.1, 69.4, 66.3, 62.3, 62.2,

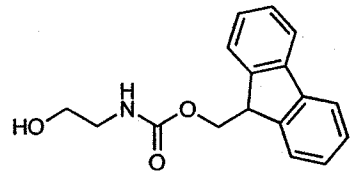
25.97, 25.95, 18.2, 18.1, -4.0, -4.1, -4.4, -4.8, -5.0, -5.1, -5.3, -5.4. HR-ESMS calcd for $C_{25}H_{45}N_3O_5Si_2Na$: 546.2790. Found: 546.2793.

2-Azido-4-*O*-benzyl-2-deoxy-3,6-di-*O*-*tert*-butyldimethylsilyl-β-D-glucopyranosyl trichloroacetimidate (5)



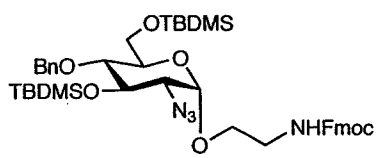
To a solution of **13** (0.47 g, 0.89 mmol) in dry dichloromethane (4 mL) with molecular sieves 4Å, potassium carbonate (0.80 g, 5.79 mmol) was added followed by acetonitrile (0.54 mL, 5.39 mmol) at rt. The mixture was stirred for 8 h at rt. On disappearance of starting material on TLC, the mixture was filtered through celite, concentrated and subjected to column chromatography. Elution with the eluent system (0.1% Et_3N) of hexane/ethyl acetate (60:1) and evaporation of organic solvents afforded the title compound as a colorless oil (0.42 g, 0.63 mmol) in 71% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.69 (s, 1H, NH), 7.40-7.24 (m, 5H. Ar), 5.65 (d, 1H, H-1, $J_{1,2}$ = 8.0 Hz), 4.82 (d, 1H, PhC$\underline{H}_2$, J = 11.0 Hz), 4.73 (d, 1H, PhC$\underline{H}_2$, J = 11.0 Hz), 3.87 (d, 2H, H-6a, H-6b, J = 2.0 Hz), 3.70-3.54 (m, 2H, H-3, H-4), 3.49 (dd, 1H, H-2, $J_{2,1}$ = 8.0 Hz, $J_{2,3}$ = 9.5 Hz), 3.40-3.34 (m, 1H, H-5), 0.97, 0.89 (s, 9H, 3 × CH_3), 0.19, 0.10, 0.06, 0.03 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 161.0 (C=N), 138.3 (C, Ar), 128.5, 127.6, 127.4 (CH, Ar), 96.7 (C-1), 90.6 ($\underline{C}Cl_3$), 77.8, 75.3 ((C-3, C-4), 76.4 (C-5), 74.7 (Ph$\underline{C}H_2$), 67.5 (C-2), 61.6 (C-6), 25.9, 25.8 (CH_3), 18.1 ($\underline{C}(CH_3)_3$), -4.1, -4.5, -4.9, -5.4 (Si$\underline{C}H_3$). HR-ESMS calcd for $C_{27}H_{45}N_4O_5NaSi_2Cl_3$: 689.1886. Found: 689.1888.

2-*N*-(9-Fluorenylmethoxycarbonylamino)-ethanol



To a cold stirring solution of ethanolamine (0.054 mL, 0.90 mmol) at 0 °C in THF, 10% aqueous sodium bicarbonate (5 mL) was added, followed by 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc-OSu, 0.30 g, 0.89 mmol). After 1 h, the turbid mixture was evaporated under reduced pressure and the residue was dissolved in ethyl acetate (40 mL). The organic mixture was washed with 1M HCl (2 × 15 mL), water (1 × 15 mL) and brine (1 × 15 mL). The organic extract was then dried over anhydrous Na_2SO_4 and filtered. On evaporation of solvent, white crystals (0.25 g, 0.87 mmol) were formed in 97% yield. ^{1}H NMR (500 MHz, CD_3OD) δ 7.73 (d, 2H, Ar, J = 7.5 Hz), 7.60 (d, 2H, Ar, J = 7.5 Hz), 7.35 (t, 2H, Ar, J = 7.5 Hz), 7.27 (t, 2H, Ar, J = 7.5 Hz), 4.75 (s, 1H, NH), 4.32 (d, 2H, J = 7.0 Hz), 4.16 (t, 1H, J = 7.0 Hz), 3.58 (t, 2H, J = 6.0 Hz), 3.23 (t, 2H, J = 6.0 Hz); ^{13}C NMR (125 MHz, CD_3OD) δ 145.3, 142.6, 128.9, 128.2, 126.2, 121.0, 67.9, 62.1, 48.5, 44.4. HR-ESMS calcd for $C_{17}H_{17}NO_3Na$: 306.1101. Found: 306.1104.

2-*N*-(9-Fluorenylmethoxycarbonylamino)-ethyl 2-azido-4-O-benzyl-2-deoxy-3,6-di-O-*tert*-butyldimethylsilyl-α-D-glucopyranoside (14)



To a stirred solution of the β-imidate **5** (0.40 g, 0.60 mmol) in dry tetrahydrofuran in the presence of molecular sieves 4Å at 0 °C. 2-*N*-(9-fluorenylmethyl oxycarbonyl)-ethanol (0.20 g, 0.71 mmol) was added followed by boron trifluoride etherate (8 μL, 0.063 mmol) as the promoter. After 1 h, The reaction was quenched with Et_3N (0.5 mL). The mixture was concentrated and purified by column chromatography with hexane/ethyl acetate (100:1→6:1). Compound **14** (0.16 g, 0.20 mmol) was obtained in 34% yield. ^1H NMR (500 MHz, $CDCl_3$) δ 7.78-7.72 (m, 2H, Ar), 7.60 (d, 2H, Ar, J = 7.8 Hz), 7.42-7.28 (m, 9H. Ar), 4.82 (d, 1H, PhC$\underline{H}_2$, J = 11.2 Hz), 4.65 (d, 1H, PhC$\underline{H}_2$, J = 11.2 Hz), 4.44-4.38 (m, 2H), 4.18-4.24 (m, 2H), 3.78-3.68 (m, 5H), 3.36-3.46 (m, 3H), 3.26-3.10 (m, 2H), 0.96, 0.88 (s, 9H, 3 × CH_3), 0.19, 0.10, 0.04, 0.02 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.2, 143.9, 138.0, 128.3, 127.6, 127.0, 102.3, 78.4, 75.8, 75.3, 74.8, 69.4, 67.9, 66.6, 62.1, 47.3, 31.6, 25.94, 25.86 18.3, 18.2, -4.00, -4.33, -5.10, -5.41. HR-ESMS calcd for $C_{42}H_{60}N_4O_7Si_2Na$: 811.3893. Found: 811.3890.

3.8 References

(1) Maruyama, T.; Toida, T.; Imanari, T.; Yu, G.; Linhart, R. J. *Carbohydr. Res.* **1998**, *306*, 35-43.

(2) Belting, M.; Borsig, L.; Fuster, M. M.; Brown, J. R.; Persson, L.; Fransson, L.-Å.; Esko, J. D. *Proc. Natl. Acad. Sci.* **2002**, *99*, 371-376.

(3) Belting, M.; Havsmark, B.; Jönsson, M.; Persson, S.; Fransson, L.-Å. *Glycobiology* **1996**, *6*, 121-129.

(4) Belting, M.; Persson, L.; Fransson, L.-Å. *Biochem. J.* **1999**, *338*, 317-323.

(5) Marks, R. M.; Lu, H.; Sundaresan, R.; Toida, T.; Suzuki, A.; Imanari, T.; Hernáiz, M. J.; Lindhart, R. J. *J. Med. Chem.* **2001**, *44*, 2178-2187.

(6) Manku, S.; Hall, D. G. *Org. Lett.* **2002**, *4*, 31-34.

(7) Misra, A. K.; Ding, Y.; Lowe, J. B.; Hindsgaul, O. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1505-1509.

(8) Schmidt, R. R. *Angew. Chem. Int. Ed.* **1986**, *25*, 212-235.

(9) Banoub, J.; Boullanger, P.; Lafont, D. *Chem. Rev.* **1992**, *92*, 1167-1195.

(10) Schmidt, R. R.; Kinzy, W. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 21-123.

(11) Herzner, H.; Reipen, T.; Schultz, M.; Kunz, H. *Chem. Rev.* **2000**, *100*, 4495-4537.

(12) Lemieux, R. U.; Ratcliffe, R. M. *Can. J. Chem.* **1978**, *57*, 1244-1251.

(13) Alper, P. B.; Hung, S.-C.; Wong, C.-H. *Tetrahedron Lett.* **1996**, *37*, 6029-6032.

(14) Holm, B.; Linse, S.; Kihlberg, J. *Tetrahedron* **1998**, *54*, 11995-12006.

(15) Poletti, L.; Fleischer, M.; Vogel, C.; Guerrini, M.; Torri, G.; Lay, L. *Eur. J. Org. Chem.* **2001**, *14*, 2727-2734.

(16) Kinzy, W.; Schmidt, R. R. *Tetrahedron Lett.* **1987**, *28*, 1981-1984.

(17) Halmos, T.; Montserret, R.; Filippi, J.; Antonakis, K. *Carbohydr. Res.* **1987**, *170*, 57-69.

(18) Berlin, W. K.; Zhang, W.; Shen, T. Y. *Tetrahedron* **1991**, *47*, 1-20.

(19) Gauffeny, F.; Marra, A.; Shun, L. K. S.; Sinaÿ, P.; Tabeur, C. *Carbohydr. Res.* **1991**, *219*, 237-240.

(20) Rademann, J.; Schmidt, R. R. *Carbohydr. Res.* **1995**, *269*, 217-225.

(21) Caputo, R.; Cassano, E.; Longobardo, L.; Palumbo, G. *Tetrahedron* **1995**, *51*, 12337-12350.

(22) Paulsen, H.; Peters, S.; Bielfeldt, T.; Meldal, M.; Bock, K. *Carbohydr. Res.* **1995**, *268*, 17-34.

Chapter 4

Identification of New and Improved Boronic Acids for the Development of Receptors and Sensors in Aqueous Recognition of Carbohydrates

4.1 Introduction

In Chapters 1 and 2, the importance of glycoconjugates in the form of glycolipids and glycoproteins was recognized because of their roles in various biological and pathological processes. These processes include inflammation, cancer metastasis and immune response among others.[1] Proteins (in particular lectins) also play a complementary role in these natural processes due to their ability to recognize glycoconjugates. Small molecules that mimic the roles of proteins such as lectins, will prove useful in the development of new therapeutics and diagnostics. Along this line, boronic acids, which can form strong reversible covalent bonds with diol-containing saccharides can fulfil this role. There has been increased interest in developing oligomeric boronic acids that can bind strongly to cell-surface carbohydrate biomarkers through the use of multivalency effects.[2,3] These oligomeric boronic acids with the ability to mimic lectins have been coined "boronolectins".[1] Oligomeric boronic acids can be derived from a monoboronic acid that has the following features:

(1) Ease of synthesis

A functional group (e.g, a carboxyl or amine group) must be present to allow conjugations of the boronic acid unit to a more complex scaffold.

(2) High solubility in water

Cell-labeling studies will require the use of an organic co-solvent to solubilize the boronic acid sensor. This can be tolerated in *in vitro* experiments but not in *in vivo* experiments. At present, the "Wulff-type" *ortho*-dialkylaminomethyl arylboronic acids[4] (Figure 4-1) are the established standards for the recognition of simple reducing sugars, but they tend to have limited solubility in aqueous solutions. As described in Chapter 1, the hydrolysis mechanism causes the benzylic amine to be protonated.

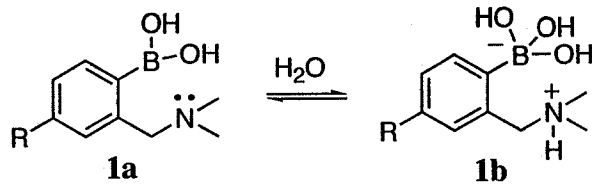


Figure 4-1. Zwitterionic nature of *ortho*-dialkylaminomethyl arylboronic acids.

(3) Ability to bind the pyranose forms of saccharides

As mentioned in Chapter 1, under aqueous conditions, saccharides tend to isomerize[5-8] from the pyranose to the furanose form, which presents vicinal planar diols for tighter binding with a boronic acid moiety. A minimum amount of angle strain is present in the resulting boronate ester. When non co-planar diols of a glycoside are forced to adopt a planar boronate ester conformation, a highly unfavorable conformational change is induced in the resulting puckered sugar ring.[9,10] Our group[6] has shown that even by designing libraries of diboronic acids with the combined effect of the "Wulff-type" arylboronic acids and electron-withdrawing cyanide groups into the arylboronic acid, no

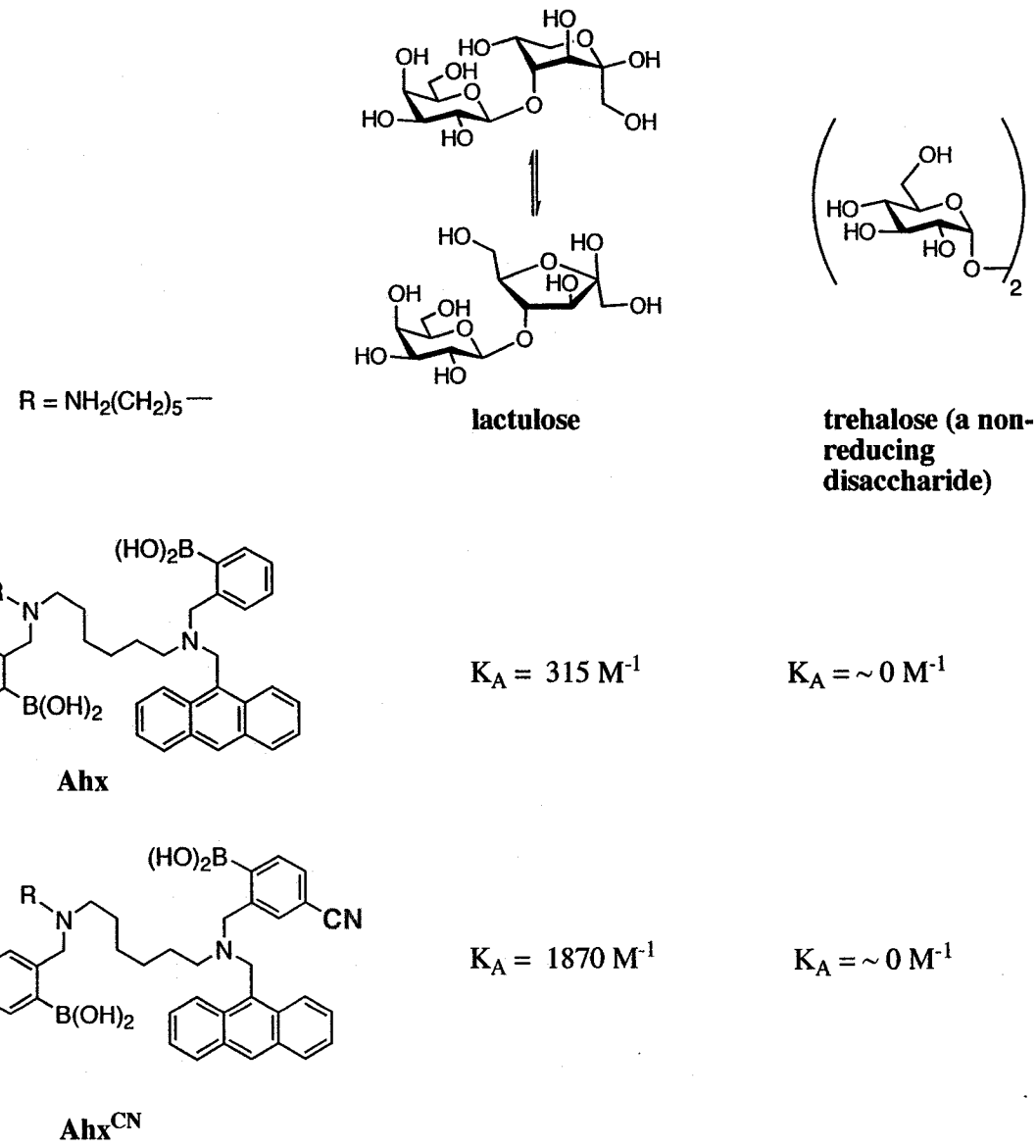


Figure 4-2. Binding constants[6] of bis-boronic acids with disaccharides.

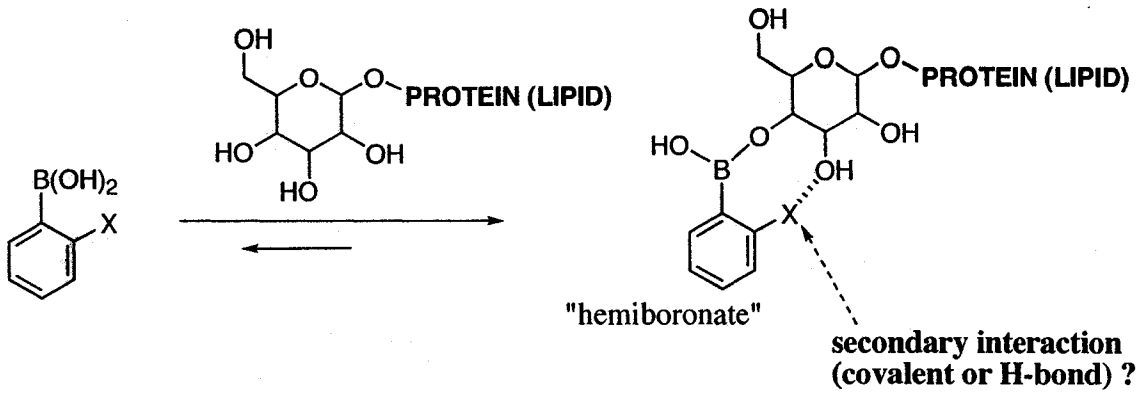
binding is observed with disaccharides (trehalose, for instance) that cannot isomerize to the five-membered furanose ring. In the case of lactulose (Figure 4-2) the 4-hydroxyl is free and isomerization to the furanose form can take place. Hence, an increase of the binding constants is observed with electron-poor arylboronic acids. However, no increase in binding constants is observed for trehalose because it is locked in the

pyranose forms. Clearly, the search for a monoboronic acid unit that forms esters with adjacent diols of hexopyranosides is still ongoing.

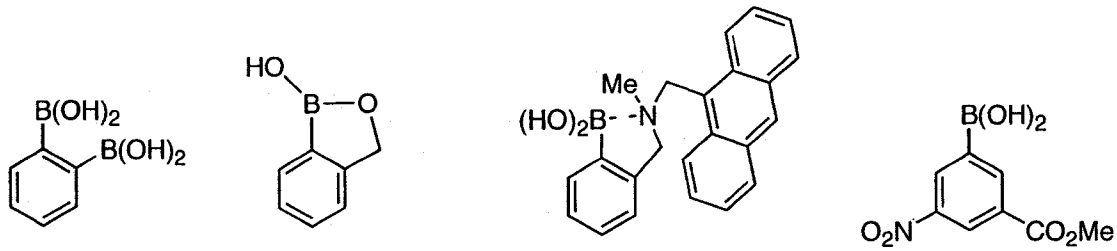
4.2 Screening of ortho-substituted arylboronic acids

To achieve recognition of the pyranose form of saccharides, we envisioned[11] that anchimeric assistance of an ortho group on the arylboronic acid could favor secondary interactions with adjacent hydroxyls in hexopyranosides. In the "hemiboronate" complex, the secondary interaction could be of covalent nature or through hydrogen-bonding forces (Figure 4-3). The ortho substituents depicted in Figure 4-3 range from electron-poor (nitro) to electron-rich functional groups (methoxy).

Among the ortho-substituted arylboronic acids shown in Figure 4-3, the most challenging to synthesize and purify was undoubtedly *ortho*-phenylenediboronic acid (**2c**). Compared to the *meta* and *para* derivatives, *ortho*-phenylenediboronic acid is less stable and more difficult to synthesize.[12] Several routes were attempted as shown in Scheme 4.1. The first route, starting from 1,2 dibromobenzene (**4**), involved the synthesis of the known compound 1,2-bis(trimethylsilyl) benzene[13,14] (**5**), which was converted to 1,2 bis-(dibromoboryl) benzene (**6**) in two steps with boron tribromide as reagent, first at –78 °C, then at 95 °C.



