INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UNIVERSITY OF ALBERTA

Synthetic and Biochemical Studies on the Active Site of the Blood Group A and B Gene-Specified Glycosyltransferases

by

Ali Mukherjee

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, 1999

UNIVERISITY OF ALBERTA

LIBRARY RELEASE FORM

NAME OF AUTHOR: **Ali Mukherjee**

TITLE OF THESIS: **Synthetic and Biochemical Studies on the Active Site of the Blood Group A and B Gene-Specified Glycosyltransferases**

DEGREE: **Doctor of Philosophy**

YEAR THIS DEGREE GRANTED: **1999**

SIGNED: Ali Mukherjee

Permanent Address:
Apt. no. 2114, 8515-112 Street,
Edmonton, Alberta
T6G 1K7

DATED: July 30, 1999

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Synthetic and Biochemical Studies on the Active Site of the Blood Group A and B Gene-Specified Glycosyltransferases** submitted by **Ali Mukherjee** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

O. Hindsgaul, supervisor

D. R. Bundle

D. L. J. Clive

L. Li

M. Peppler

P. G. Wang

DATED: JULY 30, 1999

Dedicated to my

Parents

Leela Mukherjee and Amal Kumar Mukherjee

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor Professor Ole Hindsgaul for his continued support and guidance throughout this work. I am grateful to Professor Monica M. Palcic for her advice and for the use of her facilities during the investigation and to Professor David R. Bundle for his advice and encouragement during my studies . I thank the Alberta Heritage Foundation for Medical Research for the studentship awarded to me for the past four years.

This work would not have been possible without the help and friendship of the members of our research group, Drs. Taketo Uchiyama, Asim Ray, Carles Malet, Hailong Jiao, Shahul Nilar and Ms. Suzanne Hof. In Dr. Palcic's group, I would like to thank Dr. Keiko Sujino, Ms. Catherine Compston and Dr. Adam Szpacenko for their help and advice. I would like to acknowledge the special contributions of the support staff of the Department of Chemistry, Drs. Angelina Morales, Albin Otter and Tom Nakashima. Special thanks are also due to Ms. Suzanne Hof and Mr. Xiangping Qian for their help with the manuscript and to Ms. Lynne Lechelt for her kind assistance and encouragement throughout my studies.

The creative assistance of my sister Moli and my friend Shawkat in preparing this thesis is gratefully acknowledged.

Finally, I thank all my friends in Edmonton who made my stay in this city a very memorable and enjoyable one.

ABSTRACT

Blood group A and B gene-specified glycosyltransferases, respectively the α(1,3)*N*-acetylgalactosyltransferase and the α(1,3)galactosyltransferase are enzymes that synthesize the A and B antigens (glycoproteins and glycolipids) that are found on the surfaces of erythrocytes and other cells. The A enzyme uses UDP-GalNAc while the B enzyme uses UDP-Gal as the donors. The enzymes differ in only four amino acids. The minimum acceptor structure recognized by both the enzymes is αLFuc*p*(1,2)βDGal*p*-*O*R (R = H; for R = octyl, **1**).

A series of analogs of this disaccharide, with modifications on the hydroxyl groups of the fucose moiety, were chemically synthesized to investigate the role of the hydroxyl groups in the recognition of the acceptor by these enzymes. Thus the Fuc 2, 3, 4-OH were replaced with H and OMe and the C-6' methyl with H (*arabino* analog). Evaluation of these analogs as acceptors show that modifications on the 3'-OH are tolerated by the enzymes. The 2'-OMe analog is not recognized by either as acceptor. The *arabino* analog is recognized by the A but not the B transferase. Acceptor recognition can thereby be directly ascribed to specific amino acid differences between the enzymes.

The acceptors recognized by the A transferase were used in the syntheses of modified blood group A antigens using UDP-GalNAc as donor. The *arabino* analog was used to assay a series of recombinant enzymes produced by sequentially changing the amino acids from the A to the B sequence. Although differential recognition of the acceptor by

the mutants was observed, a single amino acid can not be definitely identified as being responsible for acceptor specificity.

Photoaffinity analogs of the acceptor **1** were synthesized to covalently label the A transferase in its active site by irradiation with UV light, isolate it by lectin affinity chromatography and subsequently establish the amino acid sequence of the active site. Thus, acceptor-based photoaffinity labeling of a glycosyltransferase was achieved although the low percentage of labeling prevented conventional sequencing analysis. Mass spectrometric analysis of the labeled fragments is currently being performed.

TABLE OF CONTENTS

LIST OF SCHEMES

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

[α]	specific rotation
Ac	acetyl
AIBN	2,2'-azobisisobutyronitrile
Anal.	Analysis
Aq.	Aqueous
Ara	arabinose
APT	attached proton test
Bn	benzyl
b	broad
BSA	bovine serum albumin
Bu	butyl
c	concentration (g/100ml)
calcd.	calculated
CAN	cerium (IV) ammonium nitrate
CMP	cytosine monophosphate
CSA	camphorsulfonic acid
d	doublet
DCC	1,3-dicyclohexylcarbodiimide
DIC	diisopropylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide

DMTST	dimethyl(methylthio)sulfonium triflate
DPM	disintegrations per minute
DDT	dithiothreitol
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
equiv.	equivalent
Et	ethyl
FAB	fast atom bombardment
Fuc	fucose
Gal	galactose
GalNAc	2-acetamido-2-deoxy-galactose
GDP	guanosine diphosphate
gem	geminal
Glc	glucose
GlcNAc	2-acetamido-2-deoxy-glucose
HEPES	4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid
HOBt	1-hydroxybenzotriazole
Hz	Hertz
IR	infrared
J	coupling constant
m	multiplet
m/z	mass to charge ratio
Man	mannose

Me	methyl
mg	milligram(s)
MHz	megahertz
min	minute(s)
ml	milliliter(s)
mmol	millimole(s)
mol	mole(s)
MOPS	3-(*N*-morpholino)propanesulfonic acid
MS	mass spectrometry/molecular sieves
NDP	nucleoside diphosphate
NeuAc	*N*-acetyl neuraminic acid, sialic acid
NIS	*N*-iodosuccinimide
NMR	nuclear magnetic resonance
Ph	phenyl
pMBn	*para*-methoxybenzyl
ppm	parts per million
Pyr	pyridine
q	quartet
R_f	retention factor
rt	room temperature
s	singlet
satd	saturated
t	triplet

TBS	TRIS buffered saline
TBST	TRIS buffered saline with TWEEN
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TMS	trimethylsilyl
TRIS	*tris*-(hydroxymethyl)aminomethane
Ts	*para*-toluenesulfonyl
TWEEN	polyoxyethylene sorbitan
UDP	uridine diphosphate
vic	vicinal
Xyl	xylose

LIST OF ABBREVIATIONS-CONTINUED

AMINO ACID ABBREVIATIONS USED

A	alanine (Ala)
C	cysteine (Cys)
D	aspartic acid (Asp)
E	glutamic acid (Glu)
F	phenylalanine (Phe)
G	glycine (Gly)
H	histidine (His)
I	isoleucine (Ile)
K	lysine (Lys)
L	leucine (Leu)
M	methionine (Met)
N	asparagine (Asn)
P	proline (Pro)
Q	glutamine (Gln)
R	arginine (arg)
S	serine (Ser)
T	threonine (Thr)
V	valine (Val)
W	tryptophan (Trp)
Y	tyrosine (Tyr)

Chapter I

Introduction

1.1 *Biological roles of carbohydrates*

The surge of interest in oligosaccharide structures of biomolecules that perform critical functions in many life processes stems from the recognition of the importance of these carbohydrate motifs [1]. Although the precise mechanism of some of the actions of oligosaccharides is still uncertain, numerous studies have shown that carbohydrates are important markers for cell recognition. In addition to their well established structural, protective, and energy storing roles, carbohydrates on cell surfaces often determine the 'social behavior' of cells, i.e., how they interact with other cells [2]. Thus, they are involved in cell-cell recognition, aggregation of cells, cell differentiation and growth, immune activation, fertilization, and tumor metastasis [3]. They are recognized as signal substances as they regulate the properties and the biological activities of proteins. They serve as markers for inter-cellular trafficking by identifying the proteins and cells to be removed from circulation by trafficking receptors. They often act as cellular receptors for hormones, enzymes and bacterial toxins which lead to their adhesion and subsequent invasion (Figure 1). Leukocyte cell-adhesion molecules (LEC-CAMs) on endothelial cells recognize the oligosaccharides present on the surface of circulating leukocytes

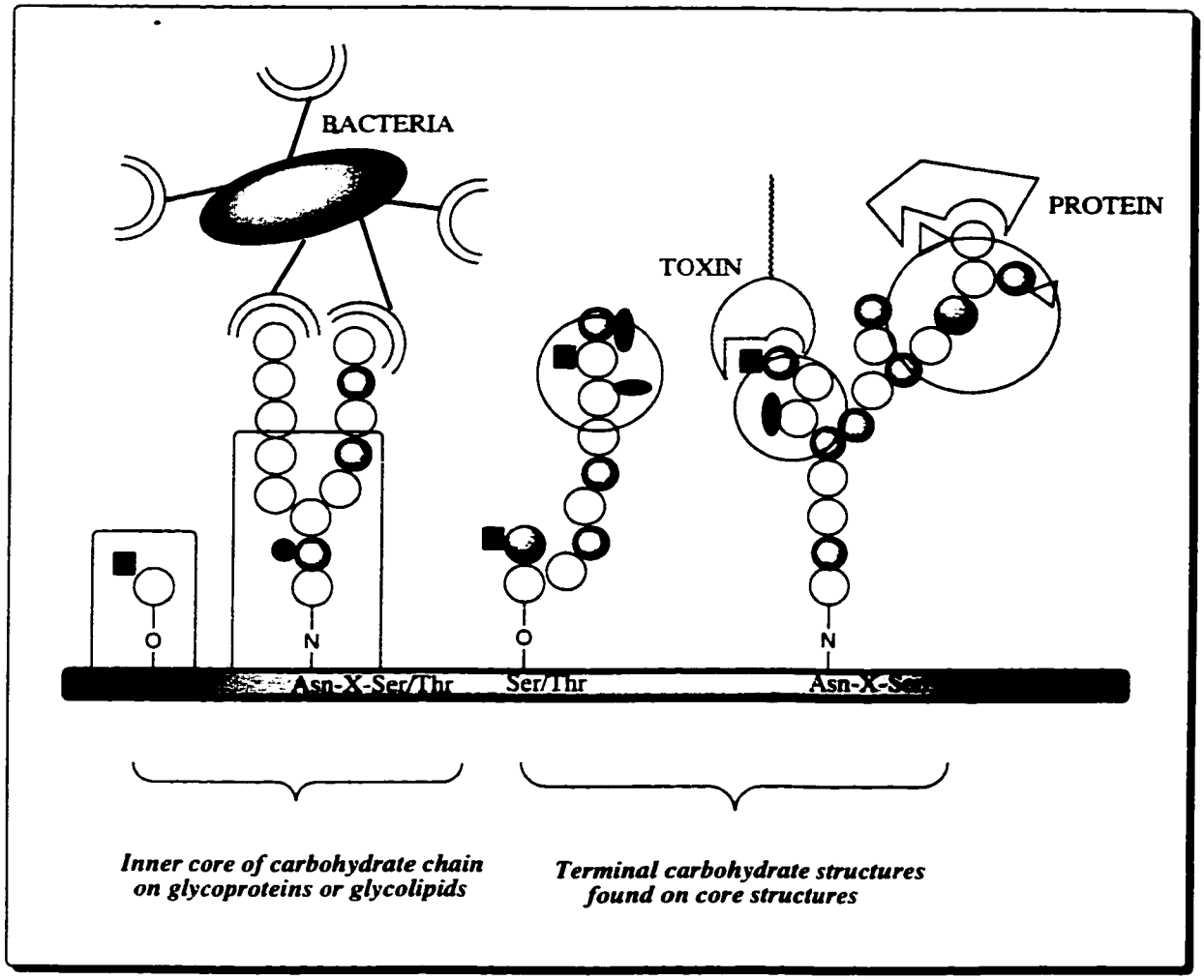


Figure 1. **Schematic representation of cell-surface glycoproteins and glycolipids with their carbohydrate epitopes mediating cellular processes. (The squares, circles and ellipsoids indicate monosaccharide residues).**

which is the first in a series of events that leads to inflammation and reperfusion injury. The importance of carbohydrate domains on glycoproteins and glycolipids is also manifested in their role as determinants in blood group typing. It is thus not surprising that understanding cellular interactions involving carbohydrates is of profound interest to researchers in the areas of glycobiology. The potential for structural diversity makes carbohydrates ideal for carrying biological information. In contrast to nucleic acids and proteins, which can only form linear assemblies, carbohydrates can form branched or linear structures with different links between them, thus enabling them to code for vast amounts of information.

1.2 *Tumor-associated carbohydrate antigens*

From the foregoing discussion, it is not surprising that abnormal expression of these carbohydrate antigens often leads to aberrant behavior of these cells and changes in the glycosylation pattern on cell surfaces have often been associated with oncogenesis. Cellular transformation into the malignant state is sometimes accompanied by the appearance of new glycoproteins that are absent or present at low levels on normal cell surfaces or ones that are native to other tissues. Several tumor-associated antigens have been shown to be carbohydrates associated with glycoproteins or glycolipids. Cells undergoing malignant transformation often manifest defects in sugar metabolic pathways, either due to anomalous glycosyltransferase or glycosidase activity or the availability of their substrates. This results in changes in glycosylation patterns [4] which can lead to

uncontrolled cell growth [5], altered cell adhesion [6], invasiveness and metastatic spread [7]. The oligosaccharide structures isolated from certain colon carcinomas showed higher degree of sialylation and fucosylation than normal cells [8]. Strong expression of the T antigen Gal(1,3)αGalNAc and the Tn antigen αGalNAc has been found in breast carcinoma [9]. Certain cancerous cell lines have been found to have a higher density of gangliosides GM_2, GM_3, GD_2 and GD_3 at the expense of the higher gangliosides [10-12]. A number of studies have shown the total or partial loss of ABH antigens from the surface of malignant cells indicating either deletion of the respective glycosyltransferases, loss of precursor structures, or excessive glycolytic activity [13, 14]. The appearance of incompatible A antigen in tumors of blood group O and B individuals has been reported in many studies [15, 16], although concrete proof for the activation of A enzyme is lacking [17]. Aberrant expression of both A and B antigens in adenocarcenoma of gastric and colonic mucosa has also been observed [18].

1.3 *Naturally occurring complex carbohydrates*

Carbohydrates are found on carrier glycans as part of biological macromolecules like glycoproteins (N-linked or O-linked) and glycolipids (O-linked) [19-21]. N-linked glycans of glycoproteins are covalently attached to asparagine (Asn) where Asn is part of the sequence Asn-X-Ser/Thr (X is any amino acid but proline; Figure 2). O-linked glycans on proteins are covalently attached to serine (Ser) or threonine (Thr), but no

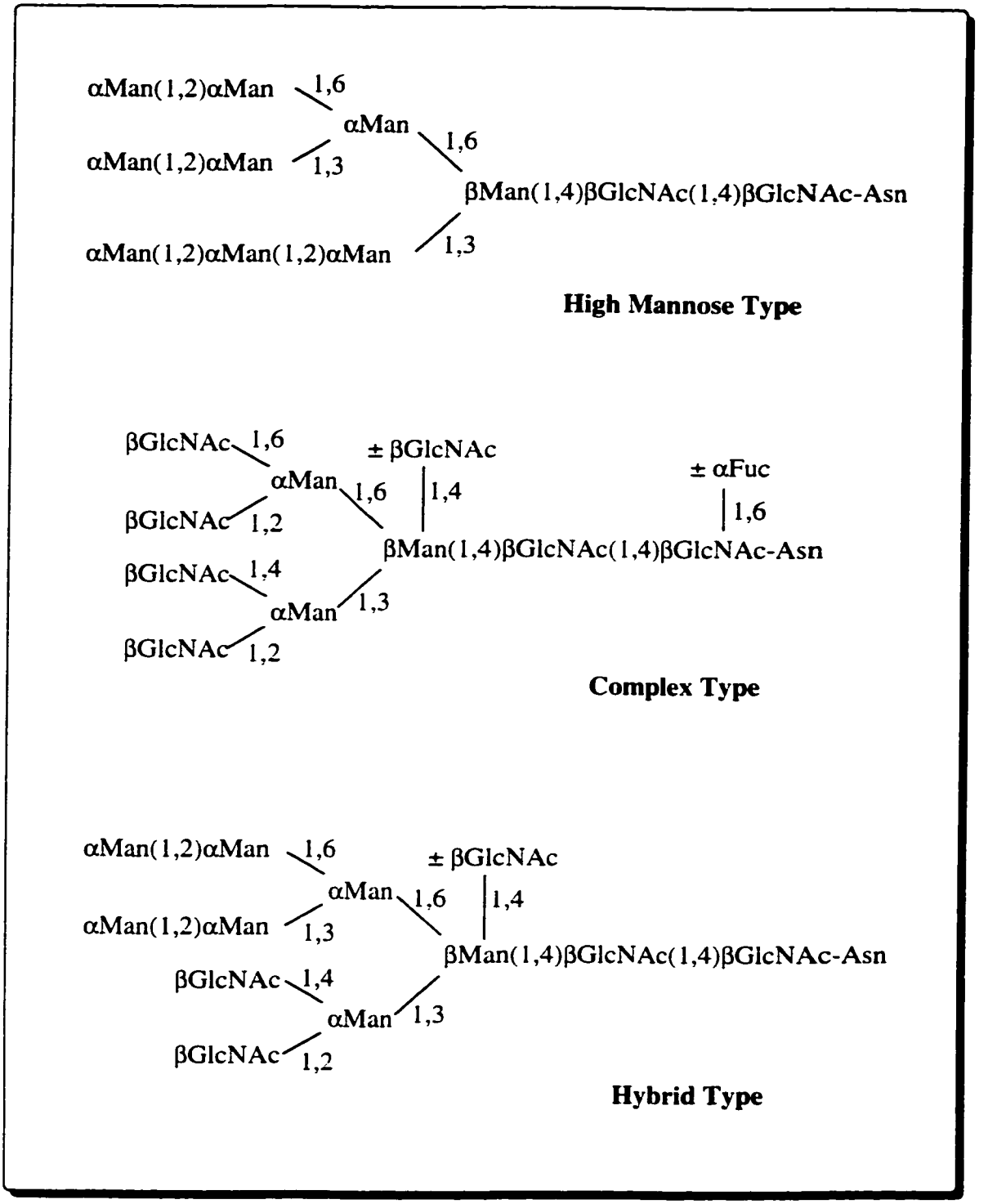


Figure 2. **Some examples of asparagine linked glycoproteins.**

consensus sequence for the peptides has been established (Figure 3). Oligosaccharides are most commonly attached to ceramide or phosphoglycerol in glycolipids.

1.3.1 *Protein- and lipid-carbohydrate linkages*

The core structure of N-linked oligosaccharides is a pentasaccharide, αMan(1,6) αMan(1,6) βMan(1,4) βGlcNAc(1,4)GlcNAc (Figure 2).

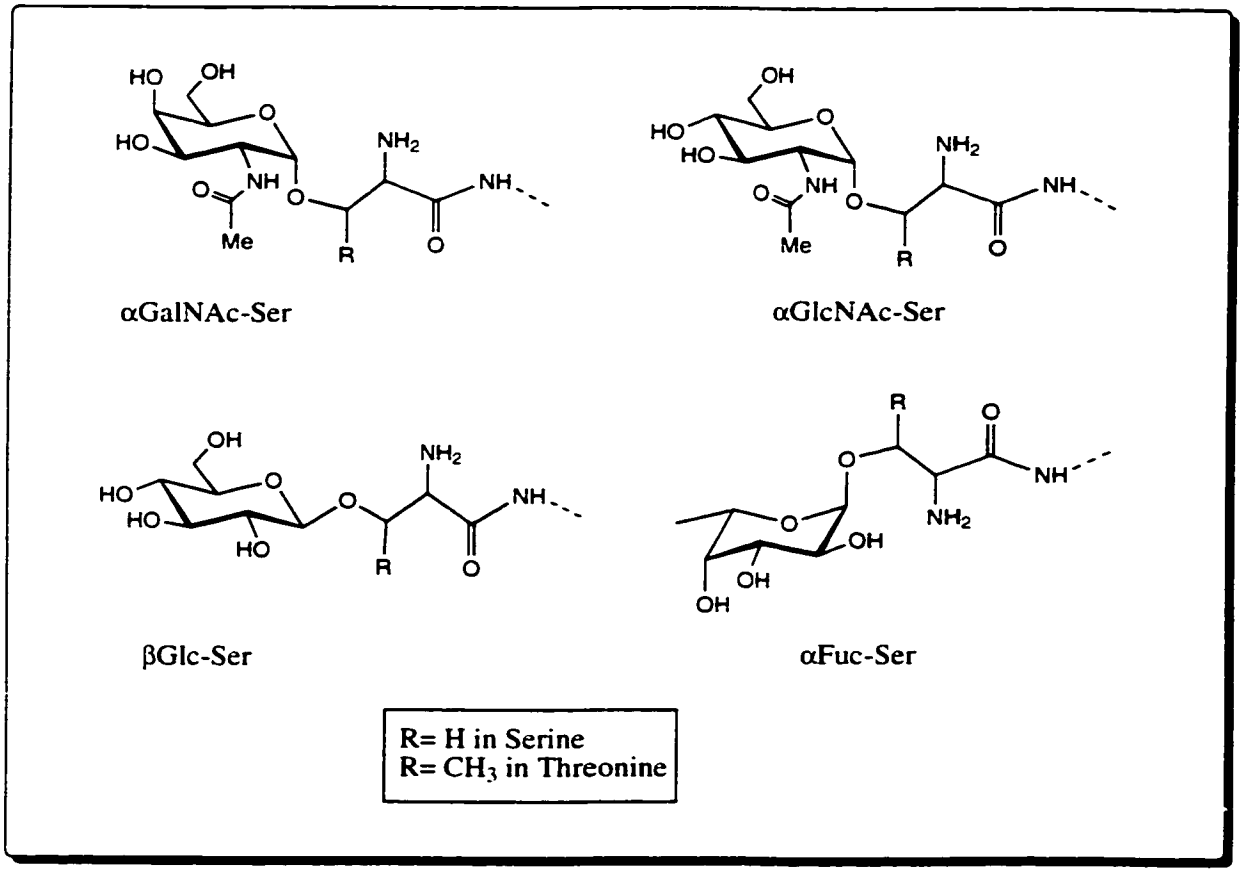


Figure 3. Some precursor structures for O-linked glycopeptides.

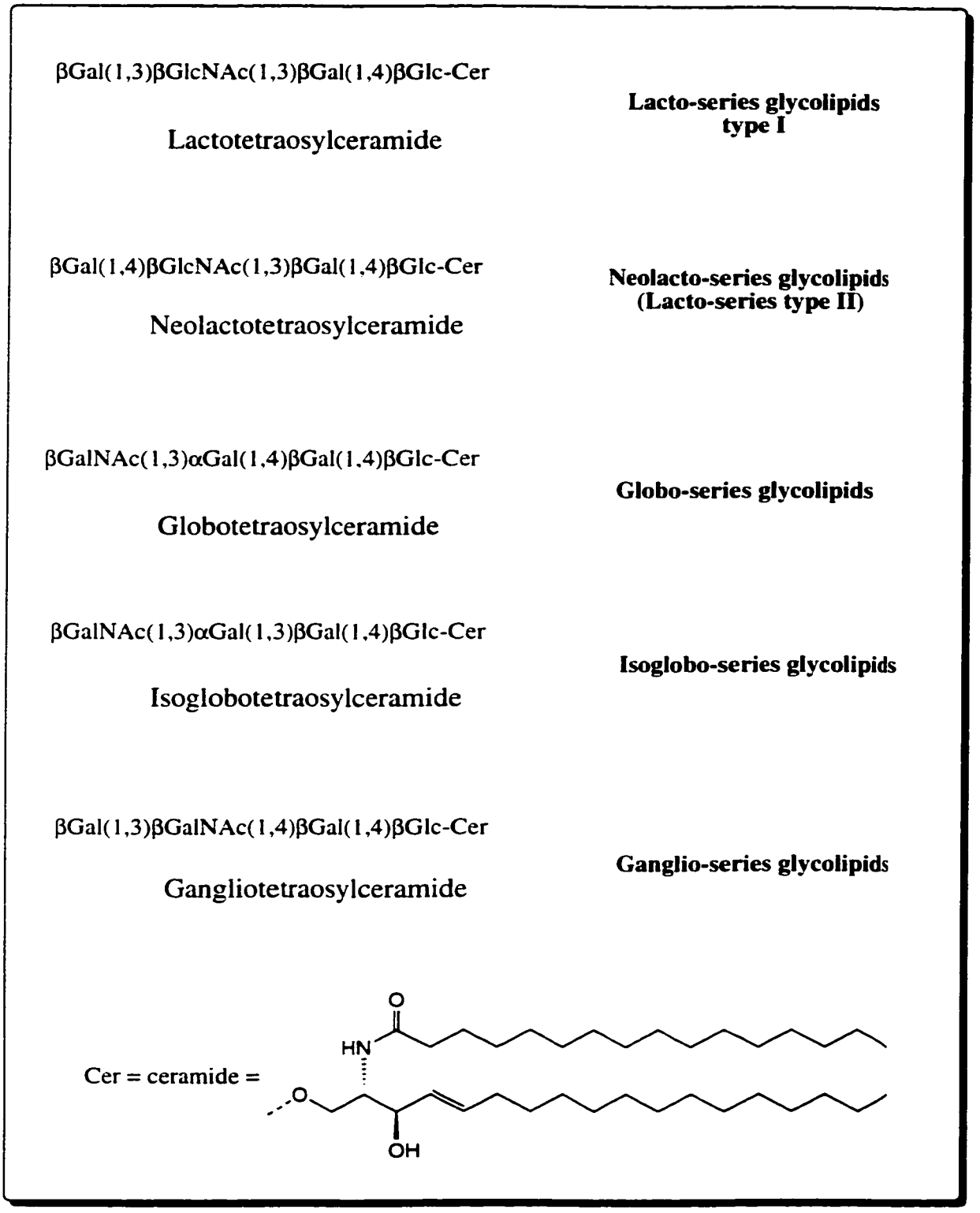


Figure 4. **Examples of glycolipid precursors.**

They are grouped into three broad classes based on the further branching of this core structure: high mannose type, which contains α-mannosyl residues on the core; complex type, which is devoid of further mannosylation; and hybrid type, which is a combination of both (Figure 2). O-linked glycans do not possess a common core structure and a number of protein-carbohydrate linkages are found (Figure 3).

Lipid-carbohydrate linkages [22] are found in both glyceroglycolipids, and glycosphingolipids. Oligosaccharides that are covalently attached to N-acyl sphingosine (ceramide) are termed glycosphingolipids and they form the largest group of the sphingolipid class. They may be classified into the following series depending on their core sugar structure: ganglio-, globo-, isoglobo-, lacto-, and neo-lacto-, all of which contain a β-lactosyl ceramide core (Figure 4).

Carbohydrates linked to phosphoglycerol form the other group of glycolipids called glycosyl phosphatidyl inositol (GPI) and they anchor the proteins they carry on their surface to the cell membranes, replacing the membrane anchoring domain of proteins [23]. The carbohydrate motif on GPI's is diverse but has a core of αMan(1,2)αMan(1,6)αMan(1,4)αGlcNAc linked to an inositol at the reducing end. It is in turn connected via a phosphate group to a glycerol unit with two long chain alkyl or acyl groups. The protein is linked to the non-reducing end through a phospho-ethanolamine moiety (Figure 5).

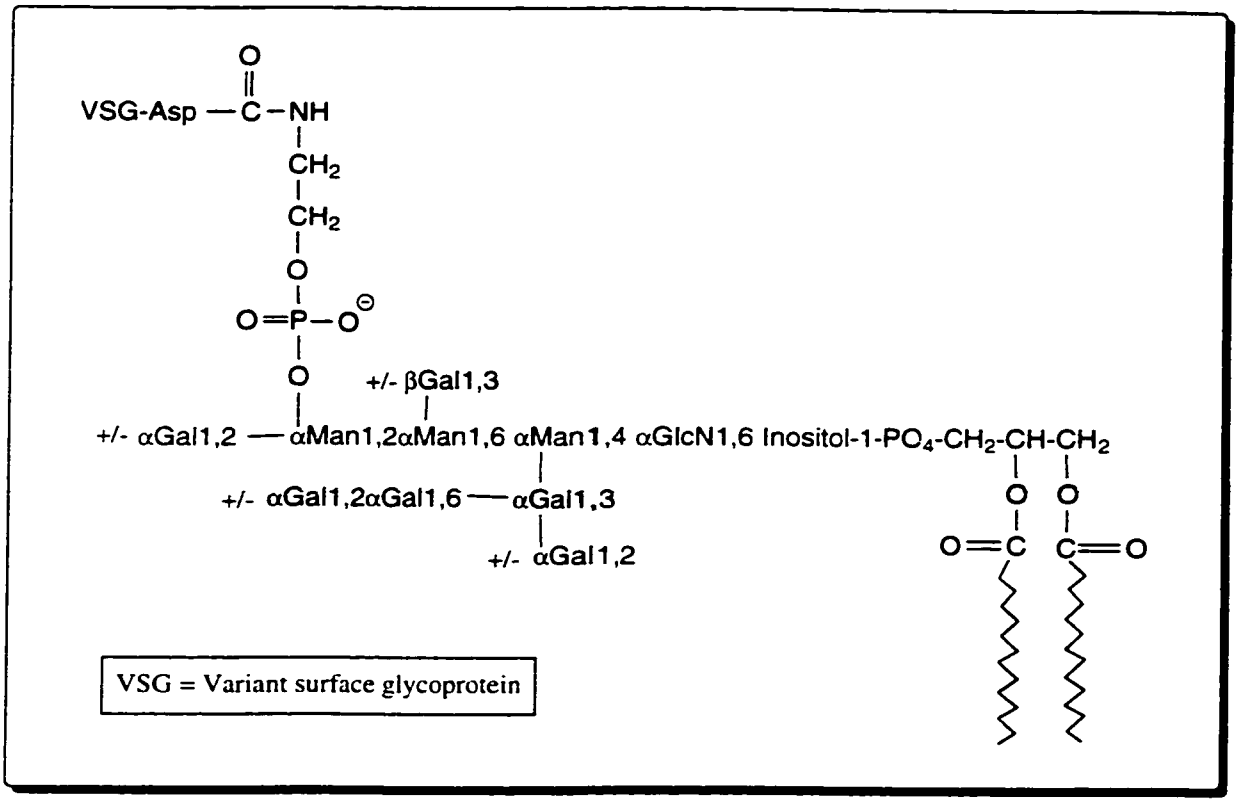


Figure 5. **Structure of a glycosylphosphatidyl inositol membrane anchor.**

1.3.2 *Blood group ABO(H) antigens*

The blood group A, B and O(H) antigens are terminal carbohydrate structures that are found on glycoprotein or glycosphingolipid molecules (Figure 6). They were the first cellular carbohydrate antigens to be characterized on human erythrocytes [24]. The first blood group system to be discovered in humans was also the ABO system in 1901. They were originally thought to be constituents only of red blood cells. Subsequently, blood group antigens were found not only on erythrocytes but also on endothelial cells, in the

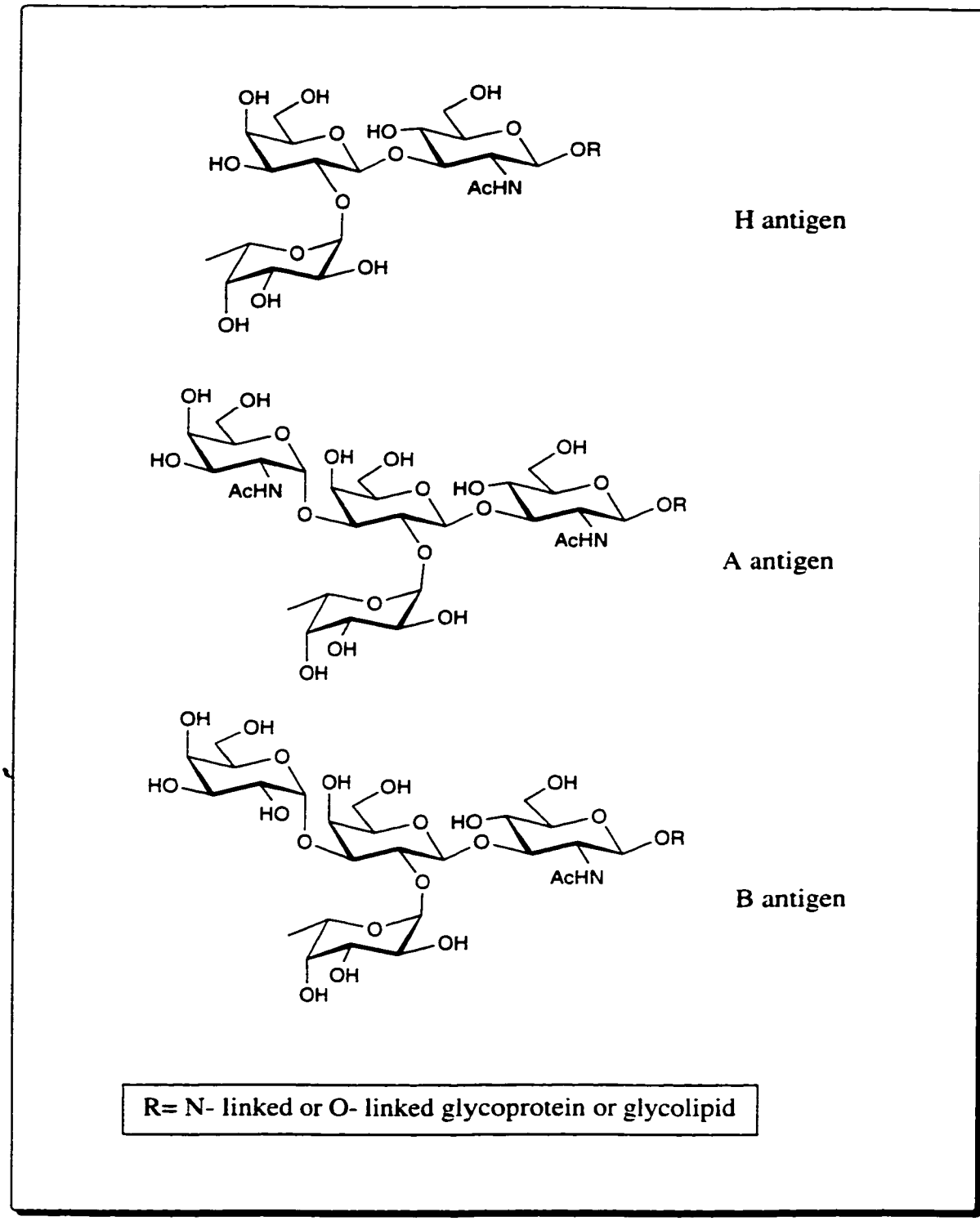


Figure 6. **Structures of blood group A, B and O(H) antigens.**

bone marrow and in soluble form in the cytoplasm, in the saliva and other body secretions. Blood groups are inherited differences in the antigens of various tissues and macromolecules, predominantly of the red cells, but also of leucocytes, platelets, plasma proteins and enzymes of various cell surfaces [25]. The A and the B antigens are major histocompatibility antigens and ABO incompatibility is a cause of transfusion reactions and transplant rejection of organs and tissue grafts. These rejections are caused by anti-A antibodies (present in B and O individuals), anti-B antibodies (present in A and O individuals) and anti- AB antibodies (present in O individuals). A individuals are further divided into A_1 and A_2 sub-types, with the former having anti-A_2 and the latter having anti-A_1 antibodies. The blood group characteristics are subject to Mendelian inheritance with A and B alleles being co-dominant over O (recessive). The amounts of the major blood group A, B, and H antigens have been shown to be different at different stages of development, and thus they are also thought to be markers for fetal development and organ differentiation [26].

1.3.3 *Biosynthesis of complex carbohydrates*

N-linked glycoproteins are biosynthesized by the sequential addition of two *N*-acetylglucosamine, nine mannose, and three glucose residues to a dolichol phosphate intermediate by the respective glycosyltransferases using the corresponding sugar nucleotides or dolichol-P-hexoses [27, 28]. Dolichol anchors the growing oligosaccharide chain to the endoplasmic reticulum (ER) membrane. The high mannose core

oligosaccharide is then transferred co-translationally to the nascent polypeptide chains by oligosaccharyl transferase. Only about one third of the Asn-X-Ser/Thr sites of eukaryotic proteins are glycosylated. The processing of the primary glycoproteins take place in the ER by the enzymatic trimming of three glucose and a mannose residue. The glycoproteins are then transported to the Golgi apparatus where D-mannose (Man) residues are removed and *N*-acetyl-D-glucosamine (GlcNAc), D-galactose (Gal), L-fucose (Fuc), and sialic acid residues are added in the different cisternae of the Golgi stack.

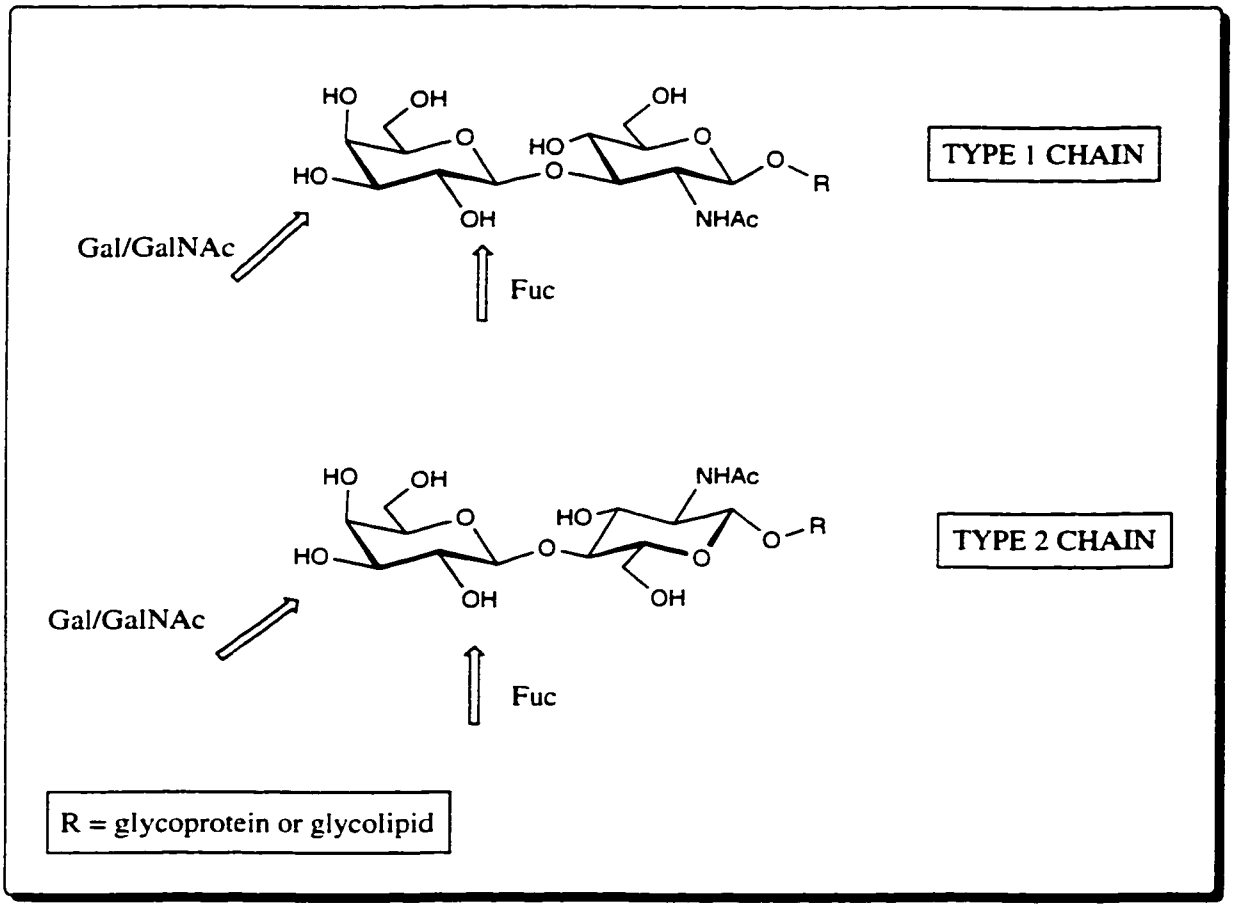


Figure 7. **Types of carbohydrate chains found on blood group A and B antigens.**

The resulting glycoproteins are sorted and transferred to their respective cellular destinations. Synthesis of O-linked oligosaccharide starts with the transfer of GalNAc (from UDP-GalNAc) to serine or threonine residues on a completed polypeptide chain. Chain elongation and sequential addition of terminal sugars like Gal, sialic acid, GlcNAc, and Fuc by their specific glycosyltransferases also occur in the Golgi complex post-translationally [29]. The glycoproteins are then incorporated into the cell membranes. Formation of glycolipids also involves the addition of a monosaccharide (usually D-glucose or Gal) from its nucleotide donor to ceramide at its C-1-OH group by the specific glycosyltransferase. Other monosaccharides are then sequentially added to this precursor in the Golgi apparatus one monosaccharide at a time.

1.3.4 *Biosynthesis of blood group A and B antigens*

The A and the B antigens are formed from the same precursor structure [30], the H active structure αFuc(1,2)βGal by the addition of GalNAc or Gal, respectively, to the terminal Gal of the type 1 and type 2 chains (shown in Figure 7) in an α1,3 linkage. The precursor structure is formed by α(1,2)fucosyltransferase which adds a Fuc to the Gal in an α1,2 linkage. The enzyme responsible for the formation of the A antigen is α(1,3)*N*-acetylgalactosaminyltransferase (E.C. 2.4.1.40), found in blood group A and AB individuals. The B antigen is formed by α(1,3)galactosyltransferase (E.C. 2.4.1.37) found in B and AB individuals (Figure 8). Both transferases can add their respective sugars only when the terminal galactose is substituted with fucose in the 2-position.

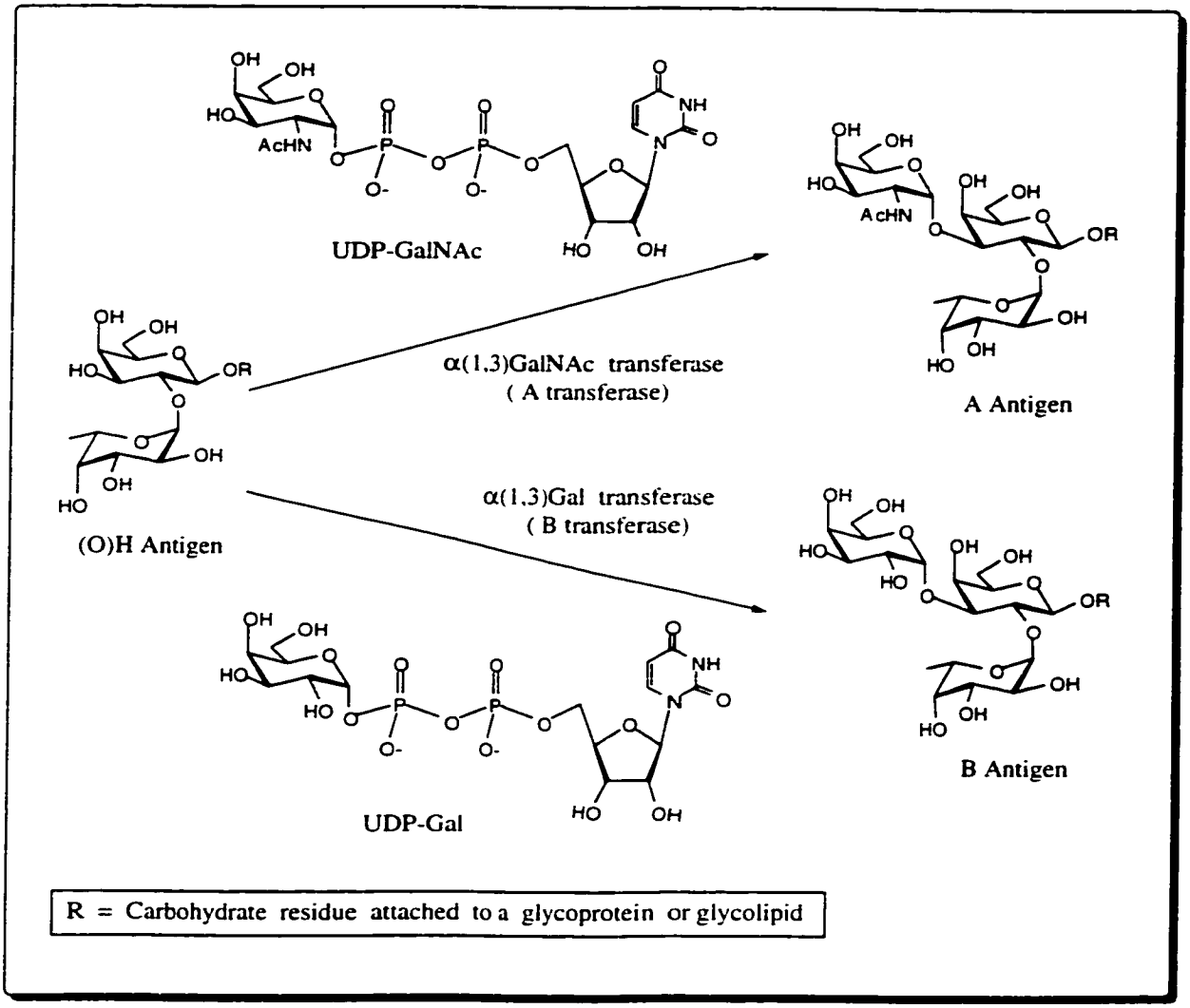


Figure 8. **Biosynthesis of blood group A and B antigens.**

These enzymes are thought to be primary protein products of the A and B genes and are structurally related. O individuals do not normally express either of these enzymes although the genes encoding these enzymes are present. ABH specific sugars are added to two main classes of oligosaccharide chains, type 1 chains have βGal(1,3)βGlcNAc sequence at the reducing end of the molecule, whereas type 2 chains have βGal(1,4)βGlcNAc terminal residue (Figure 7). On red cells, all ABH structures are

synthesized on type 2 chains with up to 80% of those being bound to proteins and the rest exist as glycolipids. In secretions, the ABH active structures are found on glycoproteins with both type 1 and type 2 chains.

1.4 *Glycosyltransferases*

The diverse oligosaccharide structures found on the cell surface are built up by a complex network of reactions mediated by glycosyltransferases and glycosidases. Glycosyltransferases catalyze a series of reactions where specific glycosidic linkages are formed in a regio- and stereospecific manner between two sugars or between a sugar and a peptide or a lipid (Figure 9). When two sugars are thus joined, an activated monosaccharide is transferred on to another monosaccharide or a growing oligosaccharide chain.

These enzymes are generally present in the Golgi compartments, but they have also been found in soluble form in body fluids. Glycosyltransferases are type II integral membrane proteins with a short amino-terminal cytoplasmic tail, a transmembrane region, a proteolytically sensitive stem region and a large catalytic domain at the C-terminal of the protein.

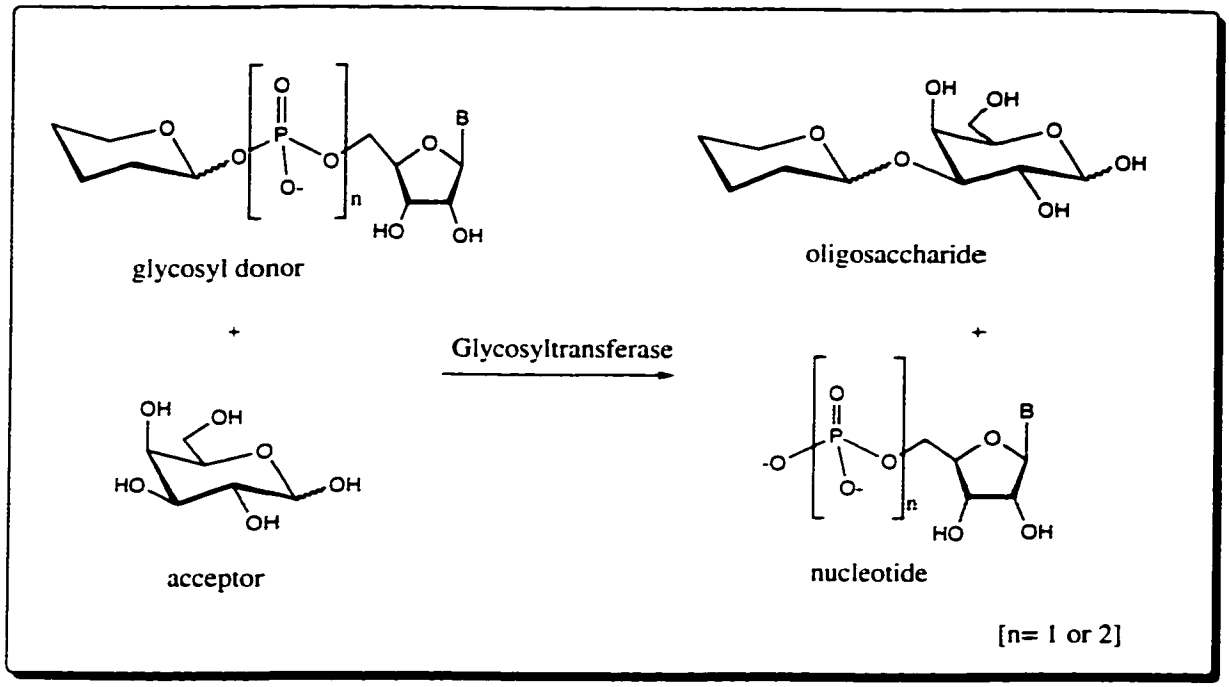


Figure 9. **Schematic representation of glycosidic bond formation by a glycosyltransferase.**

The enzymes show high specificity towards the donors they use and are named according to the sugars they transfer. It was, in fact, the discovery of the natural donor substrates for these enzymes that made the systematic investigations of their occurrence and properties possible [31]. These enzymes often, but not always, need divalent cations like Mn^{2+}, Mg^{2+} and Ca^{2+} for optimal activity. The synthesis of a complete oligosaccharide sequence depends on the sequential reactions between acceptor molecules and the glycosyltransferases which have a specific sub-cellular localization. If a glycoconjugate fails to display a particular monosaccharide on its growing chain, the subsequent sugars are not added as the enzymes responsible for their transfer fail to recognize the acceptor substrate [32]. Each glycosidic bond is formed by a different enzyme, and the concept of

one linkage-one enzyme (Figure 9), along with fact that each enzyme is coded for by a specific gene, implies that one gene is responsible for the synthesis of one glycosidic linkage [33]. Thus, it can be conceived that a multi-glycosyl transferase system acts in a concerted fashion to build up a sugar chain. The cooperative and sequential acceptor specificity of the enzymes involved in making one sugar chain led to the idea that the enzymes are arranged along physically separated assembly lines in the Golgi cisternae and that the oligosaccharide chain grows as it passes through the different compartments.

It has been suggested that apart from their role in catalyzing the transfer of glycosyl residues, glycosyltransferases on the cell surface (or ecto-glycosyltransferases) mediate recognition events like cell adhesion, cell migration, cell differentiation, bacterial adhesion and immune recognition, although these hypotheses have not received general acceptance [34, 35]. Their potential role in mediating sperm-egg binding in mice has also been suggested [36].

1.4.1 *Blood group A and B glycosyltransferases*

The blood group A and B glycosyltransferases are morphologically typical of the glycosyltransferase family of enzymes. They possess a cytoplasmic tail consisting of 16 amino acids, a transmembrane region with 21 amino acids, a stem region and a large C-terminal catalytic domain consisting of 316 amino acids located inside the Golgi apparatus [37-40]. They are both located on chromosome 9. These enzymes have one

potential site for N-glycosylation and a report suggests [41] the presence of the A antigenic structure on the A transferase.

The very low concentration of these enzymes in tissues and body fluids made isolation of pure proteins difficult and hampered structural studies using established methodologies like X-ray crystallography. In recent years, the genes coding for these enzymes have been cloned using cDNA libraries. These studies have shown that the number of amino acids is the same in both the enzymes and they differ in their amino acid sequences at only four positions [42, 43]. As shown in Figure 10, the changes are Arg123-Gly, Gly182-Ser, Leu213-Met, Gly215-Ala, in the catalytic domain of the proteins. Mutant enzymes with permutations of these variable residues have also been made to investigate the contributions of the individual amino acids to the catalytic activity of the enzymes, particularly, their donor specificities [44]. In a related study, the A, the B and the mutant transferases were expressed at high levels in *Escherichia coli* to enable kinetic studies on these enzymes [45, 46].

It has been determined that the third (Leu/Met) and the fourth (Gly/Ala) amino acids are more crucial to the donor specificities of these enzymes than the first (Arg/Gly) or the second (Gly/Ser). *In vivo*, the B transferase transfers Gal from UDP-Gal and the A transferase uses UDP-GalNAc as the donor with strict specificity. *In vitro*, however, they are known to cross react [47-49]. The disaccharide, αFuc(1,2)βGal-*O*R serves as the common acceptor for both the enzymes, R being a glycoprotein or a glycolipid *in vivo*. Various alkyl groups can be used as the aglycon without loss of acceptor activity. Prior to

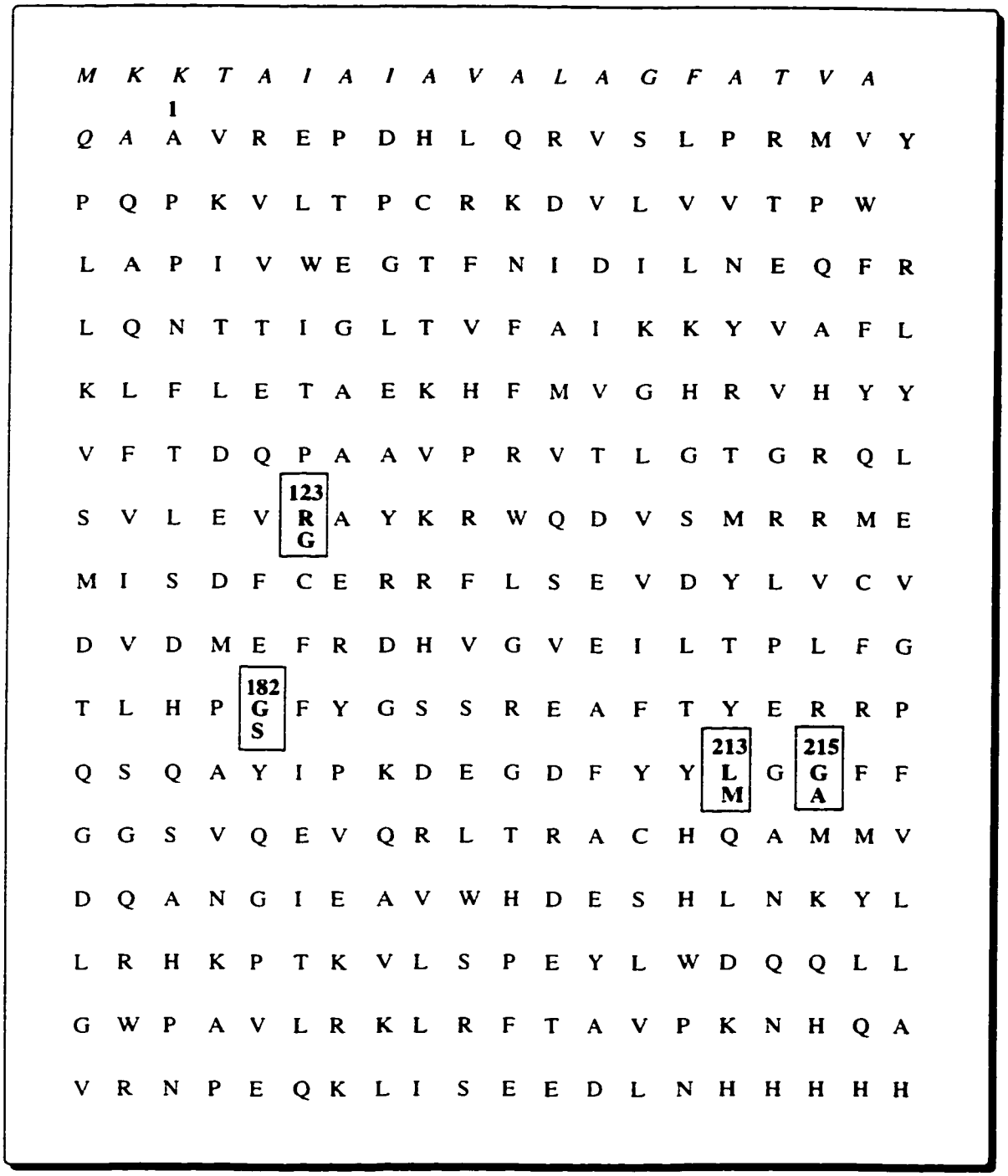


Figure 10. **Amino acid sequence of the A transferase. The difference between the A and the B enzymes is highlighted in bold. (Numbers at the top indicate positions of the amino acids).**

this study, it was not known whether these two enzymes differed in their acceptor specificity, although it had been shown that the B transferase is in general less tolerant of changes in the acceptor structure [50, 51]. The last two substitutions in the B enzyme, Met and Ala, have been shown to reduce the flexibility of the enzyme compared to the A enzyme. This could be partly responsible for its preference for the smaller UDP-Gal as donor and its lower tolerance for changes in the native acceptor.

1.5 *Sugar nucleotides*

Glycosyltransferases of the LeLoir pathway utilize nucleotide diphosphate sugars as the activated monosaccharide donors to form glycosidic linkages [52]. These are nucleoside di- or monophosphates linked to the reducing terminus of the carbohydrate through its terminal phosphate ester. There are nine sugar donors used by mammalian transferase enzymes: uridine 5'-diphosphogalacotse (UDP-Gal), uridine 5'-diphosphoglucose (UDP-Glc), uridine 5'-diphosphoglucuronic acid (UDP-GlcA), uridine 5'-diphospho-*N*- acetyl glucosamine (UDP-GlcNAc), uridine 5'-diphospho-*N*-acetylgalactosamine (UDP-GalNAc), uridine 5'-diphosphoxylose (UDP-Xyl), guanosine 5'-diphosphofucose (GDP-Fuc), guanosine 5'-diphosphomannose (GDP-Man), and cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-Neu-5-Ac) as shown in Figure 11 [53].