2a	X = H	**2e**	X = OCH_3	**2i**	X = CO_2H	**2m**	X = CH_2OCH_3
2b	X = CH_3	**2f**	X = NO_2	**2j**	X = CO_2Et	**2n**	X = CH_2OH (cyclic form)
2c	X = $B(OH)_2$	**2g**	X = NH_2	**2k**	X = $CONH_2$	**2o**	X = CH_2NMe_2
2d	X = F	**2h**	X = $NHCOCH_3$	**2l**	X = CHO	**2p**	X = CH_2 (anthr)NMe

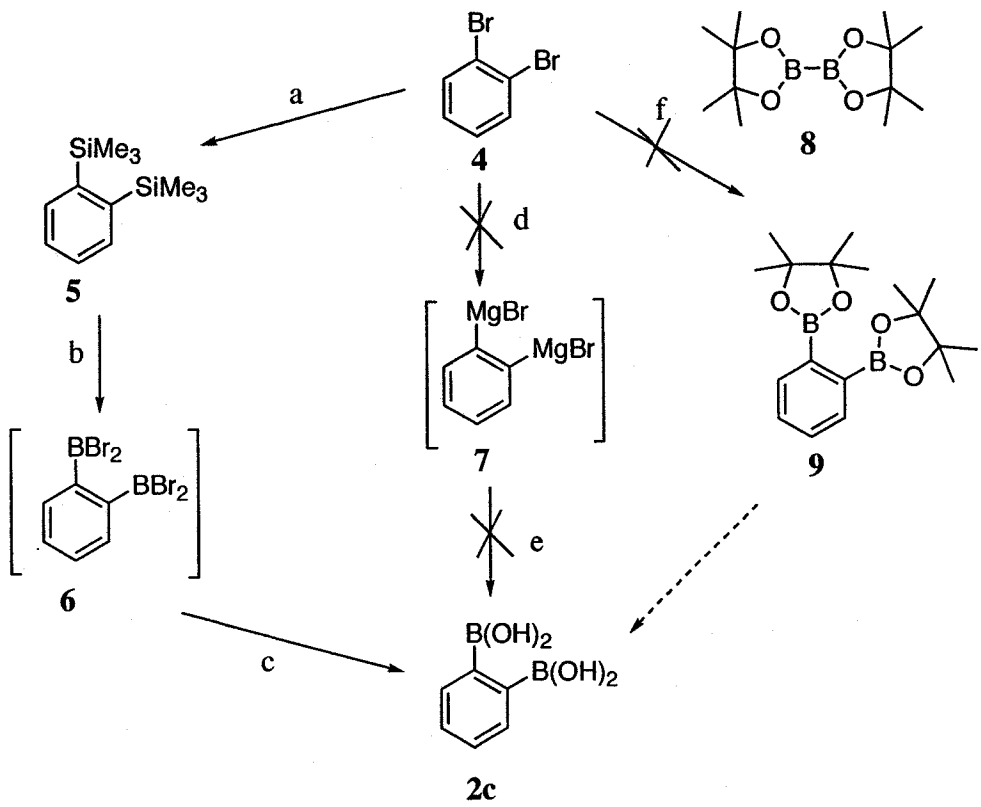


2c **2n** **2p** **3**

Figure 4-3. Secondary interactions afforded by ortho-substituted arylboronic acids.

The reaction of 1,2 bis-(dibromoboryl) benzene (**6**)[15] with methanol was carried out at 0 °C to give the easily hydrolyzable methyl ester of the diboronic acid, which then provided the crude black mixture of diboronic acid **2c** upon acidic work-up. The DEAM-polystyrene resin[16] developed by our group was used to purify the diboronic acid **2c** followed by a precipitation with hexane/ethyl acetate. The white solid of pure **2c**

was finally obtained with a low yield of 6%. Loss of material was incurred due to its low solubility in organic solvents such as ethyl acetate, tetrahydrofuran and and dichloromethane. Other attempts to synthesize *ortho*-phenylenediboronic (**2c**) failed. When the bromide groups in 1,2 dibromobenzene (**4**) were metalated to a Grignard reagent, the intermediate was made to react with tri-isopropylborate but none of the desired compound **2c** was observed.[17] The alternative procedure was adapted from a patent,[18] which involved the reaction between 1,2 dibromobenzene (**4**),



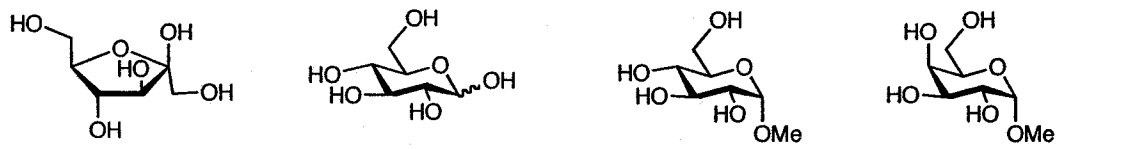
Scheme 4.1 Reactions and conditions: (a) Mg, TMSCl, HMPA, I_2, 100 °C, 72 h, 87%; (b) (i) BBr_3 (1 M in hexane), DCM, -78 °C, 4 h, (ii) BBr_3 (1 M in hexane), DCM, 95 °C, 16 h; (c) MeOH, 10% HCl, 6%; (d) Mg, THF, 60-64 °C, 6 h; (e) (i) $B(O^iPr)_3$, THF, -78 °C, 6 h, (ii) 1 M HCl; (f) $PdCl_2(dppf)$, KOAc, DMSO, 90 °C, 24 h.

bis(pinacolato) diboron **8** and [1,1'-bis(diphenylphosphino) ferrocene] dichloro-palladium (II) dichloromethane adduct at a temperature of 90 °C. However, a crude black mixture was obtained and it did not show the presence of the desired product by mass spectrometry. ^{1}H NMR analysis showed a complex mixture of aromatic side-products.

4.2.1 ARS (Alizarin Red S) competitive assay

As mentioned in Chapter 1, the ARS assay[19,20] provides a quick and colorful means of determining whether a boronic acid binds to a saccharide. The complex between the boronic acid and ARS produces a yellow colored solution, which serves as the control. When the saccharide is added, this sets up another equilibrium whereby there is complexation between the saccharide and the boronic acid, and the free ARS released produces a red color. So, the higher the binding affinity between a boronic acid and a carbohydrate molecule, the more intense is the resulting red solution.

The prearranged cis-diols in fructose account for the strong binding constant between fructose and phenylboronic acid. The binding affinity of phenylboronic acid with glucose is significantly lower. We wanted to compare the relative affinities, at pH 7.4, of a given boronic acid towards different carbohydrates, namely, fructose, glucose, methyl α-D-glucopyranoside, methyl α-D-galactopyranoside and trehalose (depicted in Figures 4-2 and 4-4). The initial colorimetric assays were done by Heidi Chau, a summer student in our group, and the most promising ones were repeated. In all cases, the reddish color was more intense



D-fructose **D-glucose** **methyl α-D-glucopyranoside** **methyl α-D-galactopyranoside**

Figure 4-4. Monosaccharides used in ARS competitive assay.

with fructose, showing the greatest binding. The ARS binding assays were done with the saccharides and *ortho*-dialkylaminomethylphenylboronic acids **2o** and **2p**. In the case of **2p**, which contains a nonpolar anthrancene ring, up to 80% methanol was required for solubilization, and no color change was observed with methyl α-D-glucopyranoside. In the case of the *ortho*-dimethylaminomethylphenylboronic acid (**2o**), 33% of methanol was used. Again, no orange color was observed indicating absence of binding. On the other hand, (2-hydroxymethylphenyl)boronic acid **2n** gave exciting results with the glycosides. An orange colored solution indicating weak binding to the model pyranosides was produced. Even the low pK_a boronic acid **3**[21] did not show any orange color. The pictures below illustrate the color changes of certain boronic acids when tested with the saccharides.

Selected assays (at pH 7.4):

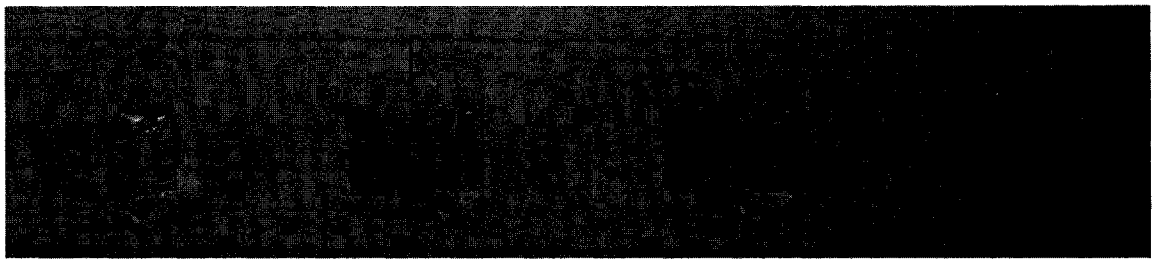


Figure 4-5. Vials of ARS (10^{-4} M in 0.1 M phosphate buffer) and cyclic boronic acid **2n** (0.02 M) containing: A, no sugar; B, 0.5 M fructose; C, 0.5 M glucose; D, 1.5 M methyl α-D-glucopyranoside.

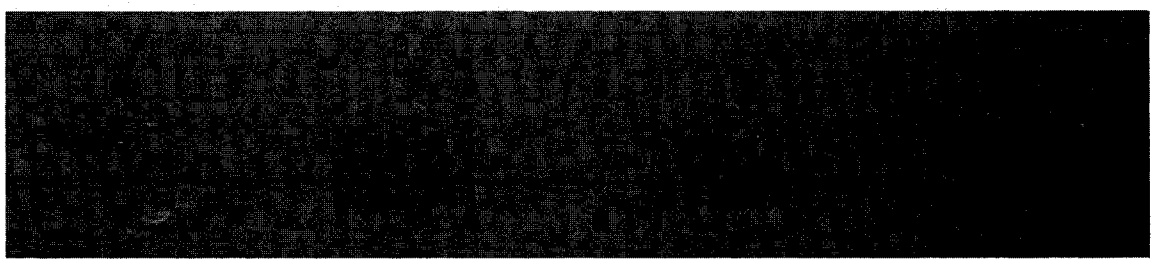


Figure 4-6. Vials of ARS (10^{-4} M in 0.1 M phosphate buffer) and phenylboronic acid (**2a**) (0.02 M) containing: A, no sugar; B, 0.5 M fructose; C, 0.5 M glucose; D, 1.5 M methyl α-D-glucopyranoside.

Figure 4-7. Vials of ARS (10^{-4} M in 0.1 M phosphate buffer) and phenylboronic acid (**2a**) (0.01 M) containing: A, no sugar; B, 0.5 M methyl α-D-galactopyranoside; C, 0.5 M methyl α-D-glucopyranoside; D, 0.5 M methyl β-D-galactopyranoside.

Figure 4-8. Vials of ARS (10^{-4} M in 0.1M phosphate buffer) and cyclic boronic acid **2n** (0.01 M) containing: A, no sugar; B, 0.5 M methyl α-D-galactopyranoside; C, 0.5 M methyl α-D-glucopyranoside; D, 0.5 M methyl β-D-galactopyranoside.

4.2.2 Summary of results of ARS competitive assays

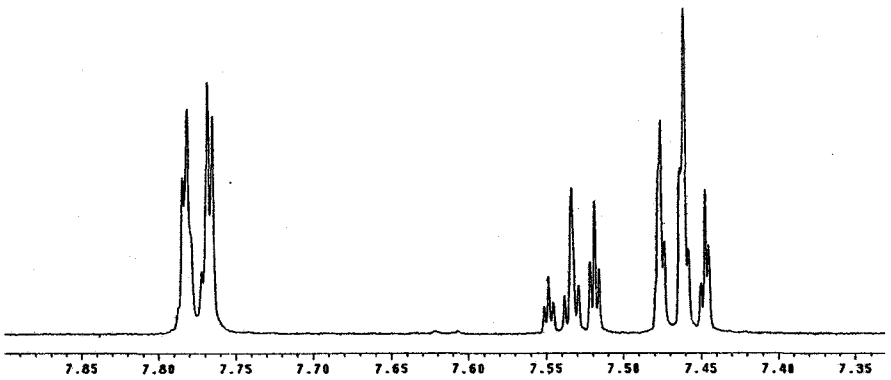
Established standard boronic acids such as **2o**, **2p** and **3** (with low pK_a values) have strong affinities with reducing sugars giving rise to red or orange solutions in ARS assays. However, they required co-solvents for solubilization and did not show any significant binding to the model methyl glycosides. Only the cyclic boronic acid **2n** indicated weak binding to the glycosides in addition to strong binding with reducing sugars. These results confirm that boronic acids with high Lewis acidities do not necessarily bind glycosides.

4.3 ^{1}H NMR studies confirming binding of cyclic boronic acid 2n with glycopyranosides

The competitive three-component ARS assay is certainly useful as a preliminary qualitative assay. To better compare the binding affinities of boronic acids toward glycosides, additional evidence was required. To this end, ^{1}H NMR titrations that would afford quantitative binding measurements at pH 7.4 were attempted. We compared the ^{1}H NMR spectra of aqueous solutions of boronic acid alone, and those of the complexed species between boronic acid and methyl α-D-glucopyranoside. The complexed boronate ester was formed by saturating the solution of boronic acid with the glycoside.

In contrast to glucose and fructose (which will be discussed later in Section 4.4), with the glycosides the bound and unbound forms of the different boronic acids were not distinguishable by ^{1}H NMR (i.e., the complexation equilibrium is faster than the NMR

(a)



(b)

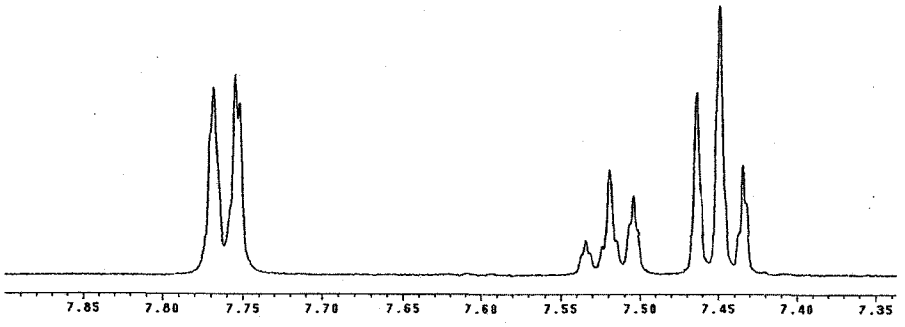
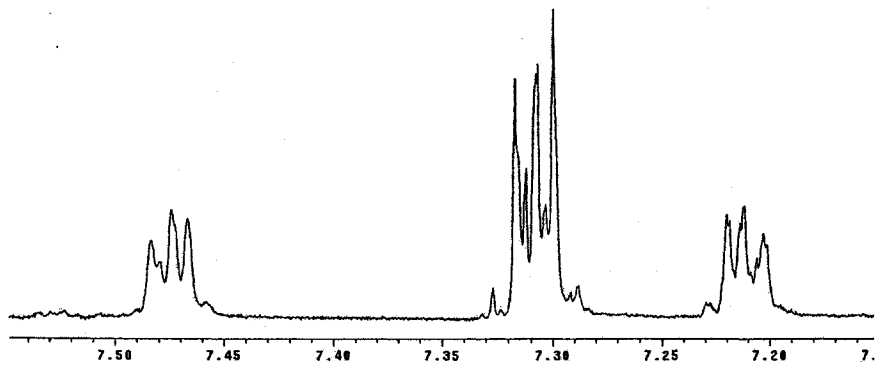


Figure 4-9. **(a)** Aromatic region of ^1H NMR (500 MHz) spectrum of a solution of phenylboronic acid (**2a**) in phosphate buffered D_2O solution (0.01 M) at pH 7.4 (b) with 0.25 M methyl α-D-glucopyranoside at pH 7.4.

time scale). Therefore, it is important to emphasize that the extent of dynamic peak broadening does not depend only on the concentrations, but also on the virtual chemical shift differences of bound *vs* unbound boronic acid, which may be large enough to allow broadening even in the case of weak binders.

(a)



(b)

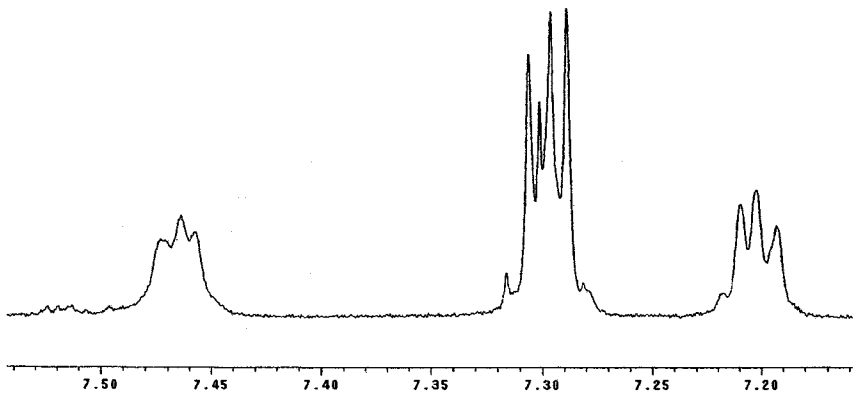


Figure 4-10. (a) Aromatic region of ^{1}H NMR (500 MHz) spectrum of a solution of "Wulff-type" boronic acid **2o** in phosphate buffered D_2O solution (0.01 M) at pH 7.4 (b) with 0.25 M methyl α-D-glucopyranoside at pH 7.4.

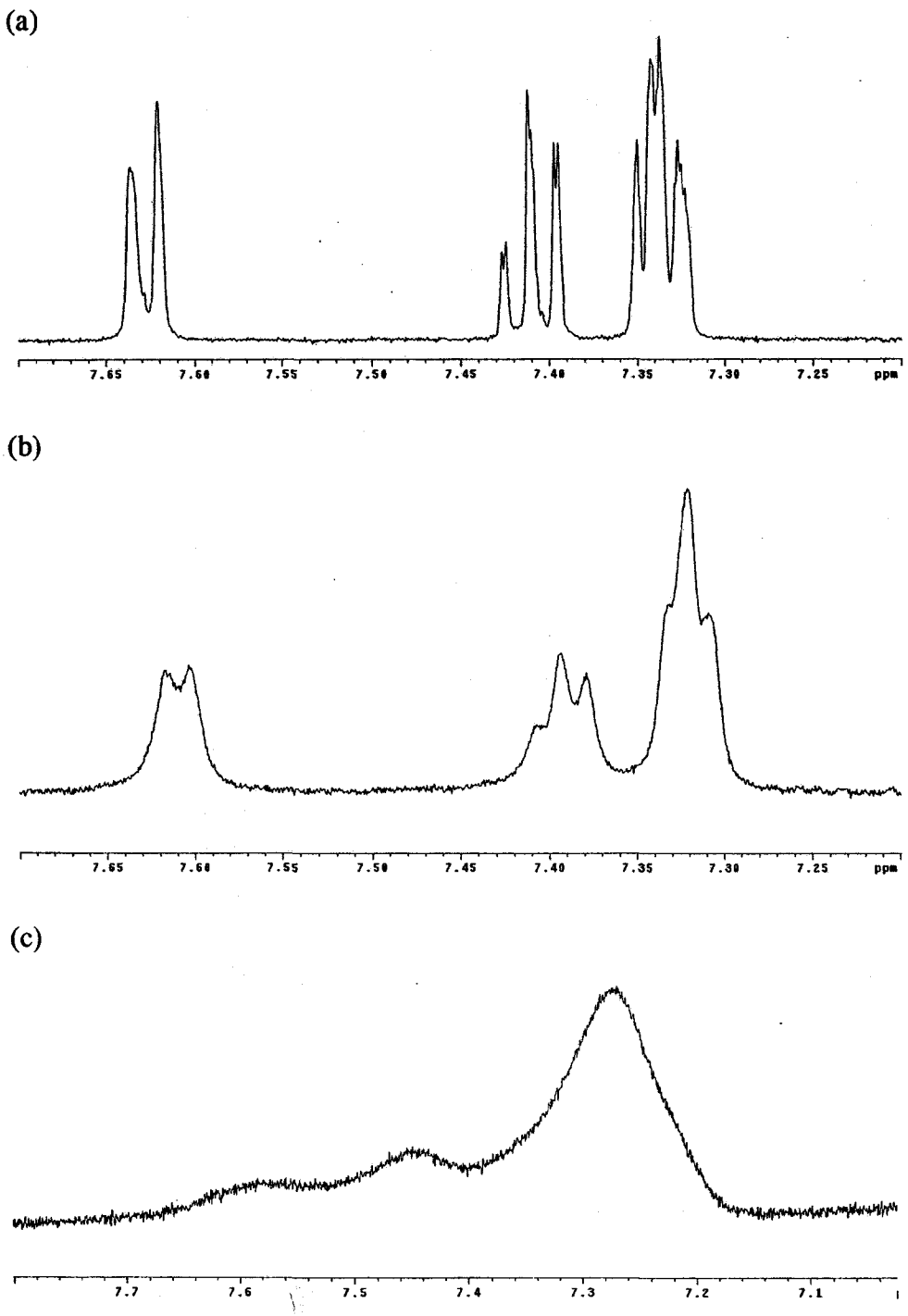
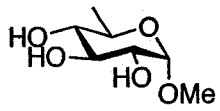


Figure 4-11. Aromatic region of ^{1}H NMR (500 MHz) spectrum of a solution of **2n** in phosphate buffered D_2O solution (0.01 M) at pH 7.4 (a) without any sugar (b) with 0.25 M methyl α-D-glucopyranoside at pH 7.4 (c) with 0.25 M methyl α-D-galactopyranoside at pH 7.4.



methyl 6-deoxy-α-D-glucopyranoside.

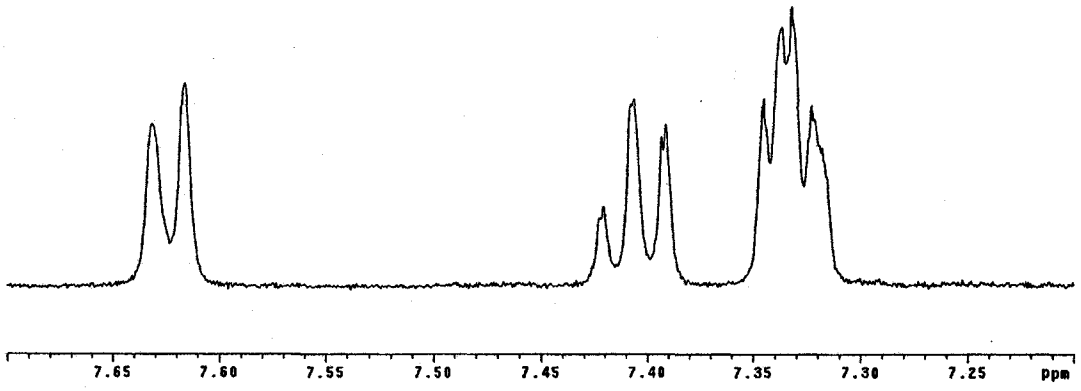
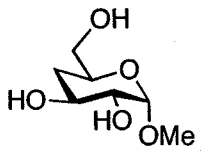


Figure 4-12. Aromatic region of ^1H NMR (500 MHz) spectrum of a solution of **2n** in phosphate buffered D_2O solution (0.01 M) at pH 7.4 with 0.25 M methyl 6-deoxy-α-D-glucopyranoside.



methyl 4-deoxy-α-D-glucopyranoside.

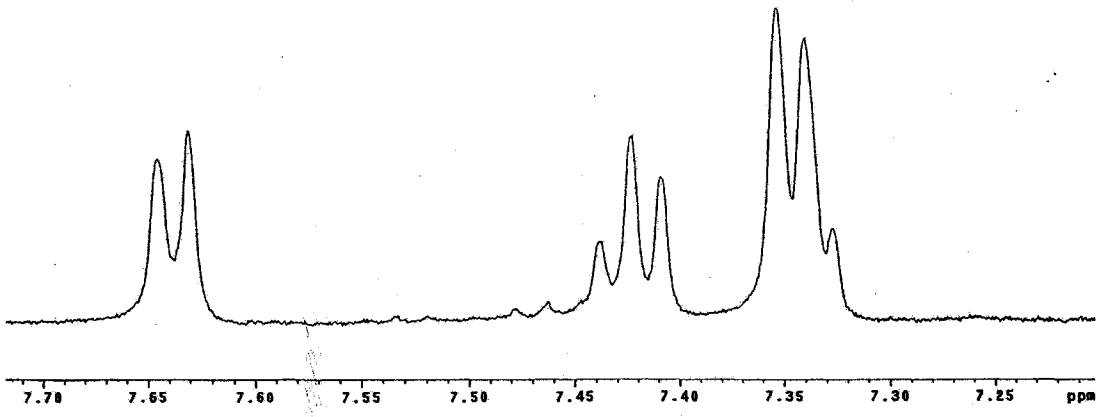


Figure 4-13. Aromatic region of ^1H NMR (500 MHz) spectrum of a solution of **2n** in phosphate buffered D_2O solution (0.01 M) at pH 7.4 with 0.25 M methyl 4-deoxy-α-D-glucopyranoside.

It can be seen by comparing the spectral data (Figures 4-9 and 4-11) that **2n** (Figure 4-11) is the only boronic acid that shows considerable peak broadening of the aromatic peaks with methyl α-D-glucopyranoside when compared with the free boronic acid. Peak broadening is even more significant with **2n** and methyl α-D-galactopyranoside (Figure 4-11(c)). These results further support the formation of a complex only in the case of **2n**.

As depicted in Figures 4-11, and 4-12, the 4- and 6-hydroxyls of the glycoside are also likely to be involved in this association as the aromatic peaks are much sharper with methyl 6-deoxy-α-D-glucopyranoside than methyl α-D-glucopyranoside. Presumably there is little or no complex formation with the 6-deoxy analogue, which is also evidenced with a negative ARS assay (*vide supra*). To confirm the role of the 4,6 diol unit in boronic acid complexation, ^{1}H NMR spectra were also taken with a solution of **2n** alone and in the presence of the 4-deoxy analogue (depicted in Figure 4-13). The ARS assay gave an orange-colored solution indicative of weak binding of the boronic acid **2n** with the 4-deoxy analogue. In Figure 4.13, we observe less amount of peak broadening of the aromatic peaks of **2n** in the presence of the 4-deoxy analogue.

Both ARS assays and ^{1}H NMR studies have shown that the 4,6 diol unit in saccharides is important in forming a complex with cyclic boronic acid **2n**. This interaction, however, may not be the only one, as in the 4-deoxy analogue, we still observe some interactions with the cyclic boronic acid **2n**. More mechanistic studies need to be performed to explain this weak binding "phenomenon".

4.4 Quantitative measurements by ^{1}H NMR

Cyclic boronic acid **2n** binds glycosides more strongly than established boronic acids. Another type of interaction should be present in the cyclic boronic acid **2n** which accounts for this exceptional binding. Moreover, this type of interaction should also be increasing the binding of cyclic boronic acid **2n** to non-reducing sugars, such as fructose and glucose. We wanted to compare quantitatively the binding constants of the cyclic boronic acid **2n** with those of phenylboronic acid and the established "Wulff-type" boronic acids by doing ^{1}H NMR tirations.

4.4.1 Methodology for K_a measurements by ^{1}H NMR

For the equilibrium between a boronic acid receptor (R) and a saccharide substrate (S), assuming a one-site binding,

$$R + S \rightleftharpoons RS$$

The ratio [RS]/[R] was found from the ratio of peaks in the ^{1}H-NMR spectrum.

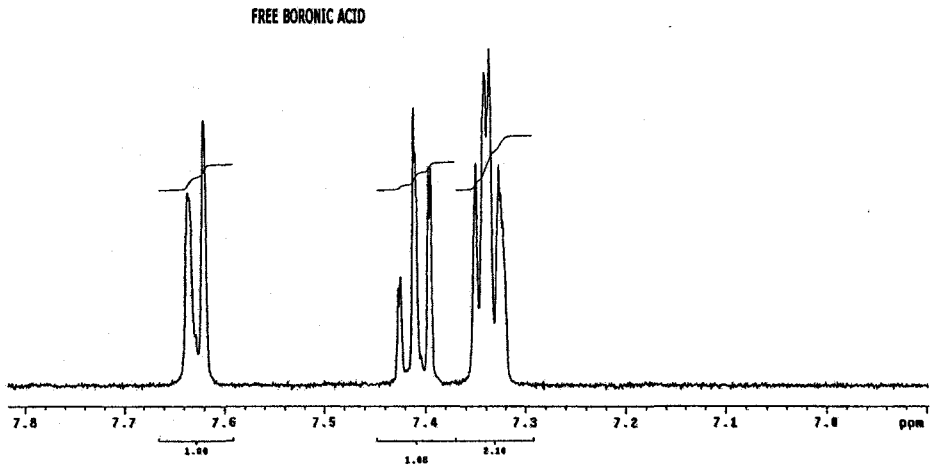
$$\theta = \frac{[RS]}{[RS] + [R]} \qquad (1)$$

The association constant K_a was determined from the Benesi-Hildebrand plot:

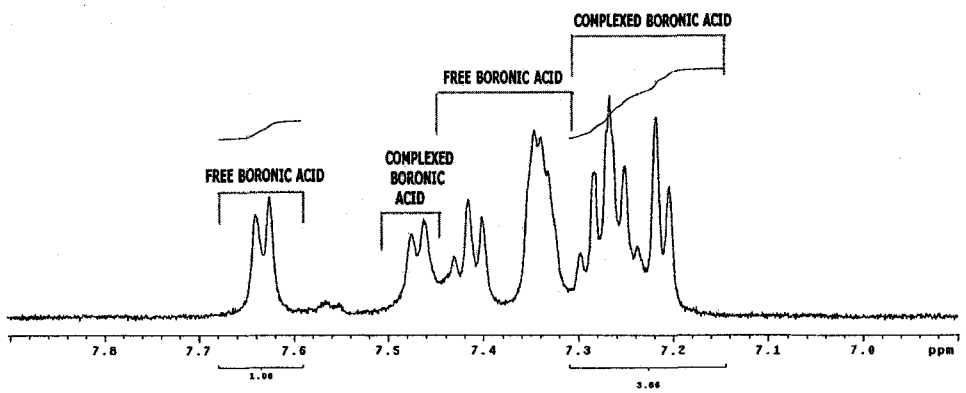
$$1/\theta = 1/K_a(1/[S]) + 1 \qquad (2)$$

where $1/\theta = 1 + [R]/[RS]$ and [S] is the substrate concentration at time t.

(a)



(b)



(c)

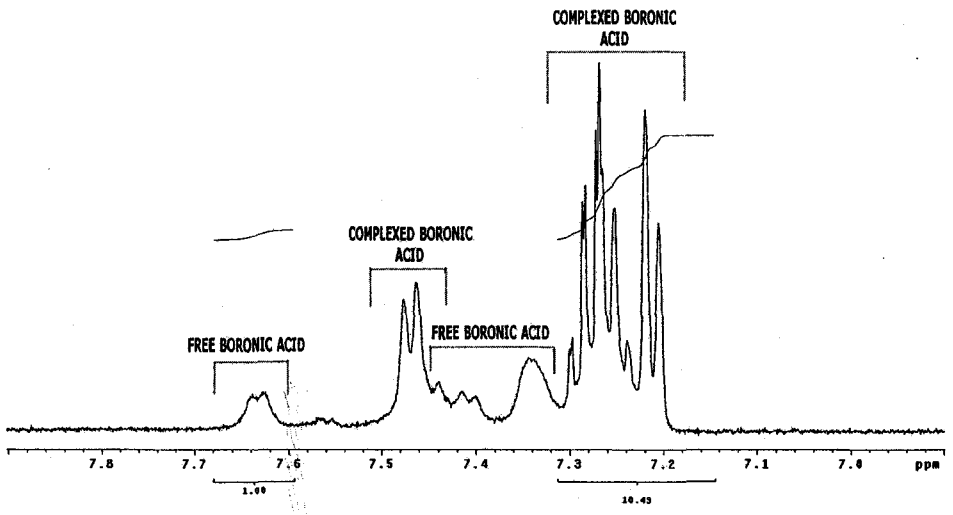


Figure 4-14. (a) ^{1}H NMR spectrum of a solution of **2n** (0.015 M) at pH 7.4, (b) ^{1}H NMR spectrum of a solution of **2n** (0.015 M) and fructose (0.012 M) at pH 7.4, (c) ^{1}H NMR spectrum of a solution of **2n** (0.015 M) and fructose (0.023 M) at pH 7.4.

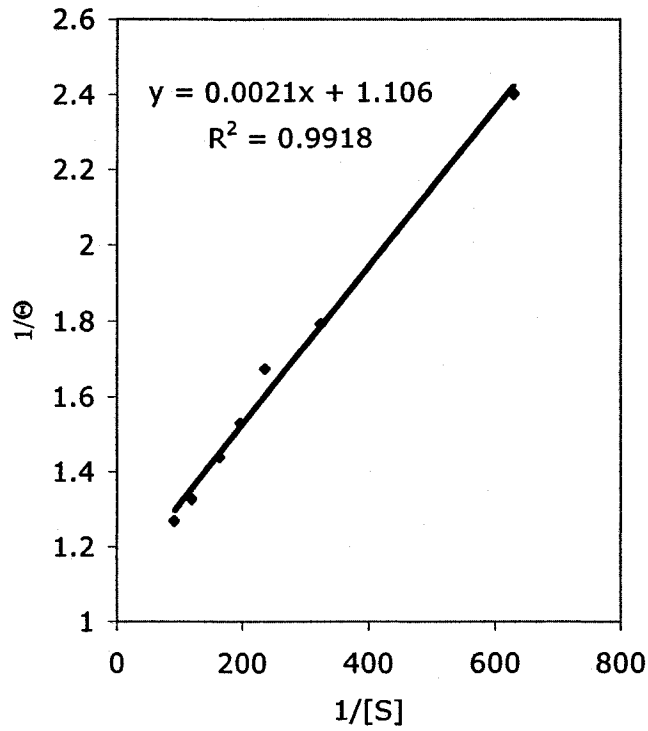


Figure 4-15 1/Θ versus 1/[S] plot for determination of K_a for (2-hydroxymethyl-phenyl) boronic acid (**2n**) (0.015 M) with fructose (8-23 mM) at pH 7.4 in 0.1 M phosphate buffered D_2O solution.

The ratio was measured directly from carefully expanded integrals of relevant peaks on the NMR spectra (see Figure 4-14). A plot of 1/θ versus 1/[S] (Equation 2) would then give the K_a value as the inverse of the intercept. Each titration was reproduced at least once. As can be seen from the example of Figure 4-15, the R^2 factor was very satisfactory.

4.4.2 Binding affinities of cyclic boronic acid 2n compared to "Wulff-type" boronic acids

For the ^{1}H NMR titrations (Table 4.1), the ideal solvent was D_2O in the absence of organic co-solvents. However, among the "Wulff-type" boronic acids, **2p** proved to be more difficult to dissolve because of the lipophilic (or hydrophobic) anthracene ring present. Hence, in the case of **2p**, 80% CD_3OD/ D_2O was used. The concentration of the buffer had to be reduced to 0.05 mM because of precipitation of the boronic acid in a buffered solution of 0.1 M. The pH of the solution was maintained at 7.4.

Table 4.1 K_a measurements[a] by ^{1}H NMR titrations.

Entry	Boronic acid	Conditions[b] (Solvent, pH)	K_a (M^{-1}) Glucose	fructose
1	$PhB(OH)_2$	D_2O, 7.4	c	79
2	$PhB(OH)_2$	D_2O, 7.8	c	130
3	**2n**	D_2O, 7.0	c	170
4	**2n**	D_2O, 7.4	17	610
5	**2n**	D_2O, 7.8	c	340
6	**2n**	80% CD_3OD/D_2O, 7.4	c	7.3
7	**2o**	4% CD_3OD/D_2O, 7.4	c	67
8	**2o**	4% CD_3OD/D_2O, 7.8	c	58
9	**2o**	33% CD_3OD/D_2O, 7.4	c	120
10	**2o**	80% CD_3OD/D_2O, 7.4	d	310
11	**2p**	80% CD_3OD/D_2O, 7.4	d	2000

[a] Values are rounded to two significant figures. Experimental errors are in the range of 3-15%. [b]In sodium phosphate monobasic buffer. [c,d] Not measured. [d] Likely below 5 M^{-1} according to the ARS qualitative assay.

The use of 0.01 M boronic acid concentration was required for ^{1}H NMR titration readings. It is known that the addition of 50% methanol to water solution results in minimal changes of pH of solution compared to 100% water.[22,23] At least two titrations were carried out except for entry 2. An average of two K_a values was used to provide the indicated association constants (Table 4.1). The values of the binding constants measured fell within 0-15% of each other.

To test the validity of our experiments, we determined the association constants of phenylboronic acid (**2a**) with fructose at pH 7.4 (entry 1) and 7.8 (entry 2). The increase in binding constant with the increase in pH followed the usual trend reported in the literature.[20] At higher pH, more boronic acid exists in the tetrahedral form (Scheme 1.1, Chapter 1) which complexes more readily with fructose. K_{tet} affects the overall binding constant (K_{eq}) more significantly because its value is of greater magnitude than $K_{trig.}$.[19,20] Therefore, with a higher proportion of anionic tetrahedral boronate form, the binding constant is increased.

The binding strengths of cyclic boronic acid **2n** with fructose was determined under various pH conditions (entries 3-5, Table 4.1). The optimal pH was deduced to be around 7.4, as the binding constant of **2n** was greatest at that pH (entry 4, Table 4.1). Therefore, depending on the nature of the buffer and the diol,[24] this monoboronic acid seems to satisfy the requirements of a water-soluble sensor with high binding constant at pH 7.4.

By comparing entries 4 and 9 in Table 4.1, cyclic boronic acid **2n** proves to complex saccharides more strongly than the "Wulff-type" boronic acid **2o** at pH 7.4. In the case of boronic acid **2p**, no direct comparison could be made as the use of 80% methanol was necessary. These conditions resulted in a lower binding constant for **2p**. We have no mechanistic data at the moment to explain this observation and we will pursue further investigations to address this problem.

For an accurate determination of binding constants for the anthracene based *ortho*-dialkylaminomethylphenylboronic acid **2p**, solubility was of prime importance. This can be seen in Table 4.1, as with an increasing amount of methanol (entries 7 and 9) there was an increase in association constant. Moreover, with the higher pH 7.8, an increase in binding constant was expected. Instead, a decrease in binding constant was observed. This could be attributed to reduced solubility of boronic acid **2p** in the aqueous solution

A solution of 80% deuterated methanol was used in entries 10 and 11 as boronic acid **2p** (Figure 4-3) was found to be highly insoluble in water. We expected to see a binding constant of the same magnitude in entries 10 and 11, as the saccharide binding was previously thought to be heavily dependent on the B-N interaction (discussed in Chapter 1). However, this proved not to be the case (binding constant in entry 11 is much larger than entry 10). It turns out that hydrophobic interactions of the anthracene ring in **2p** play a dominant role in saccharide binding.

For instance, stabilizing interactions between the carbohydrate's C-H bonds and the aromatic groups are known in the recognition of carbohydrates by both natural and artificial receptors. Crystal structures of protein-carbohydrate complexes and NMR experiments reveal stacking of aromatic residues on the less polar face of the carbohydrate.[25,26] For instance, phenol and benzene rings have been found to interact with the clusters of C-H bonds of the alpha face of methyl β-D-galactoside (Figure 4-17).[25] Consequently, it is likely that the anthracene unit of sensor **2p** is involved in similar interactions, explaining its higher binding affinity compared to **2o**.

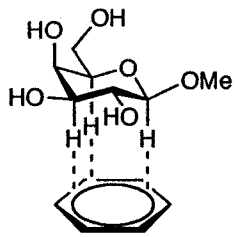


Figure 4-16. C-H/π interactions in stabilization process. [25]

4.5 Binding of cyclic boronic acid 2n to glycopyranosides

It was seen earlier (Section 4.3) that when the cyclic boronic acid **2n** binds to glycosides, no peaks of the resulting complex were distinguishable by ^{1}H NMR, implicating a fast equilibrium situation. ARS competitive experiments can provide a more sensitive means of determining binding constants as follows.

4.5.1 Methodology and examples for K_a measurements by ARS method[20]

In the complex mixture of a boronic acid receptor (R), ARS indicator (I), and sugar substrate (S), there exist the following equilibria:

$$R + I \overset{K_{ars}}{\rightleftharpoons} RI$$

$$RI + S \overset{K_a}{\rightleftharpoons} RS + I$$

where RI is the boronic acid-ARS complex and RS is the substrate complex.

Equations for association constant determinations are derived as follows:

$$1/\Delta A = (\Delta K\, p_o I_o K_{ars})^{-1}\, 1/[S] + (\Delta K\, p_o I_o)^{-1} \qquad (3)$$

where ΔK, p_o, I_o are all the parameters of the UV spectrophotometer.

$$Q = [I]/[RI] = (A_{RI} - A)/(A - A_I) \qquad (4)$$

Where A is the measured absorbance, A_{RI} is the absorbance of the receptor-indicator complex, and A_I is the absorbance of the free indicator.

$$P = [R] - 1/(QK_{ars}) - [I_0]/(Q+1) \qquad (5)$$

where $[I_0]$ is total indicator concentration (ARS).

$$[S]/P = (K_{ars}/K_a)Q + 1 \qquad (6)$$

where [S] is the substrate concentration.

The association constant of the ARS-boronic acid (K_{ars}) is the quotient of the intercept and the slope in a plot of $1/\Delta A$ versus 1/[S] (Equation 3).

Following the procedure of Wang *et al.*,[20] a 0.144 mM ARS solution was prepared in 0.1 M phosphate solution buffered at pH 7.4. A solution of **2n** (0.15 M) was prepared

so as to prepare mixtures of **2n** (0.0131-0.131 M) and UV absorbances were measured at 450 nm as shown in Figures 4.18 and 4.19. Two experiments were carried out to determine an average value of K_{ars}. To validate our experimental techniques, we reproduced the binding constant between phenylboronic acid and the ARS indicator and obtained an average value of 1290 M^{-1} compared to 1300 M^{-1} obtained in Dr. Wang's laboratory.[20] For the complexation between phenylboronic acid and fructose, the binding constant was found to be 158 M^{-1} very close to the value of 160 M^{-1} obtained by Dr. Wang's laboratory.

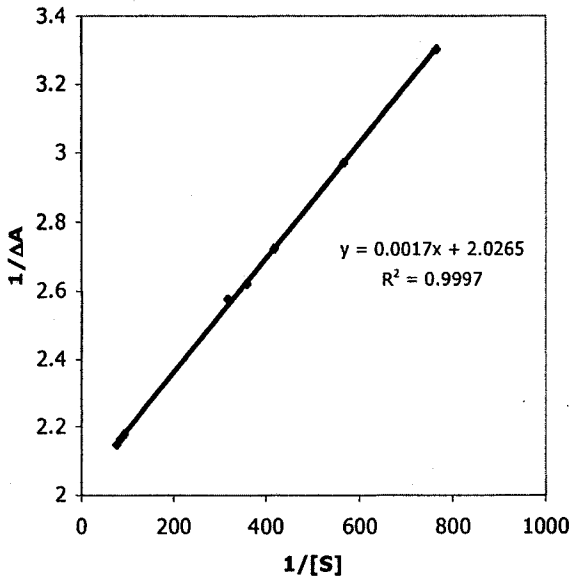


Figure 4-17. 1/ΔA versus 1/[S] for determination of ARS-boronic acid **2n** (K_{ars}) in the presence ARS (0.144 mM) and **2n** (0-0.0131 M) with data being taken at 450 nm.