UDP-Gal is an intermediate in the glycolytic pathway [54]. It is either formed *in vivo* by condensation of D-galactose-1-phosphate (Gal-1-P) with uridine triphosphate (UTP)

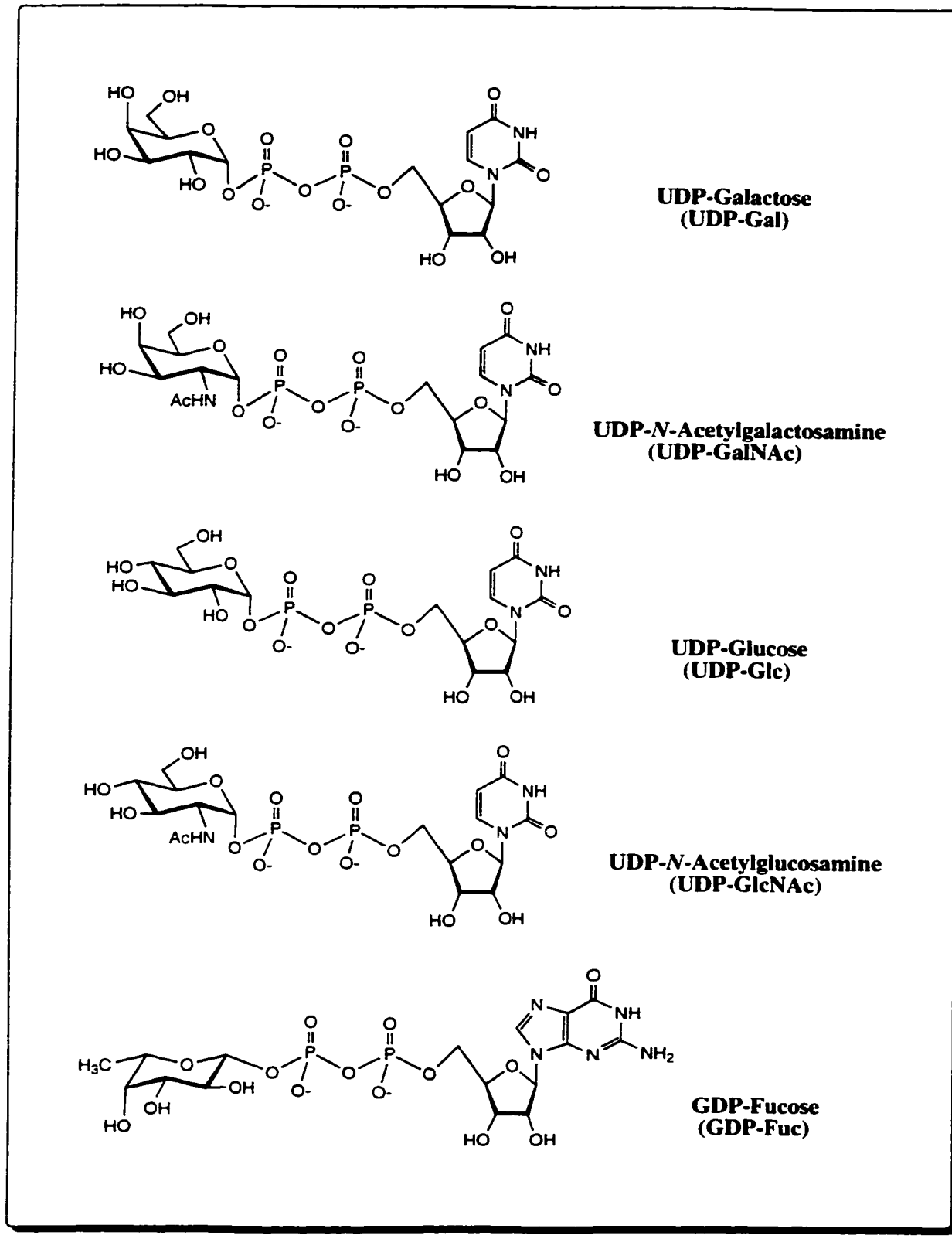


Figure 11. **Sugar nucleotide donors used in mammalian systems.**

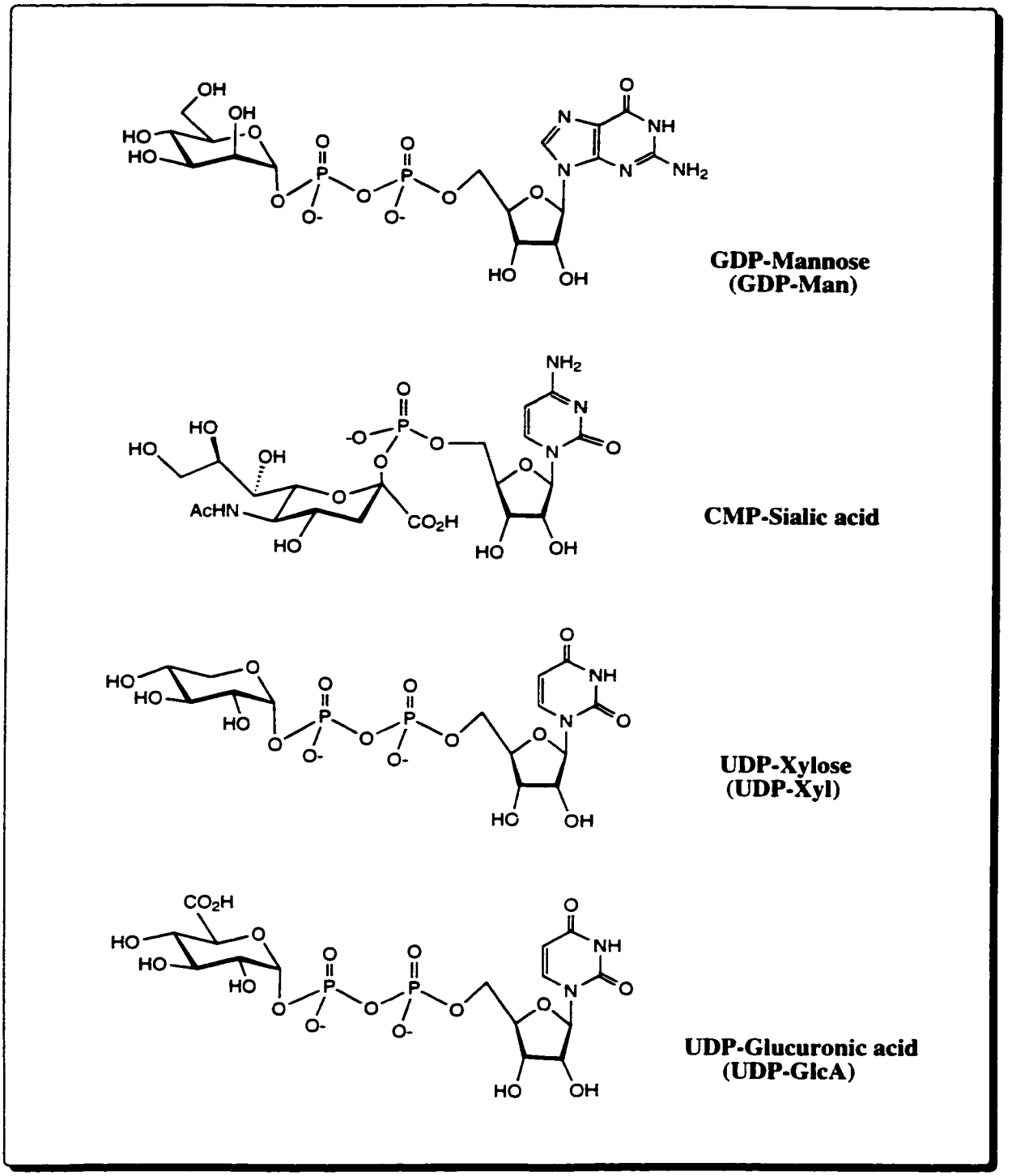


Figure 11. **(Continued) Sugar nucleotide donors used in mammalian systems.**

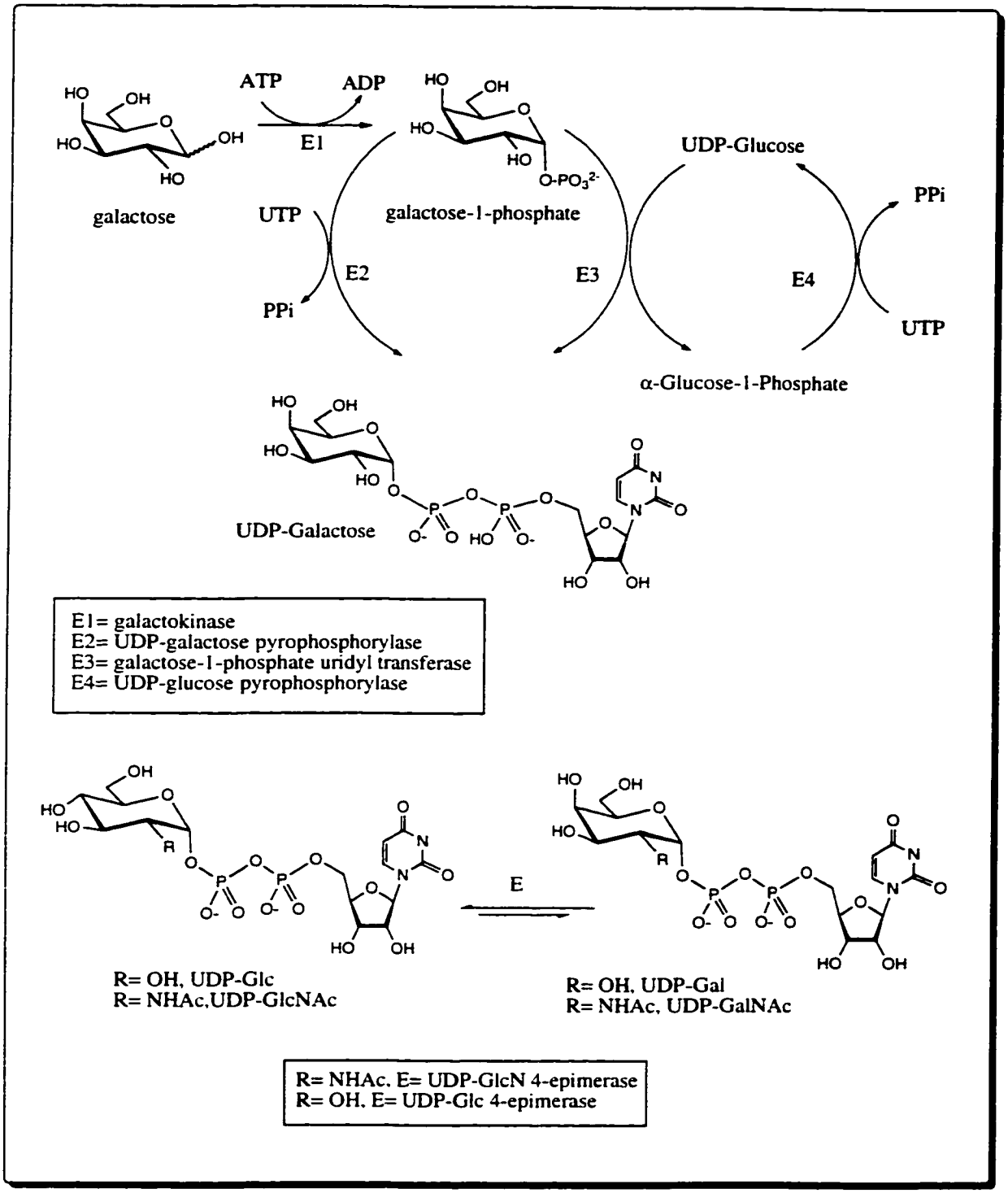


Figure 12. **Biosynthesis of sugar nucleotides.**

catalyzed by UDP-Gal pyrophosphorylase, or by the transfer of an uridyl moiety from UDP-Glc to Gal-1-P catalyzed by galactose-1-P uridyltransferase [54, 55] (Figure 12). Galactokinase phosphorylates Gal to give Gal-1-P. The latter pathway is utilized to form other nucleotide donors except CMP-Neu-5-Ac, which is formed by the action of CMP-NeuAc synthetase from CMP and Neu-5-Ac [57]. UDP-GalNAc is formed by the epimerization of UDP-GlcNAc by UDP-GlcNAc 4-epimerase as shown in Figure 12 [58].

1.6 *Chemical synthesis of oligosaccharides*

Elucidation of the biological roles of the glycans necessitates the generation of defined oligosaccharide fragments and thus requires the development of efficient methodologies for the synthesis of these compounds and their analogs. The very structural diversity that makes carbohydrates excellent carriers of biological information also makes their chemical synthesis more complicated than that of peptides and nucleotides. The construction of glycosidic linkages in a regio- and stereo-controlled manner is one of the major challenges of synthetic carbohydrate chemistry. No universal solution has yet been found for the syntheses that involve coupling between at least eight different kinds of monosaccharides and requires extensive functional group manipulation.

A number of protection and deprotection steps have to be undertaken on the acceptor moiety (a monosaccharide or an oligosaccharide block) to address the problem of regio-

control so that all but the hydroxyl group to which the bond is being formed is left free. In the block synthesis of oligosaccharides, functional group manipulation has also to be performed to obtain the donor block.

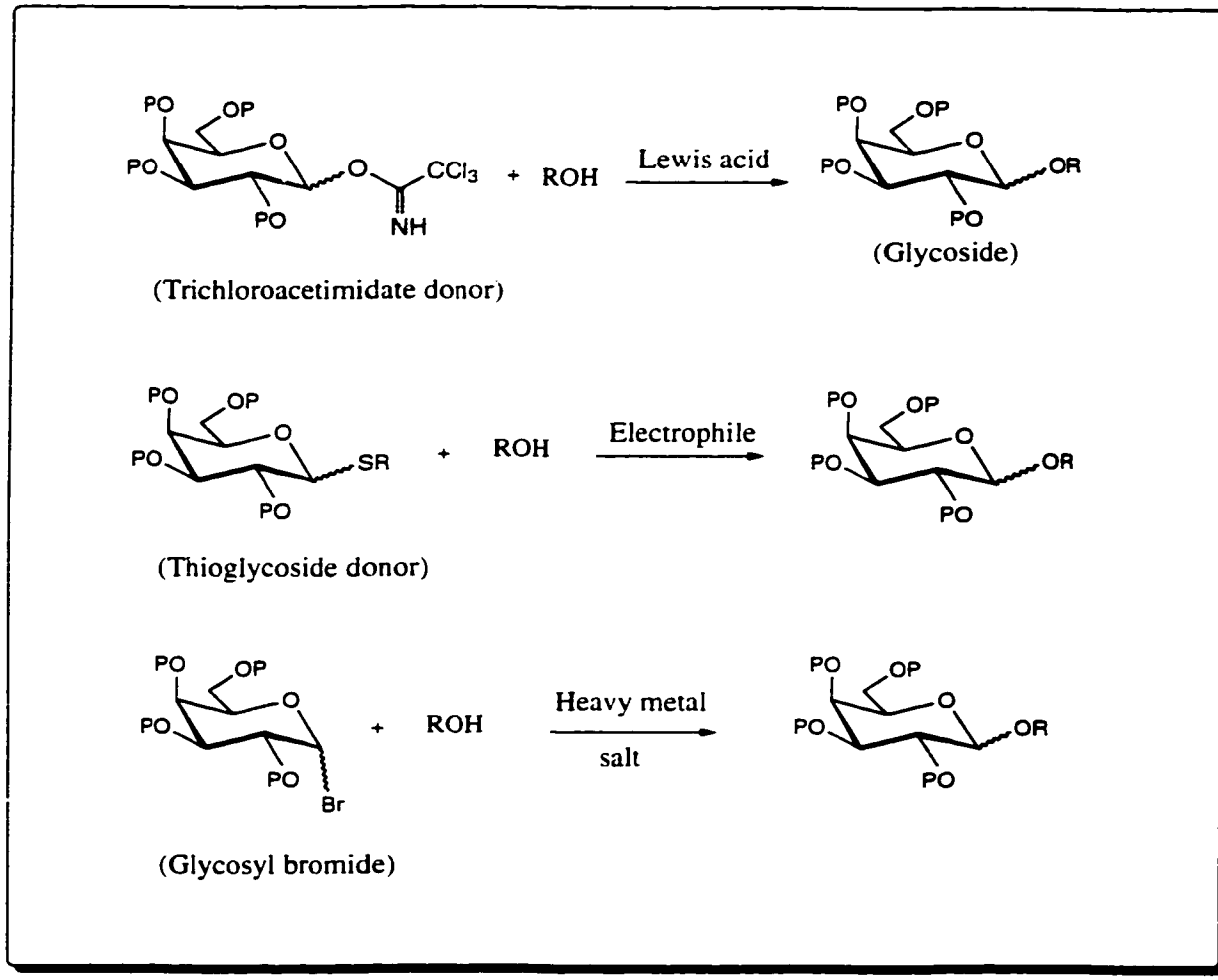


Figure 13. **Some glycosyl donors used in oligosaccharide synthesis.**

Numerous protecting groups have been developed for this purpose along with multiple activators of glycosyl donors. Protecting groups on the donor molecule, as well as the group at the anomeric center and the reagents used to activate it are important criteria to

be considered when designing a glycosyl donor. The three most frequently used glycosyl donors are the trichloroacetimidate donor, thioglycoside and glycosyl bromide (Figure 13). The first two are predominantly used in block syntheses of oligosaccharides, whereas the latter is used in couplings involving monosaccharide donors.

The syntheses reported in the present work have been performed using thiofucosides, fucosyl bromides and galactosyl bromides as donors. The following is a brief overview of the various methods that have been used to prepare and activate these donors.

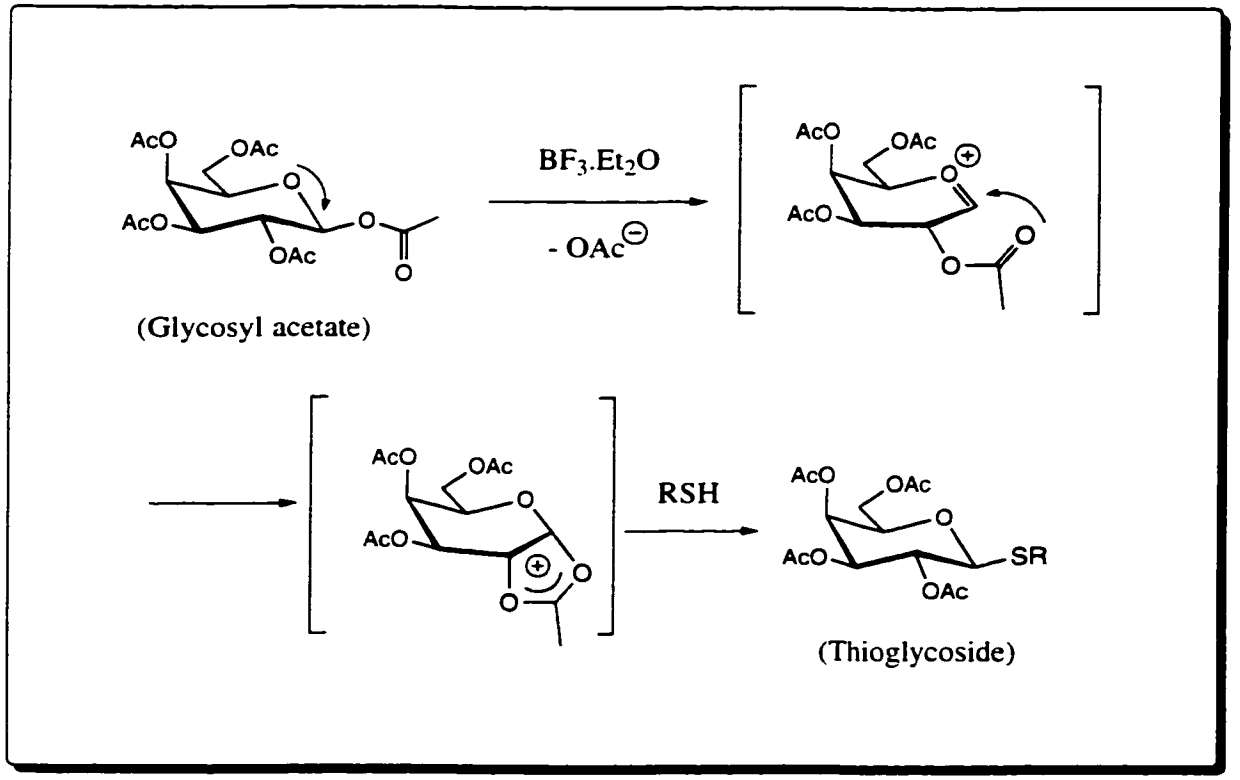


Figure 14. **Synthesis of the thioglycoside donor from galactose pentaacetate.**

1.6.1 *Synthesis of thioglycosides*

Alkyl, alkenyl and aryl thioglycosides are most often prepared from peracetylated sugars (Figure 14) or acylated glycosyl halides. In the former, thiols or their derivatives [59-61] react in presence of a Lewis acid [62, 63] to give acylated thioglycosides. In the latter, thiolate anions [64-67] are used to react with the glycosyl halide. In both cases, 1,2-*trans* thioglycosides are formed predominantly due to neighboring group participation by the acetate group.

1.6.2 *Activation of thioglycosides*

Thioalkyl or thioaryl groups are excellent temporary protecting groups for the anomeric center. They are stable to diverse conditions required for protecting group manipulation on other sugar OH groups on the sugar molecule. Thus, they can be used as glycosyl acceptors in glycosylation reactions. They can also be converted selectively to other glycosyl donors, or directly activated before coupling. When used as donors, they produce 1,2-*trans* glycosidic linkages when a participating group is present at the 2-position of the donor, and 1,2-*cis* glycosides with moderate stereoselectivity with a non-participating group (Figure 15).

Direct activation of thioglycosides has been undertaken using heavy metal salts like mercuric sulfate/acetate [68], mercuric chloride, nitrate, benzoate [69-71], phenyl

mercury trifluoromethane sulfonate [72], copper trifluoromethane sulfonate [73] and *N*-bromosuccinimide (NBS) [70, 74].

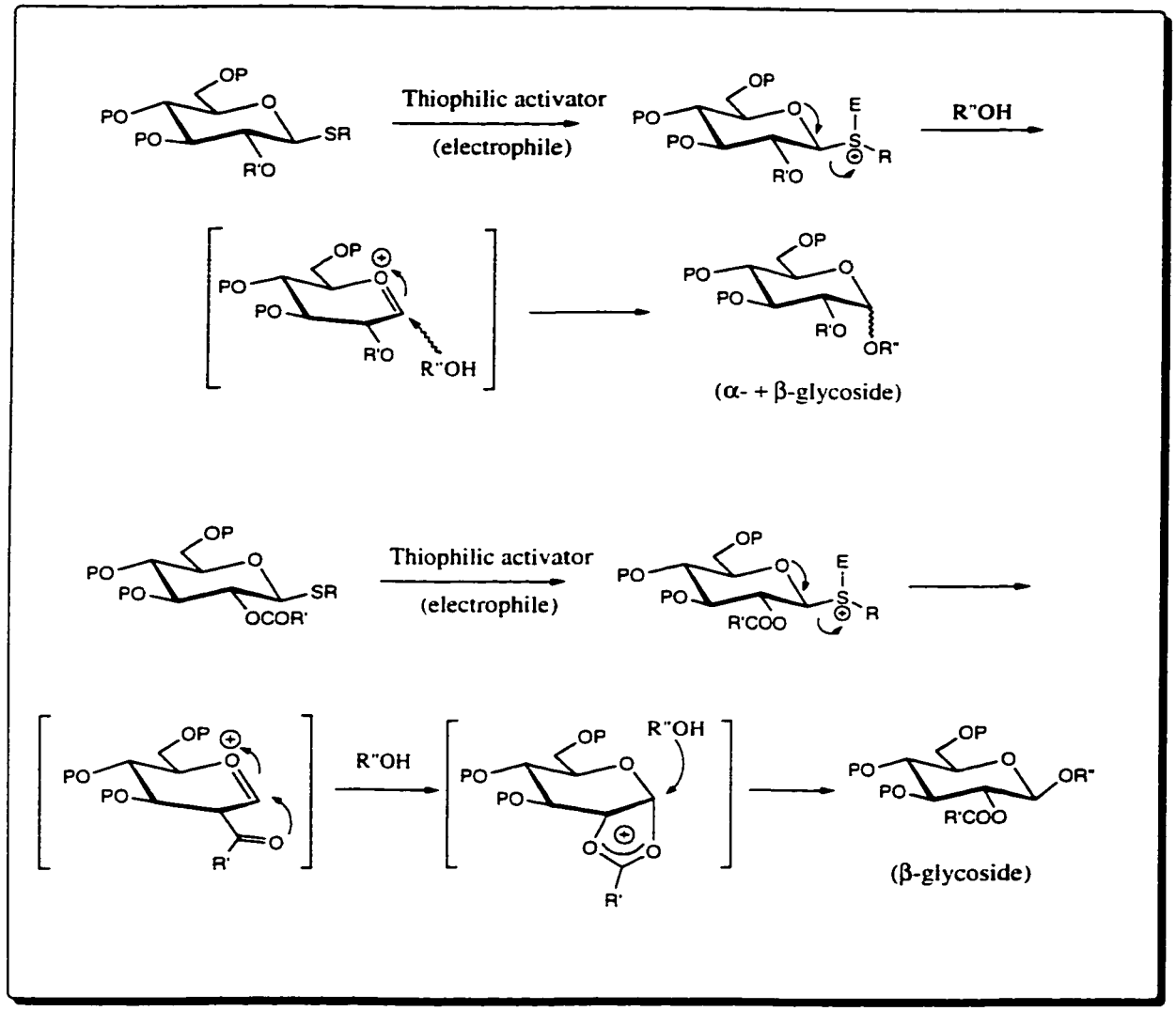


Figure 15. **Activation of thioglycosides for use as glycosyl donors.**

The anomeric configuration of the glycoside thus formed is opposite to that of the thioglycoside from which it was obtained when a participating group is present at C-2 of

the donor [68]. But the use of heavy metal salts did not find general use for activation of thioglycosides due to their low reactivity and was restricted to 2-deoxy thioglycosides and heterocyclic thioglycosides, as they are generally more reactive [75, 76].

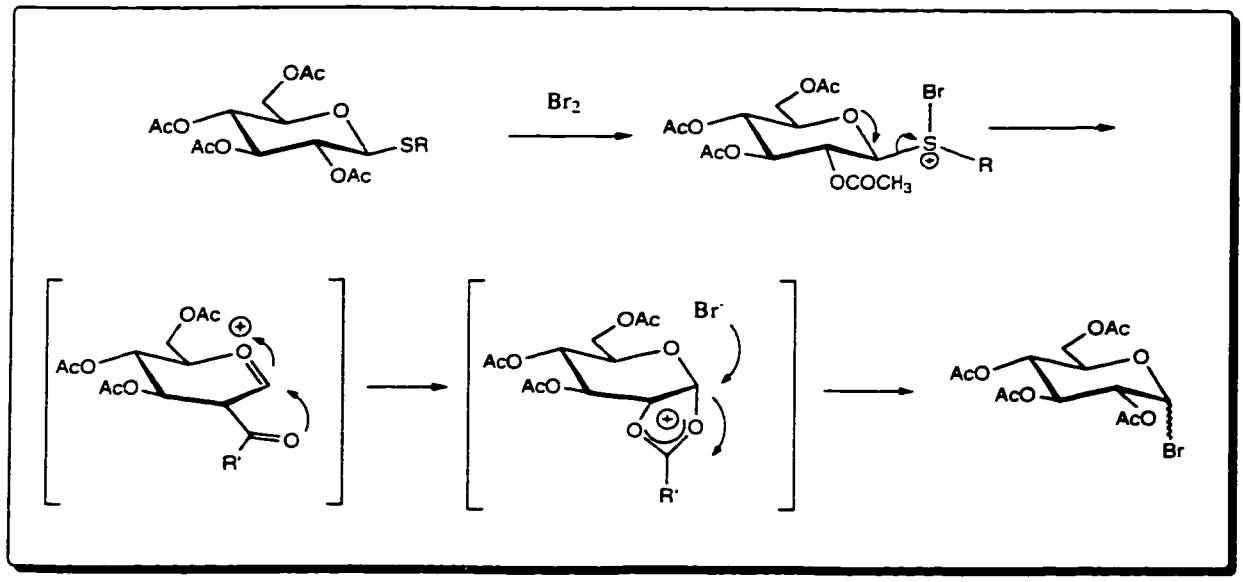


Figure 16. **Conversion of thioglycoside to glycosyl bromide.**

Thioglycosides have been used as donors by *in situ* generation of glycosyl halides (Figure 16). Activation of the sulfur atom with halogen [77], or copper (II) bromide and tetrabutylammonium bromide [78], or NBS and diethylaminosulfur trifluoride (DAST) [79] converts them to the corresponding glycosyl halides. This is followed by activation of this donor by a halophilic reagent [80-83].

The other direct method is to activate the anomeric sulfur with a thiophilic reagent in the same manner as the heavy metal promoters, but with more efficient results and in one

step. Electrophilic methylating reagents like methyl trifluoromethanesulfonate (methyl triflate), dimethyl(methylthio)sulfonium trifluoromethanesulfonate (DMTST) [84], dimethyl(thiomethyl)sulfonium tetrafluoroborate (DMTSB), methylsulfenyl triflate/ methylsulfenyl bromide [85] and methyl iodide [86] have been used to activate thioglycosides in oligosaccharide synthesis. These reagents preferentially methylate the sulfur atom to produce sulfonium ions, which are good leaving groups enabling the formation of carbocationic intermediates which are glycosylated. Although methyl triflate [87, 88] results in the formation of 1,2-*cis* glycosides with reasonable stereoselectivity with a non-participating group at the 2-position in diethyl ether as solvent, it is not suitable for use in large scale due to its toxicity. DMTST [89] was introduced by Garegg's group as a faster glycosylating reagent than methyl triflate [90] and one that avoids *O*-methylation, but it shows poor stereoselectivity except when a participating group is present adjacent to the anomeric position. This problem can be circumvented and 1,2-*cis* glycosides formed by using tetrabutylammonium bromide in the reaction mixture [78]. DMTSB [91] is used for its easier preparation and handling and lower hygroscopicity and higher stability compared to DMTST, which is prepared from methyl triflate.

N-iodosuccinimide with trifluoromethane sulfonic acid (NIS-triflic acid) [92, 93] and iodonium dicollidine perchlorate (IDCP) [94, 95] also activate the thioglycosides by producing intermediate sulfonium ions.

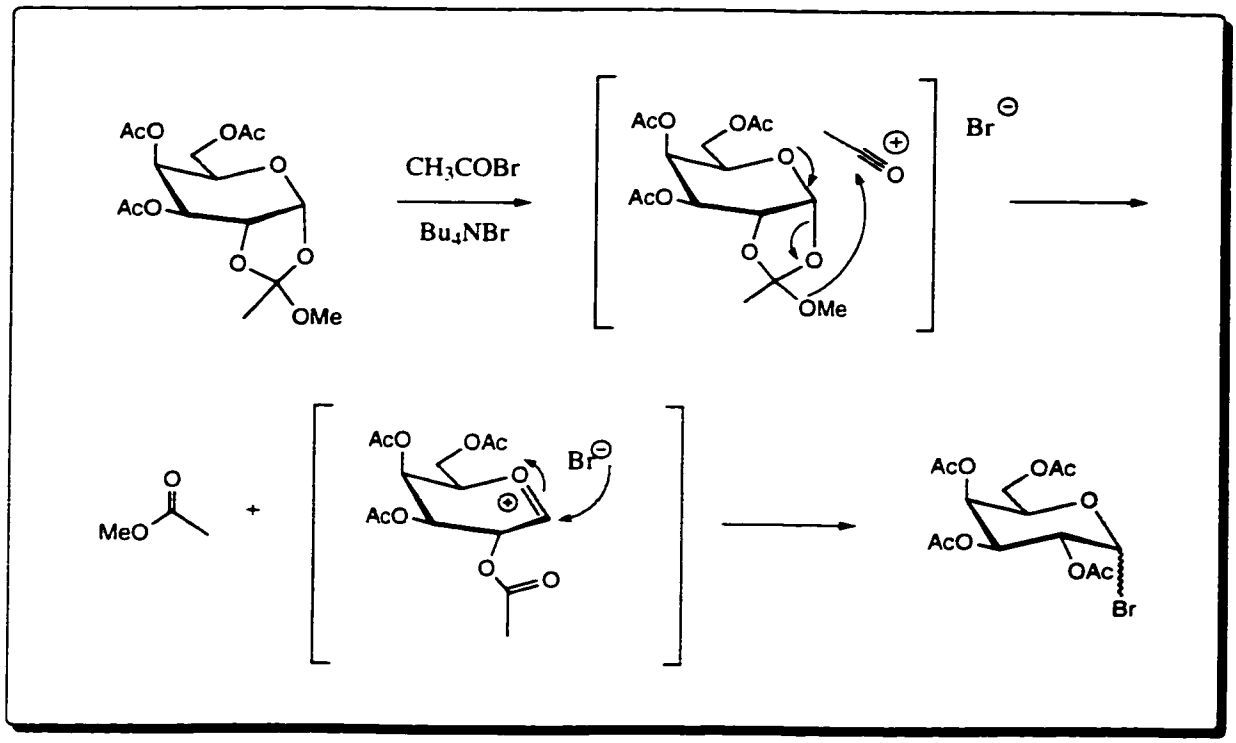


Figure 17. **Conversion of orthoester to glycosyl bromide.**

1.6.3 *Synthesis of glycosyl bromides*

Glycosyl bromides are most frequently prepared from peracylated sugars with hydrogen bromide in acetic acid solvent [96]. This method is not suitable for use when acid labile protecting groups are present. To synthesize glycosyl halides from hemiacetals, they are treated with oxalyl bromide in DMF and dichloromethane. Reaction between oxalyl bromide and DMF produces the Vilsmeyer reagent *in situ*, which reacts with the hemiacetal. Anomeric bromides can also be generated from orthoesters using acetyl bromide and TBAB [97] or trimethylsilyl bromide [98] (Figure 17). Trimethylsilylethyl (TMSET) groups are frequently used to protect the anomeric position during functional

group manipulation of glycosides [99]. They can be converted to the corresponding glycosyl bromide when treated with dibromomethylmethyl ether under zinc bromide catalysis.

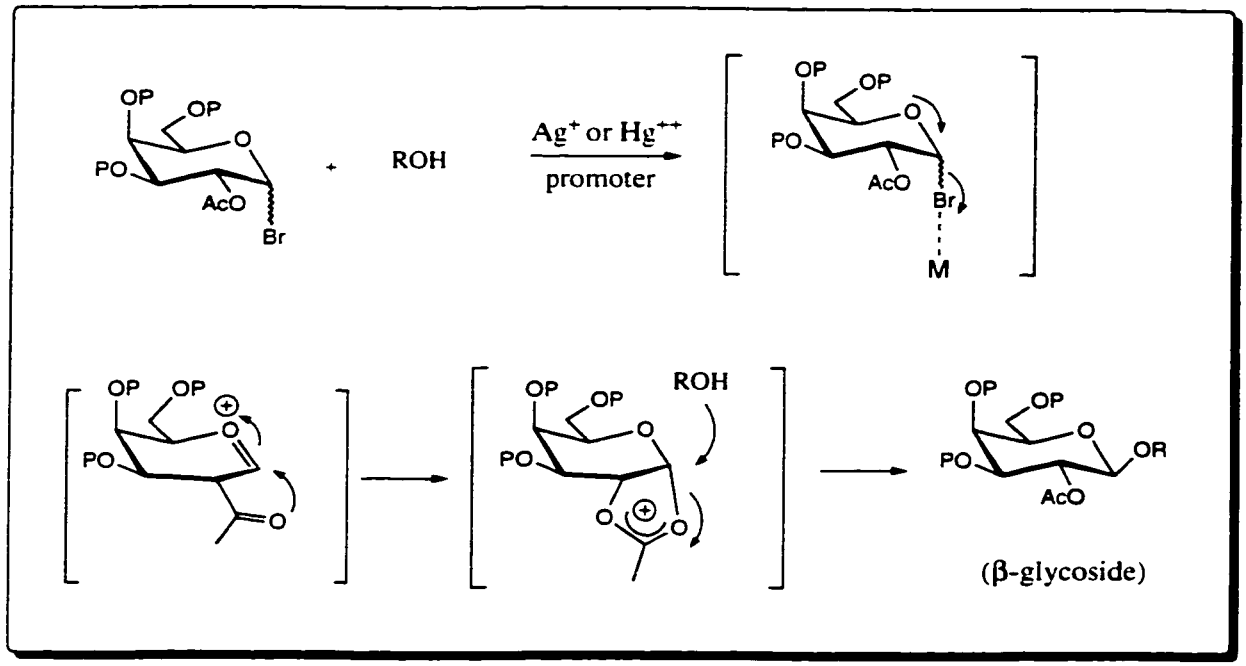


Figure 18. **Activation of glycosyl bromides with participating group at the 2-position of the donor.**

1.6.4 *Activation of glycosyl bromides*

Since their introduction in 1901 by Koenigs and Knorr [100], glycosyl bromides have been activated predominantly by mercury and silver salts to produce 1,2-*trans* glycosides when a participating group is present at C-2 of the sugar and anomeric mixtures when a

non-participating group is present (Figures 18 and 19). Ag_2O, Ag_2CO_3, $AgClO_4$, AgOTf, $AgNO_3$, Ag-silicate, $Hg(CN)_2$, $HgBr_2$ and HgI_2 and their combinations have been used

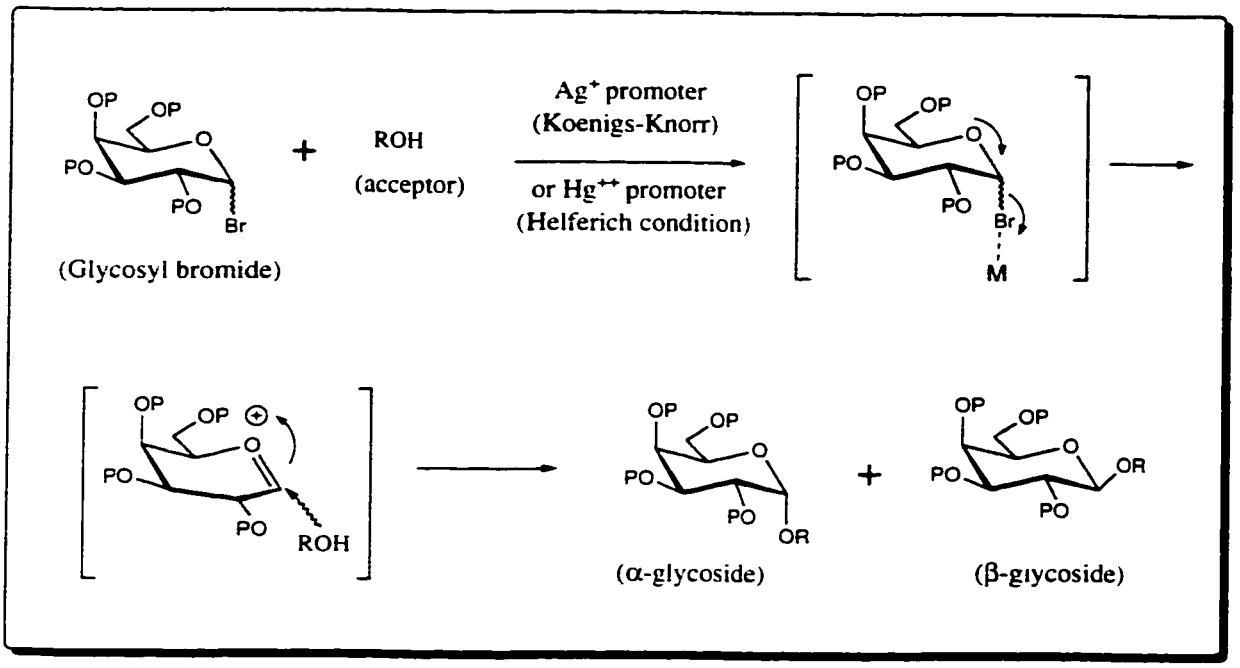


Figure 19. **Activation of glycosyl bromides using heavy metal salts.**

extensively [101-110]. These promoters require acid scavengers to remove the HBr produced and are usually carried out in presence of molecular sieves to remove water.

Lemieux introduced TBAB catalyzed glycosylation for 1,2-*cis* glycoside synthesis using glycosyl bromides [111]. This method has been one of the most commonly used for 1,2-*cis* glycoside linkage. It relies on the establishment of a rapid α/β equilibrium between the α and the β bromides and the faster rate of reaction of the more reactive β bromide to give the α glycoside (Figure 20).

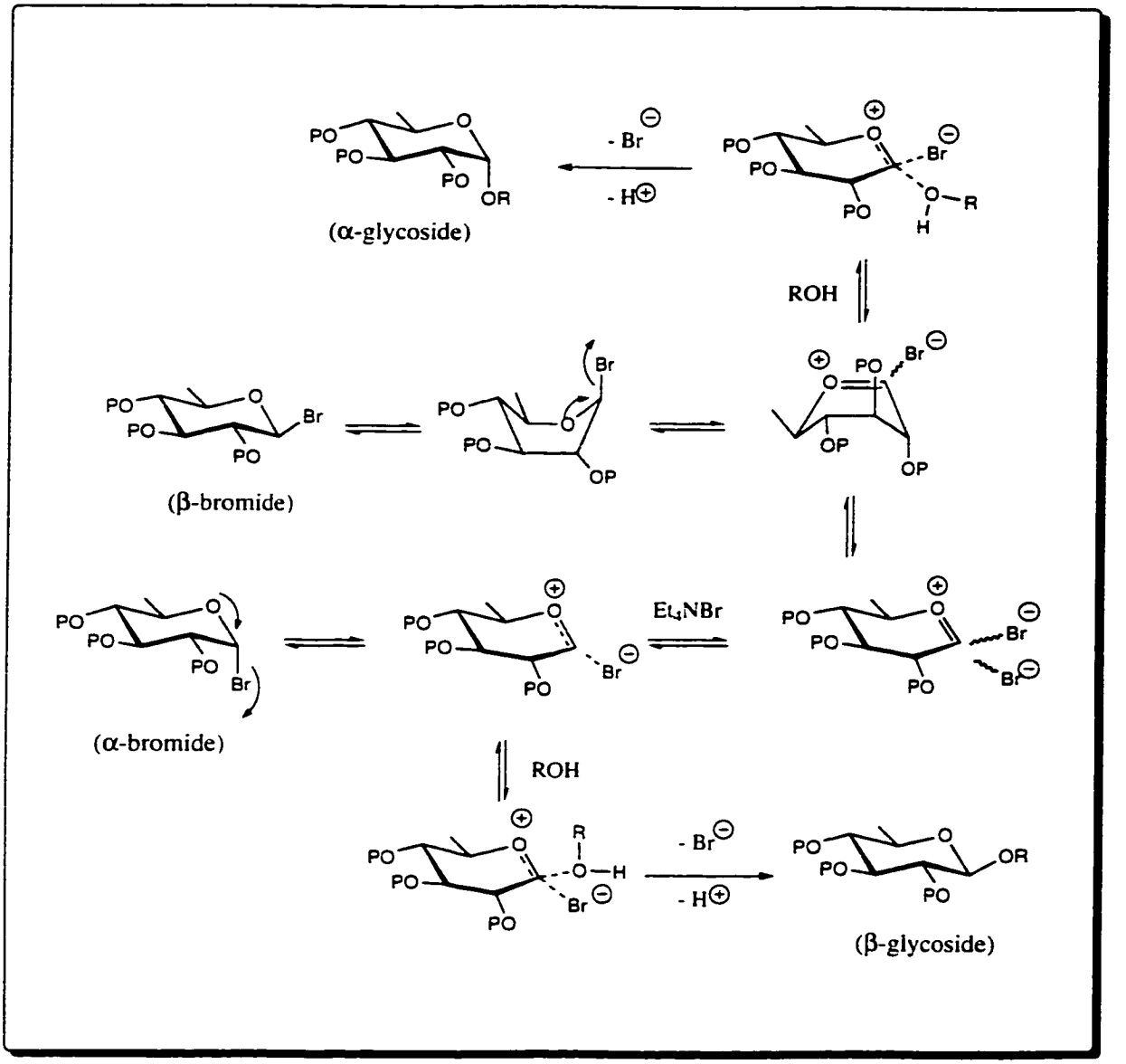


***Figure 20. In situ* anomerization of glycosyl bromides.**

The heterogeneous catalysis method was developed for the synthesis of the challenging β-mannosidic linkage formation where the insoluble silver catalyst removes the bromide from the α-face of the molecule, thereby facilitating the attack by the alcohol from the β-face [103, 104, 112] (Figure 21). Heterogeneous conditions ensure that anomerization to

the β-halide is restricted. Inversion of configuration at the anomeric position takes place when the glycosyl bromide and the alcohol are sufficiently reactive. Various Lewis acid catalysts such as borontrifluoride-etherate and tin tetrachloride [113] to activate bromides have also been used, which eliminate the use of toxic metal salts [113- 116].

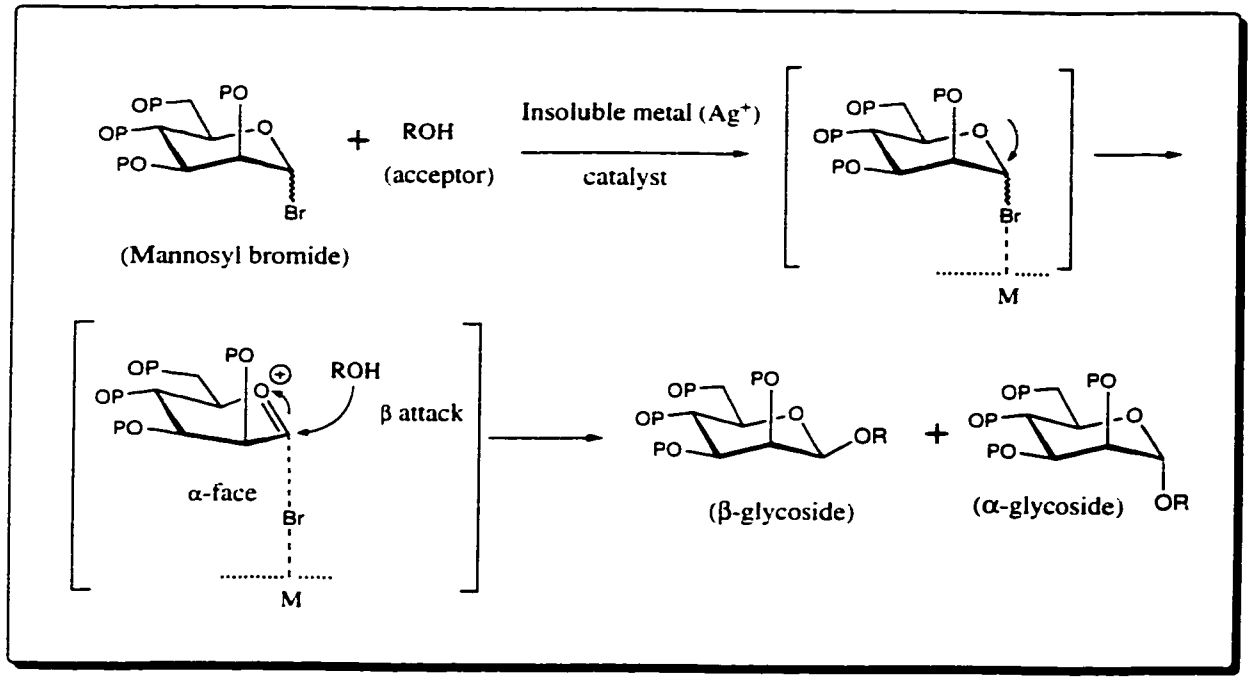


Figure 21. **Synthesis of β-mannosidic linkage under heterogeneous catalysis.**

1.7 *Active site mapping of enzymes*

Structural exploration of biologically active molecules like enzymes is challenging and numerous techniques have been developed to investigate interactions between proteins

and their ligands in biological systems. These interactions are key to the biological properties and functions of enzymes, hormones and other receptors. The importance of determining the nature of ligand-protein interaction is immense since it provides information about the active site of the protein. Several approaches have been utilized to address this issue. X-ray crystallography and/or nuclear magnetic resonance spectroscopy of the protein-ligand complex, site-directed mutagenesis of the protein, chemical mapping of 'key polar interactions' between the protein and the ligand and photoaffinity labeling of the protein in its active site have been used frequently. We have chosen the two latter approaches to study the active site of the blood group A and B glycosyltransferases.

1.7.1 *Modification of donor or acceptor substrates of enzymes*

Engineered ligands have been used quite successfully to explore the polar, steric and conformational requirements of protein-ligand complexes. Specific functional groups on the ligands have been modified to determine their contribution to the recognition and binding of ligands by enzymes. This approach is complimentary to site-directed mutagenesis where groups on the protein are modified to assess their contribution to the binding process.

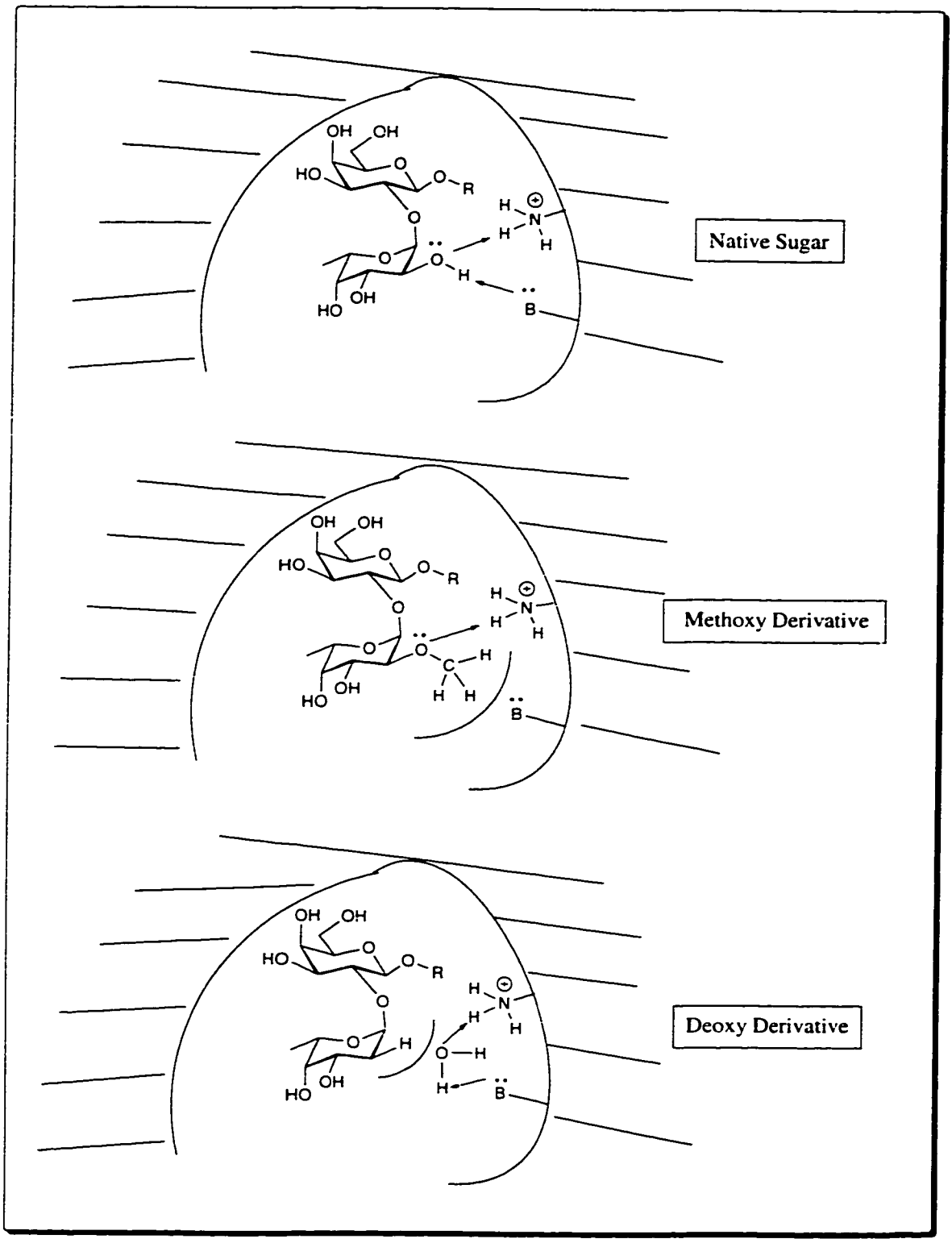


Figure 22. **Schematic representation of hydrogen bonding patterns in native, methoxy and deoxy analogs.**

When donors or acceptors of enzymes are carbohydrates, the hydroxyl groups play a major role in the hydrogen bonding process, which in turn contributes greatly to the specificity of the sugar-enzyme complex. A hydroxyl group is both a potential hydrogen bond donor and acceptor and replacing it with a functionality that is only an acceptor or a donor can identifiy the structure-function relationship of the interactions between the enzyme and its substrate.

Among the more common synthetic derivatives of the sugars used to study protein-ligand binding are their methoxy, fluoro, amino and deoxy analogs. The methoxy group is a hydrogen bond acceptor but not a donor, and it is sterically more demanding than an hydroxyl group (Figure 22). Additionally, it is more hydrophobic than the native hydroxyl group [117]. Similarly, fluorine can accept hydrogen bonds, but it is smaller in size and is more hydrophilic [118]. The amino group is nearly of the same size as the hydroxyl, but it can be protonated under physiological conditions resulting in a positively charged species. It can potentially form a hydrated complex, which is sterically more demanding. Deoxy sugars are neither hydrogen bond acceptors nor donors. Moreover, a hydrogen is nonpolar and the smallest group that can replace a hydroxyl [119, 120].

Epimeric analogs have been utilized to probe the stereo-electronic requirements of the interactions between specific hydroxyl groups and enzymes [121]. Structurally rigid

sugar analogs provide information on the conformational freedom that is allowed in a sugar-enzyme complex [122]. X-ray crystal structures of the synthetic analogs bound in the enzyme active site, or the determination of the various kinetic and thermodynamic constants of the binding of the substrate with the enzyme then allow us to determine the structural basis of the specificity of the various interactions in the active site of the enzyme.

1.7.2 *Photoaffinity labeling as a biochemical tool*

Photoaffinity labeling is another method used to gain structural information about the binding of macromolecules with their ligands. This refers to the techniques developed to reversibly bind biological receptors at the active site of photoactivable, but chemically inert, ligand analogs. It is considered a labor-intensive but reliable method for the structural investigation of binding sites of biological receptors [123-125]. This method has evolved considerably since its discovery by Westheimer's group [126], although the basic principle remains the same. A receptor ligand complex is irradiated with light of an appropriate wavelength, which results in a highly reactive species that binds to the receptor irreversibly at the site of interaction (Figure 23). If the ligands have a tag that can be identified either on SDS-PAGE, by affinity chromatography, or by mass

spectrometry, they allow the characterization of the covalently modified functional proteins through proteolysis and sequencing of the fragments that contain the bound ligand.

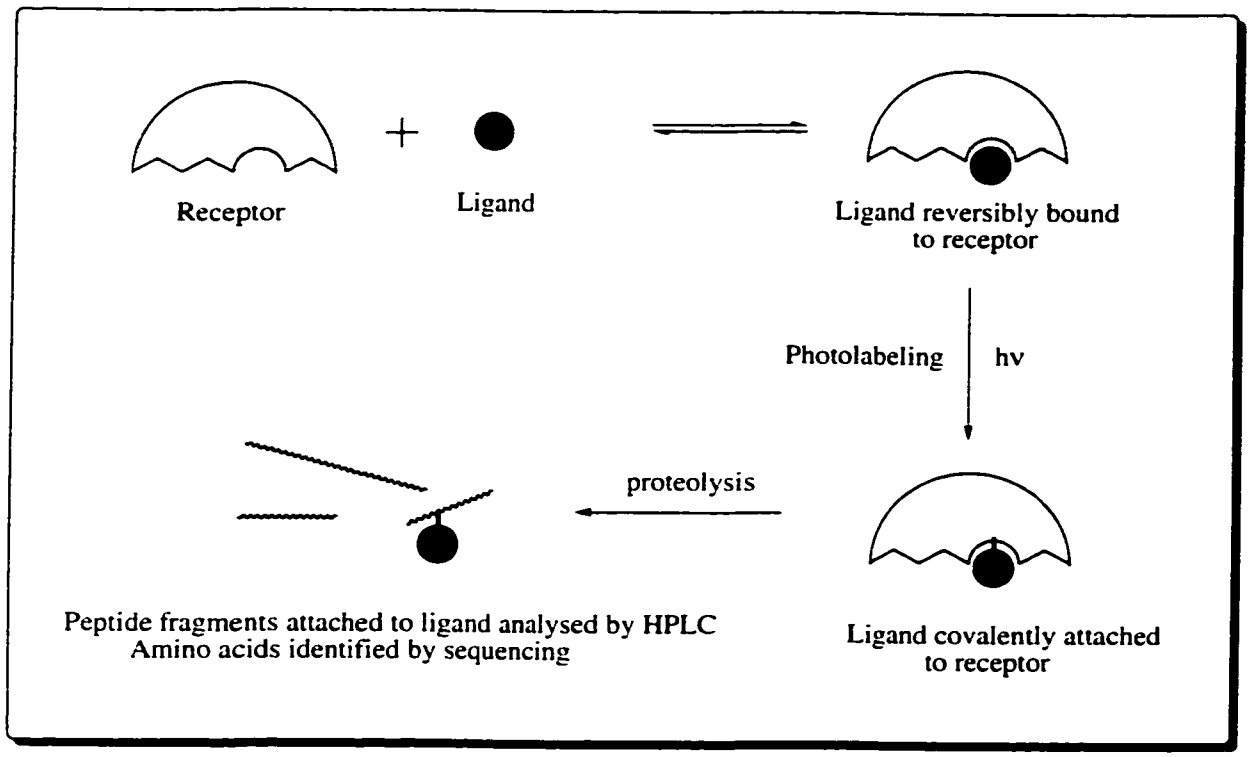


Figure 23. **Schematic representation of the photoaffinity labeling of a receptor with a ligand.**

A number of photoactivable reagents have been developed over the years that fulfill the following requirements [127]:

i) The reagents should be stable in the absence of light, but highly reactive under a light of wavelength that does not cause photolysis of the biological sample under study.

ii) Intermediates of high reactivity should be formed to enable ligands to insert indiscriminately, even into chemically inert bonds, especially in the case of low affinity ligands.

iii) They should not undergo intramolecular rearrangement and thereby form stable species that can cause unspecific labeling outside the binding site of the target receptor or to different species altogether.

iv) They must not be large enough to sterically perturb the system being studied and the photo labeling experiment must ensure that the covalent bond occurs at the binding site.

v) Additionally, the product of the photoreaction should be stable and isolable, enabling its purification and analysis.

The reagents that have been used most often are aryldiazonium salts, aryl diazirines (where the reactive species is a carbene) and aryl azides (where a nitrene is the reactive intermediate generated). These radical intermediates have also been generated from benzophenone and its derivatives, sulfur compounds and halogenated substrates [128]. All these reagents generate triplet or singlet intermediates in their excited state that are expected to behave like biradical species. Carbon electrophiles generated *in situ* from

nitrobenzene and diazonium salts have also been used in photoaffinity labeling (Figure 24).