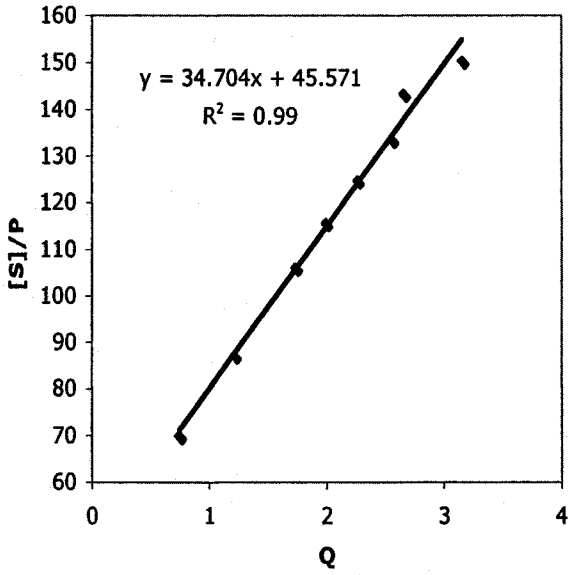


Figure 4-18. [S]/P versus Q plot for for binding of **2n** (3.11 mM) to glucose (0.13-0.42 M) in the three-component assay with the ARS solution at 0.144 mM with data at 453 nm.

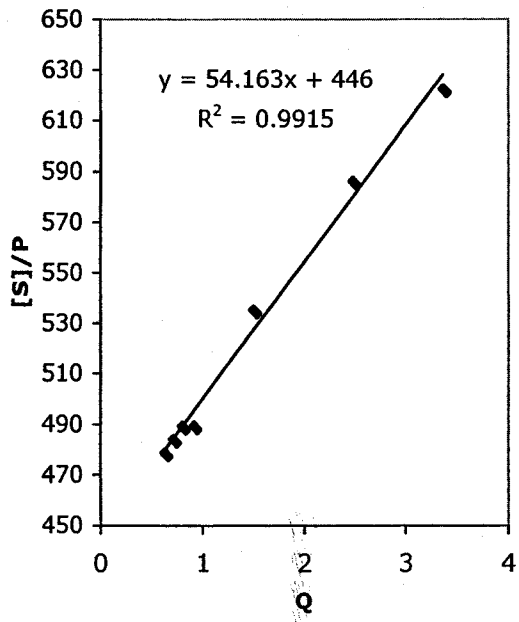


Figure 4-19. [S]/P versus Q plot for binding of **2n** (3.11 mM) to methyl α-D-glucopyranoside (0.80-1.92 M) in the three-component assay with the ARS solution at 0.144 mM with data taken at 453 nm.

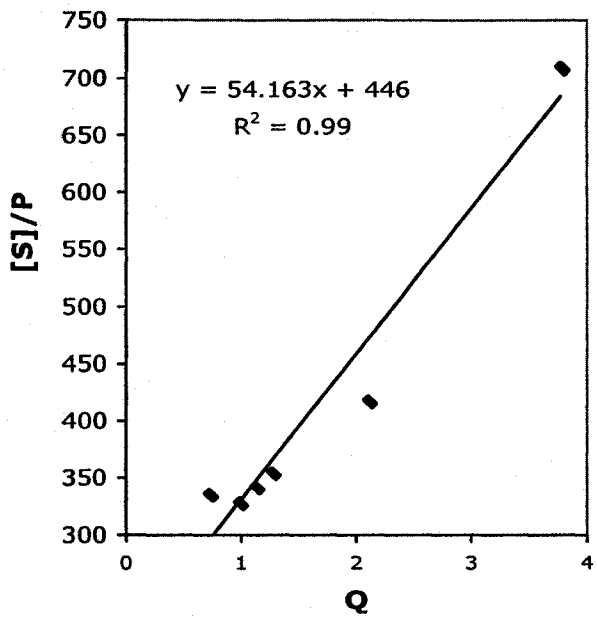


Figure 4-20 [S]/P versus Q plot for binding of **2n** (3.63 mM) to methyl β-D-glucopyranoside (0.80-1.92 M) in the three-component assay with the ARS solution at 0.144 mM with data taken at 453 nm.

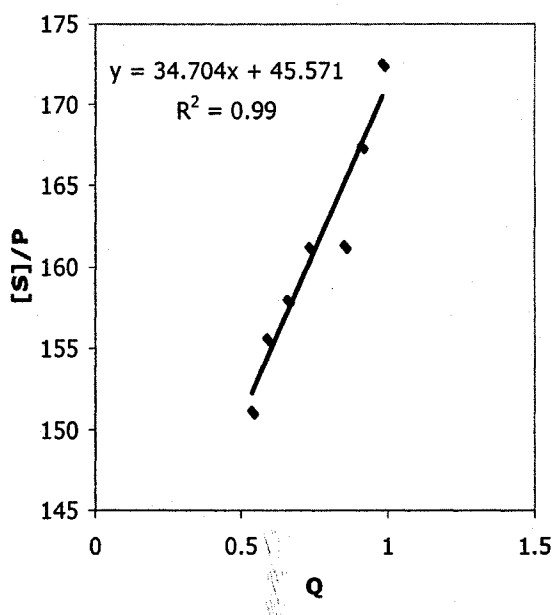


Figure 4-21. [S]/P versus Q plot for binding of **2n** (3.66 mM) to methyl α-D-galactopyranoside (0.10-0.50 M) in the three-component assay with the ARS solution at 0.144 mM with data taken at 453 nm.

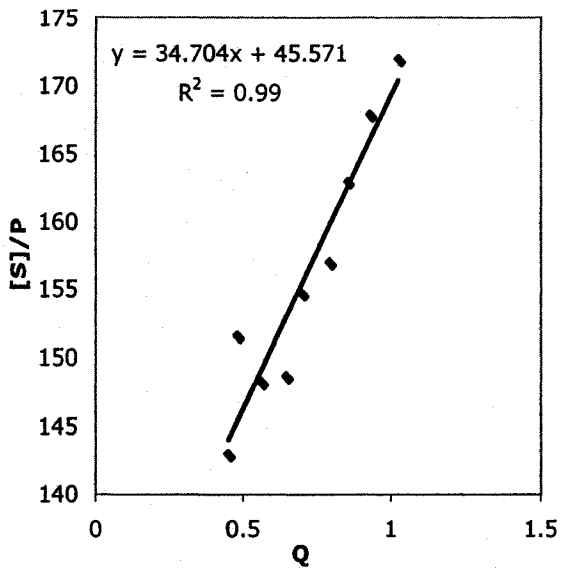


Figure 4-22. [S]/P versus Q plot for binding of **2n** (3.66 mM) to methyl β-D-galactopyranoside (0.10-0.50 M) in the three-component assay with the ARS solution at 0.144 mM with data taken at 453 nm.

According to Equation 6, the y-intercept should be 1, but in our studies, it was not so. This could be attributed to the UV instrumental parameters, and when we determined the binding constants of phenylboronic acid and fructose according to the description of Wang and co-workers, they matched the reported ones (in their case, too, the intercept was not 1).[20]

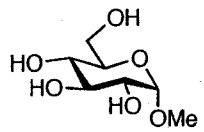
The above graphs yielded the values of binding constants listed in Table 4.2. These K_a's were measured in a sodium monophosphate buffered solution at pH 7.4. From the table, we can conclude that the functional group at the anomeric position does not influence binding with the cyclic boronic acid **2n**. Boronic acid **2n** binds with similar affinity with glucose and methyl galactopyranoside, which is rather uncommon for a

monoboronic acid. These affinities are comparable or superior to recently reported macrocyclic receptors.[27] With these values in hand, it is clear that glycopyranoside binding to the cyclic boronic acid **2n** does take place, in contrast to the ortho-substituted boronic acids, which were investigated.

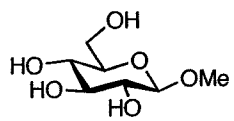
Table 4.2 K_a measurements[a] using the ARS UV Assay at neutral pH.

Diol-containing Substrates	K_a (M^{-1})
Alizarin Red. S	1200
Glucose	36
Methyl-α-D-glucopyanoside	22
Methyl-α-D-galactopyanoside	34
Methyl-β-D-glucopyanoside	22
Methyl-β-D-galactopyanoside	34

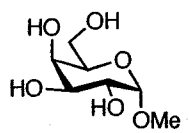
[a]Values are rounded to two significant figures. Experimental errors are in the range 3-10%.



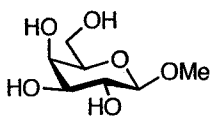
methyl α-D-glucopyranoside



methyl β-D-glucopyranoside



methyl α-D-galactopyranoside



methyl β-D-galactopyranoside

Figure 4-23. Glycopyranosides used in ARS competitive assay.

Next, we then wanted to determine the pK_a of boronic acid **2n** as a first step towards investigating the binding mode and its pyranoside-binding exceptional behavior.

4.6 pK_a determination and proposed binding mode of cyclic boronic acid 2n to saccharides

The acidity of boronic acid **2n** could play an important role in its saccharide-binding properties. The pK_a value and the geometry of the cyclic boronic acid **2n** were determined using potentiometric (Figure 4-24) and ^{11} B NMR titrations (Figure 4-25). [28] From the ^{11}B NMR curve, we can see that the boron changes its geometry from trigonal sp^2 form (32 ppm at pH 5) to the sp^3 tetrahedral form (7 ppm at pH 11). From Figure 4-24, the pK_a value obtained by potentiometric studies was determined to be 7.3, which is close to the value of 7.15 extrapolated from Figure 4-25. An average of the two values (by potentiometry and ^{11}B NMR) gave a value of 7.2 for the pK_a of boronic acid **2n**. The pK_a of the boronic acid **2n**-fructose complex was found to be 5.30 (Figure 4-26), which confirms that the boronic acid-fructose complexation is favored under

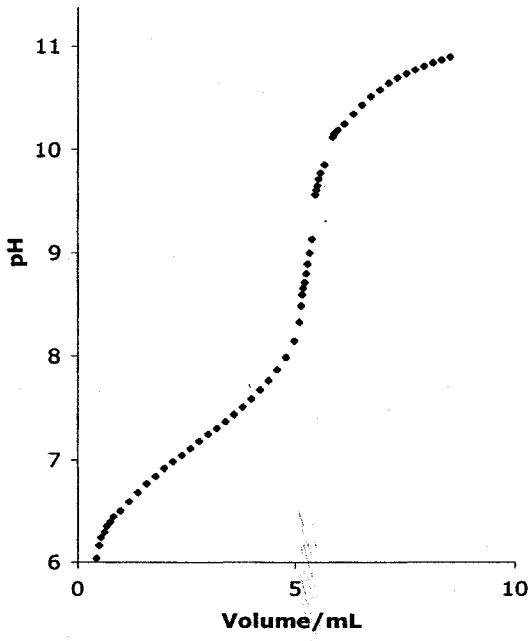


Figure 4-24. pH profile of **2n** (0.05 M) upon addition of base (0.025 M NaOH, 0.15 M NaCl in water).

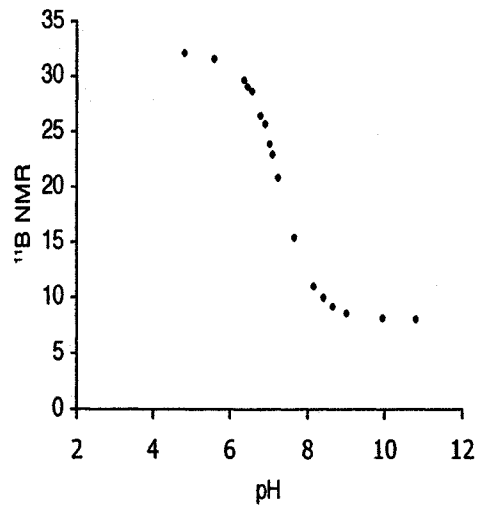


Figure 4-25. ^{11}B NMR chemical shifts of **2n** with increasing pH (10% D_2O in H_2O, 16 mM in 0.10 M phosphate buffer). Referenced to Et_2O-BF_3.

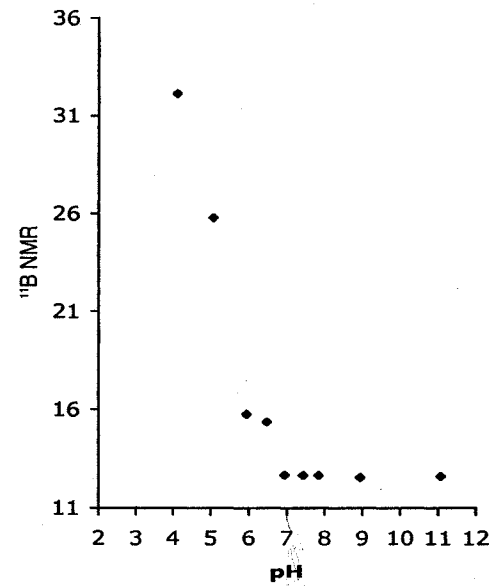


Figure 4-26. ^{11}B NMR chemical shifts of **2n** with increasing pH (10% D_2O in H_2O, 12 mM of **2n** and 120 mM of fructose in 0.10 M phosphate buffer). Referenced to Et_2O-BF_3.

physiological conditions of pH 7.4.

Lewis acidity alone cannot account for the observed saccharide binding behavior. For instance, in spite of the low pK_a value of *ortho*-dimethylaminomethylphenylboronic acid (**2o**) (6.7)[24] compared to cyclic boronic acid **2n** (7.2), the latter still has a higher binding affinity for fructose and glucose (Table 4.1). Moreover, **2o** does not complex glyco-pyranosides. Geometrical factors unique to boronic acid **2n** may explain its high binding affinity.

An X-ray crystal structure of a dimer of **2n** was reported by Zhdankin and co-workers[29] in 1999 (Figure 4-27).

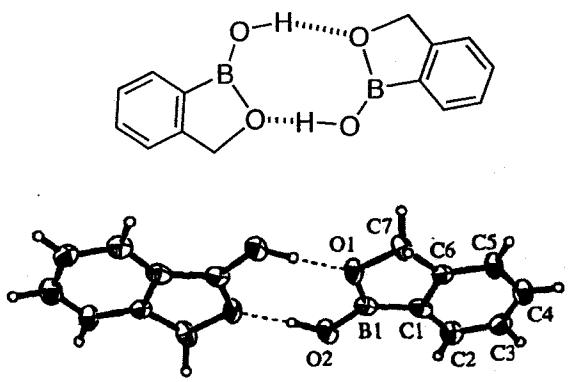
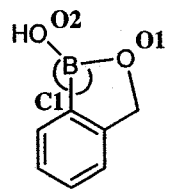


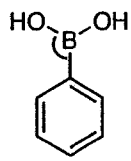
Figure 4-27. X-ray crystal structure of cyclic boronic acid **2n** (reproduced from reference 29).

Table 4.3 Structural parameters of boronic acids **2a** and **2n**.

Parameter	Observed bond angles and distances	
	Cyclic boronic acid **2n**[29]	Phenylboronic acid (**2a**)[30-32]
B-C (Å)	1.494	1. 568
B-O1 (Å)	1.408	1.378
B-O2 (Å)	1.372	1.362
O1-B-O2 (°)	118.3	-
O1-B-C1 (°)	110.5	125.0
O2-B-C1 (°)	131.2	125.0



2n, C1-B-O2 = 131.2 ^{0}
C1-B-O1 = 110.5 ^{0}



2a, C1-B-O2 = 125.0 ^{0}

Figure 4-28. Outer C-B-O angles (Table 4.3).

If we have a closer look at the structure (Figure 4-28) of boronic acid **2n** compared to phenylboronic acid (**2a**), the outer C1-B-O2 angle in **2n** is greater than in **2a**[29] due to the unusually small C-1-B-O1 dihedral angle. This distorted geometry may better accommodate the 4,6-diol of hexopyranosides compared to usual boronic acids. Moreover, the basicity of O1 is higher in **2n** than in a usual boronic acid. It was reported by Zhdankin[29] and co-workers that the five-membered ring is forced out of the plane of the benzene ring due to geometrical constraint. With inefficient overlap of oxygen O1 with boron in **2n**, O1 is richer in electrons and can participate in stronger hydrogen-

bonding interactions with the saccharide. We can imagine that with less steric hindrance and the possibility of stronger hydrogen-bonding interactions, the complex boronic acid **2n**-saccharide will be more stable giving rise to a higher binding constant.

Based on all of the above observations, including the requirement for a 4,6-diol, the following mechanism can be proposed. The boron atom of **2n** exists ($pK_a = 7.2$) largely in the sp^3 tetrahedral state at pH 7.4. When binding to saccharides, 4,6-diols of hexapyranosides form six-membered boronate esters with cyclic boronic acid **2n** and they may experience less steric strain than in normal boronic acids. This would be due to the larger cone angle C1-B-O2 in boronic acid **2n**. During boronate ester formation, there is also less loss in entropy as compared to usual boronic acids as a result of the internal alkoxy arm of **2n**. Both complexes **10** and **11** are proposed as possibilities.

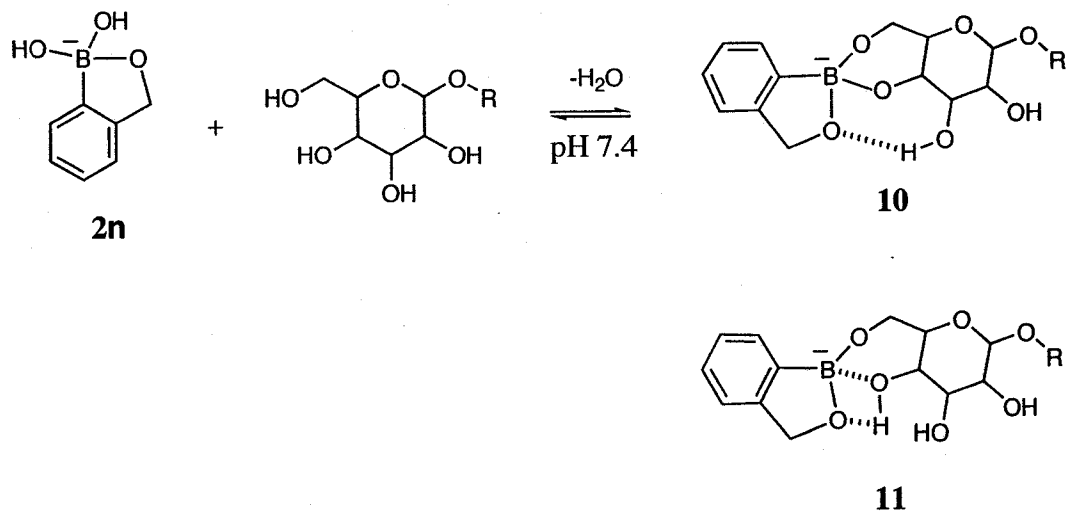


Figure 4-29. Proposed mechanism for glycopyranoside binding with boronic acid **2n**.

4.7 Design of diboronic acid-based sensors

4.7.1 Rationale for diboronic acids

Most saccharides have more than one pair of diols that could serve as binding sites with a boronic acid moiety. To further enhance binding and especially to enable discrimination between various carbohydrates, the concept of multivalent binding of carbohydrates by two or more boronic acid moities was suggested.[33] Two or more boronic acids in a certain spatial arrangement complementary to that of the saccharide can bind with high selectivity and specificity. Shinkai and co-workers have demonstrated that compound **12** with a hexamethylene spacer between two arylboronic acid moieties has high selectivity for glucose.[34] This result is important as arylboronic acids have a high intrinsic preference for D-fructose over D-glucose.

Along this line, several research groups have adopted this approach for the development of diboronic acid based fluorescent sensors[34-37] or receptors for cell-surface oligosaccharides such as sialyl Lewis X (sLex).[2,3]

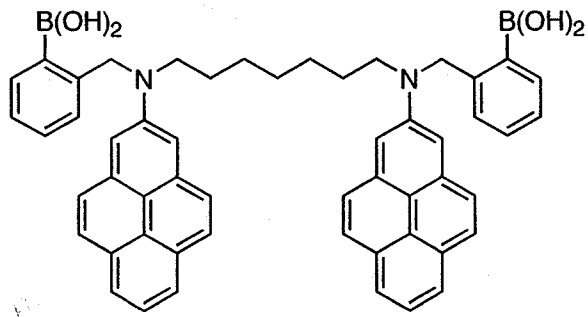


Figure 4-30. Bis-boronic acid **12**.

Therefore, to develop more effective sensors and gain better understanding of the binding mode of cyclic boronic acid **2n** with glycopyranosides, we endeavored to synthesize dimers of boronic acid **2n** (Figure 4-31) which are expected to bind with higher affinities to the hexopyranosides. Consequently, peaks of the complexed diboronic acids can be distinguishable in ^{1}H NMR spectra.

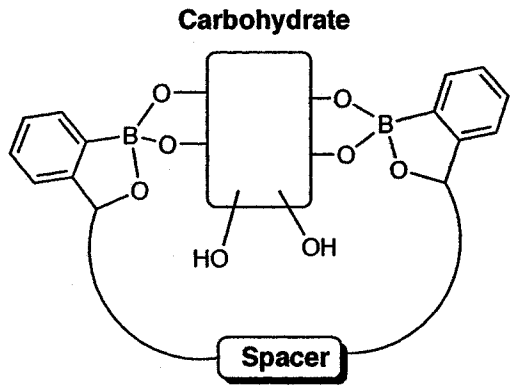
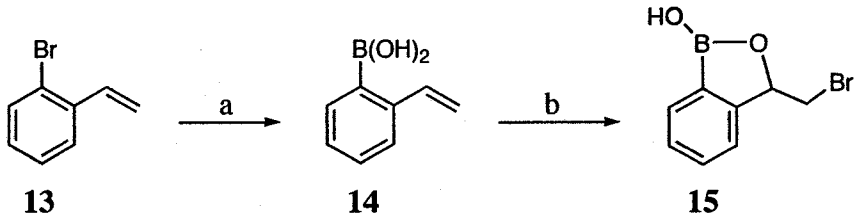


Figure 4-31. Bidentate complex of a bis-boronic acid with a carbohydrate.