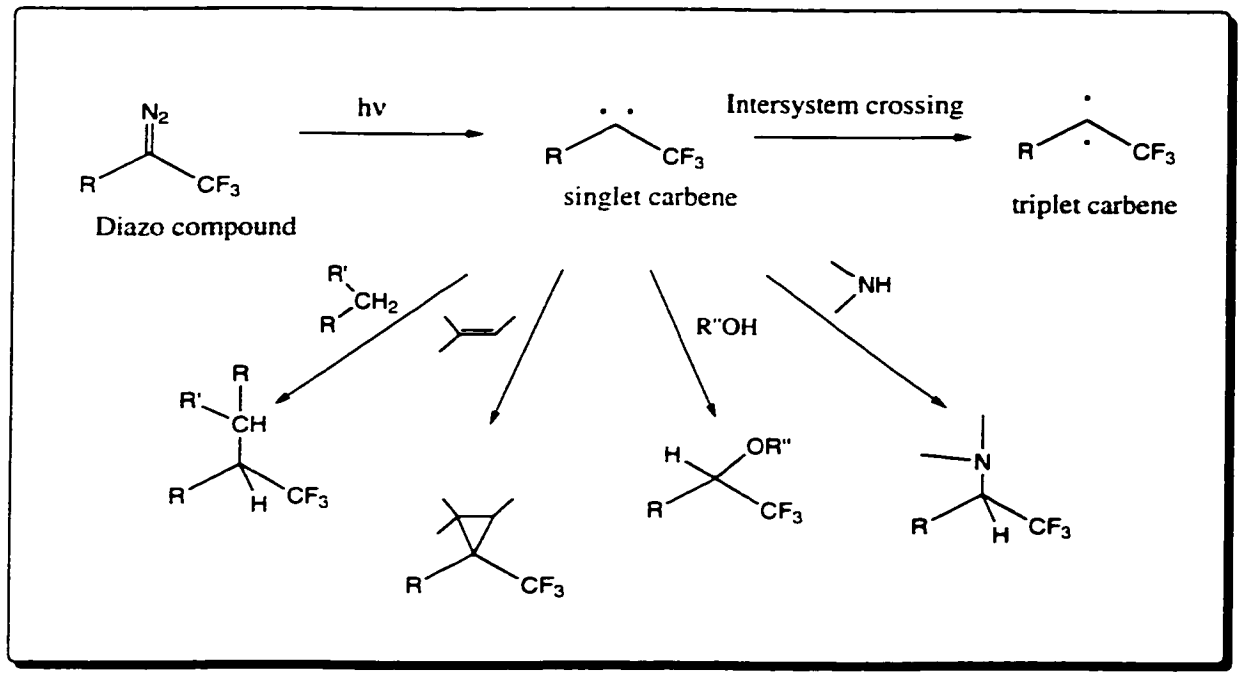


Figure 24. **Use of diazo compounds as reagents in photoaffinity labeling.**

Although arylazides were traditionally a popular choice of photoaffinity reagents, aryl ketones like benzophenone and acetophenone are being used by more and more groups as the photo probe of choice in diverse systems. Their application has been expanding especially since the discovery of 4-benzoyl-L-phenylalanine, a heterobifunctional crosslinking reagent that can be easily attached to biologically active ligands [129] (Figure 25). A study comparing some commonly used photoaffinity labeling probes

found that the benzophenone group has an extremely low probability of reacting with water compared to the other reagents (aryl azide, aryl diazirine, α-diazocarbonyl), as well

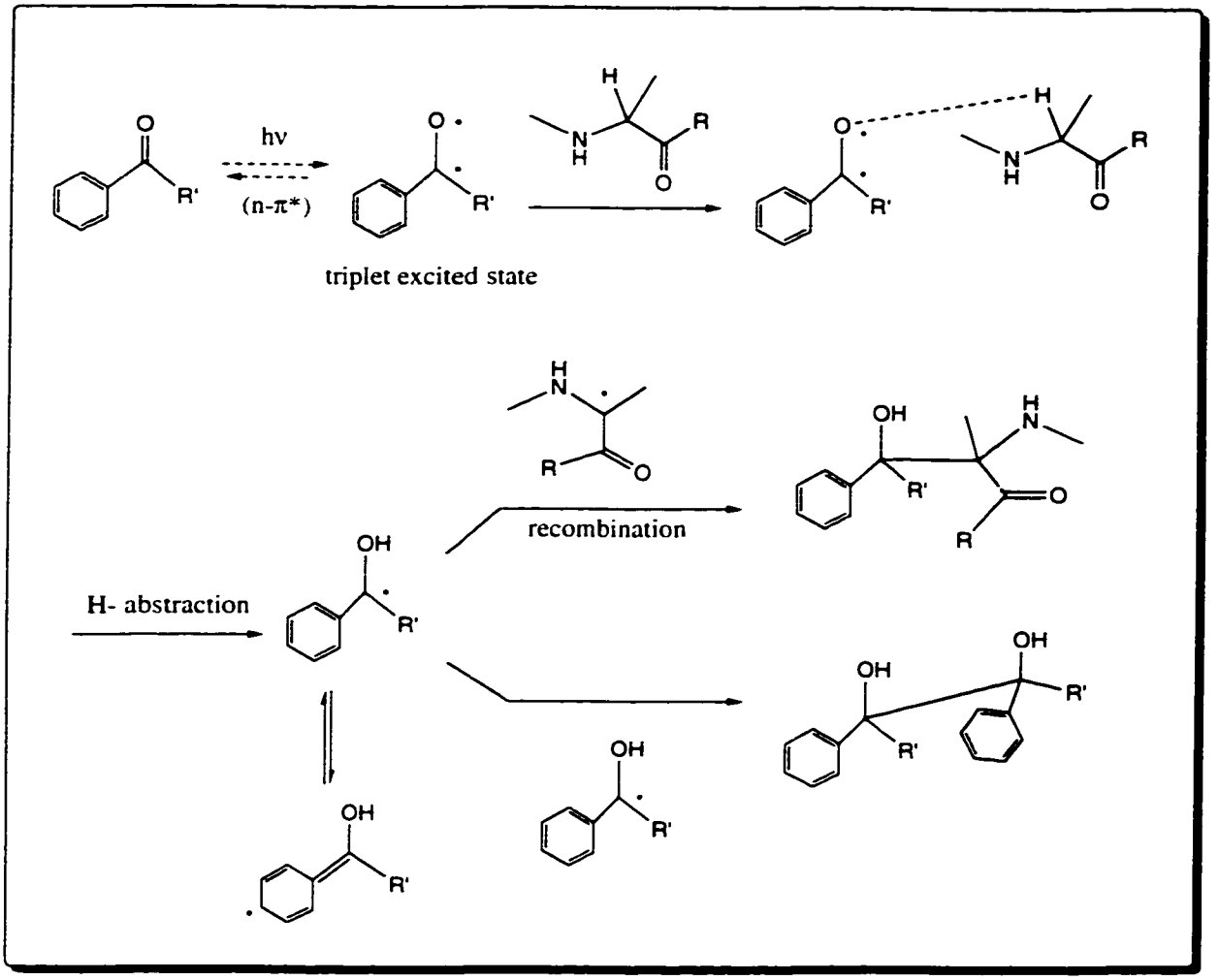


Figure 25. **Photochemistry of benzophenones.**

as possessing similar crosslinking efficiency, but required longer irradiation time [130] (Figure 25).

Some of the other advantages of this group relative to carbene and nitrene precursors are:

a) Higher chemical stability towards acids and bases.

b) High abstraction potential for hydrogen from a C-H bond, even in the presence of water, giving covalent adducts stable to chemical and enzymatic polypeptide cleavage.

c) Stability in ambient light.

d) Activation is not photo-dissociative and reversible, and the conditions are much less damaging to the proteins.

e) High efficiency covalent modification of hydrophobic regions of the protein.

Benzophenone (BP) generates an excited triplet biradical by promotion of one electron from the non-bonding orbital of oxygen to the antibonding π^* orbital of the carbonyl group upon irradiation with UV light of ~350 nm. The lifetime of electrons containing two unpaired electrons is much longer than that of a singlet. The species can then abstract hydrogen from a suitably oriented geometrically accessible (within 2.5-3.1 Å) C-H σ bond.

The ketyl radical thus generated readily combines with the alkyl radical formed by H-abstraction. If a H-donor with the required geometry is not available, the triplet state relaxes to the ground state (Figure 25). It can also undergo electron transfer from another species followed by H-abstraction.

The covalently labeled enzymes obtained from photoaffinity labeling can be examined by X-ray crystallography, spectroscopic analysis of the complex, or partial or complete hydrolysis of the labeled protein followed by mass spectrometric or HPLC analysis of the fragments. Photoaffinity labeling does not require cloning, expression and purification of large quantities of the proteins and their genetic manipulation, as in other approaches like modification by single point site–directed mutagenesis. It has the potential to give structural as well as functional information on biological receptors.

1.8 *Scope of this project*

The blood group A and B glycosyltransferases, represent an ideal system for the study of a glycosyltransferase active site and the design and synthesis of specific glycosyltransferase inhibitors since they have been sequenced and cloned [42], giving us access to substantial amounts of these enzymes. They are attractive models for this study also because they differ in their sequence by only four amino acids. A comprehensive understanding of the process of glycosyltransfer requires precise identification of the sites of molecular recognition on the protein, that is, detailed information about the key interactions between the enzyme in the active site and the glycosyl donor and the acceptor. One way of achieving this is by modifying the hydroxyl groups on the acceptor

and/or the donor structure to see which part of these compounds are required in the recognition process. In order to gain a detailed understanding of the interactions between the acceptor substrate and the enzyme, we designed and synthesized modified acceptors in order to probe the enzyme active site, an approach that has been used frequently in our group [131-135].

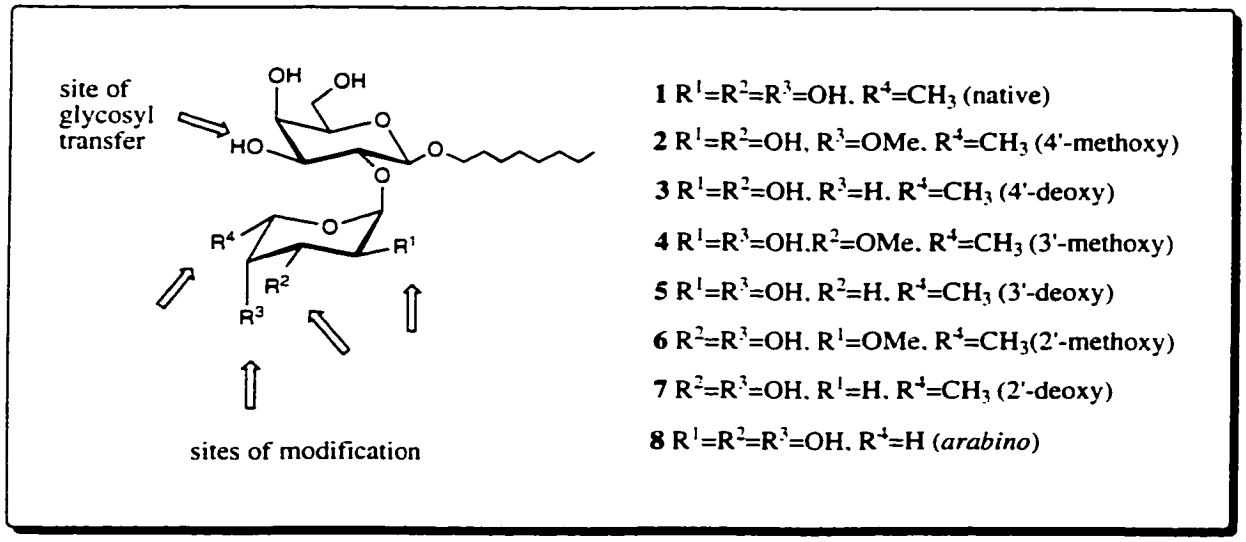


Figure 26. **Some modified disaccharides synthesized for this study.**

Glycosyltransferase inhibitors can be used as tools to study enzyme-substrate interactions [136] and may have potential as anti-tumor agents. The minimum acceptor structure recognized by the two enzymes is αLfuc*p*(1,2)βDgal*p*-*O*R [137]. The systematic modification on the galactose moiety had already been described as part of the initial work on these enzymes [50, 51]. For the present project it was decided that the hydroxyl

groups on the Fuc residue would be replaced with H and *O*-methyl groups, and the C6' methyl on the Fuc residue would be replaced by H (Figure 26).

Deoxy analogs of the acceptor would help us to determine the H-bonding interactions between the hydroxyl groups on the Fuc moiety and polar groups in the active site. Methoxy groups are potential H-bond acceptors and they also probe the steric requirements in the active site. The *arabino* analog would be prepared to determine the role of the 6'-methyl group on the fucose. The disaccharides synthesized would be tested enzymatically and kinetic studies would be performed on compounds that were good acceptors or inhibitors of the enzymes. We decided to use a hydrophobic aglycon, the octyl group since the compounds synthesized can be purified by reverse phase chromatography and they can be used in Sep-Pak assays to determine their activities [138].

Acceptors of A and B glycosyltransferases would be used in the enzymatic synthesis of modified A and B antigens. A series of recombinant enzymes were made by sequentially changing the amino acids corresponding to the A transferase to that corresponding to the B transferase [46]. We decided to assay the acceptor specificity of these mutants since both the wild type enzymes use the same acceptor.

Photoaffinity labeling is a useful technique to identify the sites of interaction between receptors and ligands and we decided to apply this methodology to our system. The acceptor or inhibitor identified in our study would be conjugated to a chromophore and used to label the enzyme active site by irradiation with ultraviolet light (Figure 27).

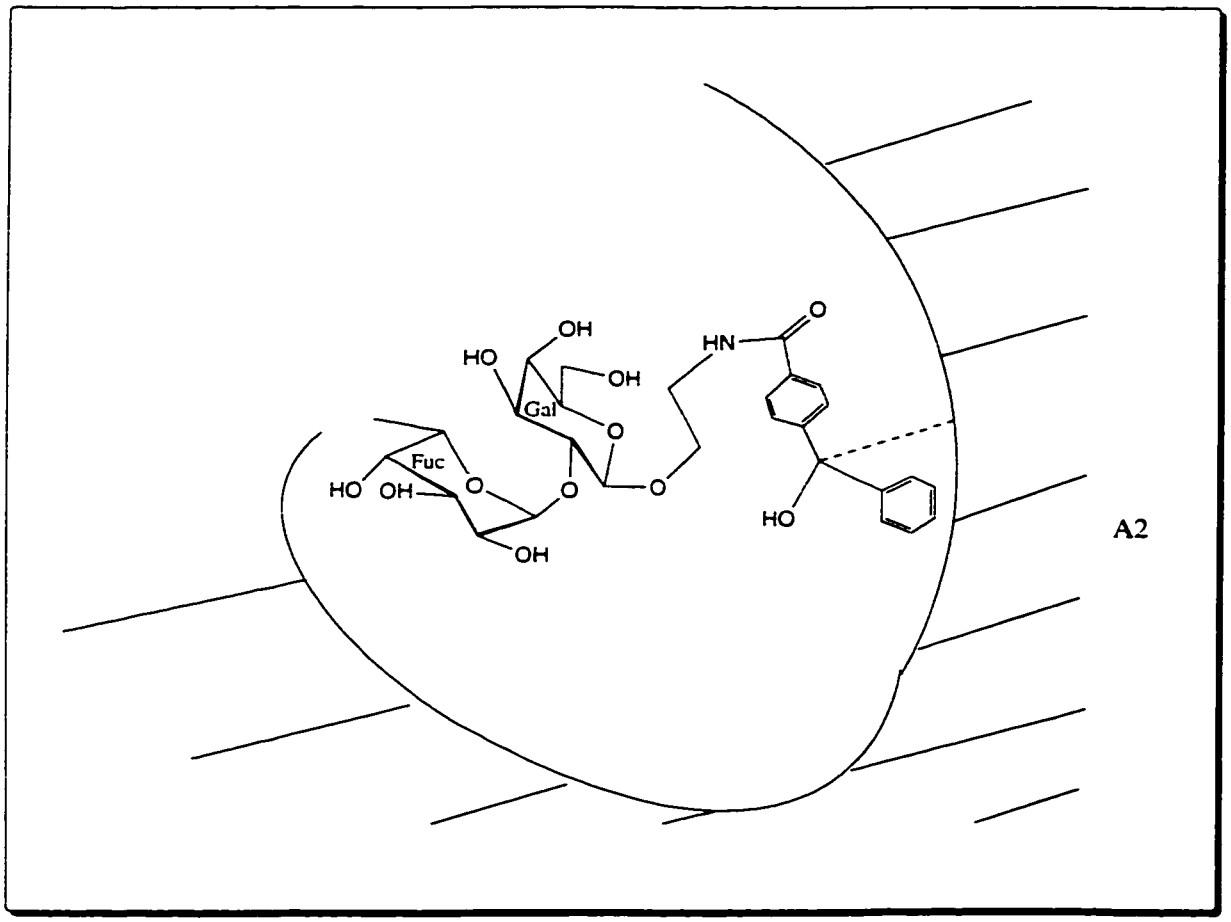


Figure 27. **Schematic representation of the photolabeling of A transferase by an acceptor modified by benzophenone.**

The labeled protein could then be identified and separated from unlabeled protein using lectins [139, 140] that recognize either the terminal monosaccharide (Fuc) or the disaccharide (O antigen). The isolated fractions could be further degraded and peptide fragments containing the probe analyzed. As the amino acid sequence of these enzymes are known, sequencing a small peptide would definitely identify its location in the original protein.

Escherichia coli cells transformed with the plasmid containing ompA-glycosyltransferase genes was made available to us by our collaborators in NRC Ottawa [45, 46]. The recombinant enzymes were produced in soluble forms in the *E. coli* periplasm by the replacement of the membrane anchoring domain by *E. coli* omp A secretion leader. They were grown and the genes expressed in our laboratory to obtain the wild type and mutant A and B glycosyltransferases that were used in the enzymatic assays and syntheses.

Chapter II

SYNTHESIS AND ENZYMATIC EVALUATION OF MODIFIED ACCEPTORS OF RECOMBINANT BLOOD GROUP A AND B GLYCOSYLTRANSFERASES

2.1 *Introduction*

The great interest generated in understanding the involvement of blood group antigens in the invasion and metastasis of tumor cells led us to take a closer look at some of the glycosyltransferases that synthesize these structures *in vivo*, viz., the blood group A and B glycosyltransferases, respectively the α(1,3)*N*-acetylgalactosaminyl and the α(1,3)galactosyltransferase. This chapter describes the synthesis of some analogs of the minimum acceptor structure recognized by these two enzymes, αLFuc*p*(1,2)βDGal*p*-*O*Octyl (**1**). Several acceptor analogs were synthesized by modifying the hydroxyl groups on the fucose residue (Figure 28) and assayed with these enzymes in order to determine the functional group specificities, i.e., the steric and hydrogen bonding requirements in the active site of the enzymes. Deoxy analogs of compound **1** were prepared to assess the H-bonding interactions between the hydroxyl groups on the Fuc moiety and polar groups in the active site. Methoxy groups can be H-bond acceptors but not donors and they also probe the steric requirements of the active site. The *arabino* analog was prepared to

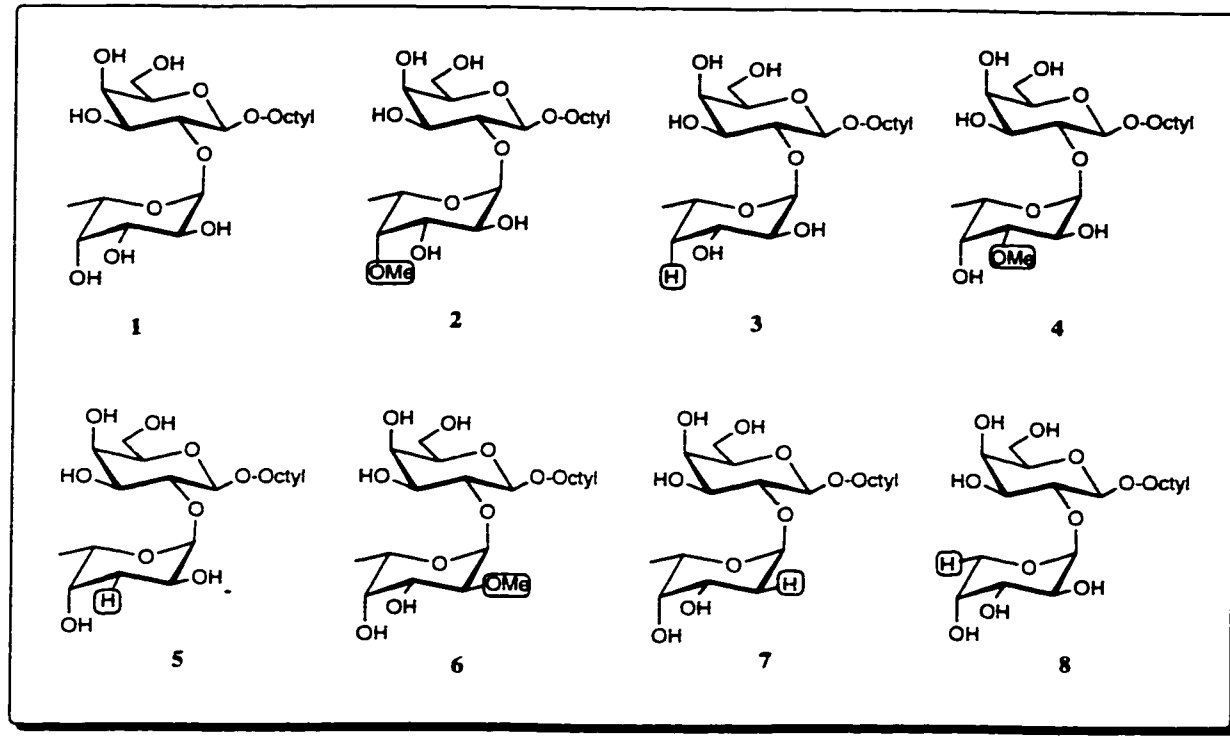


Figure 28. **Native (1) and modified acceptors (2-8) of the blood group A and B glycosyltransferases.**

determine the role of the 6'-methyl group on the Fuc residue. The octyl aglycon facilitates the purification of these compounds by reverse phase C-18 cartridges. It also permitted the use of radioactive Sep-Pak assays of these synthetic analogs with the glycosyltransferases being studied. The biochemical evaluations of these compounds are then discussed. Finally, an analysis of the results obtained is presented.

In the Sep-Pak assay, the enzyme assay mixture is transferred to a pre-washed (with methanol and water) C-18 Sep-Pak cartridge. It is washed extensively with water when the un-reacted donor and the salts are removed. The un-reacted acceptor and the product

are retained by the cartridge due to their hydrophobic octyl arm. They are then eluted with methanol and counted in a scintillation counter to determine the extent of transfer of the radioactive donor (Figure 29).

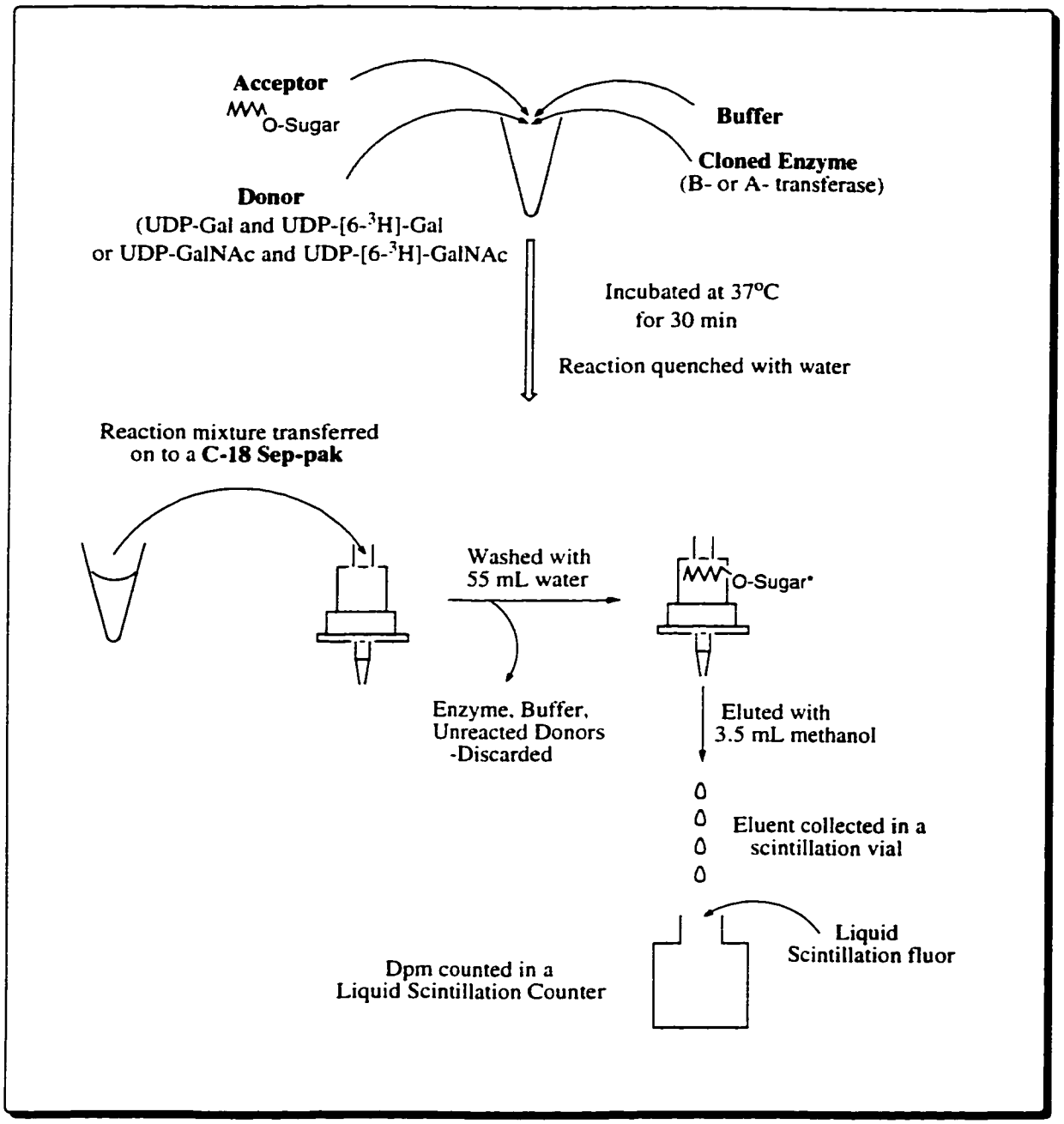


Figure 29. **Schematic representation of the Sep-Pak assay method.**

2.2 *Chemical synthesis of the disaccharide analogs*

The monosaccharide building blocks were synthesized using established protection-deprotection strategies. In order to obtain the required disaccharides modified at the 2, 3, and 4 positions of the Fuc residue, an octyl β-D-galactopyranoside (**25**) protected at the 3, 4, and 6 positions was coupled with suitably protected Fuc and Ara derivatives using halide ion catalyzed glycosylation or thioglycoside activation with dimethylmethylthiosulfonium triflate (DMTST). Instead of using individually *O*-methylated and deoxygenated fucose donors, temporary-protecting groups that can be removed after glycosylation were used (Figure 30). Methylation and deoxygenation were performed on the disaccharides followed by deprotection to obtain the target compounds. Retrosynthetically, fucose with the 3-OH or the 4-OH free can be obtained regioselectively from a common precursor, in a single step, and this route was chosen instead of preparing them individually by two separate chemical manipulations as shown in Figure 30. Phase transfer catalysis was utilized in this step, and the precursor was obtained by the hydrolysis of the isopropylidene group on the 2-*O*-benzyl derivative of fucose. The free hydroxyl groups were then protected prior to glycosylation.

2.2.1 *Preparation of the acceptor*

The synthesis of the acceptor **25** began with galactose pentaacetate which was first converted to galactosyl bromide **19** with hydrogen bromide in acetic acid and then to the

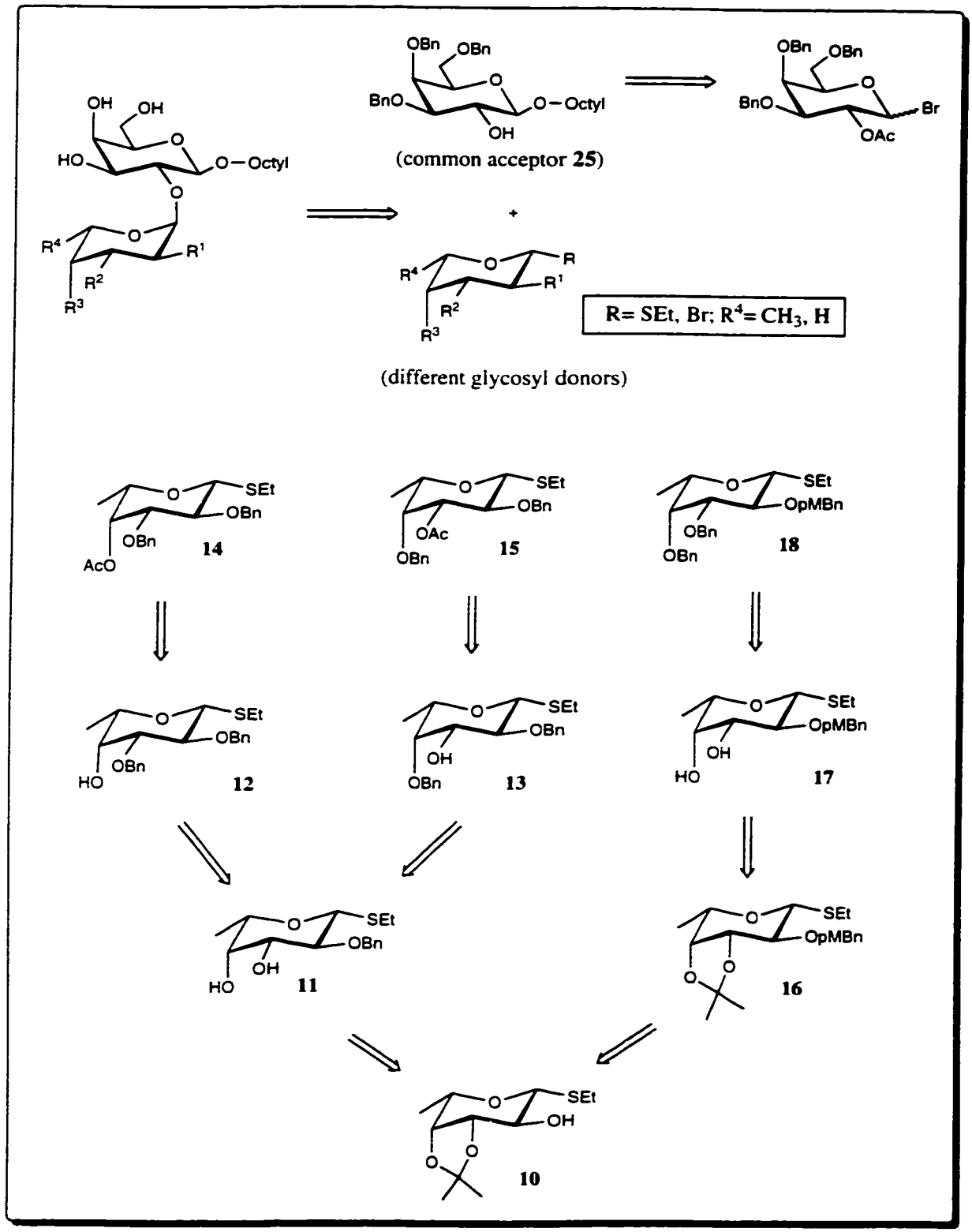
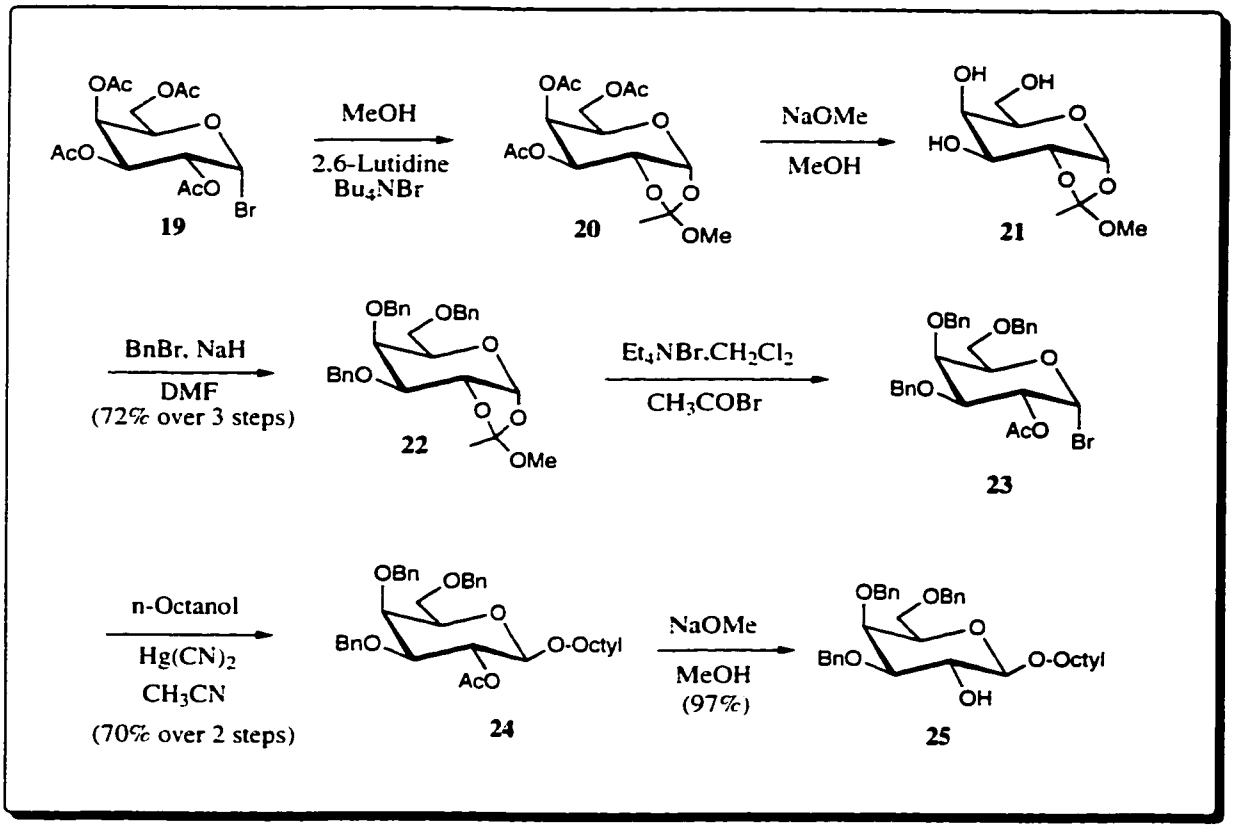
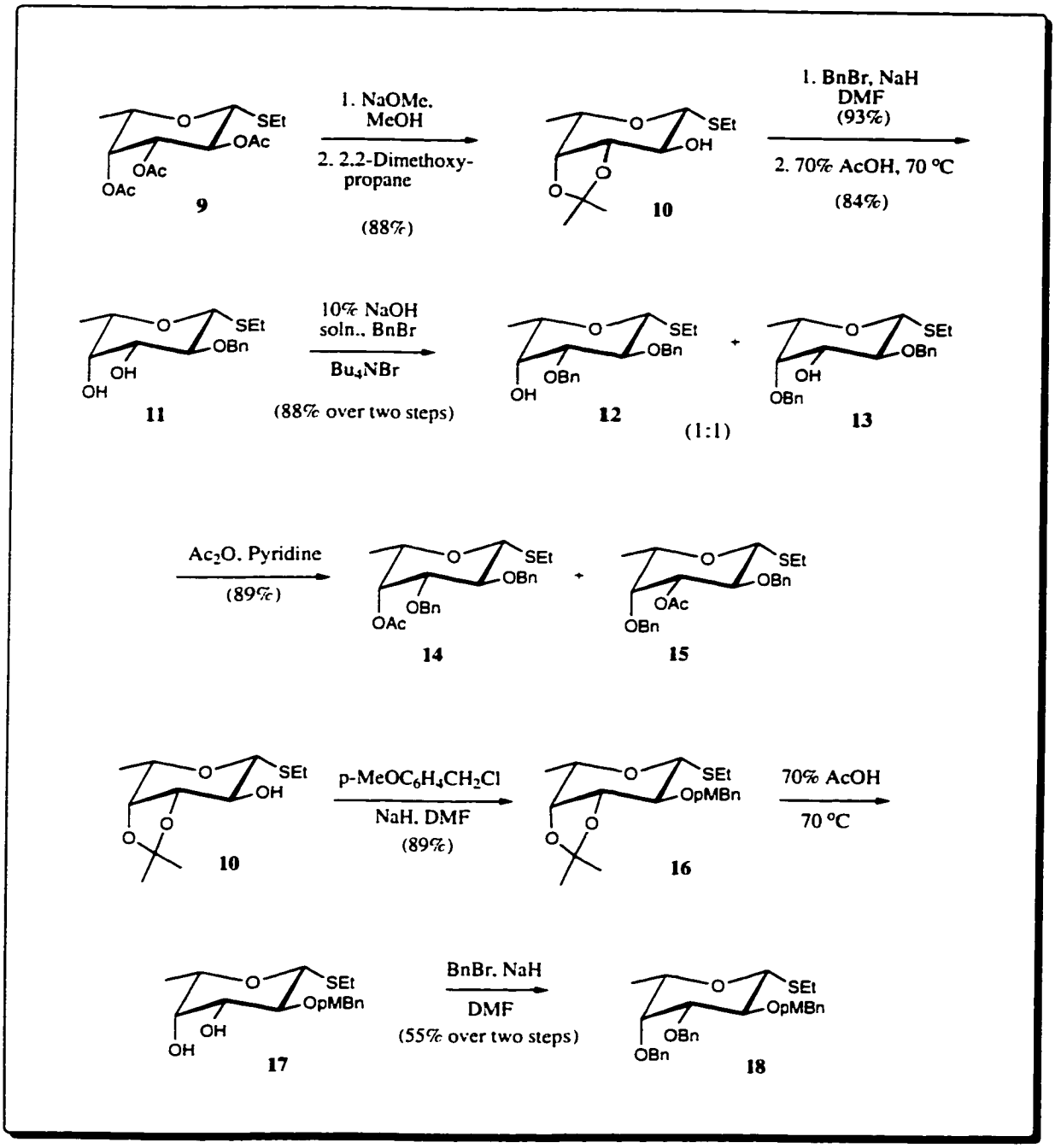


Figure 30. **Retrosynthetic analysis of the synthetic strategy utilized to prepare the modified analogs.**

orthoester **20** with methanol and lutidine [141, 142]. Compound **20** was deacetylated with sodium methoxide in methanol and benzylated with benzyl bromide to give compound **22** in an overall yield of 72%. As shown in Scheme 1, the orthoester was converted to 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-galactopyranosyl bromide (**23**) with tetrabutyl ammonium bromide and acetyl bromide [143-147]. Compound **23** was glycosylated with octanol under Helferich glycosylation conditions to give compound **24** (70% over two steps), followed by deacetylation with sodium methoxide in methanol to give **25** (97%).



Scheme 1. **Synthesis of the acceptor alcohol.**



Scheme 2. **Synthesis of the modified fucosyl donors.**

2.2.2 *Preparation of the Fuc 3-OH and 4-OH modified derivatives*

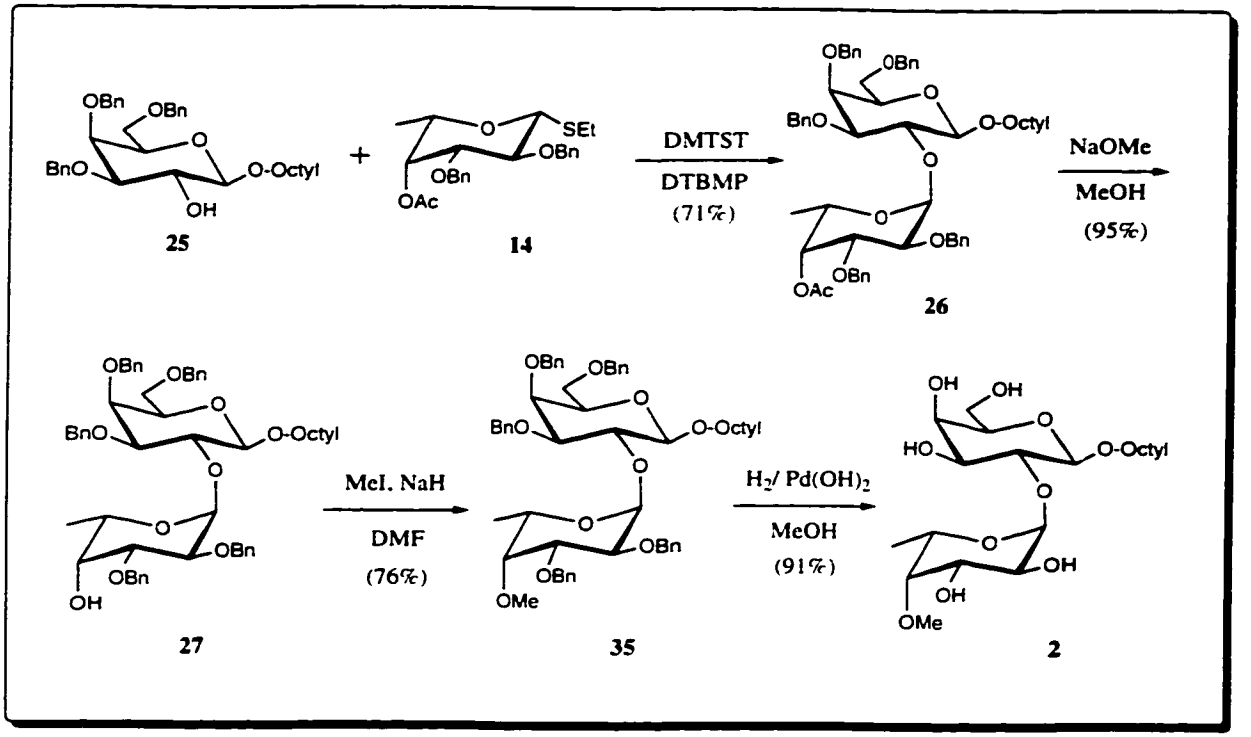
Ethyl 1-thio-fucopyranoside was treated with 2,2-dimethoxy propane and a catalytic amount of camphorsulfonic acid to give compound **10** (88%). It was alkylated with benzyl bromide (93%) and hydrolyzed [148] to give compound **11** (84%). To prepare both the analogs in one step, compound **11** was mono-benzylated under phase transfer catalysis using a quaternary ammonium salt [149], which gave both the 3-*O*-benzyl (**12**) and 4-*O*-benzyl (**13**) compounds (88%). The two compounds were acetylated (in 90% yield) to produce the donors and to ascertain the identity of these compounds (Scheme 2). Based on the downfield shift of H-3' in compound **15** and H-4' in compound **14** in their proton NMR spectra, their structures were unambiguously assigned.

2.2.3 *Preparation of the Fuc 2-OH modified derivatives*

Retrosynthetic analysis of the disaccharides leads to a fucose precursor with a non-participating protecting group at the 2-OH that would survive glycosylation conditions, and can be removed in presence of a glycosidic bond and the other protecting groups. The *para*-methoxybenzyl group was considered suitable for this purpose. Ethyl 3,4-di-*O*-isopropylidene-1-thio-β-D-galactopyranoside (**10**) was alkylated with *para*-methoxybenzyl chloride [150] to give compound **16** (70%), which was then hydrolyzed to remove the isopropylidene group and benzylated to obtain compound **18** in 55% (Scheme 2).

2.2.4 *Preparation of 2, the 4'-methoxy analog of 1*

Alcohol **25** was coupled with compound **14** to give compound **26** (71%). Compound **27** was obtained by deacetylation of **26** in sodium methoxide in methanol (95%). Compound **27** was methylated with methyl iodide to give compound **35** (76%). Compound **35** was hydrogenolyzed to give compound **2** in 91% yield (Scheme 3).



Scheme 3. **Synthesis of 2, the 4'-methoxy analog of 1.**

Barton's radical deoxygenation method [151, 152] was used to obtain all the deoxy derivatives. The precusor alcohols were first converted to the thiocarbonyl derivatives using pentafluorophenyl chlorothionoformate with pyridine and dimethyl aminopyridine

in dichloromethane. The thiocarbonates were then treated with tributyltin hydride and azobisisobutyronitrile (AIBN) to obtain the deoxygenated compounds (Figure 31).

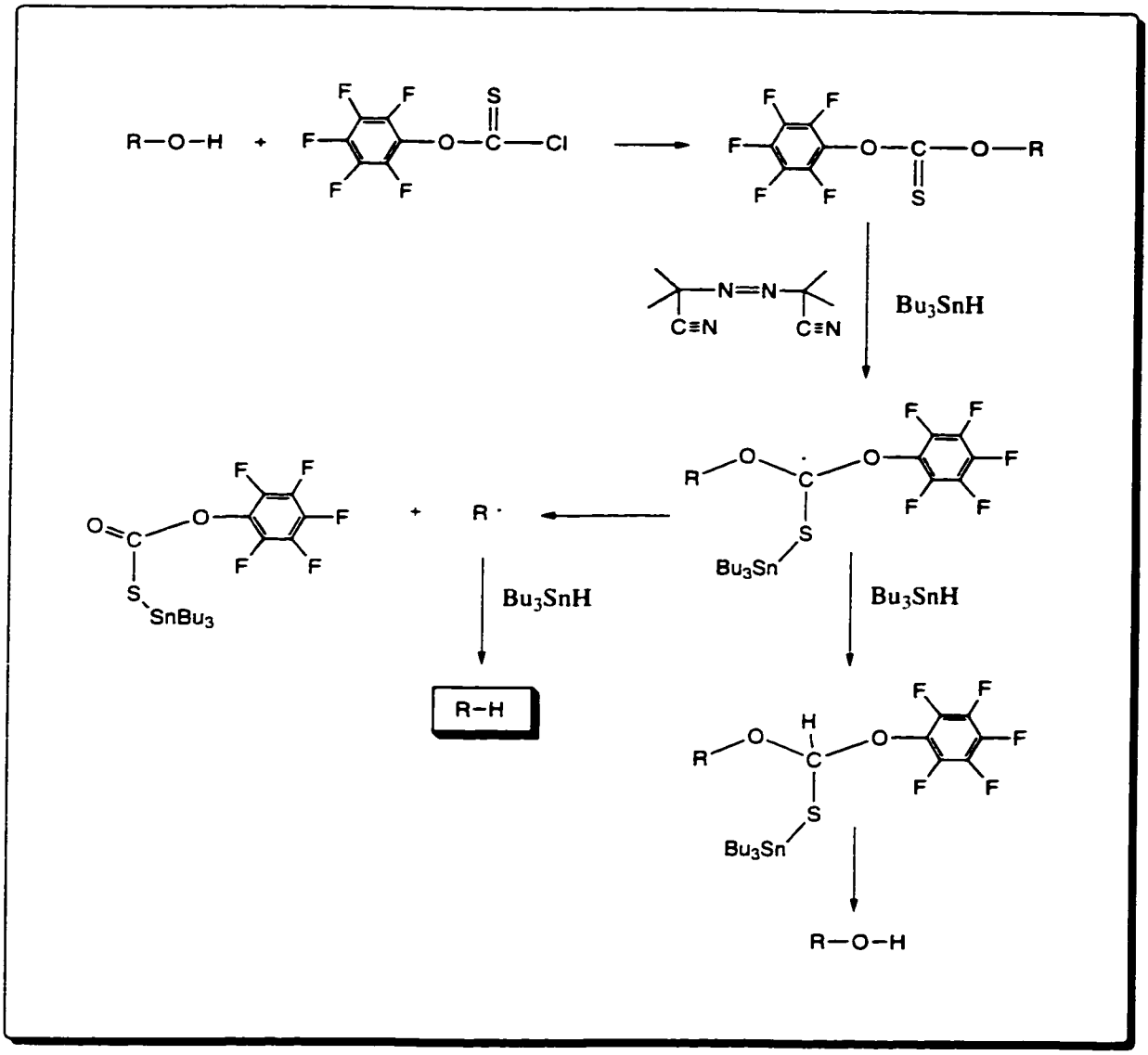
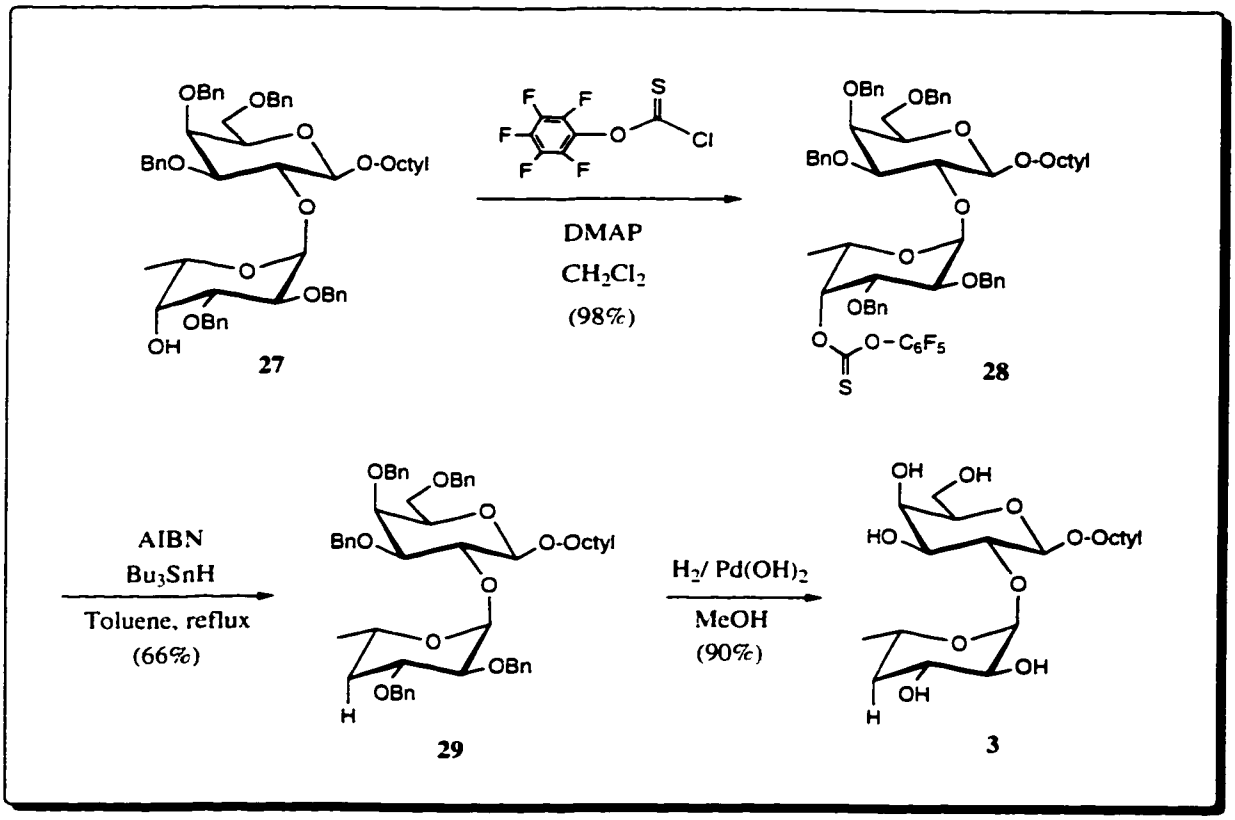


Figure 31. **Mechanism of the Barton deoxygenation reaction.**

2.2.5 *Preparation of 3, the 4'-deoxy analog of 1*

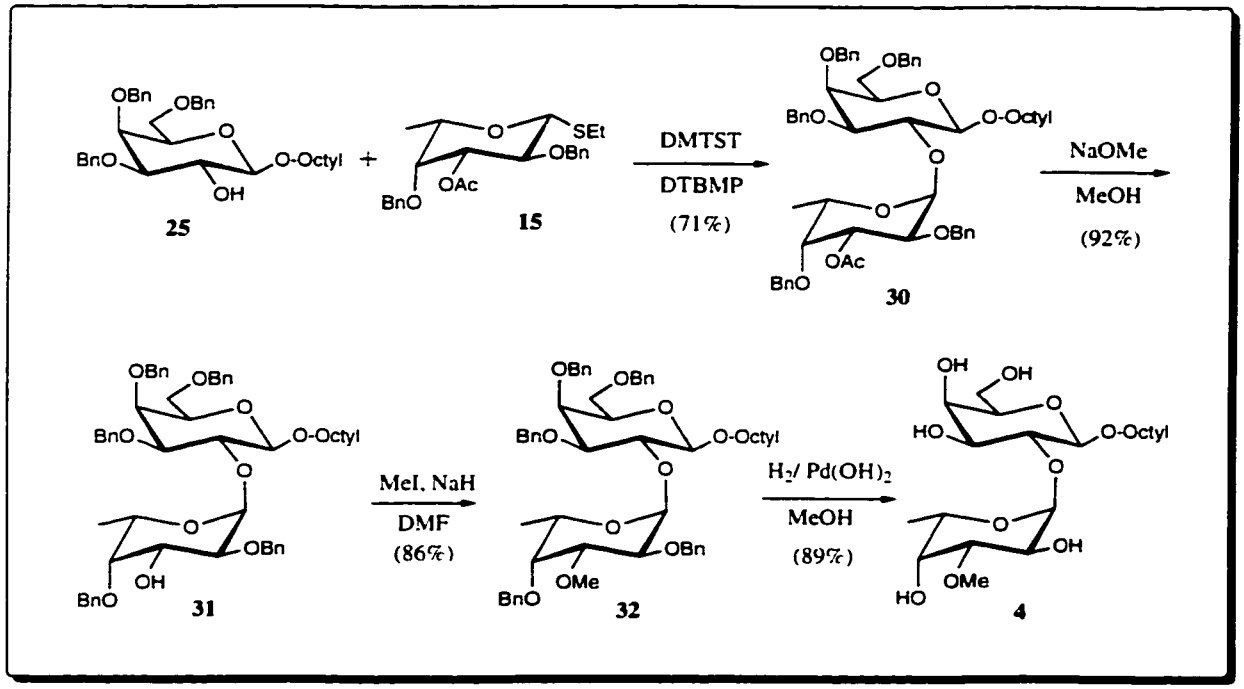
Compound **27** was converted to the pentafluorophenylchlorothiono derivative (**28**) in 98% and deoxygenated with tributyltin hydride [133, 153] to give compound **29** (66%). Debenzylation of this compound gave the disaccharide **3** in 90% (Scheme 4).



Scheme 4. **Synthesis of 3, the 4'-deoxy analog of 1.**

2.2.6 *Preparation of 4 the 3'-methoxy analog of 1*

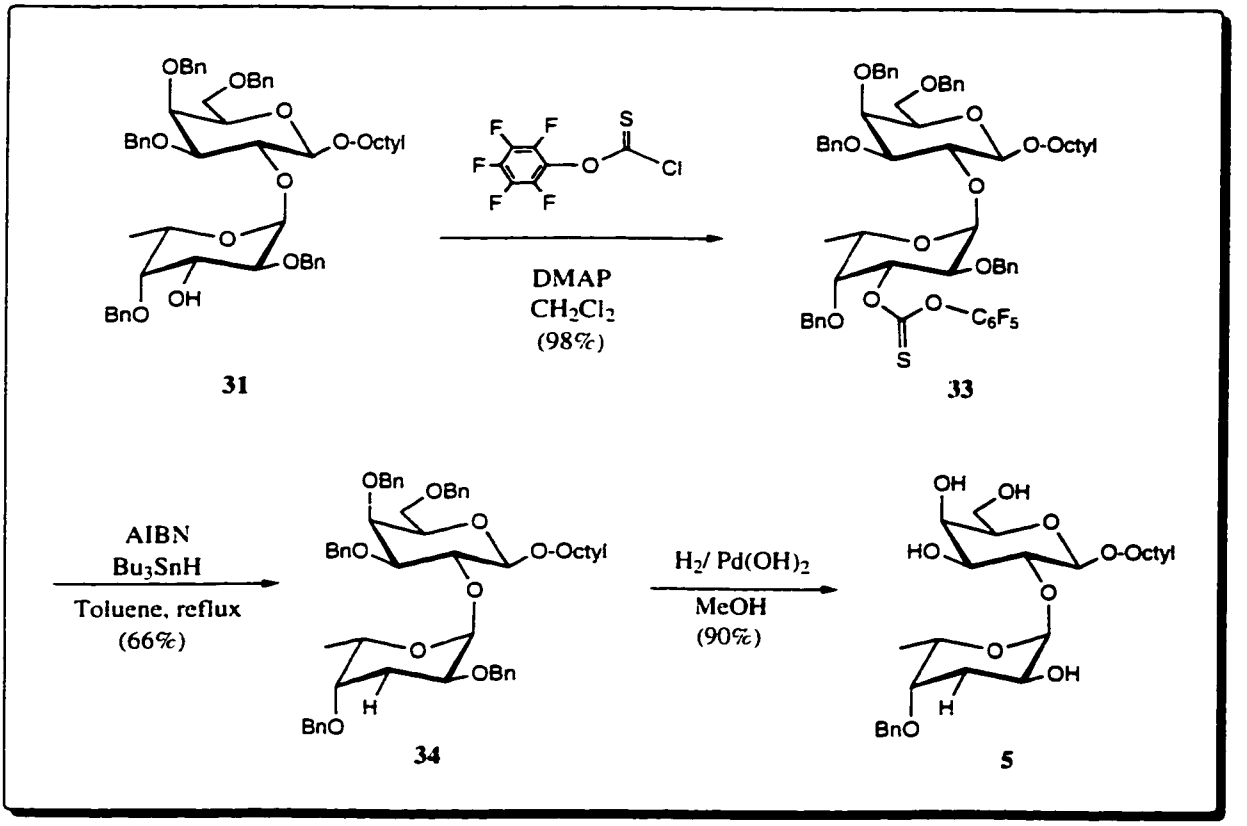
Disaccharide **30** was similarly obtained by coupling alcohol **25** with donor **15** with DMTST (71%). Compound **31** was obtained by Zemplen deacetylation of **30** in sodium methoxide in methanol (92%). Compound **31** was methylated with methyl iodide to give compound **32** (86%). It was debenzylated with hydrogen and palladium on charcoal to give the disaccharide **4** in 89% yield (Scheme 5).



Scheme 5. **Synthesis of 4, the 3'-methoxy analog of 1.**

2.2.7 *Preparation of 5, the 3'-deoxy analog of 1*

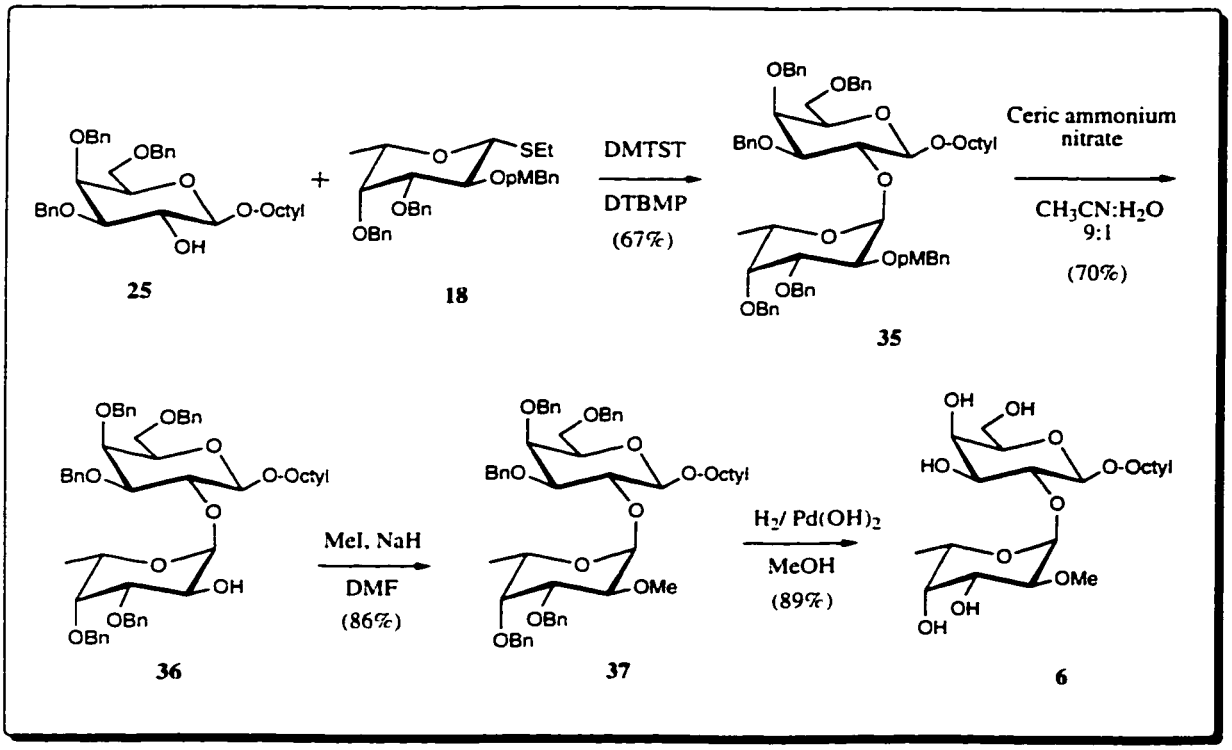
Disaccharide **34** was obtained by converting compound **31** to **33** with pentaflurophenyl chlorothionoformate and reducing it with tributyltin hydride and AIBN (66%). This was followed by hydrogenation to give compound **5** in 90% yield (Scheme 6).