4.7.2 Synthesis of monoboronic acid models

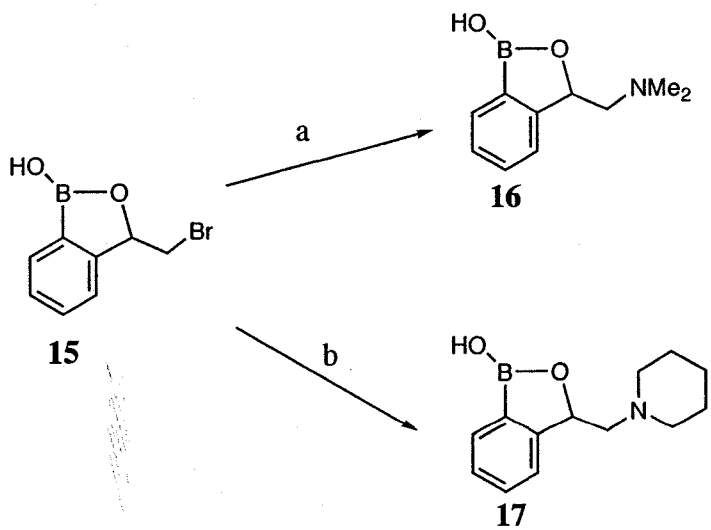
The synthesis of dimers having the benzoboroxole unit can be envisaged by introducing a reactive group at either the alkyl chain or in the aromatic ring of the benzoboroxole. To this end, we took advantage of Falck's methodology whereby 3-bromomethyl-3-H-benzo[c][1,2]oxaborol-1-ol (**15**) is synthesized (Scheme 4.2) by bromolactonization of 2-vinylbenzeneboronic acid in the presence of *N*-bromosuccinimide (NBS). We therefore synthesized 2-vinylbenzeneboronic acid (**14**) by lithiation of 2-bromostyrene with butyl lithium at a low temperature (–78 °C), followed by metal exchange with tri-isopropyl borate (Scheme 4.2).



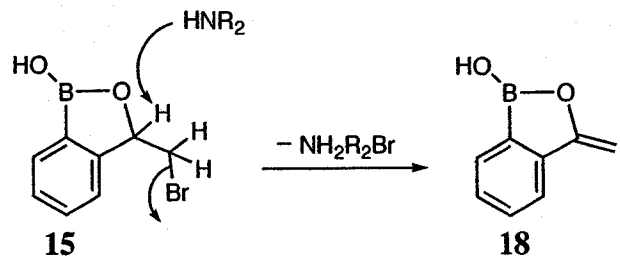
13 14 15

Scheme 4.2 Reactions and conditions: (a) (i) n-BuLi, dry THF, N_2, –78 °C, 30 min, (ii) $B(O^iPr)_3$, dry THF, 4 h; (b) NBS, 0 °C, THF/H_2O (4:1), 4 h.

Dimers of the cyclic boronic acid could be synthesized by forming amine or amide bonds with the linker. Before planning the synthesis of more complex conjugates, we wanted to synthesize monoboronic acid models that will possess the amine group or the amide group. These monomers will then be tested qualitatively using the ARS competitive assay. Thus, the *N,N* dimethyl and piperidine derivatives **16** and **17** were generated by nucleophilic substitution of the bromide ions and they were obtained in 41% yield and 49% yield respectively (Scheme 4.3). The reduced yield was attributed to the competitive elimination of the bromide ion by the base shown in Figure 4-32.



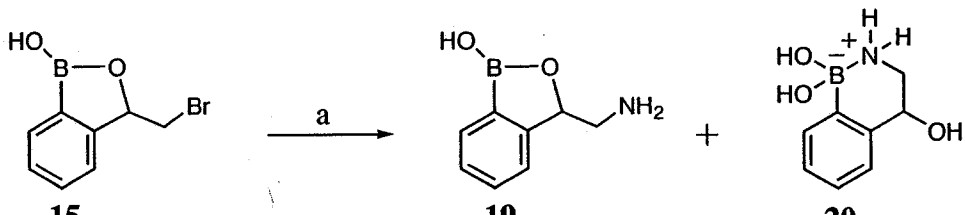
Scheme 4.3 Reagents and conditions: (a) Me_2NH, THF, rt, 12 h, 41%; (b) piperidine, THF, rt, 12 h, 49%.



15 18

Figure 4-32. Base-induced elimination of bromide ion.

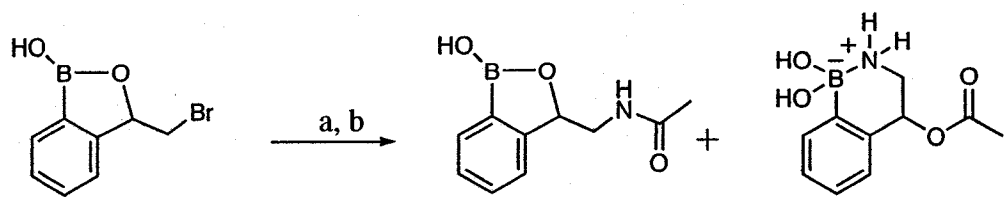
The model monomers for the diboronic acids with amide bonds in the linker will be formed by ammonolysis[38] of the bromide derivative **15** (Scheme 4.4), followed by acylation of the resulting amine (Scheme 4.5). As seen in Scheme 4.4, the desired amine **19** was obtained as an inseparable mixture of tautomers (after HPLC purification). This tautomerism originates from the amine coordination to the boron and the five-membered boroxole ring opening up to generate the free hydroxyl group. It was therefore logical to observe a mixture of amide **21** and ester **22** when the amines **19/20** was acylated with acetyl chloride at low temperatures. Care had to be taken to avoid strongly basic conditions as bromide elimination occurred as shown in Figure 4-32.



15 19 20

35 % yield as a 4:1 mixture of tautomers **19/20**

Scheme 4.4 Reagents and conditions: (a) NH_3 (7 M in methanol), μwaves, 60-80 °C, 20 min.



15 **21** **22**, B-N tautomer

37 % yield as a 8:1 mixture of **21/22**

Scheme 4.5 Reagents and conditions: (a) NH_3 (7 M in methanol), μwaves, 60-80 °C, 20 min; (b) acetyl chloride, THF, 0 °C to rt, 2 h.

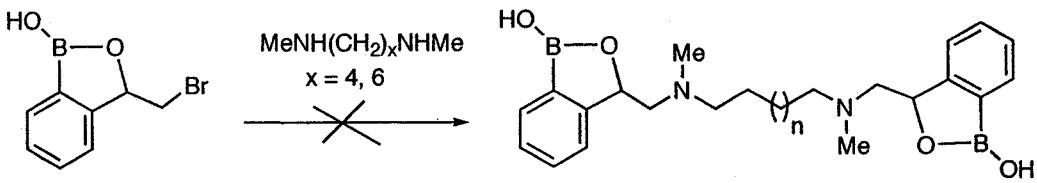
4.7.3 Binding affinity of monoboronic acid models

With the ARS qualitative assay, the amine conjugates **16** and **17** (Scheme 4.3) gave a similar color change with fructose, glucose and methyl glucopyranosides as the simple benzoboroxole unit **2n**, under same concentration conditions. The amide **21** also maintained its ability to bind glycosides, but a 25% proportion of tetrahydrofuran was required for a clear ARS solution mixture. However, the amines had a stronger color change when compared to the amide. A possible reason could be the lower pK_a of the protonated amine, which is absent in the amide linkage. The binding constants of the boronic acid derivatives **16** and **17** could not be determined quantitatively by ARS competitive assays due to limited samples.

An ^1H NMR titration experiment was attempted with the boronic acid **17** in D_2O, but the product peaks were overlapping with those of the free acid. Solvents such as DMSO or CD_3OD did not improve the situation. Since amide **21** was a mixture, its binding constant could not be accurately determined.

4.7.4 Synthesis of diboronic acids with an amine linkage

As the ability of the boronic acids **16** and **17** to bind pyranosides did not seem to be affected by the amine functional group, several attempts to synthesize the amine derivatives were made (Equation 4.1). Different bases were used, such as triethylamine, sodium bicarbonate and potassium carbonate. Solvents such as dry tetrahydrofuran and dimethylformamide were also employed. Various temperature conditions were used, such as room temperature and higher temperatures of 60 to 100 °C (Equation 4.1). In all the experiments, the predominant product was the elimination side-product **18** (shown in Figure 4-32) or the starting material that was recovered. When microwave conditions were used, the mixture consisted of boronic acid complexed with the amine (Scheme 4.6). It seemed that the boronic acid was unreactive to the amine, which may be coordinating to it rather than undergoing a nucleophilic substitution reaction to give the desired product **23**.

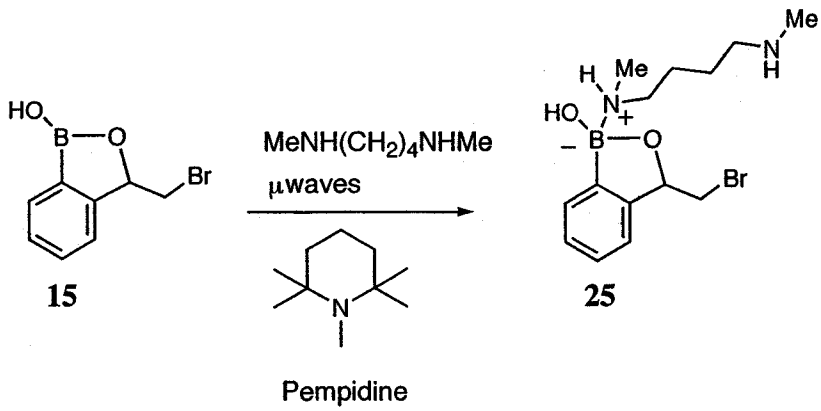


15

23, n=1

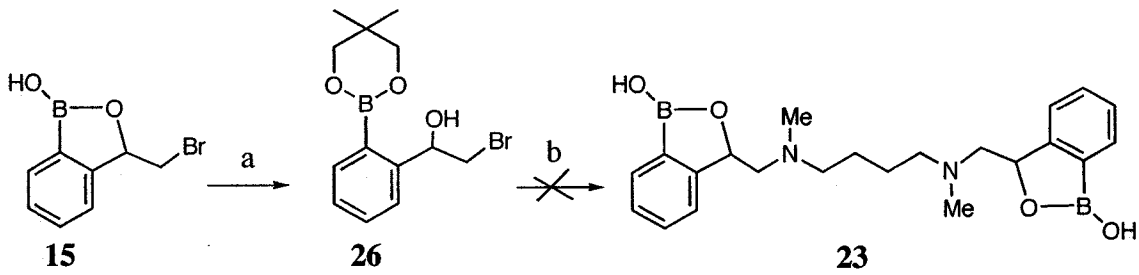
24, n=2

Equation 4.1 Attempted synthesis of diamine diboronic acids.



Scheme 4.6

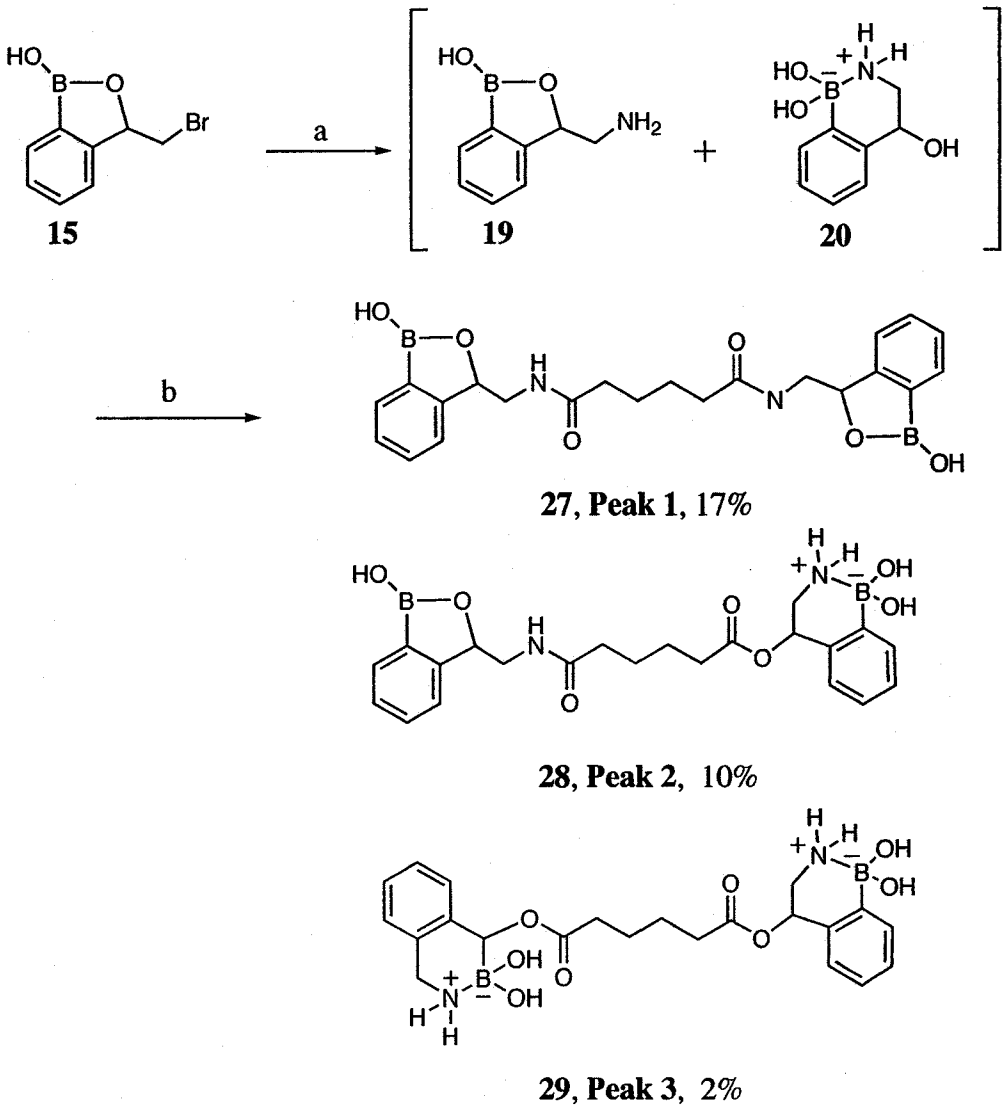
We therefore synthesized the 1,3 diol ester of boronic acid **15** (Scheme 4.7) to make the boron electron–rich and sterically hindered and thus force the amine to undergo a nucleophilic substitution reaction. Unfortunately, only the starting material was recovered after work-up.



Scheme 4.7 Reagents and conditions: (a) Neopentyl glycol, reflux in toluene, 12 h; (b) $MeNH(CH_2)_4NHMe$, THF, pempidine, 65-90 °C, 10 h.

4.7.5 Synthesis of diboronic acids with an amide linkage

All attempts at making dimeric variants of the cyclic boronic acid **2n** with the amine linkage failed. We therefore chose the other option of making the diamide **27** by condensation with adipoyl chloride in one step, generating the desired *N,N*-diacyl



Scheme 4.8 Reagents and conditions: (a) NH_3 (7 M in methanol), μwaves, 60-80 °C, 20 min; (b) adipoyl chloride, $NaHCO_3$, THF, 0 °C to rt, 4 h.

derivative in Scheme 4.8. Using microwave conditions, the bromide in boronic acid **15** was converted to the primary amine by reaction with concentrated 7 M ammonia. This amine **19** was obtained as a mixture with its tautomer **20**. The mixture was made to react with adipoyl chloride at a low temperature of 0 °C. The crude product mixture was purified by reverse phase C8 column chromatography (Figure 4-33) using water

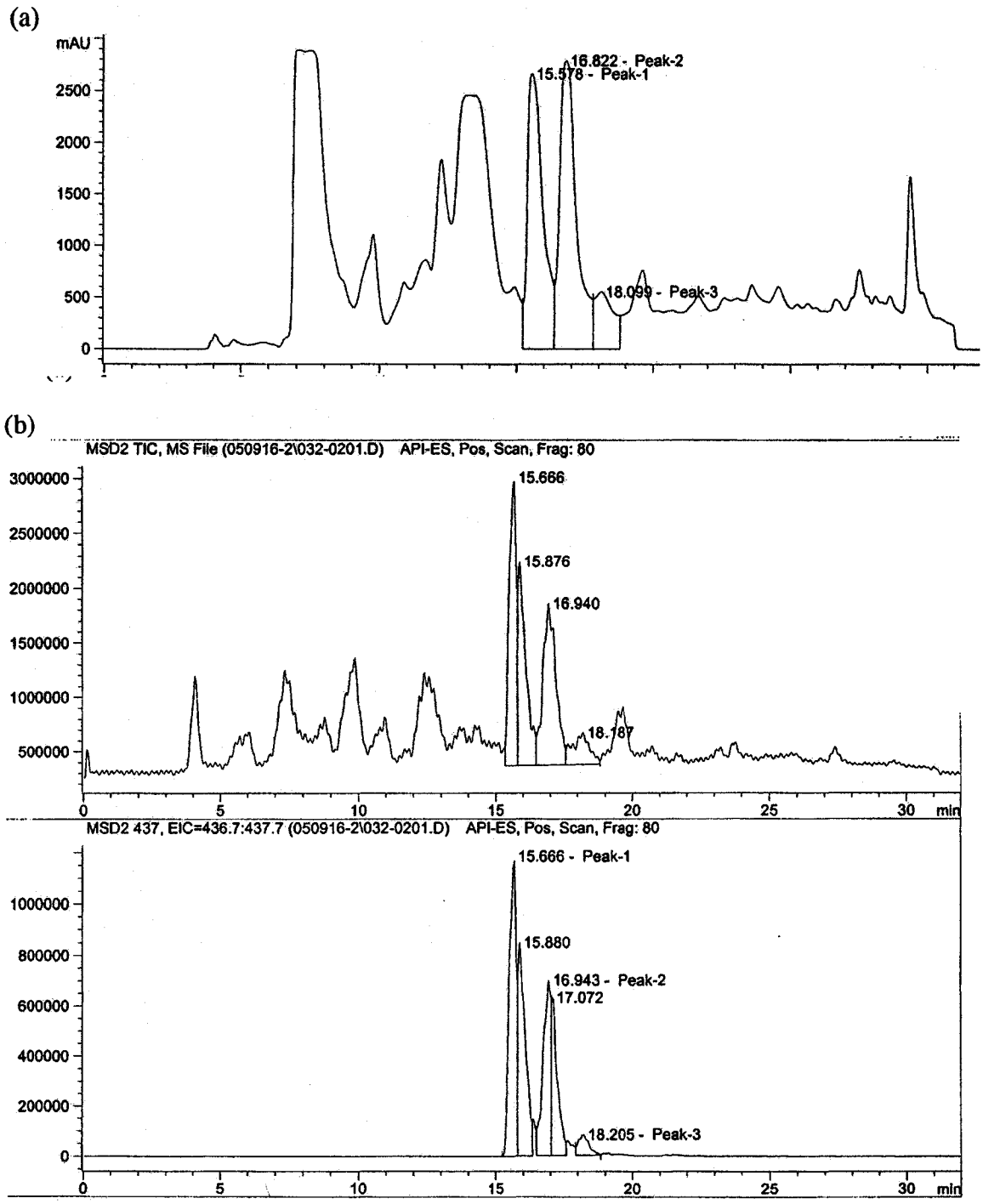


Figure 4-33. HPLC chromatograms for the purification of diboronic acids **27**-**29**. (for analytical conditions, see experimental section) with detection by (a) UV, (b) electrospray mass spectrometry.

methanol as eluting solvents (this work was performed by Mr. Eric Pelletier). To our satisfaction, the desired *N,N*-diacyl derivative **27**, **Peak 1**, eluted first during HPLC purification, and was generated in a low yield of 17%. The tautomer **20** was also generated during the ammonolysis reaction (Scheme 4.4). Thus, other diboronic acid isomers were formed under the aqueous basic conditions, namely **28** (10% yield) and **29** (2% yield). In this case as well, the low yielding step is likely the formation of the primary amine **19** because of bromide elimination under basic conditions. Due to the side-products of *O*-acylation formed, the desired diboronic acid **27** was obtained with a lower yield

4.7.6 Qualitative ARS assay and attempted ^{1}H NMR titrations with dimers 27-29

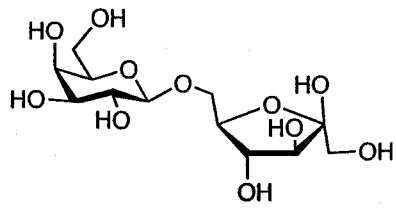
We wanted to investigate whether by dimerization, binding to a disaccharide is enhanced by cooperative binding of two diboronic acids with adjacent diols of carbohydrates. Both dimers **27** and **28** required 30% tetrahydrofuran as a co-solvent for the ARS assay, but dimer **29** did not need any. We tested the dimers with fructose, glucose, methyl α-D-glucopyranoside and trehalose. Dimers **27** and **28** displayed the same color changes as the monomer unit **2n** but the color was more intense for diboronic acid **27**. Diboronic acid **29** did not show any observable color changes with the glycosides.