Scheme 6. **Synthesis of 5, the 3'-deoxy analog of 1.**

2.2.8 *Preparation of 6, the 2'-methoxy analog of 1*

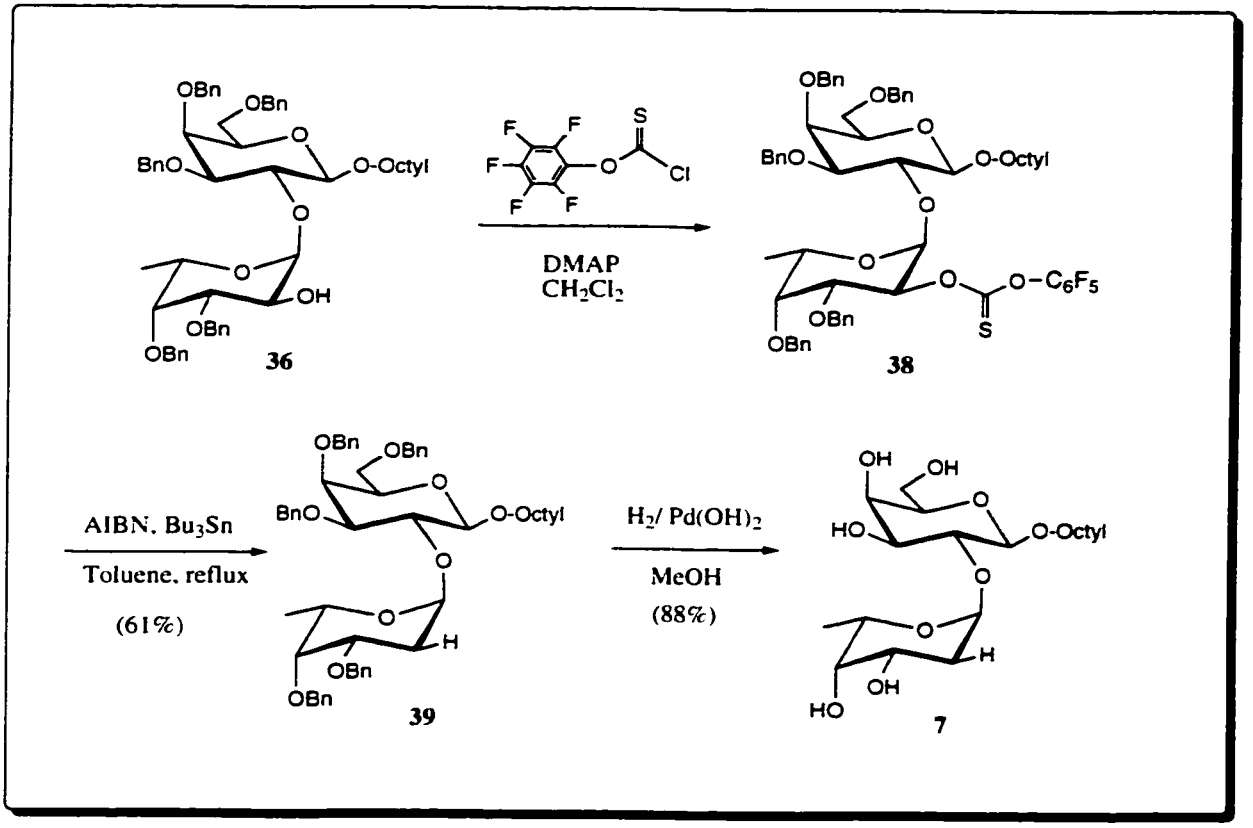
Compounds **25** and **18** were coupled by activating the thioglycoside donor (**18**) with DMTST to yield the disaccharide **35** (67%). Compound **36** was obtained from compound **35** by removing the *para*-methoxybenzyl group with ceric ammonium nitrate (70%) [154]. The hydroxyl group was methylated using methyl iodide and sodium hydride to give the disaccharide **37** (86%). Compound **37** was hydrogenated to give the target compound **6** in 89% yield (Scheme 7).



Scheme 7. **Synthesis of 6, the 2'-methoxy analog of 1.**

2.2.9 *Preparation of 7, the 2'-deoxy analog of 1*

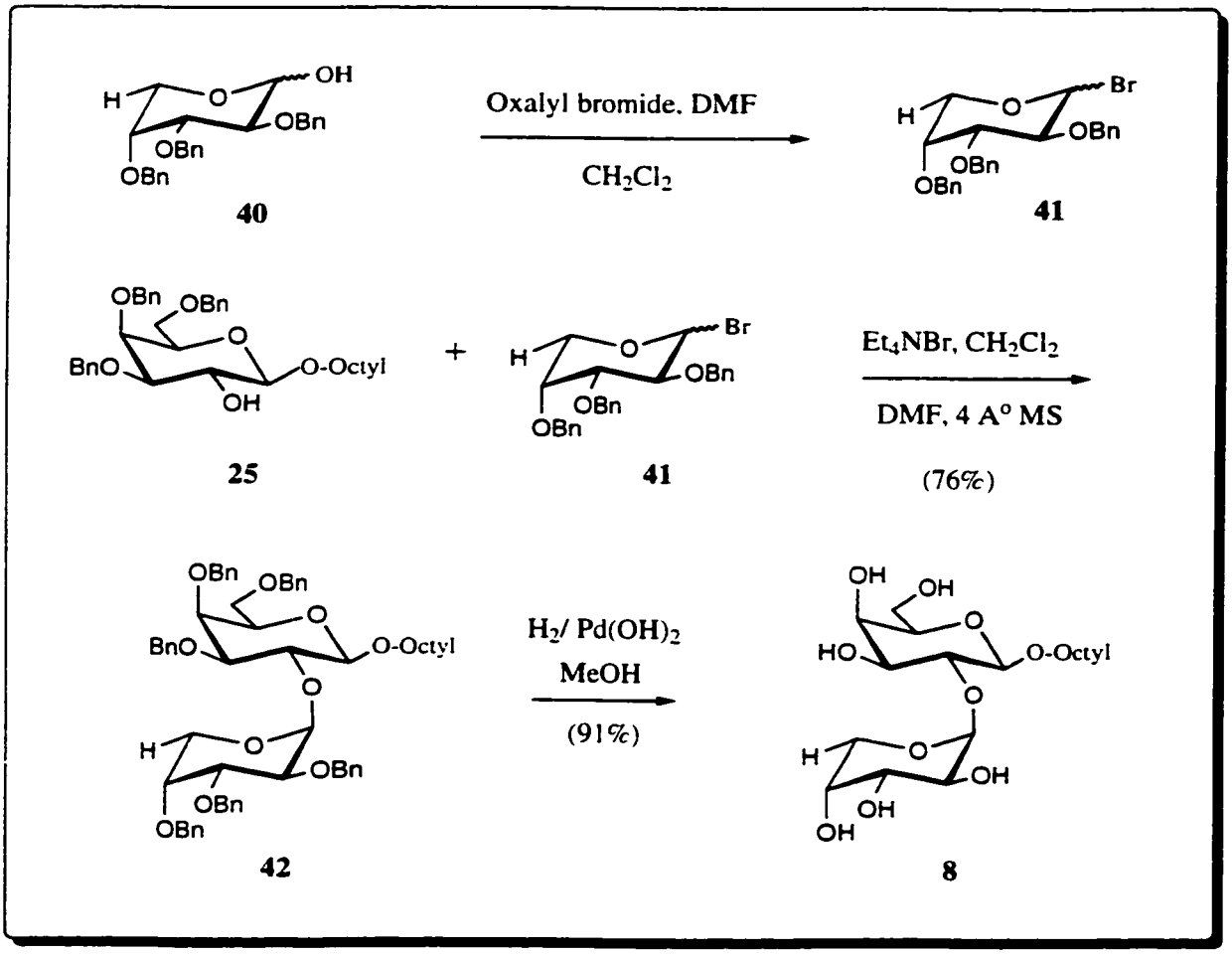
Compound **36** was acylated with pentaflurophenyl chlorothionoformate to give compound **38**. Barton deoxygenation yielded the protected disaccharide **39** in 61% over two steps, which was debenzylated by hydrogenation to give compound **7** in 88% (Scheme 8).



Scheme 8. **Synthesis of 7, the 2'-deoxy analog of 1.**

2.2.10 *Preparation of 8, the arabino-analog of 1*

2, 3, 4-Tri-*O*-benzyl-D-arabinopyranose (**40**) [155] was converted to the corresponding arabinopyranosyl bromide **41** with oxalyl bromide and DMF [147, 156] and this was directly coupled with the alcohol **25** under halide ion catalysis to give compound **42** in 78% yield, which was hydrogenolyzed to give compound **8** in 91% yield (Scheme 9).



Scheme 9. **Synthesis of 8, the *arabino* analog of 1.**

2.3 *Enzymatic assays of the disaccharide analogs*

The modified disaccharides (**2-8**) were screened in radioactive Sep-Pak assays to determine their activities as potential acceptors relative to the natural acceptor (**1**) with the recombinant blood group A and B glycosyltransferases. Compounds that were not acceptors were assayed as inhibitors. The radioactive assay technique used for this purpose quantitates the rate of transfer of radiolabeled Gal or GalNAc from the corresponding sugar nucleotides to the octyl glycosides. The potential acceptors were incubated with the A or the B enzyme and UDP-GalNAc or UDP-Gal respectively in a suitable buffer. The mixtures were then loaded onto a C-18 Sep-Pak cartridge that had been washed with methanol and water. The unreacted donor was washed with water and the products eluted with methanol. The radioactivity of the eluant was determined in a liquid scintillation counter (Figure 29). Percentage activity was calculated as the number of decays per minute (dpm) observed for each compound relative to the native acceptor. The potential inhibitors were similarly treated after incubating with the enzyme, the corresponding donor, and the native acceptor. Percentage inhibition was determined as the relative loss of activity of the native acceptor in the presence of the inhibitor.

2.3.1 *Evaluation of the analogs as potential substrates*

Preliminary screening, the results of which are shown in Table 1, shows that the A transferase can recognize all the disaccharides as acceptors to various extents except the

Table 1. **Relative acceptor activities of disaccharides 1-8 towards cloned blood group A and B glycosyltransferases.**

SUBSTRATE	A transferase (%activity)	B transferase (% activity)
2'-methoxy (**6**)	4%	1%
2'-deoxy (**7**)	66%	6%
3'-methoxy (**4**)	108%	78%
3'-deoxy (**5**)	84%	27%
4'-methoxy (**2**)	86%	11%
4'-deoxy (**3**)	57%	5%
arabino- (**8**)	75%	5%
native disaccharide (**1**)	100%	100%

* UDP-*N*-acetylgalactosamine and UDP-galactose were used as donors for the A and the B enzymes respectively.

** Both donors were used at a fixed (saturation) concentrations.

2'-methoxy derivative (**6**). All the analogs tested were less active than the native disaccharide except the 3'-methoxy derivative. For the B transferase, all the modifications caused loss of activity to some extent. The 2'-hydroxyl group is crucial to the recognition of the acceptor by the A enzyme. When it is replaced by the methoxy group, recognition is lost. This could be in part due to the steric bulk of the methoxy group at the 2- position, which is close to the active site of the enzyme and the position of

transfer of the donor. The lack of reactivity can also be explained in terms of hydrogen bonding, since replacing the OH with an OMe renders it an H-bond acceptor but not a donor.

The 2'-deoxy compound has a relative rate of about two-thirds of that of the natural acceptor, since it is no longer an H-bond donor, but it does not hinder the transfer of the donor, probably due to its minimal steric demand. The A transferase also tolerates modifications on the 3- and the 4- hydroxyl groups of fucose. The 3'-methoxy, 3'-deoxy, and 4'-methoxy compounds have relative rates that are quite close to that of the native acceptor. This probably indicates that the 3- and 4- hydroxyl groups of fucose are not close enough to the active site to be able to perturb it in any significant way.

The 3'-OH is probably not a key polar group since both the methoxy and deoxy analogs are good acceptors. It might be a hydrogen bond acceptor, since the deoxy compound loses some activity compared to the methoxy, but its contribution to the stability of the enzyme substrate complex is unlikely to be significant. The 4'-deoxy compound has a relative rate of half of that of compound **1**, which is lower than that of the methoxy derivative. This could indicate that 4'-OH is also a hydrogen bond acceptor. The *arabino* analog is an acceptor for the A transferase with a high relative rate, thus the methyl group on the fucose is not critical for recognition by the A transferase.

As observed before for the galactose modifications, the B transferase is less amenable to modifications of the hydroxyl groups on fucose as well. The 2'-OH is again crucial for

recognition, and recognition of the acceptor is lost when either modification is made. Thus, it seems likely that apart from strict steric requirements for the groups that are tolerated at this position, the 2'-OH is likely to be both a hydrogen bond acceptor and donor. The 3'-OH is again the least significantly involved in steric interactions in the active site, and since the 3'-methoxy is a good acceptor for the B enzyme, the 3'-OH is probably a hydrogen bond acceptor in the active site. No other analogs are acceptors at all or marginally so. The 4'-OH is also probably involved in hydrogen-bonding interactions in the active site. The 6'-methyl group is required for the acceptor to bind to the B transferase. Thus, although the *arabino* derivative is an acceptor for the A enzyme, it is not recognized by the B enzyme as an acceptor.

The binding constants of the synthetic analogs most often parallel the percent activities of these compounds with the exception of the 4'-methoxy and the 4'-deoxy analogs, which have K_m values of about 1140 μM and 700 μM respectively with the A transferase (Table 2). The K_m values of the other analogs range from 20 μM to 200 μM, which is about 2 to 20 fold higher than the native acceptor (see appendix Figure 54). Not surprisingly, the 3'-methoxy analog has the lowest K_m value of all the modified acceptors. It is not clear, however, why the 4'-methoxy compound is required at such a high concentration for it to bind to the enzyme.

The K_m value for the *arabino* compound (**8**) is about 203 μM, with a relative rate that is about 2.5 times higher than the native disaccharide.

The rates of the reactions in all cases are comparable to that of the natural acceptor. The 4'-methoxy compound has the highest reaction velocity among all the synthesized analogs, while the 3'-deoxy compound reacts at the slowest rate, about 2.5 times and 0.6 times of compound **1** respectively.

Table 2. **Calculated kinetic constants for the modified acceptors with blood group A and B glycosyltransferases.**

SUBSTRATE	A TRANSFERASE		B TRANSFERASE	
	K_m (μM)	V_{max} (nmole/min/μg)	K_m (μM)	V_{max} (nmole/min/μg)
2'-deoxy (**7**)	170±23	3.5±0.17	-	-
3'-methoxy (**4**)	20±3	3.9±0.4	200±25	8.1±0.9
3'-deoxy (**5**)	84±13	1.8±0.09	400±52	1.7±0.06
4'-methoxy (**2**)	1140±182	7.7±0.07	4000±640	2.29±0.16
4'-deoxy (**3**)	722±115	2.6±0.13	-	-
arabino (**8**)	208±6	7.4±0.11	-	-
native disaccharide (**1**)	12.9±1.2	2.9±0.08	110±12	0.24±0.02

The 3'-methoxy and the 3'-deoxy derivatives were the only acceptors for the B transferase with K_m values of about 200 μM and 400 μM respectively. The relative rates

of the three modified acceptors on which kinetic studies could be performed were higher than the native disaccharide. The K_m values of the other compounds could not be determined since they were insoluble at the high concentrations that were required for the assays.

2.3.2 *Evaluation of the analogs as potential inhibitors*

Since the 2'-methoxy derivative was concluded not to be an acceptor for either the A or the B transferase, it was assayed as an inhibitor for the two enzymes. The B enzyme failed to recognize it as an inhibitor. The compound showed a calculated K_i value of about 1 mM for the A transferase (Table 3).

Table 3. **Relative inhibition of disaccharide 6 toward cloned blood group A and B glycosyltransferases.**

SUBSTRATE	A transferase		B transferase
	% inhibition	K_i (mM)	% inhibition
2'-OMe (**6**)	34%	1.03	0%

2.3.3 *Possible interactions around the hydroxyl groups on fucose*

None of the modifications made on the disaccharide resulted in the loss of the acceptor property, that is, the 3-OH remained active. Therefore, the loss of or modification in the activities of the analogs was a result of the perturbation of the interactions around the site of transfer in the active site. Molecular modeling of the native disaccharide shows that the 2'-OH is in close proximity to the 3-OH group, thus, it has the most significant effect on the transfer reaction.

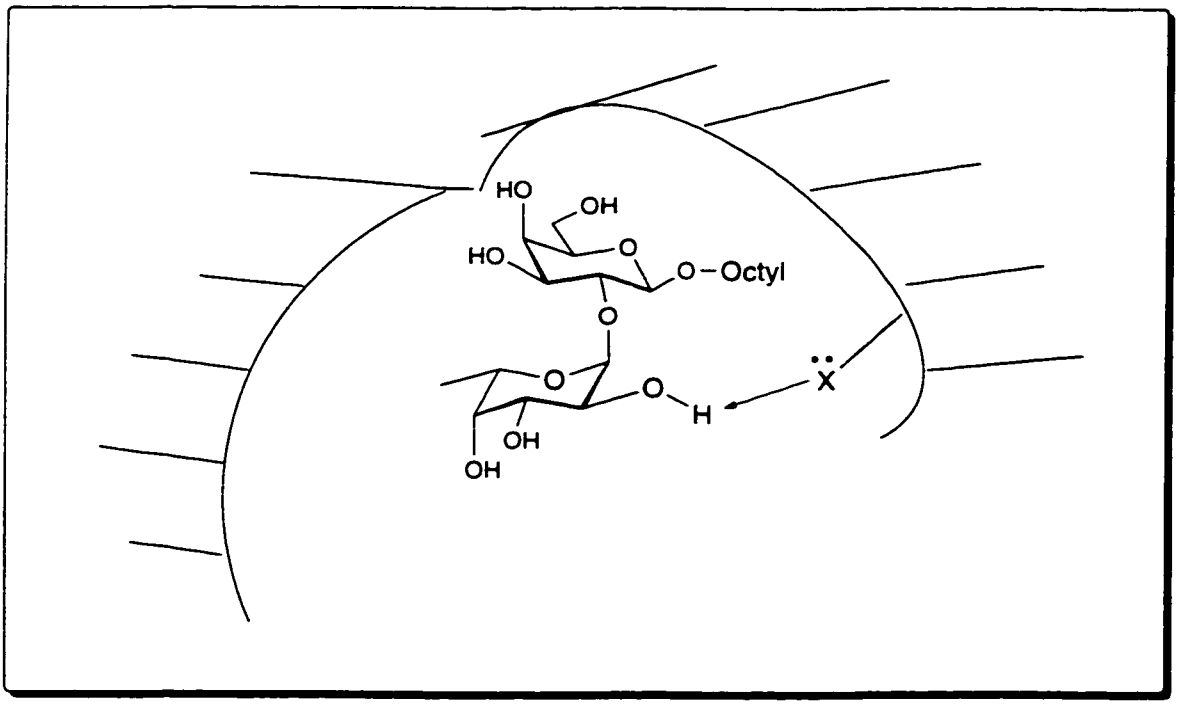


Figure 32. **Possible interactions around Fuc 2-OH.**

Since the 2'-deoxy compound is not completely inactive as an acceptor, we might postulate the presence of an amino acid residue in the active site that does not favor the presence of a bulky group at that site. It is also possible that the 2'-OH is adjacent to a basic group to which it is hydrogen bonded. This could also explain the loss of activity on going from the deoxy to the methoxy analog (Figure 32).

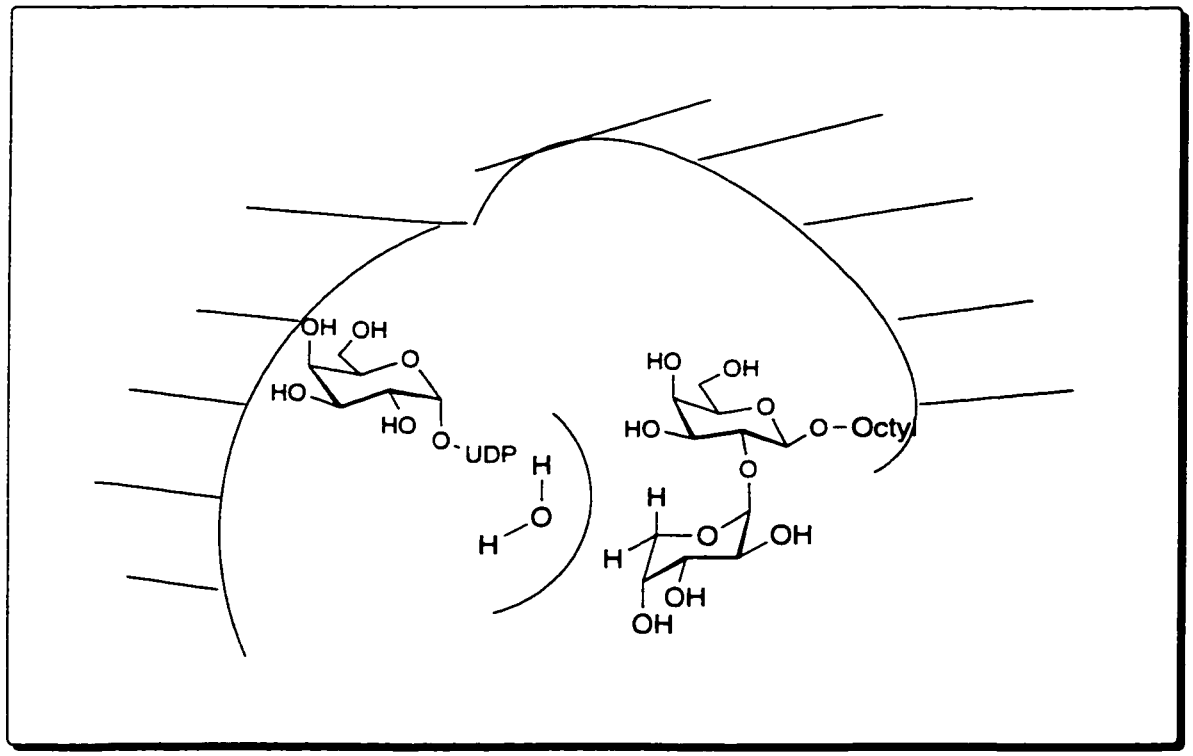


Figure 33. **Hypothetical interactions around Ara C-5.**

The *arabino* derivative was distinguished by the two enzymes and thus the enzymes demonstrate acceptor specificity. It can be postulated that the C-6' methyl group is probably involved in Van der Waals and/or hydrophobic interactions with hydrophobic amino acid residues in the active site of this enzyme (Figure 33). Absence of the methyl

group might result in the loss of such interactions or cause unfavorable interactions by allowing a water molecule to be hydrogen bonded to the donor or to a hydrophilic amino acid.

To summarize the results, the modifications on the Fuc residue show that an intact 2'-OH is necessary for recognition by the enzymes, particularly by the B enzyme. Modifications on the 3'-OH are tolerated by both enzymes, more so by the A enzyme. 4'-OH and C-6' are crucial for recognition by B, but modifications are tolerated well by the A enzyme. Thus, a fair amount of flexibility was observed in recognition of the Fuc residue by the two enzymes. The two enzymes recognized the *arabino* and the 4'-deoxy compounds differentially as acceptors. Therefore, the acceptor specificity of the enzymes was shown to be determined by their amino acid sequence

2.4 *Experimental*

2.4.1 *General methods*

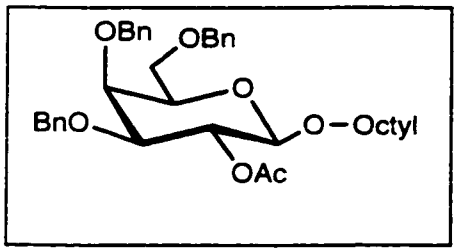
Analytical TLC was performed on Silica Gel 60-F_{254} (E. Merck, Darmstadt) with detection by quenching of fluorescence (aromatic compounds), charring with 5% sulfuric acid or ninhydrin. Unless otherwise noted, column chromatography was performed on Silica Gel 60 (E. Merck). Iatrobeads was from Iatron Laboratories Inc. (Japan). Millex-GV (0.22μm) filters were from Millipore (Missisauga, ON), C-18 Sep-Pak (reverse

phase) cartridges were from Waters Associates (Missisauga, ON). All commercial reagents were used as supplied and all chromatography solvents were distilled prior to use. Ecolite scintillation cocktail was from ICN Radiochemicals (St. Louis, MO) UDP-[6-^{3}H]-Gal (specific activity 1Ci/mmole) and UDP-[6-^{3}H]-GalNAc (specific activity 1Ci/mmole) were from American Radiolabelled Chemicals (St. Louis, MO). UDP-Gal and UDP-GalNac were from Sigma. Optical rotation measurements were done on a Perkin-Elmer 241 polarimeter at 22 ± 2 °C. ^{1}H NMR spectra were recorded at 360 megahertz (MHz) on a Bruker WM-360, 500 MHz (Varian unity 500) or 300 MHz (Varian i300) in solutions of $CDCl_3$ (proton chemical shifts referenced to residual proton signal of chloroform at δ 7.26), CD_3OD (residual proton signal of CD_2HOD at δ 3.30) or D_2O (DOH at δ 4.85 at 22 °C or referenced to external acetone at δ 2.225). ^{13}C NMR spectra were recorded at 75 MHz (Bruker AM-300), or 125 MHz (Varian unity 500) in $CDCl_3$ (δ 77.07), CD_3OD (δ 49.0) or D_2O (external acetone at δ 31.07). Fast atom bombardment (FAB) mass spectra were recorded on a Kratos AEIMS9 instrument with Xe as the bombarding gas and glycerol as the matrix. Electrospray ionization mass spectra (performed either in positive or negative ion mode) were recorded on a Zabspec Hybrid Sector-Time of Flight instrument from Micromass (Manchester, UK). The liquid carrier was infused into the electrospray source by means of a Harvard syringe pump at a flow rate of 10 μL/minute. The sample solution was introduced via a 1μL-loop-injector. Pre-purified nitrogen was used as a spray pneumatic aid and filtered air as the bath gas, heated at ca. 80 °C. For low resolution, the mass spectra were acquired by magnet scan at a rate of 5 seconds/decade. For exact mass measurements, the spectra were obtained by voltage scan over a narrow mass range. Data acquisition and processing was done using

the OPUS software package on a Digital Alpha station with VMS operating system. Elemental analyses were performed on a Carlo Erba EA1108 instrument.

2.4.2 *Synthesis of the modified acceptors*

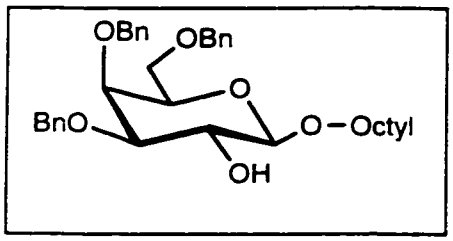
Octyl 2-*O*-acetyl-3,4,6,-tri-*O*-benzyl-β-D-galactopyranoside (**24**).



2-*O*-Acetyl-3,4.6,-tri-*O*-benzyl-β-D-galactopyranosyl bromide (13 g; 23.4 mmoles) was dissolved in 100 ml acetonitrile, and 3Å molecular sieves (powdered, 12 g), and octanol (11 ml; 70.2 mmoles) were added and the mixture stirred under argon for 20 minutes. $Hg(CN)_2$ (8.3 g; 32.8 mmoles) and $HgBr_2$ (catalytic amount) were added and the reaction mixture was stirred overnight. The mixture was diluted with three times its volume of CH_2Cl_2 and filtered over Celite. The filtrate was collected and washed successively with water, aqueous 8% KI and aqueous $NaHCO_3$. Drying (Na_2SO_4) and concentration yielded a syrup which was chromatographed (pentane-EtOAc 15:1, 10:1, 8:1, 5:1) to yield **24** (8.8 gm, 74%) as a white solid. $[\alpha]_D = +6.1°$ (c 3.3 $CHCl_3$); R_f 0.45 (3:1 hexane-EtOAc). ^1H NMR ($CDCl_3$): δ 7.2-7.4 (m, 15 H, ArC*H*), 5.35 (dd, 1 H, $J_{2,3}$ 10.0 Hz, $J_{1,2}$ 8.0 Hz, H-2), 4.94, 4.67, 4.59, 4.51, 4.46, 4.41 (d, 1 H, J_{gem} 11.5 Hz, PhCH_2), 4.32 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 3.94 (d, 1 H, $J_{3,4}$ 2.8 Hz, $J_{4,5}$ 0.8 Hz, H-4), 3.83 (dt, 1 H, J_{gem} 9.8 Hz, J_{vic} 6.9 Hz, CH_2O), 3.53-3.65 (m, 3 H, H-5, H-6b, H-6b), 3.50 (dd, 1 H, $J_{2,3}$ 10.0 Hz, $J_{3,4}$ 2.8 Hz, H-3), 3.39 (dt, 1 H, J_{gem} 9.8 Hz, J_{vic} 6.9 Hz, CH_2O), 2.02 (s, 3 H, CH_3CO), 1.52 (m, 2 H,

C*H*$_2$CH$_2$O), 1.25 (bs, 10 H, C*H*$_2$ octyl), 0.87 (t, 3 H, C*H*$_3$ octyl); ^{13}C NMR ($CDCl_3$) δ: 169.5 (*C*OCH$_3$), 138.5, 138.0, 137.9, (Ar*C*), 128.4, 128.3, 128.2, 127.9, 127.8, 127.7, 127.5, 127.4, (Ar*C*H), 101.4 (C-1), 80.4 (C-2), 74.7, 74.4, 73.7, 73.6, 72.6, 71.9, 71.5, 69.4, 68.7, (C-3, C-4, C-5, C-6, *C*H$_2$O x5), 31.8, 29.5, 29.4, 29.3, 25.9, 22.6 (*C*H$_2$ octyl), 21.0 (*C*H$_3$CO), 14.4 (*C*H$_3$ octyl). Anal. Calc. for $C_{37}H_{48}O_7$ (604.75): C, 73.53; H, 8.00. Found: C, 73.53; H, 8.15.

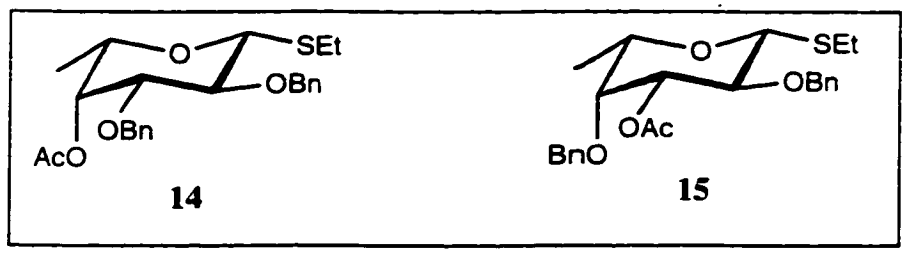
Octyl 3,4,6,-tri-*O*-benzyl-β-D-galactopyranoside (**25**).



Compound **24** (8.8 g; 14.5 mmoles) was deacetylated with sodium methoxide (0.02 M) in dry methanol (Zemplen deacetylation) to yield compound **25** as a white solid (8.8 g, 74%). $[\alpha]_D$ = -0.8° (c 1.6 $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.2-7.4 (m, 15 H, ArC*H*), 4.89, 4.73, 4.68, 4.61, 4.49, 4.43 (d, 1 H, J_{gem} 11.5 Hz, PhC*H*$_2$), 4.22 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 3.95 (dd, 1 H, $J_{1,2}$ 7.5 Hz, $J_{2,3}$ 10.0 Hz, H-2), 3.92 (d, 1 H, $J_{3,4}$ 2.8 Hz, H-4), 3.86 (dt, 1 H, J_{gem} 9.8 Hz, J_{vic} 6.9 Hz, C*H*$_2$O), 3.55-3.63 (m, 3 H, H-5, H-6a, H-6b), 3.48 (m, 1 H, C*H*$_2$O), 3.44 (dd, 1 H, $J_{2,3}$ 10.0 Hz, $J_{3,4}$ 2.8 Hz, H-3), 2.25 (d, 1 H, $J_{H\text{-}2,OH}$ 1.8 Hz, OH), 1.61 (m, 2 H, C*H*$_2$CH$_2$O), 1.28 (bs, 10 H, C*H*$_2$ octyl), 0.89 (t, 3 H, C*H*$_3$ octyl); ^{13}C NMR ($CDCl_3$) δ: 169.7 (*C*OCH$_3$), 138.2, 137.8, 137.6 (Ar*C*), 128.4, 128.5, 128.2, 128.0, 127.9, 127.8, 127.7, 127.5, 127.4, 127.3 (Ar*C*H), 102.0 (C-1), 80.2 (C-2), 74.6, 74.4, 73.6, 72.7, 72.1, 69.1, 68.0, 62.3, 56.6 (C-3, C-4, C-5, C-6, *C*H$_2$O x5), 21.05 (*C*H$_3$CO), 33.0, 31.4, 30.6, 30.4, 27.3, 23.7 (*C*H$_2$ octyl), 16.8 (C-6'), 14.4 (*C*H$_3$ octyl). ESMS for $C_{37}H_{52}O_{13}N_2Na$: Cald. Mass 585.336710; found

585.336560. Anal. Calc. for $C_{37}H_{48}O_7$ (604.75): C, 73.53; H, 8.00. Found: C, 73.53; H, 8.15. ESMS for $C_{35}H_{46}O_6Na$: Calc. 585.3192. Found 585.3185.

Ethyl 4-*O*-acetyl-2,3-di-*O*-benzyl-1-thio-α-L-fucopyranoside (**14**) and Ethyl 3-*O*-acetyl-2,3-di-*O*-benzyl-1-thio-α-L-fucopyranoside (**15**).

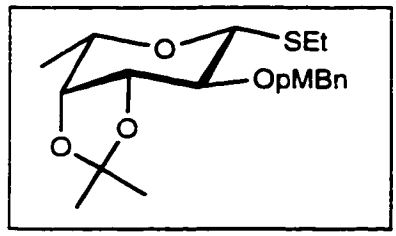


Ethyl 2-*O*-benzyl-1-thio-α-L-fucopyranoside (1.1 g; 3.35 mmole) was dissolved in 60 ml CH_2Cl_2. A 5% solution of aqueous NaOH (5 ml), BnBr (0.63 ml; 50 mmole) and Bu_4NHSO_4 (0.23 g; 0.67 mmole) were added and the mixture was stirred at room temperature for 1 day. More BnBr (0.45 ml) was added and stirring was continued for another 24 hours. After extraction with CH_2Cl_2 and washing with water, drying and evaporation of the solvent yielded a syrup which was chromatographed (pentane-EtOAc 15:1, 10:1, 5:1, 2:1) to yield compound **12** (500 mg) and **13** (600 mg) in a combined yield of 88%. Each of the two compounds was acetylated without characterization to give the required **14** and **15** respectively. Data for compound **14**: $[\alpha]_D$ = -28.8° (c 0.4 $CHCl_3$). ^1H NMR ($CDCl_3$): δ 7.2-7.4 (m, 10 H, ArC*H*), 5.38 (d, 1 H, $J_{3,4}$ 2.5 Hz, H-4) 4.81, 4.75, 4.72, 4.50 (d, 1 H, J_{gem} 11.5 Hz, PhCH_2), 4.42 (d, 1 H, $J_{1,2}$ 9.0 Hz, H-1), 3.64 (q, 1 H, $J_{5,6}$ 6.5 Hz, H-5), 3.53-3.61 (m, 2H, H-2, H-3), 2.7-2.8 (m, 2 H, SCH_2), 2.19 (s, 3 H, CH_3CO), 1.30 (t, 3 H, J_{vic} 7.5 Hz, SCH_2CH_3), 1.20 (d, 3H, $J_{5,6}$ 6.5 Hz, H-6); ^{13}C NMR ($CDCl_3$): δ 170.9 (*C*OCH_3), 138.3, 137.8 (Ar*C*), 128.4, 128.3, 128.1, 128.0 (Ar*C*H), 85.0

(C-1), 81.5, 75.8, 73.0, 71.8, 69.9 (C-3, C-4, C-5, CH_2O), 24.9, 21.0 (CH_3CO), 16.7 (C-6), 15.06 (SCH_2CH_3). Anal. Calc. for $C_{24}H_{30}O_5S$ (430.56): C, 66.95; H, 7.02. Found: C, 67.33; H, 6.92. ESMS for $C_{24}H_{30}O_5SNa$: Calc. 453.1711. Found 453.1718.

Data for compound **15**: $[\alpha]_D$ = -42.4° (c 0.4 $CHCl_3$). ^1H NMR ($CDCl_3$): δ 7.20-7.38 (m, 10 H, ArC*H*), 4.89 (dd, 1 H, $J_{2,3}$ 9.8 Hz , $J_{3,4}$ 3.0 Hz, H-3) 4.87 (d, 1 H, J_{gem} 11.5 Hz, PhCH_2), 4.57-4.67 (m, 3H, PhCH_2x 3), 4.43 (d, 1 H, $J_{1,2}$ 9.8 Hz, H-1), 3.79 (t, 1H, J 9.8 Hz, H-2), 4.89 (dd, 1 H, $J_{3,4}$ 3.0 Hz , $J_{4,5}$ 0.8 Hz, H-3), 3.60 (dq, 1 H, $J_{5,6}$ 6.5 Hz, $J_{4,5}$ 0.8 Hz, H-5), 2.7-2.8 (m, 2 H, SCH_2), 1.89 (s, 3 H, CH_3CO), 1.38 (t, 3 H, J_{vic} 7.5 Hz, SCH_2CH_3), 1.22 (d, 3H, $J_{5,6}$ 6.5 Hz, H-6); ^{13}C NMR ($CDCl_3$): δ 170.3 ($COCH_3$), 138.1, 138.0 (Ar*C*), 128.1, 128.1, 128.1, 129.1, 128.0, 127.9, 127.9, 127.8, 127.8, 127.67 (Ar*C*H), 84.8 (C-1), 77.4, 76.4, 76.2, 75.2, 74.1, (C-3, C-4, C-5, CH_2O), 24.7, 21.7 (CH_3CO), 16.7 (C-6), 14.8 (SCH_2CH_3). Anal. Calc. for $C_{24}H_{30}O_5S$ (430.56): C, 66.95; H, 7.02. Found: C, 66.99; H, 7.02. ESMS for $C_{24}H_{30}O_5SNa$: Calc. 453.171166. Found 453.171697.

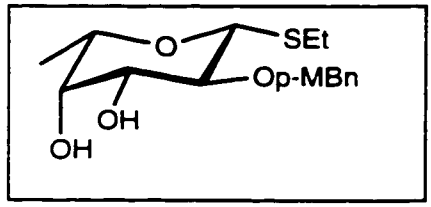
Ethyl 3,4-*O*-isopropylidene-2-*O*-paramethoxybenzyl-1-thio-β-L-fucopyranoside (**16**)



Ethyl 3,4-*O*-isopropylidene-1-thio-β-L-fucopyranoside (300 mg; 1.2 mmole) was dissolved in DMF (4 ml) and 0.115 g of 50% NaH was added and the mixture stirred in a cold water bath for 10 minutes. *para*-Methoxybenzyl chloride (0.325 ml) was added slowly and stirred for another 3 hours. After completion of the

reaction excess NaH was destroyed with MeOH. The reaction mixture was extracted with CH_2Cl_2 and washed with water and dried (Na_2SO_4) and concentrated in *vacuo*. The residue was chromatographed (pentane-ethyl acetate 10:1, 7:1, 5:1) and **16** was obtained (387 mg, 89%). $[\alpha]_D$ = -42.4° (c 0.4 $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.35 (d, 2 H, J_{ortho} 9.0 Hz, ArC*H*), 6.85 (d, 2 H, J_{ortho} 9.0 Hz, ArC*H*), 4.76 (d, 1 H, J_{gem} 11.0 Hz, PhCH_2), 4.67 (d, 1 H, J_{gem} 11.0 Hz, PhCH_2), 4.34 (d, 1 H, $J_{1,2}$ 9.5 Hz, H-1), 4.15 (dd, 1 H, $J_{2,3}$ 6.5 Hz, $J_{3,4}$ 5.5 Hz, H-3), 4.01 (dd, 1 H, $J_{3,4}$ 5.5 Hz , $J_{4,5}$ 2.0 Hz, H-4), 3.78 (dq, 1 H, $J_{5,6}$ 6.5 Hz, $J_{4,5}$ 2.0 Hz, H-5), 3.76 (s, 3 H, CH_3O), 3.39 (dd, 1H, $J_{1,2}$ 9.5Hz, $J_{2,3}$ 6.5 Hz, H-2), 2.60-2.78 (m, 2 H, SCH_2), 1.38, 1.35 (s, 3H, CH_3C) , 1.34 (d, 3H, $J_{5,6}$ 6.5 Hz, H-6), 1.27 (t, 3 H, J_{vic} 7.5 Hz, SCH_2CH_3); ^{13}C NMR ($CDCl_3$): δ 159.2 (Ar*C*OCH_3), 130.1 (Ar*C*), 129.9, 113.6 (Ar*C*H), 109.5 (Me_2C) 83.4 (C-1), 79.8, 78.6, 76.5, 73.1, 72.4, (C-3, C-4, C-5, CH_2O), 55.2 (OCH_3) 28.0, 26.4, 24.4, 16.8 (C-6), 14.8 (SCH_2CH_3). ESMS for $C_{19}H_{28}O_5SNa$: Calc. 391.1555. Found 391.1555.

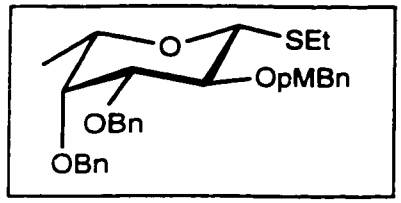
Ethyl 2-*O*-paramethoxybenzyl-1-thio-β-L-fucopyranoside (**17**).



Compound **16** (331 mg; 0.89 mmole) was dissolved in aqueous 50% HOAc (20 ml) and heated with stirring at 60 °C for 2 hours. After completion of reaction, the solvent was evaporated and subsequently co-evaporated with toluene to dryness to give a syrup. It was chromatographed with pentane-ethyl acetate (2:1) to give compound **17**. $[\alpha]_D$ = -11.2° (c 0.4 $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.32 (d, 2 H, J_{ortho} 9.0 Hz, ArC*H*), 6.88 (d, 2 H, J_{ortho} 9.0 Hz, ArC*H*), 4.88, 4.60 (d, 1 H, J_{gem} 10.5 Hz, PhCH_2), 4.36 (d, 1 H, $J_{1,2}$

9.5 Hz, H-1), 3.79 (s, 3 H, C*H*$_3$O), 4.71 (bs, 1 H, H-4), 3.58 (m, 2 H, H-3, H-5), 3.41 (t, 1H, J 9.5 Hz, H-2), 2.67-2.82 (m, 2 H, SC*H*$_2$), 2.55, 2.28 (bs, 1 H, OH), 1.31 (t, 3 H, J $_{vic}$ 7.5 Hz, SCH$_2$C*H*$_3$), 1.30 (d, 3H, J $_{5,6}$ 6.5 Hz, H-6); ^{13}C NMR ($CDCl_3$): δ 159.5 (Ar*C*OCH$_3$), 130.2 (Ar*C*), 130.0, 114.0 (Ar*C*H), 84.7 (C-1), 78.4, 75.1, 74.8, 71.7 (C-3, C-4, C-5, *C*H$_2$O), 55.2 (O*C*H$_3$), 24.9, 16.5 (C-6), 14.9 (SCH$_2$*C*H$_3$). ESMS for $C_{16}H_{24}O_5SNa$: Calc. 351.1242. Found 351.1247.

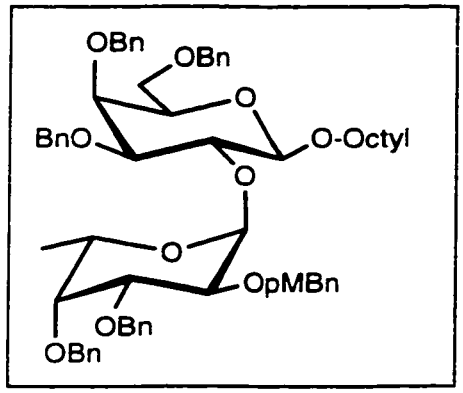
Ethyl 3,4-di-*O*-benzyl-2-*O*-paramethoxybenzyl-1-thio-β-L-fucopyranoside (**18**).



Compound **17** (250 mg; 0.89 mmole) was dissolved in DMF (5 ml), 0.2 g NaH (50% suspension in oil) was added and the mixture stirred for 10 minutes at 0 °C. Benzyl bromide (0.5 ml) was added to it slowly and stirring continued for another 3 hours at room temperature. The reaction mixture was then cooled in an ice-bath and methanol added. It was diluted with CH_2Cl_2 and washed with water. The organic layer was dried (Na_2SO_4) and evaporated *in vacuo* to obtain a syrup which was chromatographed with (pentane-ethylacetate 15:1) to obtain compound **18** (200 mg; 92% from **16**). $[\alpha]_D$ = -1.5° (c 0.4 $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.25-7.45 (m, 12 H, ArC*H*), 6.86 (d, 2 H, ArC*H*), 5.00, 4.82, 4.79, 4.47, 4.73, 4.70 (d, 1 H, J $_{gem}$ 11.5 Hz, PhC*H*$_2$), 4.38 (d, 1 H, J $_{1,2}$ 9.5 Hz, H-1), 3.81 (t, 1H, J 9.5 Hz, H-2), 3.80 (s, 3 H, C*H*$_3$O), 3.61 (dd, 1 H, J $_{3,4}$ 2.5 Hz , J $_{4,5}$ 0.9 Hz, H-4), 3.57 (dd, 1 H, J $_{2,3}$ 9.5 Hz , J $_{3,4}$ 2.5 Hz, H-3), 3.47 (dq, 1 H, J $_{5,6}$ 6.5 Hz, J $_{4,5}$ 0.9 Hz, H-5), 2.82-2.65 (m, 2 H, SC*H*$_2$), 1.35 (t, 3 H, J$_{vic}$ 7.5 Hz, SCH$_2$C*H*$_3$), 1.20 (d, 3H, J$_{5,6}$ 6.5 Hz, H-6); ^{13}C NMR ($CDCl_3$): δ 158.9 (Ar*C*OCH$_3$), 138.4, 138.2, 132.9, 130.3 (Ar*C*),

129.6, 129.1, 128.0, 127.9, 127.8, 127.2, 127.1, 126.5 (Ar*C*H), 84.6 (C-1), 84.1, 77.7, 76.3, 74.9, 74.2, 74.1, 72.4, (C-2, C-3, C-4, C-5, $C H_2O$), 54.8 (OCH_3), 24.2, 16.9 (C-6), 14.7 (SCH_2CH_3). Anal. Calc. for $C_{30}H_{36}O_5S$ (508.65): C, 70.84; H, 7.13. Found: C, 70.51; H, 7.31. ESMS for $C_{30}H_{36}O_5SNa$: Calc. 531.2181. Found 531.2179.

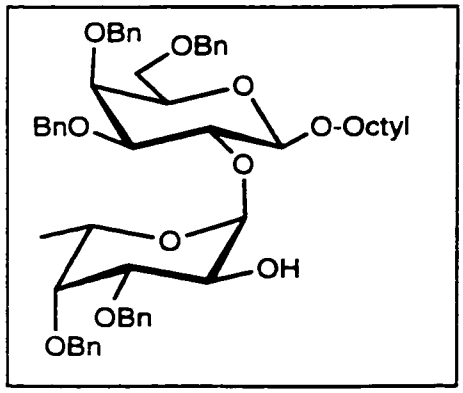
Octyl 3,4,6,-tri-*O*-benzyl-2-*O*-(3,4-di-*O*-benzyl-2-*O*-paramethoxybenzyl-α-L-fucopyranosyl)-β-D-galactopyranoside (**35**).



Compound **25** (140 mg; 0.25 mmole) and compound **18** (230 mg; 0.45 mmole; 1.8 eq) were dissolved in dry CH_2Cl_2 (10 ml) and stirred for one hour with 500 mg 4 Å MS. DMTST (356 mg; 1.38 mmole) and DTBMP (380 mg; 1.84 mmole) were then added and the mixture stirred for another 3 hours. Upon completion of the reaction as determined from the TLC of the reaction mixture, the reaction was quenched with Et_3N, filtered over Celite, and the solvent evaporated. Column chromatography of the syrup thus obtained (Pentane-EtOAc 10:1, 6:1, 4:1) gave **35** (185 mg; 67% isolated yield). $[\alpha]_D$ = -28.8° (c 0.26 $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.2-7.4 (m, 25 H, Ar*H*), 6.95, 6.61 (d, 2 H, J_{ortho} 8.6 Hz, Ar*H*), 5.69 (d, 1 H, $J_{1',2'}$ 3.56 Hz, H-1'), 4.96, 4.85, 4.82, 4.77, 4.73, 4.66 (d, 1 H, J_{gem} 11.6 Hz, PhCH_2), 4.64, 4.54, 4.53 (d, 1 H, J_{gem} 11.5 Hz, PhCH_2), 4.47 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.46 (s, 2 H, PhCH_2), 4.25 (dd, 1 H, $J_{2,3}$ 9.6 Hz, $J_{1,2}$ 7.8 Hz, H-2), 4.04 (dd, 1 H, $J_{2',3'}$ 10.2 Hz, , $J_{1',2'}$ 3.6 Hz, H-2'), 3.95-3.99 (m, 2 H, H-5', H-6a), 3.90 (dd, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 3.76 (dd, 1 H, $J_{2,3}$ 9.6 Hz, $J_{3,4}$ 2.68

Hz, H-3), 3.72 (s, 3 H, OCH_3), 3.55-3.67 (m, 5 H, H-4, H-5, H-6b, H-3', H-4'), 3.35-3.43 (m, 1 H, CH_2O), 1.53 (m, 2 H, CH_2CH$_2$O), 1.25 (bs, 10 H, CH_2 octyl), 1.14 (d, 2 H, $J_{5'6'}$ 6.5 Hz, H-6'), 0.90 (t, 3 H, J_{vic} 7.0 Hz, CH_3 octyl); ^{13}C NMR ($CDCl_3$): δ 158.9 (Ar*C*-OMe), 139.0, 138.8, 138.4, 138.1, 137.9, 130.3, 129.4 x2, 128.3, 128.2, 128.1, 127.9, 127.8, 127.5, 127.3, 126.4, 113.4 (Ar*C*), 102.1 (C-1), 97.2 (C-1'), 84.5, 79.7, 78.1, 75.1, 74.7, 74.3, 73.6, 73.3, 72.9, 72.3, 72.1, 72.0, 71.3, 69.7, 68.9, 66.1 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', *C*H$_2$O), 55.1 (*C*H$_3$O), 31.8, 29.7, 29.5, 29.3, 26.3, 22.6 (*C*H$_2$ octyl), 16.5 (C-6'), 14.1 (*C*H$_3$ octyl). ESMS for $C_{63}H_{76}O_{11}Na$: Calc. 1031.5285. Found 1031.5280.

Octyl 3,4,6-tri-*O*-benzyl-2-*O*-(3,4-di-*O*-benzyl-α-L-fucopyranosyl)-β-D-galacto-pyranoside (**36**).



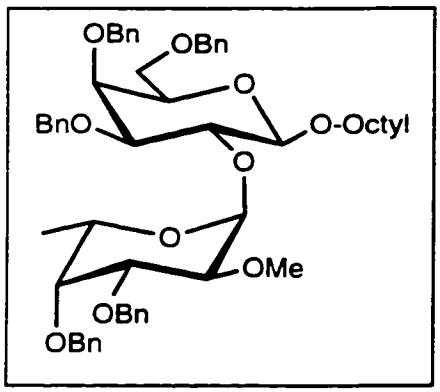
Compound **35** (150 mg; 0.148 mmole) and ceric ammonium nitrate (154 mg; 0.28 mmole) were dissolved in CH_3CN-H_2O (9:1, 10 ml) and the mixture stirred for 30 min. at room temperature. The reaction mixture was diluted with CH_2Cl_2, washed with saturated $NaHCO_3$ and water, dried (Na_2SO_4), filtered and evaporated *in vacuo* to yield a syrup which was chromatographed (pentane-EtOAc 6:1) to give compound **36** (100 mg; 76%). $[\alpha]_D$ = -11.9° (c 1.2 $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.2-7.4 (m, 25 H, Ar*H*), 5.49 (d, 1 H, $J_{1',2'}$ 3.9 Hz, H-1'), 4.93, 4.85, 4.59, 4.56, 4.47, 4.43 (d, 1 H, J_{gem} 11.44 Hz, PhCH_2), 4.69 (s, 2 H, PhCH_2), 4.65, 4.62 (d, 1 H,

J_{gem} 11.0 Hz, PhCH_2), 4.33 (d, 1 H, $J_{1,2}$ 7.77 Hz, H-1), 4.27 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 4.17 (m, 1 H, H-6a), 4.10 (dd, 1 H, $J_{2,3}$ 9.76 Hz, $J_{1,2}$ 7.77 Hz, H-2), 3.95 (bd, 1 H, $J_{3,4}$ 2.4 Hz, H-4), 3.86 (dd, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 3.70 (dd, 1 H, $J_{2',3'}$ 9.9 Hz, $J_{3',4'}$ 2.7 Hz, H-3'), 3.53-3.63 (m, 5 H, H-2', H-3, H-5, H-6b, H-4'), 3.40 (dd, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 2.44 (d, 1 H, $J_{OH,H-2'}$ 7.4 Hz, OH), 1.56 (m, 2 H, CH_2CH_2O), 1.27 (bs, 10 H, CH_2 octyl), 1.14 (d, 2 H, $J_{5'6'}$ 6.5 Hz, H-6'), 0.89 (t, 3 H, J_{vic} 7.0 Hz, CH_3 octyl); ^{13}C NMR ($CDCl_3$): δ 138.8, 138.7, 138.6, 137.6, 137.0, 128.4 x2, 128.3, 128.2, 127.9, 127.8, 127.5 (Ar*C*), 102.3 (C-1), 99.2 (C-1'), 83.7, 80.4, 77.0, 74.7, 74.3, 73.6, 73.4, 72.5, 72.4, 72.2, 69.9, 69.2, 68.2, 66.8 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', CH_2O), 31.8, 29.6, 29.5, 29.3, 26.2, 22.7 (CH_2 octyl), 16.7 (C-6'), 14.1 (CH_3 o

c

tyl). Anal. Calc. for $C_{55}H_{68}O_{10}$ (889.09): C, 74.30; H, 7.71. Found: C, 74.06; H, 7.84.

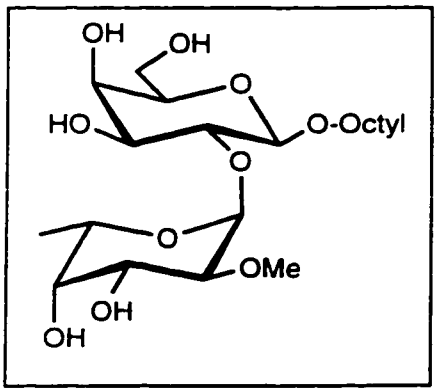
Octyl 3,4,6,-tri-*O*-benzyl-2-*O*-(3,4-di-*O*-benzyl-2-*O*-methyl-α-L-fucopyranosyl)-β-D-galactopyranoside (**37**).



Compound **36** (200 mg; 0.225 mmole) was dissolved in DMF (10 ml) and methyl iodide (0.028 ml; 0.45 mmole) and NaH (50%, 25 mg; 0.9 mmole) were added at 0 °C and the mixture stirred for 4 hours at room temperature. The reaction was quenched with methanol in the cold and the mixture diluted with CH_2Cl_2. It was washed with water, dried (Na_2SO_4) and evaporated. Column chromatography (pentane-EtOAc 10:1,

6:1) gave **37** (176 mg; 87%). $[\alpha]_D$ = -71.8° (c 3.0 $CHCl_3$). ^1H NMR ($CDCl_3$): δ 7.20-7.45 (m, 25 H, Ar*H*), 5.70 (d, 1 H, $J_{1',2'}$ 3.8 Hz, H-1'), 4.94, 4.93, 4.81, 4.78, 4.73, 4.67, 4.61, 4.59, 4.53 (d, 1 H, J_{gem} 11.5 Hz, PhCH_2), 4.44, 4.40 (d, 1 H, J_{gem} 11.8 Hz, PhCH_2), 4.39 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.19 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 4.17 (dd, 1 H, $J_{2,3}$ 9.8 Hz, $J_{1,2}$ 7.8 Hz, H-2), 3.92 (bd, 1 H, $J_{3,4}$ 2.5 Hz, H-4), 3.82-3.85 (m, 2 H, H-3, H-6a), 3.80 (dd, 1 H, $J_{2',3'}$ 10.0 Hz, $J_{1',2'}$ 3.8 Hz, H-2'), 3.72 (bd, 1 H, $J_{2',3'}$ 10.0 Hz, H-3'), 3.67 (bs, 1 H, H-4'), 3.55-3.61 (m, 2 H, H-5, H-6b), 3.52 (dd, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 3.43 (s, 3 H, OCH_3), 3.35 (dd, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 1.49 (m, 2 H, C$H_2$$CH_2$O), 1.22 (bs, 10 H, C$H_2$ octyl), 1.12 (d, 2 H, $J_{5'6'}$ 6.5 Hz, H-6'), 0.85 (t, 3 H, J_{vic} 7.0 Hz, CH_3 octyl); ^{13}C NMR ($CDCl_3$): δ 139.0, 138.8, 138.6, 138.5, 138.0, 128.4, 128.3, 128.2, 128.1, 127.9, 127.8, 127.5, 127.4, 127.3 x2, 126.7 (Ar*C*), 102.0 (C-1), 97.0 (C-1'), 84.5, 78.5, 78.2, 78.0, 77.6, 74.9, 74.3, 73.6, 73.4, 73.0, 72.9, 72.4, 71.8, 69.6, 66.6 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', *C*H_2O), 58.1 (O*C*H_3), 31.8, 29.6, 29.5, 29.3, 26.2, 22.6 (*C*H_2 octyl), 16.7 (C-6'), 14.0 (*C*H_3 octyl). Anal. Calc. for $C_{56}H_{70}O_{10}$ (903.08): C, 74.47; H, 7.81. Found: C, 74.57; H, 7.86.

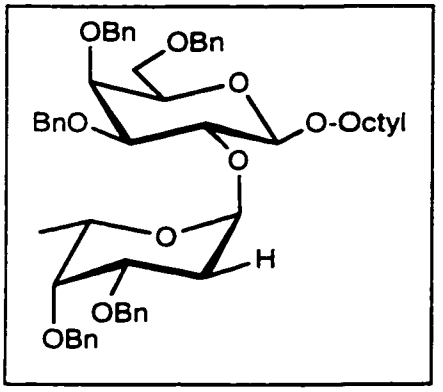
Octyl 2-*O*-(-2-*O*-methyl-α-L-fucopyranosyl)-β-D-galactopyranoside (**6**).



Compound **37** (16 mg; 0.017 mmole) was hydrogenated with hydrogen and palladium on charcoal in methanol, and purified as follows. The solution was filtered to remove the catalyst and the solvent evaporated. The crude was dissolved in water and passed though a C-18

column. It was washed with water and eluted with methanol. The fractions containing the product were pooled and the solvent was again evaporated. The residue was re-dissolved in water, passed though a 0.22 μm Millipore filter and lyophilized to give compound **6** (7 mg; 87%). ^{1}H NMR (CD_3OD): δ 5.49 (d, 1 H, $J_{1',2'}$ 3.8 Hz, H-1'), 4.41 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.35 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 3.86-3.94 (m, 2H, H-2, H-4), 3.68-3.80 (m, 5 H, H-3, H-6a, H-2', H-3', H-4'), 3.52-3.64 (m, 3 H, H-6b, C*H*$_2$O), 3.50 (s, 3 H, OCH_3), 3.45 (dd, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, C*H*$_2$O), 1.57 (m, 2 H, C*H*$_2$CH$_2$O), 1.28 (bs, 10 H, C*H*$_2$ octyl), 1.17 (d, 2 H, $J_{5',6'}$ 6.5 Hz, H-6'), 0.86 (t, 3 H, J_{vic} 7.0 Hz, C*H*$_3$ octyl); ^{13}C NMR (CD_3OD): δ 104.6 (C-1), 100.1 (C-1'), 82.3, 80.3, 76.4, 74.5, 73.9, 72.8, 71.8, 71.0, 69.5, 62.4 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', *C*H$_2$O), 61.0 (O*C*H$_3$), 38.0, 30.8, 30.6, 30.4, 27.3, 23.7 (*C*H$_2$ octyl), 16.6 (C-6'), 14.4 (*C*H$_3$ octyl). ESMS for $C_{21}H_{40}O_{10}Na$: Calc. 475.2519. Found 475.2514.