Other disaccharides (Figure 4-34) were then tested with dimer **27** and the monoboronic acid **2n**. Identical color changes were observed with the dimer **27** and the monomer. This could be attributed to the fact that with an excess of the saccharide, which is 25

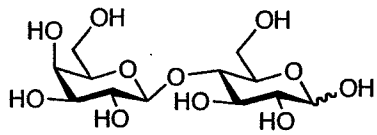
times more concentrated than the ARS-diboronic acid complex, the equilibrium of the dimer may be driven toward a 1:2 complex with saccharides rather than a 1:1 complex as hoped. Overall, it was found that the intensity of the reddish-orange color (binding to glycoside) decreases as follows:

Lactulose > lactose > sucrose ~ maltose ~ trehalose.

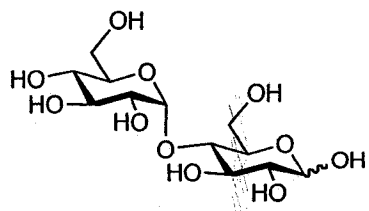
Not surprisingly, the dimer **27** and the cyclic boronic acid **2n** showed a strong preference for disaccharides having of course a fructose ring (lactulose) and next a galactopyranoside ring (lactose). Attempted ^{1}H NMR studies of the dimer **27** in a mixture of 20% deuterated tetrahydrofuran in deuterated methanol showed that product peaks with methyl α-D-galactopyranoside were broadened, just as with the monomer **2n** (Section 4.3, Figure 4-11). Consequently, binding constant with the galactopyranoside could not be determined by ^{1}H NMR.



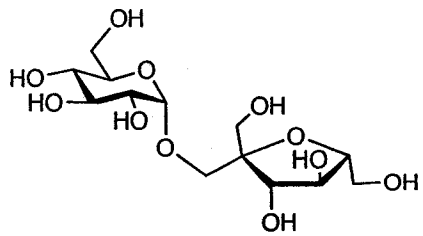
D-lactulose



D-lactose



D-maltose

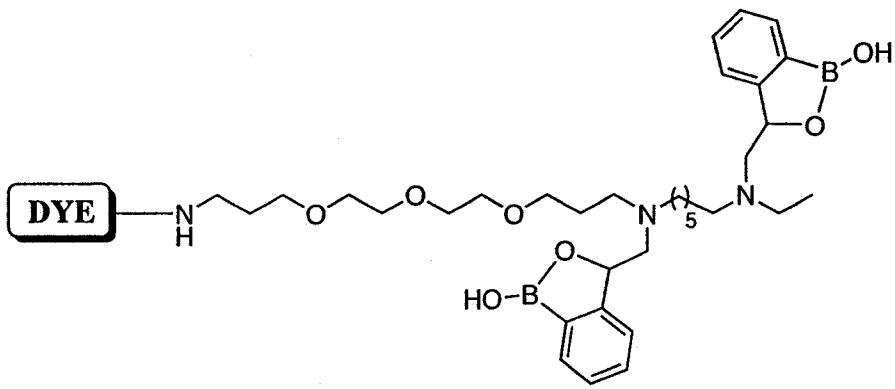


D-sucrose

Figure 4-34. Disaccharides used in the quanlitative ARS assay.

4.8 Attempted synthesis of a fluorescent diboronic acid conjugate

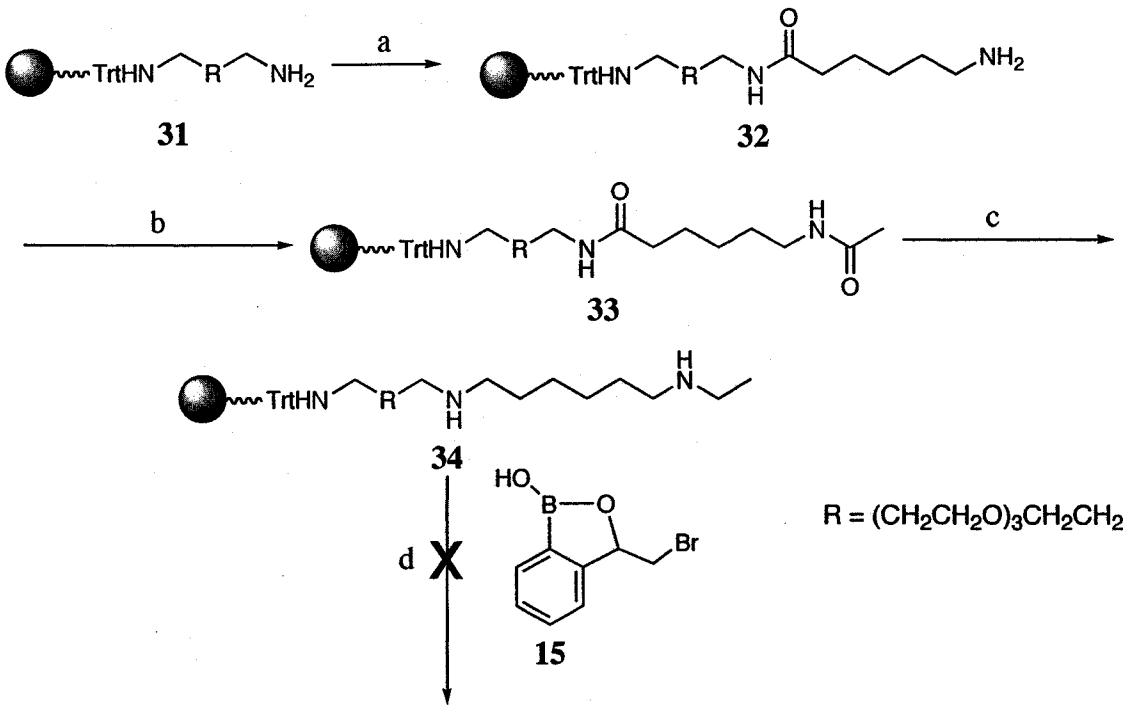
The potential of diboronic acid sensors has recently been highlighted in cell-labeling experiments[2,3] whereby the presence of a carbohydrate associated with cancer or another disease can be detected by fluorescence emission of a diboronic acid ligand binding strongly to a sialic acid containing carbohydrate. We envisaged the synthesis of such a dimer **30** (Figure 4-35) by a solid-phase methodology developed previously by our group for the synthesis of oligoboronic acid receptors.[6]



30

Figure 4-35. Fluorescently tagged diboronic acid.

In Scheme 4.9, a spacer with a terminal protected amine group was attached to chlorotritylpolystyrene resin. By coupling the amine with the Fmoc protected amino acid ε-aminocaproic acid and subsequent cleavage of the Fmoc group with piperidine, intermediate **32** was obtained. Another coupling was carried out with acetic acid under the presence of coupling agents, HOBT and HBTU. Diamide **33** was then reduced to the corresponding amine derivative **34** with a solution of sodium borohydride.



Scheme 4.9 Reagents and conditions: (a) (i) $HO_2C(CH_2)NHFmoc$, HBTU, HOBT, DMF, 12 h (ii) piperidine, DMF, 12 h; (b) CH_3CO_2H, HBTU, HOBT, DMF, 12 h; (c) (i) 1.0 M BH_3.THF, THF, 65 °C, 24-48 h, (ii) piperidine, DMF, 65 °C, 16 h; (d) pempidine, DMF or triethylamine as base, 65 °C.

Intermediate **34** was subjected to a double alkylation of the secondary amines with the bromide-containing boronic acid **15** in the presence of a base (pempidine or triethylamine). On cleavage of the product from the resin with a 5% TFA v/v solution of TFA in dichloromethane, the reduced amine was observed by ^{1}H NMR analysis and no aromatic protons were seen. Boronic acid **15** must have more likely undergone elimination rather than the expected nucleophilic substitution reaction.

4.9 Conclusions and future prospects

4.9.1 Conclusions regarding boronic acid receptors for saccharides

Boronic acids play an important role in recognizing and binding to biologically important carbohydrates that can serve as cell-markers.[22] Along this line, boronic acid based receptors for carbohydrates can be used in cancer diagnosis or as transporters of drugs into cells.[1] However, *in vivo* experiments require a boronic acid unit that is water-soluble and exhibits a high affinity towards carbohydrates at a physiological pH of 7.4. Moreover, receptors that can bind to the ubiquitous hexopyranoside units of complex carbohydrates are needed.

With the help of Alizarin Red S. competitive displacement assays,[19] we have compared and identified ortho-substituted arylboronic acids that can bind strongly to glucose and glycosides. *Ortho*-hydroxymethylphenylboronic acid (**2n**) (benzoboroxole) has broken the accepted dogma that monoboronic acids can bind only to saccharides that can isomerize to furanose forms. These five-membered ring structures present easily accessible hydroxyls such as vicinal cis-diols. In contrast to the "Wulff/Shinkai-type" *ortho*-dialkylaminomethylphenylboronic acids (**2o**, **2p**), the benzoboroxole **2n** is more water-soluble and binds glycosides at a physiological pH. In addition, **2n** exhibits higher or comparable binding constants with reducing sugars. K_a measurements by ^1H NMR experiments have shown that "Shinkai–type" boronic acid sensors benefit significantly from hydrophobic effects and not only via B-N interactions for binding reducing sugars.

The ^{1}H NMR experiments (depicted in Figures 4-12 and 4-13) performed with the 4-deoxy and 6-deoxy analogues of methyl α-D-glucopyranosides have shown the importance of the 4,6-diol unit in the complexation of *ortho*-hydroxymethylphenylboronic acid (**2n**) to the glucopyranoside. More experiments need to be done to confirm the mode of binding, possibly with receptors with more than one boroxole units.

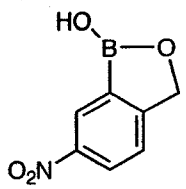
The Lewis acidity of boronic acid **2n** alone cannot explain the oserved glycoside binding as strongly Lewis acidic boronic acids such as **2o** and **3** do not bind glycopyranosides. Certainly, the low pK_a of *ortho*-hydroxymethylphenyl boronic acid (**2n**) (pKa = 7.2) plays a role as it exists largely in the tetrahedral sp^3 form, which binds more readily to the diol moieties of saccharides at a pH of 7.4. We believe that due to the inefficiency of the ether derivative **2m**, the cyclic boroxole ring of *ortho*-hydroxymethylphenyl boronic acid accounts for this exceptional glycoside binding capability. The unusually small C-B-O dihedral angle of *ortho*-hydroxymethylphenyl boronic acid opens up the cone angle in the resulting tetrahedral diol-boronate complex. This geometry may afford a better accommodation of the 4,6-diol unit of hexopyranosides compared to the usual boronic acids.

In an effort to develop more effective saccharide receptors embodying more than one boroxole unit, we synthesized dimers starting from 3-bromomethyl-3H-benzo[2] - [1,2]oxaborol-1-ol. However, under strongly basic conditions, elimination of the bromide occurred during the amination reaction, and more importantly the boroxole

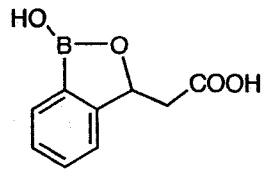
ring opens up to give the B-N form **20** with a free hydroxyl group. The latter competes with the amine during acylation reactions. Clearly, more efficient synthetic routes must be developed for the synthesis of oligomeric derivatives of the benzoboroxole **2n**. To this end, another strategy must be devised to introduce another functional group at the alkyl chain of *ortho*-hydroxymethylphenylboronic acid derivatives.

4.9.2 Future research into developing more efficient oligomeric boronic acids with boroxole rings

The synthesis of oligomers of boronic acids has been recognized as a useful strategy for developing saccharide receptors with high selectivity and binding affinity. The benzoboroxole skeleton offers several opportunities for functionalization. An alternative monoboronic acid synthesized by Snyder and Lennarz[39] is 5-nitro-boronophthalide (**35**) depicted in Figure 4-36. It contains an amino group in the aromatic ring that can react with other functional groups to assemble complex conjugates. Another possible building block is boronophthalidylacetic acid **37** (also shown in Figure 4-36). Boronic acid **36** was developed by Snyder and co-workers[40] and it has a carboxylic acid group in the alkyl chain that can form amide bonds. These boronic acids would enable conjugation of boronic acids without involving the bromide elimination and ring opening that plagued derivative **15**.



35



36

Figure 4-36. Alternative benzoboroxole building blocks.

By having access to higher affinity boronic acid receptors, more insight can be gained into the mode of binding of dimers or oligomeric derivatives with oligosaccharides at a physiological pH of 7.4. Observable changes in chemical shifts and coupling constants will be more drastic and conclusive.

Libraries of oligoboronic acid receptors can be developed with various functional groups that can act as a scaffold for attaching dye-molecules. Using the same strategy illustrated in Scheme 4.9, polyamine libraries developed by our group[6] can be treated with an aldehyde or carboxyl derivatives at the alkyl chain of the boroxole.[22] Solid-supported synthesis can furnish pure boronic acid compounds and can facititate their isolation. This is particularly essential as these boronic acids are very polar and water-soluble. In addition, they are difficult to purify by column chromatography because of their tendency to stick to silica gel. The alkyl spacer also can be varied, as length of spacers play an important role in cooperative binding to saccharides.

Current research efforts in our laboratories are being directed (work by Dr. Marie Bérubé, a post-doctoral associate) into synthesizing fluorescent reporter molecules based on the boroxole unit for cell-labeling purposes. It has been seen that among the

glycosides, the galactopyranose ring binds more tightly to *ortho*-hydroxymethyl-phenylboronic acid (**2n**). Galactopyranose rings are present in many biologically important carbohydrates such as the T-antigen (Chapter 1, Figure 1-9) whose over-expression in cells indicates the development of cancer. The Hall laboratory is committed to work toward a general approach to the selective recognition of cell-surface glycoconjugates using benzoboroxole derivatives.

4.10 Experimental

4.10.1 General

The methods described in Section 2.8.1 also apply here with the following additions. Alizarin Red S. and phenylboronic acid were purchased from Acros, and were used as received. All boronic acids except for **2o**, **2p** and **3** were purchased from Aldrich Chemical Co., Combi-blocks and Frontier Scientific respectively. The water used for the K_a studies was distilled and further purified with a filtration system. Quartz cuvettes were used in all studies and the ultraviolet absorptions were taken with a Varian-Scan 400 UV spectrophotometer. All data was plotted using Microsoft Excel. All ^{11}B NMR were taken with boron trifluoride etherate as external reference.

Microwave reactions were carried out in microwave vials in a Biotage initiator automated microwave reactor.

For HPLC analyses and purifications, detection was carried out with a UV diode array detector ($\lambda = 254$ nm), coupled to a ESI-MS detector (fragmentor voltage: 80 eV).

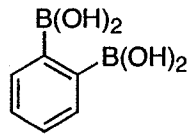
In most cases, the quatenary aromatic carbon bound to boron is not seen in the carbon NMR due to extensive peak broadening.

4.10.2 Experimental procedures

1,2-Bis(trimethylsilyl)benzene (5)[41]

It was prepared using the same conditions as Kitamura and co-workers,[14] with a different starting material, 1,2-dibromobenzene, instead of 1,2-dichlorobenzene. The spectral data matched the reported ones. Yield: 67%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.69 (dd, 2H, J = 5.8, 8.8 Hz), 7.34 (dd, 2H, J = 5.8 Hz, 8.8 Hz), 0.386 (s, 3H), 0.385 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 146.0 (ArC), 135.2, 127.7 (ArCH), 1.9 ($\underline{C}H_3$). HR-MS (EI) calcd for $C_{12}H_{22}Si_2$: 222.1260. Found: 222.1258 $[M]^+$.

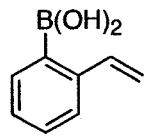
1,2-Phenylenediboronic acid (2c)



The following procedure was modified from the one reported by Kaufmann.[15] To a stirred solution of compound **5** (0.60 g, 2.70 mmol) in dry DCM (5 mL), was added 1M solution of boron tribromide (7 mL, 7.00 mmol) in DCM, at –78 °C. The solution was stirred for 4 h and allowed to warm to room temperature. After evaporation of the solvent, dry dichloromethane (5 mL) was again added, followed by 1M solution of boron tribromide (7 mL, 7.00 mmol) in DCM. The mixture was heated at 95 °C for 16 h. The solvent was again evaporated and the mixture was dissolved in methanol (3 × 5 mL) and the solvent was removed using rotary evaporator. 10% Hydrochloric acid (1 mL) was added for complete hydrolysis of the methyl esters of the diboronic acid.

After removal of solvent and drying under vacuum for 4 h, the black solid was subjected to DEAM-resin purification.[16] A solution of the black crude mixture in dry tetrahydrofuran (15 mL) was added to DEAM-PS (2.35 g, 2.70 mmol, 1 equiv, experimental loading: 1.15 mmol g^{-1}) in a pp vessel. The reaction suspension was shaken for 1 h at room temperature and the pp vessel was drained. Washing of the resin was carried out with dry tetrahydrofuran (3 × 10 mL). The resin-bound diboronic acid **2c** was cleaved by vortexing the resin with 20% H_2O/THF (3 × 10 mL) for 1 min at room temperature. The product containing solution was drained. The filtrates were combined, concentrated under reduced pressure and dried under high vacuum for 6 h to afford a pale brown solid (60 mg). Attempted precipitation with ethyl acetate/hexane and 1% water yielded a pale yellow solid (0.026 g, 0.16 mmol, 6%), which was recovered after filtration. ^{1}H NMR (500 MHz, CD_3OD/D_2O: 4/1) δ 7.90-7.66 (m, 2H), 7.44-7.30 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD/D_2O: 4/1) δ 134.9, 134.8, 128.7; ^{11}B NMR (128 MHz, CD_3OD/D_2O: 4/2) δ 28.6. HR-ESMS calcd for $C_6H_8B_2O_4Cl$: 201.0292. Found: 201.0291 $[M+Cl]^-$.

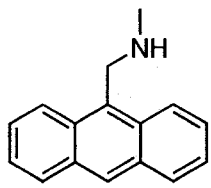
2-Vinylbenzeneboronic acid (15)[42]



*n*BuLi (1.58 M in hexanes, 5.46 mL) was added to a solution of bromostyrene (1.00 mL, 3.74 mmol) in dry tetrahydrofuran (25 mL) cooled to -78 °C. The mixture was stirred for 40 min, then triisopropylborate (3.78 mL, 16.4 mmol) was added slowly to

the solution over 30 min at -78 °C. The solution was allowed to warm to room temperature for 4 h. The reaction was quenched by adding a 50% solution of ammonium chloride in water to the mixture at 0 °C. Tetrahydrofuran was removed by evaporation and the aqueous mixture was extracted with diethyl ether (60 mL). The organic phase was washed with water (2 × 40 mL) and brine (1 × 40 mL). The solvent was removed under reduced pressure and the resulting white crystals (1.21 g, 3.74 mmol, quantitative yield) were used for the next step without purification. ^{1}H NMR (400 MHz, DMSO/D_2O: 98/2) δ 7.60-7.58 (m, 1H), 7.43 (dd, 1H, *J* = 1.2 Hz, 7.2 Hz), 7.36-7.28 (m, 1H, *J* = 1.2 Hz, 7.6 Hz, 8.8 Hz), 7.21 (dt, 1H, *J* = 1.2 Hz, 7.6 Hz, 8.8 Hz), 7.09 (dd, 1H, *J* = 9.6 Hz, 17.6 Hz), 5.67 (dd, 1H, *J* = 1.2 Hz, 17.6 Hz), 5.20 (dd, 1H, *J* = 1.2 Hz, 11.0 Hz); ^{13}C NMR (100 MHz, DMSO/D_2O: 98/2) δ 140.6, 138.0, 133.3, 129.0, 126.9, 124.3, 114.4; ^{11}B NMR (128 MHz, DMSO/D_2O: 98/2) δ 29.7. HR-ESMS calcd for $C_8H_8BO_2$: 147.0611. Found: 147.0613 $[M-H]^-$.

9-(*N*-methylaminomethyl)anthracene

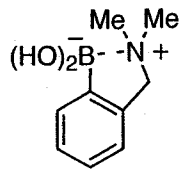


A solution of 9-anthraldehyde (0.69 g, 3.33 mmol) was made in dry methanol. Methylamine hydrochloride (0.25 g, 3.70 mmol) was added to the solution followed by triethylamine (6 mL). The mixture was stirred with molecular sieves (3Å) and heated at 40 °C for 4 h. The mixture was cooled and sodium borohydride (0.33 g, 8.77 mmol) was added. The resulting solution was stirred at room temperature for 6 h. The mixture

was filtered through celite and dissolved in dichloromethane (60 mL). The organic solution was washed with water (2 × 30 mL) and brine (1 × 30 mL). After drying over anhydrous sodium sulfate and filtration, the solvent was removed by evaporation and the leftover yellow oil was triturated with hexane and cooled in ice. Filtration afforded a yellow colored solid (0.73 g, 3.30 mmol, 99% yield). The ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.42 (s, 1H), 8.36 (d, 2H, J = 8.5 Hz), 8.02 (d, 2H, J = 8.5 Hz), 7.55 (t, 2H, J = 7.8 Hz), 7.47 (t, 2H, J = 8.0 Hz), 4.71 (s, 2H), 2.67 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 131.5, 130.4, 129.2, 127.2, 126.1, 124.9, 124.9, 124.1, 47.7, 37.0. HR-ESMS calcd for $C_{16}H_{16}N$: 222.1278. Found: 222.1276 $[M+H]^+$.

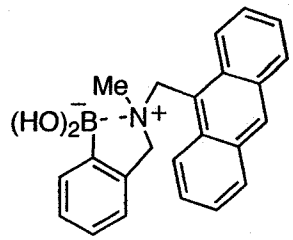
Compounds **20** and **2p** were prepared according to literature procedures.[43]

N,N dimethylaminomethylphenyl boronic acid (2o)



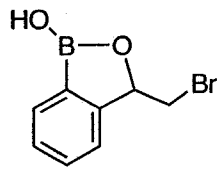
Compound **2o**, yield: 79%; ^{1}H NMR (500 MHz, CD_3OD) δ 7.74 (brd, 1H, J = 5 Hz), 7.42-7.24 (m, 3H), 4.22 (s, 2H), 2.71 (s, 6H); ^{13}C NMR (125 MHz, CD_3OD) δ 138.2, 135.1, 130.4, 129.5, 64.6, 44.1; ^{11}B NMR (128 MHz, CD_3OD) δ 18.0. HR-ESMS calcd for $C_9H_{14}BNO_2$(Na): 202.1010. Found: 202.1011 $[M+Na]^+$.

N-(9-Anthrylmethyl)-N-methylaminomethylphenyl boronic acid(2p)



Compound **2p,** yield: 60%; ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$: 9/1) δ 8.36 (s, 1H), 8.06-7.88 (m, 5H), 7.44-7.32 (m, 7H), 4.41 (s, 2H), 3.86 (s, 2H), 3.25 (br s, 2H), 2.18 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$: 9/1) δ 141.4, 135.7, 135.4, 131.3, 131.2, 131.0, 130.0, 129.0, 128.1, 127.74, 127.70, 125.8, 124.8, 124.4, 65.4, 50.3, 41.8; ^{11}B NMR (128 MHz, $CDCl_3/CD_3OD$: 9/1) δ 28.8. HR-ESMS calcd for $C_{23}H_{23}BNO_2$: 356.1816. Found: 356.1819 $[M+H]^+$.

3-Bromomethyl-3H-benzo[2][1,2]oxaborol-1-ol (15)



Compound **16** was made from a known procedure[44] and its spectral characteristics matched the reported data.

Analysis and purification of boronic acids 16, 17, 19, 20, 27, 28, 29 by HPLC:

LCMS/UV analyses were performed on a Hewlett-Packard/Agilent 1100MSD by means of one of the following separation methods.

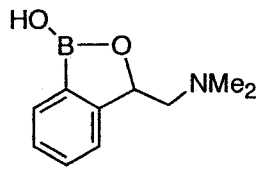
Method A : Column: SB-C8 semi-preparative, 3.5 μm, 9.4 × 250 mm; eluent: 0% MeOH (0.05% formic acid) to 25% MeOH (0.05% formic acid) over 15 min, 80%

MeOH (0.05% formic acid) over 10 min, hold for 5 min and 0% MeOH (0.05% formic acid) over 7 min at a flow rate of 3.0 mL/min for the main pump and 0.4 mL at the post pump. Peaks were collected based on UV absorptions at 254 nm.

Method B: Semi-preparative purification: Column: SB-C8 semi-preparative, 3.5 μm, 9.4 × 250 mm; eluent: 40% MeOH (0.05% formic acid) to 80% MeOH (0.05% formic acid) over 25 min, then hold for 2 min at a flow rate of 3.0 mL/min for the main pump and 0.4 mL at the post pump. Post run of 5 min, with automated collection of peaks based on UV absorptions at 254 nm.

Analysis: Column: SB-C8, 3.5 μm, 4.6 × 50 mm using the same elution protocol as above but at a flow rate of 1 ml/min over a run time of 27 min.

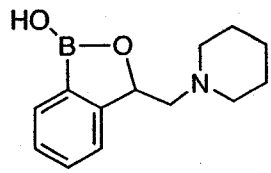
3-Dimethylaminomethyl-3*H*-benzo[*c*][1,2]oxaborol-1-ol (16)



Compound **15** (0.056 g, 0.25 mmol) was made into a solution with dry tetrahydrofuran (1 mL) at room temperature and dimethylamine (2 M in methanol, 1.1 mL) was added, followed by a two drops of triethylamine. The mixture was allowed to stir for 12 h, and the solvent was removed under reduced pressure. The crude (0.022 g) was subjected to HPLC purification to yield a white solid (0.019 g, 0.10 mmol) in 41% yield. Semi-preparative purification was performed using *Method A*. ^{1}H NMR (500 MHz, $CDCl_3$ with a drop of D_2O) δ 7.67 (d, 1H, J = 7.2 Hz), 7.41 (dt, 1H, J = 1.2 Hz, 7.6 Hz), 7.26-

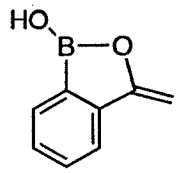
7.36 (m, 2H), 5.40 (dd, 1H, J = 2.4 Hz, 9.2 Hz), 3.19 (dd, 1H, J = 2.4 Hz, 13.4 Hz), 2.63 (s, 6H), 2.57 (dd, 1H, J = 9.2 Hz, 13.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$ with a drop of D_2O) δ 153.0, 130.63, 130.55, 127.9, 121.2, 77.6, 64.2, 44.6; ^{11}B NMR (100 MHz, $CDCl_3$ with a drop of D_2O) δ 30.0. HR-ESMS calcd for $C_{10}H_{15}BNO_2$: 192.1190. Found: 192.1197 $[M+H]^+$.

3-Piperidin-1-ylmethyl-3*H*-benzo[*c*][1,2]oxaborol-1-ol (17)



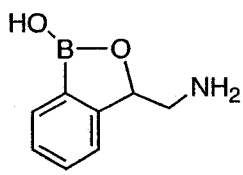
The same procedure as for the boronic acid **16** was used here with piperidine as the amine. A white solid was obtained in 49% yield after HPLC purification. Semi-preparative purification was performed using *Method A*. ^{1}H NMR (400 MHz, $CDCl_3$ with a drop of D_2O) δ 7.44 (d, 1H, J = 7.2 Hz), 7.08-7.22 (m, 3H), 5.30 (dd, 1H, J = 2.2 Hz, 7.2 Hz), 3.36 (dd, 1H, J = 2.8 Hz, 13.6 Hz), 2.94 (apparent triplet, 4 H, J = 5.2 Hz), 2.73 (dd, 1H, J = 7.6 Hz, 13.6 Hz), 1.58-1.74 (m, 4H), 1.43 (ddd, 2H, J = 6.0, 11.6 Hz, 17.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$ with a drop of D_2O) δ 150.0, 130.0, 129.3, 127.6, 121.0, 75.4, 63.0, 54.1, 22.8, 21.5; ^{11}B NMR (100 MHz, $CDCl_3$ with a drop of D_2O and one drop of CD_3OD) δ 20.6. HR-ESMS calcd for $C_{13}H_{19}BNO_2$: 232.1503. Found: 232.1504 $[M+H]^+$.

3-Methylenebenzo[*c*][1,2]oxaborol-1(3*H*)-ol (18)

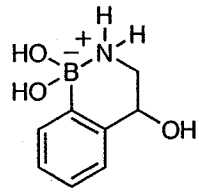


The spectra of the elimination product **18** were as follows: ^{1}H NMR (500 MHz, $CDCl_3$ with a drop of D_2O) δ 8.06 (d, 1H, J = 7.5 Hz), 7.60 (dt, 1H, J = 1.0 Hz, 7.5 Hz), 7.42-7.38 (m, 2H), 7.04 (d, 1H, J = 5.5 Hz), 6.31 (d, 1H, J = 5.5 Hz) ; ^{13}C NMR (125 MHz, $CDCl_3$ with a drop of D_2O) δ 142.4, 141.9, 132.8, 132.3, 126.3, 125.4, 109.9; ^{11}B NMR (100 MHz, $CDCl_3$ with a drop of D_2O) δ 19.0. HR-ESMS calcd for $C_8H_6BO_2$:145.0455. Found:145.0454 $[M+H]^+$.

3-Aminomethyl-3H-benzo[*c*][1,2]oxaborol-1-ol (19) and 3,4-dihydro-2-aza-1-bora-naphthalene-1,1,4-triol (20)



19



B-N tautomer **20**

This preparation was adapted from a reported experiment.[38] A solution of 7 M ammonia in methanol (Aldrich; 2 mL) was added to **16** (0.15 g, 0.67 mmol) in a 5 mL microwave vial. The mixture was heated at 60 °C for 10 min, and after the removal of the solvent, another 2 mL of 7 M ammonia in methanol was added. The mixture was then heated again under microwave conditions at 80 °C for 10 min. On evaporation of solvent, the solid was purified by HPLC to give a mixture of **19/20** (0.038 g, 0.24 mmol, 35%) as a white solid. Semi-preparative purification was performed using

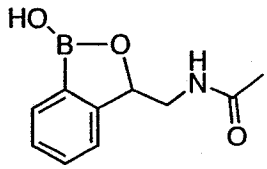
Method A. The amine compounds existed as a 4:1 inseparable mixture of 3-aminomethyl-3H-benzo[*c*][1,2]oxaborol-1-ol **19** and the B-N tautomer **20**.

^{1}H NMR of 3-aminomethyl-3H-benzo[*c*][1,2]oxaborol-1-ol **19**:(500 MHz, D_2O) δ 7.30-7.80 (m, 4H), 5.39 (brs, 1H), 3.58 (d, J = 13.0 Hz, 1H), 3.09 (dd, 1H, J = 7.0 Hz, 13.0 Hz); ^{13}C NMR (125 MHz, D_2O) δ 151.5 (ArC), 132.3, 131.4, 129.4, 122.5 (ArCH), 77.6 (CH), 44.8 (CH_2); ^{11}B NMR (128 MHz, D_2O) δ 28.9. HR-ESMS calcd for $C_8H_{11}BNO_2$: 164.0877. Found: 164.0878 $[M+H]^+$.

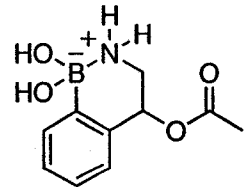
^{1}H NMR of the B-N coordinated tautomer **20**: (500 MHz, D_2O) δ 7.30-7.80 (m, 4H), 4.49 (t, 1H, J = 2.3 Hz), 4.35-4.27 (m, 2 H); ^{13}C NMR (125 MHz, D_2O) δ 139.5 (ArC), 134.0, 132.5, 130.5, 128.9 (ArCH), 65.5 (CH_2), 51.6 (CH); ^{11}B NMR (128 MHz, D_2O) δ 24.3.

The structures of the B-O and B-N coordinated tautomers were based on the following: In the B-N coordinated form **20**, the CH_2 protons at 4.35-4.27 ppm did not have any long distance correlations with the aromatic protons and the CH proton of **20** being closer to the aromatic ring did. In the B-O coordinated form in 3-aminomethyl-3H-benzo[*c*][1,2]oxaborol-1-ol (**19**), the protons of the CH_2 and CH groups are close to the aromatic rings and they both exhibit spatial correlations with the aromatic protons as seen in the TROESY spectrum.

***N*-(1-Hydroxy-1,3-dihydro-benzo[*c*][1,2]oxaborol-3-ylmethyl)-acetamide (21) and acetic acid 1,1-dihydroxy-3,4-dihydro-2-aza-1-bora-naphthalen-4-yl ester (22)**



21

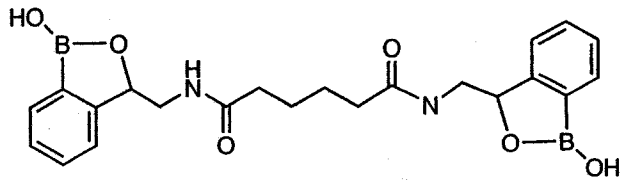


22

The amines **19/20** were formed using the above microwave procedure from compound **15** (0.19 g, 0.82 mmol) and used without any purification. The amines **19/20** were made into a solution with dry tetrahydrofuran (1 mL) and a few drops of triethylamine (0.2 mL) was added at 0 °C. Acetyl chloride (0.64 mL, 9.00 mmol) was then added and the mixture was allowed to warm to room temperature for 2 h. The solvent was removed under reduced pressure and HPLC purification afforded **21** as an inseparable 8:1 mixure (0.060 g, 0.30 mmol, 37%) of the desired product with the by-product, B-N isomer **22**. Semi-preparative purification and analysis were performed using *Method B*.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70 (d, 1H, J = 7.0 Hz), 7.40-7.28 (m, 3H), 5.23 (dd, 1H, J = 3.5 Hz, 8.0 Hz), 5.12-5.17 (m, 1H, B-N form), 4.18-4.28 (m, 2H, B-N form), 4.03-4.10 (m, 1H), 3.03 (ddd, 1H, J = 3.5 Hz, 8.0 Hz, 12.5 Hz), 1.96 (s, 3H, B-N form), 1.95 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.0, 170.2 (B-N form), 153.5 (Ar<u>C</u>), 131.9 (Ar<u>C</u>H, B-N form), 131.1, 130.6 (Ar<u>C</u>H), 128.0 (Ar<u>C</u>H, B-N form), 127.9 (ArCH), 126.8 (ArCH, B-N form), 121.5 (ArCH), 80.4 (CH), 67.6 (CH, B-N form), 49.4 (CH_2), 44.8 (CH_2, B-N form), 23.1, 23.0 (<u>C</u>H_3); ^{11}B NMR (128 MHz, $CDCl_3$) δ 31.6, 27.7 (B-N form). HR-ESMS calcd for $C_{10}H_{12}BNO_3$(Na): 228.0802. Found: 228.0803 $[M+Na]^+$.

N^1,N^6-Bis((1-hydroxy-1,3-dihydrobenzo[*c*][1,2]oxaborol-3-yl)methyl)adipamide (27), 1-hydroxy-1,2,3,4-tetrahydrobenzo[*c*][1,2]azaborinin-4-yl 6-((1-hydroxy-1,3-dihydrobenzo[*c*][1,2]oxaborol-3-yl)methylamino)-6-oxohexanoate (28) and 1-hydroxy-1,2,3,4-tetrahydrobenzo[*c*][1,2]azaborinin-4-yl-3-hydroxy-1,2,3,4-tetrahydrobenzo[*d*][1,2]azaborinin-4-yl adipate (29)



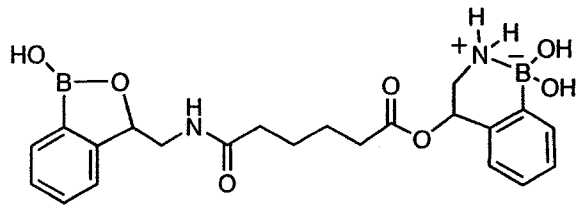
27

3-aminomethyl-3H-benzo[*c*][1,2]oxaborol-1-ol (**19**) (0.50 g, 3.23 mmol) was synthesized under microwave conditions as above and used as a crude substrate without further purification. After evaporation of methanol, dry tetrahydrofuran (6 mL) was added followed by saturated aqueous $NaHCO_3$ (6 mL) at 0 °C. Adipoyl chloride (0.16 mL, 1.08 mmol) was added and the mixture was stirred for 4 h with the temperature slowly rising to rt. After completion of the reaction, the solvent was removed under vacuum and purified by HPLC to give three fractions in increasing order of the retention time, **Peaks 1-3**. The first fraction to be detected, **Peak 1**, was assigned to the structure of the desired *N*,*N*-diacyl product **27**. Semi-preparative purification and analysis were performed using *Method B*.

Peak 1, Compound **27**: yield, 17%; ^{1}H NMR (500 MHz, $CDCl_3$ with 2 drops of CD_3OD and 1 drop of D_2O) δ 7.63 (d ,1H, J = 7.0 Hz), 7.39 (t, 2H, J = 7.5 Hz), 7.23-7.35 (m,

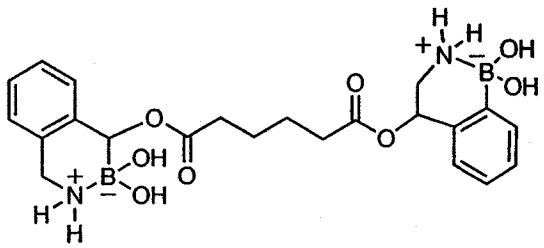
4H), 5.19 (dd, 1H, J = 2.5 Hz, 7.0 Hz,), 3.90 (dt, 1H, J = 2.5 Hz, 14.5 Hz), 3.13 (ddd, 1H, J = 2.9, 7.5, 10.5 Hz), 1.98-2.15 (m, 4H), 1.29-1.48 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$ with 2 drops of CD_3OD and 1 drop of D_2O) δ 174.2 (C=O), 153.5 (ArC), 131.0, 130.4, 127.8, 121.5 (ArCH), 80.4 (CH), 44.2, 35.6, 24.8 (CH_2); ^{11}B NMR (128 MHz, $CDCl_3$ with 2 drops of CD_3OD and 1 drop of D_2O) δ 29.7. HR-ESMS calcd for $C_{22}H_{26}B_2N_2O_6$(Na): 459.1869. Found: 459.1879 $[M+Na]^+$.

Peak 2, compound **28**:



Yield, 10%; ^{1}H NMR (500 MHz, $CDCl_3$ with 3 drops of CD_3OD and 1 drop of D_2O) δ 7.54-7.64 (m, 2H), 7.14-7.38 (m, 6H), 5.11 (dd, 1H J = 2.5 Hz, J = 7.0 Hz), 5.04-4.98 (m, 1H), 4.02-4.12 (m, 2H), 3.79 (dt, J = 3.0 Hz,1H, J = 14.0 Hz), 3.06 (ddd, 1H, J =, 7.5; 12.5 Hz), 1.98-2.10 (m, 4H), 1.34-1.48 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$ with 3 drops of CD_3OD and 1 drop of D_2O) δ 174.3, 173.7 (C=O), 153.5, 144.3 (ArC), 132.8, 131.7, 130.9, 130.3, 127.7, 127.6, 126.1, 121.4 (ArCH), 80.3 (CH), 67.2 (CH_2), 49.4 (CH), 44.2, 44.1, 35.5, 35.43, 35.41, 24.89, 24.87, 24.8, 24.7 (CH_2); ^{11}B NMR (128 MHz, $CDCl_3$ with 3 drops of CD_3OD and 1 drop of D_2O) δ 31.4, 27.7. HR-ESMS calcd for $C_{22}H_{26}B_2N_2O_6$(Na): 459.1869. Found: 459.1867 $[M+Na]^+$.

Peak 3, compound **29**:

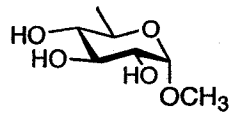


Yield, 2%; ^{1}H NMR (500 MHz, $CDCl_3$ with 2 drops of CD_3OD and 1 drop of D_2O) δ 7.70-7.60 (m, 2H), 7.42-7.18 (m, 6H), 5.06 (br s, 1H), 4.12 (br s, 2H), 2.18-2.04 (m, 4H), 1.60-1.50 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$ with 2 drops of CD_3OD and 1 drop of D_2O) δ 173.8 (C=O), 144.5 (ArC), 133.1, 132.0, 131.26, 131.25, 130.7, 128.0, 126.5, 121.7 (ArCH), 67.6 (CH_2), 49.6 (CH), 35.8, 25.0 (CH_2); ^{11}B NMR (128 MHz, $CDCl_3$ with 2 drops of CD_3OD and 1 drop of D_2O) δ 27.1. HR-ESMS calcd for $C_{22}H_{26}B_2N_2O_6Na$: 459.1869. Found: 459.1871 $[M+Na]^+$.

Peak 1 is assigned to be structure **27** due to the following reasons:

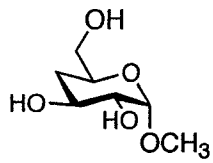
1. It displays the symmetrical pattern of monomer **2n** where the CH_2 protons are diastereotopic with different chemical shifts.
2. The diastereotopic CH_2 protons show each a ddd at 3.13 ppm and at 3.90 ppm, showing the existence of an extra coupling with the NHAc of the spacer.
3. From the ^{11}B NMR spectrum, **Peak 1** has a ^{11}B shift at 29.7 ppm, similar to the ^{11}B shift of **2n** at 32.0 and of **15** at 31.5 ppm, under similar solvent conditions. In contrast, **Peak 2** has a much lower shift at 27.7 ppm.

Methyl 6-deoxy-α–D-glucopyranoside



Methyl 6-deoxy-α-D-glucopyranoside is a known compound, synthesized by benzylidenation[45] of methyl α-D glucopyranoside, NBS[46-48] ring opening of the ring followed by catalytic hydrogenation[47] to give the deoxy sugar whose spectral data[49] matched those found in the literature. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.44 (d, 1H, H-1, *J* = 3.6 Hz), 3.34-3.46 (m, 2H, H-5, H-3), 3.24 (dd, 1H, H-2, *J* = 2.0, 4.0 Hz), 3.19 (s, 3H, CH_3), 2.84 (t, 1H, H-4, *J* = 9.2 Hz), 1.05 (d, 3H, *J* = 6.4 Hz, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 99.3 (C-1), 76.7 (C-4), 75.3 (C-3), 71.9 (C-2), 67.0 (C-5), 54.6 (CH_3), 16.9 (C-6, CH_3). HR-ESMS calcd for $C_7H_{14}O_5$(Na): 201.0733. Found: 201.0732 $[M+Na]^+$.