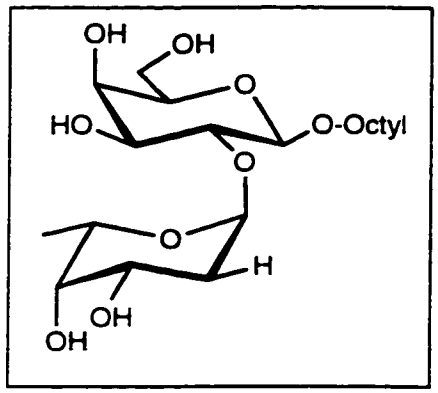
Octyl 3,4,6-tri-*O*-benzyl-2-*O*-(3,4-di-*O*-benzyl-α-L-xylo-hexopyranosyl)-β-D-galactopyranoside (**39**).



Compound **36** (50 mg; 0.09 mmole) was dissolved in CH_2Cl_2 (10 ml) and 4-dimethyl aminopyridine (55 mg; 0.45 mmole) and pentafluorophenylchlorothionoformate (42 μl; 0.26 mmole) were added in cold and stirred overnight at room temperature. After completion of the reaction, the reaction mixture was washed successively with aqueous 5% HCl, saturated aqueous $NaHCO_3$ and water and then dried. The solvent

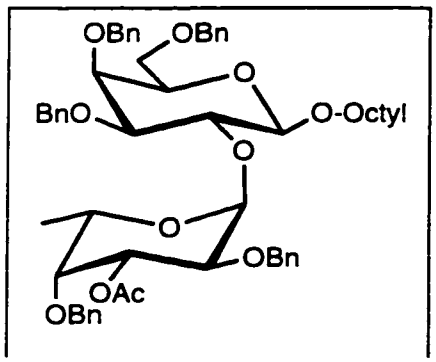
was evaporated *in vacuo* and this was taken to the next step as follows. Tributyltin hydride (39 µl; 0.52 mmole) and azobisisobutyronitrile (AIBN) (42.3 mg; 0.25 mmole) were added to it in toluene and refluxed at 130 °C bath temperature for 4 hours. Solvent was evaporated and chromatographed (pentane-EtOAc 6:1) to give compound **39** (50 mg; 57%). $[\alpha]_D$ = -10.4° (c 0.74 $CHCl_3$). ^1H NMR ($CDCl_3$): δ 7.12-7.40 (m, 25 H, Ar*H*), 5.50 (d, 1 H, $J_{1',2a'}$ 3.7 Hz, H-1'), 4.95, 4.84, 4.68, 4.66, 4.57, 4.49 (d, 1 H, J_{gem} 11.6 Hz, PhCH_2), 4.58, 4.42 (s, 2 H, PhCH_2), 4.24 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.22 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 4.06 (dd, 1 H, $J_{2,3}$ 9.8 Hz, $J_{1,2}$ 7.8 Hz, H-2), 3.92 (bd, 1 H, $J_{3,4}$ 2.6 Hz, H-4),), 3.84 (dd, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 3.59-3.64 (m, 2 H, H-3', H-6a), 3.58 (bs, 1 H, H-4'), 3.53-3.56 (m, 2 H, H-5, H-6b), 3.36 (dd, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 2.09-2.18 (ddd, 1 H, J_{gem} 16.3 Hz, $J_{2'a,3'}$ 12.2 Hz, $J_{2'a,1'}$ 3.7 Hz, H-2'a), 1.89 (m, 1 H, H-2'b), 1.60 (m, 2 H, CH_2CH_2O), 1.25 (bs, 10 H, CH_2 octyl), 1.12 (d, 2 H, $J_{5',6'}$ 6.5 Hz, H-6'), 0.89 (t, 3 H, J_{vic} 6.9 Hz, CH_3 octyl); ^{13}C NMR ($CDCl_3$): δ 139.5, 1349.4, 139.3, 139.2, 138.9, 128.4, 128.3, 128.1, 127.9, 127.8, 127.7, 127.5, 127.2 (Ar*C*), 102.5 (C-1), 98.1 (C-1'), 84.2, 82.7, 77.2, 76.5, 74.4, 74.1, 73.6, 73.5, 72.9, 72.6, 72.1, 70.3, 69.4, 68.9 (C-2, C-3, C-4, C-5, C-6, C-3', C-4', C-5', *C*H_2O), 33.8 (C-2'), 29.5, 29.4, 29.3, 27.3, 26.2, 22.6 (*C*H_2 octyl), 17.2 (C-6'), 14.1 (*C*H_3 octyl). Anal. Calc. for $C_{55}H_{69}O_9$ (873.05): C, 75.66; H, 7.85. Found: C, 74.39; H, 7.88.

Octyl 2-*O*-(α-L-xylo-hexopyranosyl)-β-D-galactopyranoside (**7**).



Compound **39** (25 mg; 0.028 mmole) was hydrogenated as described for **37**. After the usual work-up, it was chromatographed (CH_2Cl_2-MeOH 10:1) and then purified as described for compound **6** to give compound **7** (10 mg; 84%). ^{1}H NMR (CD_3OD): δ 5.38 (dd, 1 H, J_{vic} 2.2, 2.1 Hz, H-1'), 4.31 (q, 1 H, $J_{5',6'}$ 6.6 Hz, H-5'), 4.25 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 3.86-3.96 (m, 2H, H-3', CH_2O), 3.62-3.78 (m, 4 H, H-2, H-4, H-5, H-6a), 3.58 (dd, 1 H, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 3.4 Hz, H-3), 3.44-3.53 (m, 3 H, H-4', H-6b, CH_2O), 1.80-1.88 (m, 2 H, H-2'), 1.52-1.60 (m, 2 H, CH_2CH$_2$O), 1.26-1.36 (m, 10 H, CH_2 octyl), 1.17 (d, 2 H, $J_{5',6'}$ 6.6 Hz, H-6'), 0.88-0.92 (m, 3 H, CH_3 octyl); ^{13}C NMR (CD_3OD): δ 104.1 (C-1), 101.8 (C-1'), 80.6, 76.4, 73.8, 72.1, 71.2, 70.8, 69.9, 69.5, 62.4 (C-2, C-3, C-4, C-5, C-6, C-3', C-4', C-5', CH_2O). 35.2 (C-2'), 31.6, 30.8, 30.5, 30.4, 27.3, 23.7 (CH_2 octyl), 17.0 (C-6'), 14.4 (CH_3 octyl). ESMS for $C_{20}H_{38}O_9Na$: Calc. 445.2413. Found 445.2414.

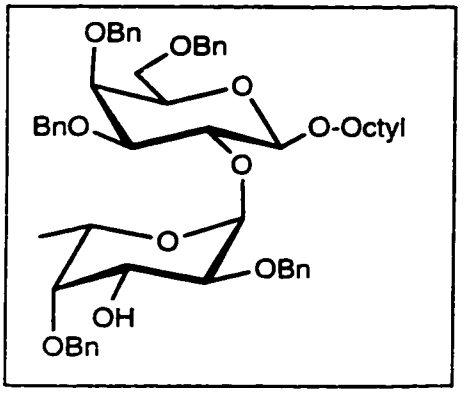
Octyl 3,4,6-tri-*O*-benzyl-2-*O*-(3-*O*-acetyl-2,4-di-*O*-benzyl-α-L-fucopyranosyl)-β-D-galactopyranoside (**30**).



Compound **25** (146 mg; 0.26 mmole) and **15** (200 mg; 0.46 mmole; 18 eq) were dissolved in dry CH_2Cl_2 (10 ml) and the mixture stirred for 1 hour with 500 mg 4 Å

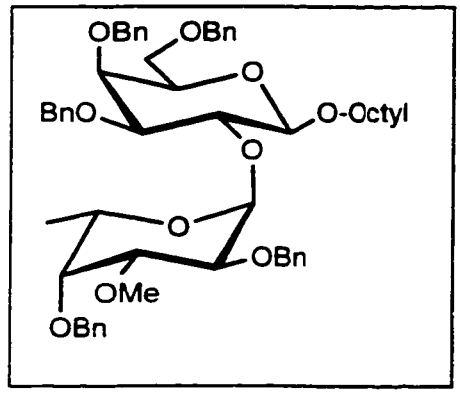
MS. DMTST (356 mg; 1.38 mmole) and DTBMP (380 mg; 1.84 mmole) were then added and stirring continued for another 3 hours. Upon completion, the reaction mixture was filtered over Celite, washed with (Na_2SO_4) and evaporated. Column chromatography (pentane-EtOAc 10:1, 6:1, 4:1) gave compound **30** (200 mg; 82% based on alcohol). $[\alpha]_D$ = -36.8° (c 1.9 $CHCl_3$). ^1H NMR ($CDCl_3$): δ 7.20-7.40 (m, 25 H, Ar*H*), 5.74 (d, 1 H, $J_{1',2'}$ 3.72 Hz, H-1'), 5.31 (dd, 1 H, $J_{2',3'}$ 10.6 Hz, $J_{3',4'}$ 2.8 Hz, H-3'), 4.85, 4.77 (d, 1 H, J_{gem} 11.6 Hz, PhCH_2), 4.40-4.60 (m, 8 H, PhCH_2), 4.22 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1), 4.14 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 4.09 (m, 1 H, H-6a), 4.00 (dd, 1 H, $J_{2',3'}$ 9.5 Hz, $J_{1',2'}$ 3.7 Hz, H-2'), 3.97 (bs, 1 H, H-4),), 3.92 (m, 1 H, CH_2O), 3.76 (bs, 1 H, H-4'), 3.73 (dd, 1 H, $J_{2,3}$ 9.0 Hz, $J_{3,4}$ 2.6 Hz, H-3), 3.68-3.58 (m, 3 H, H-5, H-6b, H-2), 3.52-3.45 (m, 1 H, CH_2O), 2.05(s, 3 H, CH_3CO), 1.60 (m, 2 H, CH_2CH_2O), 1.35 (bs, 10 H, CH_2 octyl), 1.20 (d, 2 H, $J_{5',6'}$ 6.5 Hz, H-6'), 0.95 (t, 3 H, J_{vic} 6.9 Hz, CH_3 octyl); ^{13}C NMR ($CDCl_3$): δ 170.5 (CH$_3$*C*O), 139.2, 139.2, 139.1, 138.8, 138.4, 138.0, 133.3, 128.5, 128.4, 128.3, 128.2, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 127.7 x2, 127.5, 127.4, 127.3, 127.0 (Ar*C*), 103.5 (C-1), 99.1 (C-1'), 81.5, 76.6, 75.9, 75.3, 74.5, 74.4, 73.1, 72.9, 70.4, 69.9, 68.0 (C-2, C-3, C-4, C-5, C-6, C-3', C-4', C-5', *C*H$_2$O), 31.8, 29.5, 29.3, 29.2, 26.0, 22.6 (*C*H$_2$ octyl), 20.9 (C*H*$_3$CO), 16.6 (C-6'), 14.1 (*C*H$_3$ octyl). ESMS for $C_{57}H_{70}O_{11}Na$: Calc. 953.4815. Found 953.4817.

Octyl 3,4,6-tri-*O*-benzyl-2-*O*-(2,4-di-*O*-benzyl-α-L-fucopyranosyl)-β-D-galactopyranoside (**31**).



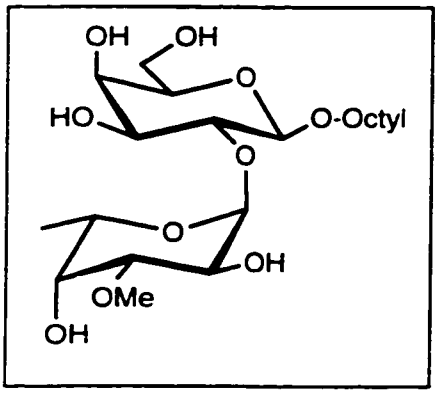
Compound **30** (160 mg; 0.172 mmole) was deacetylated by the Zemplen method and after the usual workup, evaporation of the solvent and column chromatography of the residue (pentane-EtOAc 10:1, 4:1) gave compound **31** (140 mg; 90% from **30**). $[\alpha]_D$ = -19.0° (c 2.4 $CHCl_3$). ^1H NMR ($CDCl_3$): δ 7.18-7.43 (m, 25 H, Ar*H*), 4.97, 4.96,, 4.89, 4.86, 4.69, 4.68, 4.64, 4.57, 4.47, 4.42 (d, 1 H, J_{gem} 11.67 Hz, PhCH_2), 4.83 (d, 1 H, $J_{1',2'}$ 4.03 Hz, H-1'), 4.29 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.22 (dd, 1 H, $J_{2,3}$ 9.1 Hz, $J_{1,2}$ 7.8 Hz, H-2), 3.88 (bd, 1 H, $J_{3,4}$ 2.8 Hz, H-4), 3.82 (m, 1 H, CH_2O), 3.38-3.62 (m, 7 H, H-3, H-5, H-6a, H-6b, H-2', H-3', H-4'), 3.44 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 3.34 (m, 1 H, CH_2O), 2.32 (d, 1 H, $J_{H\text{-}2',OH}$ 4.3 Hz, OH), 1.55 (m, 2 H, C$H_2$$CH_2$O), 1.25 (bs, 10 H, C$H_2$ octyl), 1.22 (d, 2 H, $J_{5',6'}$ 6.4 Hz, H-6'), 0.89 (t, 3 H, J_{vic} 6.9 Hz, CH_3 octyl); ^{13}C NMR ($CDCl_3$): δ 138.6, 138.3, 137.9, 137.8, 128.4, 128.2, 128.1, 128.1, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 127.5, 127.5, 127.3, 127.3, 127.1 (Ar*C*), 103.21 (C-1), 97.1 (C-1'), 81.9, 79.9, 76.0, 75.3, 74.4, 73.7, 73.6, 73.3, 73.0, 72.4, 72.3, 71.8, 71.5, 71.0, 70.4, 69.8, 68.7 (C-2, C-3, C-4, C-5, C-6, C-3', C-4', C-5', *C*H_2O), 31.8, 29.6, 29.4, 29.2, 26.2, 22.6 (*C*H_2 octyl), 16.5 (C-6'), 14.0 (*C*H_3 octyl). ESMS for $C_{55}H_{68}O_{10}Na$: Calc. 911.4710. Found 911.4707.

Octyl 3,4,6-tri-*O*-benzyl-2-*O*-(2,4-di-*O*-benzyl-3-*O*-methyl-α-L-fucopyranosyl)-β-D-galactopyranoside (**32**).



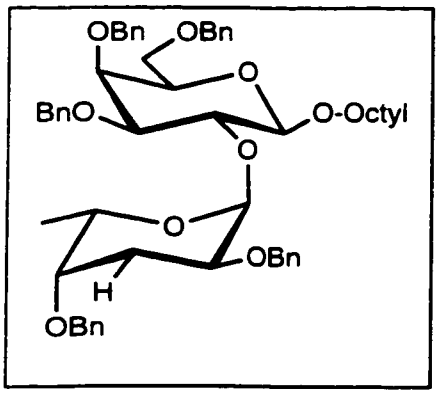
Compound **31** (160 mg; 0.172 mmole) was methylated with MeI (25 ml; 0.4 mmole) and NaH (13 mg; 0.45 mmole) in DMF. Work-up and column chromatography (pentane-EtOAc 10:1, 4:1) gave compound **32** (140 mg; 90% from **31**). $[\alpha]_D$ = -8.5° (c 2.8 $CHCl_3$). ^1H NMR ($CDCl_3$): δ 7.15-7.47 (m, 25 H, Ar*H*), 5.69 (d, 1 H, $J_{1',2'}$ 3.7 Hz, H-1'), 4.95, 4.82, 4.76, 4.64, 4.61, 4.57 (d, 1 H, J_{gem} 11.5 Hz, PhCH_2), 4.54, 4.46 (s, 2 H, PhCH_2), 4.45 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.44 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 4.24 (dd, 1 H, $J_{2,3}$ 10.0 Hz, $J_{1,2}$ 7.8 Hz, H-2), 3.98 (dd, 1 H, $J_{2',3'}$ 9.5 Hz, $J_{1',2'}$ 3.7 Hz, H-2'), 3.96 (bs, 1 H, H-4), 3.92 (dt, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 3.81 (s, 3 H, OCH_3), 3.52-3.75 (m, 6 H, H-3, H-5, H-6a, H-6b, H-3', H-4'), 3.41 (dt, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 1.58 (m, 2 H, CH_2CH$_2$O), 1.30 (bs, 10 H, CH_2 octyl), 1.15 (d, 2 H, $J_{5',6'}$ 6.5 Hz, H-6'), 0.91 (t, 3 H, J_{vic} 6.9 Hz, CH_3 octyl); ^{13}C NMR ($CDCl_3$): δ 138.9, 138.5, 138.4, 138.1, 137.9, 128.4, 128.3, 128.2, 128.1 x2, 128.0, 127.9, 127.8, 127.7, 127.5, 127.4, 127.3, 127.2, 126.3 (Ar*C*), 102.0 (C-1), 97.2 (C-1'), 84.4, 81.5, 75.4, 74.6, 74.3, 73.6, 73.3, 69.6, 68.9, 66.1 (C-2, C-3, C-4, C-5, C-6, C-3', C-4', C-5', *C*H$_2$O), 58.2 (O*C*H$_3$), 31.8, 29.7, 29.5, 29.3, 26.3, 22.6 (*C*H$_2$ octyl), 16.5 (C-6'), 14.0 (*C*H$_3$ octyl). Anal. Calc. for $C_{56}H_{70}O_{10}$ (903.16): C, 74.47; H, 7.81. Found: C, 74.32; H, 7.84.

Octyl 2-*O*-(3-*O*-methyl-α-L-fucopyranosyl)-β-D- galactopyranoside (**4**).



Compound **32** (21.7 mg; 0.024 mmole) was hydrogenated as described for compound **33**. The usual work up and chromatography on C-18 Sep-Pak cartridges gave compound **4** (10 mg; 92%). ^{1}H NMR (CD_3OD): δ 5.19 (dd, 1 H, $J_{1',2'}$ 3.9 Hz, H-1'),), 4.33 (d, 1 H, $J_{1,2}$ 7.0 Hz, H-1), 4.27 (q, 1 H, $J_{5',6'}$ 6.6 Hz, H-5'), 3.90 (dt, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, C*H*$_2$O), 3.80-3.85 (m, 3H, H-3, H-4', H-6a), 3.62-3.76 (m, 4 H, H-2, H-4, H-5, H-2'), 3.46-3.54 (m, 2 H, H-6b, C*H*$_2$O), 3.45 (s, 3H, OC*H*$_3$), 3.41 (dd, 1 H, $J_{2',3'}$ 10.0 Hz, $J_{3',4'}$ 3.2 Hz, H-3'), 1.56-1.64 (m, 2 H, C*H*$_2$CH$_2$O), 1.28-1.38 (m, 10 H, C*H*$_2$ octyl), 1.20 (d, 2 H, $J_{5',6'}$ 6.6 Hz, H-6'), 0.90 (t, 3 H, J_{vic} 6.7 Hz, C*H*$_3$ octyl); ^{13}C NMR (CD_3OD): δ 104.3 (C-1), 101.2 (C-1'), 84.3, 79.7, 76.4, 73.8, 71.9, 71.5, 71.1, 69.5, 68.6, 62.4 (C-2, C-3, C-4, C-5, C-6, C-3', C-4', C-5', *C*H$_2$O), 57.2 (O*C*H$_3$), 33.0, 30.8, 30.6, 30.4, 27.3, 23.7 (*C*H$_2$ octyl), 16.7 (C-6'), 14.4 (*C*H$_3$ octyl). ESMS for $C_{21}H_{40}O_{10}Na$: Calc. 475.2519. Found 475.2513.

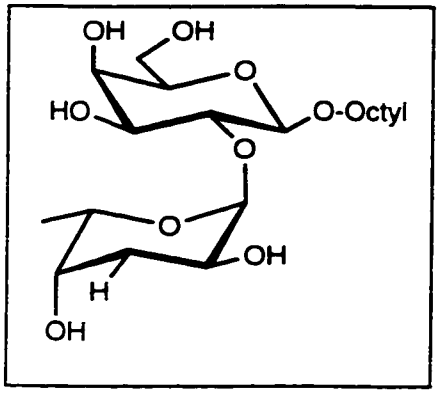
Octyl 3,4,6-tri-*O*-benzyl-2-*O*-(2,4-di-*O*-benzyl-α-L-xylo-hexopyranosyl)-β-D-galactopyranoside (**34**).



Compound **31** was acetylated with pentafluorophenyl-chlorothionoformate (126 µl; 0.78 mmole) and 4-dimethylamino pyridine (100 mg; 0.84 mmole) in dry CH_2Cl_2 (10 ml). After 14 hours the reaction mixture was

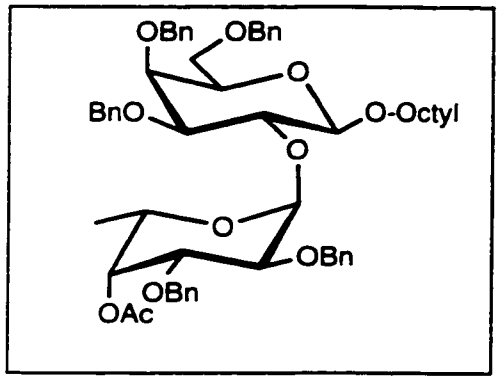
extracted with CH_2Cl_2, washed with aqueous 0.5% HCl, saturated $NaHCO_3$, and water, dried and concentrated to a syrup. This was directly deoxygenated as follows. To the syrup (109 mg) dissolved in toluene (10 ml), tributyltin hydride (130 µl; 0.48 mmole) and azobisisobutyronitrile (40 mg; 0.24 mmole) were added and the mixture was refluxed at 130 °C for 4 hours. Evaporation of the solvent left a syrup, which was dissolved in acetonitrile and washed with hexane. The acetonitrile layer was concentrated *in vacuo* and chromatographed (pentane-EtOAc 6:1, 4:1) to obtain **34** as a white solid (225 mg; 89%). $[\alpha]_D$ = -2.9° (c 0.8 $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.12-7.40 (m, 25 H, Ar*H*), 5.12 (d, 1 H, $J_{1',2a'}$ 3.3 Hz, H-1'), 4.86, 4.80, 4.64, 4.56, 4.30 (d, 1 H, J_{gem} 11.6 Hz, PhCH_2), 4.36-4.50 (m, 5 H, PhCH_2), 4.25 (d, 1 H, $J_{1,2}$ 7.97 Hz, H-1), 3.95 (bd, 1 H, $J_{3,4}$ 2.0 Hz, H-4), 3.88 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 3.77 (dd, 1 H, $J_{2',3'}$ 10.6 Hz, $J_{1',2'}$ 3.3 Hz, H-2'), 3.72 (dd, 1 H, $J_{2,3}$ 9.8 Hz, $J_{3,4}$ 2.0 Hz, H-3),), 3.55-3.65 (m, 5 H, H-2, H-5, H-6a, H-4', CH_2O), 3.35-3.43 (m, 2 H, H-6b, CH_2O), 2.05 (m, 1 H, H-3'a), 1.85 (t, 1 H, J 10.5 Hz, H-3'b), 1.50 (m, 2 H, CH_2CH_2O), 1.25 (bs, 10 H, CH_2 octyl), 1.15 (d, 2 H, $J_{5'6'}$ 6.5 Hz, H-6'), 0.85 (t, 3 H, J_{vic} 6.9 Hz, CH_3 octyl); ^{13}C NMR ($CDCl_3$): δ 138.5, 138.3, 137.9, 130.6, 128.9, 128.5, 128.4, 128.3, 128.2, 128.2, 128.1, 127.9, 127.8, 127.7, 127.5, 127.4, 127.3, 126.4, 124.6 (Ar*C*), 102.2 (C-1), 96.4 (C-1'), 76.0, 75.7, 74.4, 73.6, 73.4, 72.4, 72.3, 71.5, 71.0, 70.6, 70.3, 69.7, 69.5, 65.9 (C-2, C-3, C-4, C-5, C-6, C-2', C-4', C-5', *C*H_2O), 31.8 (C-3'), 29.7, 29.5, 29.2, 27.5, 26.2, 22.6 (*C*H_2 octyl), 16.3 (C-6'), 14.1 (*C*H_3 octyl). ESMS for $C_{55}H_{68}O_9Na$: Calc. 895.4761. Found 895.4757.

Octyl 2-*O*-(α-L-xylo-hexopyranosyl)-β-D- galactopyranoside (**5**).



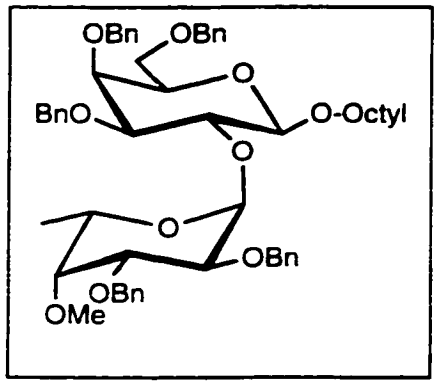
Compound **34** (15 mg; 0.017 mmole) was dissolved in 10 ml EtOH and 10 mg $Pd(OH)_2$ on charcoal was added. **34** was hydrogenated at room temperature and atmospheric pressure overnight. After completion of the reaction, the catalyst was filtered off on a 0.22 μm Millipore filter, solvent evaporated, the residue dissolved in water, passed through Waters C-18 Sep-Pak cartridges and eluted with 50% methanol in water. This was evaporated, redissolved in water, filtered on 0.22 μm Millipore filter, and lyophilized to give compound **5** (6.8 mg; 94%). ^{1}H NMR (CD_3OD): δ 5.14 (dd, 1 H, $J_{1',2'}$ 3.5 Hz, H-1'), 4.33 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.22 (q, 1 H, $J_{5',6'}$ 6.6 Hz, H-5'), 3.98 (ddd, 1 H, $J_{2',3a'}$ 17.0 Hz, $J_{1',2'}$ 3.5 Hz, $J_{2'3'b}$ 2.2 Hz, H-2'), 3.88 (dt, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, C*H*$_2$O), 3.81 (m, 1 H, H-6a), 3.62-3.76 (m, 5H, H-2, H-3, H-4, H-5, H-4'), 3.44-3.58 (m, 2 H, H-6b, C*H*$_2$O), 1.85-2.00 (m, 2 H, H-3a, H-3'b), 1.60 (m, 2 H, C*H*$_2$CH$_2$O), 1.25-1.40 (m, 10 H, C*H*$_2$ octyl), 1.12 (d, 2 H, $J_{5'6'}$ 6.6 Hz, H-6'), 0.90 (t, 3 H, J_{vic} 6.8 Hz, C*H*$_3$ octyl); ^{13}C NMR (CD_3OD): δ 104.9 (C-1), 101.4 (C-1'), 77.7, 75.5, 75.1, 72.5, 71.4, 70.4, 69.0, 66.2, 61.5 (C-2, C-3, C-4, C-5, C-6, C-2', C-4', C-5', *C*H$_2$O), 37.8 (C-3'), 32.1, 29.9, 29.6, 29.5, 26.4, 23.0 (*C*H$_2$ octyl), 16.5 (C-6'), 14.3 (*C*H$_3$ octyl). ESMS for $C_{20}H_{38}O_9Na$: Calc. 445.2413. Found 445.2417.

Octyl 3,4,6-tri-*O*-benzyl-2-*O*-(4-*O*-acetyl-2,3-di-*O*-benzyl-α-L-fucopyranosyl)-β-D-galactopyranoside (**26**)



Compounds **25** (145 mg; 0.26 mmole) and **14** (200 mg; 0.46 mmole; 1.8 eq) were coupled with DMTST as described for compound **30**. The usual work-up and column chromatography (pentane-EtOAc 10:1, 6:1, 4:1) gave compound **26** (170 mg; 69%). $[\alpha]_D$ = -42.2° (c 0.76 $CHCl_3$). ^1H NMR ($CDCl_3$): δ 7.20-7.40 (m, 25 H, Ar*H*), 5.69 (d, 1 H, $J_{1',2'}$ 3.5 Hz, H-1'), 5.42 (bd, 1 H, $J_{3',4'}$ 2.5 Hz, H-4'), 4.93, 4.85, 4.82, 4.78, 4.75, 4.67, 4.65 (d, 1 H, J_{gem} 11.5 Hz, PhCH_2), 4.44-4.58 (m, 3 H, PhCH_2), 4.27 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.1 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 4.00 (dd, 1 H, $J_{2,3}$ 10.5 Hz, $J_{3,4}$ 3.0 Hz, H-3), 3.85-3.95 (m, 3 H, H-2, H-4, H-6a), 3.77 (dd, 1 H, $J_{2',3'}$ 10.0 Hz, $J_{1',2'}$ 3.5 Hz, H-2'), 3.72 (dd, 1 H, $J_{2',3'}$ 10.0 Hz, $J_{3',4'}$ 2.5 Hz, H-3'), 3.51-3.64 (m, 3 H, H-5, H-6b, CH_2O), 3.40 (dt, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 2.10 (s, 3 H, CH_3CO), 1.60 (m, 2 H, CH_2CH$_2$O), 1.30 (bs, 10 H, CH_2 octyl), 1.12 (d, 2 H, $J_{5'6'}$ 6.5 Hz, H-6'), 0.88 (t, 3 H, J_{vic} 6.9 Hz, CH_3 octyl); ^{13}C NMR ($CDCl_3$): δ 159.7 (CH$_3$*C*O), 129.3, 139.2, 138.8, 138.1, 138.0, 128.4, 128.3, 128.1, 128.0, 127.9 x2, 127.8, 127.7, 127.6, 127.4, 127.3, 127.2 x2, (Ar*C*), 103.5 (C-1), 99.3 (C-1'), 81.1, 79.5, 79.4, 76.8, 75.3, 74.9, 74.2, 74.0, 74.0, 73.3, 72.7, 71.3, 71.0, 70.0, 69.7 (C-2, C-3, C-4, C-5, C-6, C-3', C-4', C-5', *C*H$_2$O), 32.3, 30.3, 30.2, 30.0, 29.2, 27.2 (*C*H$_2$ octyl), 21.7 (*C*H$_3$CO), 16.7 (C-6'), 14.6 (*C*H$_3$ octyl). ESMS for $C_{57}H_{70}O_{11}Na$: Calc. 953.4815. Found 953.4815.

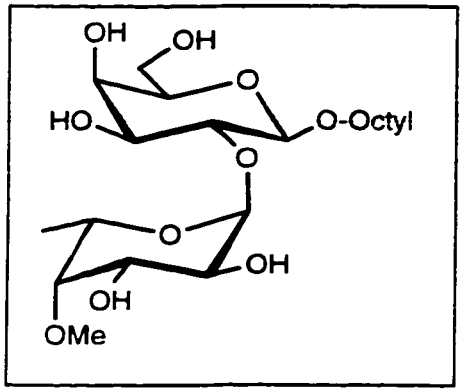
Octyl 3,4,6-tri-*O*-benzyl-2-*O*-(2,3-di-*O*-benzyl-4-*O*-methyl-α-L-fucopyranosyl)-β-D-galactopyranoside (**35**)



Compound **26** (140 mg; 0.15 mmole) was dissolved in dry methanol (10 ml) and 2 ml of a 0.5 M solution of sodium methoxide in methanol was added to it and the mixture stirred at room temperature for 4 hours. The reaction mixture was neutralized with Dowex 50 H^+ resin and filtered. The solvent was evaporated to obtain a syrup (compound **27**) which was methylated as described for compound **32** to obtain compound **35** (102 mg; 75% from **26**). $[\alpha]_D$ = -47.2° (c 1.1 $CHCl_3$). ^1H NMR ($CDCl_3$): δ 7.20-7.40 (m, 25 H, Ar*H*), 5.71 (d, 1 H, $J_{1',2'}$ 2.4 Hz, H-1'), 4.84, 4.83, 4.78, 4.76, 4.72, 4.61, 5.59, 4.55 (d, 1 H, J_{gem} 11.5 Hz, PhCH_2), 4.52 (s, 2 H, PhCH_2), 4.47 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.43 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 4.25 (dd, 1 H, $J_{2,3}$ 10.0 Hz, $J_{1,2}$ 7.8 Hz, H-2), 3.97 (bd, 1 H, $J_{3,4}$ 2.5 Hz, H-4), 3.94 (m, 1 H, H-6a), 3.91 (dd, 1 H, $J_{2',3'}$ 9.8 Hz, $J_{1',2'}$ 2.4 Hz, H-2'), 3.73 (dd, 1 H, $J_{2',3'}$ 9.8 Hz, $J_{3',4'}$ 2.5 Hz, H-3'), 3.55-3.65 (m, 5 H, H-2, H-3, H-5, H-6a, CH_2O), 3.61 (s, 3 H, OCH_3), 3.40 (dt, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 1.55 (m, 2 H, CH_2CH_2O), 1.32 (bs, 10 H, CH_2 octyl), 1.21 (d, 2 H, $J_{5',6'}$ 6.5 Hz, H-6'), 0.90 (t, 3 H, J_{vic} 6.9 Hz, CH_3 octyl); ^{13}C NMR ($CDCl_3$): δ 138.9, 138.4, 138.3, 138.0, 137.9, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.6, 127.4, 127.3, 127.2, 127.2, 127.0, 126.35 (Ar*C*), 103.6 (C-1), 97.0 (C-1'), 84.5, 80.9, 79.2, 76.0, 74.3, 73.6, 73.3, 72.9, 72.8, 72.6, 72.1, 71.2, 70.3, 70.0, 69.7 (C-2, C-3, C-4, C-5, C-6, C-3', C-4', C-5', *C*H_2O), 61.6 (O*C*H_3), 31.8, 29.7, 29.5, 29.3, 26.2, 22.6 (*C*H_2 octyl), 16.2 (C-6'), 14.0

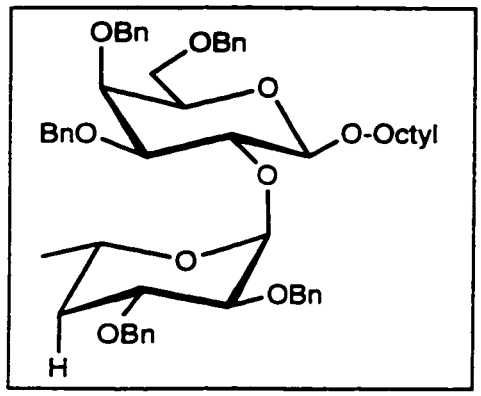
(CH_3 octyl). Anal. Calc. for $C_{56}H_{70}O_{10}$ (903.18): C, 74.47; H, 7.81. Found: C, 74.54; H, 7.84.

Octyl 2-*O*-(4-*O*-methyl-α-L-fucopyranosyl)-β-D-galactopyranoside (**2**).



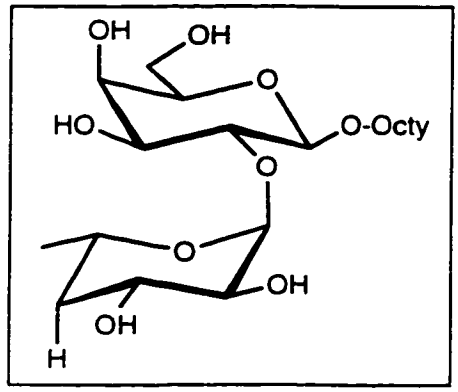
Compound **35** (80 mg; 0.088 mmole) was hydrogenated in a flow of hydrogen overnight as described for **37** to give **2** (36 mg; 90%) after purification by reverse-phase chromatography. ^{1}H NMR (CD_3OD): δ 5.17 (d, 1 H, $J_{1',2'}$ 3.8 Hz, H-1'), 4.40 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 4.28 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 3.85-3.93 (m, 2 H, H-4, CH_2O), 3.78-3.83 (m, 2H, H-4', H-6a), 3.69-3.74 (m, 3 H, H-6b, H-2', H-3'), 3.61-3.67 (m, 2 H, H-2, H-3), 3.56 (s, 3H, OCH_3), 3,45-3.51 (m, 2 H, H-5, CH_2O), 1.55-1.64 (m, 2 H, CH_2CH_2O), 1.26-1.36 (m, 10 H, CH_2 octyl), 1.19 (d, 2 H, $J_{5'6'}$ 6.5 Hz, H-6'), 0.0.87-0.93 (m, 3 H, CH_3 octyl); ^{13}C NMR (CD_3OD): δ 104.4 (C-1), 101.5 (C-1'), 84.5, 83.4, 79.0, 76.5, 75.7, 72.2, 70.9, 70.8, 70.4, 67.9 (C-2, C-3, C-4, C-5, C-6, C-3', C-4', C-5', CH_2O), 62.38 (OCH_3), 32.9, 30.9, 30.5, 30.3, 27.2, 23.6 (CH_2 octyl), 16.8 (C-6'), 14.4 (CH_3 octyl). ESMS for $C_{21}H_{40}O_{10}Na$: Calc. 475.2519. Found 475.2512.

Octyl 3,4,6-tri-*O*-benzyl-2-*O*-(2,3-di-*O*-benzyl-α-L-xylo-hexopyranosyl)-β-D-galactopyranoside (**29**).



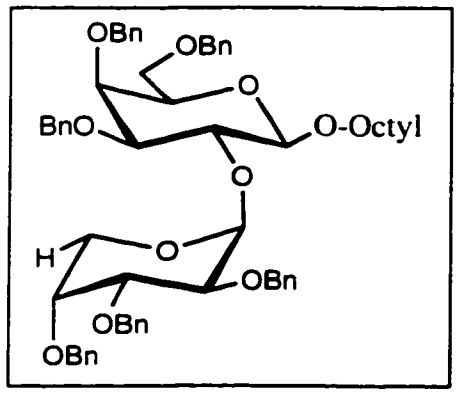
Compound **26** (483 mg; 0.52 mmole) was deacetylated to give **27** as before and then acylated with pentafluorophenylchlorothionoformate (175 µl; 1.08 mmole) and DMAP (250 mg; 0.05 mmole) in CH_2Cl_2 (**28**). Compound **28** was deoxygenated with AIBN (88 mg; 0.52 mmole) and Bu_3SnH (700 µl; 2.5 mmole) as described for the synthesis of **34** to give **29** (207 mg; 83% from alcohol) after column chromatography (pentane-EtOAc 10:1, 6:1).$[\alpha]_D$ = -15.2° (c 0.9). ^{1}H NMR ($CDCl_3$): δ 7.20-7.40 (m, 25 H, Ar*H*), 5.69 (d, 1 H, $J_{1',2'}$ 3.6 Hz, H-1'), 4.85, 4.79, 4.74, 4.66, 4.64, 4.60, 4.56, 4.49 (d, 1 H, J_{gem} 11.5 Hz, PhCH_2), 4.47 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.45 (s, 2 H, PhCH_2), 4.25 (dd, 1 H, $J_{2,3}$ 9.8 Hz, $J_{1,2}$ 7.8 Hz, H-2), 3.89 (m, 3 H, H-4, H-6a, CH_2O), 3.76 (dd, 1 H, $J_{2,3}$ 9.8 Hz, $J_{3,4}$ 2.7 Hz, H-3) 3.57-3.65 (m, 4 H, H-5, H-6b, H-2', H-3'), 3.40-3.45 (m, 2 H, H-5', CH_2O), 2.10 (m, 1 H, H-4'a), 1.05 (m, 1 H, H-4'b), 1.59 (m, 2 H, CH_2CH$_2$O), 1.30 (bs, 10 H, CH_2 octyl), 1.16 (d, 2 H, $J_{5',6'}$ 6.5 Hz, H-6'), 0.90 (t, 3 H, J_{vic} 6.9 Hz, CH_3 octyl); ^{13}C NMR ($CDCl_3$): δ 139.0, 138.4, 138.2, 138.1, 137.9, 128.4, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.5, 127.3, 127.2, 126.4 (Ar*C*), 102.2 (C-1), 97.3 (C-1'), 84.5, 80.1, 75.5, 74.3, 73.6, 73.3, 72.3 x2, 72.2 x2, 72.1, 71.3, 69.8, 68.9, 63.3 (C-2, C-3, C-4, C-5, C-6, C-2', C-3' C-5', *C*H$_2$O), 39.1 (C-4'), 31.8, 29.7, 29.5, 29.3, 26.2, 22.6 (*C*H$_2$ octyl), 20.8 C-6'), 14.0 (*C*H$_3$ octyl). Anal. Calc. for $C_{55}H_{69}O_9$ (873.54): C, 75.66; H, 7.85. Found: C, 75.67; H, 7.69.

Octyl 2-*O*-(α-L-xylo-hexopyranosyl)-β-D-galactopyranoside (**3**).



Compound **29** (60 mg; 0.068 mmole) was hydrogenated and purified as before to give compound **3** (26.6 mg; 91%). ^{1}H NMR (CD_3OD): δ 5.16 (d, 1 H, $J_{1',2'}$ 3.8 Hz, H-1'), 4.31 (d, 1 H, $J_{1,2}$ 7.1 Hz, H-1), 4.24 (m, 1 H, H-5'), 3.89 (dt, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 3.77-3.84 (m, 2 H, H-3', H-4), 3.70-3.74 (m, 2H, H-6a, H-2'), 3.61-3.67 (m, 2 H, H-2, H-3), 3.46-3.55 (m, 3 H, H-5, H-6b, CH_2O), 1.91 (ddd, 1 H, J_{gem} 12.7 Hz, J_{vic} 4.7 Hz, 2.3 Hz, H-4'a), 1.54-1.64 (m, 2 H, CH_2CH_2O), 1.22-1.38 (m, 11 H, H-4'b, CH_2 octyl), 1.13 (d, 1 H, $J_{5',6'}$ 6.3 Hz, H-6'), 0.86-0.94 (m, 3 H, CH_3 octyl); ^{13}C NMR (CD_3OD): δ 104.1 (C-1), 97.3 (C-1'), 80.6, 77.0, 76.3, 75.4, 73.7, 71.9, 71.1, 69.5, 62.4 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-5', CH_2O), 41.9 (C-4'), 33.0, 30.8, 30.5, 30.4, 27.2, 23.7 (CH_2 octyl), 21.1 (C-6'), 14.4 (CH_3 octyl). ESMS for $C_{20}H_{38}O_9Na$: Calc. 445.2413. Found 445.2419.

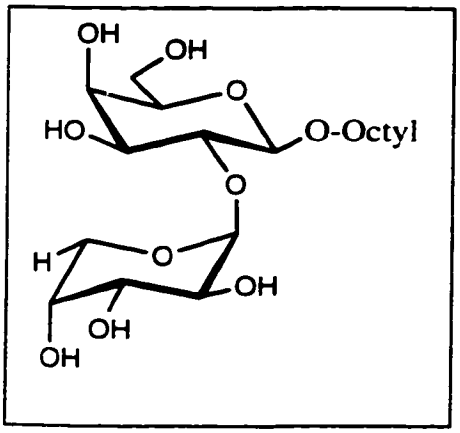
Octyl 3,4,6-tri-*O*-benzyl-2-*O*-(2,3,4-tri-*O*-benzyl-β-D-arabinopyranosyl)-β-D-galactopyranoside (**42**).



2,3,4-Tri-*O*-benzyl-D-arabinopyranose (**40**; 238 mg; 0.566 mmole) was dissolved in CH_2Cl_2 (2 ml) and DMF (22.3 µl) was added to it with stirring. Oxalyl bromide (0.184 ml; 1.3 mmole) was added drop-wise and stirring was continued for 20 minutes after

evolution of gas ceased. The reaction mixture was washed with water, dried, solvent partially evaporated and dried over 4 Å MS. The resulting syrup was transferred to the reaction vessel containing compound **25** (68 mg; 0.12 mmole), Bu_4NBr (72 mg; 0.22 mmole) and 1g 4 Å MS in 2:1 CH_2Cl_2-DMF (15 ml) and stirred for 2 days. The mixture was filtered over Celite, and the solvents evaporated. Column chromatography (pentane-EtOAc 10:1) gave **42** (100 mg; 78% based on alcohol). $[\alpha]_D$ -53.8° (c 1.6 $CHCl_3$). ^1H NMR ($CDCl_3$): δ 7.20-7.40 (m, 30 H, Ar*H*), 5.78 (d, 1 H, $J_{1',2'}$ 3.8 Hz, H-1'), 4.85, 4.77, 4.75, 4.72, 4.63, 4.55, 4.49, 4.45 (d, 1 H, J_{gem} 11.5 Hz, PhCH_2), 4.74, 4.61 (s, 2 H, PhCH_2), 4.46 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.25 (dd, 1 H, $J_{2,3}$ 10.0 Hz, $J_{,2}$ 7.8 Hz, H-2), 4.08 (dd, 1 H, $J_{2',3'}$ 10.0 Hz, $J_{1',2'}$ 3.8 Hz, H-2'), 3.98 (dd, 1 H, $J_{2,3}$ 10.0 Hz, $J_{3,4}$ 3.0 Hz, H-3), 3.94 (m, 1 H, H-4, H-6a). 3.89 (dt, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, CH_2O), 3.80 m, 1 H, H-5'a), 3.74 (dd, 1 H, $J_{2',3'}$ 9.8 Hz, $J_{3',4'}$ 2.8 Hz, H-3'), 3.56-3.69 (m, 4 H, H-4, H-6b, H-4', H-5'b), 3.42 (m, 1 H, CH_2O), 1.56 (m, 2 H, CH_2CH_2O), 1.26 (bs, 10 H, CH_2 octyl), 0.91 (t, 3 H, J_{vic} 6.9 Hz, CH_3 octyl); ^{13}C NMR ($CDCl_3$): δ 138.8, 138.6, 138.5, 138.4, 138.0, 137.9, 128.5, 128.4, 128.3, 128.2, 128.1 x3, 128.0, 127.9, 127.8, 127.7 x2, 127.6, 127.5, 127.4, 127.3, 127.2, 126.5 (Ar*C*), 102.1 (C-1), 97.5 (C-1'), 84.4, 77.6, 76.0, 74.4 x2, 73.6, 73.3, 73.0, 72.4, 72.4, 72.1, 71.5, 71.5, 69.6, 68.8 (C-2, C-3, C-4, C-5, C-6, C-2', C-3' C-4', CH_2O), 60.3 (C-5'), 31.8, 29.7, 29.4, 29.2, 26.2, 22.6 (CH_2 octyl), 14.1 (CH_3 octyl). ESMS for $C_{61}H_{72}O_{10}Na$: Calc. 987.5502. Found 987.5502.

Octyl 2-*O*-(β-D-arabinopyranosyl)-β-D-galactopyranoside (**8**).



Compound **42** (50 mg; 0.052 mmole) was hydrogenated as described before and purified in the usual way to give compound **8** (20 mg; 91%). ^{1}H NMR (CD_3OD): δ 5.21 (d, 1 H, $J_{1',2'}$ 2.75 Hz, H-1'), 4.32 (d, 1 H, $J_{1,2}$ 7.23 Hz, H-1), 4.15 (dd, 1 H, J_{gem} 11.72 Hz, $J_{4',5'a}$ 1.5 Hz, H-5'a), 3.89 (dt, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, C*H*$_2$O), 3.80-3.84 (m, 2 H, H-3', H-3), 3.76-3.79 (m, 2H, H-4, H-2'), 3.70-3.74 (m, 2 H, H-4', H-5'b), 3.45-3.58 (m, 3 H, H-2, H-5, C*H*$_2$O), 1.59 (m, 2 H, C*H*$_2$CH$_2$O), 1.30 (m, 10 H, C*H*$_2$ octyl), 0.90 (m, 3 H, C*H*$_3$ octyl); ^{13}C NMR (CD_3OD): δ 103.5 (C-1), 98.5 (C-1'), 77.6, 75.6, 73.0, 72.4, 71.8, 71.6, 70.4, 69.1, 68.9 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', *C*H$_2$O), 61.5 (C-5'), 32.0, 29.8, 29.5, 29.4, 26.3, 22.9 (*C*H$_2$ octyl), 14.3 (*C*H$_3$ octyl). ESMS for $C_{19}H_{36}O_{10}Na$: Calc. 447.2206. Found 447.2202.

2.4.3 *Enzyme kinetics*

The enzymatic assays performed were based on a method developed that uses the rate of transfer of sugar donors incorporated with a radioisotope onto an acceptor. All the compounds synthesized had a hydrophobic aglycon and therefore C-18 (reverse phase) cartridges were used to separate the products from unreacted donors.

The recombinant enzymes were expressed in *E. coli* and the isolation and purification was performed according to established protocol [45, 46]. They were stored at -70 °C with 1 mg/ml bovine serum albumin (BSA) until used.

All reactions were carried out in 450 µL microfuge tubes in a total volume of 33 µL. The modified derivatives were screened at 1 mM concentrations with the A and B enzymes, all activity being measured relative to the natural acceptor [Octyl 2-*O*-(α-L-fucopyranosyl)-β-D-galactopyranoside (**1**)]. All assays were carried out in 35 mM sodium cacodylate buffer containing 0.02 M $MnCl_2$ and 1 mg/ml BSA at 37 °C. The reactions were carried out for 30 min before being quenched with water. The reaction mixture was transferred to a C-18 Sep-Pak cartridge pre-equilibrated with methanol and water. The cartridge was washed with water to remove unreacted donor until background counts were obtained. The products were then eluted with methanol (3.5 ml) and the radioactivity in dpm (decays per minute) was measured in a liquid scintillation counter.

UDP-GalNAc (0.152 mM) and UDP-[6-^{3}H]GalNAc (0.2 µCi) were used as donors for the assay with the A transferase. The acceptors were used in concentrations of 1 mM. 0.25 µL of the enzyme (0.165 mg/ml) was used with 0.15 mM of UDP-GalNAc. When tested as inhibitors, the analogs were used in concentrations of 1 mM with 0.01 mM of the native acceptor **1**.

Compound **1** was tested at concentrations of 0.12, 0.06, 0.03, 0.015, 0.0075, 0.00375 mM for kinetic studies and under the conditions stated above K_m was determined to be 1.00, 0.750, 0.500, 0.250, 0.125, 0.0625, 0.03125 mM. K_m determination for compound **3** was

carried out at concentrations of 5.00, 2.50, 1.250, 0.625, 0.3125, 0.15625, 0.07812, 0.03906, 0.01953 mM. To determine the K_m value of compound **4**, it was used in concentrations of 1.00, 0.500, 0.250, 0.125, 0.0625, 0.03125, 0.0156, 0.0078, 0.0039 mM. Compounds **2** and **5** were assayed in concentrations of 1.00, 0.500, 0.250, 0.125, 0.0625, 0.03125, 0.0156, 0.0078 mM and compound **7** at concentrations of 0.250, 0.125, 0.0625, 0.03125, 0.0156, 0.0078, 0.0039 mM for K_m value determination.

UDP-Gal (0.6 mM) and UDP-[6-^{3}H]Gal (0.2 μCi) were used for assays with the B transferase. The acceptors were used in concentrations of 1 mM. 0.1 μL of the enzyme (0.997 mg/ml) was used with 0.15 mM of UDP-Gal. To test the inhibitory activities of potential inhibitors, the analogs were added in concentrations of 0.5 mM to 0.1 mM of the native acceptor **1**.

To determine K_m values for compound **4**, it was used in concentrations of 0.4125, 0.2062, 0.0625, 0.03125, 0.0156, 0.0078, 0.0039 mM. K_m determination for compound **5** was carried out at concentrations of 4.00, 2.00, 1.00, 0.500, 0.250, 0.125, 0.0625, 0.03125, 0.0156 mM. The data obtained were then fit to the Michaelis-Menten equation using un-weighted nonlinear regression with the Sigma Plot 4.0 Program (Jandel Scientific) to estimate the kinetic parameters.

Chapter III

EVALUATION OF MODIFIED BLOOD GROUP O (H) DISACCHARIDES AS ACCEPTORS FOR WILD TYPE AND MUTANT BLOOD GROUP A AND B GLYCOSYLTRANSFERASES

3.1 *Introduction*

As discussed in chapter II, enzymatic assays of the synthesized disaccharides revealed that the *arabino* analog was a good acceptor for the A but not for the B transferase. These enzymes utilize different sugar donors for these reactions, and they differ in their primary sequence by four amino acids. The acceptor normally used, however is the same for both the enzymes; αFuc(1,2)βGal-*O*R, where R is a glycolipid or glycoprotein. Our results showed that the amino acid sequence forms the basis for the differential recognition of the acceptor by the A and the B enzymes. The *arabino* analog (**8**) was therefore used in radiochemical assays with the mutant enzymes of blood group A and B transferases. Two other modified disaccharides were determined to be good acceptors of the A transferase, the 3'-methoxy compound (**4**) and the 4'-methoxy compound (**2**). These two compounds,

along with compound **8** were used as acceptors of the A transferase in enzymatic syntheses.

		176	235	266	268
A2	**AAAA**	**Arginine**	**Glycine**	**Leucine**	**Glycine**
A3	**BAAA**	Glycine	Glycine	Leucine	Glycine
A4	**BBAA**	Glycine	Serine	Leucine	Glycine
A5	**BBBA**	Glycine	Serine	Methionine	Glycine
A6	**BBAB**	Glycine	Serine	Leucine	Alanine
A7	**BABA**	Glycine	Glycine	Methionine	Glycine
A8	**ABBA**	Arginine	Serine	Methionine	Glycine
A9	**AABA**	Arginine	Glycine	Methionine	Glycine
A10	**ABAB**	Arginine	Serine	Leucine	Alanine
B	**BBBB**	**Glycine**	**Serine**	**Methionine**	**Alanine**

Figure 34. **Differences in the amino acid residues of the A, B and mutant enzymes. (The numbers at the top indicate the positions of the amino acids that differ).**

The blood group A and B transferase enzymes have been cloned and expressed in *Escherichia coli*, along with several recombinant mutants obtained by sequentially altering the amino acids from the blood group A to the blood group B enzyme (Figure 34).

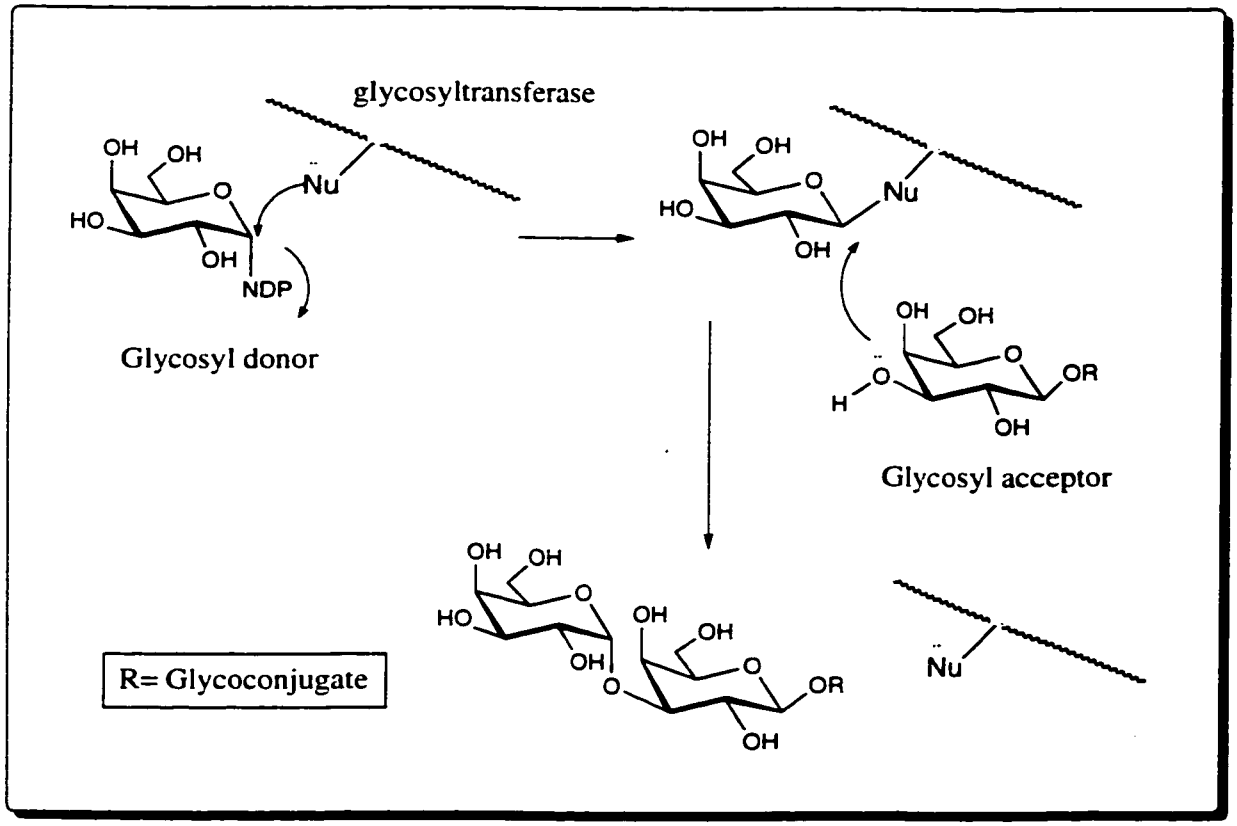


Figure 35. **Mechanism of enzymatic transfer of a monosaccharide from a sugar nucleotide donor to an acceptor with overall retention of configuration.**

Up to three amino acids of glycosyltransferase A were substituted with the corresponding amino acids of glycosyltransferase B (Arg 176→ Gly, Gly 235→Ser, and Leu 266→

Met) and the mutants were named based on what amino acids they have at the four positions where the A and the B enzymes differ. Thus wild type A2 has the sequence Arg 176, Gly 235, Leu 266 and Gly 268, or AAAA, A3 has the sequence Gly 176, Gly 235, Leu 266, and Gly 268 or BAAA and so on. These enzymes are similar to other known glycosyltransferases [157], but they transfer sugar donors with retention of configuration at the anomeric center (Figure 35), whereas most glycosyltransferases transfer donors with overall inversion of configuration [158, 159] (Figure 36).

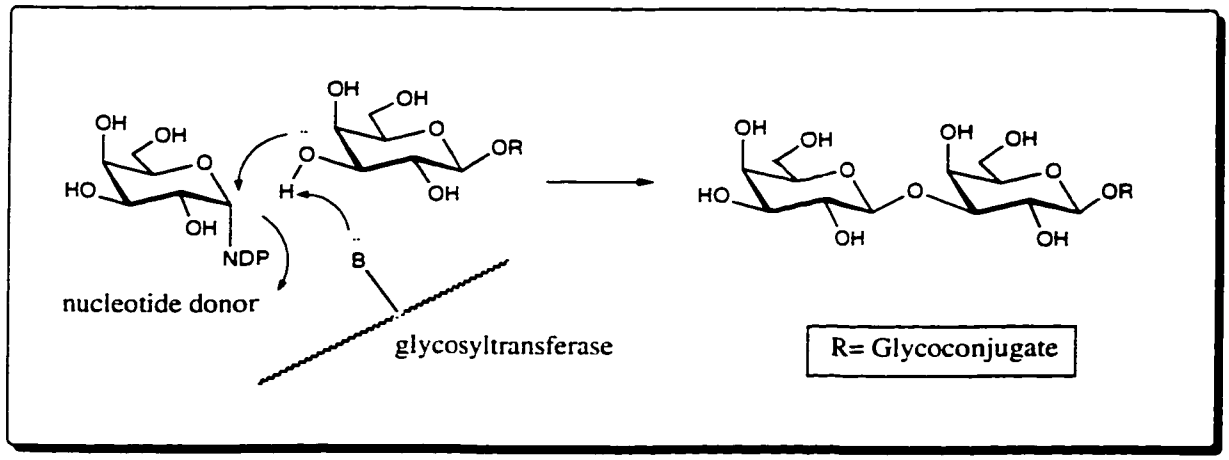


Figure 36. **Mechanism of glycosyl transfer with overall inversion of configuration.**

Three modified disaccharides **2** (4'-methoxy), **4** (3'-methoxy), and **8** (*arabino*) were used to synthesize the modified A antigens, compounds **43**, **44** and **45** using UDP-GalNAc (**46**) as the sugar donor with the A transferase (Figure 37). We determined by radioactive enzyme assays as described in chapter II that the 4'-methoxy, 3'-methoxy and the *arabino* derivatives have acceptor activity greater than 75% of that of the native acceptor (**1**) with the A transferase (Table 4). As the A enzyme showed good activity with these

compounds, they were used as acceptors in enzymatic syntheses to verify that the expected trisaccharide products had indeed formed.

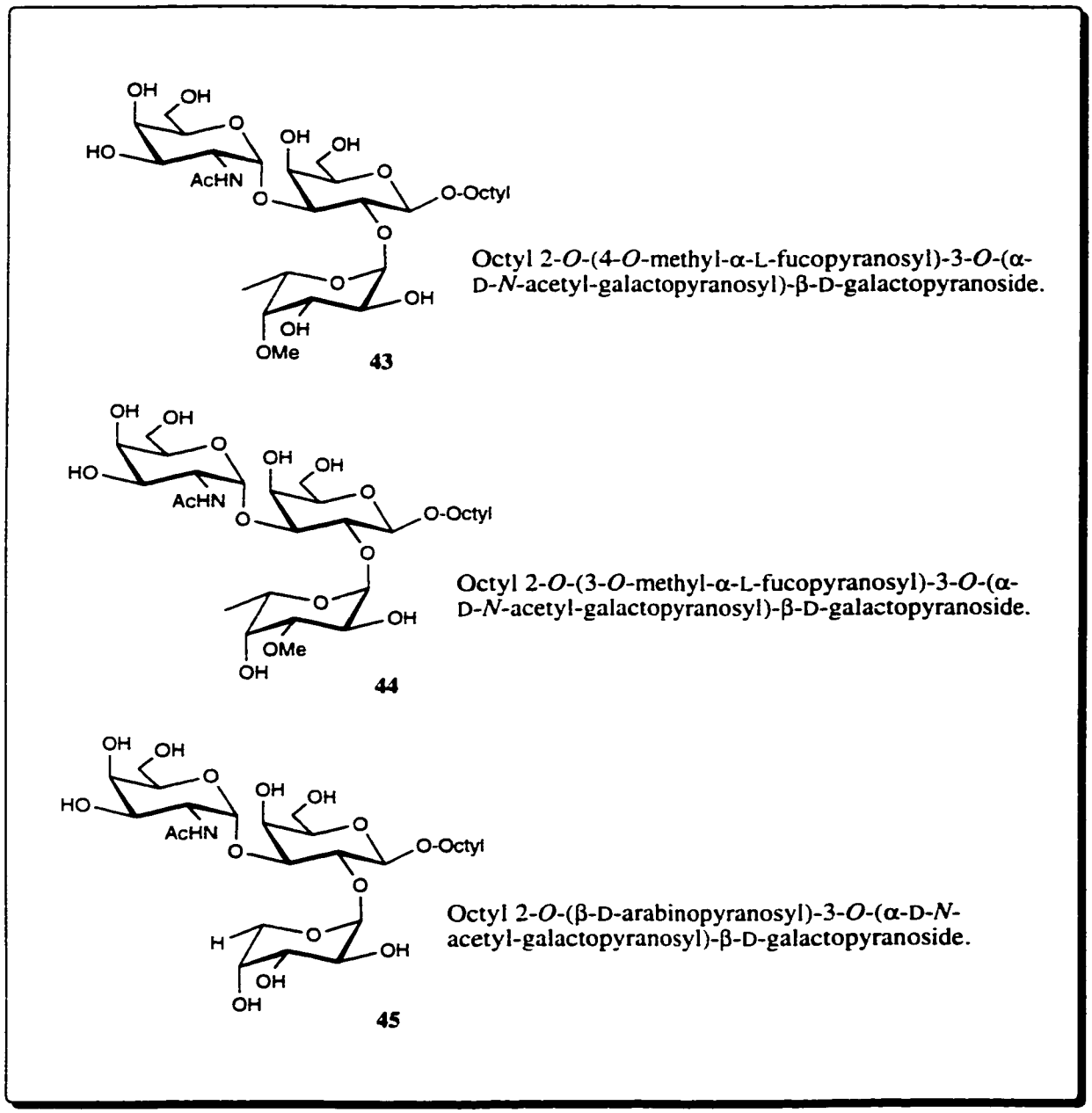


Figure 37. **Enzymatically synthesized trisaccharides.**

The *arabino* analog was found to distinguish between the A and the B enzymes. It was therefore used as an acceptor with all the mutants in order to determine which of the four amino acids is most important in differentiating between A and B specificity, i.e., to determine which of the four amino acids is responsible for differential recognition of the acceptor.

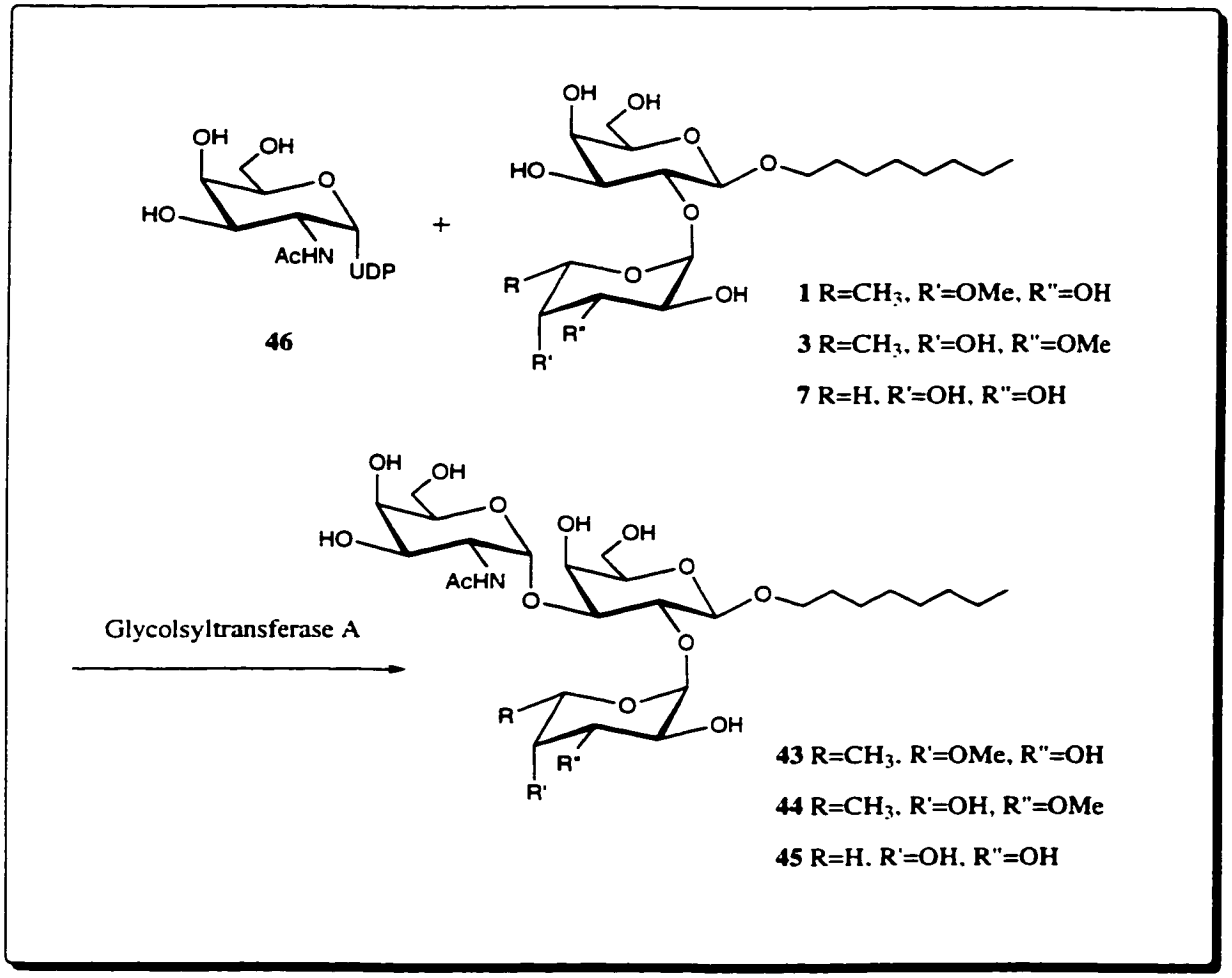
Table 4. **Kinetic constants and relative activities of the modified acceptors. (UDP-GalNAc was the donor for the A transferase and UDP-Gal was the donor for the B transferase.**

SUBSTRATES	A TRANSFERASE			B TRANSFERASE		
	% Activity (Relative to **1**)	K_m (μM)	V_{max} (nmole/min/μg)	% Activity (Relative to **1**)	K_m (μM)	V_{max} (nmole/min/μg)
1 (4'-methoxy)	86%	1140± 182	7.7± 0.07	11%	-	-
3 (3'-methoxy)	108%	20± 3	3.9± 0.4	70%	200± 25	8.1± 0.9
7 (*arabino*)	75%	208± 6	7.4± 0.11	5%	-	-

(- indicates that the values were not determined).

3.2. *Enzymatic synthesis of the trisaccharides*

Octyl 2-*O*-(4-*O*-methyl-α-L-fucopyranosyl)-β-D-galactopyranoside (**2**), octyl 2-*O*-(3-*O*-methyl-α-L-fucopyranosyl)-β-D-galactopyranoside (**4**), octyl 2-*O*-(3-*O*-methyl-β-D-arabinopyranosyl)-β-D-galactopyranoside (**8**) were synthesized as described in chapter II.



Scheme 10. **Enzymatic synthesis of the trisaccharides.**

The synthetic disaccharides used in this study as modified acceptors were all octyl glycosides in order to facilitate purification of these compounds and the enzymatically synthesized products by reverse phase chromatography on C-18 silica gel.

The enzymatic syntheses were performed according to an established protocol [45, 46] at 37 °C for two to three days at pH 6.9 (Scheme 10). The starting disaccharides were individually dissolved in 100 µl 10 x assay buffer (35 mM sodium cacodylate, 15 mM $MnCl_2$, 1 mg/ml BSA, pH 6.9), and 1.5 equivalent UDP-GalNAc, 2 µl 50 mg/ml BSA, 1 µl alkaline phosphatase (1 U/µl), 100 µl A enzyme (0.162 mg/µl of protein) and 787 µl of deionized water were added and the mixture was incubated at 37 °C. After 1 day, more of the donor was added depending on the progress of the reaction, and the reaction was continued until conversion to product was complete (TLC). The reaction mixtures were loaded onto a Waters C-18 Sep-Pak cartridge. The inorganic salts, the excess UDP-GalNAc and the enzyme were removed by passage through the Sep-Pak cartridge by washing with water, as the hydrophobic aglycon causes the glycosides to bind to C-18 silica. The compounds were eluted with 50-60% methanol in water. The solvent was evaporated, the residue was re-dissolved in deionized water, passed through a 0.22 µm Millipore filter and the filtrate lyophilized to provide the desired products in yields of 75-80%. ^1H NMR (see appendix Figures 55-57) and electrospray mass spectral data confirmed the structures of the trisaccharides. NMR chemical shift assignment was done based on data published previously [160]. Lemieux and coworkers extensively examined the structure of the A and the B antigenic determinants and other related structures and reported the detailed NMR chemical shifts for these compounds. For the A trisaccharide,

the signal for H-1 (βGal) was at δ 4.77, H-1' (αGalNAc) at δ 5.44 and H-1" (αFuc) at δ 5.58 ppm. These chemical shifts were measured in D_2O (referenced to 1% external acetone at δ 2.48 ppm) at 270 MHz. The currently accepted chemical shift for external acetone in D_2O is δ 2.225, which is 0.255 ppm lower than the value used by Lemieux and coworkers. The corresponding values for the modified A trisaccharides obtained in this study (Section 3.5.2) are between δ 4.54 and 4.64 for H-1 of βGal, δ 5.18 for αGalNAc (H-1') and between 5.31 and 5.36 for αFuc/βAra (H-1"). The differences in the chemical shifts can be explained by subtracting 0.255 ppm from the proton chemical shifts of the earlier study, when values of δ 4.515 (4.77-0.255), δ 5.105 and δ 5.325 respectively are obtained for the same protons, indicating the formation of the expected products. Similar studies on the B antigenic trisaccharide [160] showed the signal for H-1 (βGal) at δ 4.74, H-1' (αGal) at δ5.48 and H-1" (αFuc) at δ 5.53 ppm. A recent study [161] reports the proton chemical shifts of the B trisaccharide at δ 4.465, 5.237 and 5.206 respectively (recorded at 600 MHz in D_2O referenced to 0.1% external acetone set at 2.225 ppm). This discrepancy can also be explained as before. Although the NMR spectra recorded for this study were recorded at 500 MHz in methanol-D_6, they are in good agreement with the chemical shifts reported in the literature [160].

3.3 *Enzyme assays*

The *arabino* derivative was evaluated at 1 mM concentrations with recombinant A, B and the mutant enzymes in A transferase buffer. All activities measured are reported relative

to the natural acceptor **1** (Table 5). UDP-GalNAc and UDP-[6-^{3}H]GalNAc were used as donors for assays with the A transferase and the mutant enzymes. UDP-Gal and UDP-[6-^{3}H]Gal were used for assays with the B transferase. Incubations were carried out for 30 min. Reaction mixtures were quenched with water, transferred to a C-18 Sep-Pak (pre-equilibrated by washing with methanol and water) and washed with water to remove the unreacted tritiated donors. Products were eluted with methanol (3.5 ml) and the radioactivity quantitated in a liquid scintillation counter.

Table 5. **Percent activities of the *arabino* derivative (8) relative to the natural acceptor 1 with the wild type and mutant transferases.**

ENZYME	% ACTIVITY OF THE *ARABINO*-ANALOG (**8**)
A2 (wild type)	75%
A3	39%
A4	23%
A5	28%
A6	31%
A7	56%
A8	12%
A9	59%
A10	41%
B (wild type)	5%

3.4 *Analysis of the results*

As seen from results obtained earlier and summarized in Table 4, the *arabino* disaccharide (**8**) is a good acceptor for the A transferase but not the B transferase. The two enzymes differ by four amino acids that form the basis for the differentiation between the donors by the two enzymes. From our studies, we have found that the amino acid sequence in these enzymes determine acceptor specificity as does donor specificity. Thus, the methylene group on arabinose is acceptable by the enzyme with one sequence, but not by the other. In order to find out which of these four amino acids is responsible for this discrimination, i.e. why a fucose residue is distinguished from an arabinose residue, we tested this acceptor with several cloned mutants of the A and B transferases expressed in *E. coli*. The activity of **8** with these enzymes was determined by radioactive assay where the rate of transfer of ^{3}H labelled GalNAc from UDP-[6-^{3}H]-GalNAc to the acceptor was quantitated. The initial results (Table 5) show that a single amino acid cannot be identified as being responsible for the change in activity from A to B. It is likely that all the four amino acids come together in the binding site to recognize the arabino derivative as an acceptor in one case (A enzyme, sequence AAAA) but not in the other (B enzyme, sequence BBBB).

In all the mutants, change of one amino acid from A to B causes partial loss of recognition of this acceptor by the enzyme, with recognition being completely abolished with the B enzyme. However, the second of the four amino acids that differ between the two glycosyltransferases might play a crucial role in the recognition of the acceptor. All

the enzymes with glycine at position 235 have activities with the *arabino* derivative higher compared to the ones with serine at that position (Table 5). The methyl group on

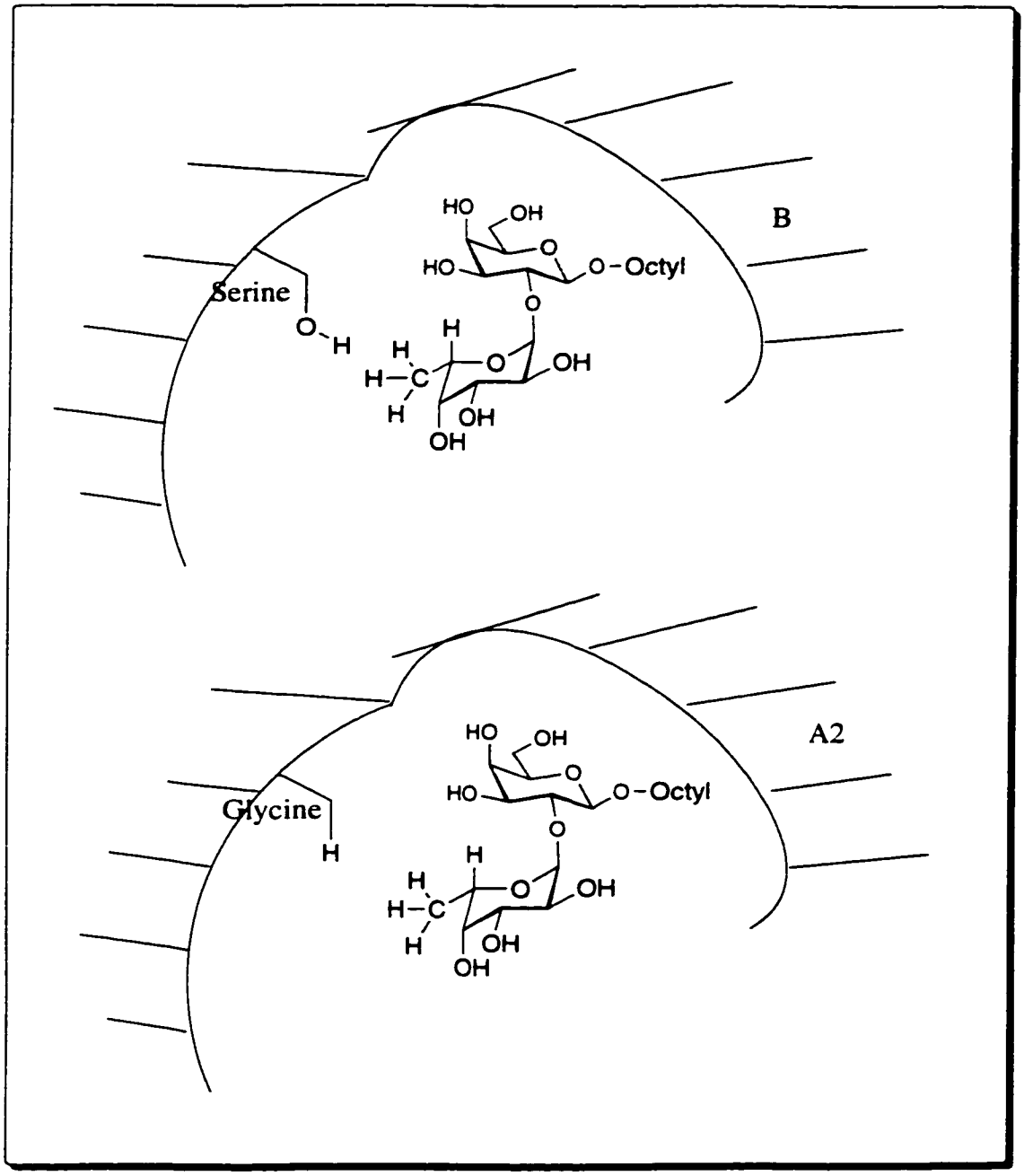


Figure 38. **Hypothetical interactions around C-6' (methyl) of fucose.**

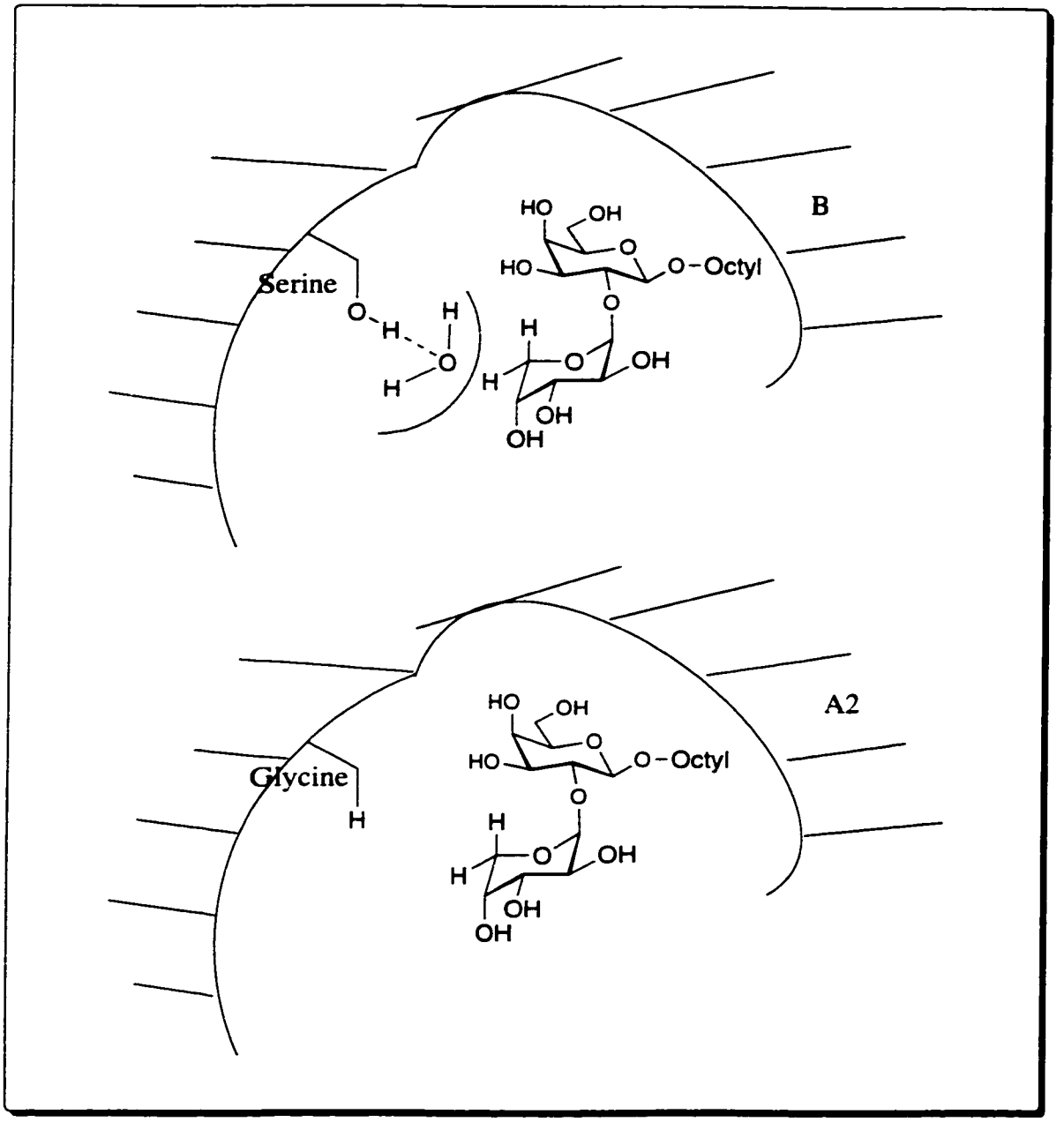


Figure 39. **Hypothetical interactions around C-5' (methylene) of arabinose.**

fucose is tolerated by both the A and the B enzymes, so it can be assumed that unfavorable interactions are absent in the active site in the presence of the fucosylated

acceptor irrespective of whether glycine or serine is at position 235 (Figure 38). Glycine may be involved in some favorable interaction with the methylene group on arabinose; at least there is no unfavourable interaction. When the methyl group is changed to an H, the change from glycine to serine leads to unfavorable interactions resulting in its rejection as an acceptor by the enzymes (Figure 39).

In summary, we have found that disaccharide acceptor analogs that are recognized by the blood group A glycosyltransferase as acceptors are used by the enzyme to transfer GalNAc from the sugar donor onto the acceptors to form trisaccharide product analogs. It has also been found that as with donor specificity, acceptor specificity is also determined by the amino acid sequence in these enzymes, but no particular amino acid can be identified as being solely responsible for the differential recognition of the acceptor by the enzymes.

3.5 *Experimental*

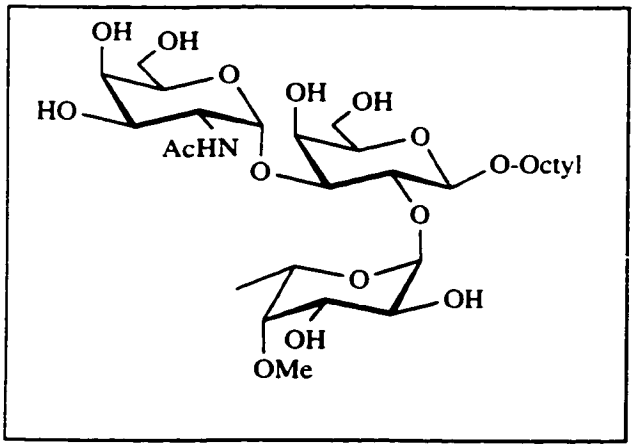
3.5.1 *General methods*

Analytical TLC was performed on Silica Gel 60-F_{254} (E. Merck, Darmstadt) with detection by charring with 5% sulfuric acid. Millex-GV (0.22μm) filters were from Millipore (Missisauga, ON), C-18 Sep-Pak cartridges were from Waters Associates (Missisauga, ON). Ecolite scintillation cocktail was from ICN Radiochemicals (St. Louis, MO) UDP-[6-^{3}H]-Gal (specific activity 1Ci/mmole) and UDP-[6-^{3}H]-GalNAc (specific

activity 1 Ci/mmole) were from American Radiolabelled Chemicals (St. Louis, MO). Bovine serum albumin, UDP-Gal and UDP-GalNAc were from Sigma. ^{1}H NMR spectra were recorded on 500 MHz (Varian Unity 500) in D_2O. Electrospray mass spectra were recorded on a Zabspec instrument from Micromass (Manchester, UK). Wild type recombinant enzymes and mutants were isolated from *E. coli* harboring plasmids containing the corresponding nucleotide sequences. They were purified by ammonium sulphate precipitation followed by affinity chromatography on a UDP-hexanolamine column by eluting with sodium cacoylate buffer (pH 6.9) containing uridinediphosphate [45, 46].

3.5.2 *Enzymatic syntheses*

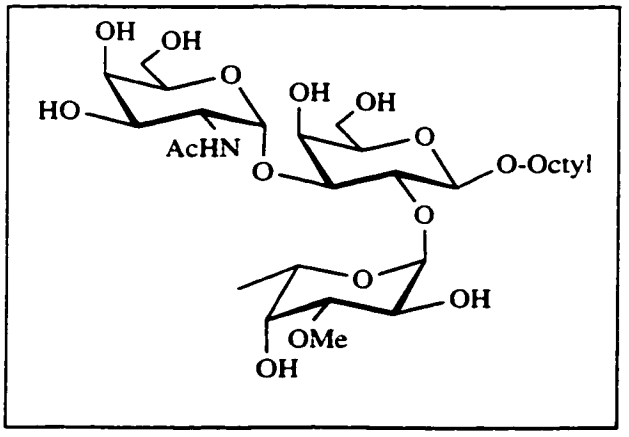
Octyl 2-*O*-(4-*O*-methyl-α-L-fucopyranosyl)-3-*O*-(α-D-*N*-acetyl-galactopyranosyl)-β-D-galactopyranoside (**43**).



Octyl 2-*O*-(4-*O*-methyl-α-L-fucopyranosyl)-β-D-galactopyranoside (1 mg) was dissolved in 100 µl 10 x assay buffer (35 mM sodium cacodylate, 15 mM $MnCl_2$, 1 mg/ml BSA, pH 6.9), 1.5 equivalent UDP-GalNAc, 2 µl 50 mg/ml BSA, 1 µl alkaline phosphatase (1 U/µl), 10 µl 0.5 M sodium cacodylate (pH 7.5), 100 µl

A enzyme (0.162 mg/μl of protein), 787 μl of deionized water and incubated at 37 °C. After 1 day another 1.5 equivalent of the donor was added and the reaction was continued till complete (TLC). The reaction mixture was directly loaded on a Waters C-18 Sep Pak cartridge, washed extensively with water, and the compound eluted with 60% methanol in water. The solvent was evaporated, redissolved in deionized water, passed through a 0.22 μm Millipore filter and the filtrate lyophilized to provide a white solid (1 mg) in about 75% yield. R_f (CH_2Cl_2:MeOH:H_2O 65:35:2) 0.2. Selected ^1H NMR data ($CDCl_3$): δ 5.31 (d, 1H, $J_{1''2''}$ 4.0 Hz, H-1", α-Fuc), 5.18 (d, 1H, $J_{1'2'}$ 3.5 Hz, H-1', α-GalNAc), 4.64 (d, 1H, J_{12} 7.6 Hz, H-1, β -Gal), 2.05 (s, 3H, N*Ac*). FAB Mass: m/z 656.2 $[M+H]^+$, 678.2 $[M+Na]^+$. ESMS for $C_{29}H_{53}NO_{15}Na$: Calc. 678.331290. Found 678.332061.

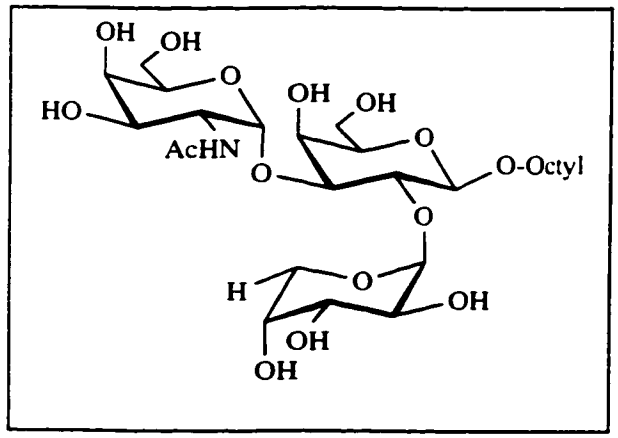
Octyl 2-*O*-(3-*O*-methyl-α-L-fucopyranosyl)-3-*O*-(α-D-*N*-acetyl-galactopyranosyl)-β-D-galactopyranoside (**44**).



Octyl 2 -*O*-(3-*O*-methyl-α-L-fucopyranosyl)-β-D-galactopyranoside (1.2 mg was taken in 100 μl 10 x assay buffer with 1.5 equivalent UDP-GalNAc, 2 μl 50 mg/ml BSA, 1 μl alkaline phosphatase (1 U/μl) 100 μl A enzyme (0.162 mg/μl of protein), 787 μl of deionized water and incubated at 37 °C. 1.5 equivalent of the donor was added every day for three until the reaction was complete (TLC). The reaction

mixture was directly loaded on a Waters C-18 Sep Pak cartridge, washed extensively with water, and the compound eluted with 60% methanol in water. The solvent was evaporated, redissolved in deionized water, passed through a 0.22 μm Millipore filter and the filtrate lyophilized to provide 1.4 mg of a white solid (80%). R_f (CH_2Cl_2:MeOH:H_2O 65:35:2) 0.23. Selected ^1H NMR data ($CDCl_3$): δ 5.32 (d, H, $J_{1''-2''}$ 4.0 Hz, H-1", α-Fuc), 5.18 (d, 1H, $J_{1'-2'}$ 3.7 Hz, H-1', α- GalNAc), 4.54 (d, 1H, J_{12} 7.9 Hz, H-1, β -Gal), 2.05 (s, 3H, N*Ac*). FAB Mass: m/z 656.2 $[M+H]^+$,678.2 $[M+Na]^+$. ESMS for $C_{29}H_{53}NO_{15}Na$: Calc. 678.331290. Found 678.331018.

Octyl 2-*O*-(β-D-arabinopyranosyl)-3-*O*-(α-D-*N*-acetyl-galactopyranosyl)-β-D-galacto-pyranoside (**45**).



Octyl 2-*O*-(β-D-arabinopyranosyl)-3-*O*-(α-D-*N*-acetyl-galactopyranosyl)-β-D-galacto-pyranoside (1.5) mg was taken in 100 μl 10 x assay buffer, 1 equivalent UDP-GalNAc, 2 μl 50 mg/ml BSA, 1 μl alkaline phosphatase (1 U/μl), 100 μl A enzyme (0.162 mg/μl of protein), and 787 μl of de-ionized water was added to it and incubated at 37 °C. 1.5 equivalent of the donor was added after one day and 1 equivalent on the second day. The reaction was continued until complete. The reaction mixture was loaded on a Waters C-18 Sep Pak cartridge, washed extensively with water, and the compound

eluted with 50% methanol in water. The solvent was evaporated, redissolved in deionized water, passed through a 0.22 μm Millipore filter and the filtrate lyophilized to provide a white solid (1.6 mg) in 75% yield. R_f (CH_2Cl_2:MeOH:H_2O 65:35:2) 0.26. Selected ^1H NMR data (CD_3OD): δ 5.36 (d, 1H, $J_{1''2''}$ 3.7 Hz, H-1", β-Ara), 5.18 (d, 1H, $J_{1'2'}$ 2.4 Hz, H-1', β-GalNAc), 4.54 (d, 1H, J_{12} 7.8 Hz, H-1, β -Gal), 2.05 (s, 3H, N*Ac*). FAB Mass: m/z 628.2 $[M+H]^+$, 650.2 $[M+Na]^+$. ESMS for $C_{27}H_{49}NO_{15}Na$: Calc. 650.299990. Found 650.299276.

3.5.3 *Enzyme assays*

All the assays were carried out in A transferase buffer containing 35 mM sodium cacodylate, 20 mM $MnCl_2$, 1 mg/ml BSA (pH 6.9) in a total volume of 33 μl. The incubations were carried out for 30 minutes. UDP-GalNAc was used as the donor for all the enzymes except the B enzyme. For the B enzyme, 0.60 mM UDP-Gal and 0.2 μCi UDP-[6-^{3}H]Gal was used as donors with 0.02 μl (0.997 mg/ml) B enzyme. A2 enzyme (0.28 μl; 0 165 mg/ml) was used with 0.15 mM UDP-GalNAc. A3 enzyme (0.00125 μl; 0.483 mg/ml) was used with 0.15 mM donor. A4 (0.01 μl; 0.170 mg/ml) was used with 0.35 mM donor. A5 (0.05 μl; 0.107 mg/ml) was used with 0.65 mM donor. A6 (0.05 μl; 0.107 mg/ml) was used with 0.60 mM donor. A7 (0.1 μl; 0.165 mg/ml) was used with 1.0 mM donor. A8 (0.0125 μl; 0.288 mg/ml) was used with 0.60 mM donor. A9 (0.40 μl; 0.042 mg/ml) was used with 0.60 mM donor. A10 (0.325 μl; 0.0575 mg/ml) was used with 0.60 mM donor. For the assays with UDP-GalNAc, 0.2 μCi UDP-[6-^{3}H]GalNAc was used.

Chapter IV

SYNTHESIS OF PHOTOAFFINITY LABELED INHIBITOR ANALOGS

4.1 *Introduction*

This chapter describes the synthesis and enzymatic evaluation of photoaffinity labeled disaccharide based inhibitors of the A and the B enzymes starting from the previously reported 3-amino disaccharide [50, 51].

4.2 *Design of the photoactivable inhibitors*

X-ray crystallography of glycosyltransferase A or B would require over-expression of these enzymes and their isolation in a homogeneous form. With the technology for the cloning and expression of glycosyltransferases advancing rapidly [162], this is foreseeable in the near future. Glycosyltransferases A and B have been cloned and expressed in *E. coli*, but thus far, they have not been produced in quantities that would allow the use of this technology. In the absence of a crystal structure of either the A or the B glycosyltransferase, one way of investigating the active site is by probing the

enzyme with modified acceptors/donors to determine the interactions between the enzyme and the substrate as detailed in chapter II. Another way is to use an acceptor or an inhibitor to covalently tag the enzyme at its active site.

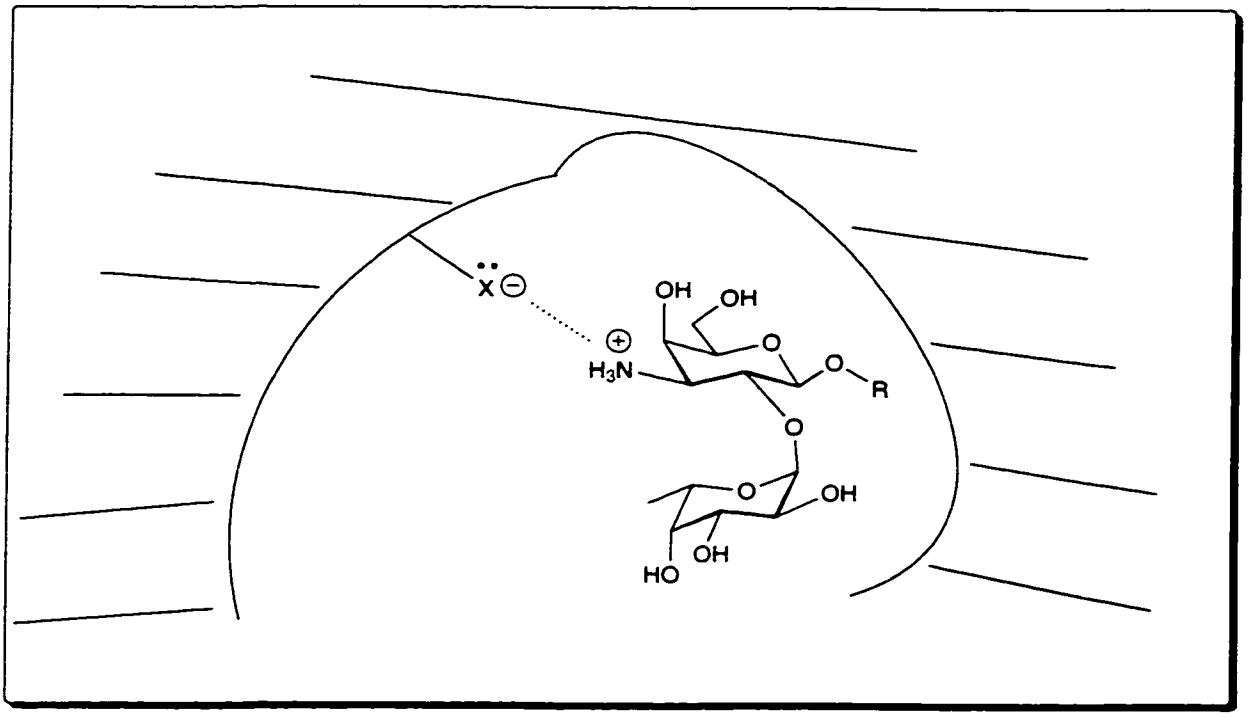


Figure 40. **Schematic representation of the interaction of a potential nucleophile with the 3-amino group.**

Previous work done in our laboratory aimed at developing specific glycosyltransferase inhibitors of the A and the B transferases had resulted in the synthesis of a submicromolar inhibitor of the serum A transferase, compound **47**. It was suggested that inhibition was caused by an interaction between a negatively charged residue in

proximity to OH-3 of the galactose residue and the protonated amine in the active site leading to the inactivation of the enzyme [163] (Figure 40).

We first decided to modify the 3-amino group into an iodoacetamido group in order to determine whether a catalytic active base or nucleophile might be present in the active site of the A enzyme. In the presence of such a functionality, the iodoacetamido group might serve as a covalent irreversible inhibitor. Displacement of the iodine by the reactive base would therefore result in the deactivation of the enzyme (Figure 41).

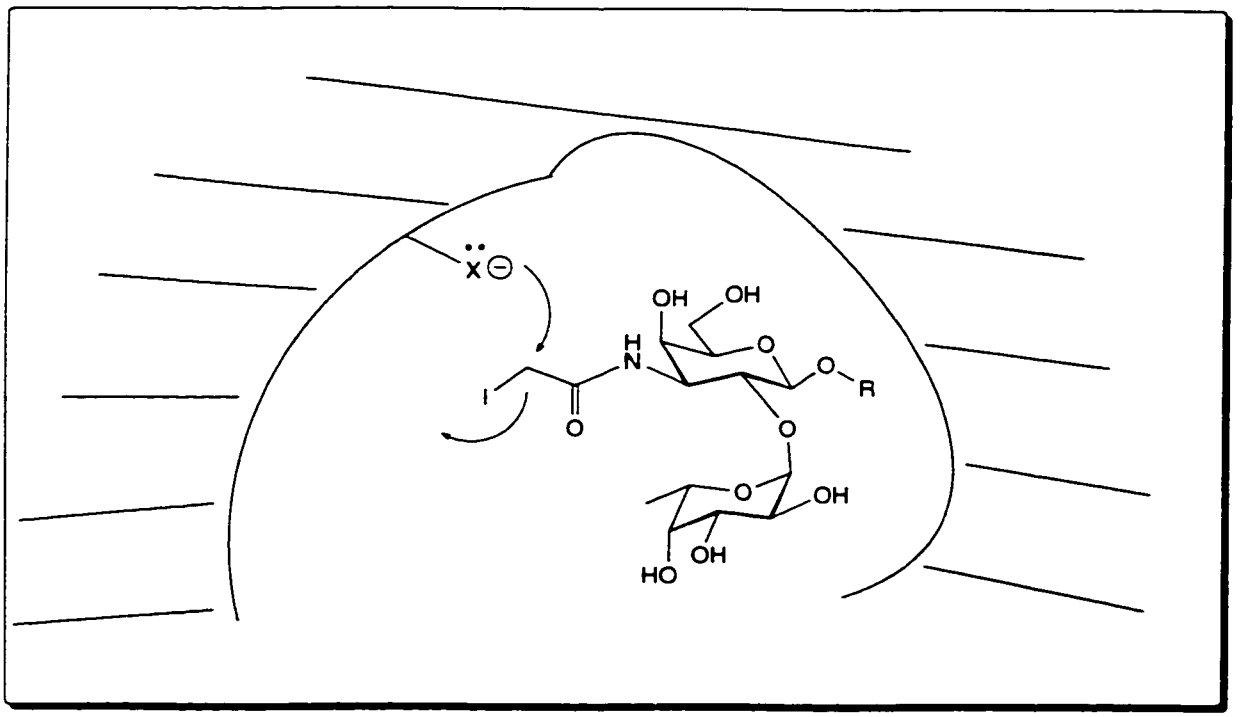


Figure 41. **Schematic representation of the reaction of a potential nucleophile in the catalytic site of the enzyme with the iodoacetamido compound.**

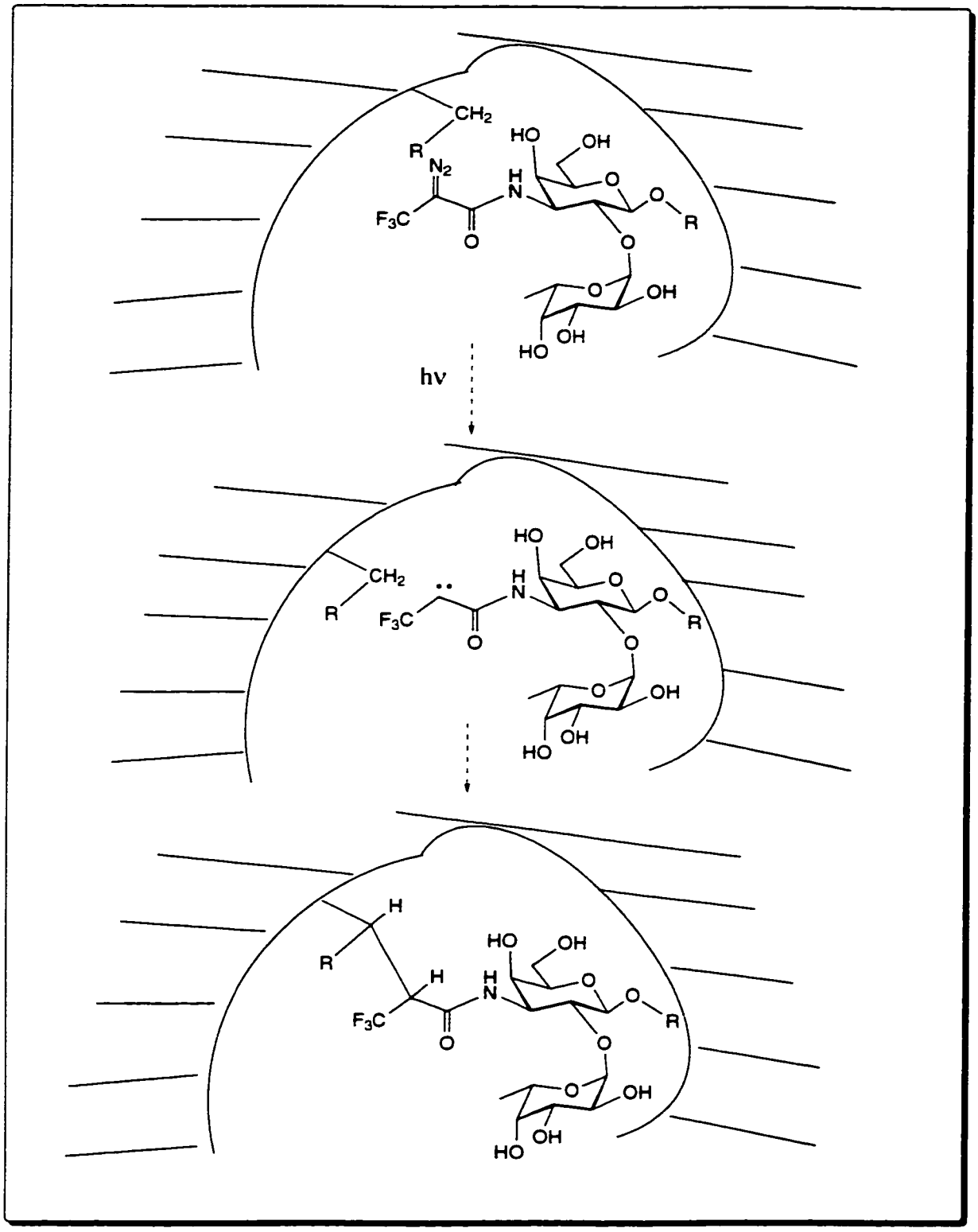


Figure 42. **Schematic representation of the potential reaction of a carbene intermediate formed from a diazo compound in the active site of the enzyme.**

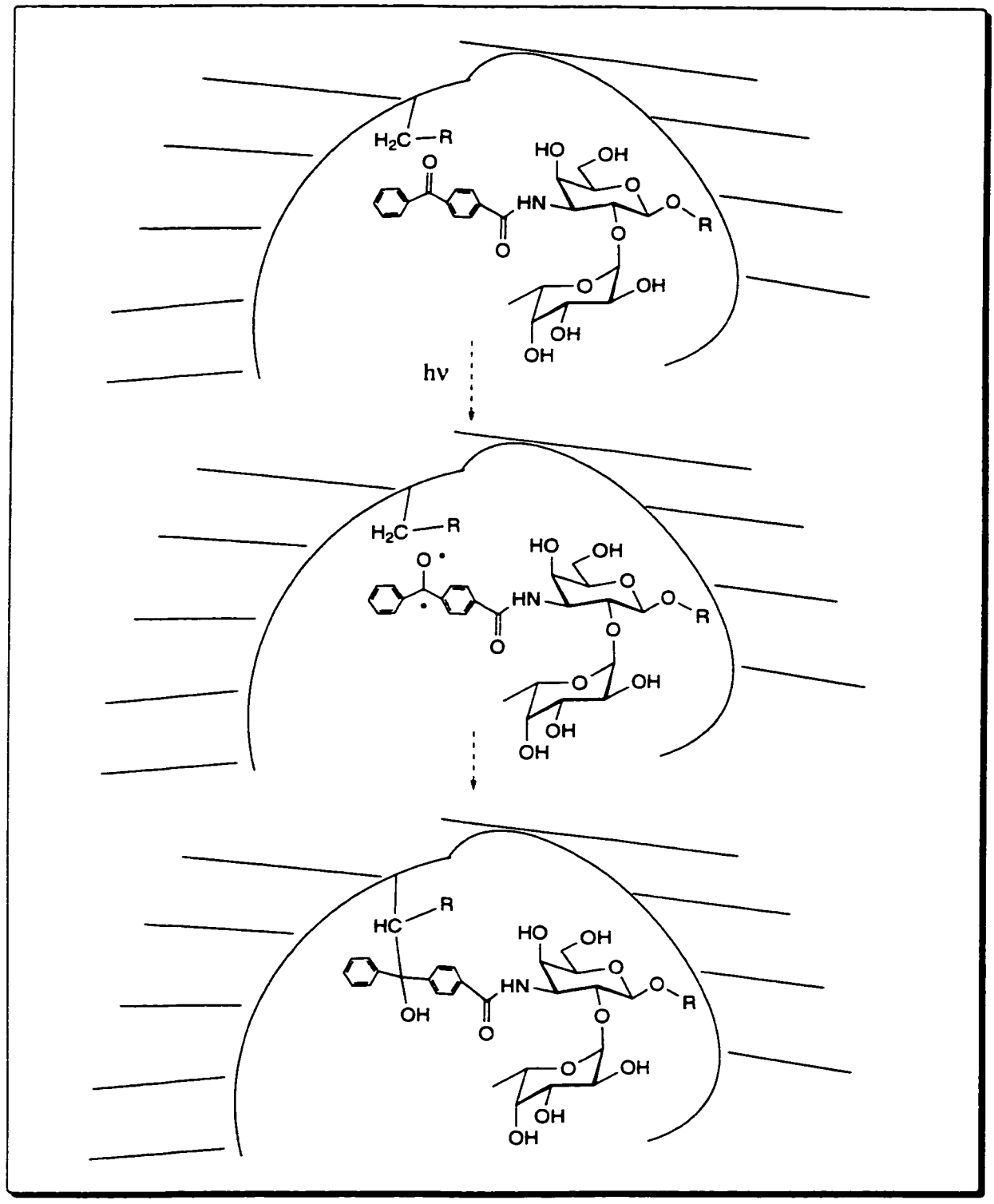


Figure 43. **Schematic representation of the potential reaction of a benzoylbenzoic acid derivative in the active site of the enzyme.**

An alternate approach was to label the enzyme with a conventional photolabile group such as a diazoketone that forms a carbene upon irradiation with light. The singlet carbene species are electron deficient and therefore strong electrophiles. They can react rapidly with double bonds, heteroatoms with non-bonded electron pairs and even aliphatic C-H bonds (Figure 42). The diazo group has been used previously to study insect hormones through photo-insertion [128]. The potential problems are the hydrolysis or reduction of such a compound during assay and its photosensitivity.

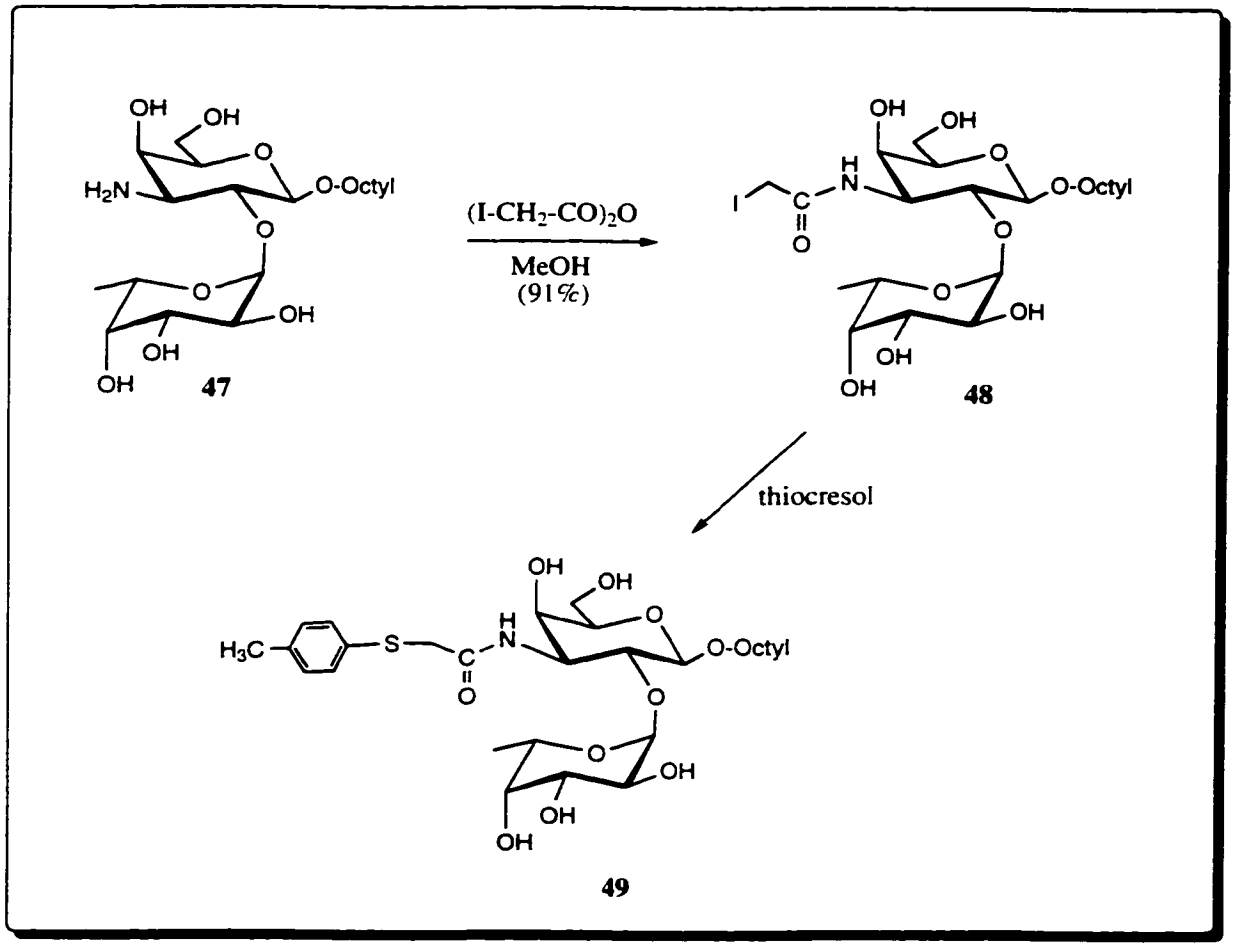
The next target was a benzoyl benzoic acid derivative, which gives a triplet biradical upon excitation that is known to abstract H efficiently (Figure 43). Since C-H bonds are cleaved more readily than O-H bonds, reaction with aliphatic residues can occur in the presence of water. This is particularly useful, as water is abundant in the environment of the enzyme under the conditions in which the experiments are performed [129].

4.2.1 *Synthesis of acceptor analogs modified at C-3 of the galactosyl residue.*

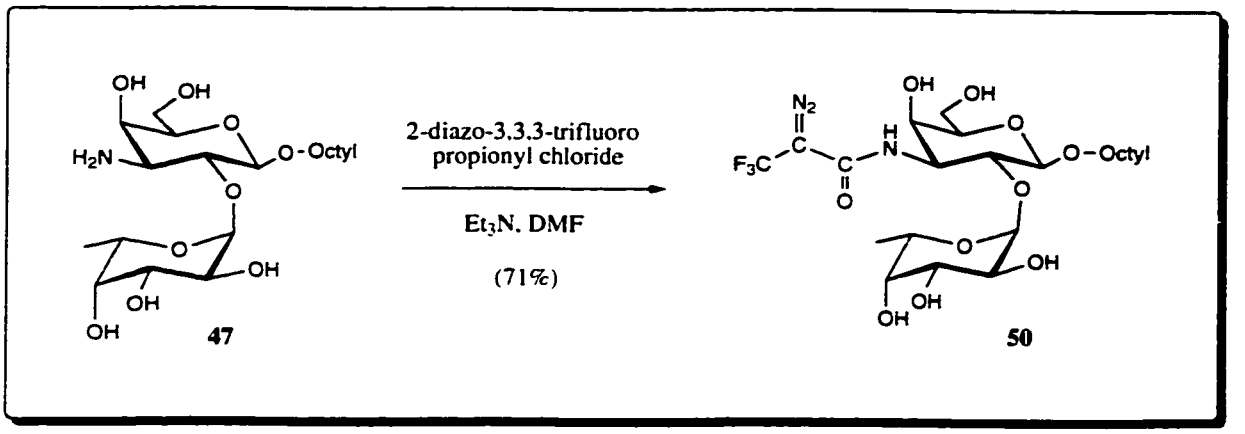
The syntheses of the modified inhibitors was accomplished using compound **47** as the starting material and employing established methodologies of coupling a carboxylic acid or acid chloride or anhydride with a primary amine in presence of a coupling reagent. The 3-iodoacetamido derivative **48** was synthesized by reacting compound **47** with iodoacetic

anhydride in methanol. This was a straightforward reaction and gave the desired compound in 91% yield (Scheme 11).

To confirm the presence of the iodoacetamido group, compound **48** was reacted with thiocresol, a nucleophile, to obtain an aromatic derivative (**49**). This compound is easily characterized by ^{1}H NMR.

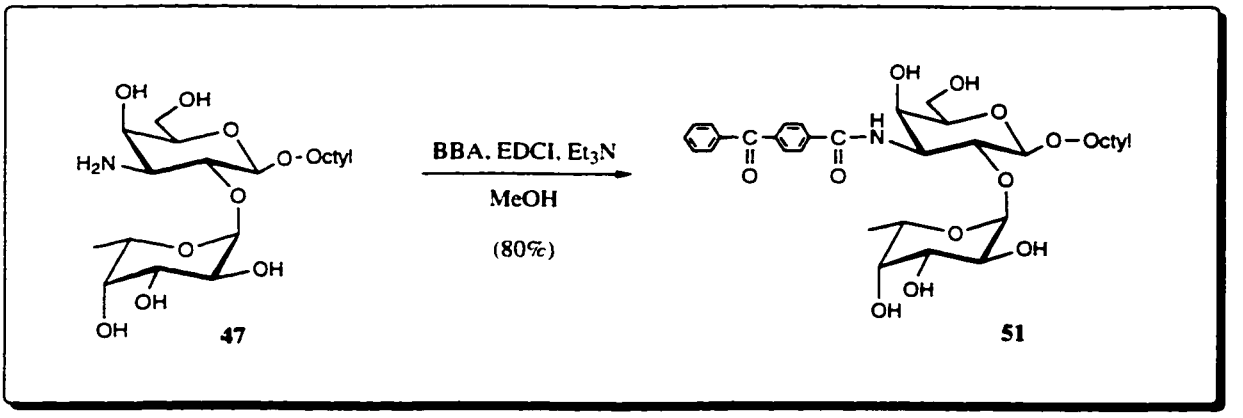


Scheme 11. **Synthesis of the iodoacetamido derivative 48.**



Scheme 12. **Synthesis of the diazo derivative 50.**

The 3-(2-azido-3,3,3-trifluoro)acetamido derivative (compound **50**) was prepared from compound **47** and 2-azido-3,3,3-trifluoro-propionylchloride in dimethylformamide (DMF) in 71% yield (Scheme 12).



Scheme 13. **Synthesis of the benzoylbenzoic acid derivative 51.**

The 3-(4-benzoyl)benzamido (BBA) derivative (**51**) was prepared from the corresponding acid and compound **47** using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDCI) as the coupling reagent in methanol and triethylamine as base in 80% yield (Scheme 13). In the absence of an activating agent, the product could be obtained only in very poor yields as activation of the carboxylic acid was required. Other carbodiimides, such as, diisopropylcarbodiimide (DIC) and dicyclohexylcarbodiimide (DCC), gave similar yields.

Table 6. **Percentage inhibition of the modified inhibitors with the A and the B transferases.**

COMPOUND	% INHIBITION	
	A Transferase	B Transferase
Compound **47**	96.7%	60%
Compound **48**	81.8%	66%
Compound **50**	0%	0%
Compound **51**	32%	38%

4.2.2 *Enzymatic evaluation*

The three synthetic derivatives prepared by modifying the 3-amino group of compound **47** were screened as potential inhibitors of the A and the B transferases. Their inhibitory

potency were in the range of 0-38% (Table 6). Thus they turned out to be poorer inhibitors than compound **47** for the A transferase and did not inhibit B transferase to any significant extent. Their K_i values were determined to be between 0.1 mM and 1.1 mM for the A enzyme and these calculations were made assuming competitive inhibition (Table 7).