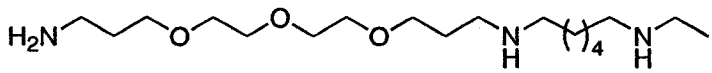
Methyl 4-deoxy-α-D-glucopyranoside



Methyl 4-deoxy-α-D-glucopyranoside is a known compound, prepared by S_N2 displacement of the 4-methylsulfonyloxy group of methyl 2,3,6-tri-*O*-benzoyl-4-deoxy-4-*O*-methylsulfonyl-α-D-glucopyranoside[50] by iodide ions and subsequent reduction[51] and debenzoylation of the resulting methyl 2,3,6-tri-*O*-benzoyl-4-deoxy-4-iodo-α-D-glucopyranoside. The spectral data of the deoxy sugar matched those found in the literature.[52] ^{1}H NMR (500 MHz, D_2O) δ 4.83 (d, 1H, H-1, *J* = 4.0 Hz), 3.94-3.86 (m, 2H, H-5, H-3), 3.66 (dd, 1H, H-6a, *J* = 3.0, 12.0 Hz), 3.58 (dd, 1H, H-6b, *J* = 6.5, 12.0 Hz), 3.47 (dd, 1H, H-2, *J* = 4.0, 9.5 Hz), 3.40 (s, 3H, CH_3), 1.97 (ddd, 1H, H-4eq, *J* =

2.0, 5.0, 7.0 Hz), 1.42 (q, 1H, H-4ax, J = 2.0, 5.0, 7.0 Hz); ^{13}C NMR (125 MHz, D_2O) δ 100.8 (C-1), 73.9 (C-2), 69.5, 69.1 (C-3, C-5), 64.6 (C-4), 35.0 (CH_3). HR-ESMS calcd for $C_7H_{14}O_5$(Na): 201.0733. Found: 201.0734 $[M+Na]^+$.

***N*-(3-{2-[2-(3-Amino-propoxy)-ethoxy]-ethoxy}-propyl)-*N*'-ethyl-hexane-1,6-diamine**



The polyamine was prepared and cleaved from the tentagel resin following the reported procedure on solid-phase synthesis.[6] ^{1}H NMR of crude (400 MHz, $CDCl_3$) δ 3.65-3.54 (m, 8H), 3.18 (m, 10H), 2.20-1.55 (m, 5H), 1.47-1.34 (m, 2H). HR-ESMS calcd for $C_{18}H_{42}N_3O$):348.3220. Found:348.3222 $[M+H]^+$.

4.10.3 Methodology for screening ortho-substituted arylboronic acids (qualitative ARS assay)[20]

Solution A: 50 mL of 10^{-3} M stock solution of ARS solution in 0.10 M sodium phosphate monobasic buffer was diluted 10-fold with 0.10 M sodium phosphate monobasic buffer in a 500 mL volumetric flask. The pH of the solution was adjusted to 7.4 with 4 M NaOH (a portable pH meter was used, which gave pH values within 0.01 units). The resultant solution containing 10^{-4} M solution of ARS in 0.10 M phosphate buffer at pH 7.4 would be referred to as solution A.

Solution B: The controls were prepared by dissolving the boronic acids (0.10 mmol) in solution A in a 5 mL volumetric flask to give 0.02 M solution with respect to the

boronic acid. The pH was adjusted to 7.4 with 4 M NaOH before diluting to the 5 mL mark with ARS solution A. For boronic acid **3**, a 20% THF/H_2O solution was employed.

Colorimetric assays were done with these solutions except for the less soluble boronic acids that required 10-33% methanol as solvent. The sugar solutions (0.5 M) were prepared by adding 0.5 mmol of sugar to the control solution B in 1 mL volumetric flasks. The pH was adjusted to 7.4 with 4 M NaOH.

In the case of boronic acid **3**, no change of coloration was seen with methyl glycosides.

4.10.4 Determination of K_a for boronic acid 2n

The deuterated buffer solution was made by evaporating off an aqueous solution of 0.10 M phosphate monobasic solution (at pH 7.4) three times with D_2O, and again the pH was adjusted to pH 7.4 (with 4N NaOH or saturated sodium phosphate monobasic solution). Due to the hygroscopic nature of D_2O, a certain amount of water as H_2O was always present as 1-2%.

The boronic acid **2n** (0.38 mmol) was dissolved in 25 mL of deuterated buffer solution in a volumetric flask, and before diluting to the final volume the pH was adjusted to 7.4 (solution A, 15 mM). A solution of the sugar (0.15 M) was made by dissolving 0.15 mmol of sugar in 1 mL of the solution A, and the pH was again adjusted to 7.4. To 700 μL of solution A was added 40 μL of the sugar solution, and afterwards aliquots of 10 μL were added to obtain a range of sugar concentrations in the range of 8-26 mM and

until the boronic acid solution was saturated with the sugar and the percentage error for calculating the complexed boronic acid/free acid ratio (Figure 4-14) is too high (i.e., a ratio in the range 0.5-4.0 was employed).

4.10.5 Methodology and examples for K_a measurements by ARS method[5,6]

Following the procedure of Wang *et al.*,[5] a 0.144 mM ARS solution was prepared in 0.1 M phosphate solution buffered at pH 7.4. A solution of **2n** (0.015 M) was prepared so as to prepare mixtures of **2n** (0.00131-0.0131 M) and UV absorbances were taken at 450 nm. Two experiments were carried out to determine an average value of K_{ars}.

4.10.6 Binding of 2n with glucose in the three-component ARS assay

The above **2n** solution (15 mM) in ARS was diluted with the ARS solution (0.144 mM) to give a 3.1 mM solution in the phosphate buffered solution at pH 7.4. 5 mL of this solution was used to make a 2.0 M glucose solution at pH 7.4. By mixing the two solutions together in the UV cuvette, a range of glucose concentrations (0.13-0.42 M) was obtained. The absorbances were determined at 453 nm and Equations 5 and 6 were used to find K_a.

4.10.7 Binding of 2n with methyl α- and β-D-glucopyranosides in the three-component assay

A solution of **2n** in ARS solution (15 mM) was diluted with the ARS solution (0.144 mM) to give 3.1 mM solution in the phosphate buffered solution at pH 7.4. Then, 5 mL of this solution was used to make 2.0 M glycoside solution at pH 7.4. By mixing the

two solutions together in the UV cuvette, a range of sugar concentrations (0.8–2.0 M) was obtained, thus giving values for Q.

4.10.8 Binding of 2n with methyl α- and β-D-galactopyranosides in the three-component assay

2n (0.037 mmol) was dissolved in 10 mL of ARS solution (0.144 mM) to give a 3.66 mM phosphate buffered solution at pH 7.4. Then, 5 mL of this solution was used to make 2.0 M glycoside solution at pH 7.4. By mixing the two solutions together in the UV cuvette, a range of sugar concentrations (0.1-0.5 M) was obtained, thus giving values for Q.

4.10.9 Measurement of pK_a of cyclic boronic acid 2n

A 0.02 M NaOH solution was prepared by dilution of a 1:1 mixture of NaOH:water (for precipitation of carbonates as carbonic acid could influence the pK_a measurement under acidic conditions). This solution was used in the titration of 10 mL of 0.01 M **2n**. Potentiometric studies were conducted with an autotitrator. A Metrohm combined pH glass electrode (Ag/AgCl) with 3 M KCl internal filling was used. Measurements were taken at 25 °C. About 70 data points were collected for each titration, and the pK_a (the pH at half the equivalence volume) was determined by manual extrapolation. Two independent titrations were used to determine the pK_a of **2n**.

5.0 References

(1) Yan, J.; Fang, H.; Wang, B. *Med. Res. Rev.* **2005**, *25*, 490-520.

(2) Yang, W.; Gao, S.; Gao, X.; Karnati, V. V. R.; Ni, W.; Wang, B.; Hooks, W. B.; Carson, J.; Weston, B. *Bioorg. Med. Chem. Lett* **2002**, *12*, 2175-2177.

(3) Yang, W.; Fan, H.; Gao, X.; Gao, S.; Ni, W.; Hooks, W. B.; Carson, J.; Weston, B.; Wang, B. *Chemistry & Biology* **2004**, *11*, 439-448.

(4) Wulff, G. *Pure Appl. Chem.* **1982**, *54*, 2093-2102.

(5) Bielecki, M.; Eggert, H.; Norrild, J. C. *J. Chem. Soc. Perkin Trans. 2* **1999**, *449-455*.

(6) Stones, D.; Manku, S.; Lu, X.; Hall, D. G. *Chem. Eur. J.* **2004**, *10*, 92-100.

(7) Nagai, Y.; Kobayashi, K.; Toi, H.; Aoyama, Y. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 2965-2971.

(8) Arimori, S.; Phillips, M. D.; James, T. D. *Tetrahedron Lett.* **2004**, *45*, 1539-1542.

(9) Kuivila, H. G.; Keough, A. H.; Soboczenski, E. J. *J. Org. Chem.* **1954**, *19*, 780-783.

(10) Sugihara, J. M.; Bowman, C. M. *J. Am. Chem. Soc.* **1958**, *80*, 2443-2446.

(11) Dowlut, M.; Hall, D. G. *J. Am. Chem. Soc.* **2006**, *128*, 4226-4227.

(12) Clement, R. *C. R. Hebd. Seances Acad. Sci., Ser. C* **1966**, *263*, 1398-1400.

(13) Bourgeois, P.; Calas, R. *J. Organomet. Chem.* **1975**, *84*, 165-175.

(14) Kitamura, T.; Yamane, M.; Inoue, K.; Todaka, M.; Fukatsu, N.; Meng, Z.; Fujiwara, Y. *J. Am. Chem. Soc.* **1999**, *121*, 11674-11679.

(15) Kaufmann, D. *Chem. Ber.* **1987**, *120*, 901-905.

(16) Gravel, M.; Thompson, K. A.; Bérubé, C.; Hall, D. G. *J. Org. Chem.* **2002**, *67*, 3-15.

(17) Nielsen, D. R.; McEwen, W. E. *J. Org. Chem.* **1957**, *79*, 3081-3084.

(18) Stolowitz, M. L.; Guisheng, L.; Wiley, J. P. *Preparation of 1,2-phenylenediboronic acid bioconjugates for reagents and complexes*: USA, 2002; pp 24.

(19) Springsteen, G.; Wang, B. *Chem. Commun.* **2001**, 1608-1609.

(20) Springsteen, G.; Wang, B. *Tetrahedron* **2002**, *58*, 5291-5300.

(21) Mulla, H. R.; Agard, N. J.; Basu, A. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 25-27.

(22) Yang, W.; Yan, J.; Springsteen, G.; Deeter, S.; Wang, B. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1019-1022.

(23) Bates, R. G. *Determination of pH*; John Wiley & Sons: London, 1964.

(24) Yan, J.; Springsteen, G.; Deeter, S.; Wang, B. *Tetrahedron* **2004**, *60*, 11205-11209.

(25) Fernádez-Alonso, M.; Cañada, F. J.; Jiménez-Barbero, J.; Cuevas, G. *J. Am. Chem. Soc.* **2005**, *127*, 7379-7386.

(26) Mazik, M.; Cavga, H.; Jones, P. G. *J. Am. Chem. Soc.* **2005**, *127*, 9045-9052.

(27) Klein, E.; Crump, M. P.; Davies, A. P. *Angew. Chem. Int. Ed.* **2005**, *44*, 298-302.

(28) Wiskur, S. L.; Lavigne, J. J.; Ait-Haddou, H.; Lynch, V. M.; Chiu, Y. C.; Canary, J. W.; Anslyn, E. V. *Org. Lett.* **2001**, *3*, 1311-1314.

(29) Zhdankin, V. V.; Persichini III, P. J.; Zhang, L.; Fix, S.; Kiprof, P. *Tetrahedron Lett.* **1999**, *40*, 6705-6708.

(30) Greenwood, N. N.; Earnshaw, A. *Chemistry of the Elements*; Pergamon: Oxford, England, 1984.

(31) Rettig, S. J.; Trotter, J. *Can. J. Chem.* **1977**, *55*, 3071-3975.

(32) Weese, K. J.; Bartlett, R. A.; Murray, B. D.; Olmstead, M. M.; Power, P. P. *Inorg. Chem.* **1987**, *26*, 2409-2413.

(33) Tsukagoshi, K.; Shinkai, S. *J. Org. Chem.* **1991**, *56*, 4089-4091.

(34) Sandanayake, K. R. A. S.; James, T. D.; Shinkai, S. *Chem. Lett.* **1995**, *7*, 503-504.

(35) Eggert, H.; Frederiksen, J.; Morin, C.; Norrild, J. C. *J. Org. Chem.* **1999**, *64*, 3846-3852.

(36) Arimori, S.; Consiglio, G. A.; Philips, M. D.; James, T. D. *Tetrahedron Lett.* **2003**, *44*, 4789-4792.

(37) Suri, J. T.; Cordes, D. B.; Cappuccio, F. E.; Wessling, R. A.; Singaram, B. *Langmuir* **2003**, *19*, 5145-5152.

(38) Saulnier, M. G.; Zimmermann, K.; Struzynski, C. P.; Sang, X.; Velaparthi, U.; Wittman, M.; Frennesson, D. B. *Tetrahedron Lett.* **2004**, *45*, 397-399.

(39) Lennarz, W. J.; Snyder, H. R. *J. Am. Chem. Soc.* **1960**, *82*, 2172-2175.

(40) Cummings, W. M.; Cox, C. H.; Snyder, H. R. *J. Org. Chem.* **1969**, *34*, 1669-1674.

(41) Furderer, P.; Gerson, F.; Krebs, A. *Helv. Chim. Acta* **1977**, *60*, 1226-1232.

(42) Dale, W. J.; Rush, J. E. *J. Org. Chem.* **1962**, *27*, 2598-2603.

(43) Wiskur, S. L.; Lavigne, J. J.; Metzger, A.; Tobey, S. L.; Lynch, V.; Anslyn, E. V. *Chem. Eur. J.* **2004**, *10*, 3792-3804.

(44) Falck, J. R.; Bondlela, M.; Venkataraman, S. K.; Srinivas, D. *J. Org. Chem.* **2001**, *66*, 7148-7150.

(45) Wallace, P. A.; Minnikin, D. E. *Carbohydr. Res.* **1994**, *263*, 43-59.

(46) Hanessian, S. *Carbohydr. Res.* **1966**, *2*, 86-88.

(47) Probert, M. A.; Zhang, J.; Bundle, D. R. *Carbohydr. Res.* **1996**, *296*, 149-170.

(48) Baer, H. H.; Hanna, H. R. *Carbohydr. Res.* **1982**, *102*, 169-183.

(49) Cicero, D.; Varela, O.; de Lederkremer, R. M. *Tetrahedron* **1990**, *46*, 1131-1144.

(50) Reist, E. J.; Spencer, R. R.; Calkins, D. F.; Baker, B. R.; Goodman, L. *J. Org. Chem.* **1965**, *30*, 2312-2317.

(51) Sinha, S. K.; Brew, K. *Carbohydr. Res.* **1980**, *81*, 239-247.

(52) Bock, K.; Duus, J. Ø. *J. Carbohydr. Chem.* **1994**, *13*, 513-543.

Chapter 5

Thesis Summary and Conclusions

The thesis is divided into three main parts with a common trend being the development of receptors for carbohydrates. In Chapter 1, we have seen that due to the prevalent protein-carbohydrate interactions in nature, glycoconjugate libraries are gaining tremendous popularity. Fluorescence is commonly used to study these interactions and the different properties of common dyes are discussed succinctly. The importance of sulfated carbohydrates is also emphasized due to the important roles they play in the medical field. Receptors for these carbohydrates could play an important role in the treatment of cancer. The use of boronic acid based sensors and receptors is also discussed as they involve reversible interactions and can thus serve as protein (lectin) mimics. The major drawbacks associated with the currently used boronic acids are also discussed, namely poor solubility of "Wulff/Shinkai-type" boronic acids in aqueous media. Likewise, strong binding of monoboronic acids to saccharides is limited to saccharides that can isomerize to the furanose forms having readily accessible vicinal cis diols (thus forming less strained boronate esters).

In Chapter 2, the study of labeled carbohydrates interacting with lectins conjugated to a solid-support shows that succinimidyl 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoate (NBD-X) dye provides by far the lowest level of nonspecific interactions with immobilized proteins. Commonly used labeling reagents constituted of charged

aromatic groups, for instance, FITC and TAMRA dyes, induce non-specific interactions with both the proteins and the agarose based solid support. Studies conducted with europium labeled carbohydrates failed because of the problem of visualization of the europium fluorescence, which was hard to localize on the beads even in the presence of the DELFIA enhancement solution. ELISA assays gave disappointing results due to the inability to coat the special fluorescent microplates with the lectins.

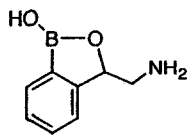
Chapter 3 dealt with the synthetic steps and problems encountered in synthesizing a sulfated disaccharide as a model for heparin, an anticoagulant drug. It was shown that even if silyl groups were good at selectively protecting the 3- and 6- hydroxyls of glycols, they were not compatible with azidonitration reactions. In these reactions where an azido group is introduced at the 2-position, the silyl groups were cleaved, making this synthetic step low yielding and the desired product hard to purify. In subsequent glycosylation reaction with a protected aminoethyl linker, loss of azide was induced under Lewis acidic conditions. Two amino groups in the same molecule and the presence of chemically sensitive silyl groups limited the synthetic methodology, which was eventually abandoned due to its slow progress.

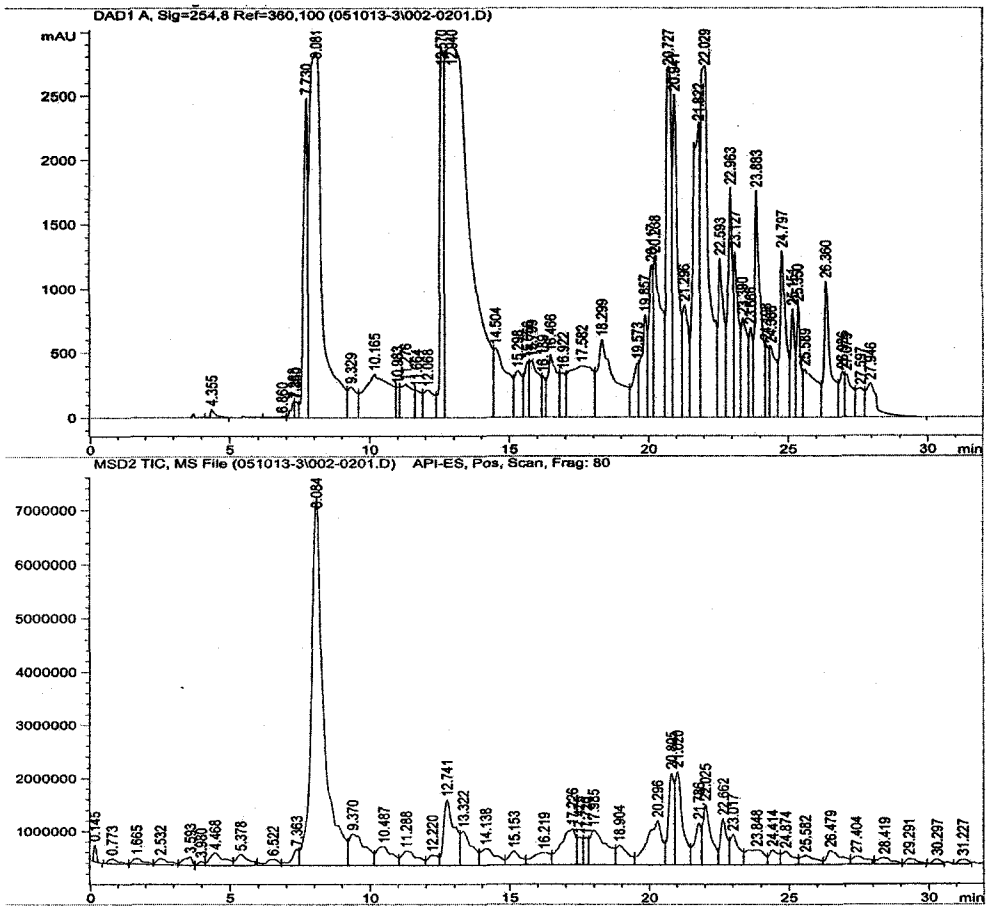
In Chapter 4, our research efforts focused on identifying new monoboronic acids that bind strongly to carbohydrates under aqueous physiological conditions. The long-term goal is the development of 'oligomers' of these boronic acids for improved binding potency and higher selectivity. A preliminary qualitative test was first carried out to determine the binding strength between the boronic acid and the carbohydrate

molecules using the catechol dye Alizarin Red S. Then, NMR titrations were carried out to determine the binding constants between the boronic acid and the carbohydrate molecules. Our results show that 2-(hydroxymethylphenyl) boronic acids (benzoboroxoles) stand out as excellent binding agents for monosaccharides, including glucose and these boronic acids exceptionally bind to model glycosides. To explain such binding selectivity, structural and mechanistic studies were carried out. pH profiles of the boronic acids were determined so as to provide insight into the geometry of the boronic acids when bound to the saccharides. *Ortho*-hydroxymethyl phenylboronic acid was shown to be superior to the well-established dialkylaminomethylphenylboronic acids ("Wulff-type") analogues. This class of benzoboroxoles were shown to bind glycopyranosides mainly using the 4,6-diols of carbohydrates. As the majority of glycoconjugates present free 4,6-diols, there is great scope to develop a novel class of benzoboroxole-based receptors that will be compatible with physiological conditions and bind specifically to hexopyranosides. Preliminary efforts for oligosaccharide synthesis utilized 3-bromomethyl-3H-benzo[2]-[1,2]oxaborol-1-ol as starting material. Unfortunately, S_N2 elimination of bromide ions under base conditions limited the usefulness of these reactions. Benzoboroxole derivatives with more stable functional groups are known in literature and can provide promising routes for the synthesis of sensors containing more than one benzoboroxole units.

Appendices

HPLC chromatograms for purification of amine 19





HPLC chromatograms for re-injection analysis of Peak 1 (diboronic acid 27)