Table 7. **K_i values of the synthetic inhibitors.**

COMPOUND	K_i (mM)	
	A Transferase	B Transferase
Compound **47**	0.017 mM	0.31 mM
Compound **48**	0.11 mM	0.129 mM
Compound **50**	–	–
Compound **51**	1.1 mM	0.8 mM

The decrease in inhibition upon modification of the primary amine can be attributed in all the cases to an increase in the steric bulk of the inhibitor. This could cause a change in the conformation of the compound in the active site of the enzymes, resulting in the inability of the catalytic functionality to react with the amides. In addition, all the derivatives synthesized were amides, which are not protonated as is the original amine under physological conditions.

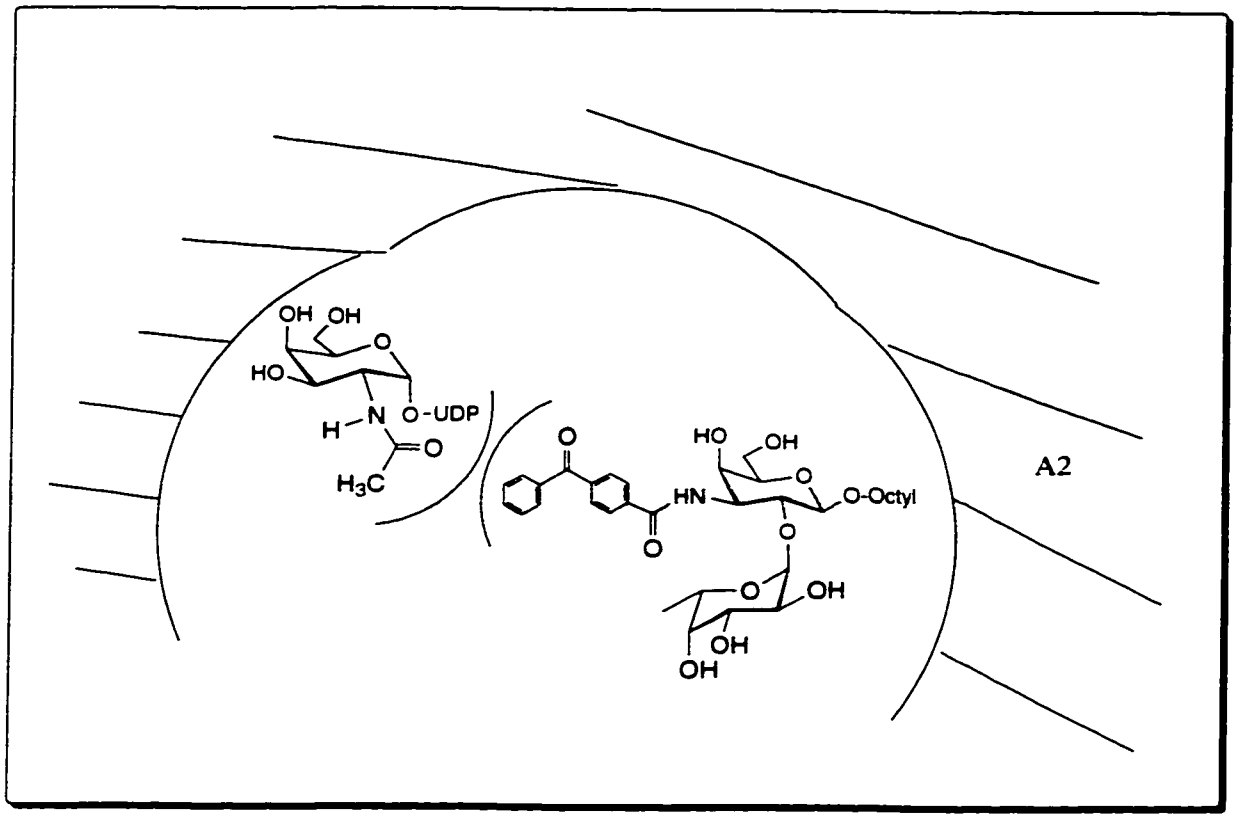


Figure 44. **Postulated interactions between the donor and the potential inhibitors in the active site of the A enzyme.**

The higher relative inhibition of the B transferase compared to the A transferase by the benzoylbenzoic acid analog can be attributed to its steric bulk. The enzyme is believed to bind to the donor before the acceptor [164] and the smaller group on C-2' of UDP-Gal compared to UDP-GalNAc implies that the B transferase has more room to bind larger substrates than A. The binding of an inhibitor with a larger group on C-3 (site of transfer) is easier for the B enzyme since it has an hydroxyl group adjacent to C-1 of donor (site of reaction) than for A which has an acetamido group (Figure 44).

The 3-iodoacetamido derivative of compound **47** was the best inhibitor among the compounds tested and was therefore used in a time dependent inhibition assay where we tried to determine if pre-incubating the modified inhibitor and the enzyme before adding the acceptor caused any increase in inhibition under the same conditions as when all three are incubated together. The assays indicated that the pre-incubation caused only marginally higher inhibition of the enzyme (data not shown). The 3-substituted compounds **48**, **50** and **51** had high K_i values (Table 7) and were judged to be inappropriate for photolabeling studies.

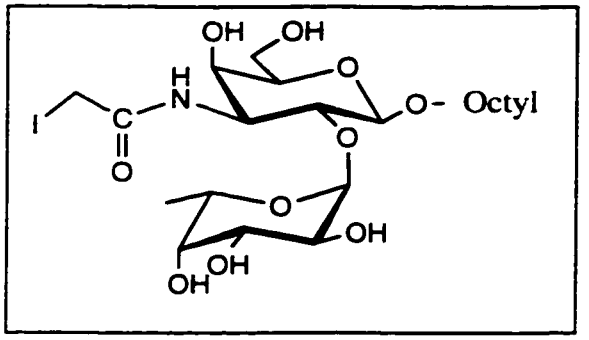
4.3 *Experimental*

4.2.2 *General methods*

The general methods used were as described in the experimental section of chapter II and chapter III.

4.3..2 *Chemical synthesis of the modified inhibitors*

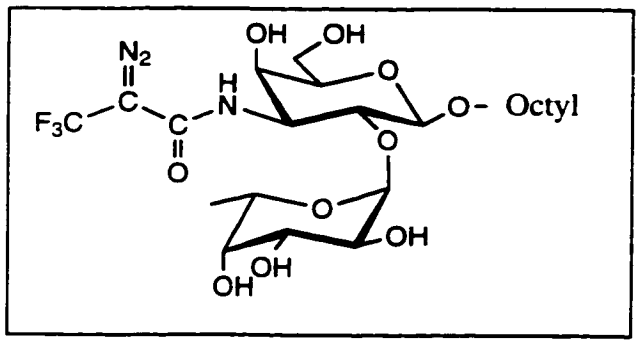
Octyl 3-deoxy-3-(2-iodoacetamido)-2-*O*-(α-L-fucopyranosyl)-β-D-galactopyranoside (**48**).



Octyl 3-amino-3-deoxy-2-*O*-(α-L-fucopyranosyl)-β-D-galactopyranoside (**47**) (12 mg; 0.027 mmole) was dissolved in dry methanol (1 ml) and iodoacetic anhydride (0.0194 g; 2 equivalent) was added to it and the mixture stirred at room temperature for 30 min. After completion, cold water was added to hydrolyze the unreacted anhydride and the solvent evaporated and coevaporated in vacuo with toluene. It was dissolved in water and loaded onto C-18 Sep-Pak, washed with water, and then eluted with 50% methanol. The solvent was evaporated, the product re-dissolved in water, and passed through 0.22 μm Millipore filter. The sample was then lyophilized to give a white powder. Yield 15 mg (91%); R_f (CH_2Cl_2:MeOH 5:2) 0.61. ^{1}H NMR (CD_3OD): δ 5.09 (d, 1 H, J 1',2' 2.6 Hz, H-1'), 4.48 (d, 1 H, J 1,2 7.7 Hz, H-1), 4.32 (q, 1 H, J 5',6' 6.5 Hz, H-5'), 4.06 (dd, 1 H, $J_{2,3}$ 10.0 Hz, $J_{3,4}$ 3.0 Hz, H-3), 3.98 (m, 1 H, C*H*$_2$O), 3.92 (d, 1 H, $J_{3,4}$ 3.0 Hz, H-4), 3.85 (m, 1 H, H-6a), 3.79 (d, 1 H, $J_{2,3}$ 10.0 Hz, $J_{1,2}$ 7.7 Hz, H-2), 3.76 (m, 5 H, H-2', H-5, H-6b, IC*H*$_2$CO x2), 3.69 (bs, 1 H, H-4'), 3.61 (m, 2 H, C*H*$_2$O), 1.55 (m, 2 H, C*H*$_2$CH$_2$O), 1.4 (bs, 10 H, C*H*$_2$ octyl), 1.23 (d, 2 H, $J_{5'6'}$ 6.6 Hz, H-6'), 0.95 (t, 3 H, C*H*$_3$ octyl); ^{13}C NMR (CD_3OD): 171.4 (*C*ONH), 104.3 (C-1), 101.0 (C-1'), 77.3, 75.7, 73.6, 71.6, 70.8, 70.4, 68.6, 68.0, 62.3, 57.2 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', *C*H$_2$O), 33.0, 31.4, 30.6, 30.4, 27.3, 23.7 (*C*H$_2$ octyl), 16.8 (C-6'), 14.4 (*C*H$_3$ octyl), -1.3 (*C*H$_2$I). ESMS for $C_{22}H_{40}NO_{10}INa$: Calc. 628.159469. Found 628.159877.

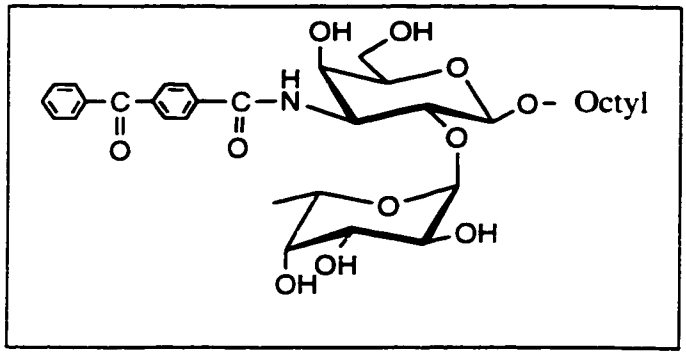
In order to determine the presence of the electrophilic iodomethelene–moiety, 2 mg of compound **48** was reacted with 0.8 mg of thiocresol and trace amount of Et_3N in MeOH. Purification of the reaction mixture was done on C-18 Sep-Pak as before. The product (**49**) was lyophilized and characterized to confirm the formation of the parent compound. ^{1}H NMR (CD_3OD): δ 7.35 (d, 2 H, J_{ortho} 8.4 Hz, Ar*H*), 7.13 (d, 2 H, J_{ortho} 8.4 Hz, Ar*H*), 5.31 (d, 1 H, $J_{1',2'}$ 3.8 Hz, H-1'), 4.38 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1), 4.24 (q, 1 H, $J_{5',6'}$ 6.6 Hz, H-5'), 4.03 (dd, 1 H, $J_{2,3}$ 10.3 Hz, $J_{3,4}$ 3.0 Hz, H-3), 3.88 (dt, 1 H, J_{gem} 10.0 Hz, J_{vic} 7.0 Hz, C*H*$_2$O), 3.76 (d, 1 H, $J_{3,4}$ 3.0 Hz, H-4), 3.64-3.71 (m, 3 H, H-2, H-5, H-6a), 3.58-3.64 (m, 5 H, H-2', H-3', H-4', SC*H*$_2$CO x2), 3.51-3.57 (m, 2 H, H-6b, C*H*$_2$O), 2.3 (s, 3 H, ArC*H*$_3$), 1.54-1.64 (m, 2 H, C*H*$_2$CH$_2$O), 1.24-1.38 (m, 10 H, C*H*$_2$ octyl), 1.16 (d, 2 H, $J_{5'6'}$ 6.6 Hz, H-6'), 0.90 (t, 3 H, C*H*$_3$ octyl); ^{13}C NMR (CD_3OD): 171.9 (*C*ONH), 139.0, 135.0 (Ar*C*), 130.8, 129.8 (Ar*C*H), 104.2 (C-1), 101.2 (C-1'), 77.4, 76.0, 73.6, 71.6, 70.8, 70.30, 69.1, 68.0, 62.3, 56.6 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', *C*H$_2$O), 39.6 (S*C*H$_2$CO), 33.0, 30.9, 30.5, 30.4, 27.2, 23.7 (*C*H$_2$ octyl), 21.0 (Ar*C*H$_3$), 16.8 (C-6'), 14.4 (*C*H$_3$ octyl). ESMS for $C_{29}H_{47}NO_{10}SNa$: Calc. 624.281839. Found 624.282063.

Octyl 3-deoxy-3-(2–azido-3,3,3-trifluoro) propionamido-2-*O*-(α-L-fucopyranosyl)-β-D-galactopyranoside (**50**).



Octyl 3-amino-3-deoxy-2-*O*-(α-L-fucopyranosyl)-β-D-galactopyranoside (7 mg; 0.41 mmole) was dissolved in DMF (1 ml) and 2-diazo-3,3,3-trifluoro-propionyl chloride (7 mg; 0.041 mmole) was added to it. Triethylamine was added and the mixture stirred for 15 min. The reaction mixture was evaporated in *vacuo* and passed through C-18 Sep-Pak as described before. Lyophilization yielded a pale yellow powder (7 mg; 71%). Selected ^{1}H NMR data (CD_3OD): δ 5.45 (d, 1 H, $J_{1',2'}$ 2.74 Hz, H-1'), 4.67 (dd, 1 H, $J_{2,3}$ 10.68 Hz, $J_{3,4}$ 3.05 Hz, H-3), 4.66 (d, 1 H, $J_{1,2}$ 7.63 Hz, H-1), 4.54 (q, 1 H, $J_{5',6'}$ 6.6 Hz, H-5'), 3.88 (dt, 1 H, $J_{2',3'}$ 10.68 Hz, $J_{1',2'}$ 7.63 Hz, H-2), 1.54-1.64 (m, 2 H, CH_2CH_2O), 1.24-1.38 (m, 10 H, CH_2 octyl), 1.16 (d, 2 H, $J_{5',6'}$ 6.6 Hz, H-6'), 0.90 (t, 3 H, CH_3 octyl); ^{13}C NMR (CD_3OD): 169.7 (*C*ONH), 153.9, 150.1, 104.4 (C-1), 100.2 (C-1'), 88.4, 77.7, 75.9, 73.6, 71.7, 70.8, 70.1, 67.8, 68.0, 62.4, 61.6 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', (CH_2O), 33.0, 30.9, 30.5, 30.4, 27.2, 23.7 (CH_2 octyl), 16.7 (C-6'), 14.4 (CH_3 octyl). ESMS for $C_{23}H_{38}N_3O_{10}F_3Na$: Calc. 596.240699. Found 596.241447.

Octyl 3-deoxy-3-(4-benzoyl) benzamido-2-*O*-(α-L-fucopyranosyl)-β-D-galacto-pyranoside (**51**).



Compound **47** (5.2 mg; 0.01 mmole) was dissolved in methanol (1 ml) and Et_3N (85 μL) was added. 4-Benzoyl benzoic acid (10 mg; 0.02 mmole) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) (44 mg; 0.23 mmole) were added and the mixture stirred overnight. After completion of the reaction as determined by TLC, the reaction mixture was diluted with CH_2Cl_2 and washed with water. After drying and evaporation of the solvent, column chromatography on Iatrobeads with CH_2Cl_2-MeOH (20:1, 10:1) gave 5.5 mg of compound **51** (80%); R_f (CH_2Cl_2:MeOH 30:3) 0.49. ^{1}H NMR (CD_3OD): δ 8.16 (d, 2 H, J_{ortho} 8.9 Hz, Ar*H*), 7.08 (d, 2 H, J_{ortho} 8.9 Hz, Ar*H*), 7.82 (m, 3 H, Ar*H*), 7.65 (m, 2 H, Ar*H*), 5.12 (d, 1 H, $J_{1',2'}$ 3.8 Hz, H-1'), 4.49 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.23 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 4.21 (dd, 1 H, $J_{2,3}$ 10.0 Hz, $J_{3,4}$ 3.0 Hz, H-3), 3.13 (d, 1 H, $J_{3,4}$ 3.0 Hz, H-4), 3.94 (m, 2 H, H-6a, C*H*$_2$O), 3.64-3.71 (m, 2 H, H-5, H-6b), 3.56-3.68 (m, 5 H, H-2, H-2', H-3', H-4', C*H*$_2$O), 1.52-1.63 (m, 2 H, C*H*$_2$CH$_2$O), 1.27-1.36 (m, 10 H, C*H*$_2$ octyl), 1.19 (d, 2 H, $J_{5',6'}$ 6.5 Hz, H-6'), 0.93 (t, 3 H, C*H*$_3$ octyl); ^{13}C NMR (CD_3OD): 102.1 (*C*O), 170.2 (*C*ONH), 139.1, 134.1, 134.1 (Ar*C*), 131.0, 130.9, 130.7, 130.6, 129.6, 128.8, 128.6 (Ar*C*H), 104.7 (C-1), 101.4 (C-1'), 77.3, 76.1, 73.7, 71.6, 71.0, 70.6, 68.5, 68.3, 62.4, 57.6 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', *C*H$_2$O), 33.0, 30.9, 30.6, 30.4, 27.2, 23.7 (*C*H$_2$ octyl), 16.8 (C-6'), 14.4 (*C*H$_3$ octyl). ESMS for $C_{34}H_{47}O_{11}N_1Na_1$: Calc. 668.304682. Found 668.304401.

4.2.2 *Enzymatic testing*

The modified inhibitors were tested as inhibitors of both the A and the B glycosyltransferases. The radiochemical assays were performed as described in chapter II. All the assays were done in a total volume of 33 µl in buffer containing 35 mM sodium cacodylate, 15 mM $MnCl_2$, 1 mg/ml BSA, pH 6.9.

For the A transferase, 0.1 µl of the enzyme (0.165 mg/ml) was used with 0.2 µCi UDP-[6-^{3}H]-GalNAc and 0.15 mM UDP-GalNAc. The native acceptor of the enzymes was present at a concentration of 0.1 M and the potential inhibitors were at a concentration of 1 M. For the B transferase, 0.01 µL of the enzyme (0.997 mg/ml) was used with 0.2 µCi UDP-[6-^{3}H]-Gal and 0.6 mM UDP-Gal. The native acceptor of the enzymes was present at a concentration of 0.05 M and the potential inhibitors were at a concentration of 0.5 M.

The reaction mixtures were incubated for 30 min. and then quenched with water. They were transferred onto C-18 Sep-Pak cartridges that had been pre-equilibrated with methanol and water. The cartridges were washed with water until background counts were obtained. The radiolabeled product was eluted with 3.5 ml methanol and the eluent counted in a liquid scintillation counter where the radioactivity was measured in dpm. The loss in radioactivity in the presence of an inhibitor was measured as a percentage of the total radioactivity of the control assay mixture when only the native acceptor is present.

Chapter V

SYNTHESIS OF PHOTOAFFINITY LABELED ACCEPTOR ANALOGS MODIFIED ON THE AGLYCON

5.1 *Introduction*

This chapter describes the synthesis of aglycon derivatives of the native acceptor of the blood group A and B transferases. The photoaffinity analogs were used to bind to the blood group A glycosyltransferase in its active site and photolabel it.

5.2 *Use of photoaffinity acceptor analogs*

As described in chapter IV, photolabeling of the A and the B enzymes using the photoaffinity analog inhibitors was not attempted due to their low affinity for the enzymes. For successful photolabeling, submicromolar binding constants for the analogs are desirable. It was therefore decided that the native disaccharide derivatized in the aglycon with benzoylbenzoic acid would be used because of the advantages such compounds present as discussed in chapter I.

The glycoprotein or the glycolipid aglycon on the naturally occuring αFuc(1,2)βGal substrate structure is not required for recognition of the acceptor by the A or the B transferases in *in vivo* assays. Thus, in all the previous syntheses of the acceptor analogs, we had used an octyl arm as the aglycon, taking advantage of its hydrophobicity to purify the compounds on C-18 Sep-Pak columns, and to test the compounds in radioactive Sep-Pak assays.

5.2.1 *Determination of the role of the aglycon in the recognition of the acceptor*

Since attaching photoactive groups to the 3-amino group of the inhibitor **47** resulted in loss of acceptor recognition, we examined whether these would be better tolerated on the aglycon. Before preparing such photo-probes, we needed to determine whether changing the aglycon from octyl to other groups would be tolerated by these enzymes.

We therefore tested the disaccharides [165, 166], αFuc(1,2)Gal-*O*H (**52**), αFuc(1,2)βGal-*O*Me (**53**), as acceptors for both these enzymes. Since these compounds lack the hydrophobic octyl aglycon, the Sep-Pak method could not be used to determine the extent of transfer of tritiated sugar-nucleotide donor by the enzymes. A different protocol for quantitation had to be adopted [167]. We used ion-exchange columns to elute the products of transfer and counted them in a liquid scintillation counter. Biorad $AG_1^{®}$-X8 anion-exchange resin columns effectively retained negatively charged sugar nucleotides (UDP-[6-^{3}H]GalNAc and UDP-[6-^{3}H]Gal). The results of the assays are shown in Table

8 which shows that the methyl glycoside is as active as the octyl glycoside with the A transferase. There is marginal decrease in activity with the B transferase. Compound **52**, however, shows only about 50% activity with the A transferase. One explanation for this could be that only one of the two anomers of this compound is recognized as an acceptor by this enzyme. Activity with the B enzyme is about 70% of the activity of the octyl glycoside.

Table 8. **Percentage activities of the native disaccharides with different aglycons.**

COMPOUNDS	% ACTIVITY	
	A Transferase	B Transferase
αFuc(1,2)βGal-OOctyl (**1**)	100%	100%
αFuc(1,2)βGal-OMe (**53**)	100%	88%
αFuc(1,2)Gal-OH (**52**)	54%	71%

5.2.2 *Design of the modified analogs*

As seen from the data in Table 8, the aglycon structure has only a modest effect on the activity of the native disaccharide with the A and the B transferases. We therefore, decided to install the photoactivable benzoylbenzoic acid group on the aglycon. We believed that the aromatic moiety would not significantly alter the binding of these disaccharides in the active site.

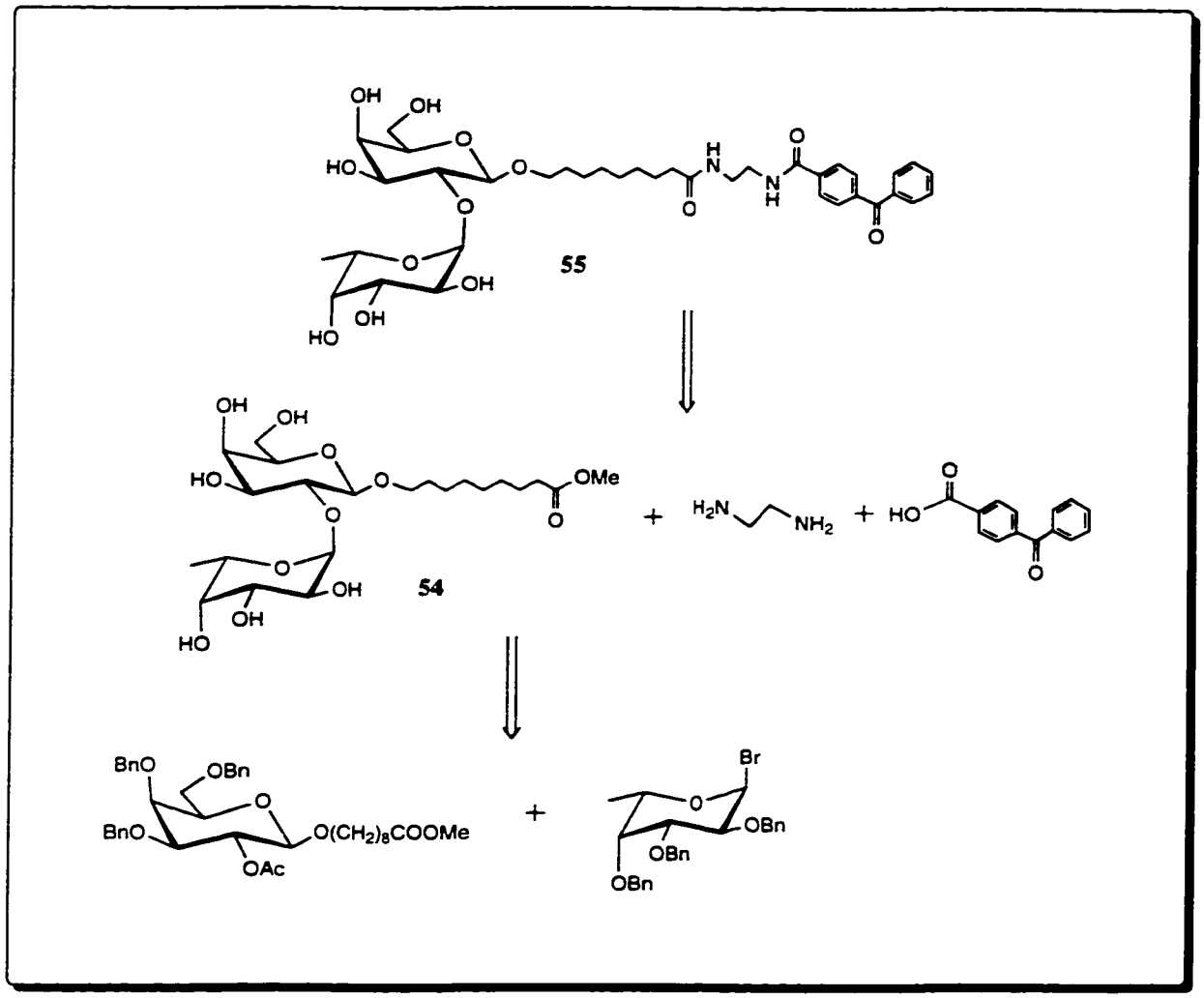


Figure 45. **Retrosynthetic analysis of photoaffinity analog 55.**

To prepare the disaccharide **55**, the disaccharide with the 8-methoxycarbonyl octyl arm (**54**) was deemed suitable since the ester carbonyl would provide the necessary electrophilic center to react with a diamino compound. The second amino functionality would be used to react with benzoylbenzoic acid (Figure 45).

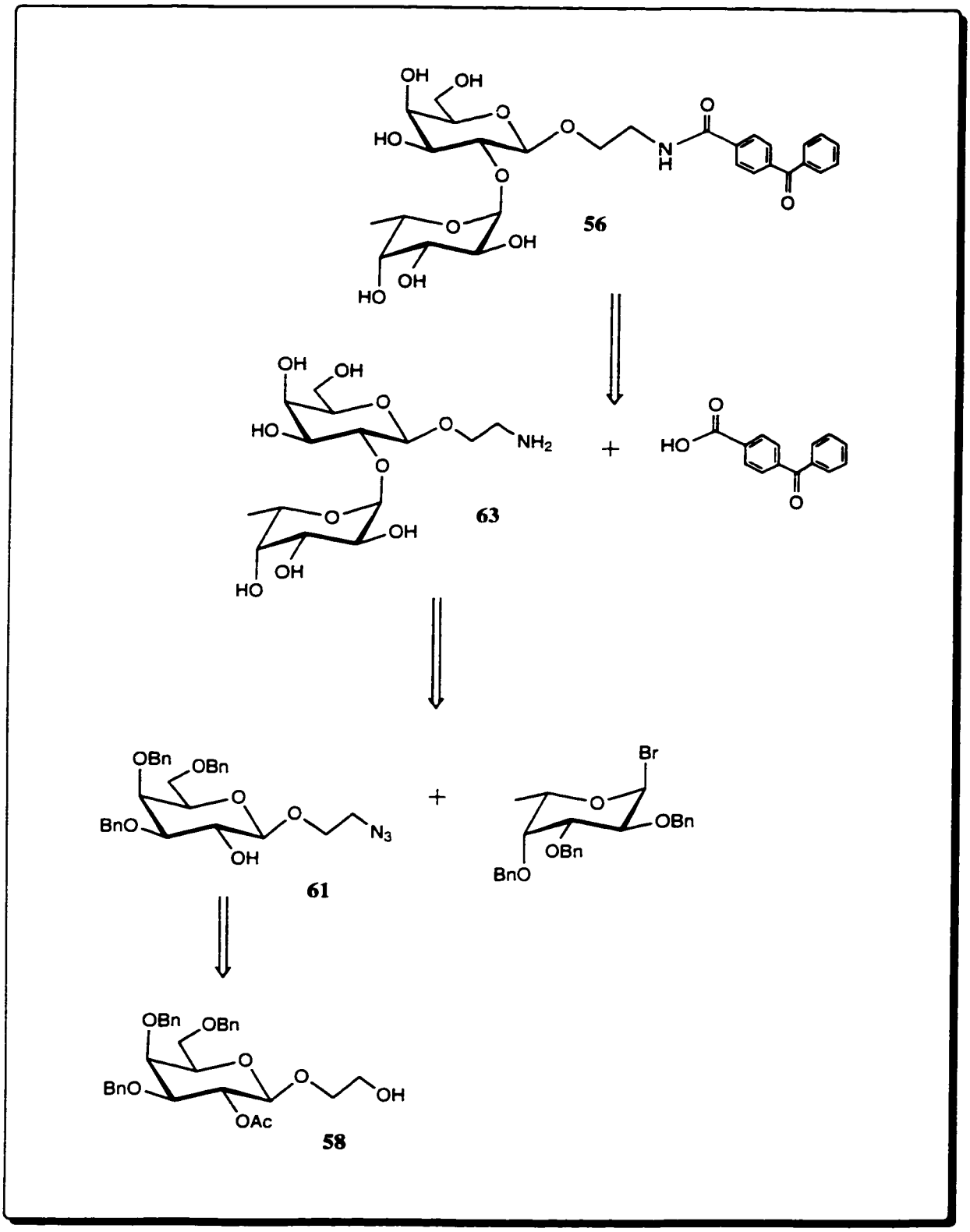


Figure 46. **Retrosynthetic analysis of photoaffinity analog 56.**

The shorter aglycon could be obtained by coupling an aminoethane moiety with benzoylbenzoic acid. Ethanediol was used to first form the galactoside, and then the other hydroxyl was converted to an azido group. Fucosylation followed by hydrogenation afforded the reqiured acceptor **56** (Figure 46).

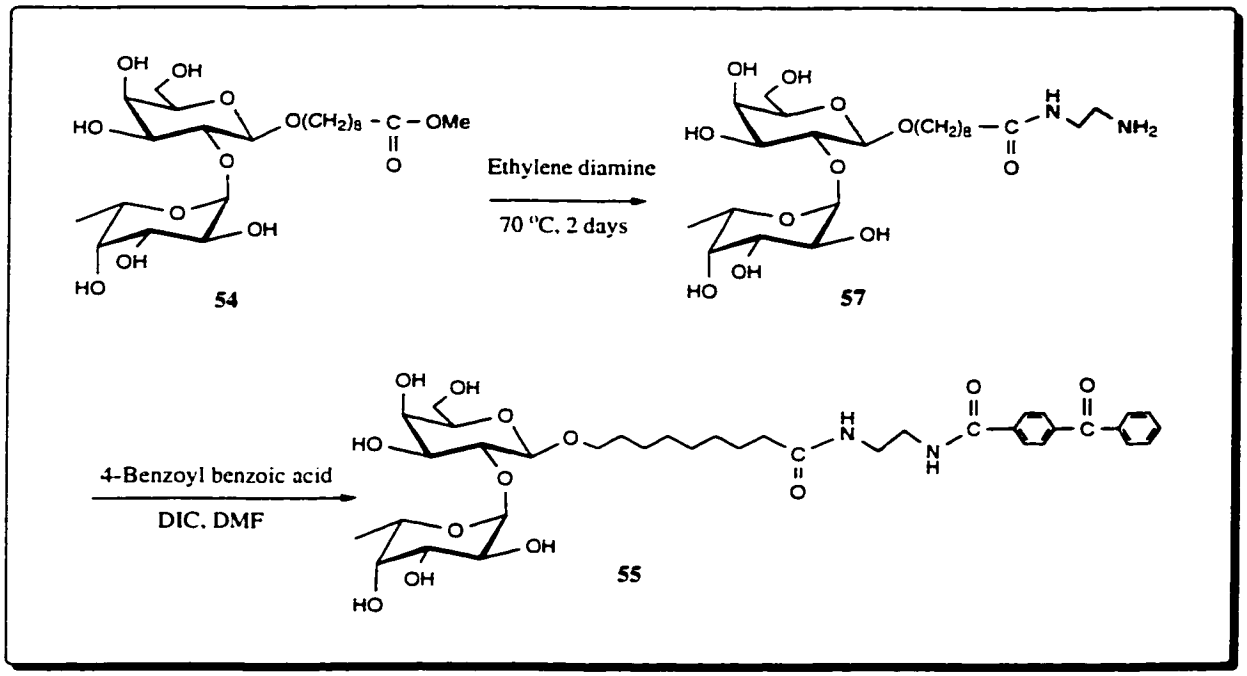
5.2.3 *Computational studies on the modified acceptors*

In order to determine the length of the modified aglycons and the distance of the carbonyl chromophore from the site of transfer, we performed molecular modeling of the disaccharides that we had designed by computer generated simulations of the minimum energy conformations of these structures.

The Insight © II molecular modeling system was used to view and rotate the molecules. The molecules were built using the Biopolymer module within the Insight suite of software. After the molecules were built, they were minimized using the Discover® module within Insight. The force field used for the minimization was AMBER PLUS. The aglycon did not seem to have any significant effect on the conformation of the disaccharides, which was confirmed by their ^{1}H NMR data. As measured from the minimized structures obtained from the computer simulations, the distance between the carbonyl on the benzophenone moiety and the 3-OH of the acceptor (site of transfer) is about 11 Å for compound **55** and about 25 Å for compound **56**.

5.2.4 *Synthesis of the modified acceptor analogs*

The known disaccharide **54** was used to prepare the amino compound **57** by heating it at 70 °C for two days with 1,2-diaminoethane [168,169].

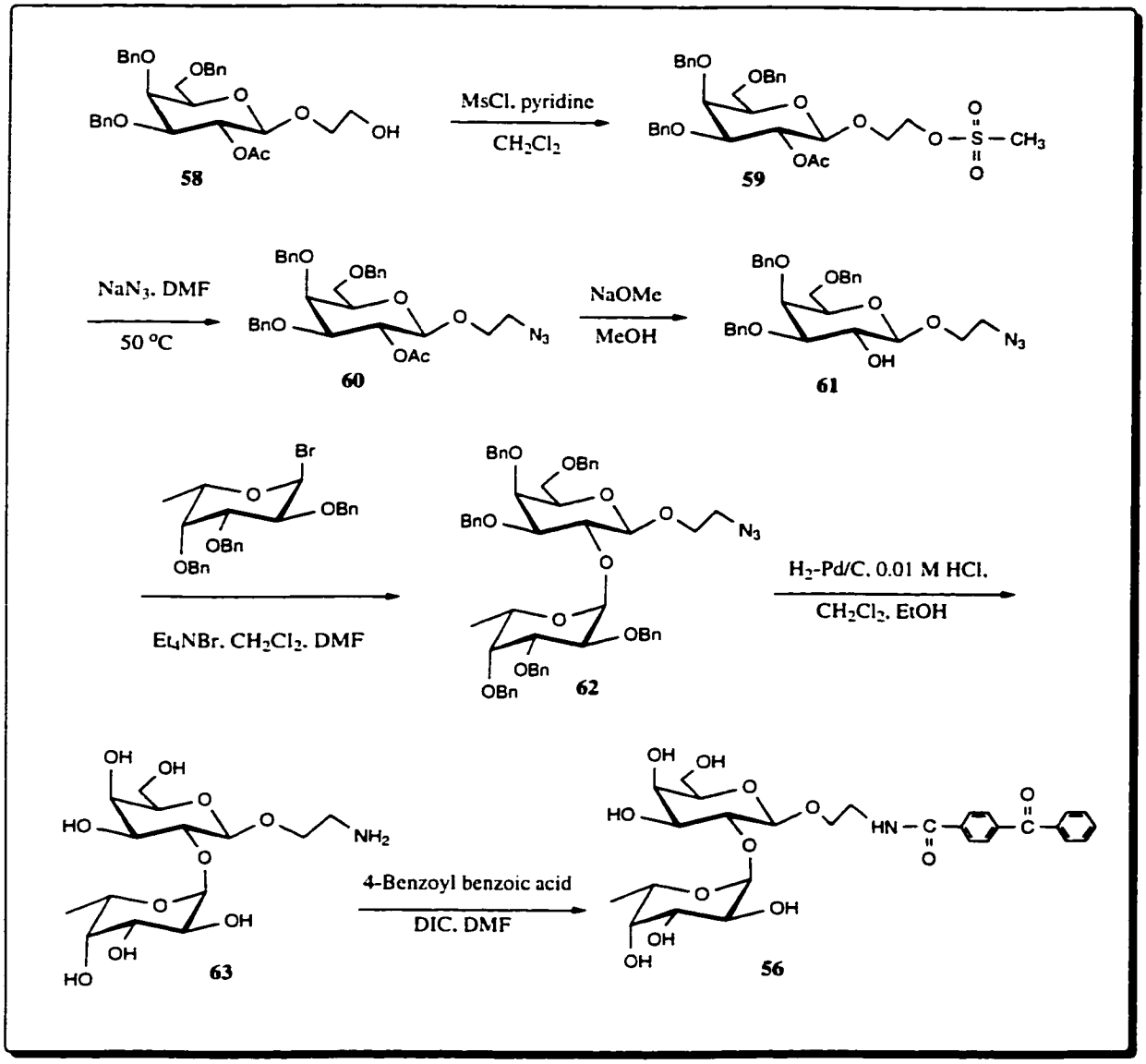


Scheme 14. **Synthesis of compound 55.**

Compound **57** was coupled with 4-benzoylbenzoic acid in presence of DIC and 1-hydroxybenzotriazole (HOBt) in DMF to give compound **55** in 70% yield. Formation of the amide required the presence of HOBt in the reaction mixture (Scheme14).

The synthesis of compound **56** started with 2-*O*-actyl-3,4,6-tri-*O*-benzyl-D-galactopyranosyl bromide (**23**) which was coupled with ethylene glycol in acetonitrile under Helferich conditions with mercuric cyanide in 75% yield. The hydroxyethyl

glycoside thus formed (**58**) was mesylated with methanesulfonyl chloride in pyridine in 95% yield to give compound **59**. It was directly heated at 50 °C overnight with sodium azide in DMF to give the azidoethyl glycoside **60** in 83% yield. Zemplen deacetylation



Scheme 15. **Synthesis of compound 56.**

(to give compound **61**), followed by fucosylation with 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl bromide [147, 170] under halide ion catalyzed conditions gave the protected disaccharide **62** in 72% yield. Hydrogenolysis with H_2 and palladium on charcoal in ethanol-dichloromethane (4:1) and 100 μL 1 M HCl occurred along with reduction of the azido group and yielded the desired amino compound **63** (82%). Compound **63** was coupled with 4-benzoylbenzoic acid as before to give the target compound **56** in 80% yield (Scheme 15). Activation of the carboxylic acid did not require HOBt for this reaction. Although DIC was used, other coupling reagents such as EDCI and DCC gave similar yields.

Table 9. **Percentage activities of compounds 55 and 56 relative to the native acceptor 1.**

COMPOUNDS	% ACTIVITY	
	A Transferase	B Transferase
αLFuc(1,2)βDGal-O$(CH_2)_8CH_3$ (Compound **1**)	100%	100%
αLFuc(1,2)βDGal-O$(CH_2)_8CONHCH_2CH_2NHCOC_6H_4COC_6H_5$ (Compound **55**)	100%	82%
αLFuc(1,2)βDGal-O$CH_2CH_2NHCOC_6H_4COC_6H_5$ (Compound **56**)	100%	75%

5.2.5 *Enzymatic evaluations*

Compounds **55** and **56** were screened as acceptors for both the glycosyltransferases (Table 9) and they were found to be good acceptors of the A enzyme. The B transferase, however was less tolerant of the modification, as has been shown in chapter II. It was

Table 10. **Kinetic constants for the modified disaccharides. (ND indicates not determined)**

COMPOUNDS	K_m(μM)	
	A Transferase	B Transferase
αFuc(1,2)βGal-O$(CH_2)_8CH_3$ (Compund **1**)	12.9±1.2 μM	110±12 μM
αFuc(1,2)βGalO$(CH_2)_8CONHCH_2CH_2NHCOC_6H_4COC_6H_5$ (Compound **55**)	39.7±5.4 μM	ND
αFuc(1,2)βGal-O$CH_2CH_2NHCOC_6H_4COC_6H_5$ (Compound **56**)	90±20 μM	ND

therefore decided to use the A enzyme for photoaffinity labeling experiments. Compounds **55** and **56** had K_m values of about 40 and 90 μM respectively with the A transferase (Table 10). Compound **55** showed a four-fold increase in the K_m value and it had a lower V_{max} value compared to the native acceptor (0.38 nmoles/min/μg).

Compound **56** had a V_{max} of 0.54 nmoles/min/μg. Since both the compounds have similar affinities for the A transferase, we decided to use compound **56** in photolabeling experiments because it has a shorter arm and the probe would therefore be closer to the active site.

5.2.6 *Irradiation experiments with the A transferase*

Having determined the kinetic constants of the acceptors **55** and **56** with the A transferase, these compounds were used to label the enzyme, potentially in its active site. For the enzyme to bind to the acceptor, the presence of the donor is necessary [164]. We could not use UDP-GalNAc, since the enzyme would turn it over to form the trisaccharide product faster than the rate at which labeling might occur. UDP is a strongly binding inhibitor of the enzyme with a K_i value of 8.6 μm [171], and was considered a suitable replacement for the donor. Thus the enzyme and the acceptor were incubated with UDP and irradiated with UV light at 365 nm.

To determine the effect of UV light on the enzyme, the irradiated mixture containing the enzyme, UDP, and the photoaffinity analog was used to perform a standard radiochemical assay with the native acceptor and donor. Loss in activity of the enzyme (indicated by lower dpm value) in the presence of the photoaffinity analog would have to be caused by covalent linkage between this analog and the enzyme. It was first

determined that irradiation of the enzyme by itself does not cause loss of enzyme activity (data not shown).

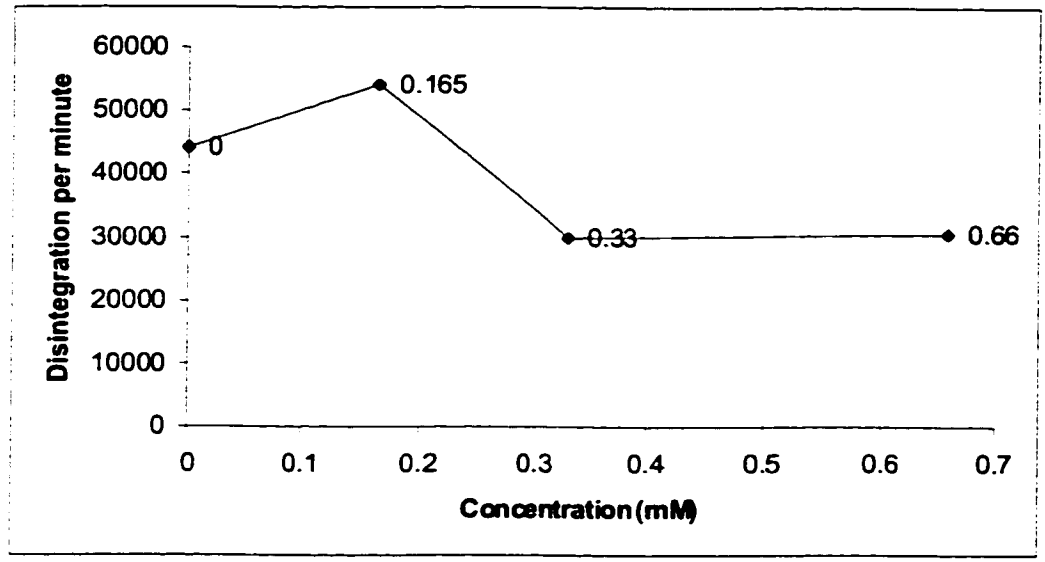


Figure 47. **Concentration dependence of the inactivtion of A2 by irradiation with UV light.**

These experiments were performed at different concentrations of the acceptor [172] to determine if photolabeling followed saturation kinetics (Figure 47). Different irradiation times were also used to find the optimum conditions for these experiments (Figure 48). The results show that inactivation of the enzyme due to irradiation in the presence of the acceptor followed saturation kinetics.

Maximum inactivation was achieved when the modified acceptor was used at concentrations of 0.3 mM to 0.6 mM (Figure 47). No inactivation was observed when the

assays were performed either in the presence of the native acceptor, or no acceptor was used during UV radiation. The reaction was found complete after 30 minutes (Figure 48).

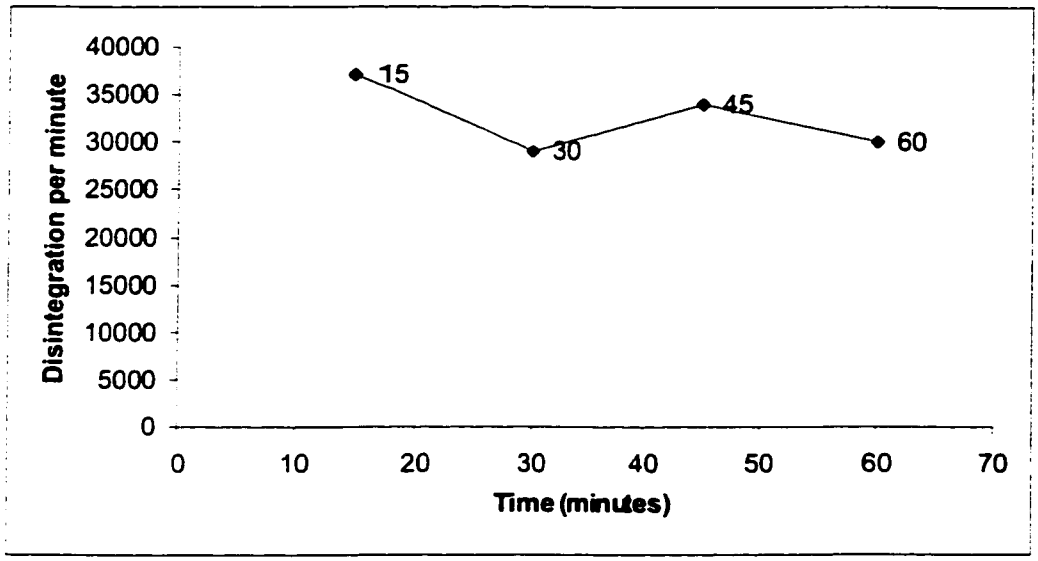


Figure 48. **Effect of time of irradiation on the inactivation of A2.**

5.2.7 *Radioisotope labeling of the A transferase*

Several techniques have been used to quantitate the percentage of the enzyme labeled [172-174]. In most cases, a radioisotope is incorporated in the photolabel that can be later identified. Our primary tag, the disaccharide, was not radiolabeled since our objective was to isolate enzymes linked to the disaccharide utilizing immobilized lectins that recognize the O(H) antigenic disaccharide. To perform a preliminary assay of the reaction mixture to determine the success and the extent of photolabeling, we used a

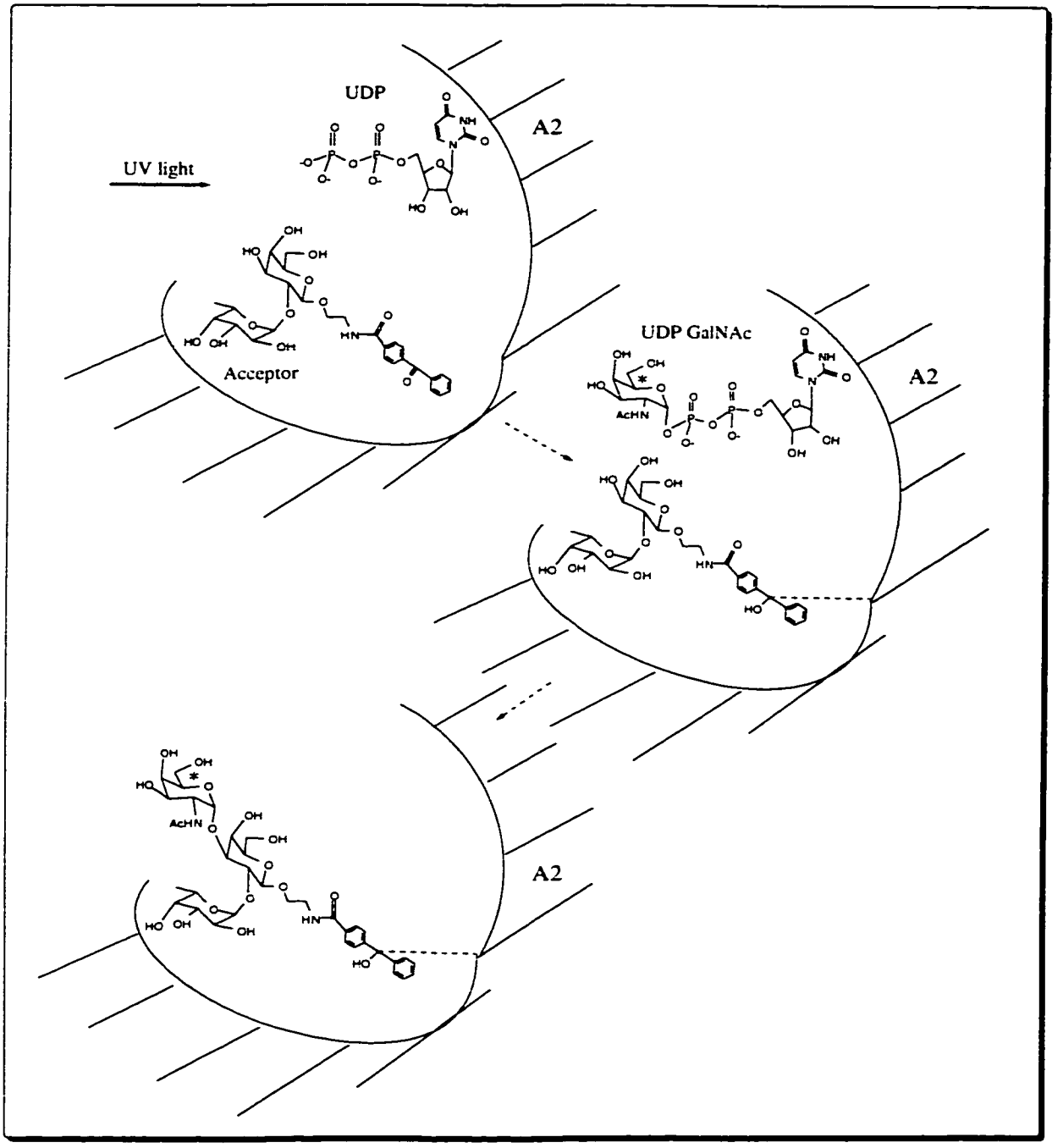


Figure 49. **Schematic representation of radiolabeling of the A transferase.**

second enzymatic reaction to incorporate tritium into the enzyme as shown in Figure 49. UDP-[6-^{3}H]-GalNAc was used as the donor to be transferred to the acceptor by the enzyme (added in excess after photolabeling) after the acceptor was covalently attached to the enzyme. After irradiation, the mixture was dialyzed to remove UDP as it is inhibitory towards the enzyme. UDP-[6-^{3}H]-GalNAc, UDP-GalNAc and the A enzyme were added and the mixture incubated at 37 °C for 30 min to incorporate the radioisotope (Figure 49). The mixture was further dialyzed to remove all UDP-[6-^{3}H]-GalNAc, and then the radioactivity counted. If the enzyme was indeed covalently attached to the disaccharide, it would be radiolabeled in the second reaction, and show high radioactivity. If there is no covalent labeling, only background counts would be obtained.

Table 11. **Relative incorporation of radioisotope into the A transferase upon irradiation with UV light in the presence and absence of the photoaffinity analog.**

CONDITION FOR THE ASSAY	RELATIVE RADIOACTIVITY
Compound **56**	10
Compound **56** with UV Radiation	100
Native Acceptor (compound **1**)	9
Native Acceptor (compound **1**) with UV Radiation	21
Buffer	140
Buffer with UV Radiation	95

As seen from Table 11, the enzyme was radiolabeled in the presence of the photoaffinity analog, but not in the presence of the native acceptor. It is difficult to speculate as to why such a high incorporation of radioisotope into the enzyme is observed in the control experiment (buffer only). This could be due to non-specific binding of the donor with the enzyme in the absence of an acceptor when the combining site of the enzyme is unoccupied, or a due to side reactions of the donor or its degraded products with the enzyme.

5.2.8 *Lectin affinity chromatography*

Lectins have been known to bind to animal cell glycoconjugates and induce the agglutination of animal cells, and they have been extensively studied, most notably by N. Sharon's group [175]. The inhibition of this lectin-induced agglutination by various carbohydrate residues has been used to define the carbohydrate binding specificities of lectins. Lectins can selectively agglutinate specific blood groups and are used for the isolation and characterization of blood group antigens [176]. Immobilized lectins have been used extensively for the affinity purification of glycoconjugates [177, 178]. The lectin bound molecules are eluted with high concentrations of simple sugars. We expected that the lectins that agglutinate the blood group O(H) antigenic structures might also recognize the disaccharide attached to the enzyme through photoaffinity labeling. Therefore, upon passing the reaction mixture containing the enzyme (bound to the photo-

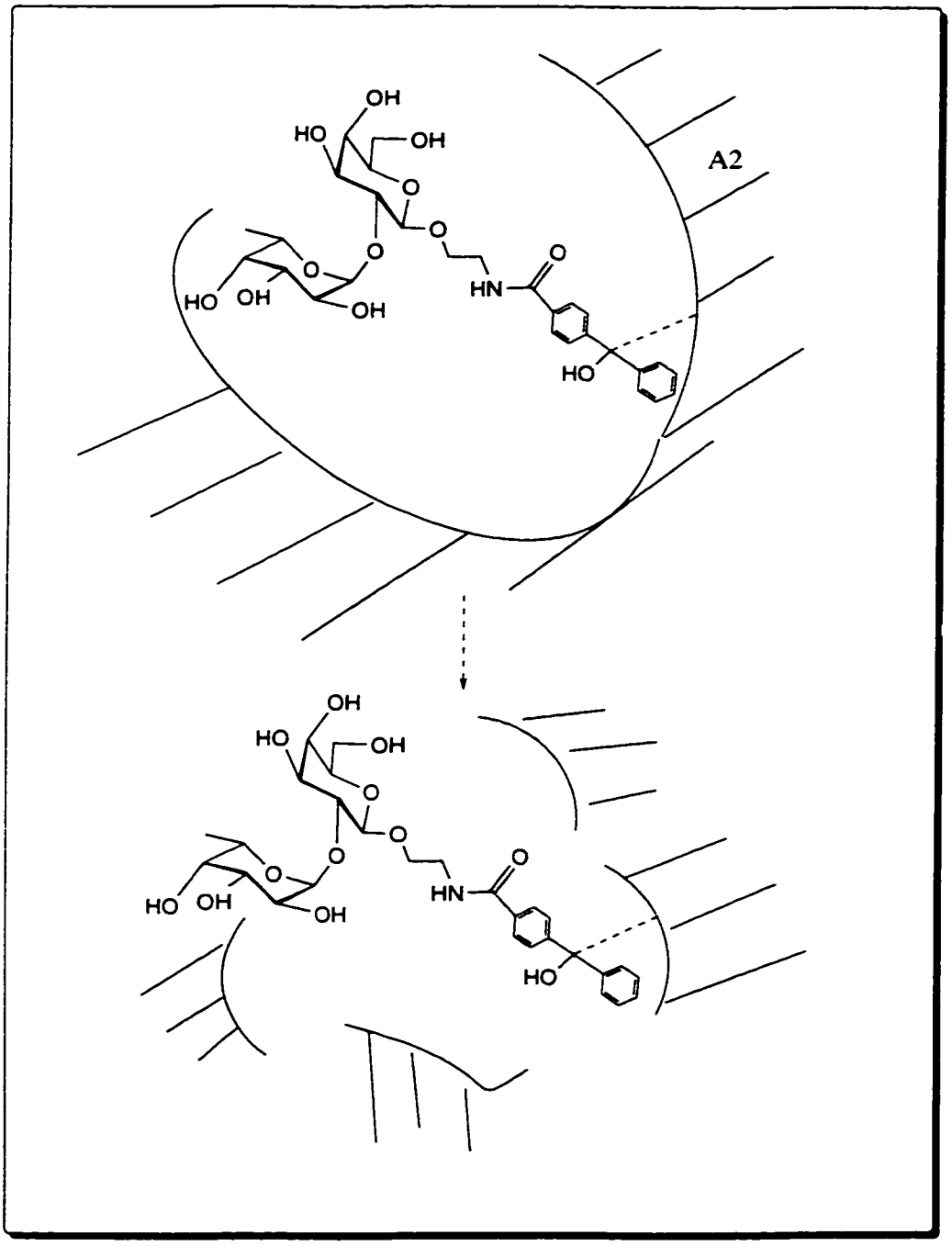


Figure 50. **Schematic representation of tryptic digestion of a photolabeled protein to obtain peptide fragments covalently attached to the disaccharide.**

affinity analog disaccharide) through the immobilized lectin, those protein molecules that are photo-labeled would be retained, and those that are not would come out with the flow-through. The bound conjugates could then be eluted by the sugars specific to the lectins. There are various groups of lectins categorized based on their carbohydrate specificity. We decided to use two L-fucose binding lectins, *Lotus tetragonolobus* (Lotus) isolated from Asparagus Pea [179], and *Ulex europeus* I (Ulex) isolated from Gorse/Furze Seed. Lotus is inhibited by L-fucose and the blood group H active substance, but not by A-, B-, or Le^a active material. Ulex interacts with blood group H antigen and other $\alpha(1,2)$ linked fucose residues. Thus immobilized Lotus and Ulex (conjugated to Sepharose) could be used to isolate the enzyme fraction that is covalently linked to disaccharide **56**. The enzyme (after irradiation in the presence of **56**) was chromatographed on the columns but were not retained by either of the two lectins. Compound **56** itself is retained (data not shown) by both the immobilized lectins and eluted with an aqueous solution of fucose. The reason for the loss of recognition is not known. It seems possible that the disaccharide is not exposed sufficiently when bound to another protein for the lectin to recognize it. In addition, the direct conjugation of the lectin to the solid support may cause steric hindrance, or may alter its binding site, preventing it from binding the carbohydrate. The enzyme-acceptor conjugate was therefore proteolyzed with trypsin to expose the disaccharide to the lectin (Figure 50). This procedure also failed to yield glycoprotein fragments that bound to either of the lectins.

5.2.9 *Immunoblotting*

Western Blot or immunoblotting can be used to detect proteins containing specific tags by regents that can detect these tags. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the irradiated enzyme was run [180] and the protein bands on the gel were transferred onto a polyvinyledine-difluoride (PVDF) membrane after electrophoresis [181]. The membrane was incubated with buffer containing BSA to reduce the background. Alkaline phosphatase or gold conjugated Lotus or Ulex lectin was then used as a specific probe for the detection of the protein-carbohydrate complex on the membrane. Ulex conjugated to gold showed precipitation of gold on the bands corresponding to the A enzyme as well as BSA [182]. Thus non-specific binding was observed. Samples of tryptic digest [183] of the enzyme was also not detected by immunoblotting. A probable reason could be that anionic detergents like SDS may have caused denaturation of the lectin or dissociation of the lectin subunits, rendering it inactive.

5.2.10 *Biotinylation*

Although glycoproteins are frequently detected using lectins, lack of lectin binding does not automatically indicate the absence of the corresponding carbohydrate residue. Another approach for the detection of glycoproteins that has great potential is the use of derivatives of biotin like biotin hydrazide [184]. They can be used to target the

carbohydrate groups or carboxyl groups on macromolecules and can be detected by Avidin or Streptavidin. Oxidative treatment of a glycoprotein that has a *cis*-diol in its carbohydrate residue would generate aldehydes, which would react with biotin hydrazide through the $NHNH_2$ group (Figure 51).

Since both galactose and fucose contain a *cis*-diol, the sugar moiety was oxidized with sodium *meta*-periodate after photoaffinity labeling of the enzyme. The aldehydes produced were reacted with the hydrazide. The hydrazone formed is stable between pH 2 and 10.

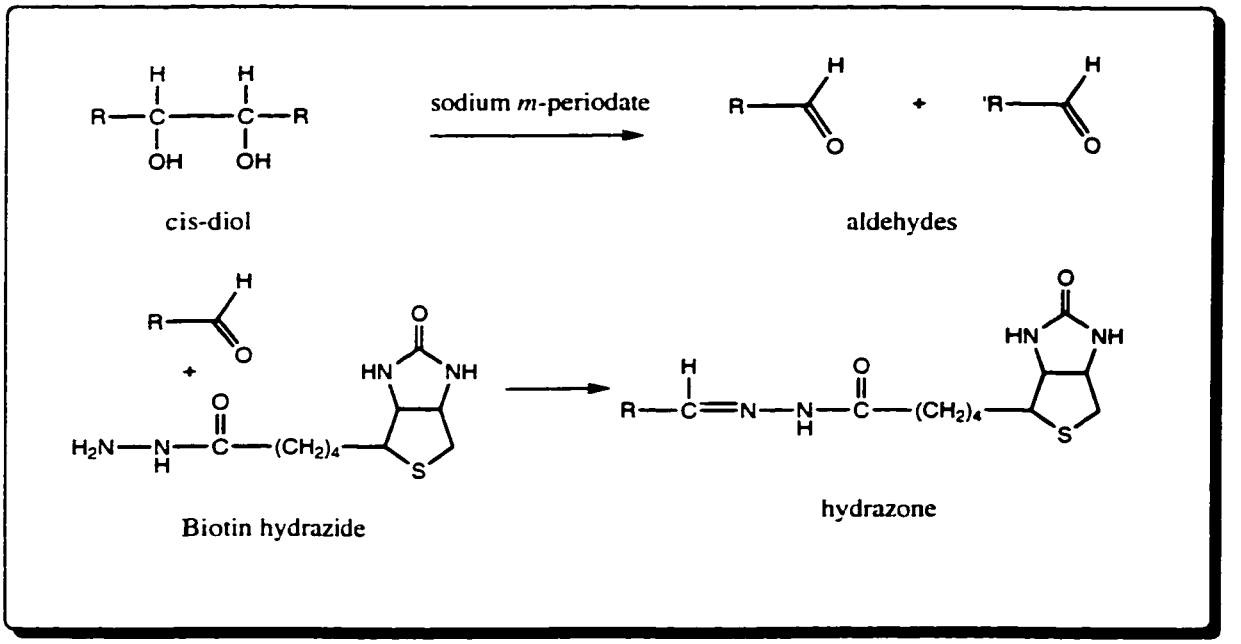


Figure 51. **Reaction of biotin hydrazide with an aldehyde.**

Biotin-LC-hydrazide was used for the reaction since it is more sensitive towards detection by Streptvidin. After dialysis and concentration of the sample, it was subjected to SDS-PAGE as before and blotted onto PVDF membrane. After blocking and washing the membrane, it was incubated with Streptavidin conjugated to colloidal gold for detection of the bands containing biotin. The results were similar to that obtained with the lectins, only non-specific immuno-precipitation was observed.

5.2.11 *SDS-PAGE analysis*

When the experiments were first designed, it was decided that lectin affinity would be used to isolate any photo-labeled enzyme. Therefore, our photo-affinity analog was devoid of any radioactive tag. Since that approach failed, we decided to use the reaction of the disaccharide with UDP-[6-^{3}H]-GalNAc in the presence of the enzyme to create a residue that can be identified by techniques like autoradiography, fluorography, or scintillation counting. The sample was dialyzed after irradiation, reacted with the donor, and dialyzed again. SDS-PAGE was done on it and bands at 10, 000 Dalton intervals were cut out and the protein extracted from each of them separately. They were individually counted in a liquid scintillation counter. The band corresponding to 30,000-40,000 Daltons showed between 100-300% increase in radioactivity compared to other the bands, indicating incorporation of radioisotope into the A enzyme.

5.2.12 *Mass spectrometric analysis*

The molecular weight of the protein under study, the A transferase, is about 37,000 Daltons. If the carbohydrate is covalently bound to the enzyme, it would result in the increase in the molecular weight of the conjugate. This aspect could be utilized by a mass spectrometer to determine the presence of the photo-affinity label on the enzyme [185]. The reaction mixture, after dialysis to remove inorganic salts, was subjected to electrospray mass spectrometry. The results obtained were ambiguous, since the molecular weight of the disaccharide (600 Dalton), was within the range of error of the instrument. At present, our collaborators in San Francisco, California, are analyzing mass spectral data obtained from the partial digest of labeled A enzyme with trypsin. The identification of the peptides will be based on the molecular weight of the fragments, the protein sequence, and the known cleavage specificity of trypsin.

To summarize:

The results from the irradiation experiments demonstrate that the enzyme is not deactivated by UV light itself.

It loses activity only if the photoaffinity labeled acceptor analog is present during irradiation. This indicates that either the acceptor covalently binds to the active site of the enzyme rendering it incapable of catalyzing subsequent reactions between UDP-GalNAc and the native acceptor, or the acceptor forms a non-dialyzable complex with the enzyme forming an inactive species.

The loss of enzyme activity is dependant on the concentration of the acceptor and maximum inactivation is reached at acceptor concentration of 0.33 mM indicating saturation of the combining site of the enzyme.

Radiolabeling of the enzyme using tritiated donor is only possible if the acceptor is linked to the enzyme either covalently, or through some stable interaction resulting in a non-dialyzable complex. Only a small amount of background radioactivity is observed in the absence of UV light and in the presence of the native acceptor. Therefore, the photoaffinity acceptor fails to insert in the absence of UV light, as does any compound lacking the benzoylbenzoic acid moiety.

The above evidence strongly suggest specific covalent insertion of the modified acceptor into the active site of the enzyme. However, since the products of photolabeling could not be isolated, conclusive proof of photolabeling of the A transferase with the photoaffinity acceptor analog has to await sequencing studies currently in progress.

5.3 *Experimental*

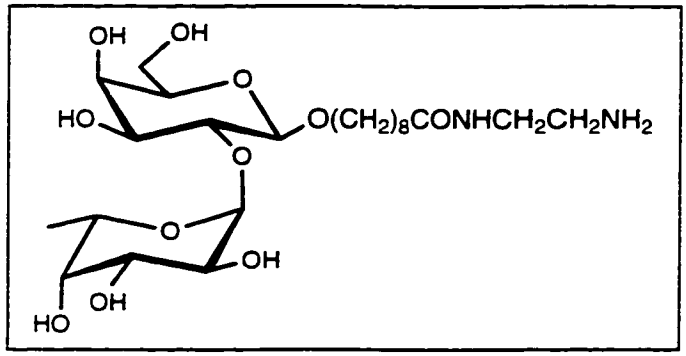
5.3.1 *General methods*

The general methods used are as described in chapter II. *Lotus tetragonolobus* and *Ulex europeus* I lectin gels and conjugates were purchased E Y Laboratories Inc. (San Mateo). The pre-cast gels used for SDS-PAGE, PVDF membranes (0.45 μm) used for Western

Blotting and AG$_1^{®}$-X8 anion-exchange resin (50-100 mesh, chloride form) were from Bio-Rad. Biotin LC Hydrazide and Slide-A-Lyzer dialysis kits were from Pierce. BSA, Tris, HEPES, and MOPS were from Sigma. 365 nm UV lamp used for the irradiation was from Mandel Laboratories. Trypsin (sequencing grade) was from Boehringer Mannheim.

5.3.2 *Chemical synthesis*

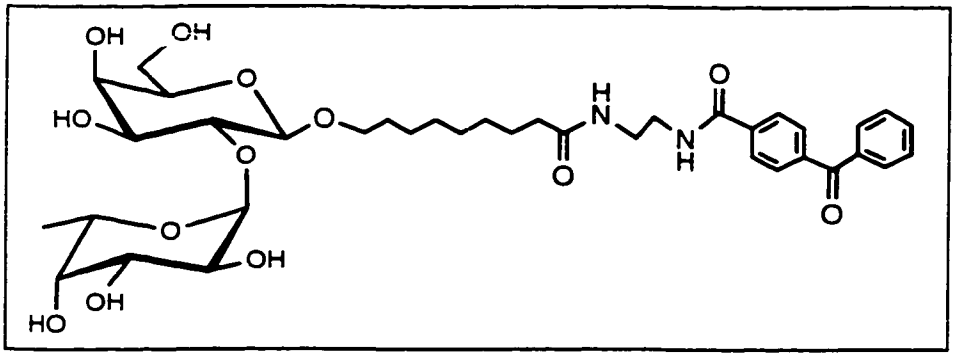
8-[*N*-(2-aminoethyl) amido]octyl 2-*O*-(α-L-fucopyranosyl)-β-D-galactopyranoside (**57**).



8-(methoxycarbonyl) octyl 2- *O*-α-L–fucopyranosyl-β-D-galactopyranoside (**54**; 40 mg; 0.08 mmole) was dissolved in neat ethylenediamine (8 ml) and heated in a sealed tube at 70 °C for 2 days. After completion of the reaction, it was diluted with milli-Q water, and passed through C-18 Sep-Pak. The aqueous flow through was loaded onto a fresh C-18 Sep-Pak cartridge, washed with water and eluted with methanol. The eluants were combined and the methanol evaporated. The residue was dissolved in de-ionized water, the solution passed through a Millipore filter, and the filtrate was lyophilized to provide a white powder (45mg; 75%). ^{1}H NMR (CD_3OD): δ 5.2 (bs, 1 H, H-1'), 4.3 (m, 2 H, H-1, H-5'), 3.88 (m, 1 H, C*H*$_2$O), 3.82 (bd, 1 H, J $_{3,4}$ 2.8 Hz, H-4), 3.70 (m, 7 H, H-2, H-5, H-a, H-2', H-3', H-4', C*H*$_2$O), 3.5 (m, 2 H, H-3, H-6b), 3.2 (t, 2 H, J $_{vic}$

5.5 Hz, NH-C*H*$_2$-CH$_2$-NH), 2.2 (t, 2 H, J $_{vic}$ 5.5 Hz, NH-CH$_2$-C*H*$_2$-NH), 1.6 (bt, 2 H, J 6.2 Hz, C*H*$_2$CH$_2$O), 1.3 (bs, 10 H, C*H*$_2$ octyl), 1.2 (d, 3 H, J $_{5',6'}$ 6.6 Hz, H-6'). ^{13}CNMR : δ 169.5 (*C*ONH), 103.5 (C-1), 101.5 (C-1'), 78.9, 76.5, 75.7, 73.7, 71.7, 70.7, 70.6, 70.4, 67.8, 62.3 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', *C*H$_2$O), 43.6 (*C*H$_2$NH), 41.8 (*C*H$_2$NH$_2$), 37.1 (*C*H$_2$CO), 30.9, 30.4, 30.3, 30.2, 27.2, 26.9 (*C*H$_2$ octyl), 16.8 (C-6'). ESMS for $C_{23}H_{45}N_2O_{11}$: Calc. 525.302336. Found 525.301802.

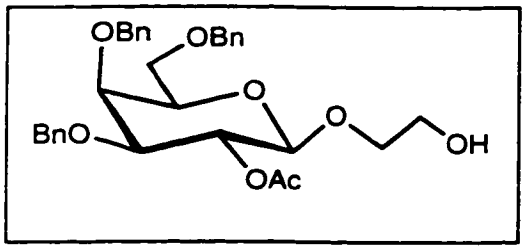
8-*N*-[2-(4-benzoylbenzamido)-ethyl]-amidooctyl 2-*O*-(α-L-fucopyranosyl)-β-D-galacto-pyranoside (**55**).



The disaccharide (**57**; 10 mg) was dissolved in DMF (2 ml), diisopropyl carbodiimide (10 μl) and 4-benzoylbenzoic acid (8 mg) were added and the mixture stirred at room temperature for 1 h. HOBt (5 mg) was added and the reaction continued overnight. After completion of the reaction, the reaction mixture was dried and purified on Iatrobeads using CH_2Cl_2:MeOH 10:1. The compound was collected and dissolved in H_2O, passed through Millipore filter, and lyophilized to get compound **55** in 70% yield. ^{1}H NMR (CD_3OD): δ 7.5-8.0 (m, 9 H, Ar*H*), 5.2 (bs, 1 H, H-1'), 4.3 (m, 2 H, H-1, H-5'), 3.86 (m, 1 H, C*H*$_2$O), 3.82 (bd, 1 H, J $_{3,4}$ 2.8 Hz, H-4), 3.70 (m, 7 H, H-2, H-5, H-6a, H-2', H-3', H-4', C*H*$_2$O), 3.5,(m, 6 H, H-3, H-6b, NH-C*H*$_2$-C*H*$_2$-NH), 2.2 (t, 2 H, J $_{vic}$ 7.4 Hz, C*H*$_2$CO),

1.6 (bt, 2 H, J 6.2 Hz, CH_2CH$_2$O), 1.3 (bs, 10 H, CH_2 octyl), 1.2 (d, 3 H, J $_{5',6'}$ 6.5 Hz, H-6'). ^{13}C NMR (CD_3OD): δ197.6 (*C*O), 176.9 (*C*ONH), 169.5 (*C*ONH), 141.3, 139.0, 138.3 (Ar*C*), 134.1, 131.0, 130.9, 129.6, 128.4 (Ar*C*H), 103.5 (C-1), 101.5 (C-1'), 78.9, 76.5, 75.7, 73. 7 x2, 70.6 x2, 70.4, 67.7, 62.4 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', *C*H$_2$O), 41.1, 39.8, 37.1 (*C*H$_2$CO), 30.9, 30.4, 30.2, 27.2, 26.9, 22.6, (*C*H$_2$ octyl), 16.1 (C-6'). ESMS for $C_{37}H_{52}O_{13}N_2Na$: Cald. 755.336710. Found 755.336560.

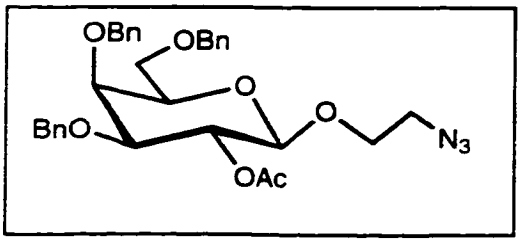
2-Hydroxyethyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranoside (**58**).



2-*O* -acetyl-3,4,6 tri-*O* benzyl-β -D-galactopyranosyl bromide (1.9 g; 3.35 mmole) was dissolved in CH_3CN (15 ml) and 3 Å MS (2 g), $Hg(CN)_2$ (1.2 g; 4.685 mmole) were added to it and the mixture stirred under Argon for 30 min. Ethelyne glycol (3.8 ml; 6.7 mmole) was added and the reaction mixture stirred overnight at room temperature. After work up (1) it was chromatographed with 3:1 pentane-ethylacetate to obtain 1.6 g of compound **11** in 75% yield. $[\alpha]_D$ = +6.1° (c 3.3 $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.2-7.4 (m, 15 H, Ar*C*H), 5.38 (dd, 1 H, J $_{2,3}$ 10.1 Hz, J $_{1,2}$ 7.9 Hz, H-2), 4.92, 4.66, 4.62, 4.56 (d, 1 H, J $_{gem}$ 11.5 Hz, PhCH_2), 4.40 (s, 2 H, PhCH_2 x2), 4.36 (d, 1 H, J $_{1,2}$ 7.9 Hz, H-1), 3.90 (d, 1 H, J $_{3,4}$ 2.8 Hz, H-4), 3.82 (m, 2 H, H-6a, CH_2O), 3.68 (m, 4 H, H-5, H-6b, CH_2OH x2), 4.06 (dd, 1 H, J $_{2,3}$ 10.1 Hz, J $_{3,4}$ 2.8 Hz, H-3), 3.53 (m, 1 H, CH_2O), 2.80 (s, 1 H, OH), 2.02 (s, 3 H, CH_3CO); ^{13}C NMR ($CDCl_3$): δ 169.7 (*C*OCH_3), 138.2, 137.8, 137.6 (Ar*C*), 128.4, 128.5, 128.2, 128.0, 127.9, 127.8, 127.7, 127.5, 127.4, 127.3 (Ar*C*H), 102.0 (C-1), 80.2 (C-2),

74.6, 74.4, 73.6, 72.7, 72.1, 69.1, 68.0, 62.3, 56.6 (C-3, C-4, C-5, C-6, CH_2O x 5), 21.0 (CH_3CO). ESMS for $C_{31}H_{36}O_8Na$: Cald. 559.230788. Found 559.229936.

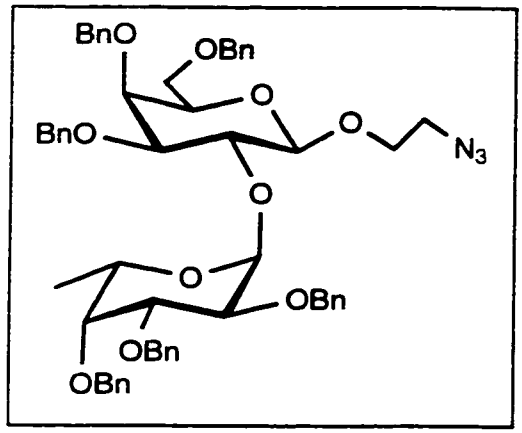
2-Azido ethyl 2-*O*-acetyl-3,4,6 tri-*O*-benzyl-β-D-galactopyranoside (**60**).



Compound **58** (400 mg; 0.75 mmole) was dissolved in CH_2Cl_2 (2 ml) and pyridine (363 µl; 4.5 mmole) was added to it in cold. Methanesulfonyl chloride (346 µl; 4.4 mmole) was added and the mixture stirred for 3 hr. After completion of the reaction, excess CH_3SO_2Cl was destroyed with methanol, solvent evaporated and co-evaporated with toluene. The residue (compound **59**) was dissolved in DMF (2 ml), NaN_3 (1g; 15.5 mmole) was added and heated at 50 °C with stirring overnight. After reaction, it was evaporated, diluted with CH_2Cl_2, washed with water, dried and concentrated to a syrup. It was chromatographed (pentane-ethylacetate 6:1) to yield compound **60** (350 mg; 83%); $[\alpha]_D$ = - 12.6° (c 4.6 $CHCl_3$). IR ($CHCl_3$): 2104 cm^{-1} (N_3), 1747 cm^{-1} (CH_3CO); ^1H NMR ($CDCl_3$): δ 7.2-7.4 (m, 15 H, ArC*H*), 5.40 (dd, 1 H, $J_{2,3}$ 10.0 Hz, $J_{1,2}$ 7.9 Hz, H-2), 4.94, 4.68, 4.58, 4.52 (d, 1 H, J_{gem} 11.5 Hz, PhCH_2), 4.40-4.45 (m, 2 H, PhCH_2 x2), 4.32 (d, 1 H, $J_{1,2}$ 7.9 Hz, H-1), 3.92-3.96 (m, 2 H, H-4, CH_2O), 3.60-3.66 (m, 3 H, H-5, H-6a, CH_2O), 3.52 (dd, 1 H, $J_{2,3}$ 10.1 Hz, $J_{3,4}$ 2.8 Hz, H-3), 3.42 (m, 2 H, H-6b, CH_2N_3), 4.22 (dt, 1 H, J_{gem} 13.3 Hz, J_{vic} 4.4 Hz, CH_2N_3) 2.05 (s, 3 H, CH_3CO); ^{13}C NMR ($CDCl_3$): δ 169.5 ($COCH_3$), 138.3, 137.85, 137.7 (Ar*C*), 128.4, 128.2, 127.8 x2, 127.7, 127.5, 127.4 (Ar*C*H), 101.0 (C-1), 80.2 (C-2), 74.4, 73.7, 73.5, x2, 72.0, 70.8, 68.6, 67.3, 50.5 (C-3, C-4, C-5, C-6, CH_2O

x4, CH_2N_3), 20.9 (CH_3CO). ESMS for $C_{31}H_{35}O_7N_3Na$: Cald. 584.237271. Found 584.237064.

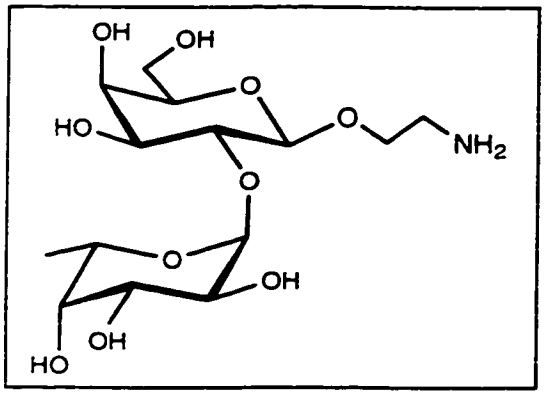
2-Azidoethyl 2-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-3,4,6-tri-*O*-benzyl-β-D-galactopyranoside (**62**).



Compound **60** (490 mg; 0.94 mmole) was deacetylated in methanol and sodium methoxide to give compound **61**. After evaporation of the solvent the mixture was dried, dissolved in CH_2Cl_2 (50 ml) and DMF (5 ml), and 4 Å MS (1.4 g) were added to it. It was stirred under argon for 1 hr before 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl bromide (1 gm; 2.3 mmole) was added to it in CH_2Cl_2 (5 ml) and stirred for a further 24 hr. After completion of the reaction, the molecular sieves were filtered over Celite, the filtrate washed with aq. $NaHCO_3$ and water, and evaporated. It was chromatographed (pentane-EtOAc 10:1, 6:1, 4:1, 3:1) to give 640 mg (72%) of **62**. $[\alpha]_D$ = - 49.1° (c 1.4 $CHCl_3$). ^1H NMR ($CDCl_3$): δ 7.2-7.4 (m, 30 H, Ar*H*), 5.68 (d, 1 H, $J_{1',2'}$ 2.65 Hz, H-1'), 4.93, 4.82, 4.80, 4.74, 4.72, 4.61, 4.59, 4.52 (d, 1 H, J_{gem} 11.5 Hz, PhCH_2), 4.50 (m, 3 H, PhCH_2 x3), 4.48 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1) 4.42 (m, 2 H, PhCH_2, H-5'), 4.24 (dd, 1 H, $J_{2,3}$ 9.6 Hz, $J_{1,2}$ 7.8 Hz, H-2), 3.78 (m, 2 H, H-2', H-4), 3.72 (bd, 1 H, $J_{3',4'}$ 2.66 Hz, H-4'), 3.72 (dd, 1 H, $J_{2',3'}$ 10.0 Hz, $J_{3',4'}$ 2.65 Hz, H-3'), 3.70 (dd, 1 H, $J_{2,3}$ 9.6 Hz, $J_{3,4}$ 2.3 Hz, H-3), 3.57 (m, 4 H, H-5, H-6a, H-6b, CH_2O), 3.40 (m, 1 H, CH_2-N_3), 1.21 (d, 3 H, $J_{5',6'}$ 6.5 Hz, H-6'). ^{13}C NMR ($CDCl_3$):

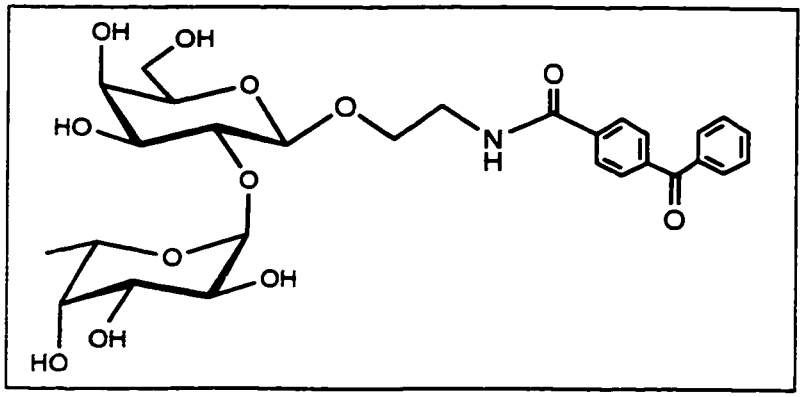
δ 139.0, 138.9, 138.3 x2, 138.0, 137.8 (Ar*C*), 128.5, 128.4, 128.3 x2, 127.97 x2, 127.8, 127.6, 127.4, 127.2, 126.4 (Ar*C*H), 104.0 (C-1), 101.3 (C-1'), 84.3, 79.6, 78.1, 75.7, 74.8, 74.4, 73. 6, 73.5, 72.9, 72.8, 72.0, 71.4, 68.8, 66.9, 66.4, 50.9 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', *C*H_2O x7, *C*H_2N_3), 16.5 (C-6'). ESMS for $C_{56}H_{61}O_{10}N_3Na$: Cald. 958.425466. Found 958.424233.

2-Amino ethyl 2-*O*-(α-L-fucopyranosyl) β-D-galactopyranoside (**63**).



Compound **62** (400 mg; 0.11 mmole) was dissolved in EtOH-CH_2Cl_2 4:1 (10 ml) and 100 µL of 1 M HCl and 10 mg of Pd-C was added to it and the mixture stirred for 4 hr under a stream of H_2. After the catalyst was filtered, solvent was evaporated, dissolved in milli-Q H_2O, loaded on C-18 Sep-Pak and washed with water. The compound was eluted with MeOH to give **63** in 82% yield (40 mg). ^{1}H NMR (CD_3OD): δ 5.09 (d, 1 H, $J_{1',2'}$ 2.84 Hz, H-1'), 4.43 (d, 1 H, $J_{1,2}$ 7.28 Hz, H-1), 4.17 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 3.98 (m, 2 H, H-6a, C*H*$_2$O), 3.84 (bd, 1 H, $J_{3,4}$ 2.4 Hz, H-4), 3.68 (m, 7 H, H-2, H-3, H-5, H-6b, H-2', H-3', H-4'), 3.16 (m, 2 H, C*H*$_2$-NH_2), 1.2 (d, 3 H, $J_{5',6'}$ 6.5 Hz, H-6'). ^{13}CNMR (CD_3OD): δ 103.7 (C-1), 102.5 (C-1'), 81.0, 76.7, 74.7, 73.6, 71.7, 70.8, 70.7, 68.5, 66.9, 62.5 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', *C*H_2O), 41.2 (*C*H_2NH_2), 16.8 (C-6'). ESMS for $C_{14}H_{28}O_{10}N$: Cald. 370.171322. Found 370.171709.

2-(4-Benzoylbenzamido) ethyl 2-*O*-α-L-fucopyranosyl-β-D-galactopyranoside (**56**).



The disaccharide **63** (20 mg) was dissolved in DMF (4 ml), diisopropyl carbodiimide (20 µl) and 4-Benzoylbenzoic acid (13 mg) were added and the mixture stirred at room temperature for 2 h. HOBt (20 mg) was added and the reaction continued overnight. After completion of the reaction, the reaction mixture was dried and purified on iatrobeads using CH_2Cl_2: MeOH 10:1. The compound was collected and dissolved in H_2O, passed through a Millipore filter, and lyophilized to get compound **56** in a yield of 80%. ^{1}H NMR (CD_3OD): δ 8.10 (m, 2 H, Ar*H*), 7.85-7.95 (m, 3 H, Ar*H*), 7.75 (m, 2 H, Ar*H*), 7.60-7.65 (m, 2 H, Ar*H*), 5.25 (d, 1 H, $J_{1',2'}$ 2.9 Hz, H-1'), 4.50 (d, 1 H, $J_{1,2}$ 7.85 Hz, H-1), 4.30 (q, 1 H, $J_{5',6'}$ 6.5 Hz, H-5'), 4.15 (m, 1 H, H-6a), 3.97 (m, 1 H, C*H*$_2$O), 3.93 (bd, 1 H, $J_{3,4}$ 2.8 Hz, H-4), 3.77 (m, 5 H, H-2, H-3, H-5, H-2', H-3'), 3.70 (m, 2 H, H-4', H-6b) 3.40 (m, 2 H, C*H*$_2$-NH), 1.25 (d, 3 H, $J_{5',6'}$ 6.5 Hz, H-6'). ^{13}CNMR (CD_3OD): δ 141.4, 140.8, 139.8, 134.1, 131.0, 131.0, 129.6, 128.5, 103.6 (C-1), 102.1 (C-1'), 80.4, 76.6, 75.2, 73.5, 71.7, 70.7, 70.1, 68.8, 68.4, 62.4 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-5', *C*H_2O), 41.5 (*C*H_2NH), 16.7 (C-6'). ESMS for $C_{28}H_{35}O_{12}NNa$: Cald. 600.205696. Found 600.205662.

5.3.2 *Isolation and purification of the A transferase*

The growth of *E.coli* and purification of the enzyme were performed using a modification of a literature procedure [45, 46]. Plasmids harboring the wild-type ompA-GTA gene were used to transform *E. coli* TG-1 cells. To produce the recombinant glycosyltransferase protein, the *E. coli* strain containing the plasmid (obtained from NRC, Ottawa) was grown at 30 °C in M-9 minimal medium supplemented with 0.4 % casamino acids and 100 µg/ml ampicillin. After 18-24 h, the cultures were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and made up to 1 x TB (12 g of tryptone, 24 g of yeast extract, and 4 ml of glycerol/liter of culture), a growth medium that maximizes cell density. The cultures were harvested 48-64 h later, and the cell extracts were obtained by French Pressing the cells. Cells from 2 liters of culture were taken in 60 ml of ice-cold resuspension buffer (20 mM tris-HCL, 1 mM EDTA, 1 mM dithiothreitol (DTT)), pH 7.2 and disrupted under high pressure. Protein activity was analyzed by radioactive Sep-Pak assays as described previously.

Soluble proteins were purified from the extracts by precipitating with ammonium sulfate. The 20-60 % ammonium sulfate fraction was resuspended in 1.5-3.0 ml of column buffer [20 mM Tris (pH 7.2), 1 mM DTT, 0.5 M NaCl] and dialyzed extensively against the same buffer. The extracts were made up to 2 mM $MnCl_2$, spun in a microcentrifuge for 5 min to remove any insoluble material and loaded onto a 10 ml UDP-hexanolamine-Sepharose column at 0.4 ml/h with column buffer containing 2 mM $MnCl_2$ and washed extensively until the A280 nm was at background levels. GTA was eluted with column

buffer containing 20 mM Tris, 1 mM DTT, 0.5 M NaCl, 20 mM $MnCl_2$ and 5 mM UDP. Since UDP has a strong absorbance at 280 nm, the presence of protein in the column fractions was determined by taking 10 µl of each fraction and performing a radioactive assay. The purification was also achieved using a different protocol. In a modification of the above procedure, the extracts in 20 mM MOPS, 1 mM EDTA, 1 mM DTT (pH 7.0) were centrifuged and the supernatant loaded onto a SP-Sepharose column in 20 mM MOPS, 1 mM DTT, pH 7.0. The protein was eluted in buffer containing 0.5 M NaCl, 5 mM $MnCl_2$ and the elution monitored by a UV monitor. The eluant was loaded onto a UDP-hexanolamine column, washed with the same buffer and eluted with buffer containing 10 mM UDP, 20 mM $MnCl_2$. The pooled column fractions (3 ml each) containing pure GTA enzyme were concentrated to 600-1000 µl volumes using Slide-A-Lyzer units, dialyzed extensively against 20 mM HEPES and 50 mM NaCl (pH 6.8) containing 1 mM dithiothreitol before use in the enzymatic experiments. The enzyme was stored at -70 °C with 1 mg/ml BSA. The protein concentration of all the enzymes was determined using the Bradford method with bovine γ globulin as a standard (Bio-Rad).

5.3.4 *Enzyme assay*

The enzymatic assays to determine the acceptor ability of the photoaffinity analogs were done as described in chapter II. The compounds were tested at concentrations of 1 mM with the A and the B transferase in radioactive Sep-Pak assays. Since both the compounds were acceptors, they were not tested as inhibitors. For the determination of

the K_m values with the A transferase, compound **55** was assayed in concentrations of 0.250, 0.125, 0.0625, 0.0313, 0.0156, 0.0078, 0.0039, 0.0019, and 0.00098 mM. Compound **56** was used in concentrations of 0.500, 0.250, 0.125, 0.0625, 0.0313, 0.0156, 0.0078, 0.0039, 0.0019, and 0.00098 mM. The data was fit into the Sigma Plot program to obtain the kinetic parameters.

Compounds **52** and **53** were assayed as follows. Glass pipettes were packed with $AG_1^{®}$-X8 anion-exchange resin (50-100 mesh, chloride form) and washed with methanol and water. The assay mixtures were separately loaded onto them and the products were eluted with 3.5 ml water each. The eluant was directly counted in a scintillation counter.

5.3.5 *Photolabeling of the A transferase*

The photolabeling of the enzyme was done following procedures reported in the literature [186]. The acceptor was used at concentrations of 0 mM to 0.66 mM to determine the saturation concentration, which was found to be 0.33 mM. This concentration was used for all subsequent experiments with 1 mg of the A transferase. The irradiation was performed for between 15-60 min. to determine the optimum time, which was found to be 45 min. UDP was used in a concentration of 86 mM, which is 10 times the K_i value of UDP for this enzyme. The reactions were done in A transferase buffer containing 0.35 M sodium cacodylate, 0.2 M $MnCl_2$, 10 mg/ml BSA, pH 6.9. The enzyme, the acceptor and UDP were incubated at 37 °C for 1 hr before being cooled on ice and placed under a hand

held UV lamp. For all further manipulations, this mixture was dialyzed before use unless otherwise noted. Between 0.1 and 0.2 μCi of UDP-[6-^{3}H]-GalNAc and 0.152 mM of UDPGalNAc were used in the experiments where the enzymes were further radiolabeled. This was done at 37 °C using excess of the enzyme.

5.3.6 *Tryptic digest of the enzyme*

Tryptic proteolysis of the enzyme was performed in 0.1 M Tris-HCl buffer, pH 8.48. Trypsin (in 1 mM HCl):substrate ratio of 1:100 was used and the mixture was incubated for between 30 min. to 3 h. The reaction was quenched with 2% trifluoroacetic acid, dialyzed, and used in either lectin affinity chromatography or SDS-PAGE.

5.3.7 *Biotinylation*

After irradiation, the sample was dissolved in 20 μl of 0.1 M sodium acetate buffer with 0.02% sodium azide (pH 5.5). 10 μl of 30 mM freshly prepared Na *m*-periodate solution was added to it and incubated in the dark for 30 min. at room temperature. Excess Na *m*-periodate was removed by dialysis in 0.1 M sodium acetate buffer. 10 μl of 5 mM biotin-LC hydrazide solution was added to the reaction mixture and incubated at room temperature for 1 h. The reaction was terminated by adding 50 μl of 0.1 M Tris, pH 7.5. The unreacted biotin was removed by centrifuging the product.

5.3.8 *Lectin column*

The commercial lectin gels were packed into mini columns as follows. The gel was mixed with buffer containing10 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ (pH 8.0). The slurry was de-gassed for 10 min. in a filter flask and then poured into a mini column. After the liquid was drained out, the gel was washed with 10 column volumes of the same buffer (degassed). The sample was dissolved in 0.5 ml of the same buffer and applied to the column. It was washed with the same buffer and the glycoprotein eluted with 50 mM L-fucose in phosphate-buffered saline (10 mM, pH 6.8).

5.3.9 *SDS-PAGE and immunoblotting*

SDS-PAGE was performed using an established protocol [180]. The proteins were visualised by staining with Coomassie Blue after electrophoresis. When used for Western blotting onto PVDF membrane, the gel was transferred onto a PVDF membrane and blotting was done in buffer containing 25 mM Tris, 192 mM glycine, 20% v/v methanol (pH 8.3) at 4 °C. The membrane was blocked with Tris-buffered saline (TBS) containing 3% w/v BSA (pH 7.5). This was followed by washing with TBS with 0.05% w/v Tween 20, pH 7.5 (TBST), TBS, and water. The membrane was then incubated overnight at room temperature with colloidal gold conjugated to *Lotus tetragonolobus* or *Ulex europeus*-I when the free disaccharide was used to label the enzyme, or with Streptavidin

conjugated to colloidal gold or alkaline phosphatase when the biotinylated derivative of the carbohydrate was prepared.

Chapter VI

CONCLUSIONS AND FUTURE PROSPECTS

Our synthetic studies into the acceptor binding site of blood group A and B glycosyltransferases have shown that the fucosyl 2-OH is involved in 'key polar interactions' in the active site of these enzymes. The interaction is mostly steric in nature, since the replacing the hydroxyl group by an H results in only partial loss of activity. The 3'-OH is not involved in any significant interactions, since both the methoxy and the deoxy analogs are tolerated by the enzymes. The 4'-methoxy analog **2** is an acceptor, but not the deoxy derivative, indicating that the hydroxyl group on the native sugar may be an important hydrogen bond acceptor. We have demonstrated that the amino acid sequence is responsible for not only donor, but also acceptor specificity. To our knowledge, the 4'-methoxy and the *arabino* analogs of **1**, **2** and **8** respectively, are the first acceptor analogs to be distinguished by the two enzymes. Confirming earlier findings, the B enzyme was found less tolerant of changes in the acceptor structure.

Through biochemical investigations we determined that the disaccharide acceptor analogs that are recognized by the blood group A glycosyltransferase as acceptors (as determined by Sep-Pak assays) are used by the enzyme to transfer GalNAc from the sugar donor onto the modified acceptors to form trisaccharide products.

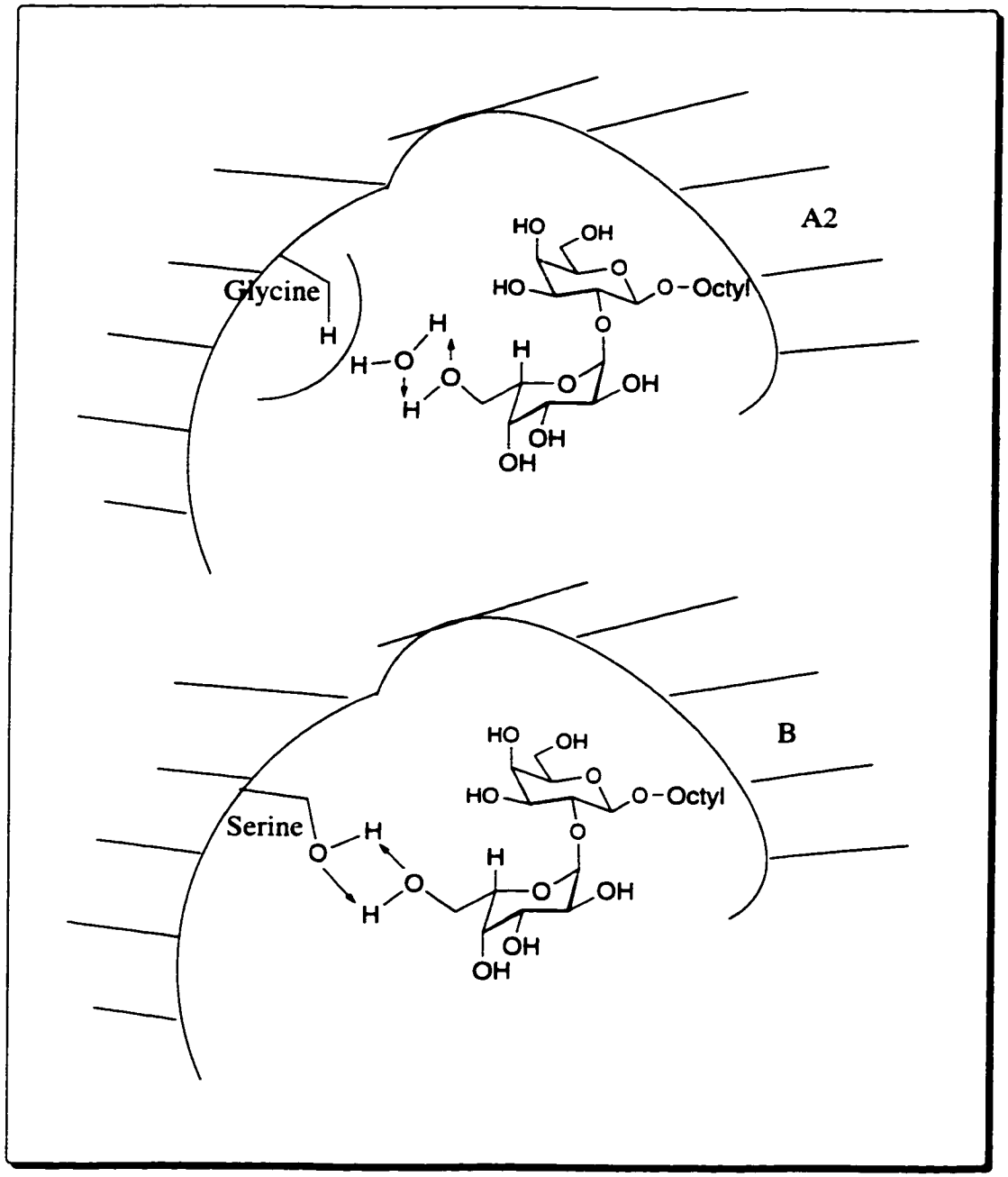


Figure 52. **Hypothetical interactions around the hydroxymethyl group of the potential acceptor 64 with the A and the B transferases.**

Enzymatic testing of the wild type and the mutant enzymes with the *arabino* analog points to the possibility that the second amino acid from the N-terminus, which differs between the A and the B enzymes might be involved in some interaction with the methyl group on fucose and play a crucial role in recognizing the acceptor. Clear conclusions could not be drawn on the correlation between the primary and the secondary amino acid sequence of the enzymes and it acceptor specificity. Further synthetic and biochemical studies might present a clearer picture. Replacing the fucose with an L-galactose (compound **64**) would present a hydroxymethyl group to this amino acid (glycine in A, serine in B at position 235). It would be interesting to see if the A transferase still recognizes this acceptor and if the serine on the B enzyme is involved in any hydrophilic interactions with the hydroxyl group on C-6' of **64** making it more acceptable to the enzyme (Figure 52). Further assays can then be performed with the mutants to identify the amino acids that recognize the acceptor.

Although the B enzyme does not tolerate modifications on the acceptor as well as the A, inhibitory potency of some of our inhibitors was found to be higher for this enzyme.

Our efforts to photolabel the A transferase were met with limited success. Incorporation of a radioisotope into our photoaffinity analog was necessary to prove the labeling of the A enzyme, but this was done indirectly, resulting in low incorporation of the radioisotope into the enzyme. The radioisotope used was tritium, which resulted in low counts. This was not conducive to autoradiography, and isolation of the radiolabeled enzyme for structural investigation was not possible. We were therefore unable to characterize the

radiolabeled fragments. At present, the enzyme labeled with compound **56** is being sequenced by our collaborators at the University of California at San Francisco. Given the novelty of the technique, the content and the significance of the information that can be obtained is difficult to predict. Nonetheless, to the best of our knowledge, this is the first instance of photolabeling of a glycosyltransferase by an acceptor analog. Previously, photoaffinity analog of only the donor has been used to label a fucosyltransferase [187].

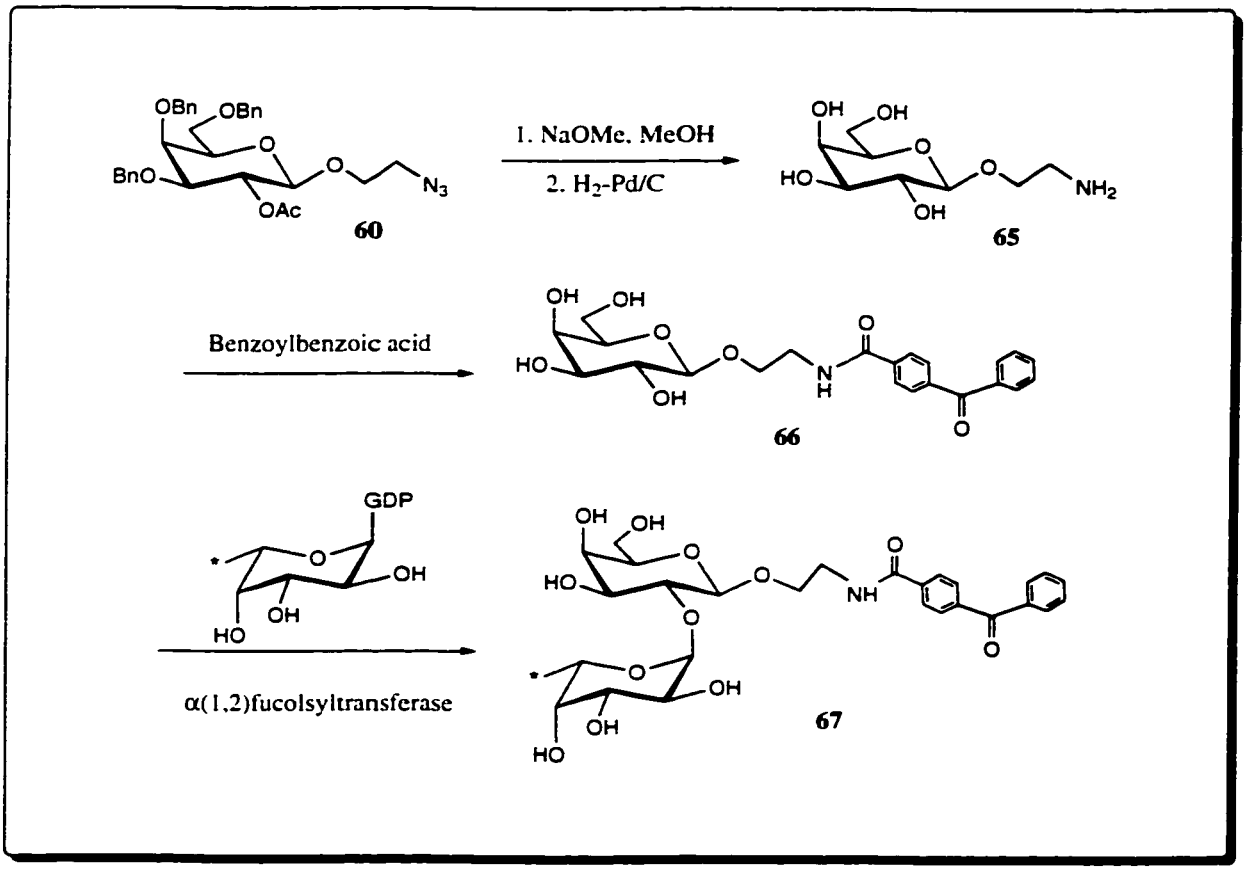


Figure 53. **Synthesis of the radiolabeled photoaffinity acceptor.**

If the radiolabeling is done chemo-enzymatically, the use of C-14 labeled GDP-fucose will enhance the sensitivity of the methods used (autoradiography or scintillation counting). One of the intermediates (**60**) used for the synthesis of the photoaffinity label **56**, after introduction of the benzoylbenzoic acid moiety, could be used as an acceptor with α(1,2)fucosyltransferase to transfer fucose from GDP-[^{14}C]-Fuc (Figure 53). This radiolabeled acceptor (**67**) could then be used to photolabel the enzyme and the labeled enzyme could be used for autoradiography and subsequently in sequencing studies. Using a radiolabeled probe to photolabel the enzyme would enhance the incorporation of the radioisotope, which would lead to more efficient isolation of labeled enzyme.

There have been two reports of X-ray crystal structures of glycosyltransferases, a glucosyltransferase [188] and a nucleotide-diphospho-sugar transferase [189] which show their donor and acceptor binding domains. X-ray crystal structures of the A and the B transferases, preferably with the acceptor substrate bound in their active site, would provide more information on the acceptor binding site of these enzymes. Our collaborators Professor Monica M. Palcic and Dr. Nina L. Seto (NRC, Ottawa) are working towards large-scale isolation of the A and the B transferases. Once these enzymes are obtained in sufficient homogeneity, crystal structures of the A and the B enzymes with the *arabino* analog bound in their active site would give us a better insight into the correlation between the amino acid sequence and the acceptor specificity of the enzymes.

Chapter VII

BIBLIOGRAPHY

1. A. Varki, *Glycobiology*, **1993**, *3*, 97-130.
2. N. Sharon and H. Lis, *Scientific American*, **1993**, 82-29.
3. T. Feizi, *Nature*, **1985**, *314*, 53-57.
4. E. F. Hounsell, M. Young, M. J. Davies, *Clinical Science*, **1997**, *93*, 287-293.
5. R. J. Coffey, G. D. shipley, H. L. Moses, *Cancer Res.*, **1986**, 1164-1169.
6. G. Poste, I. J. fidler, *Nature*, **1980**, *283*, 139-146.
7. S. C. Barnett, S. A. Eccles, Clin. Expt. Metastasis, **1984**, *2*, 297-310.
8. E. Nudelman, S. B. Levery, T. Kaizu, S. Hakomori, *J. Biol. Chem.*, **1986**, *261*, 11247-11253.
9. G. F. Springer, P. R. Desai, E. F. Scanlon, *Cancer*, **1976**, *37*, 169-176.
10. T. Tai, J. C. Paulsen, L. D. Cahan, R. F. Irie, *Proc. Natl. Acad. Sci. (USA)*, **1983**, *80*, 5392-5396.
11. A. Singhal, S.-I. Hakomori, *Bioessays*, **1990**, *12*, 223-230.
12. H. F. Oettgen, Ed., 1989, Ganglioside and Cancer, New York, VCH Publishers.
13. K. Watanabe. S. -I. Hakomori, *J. Expl. Med.*, **1976**, *144*, 644-654.

14. B. R. Juhl, S. H. Hartzen, B. Hainau, *Cancer*, **1986**, *57*, 1768-1775.

15. I Hakkinen, *J. Natl. Cancer Inst.*, **1970**, *44*, 1183-1193.

16. H. clausen, S.-I. Hakomori, N. Graem, E. Dabelsteen, *J. Immunol.*, **1986**, *136*, 326-330.

17. T. Feizi, *Cancer Surveys*, **1985**, *4*, 245-269.

18. H. Denk, G. Tappeiner, A. Davidowitz, R. Eckerstorfer, J. H. Holzner, *J. Natl. Cancer Inst.*, **1974**, *53*, 933-942.

19. H. Schachter, *Clin. Biochem.*, **1984**, *17*, 3-14.

20. H. A. Kaplan, J. K. Welpy, W. J. Lennarz, *Biochem. et Biophys. Acta*, **1987**, *906*, 161-173.

21. H. Schachter, I. Brockhausen, The biosynthesis of branched O-glycans. In Mucus and Related Topics, Eds. E. Chantler and N. A. Ratcliffe, **1989**, *43*, 1-26.

22. G. van Echten and K. Sandhoff, *J. Biol. Chem.*, **1993**, *268*, 5341-5344.

23. M. A. J. Ferguson, S. W. Homans, R. A. Dwek, T. W. Rademacher, *Science*, **1988**, *239*, 753-759.

24. W. M. Watkins, *Adv. Hum. Gen.*, **1980**, *10*, 1-136.

25. C. Green, *FEMS Microbiol. Immunol.*, **1989**, *47*, 321-330.

26. A. E. Szulman, *Curr. Topics Dev. Biol.*, **1982**, *60*, 127-145.

27. V. E. Dube, *Cancer and Metastasis Rev.*, **1987**, *6*, 541-557.

28. W. G. Dunphy, R. Brands, J. E. Rothman, *Cell*, **1985**, *40*, 463-472.

29. D. C. Johnson, P. G. Spear, *Cell*, **1983**, 987-997.

30. W. M. Watkins, P. Greenwell, A. D. Yates, *Immunological Comm.*, **1981**, *10(2)*, 83-100).

31. W. M. Watkins, *Carbohydr. Res.*, **1986**, *149*, 1-12.

32. M. Pierce, E. A. Turley, S. Roth, Intl. Rev. Cytol., **1980**, *65*, 1-47.

33. R. Kleene, E. G. Berger, *Biochim. et Biophys. Acta*, **1993**, *1154*, 283-325.

34. S. Roth, E. J. Mcguire, S. Roseman, *J. Cell Biol.*, **1971**, *51*, 525-536.

35. M. Pierce, E. A. Turley, S. Roth, B. D. Shur, S. Evans, Q. Lu, *Glycoconjugate J.*, **1998**, *15*, 537-548.

36. D. J. Miller, M. B. Macek, B. D. Shur, *Nature*, **1992**, *357*, 589-593.

37. H. Schachter, *Curr. Opin. Struct. Biol.*, **1991**, *1*, 755-765.

38. J. C. Paulson, K. J. Colley, *J. Biol. Chem.*, **1989**, *264*, 17615-17618.

39. J. Roth, D. J. Taatjes, J. Weinstein, J. C. Paulson, P. Greenwell, W. M. Watkins, *J. Biol. Chem.*, **1986**, *261*, 14307-14312.

40. J. Roth, P. Greenwell, W. M. Watkins, *Eur. J. Biochem*, **1988**, *46*, 105-112.

41. T. White, U. Mandel, T. F. Orntoft, E. Dabelsteen, J. Karkov, M. Kubeja, S.-I. Hakomori, H. Clausen, *Biochemistry*, **1990**, *29*, 2740-2747.

42. F.-I. Yamamoto, H. Clausen, T. White, J. Marken, S.-I. Hakomori, *Nature*, **1990**, *345*, 229-233.

43. F. Yamamoto, S.-I. Hakomori, *J. Biol. Chem.*, **1990**, *265*, 19257-19262.

44. F.-I. Yamaoto, J. Marker, T. Tsuju, T. White, H. Clausen, S.-I. Hakomori, *J. Biol. Chem.* **1990**, *265*, 1146-1151.

45. N. O. L. Seto, M. M. Palcic, O. Hindsgaul, D. R. Bundle, S. A. Narang, *Eur. J. Biochem.*, **1995**, *234*, 323-328.

46. N. O. L. Seto, M. M. Palcic, C. A. Compston, H. Li, D. R. Bundle, S. A. Narang, *J. Biol. Chem.*, **1997**, *272*, 14233-14238.

47. A. D. Yates, J. Feeney, A. S. R. Donald, W. M. Watkins, *Carbohydr. Res.*, **1984**, *130*, 251-260.

48. P. Greenwell, A. D. Yates, W. M. Watkins, *Carbohydr. Res.*, **1986**, *149*, 149-170.

49. A. D. Yates, W. M. Watkins, *Biochem. Biophys. Res. Comm.*, **1982**, *109*, 958-965.

50. T. L. Lowary, O. Hindsgaul, *Carbohydr. Res.*, **1993**, *249*, 163-195.

51. T. L. Lowary. O. Hindsgaul, *Carbohydr. Res.*, **1994**, *251*, 33-67.

52. J. E. Heidlas, W. J. Lees, G. M. Whitesides, *J. Org. Chem.*, **1992**, *57*, 152-157.

53. J. E. Heidlas, K. W. Williams, G. M. Whitesides, *Acc. Chem. Res.*, **1992**, *25*, 307-314.

54. R. Caputto, L. F. Le Loir, C. E. Cadini, A. C. Paladini, *J. Biol. Chem.*, **1950**, *184*, 333-350.

55. H. M. Kalckar, *Adv. Enzymol.*, **1958**, *20*, 111-134.

56. H. D. Abraham, R. R. Howell, *J. Biol. Chem.*, **1969**, *244*, 545-550.

57. H. J. M. Gijsen, L. Qiao, W. Fitz, C.-H. Wong, *Chem. Rev.*, **1996**, *96*, 443-473.

58. L. Glaser, *J. Biol. Chem.*, **1959**, *234*, 2801-2805.

59. V. Pozsgay, H. J. Jennings, *Tetrahedron. Lett.*, **1987**, *28*, 1375-1376.

60. T. Ogawa, M. Matsui, *Carbohydr. Res.*, **1977**, *54*, c17-c21.

61. R. K. Jain, K. L. Matta, *Carbohydr. Res.*, **1990**, *208*, 51-58.

62. M. O. Contour, J. Defaye, M. Little, E. Wong, *Carbohydr. Res.*, **1989**, *193*, 283-287.

63. F. Dasgupta, P. J. Garegg, *Acta Chem. Scand.*, **1989**, *43*, 471-475.

64. W. Sneider, J. Sepp, O. Stiehler, *Chem. Ber.*, **1918**, *51*, 220-234.

65. B. Helferich, H. Grunewald, F. Langenhoff, *Chem. Ber.*, **1953**, *86*, 873-875.

66. M. Yde, C. K. Debruyne, *Carbohydr. Res.*, **1973**, *26*, 227-229.

67. V. Pedretti, A. Veyrieres, P. Sinay, *Tetrahedron*, **1990**, *46*, 77-88.

68. R. J. Ferrier, R. Y. Hay, N. Vethaviyasar, *Carbohydr. Res.*, **1973**, *27*, 55-61.

69. T. Y. R. Tsai, H. Jin, K. Wiesner, *Can. J. Chem.*, **1984**, *62*, 1403-1405.

70. S. Hanessian, C. Baquet, N. Lehong, *Carbohydr. Res.*, **1980**, *80*, C17-C22.

71. J. W. van Cleve, *Carbohydr. Res.*, **1979**, *70*, 161-164.

72. P. J. Garegg, C. Henrichson, T. Norberg, *Carbohydr. Res.*, **1983**, *116*, 162-165.

73. T. Mukaiyama, T. Nakatsuka, S. Shoda, *Chem. Lett.*, **1979**, 487-490.

74. K. C. Nicolau, S. P. Seitz, D. P. Papahatjis, *J. Am. Chem. Soc.*, **1982**, *105*, 2430-2434.

75. K. Wiesner, T. Y. R. Tsai, H. Jin, Helv. *Chem.. Acta.* **1985**, *68*, 300-314.

76. P. G. M. Wuts, S. S. Biglow, *J. Org. Chem.*, **1983**, *48*, 3489-3493.

77. S. Koto, T. Uchida, S. Zen, *Chem. Lett.*, **1972**, 1049-1052.

78. S. Sato, M. Mori, Y. Ito, T. Ogawa, *Carbohydr. Res.*, **1986**, *155*, C6-C10.

79. K. C. Nicolau, R. E. Dolle, D. P. Paphatjis, J. L. Randall, *J. Am. Chem. Soc.*, **1984**, *106*, 4189-4192.

80. F. Weygand, H. Ziemann, H. J. Bestmann, *Chem. Ber.*, **1958**, *91*, 2534-2536.

81. F. Weygand, H. Ziemann, *Justus Liebigs Ann. Chem.* **1962**, *657*, 179-198.

82. S. Koto, T. Uchida, S. Zen, *Bull. Chem. Soc. Japan*, **1973**, *46*, 2520-2553.

83. K. Suzuki, H. Maeta, T. Suzuki, T. Matsumoto, *Tetradedron Lett.*, **1989**, *30*, 6879-6882.

84. P. Fugedi, P. J. Garegg, *Carbohydr. Res.*, **1986**, *149*, C9-C12.

85. F. Dasgupta, P. J. Garegg, *Carbohydr. Res.*, **1988**, *177*, C13-C17.

86. G. V. Reddy, V. R. Kulkarni, H. B. Mereyala, *Tetrahedron Lett.*, **1989**, *30*, 4283-4286.

87. H. Lonn, *Carbohydr. Res.*, **1985**, *139*, 115-121.

88. H. Lonn, *Carbohydr. Res.*, **198**, *135*, 105-113.

89. M. Ravenscroft, R. M. G. Roberts, J. G. Tillet, *J. Chem. Soc. Perkin Trans.*, **1982**, *2*, 1569-1572.

90. P. J. Garegg, Adv. Carbohydr. *Chem. Biochem.*, **1997**, *52*, 179-205.

91. P.-M. Aberg, L. Blomberg, H. Lonn, T. Norberg, *J. Carbohydr. Chem.*, **1994**, *13*, 141-161.

92. G. H. Veeneman, S. H. van Leeuwen, J. H. van Boom, *Tetrahedron Lett.*, **1990**, *31*, 1331-1334.

93. P. Konradsson, U. E. Udodong, B. Fraser-Reid, *Tetrahedron Lett.*, **1990**, *31*, 4313-4316.

94. G. H. Veeneman, J. H. van Boom, *Tetrahedron Lett.*, **1990**, *31*, 275-278.

95. A. Marra, J. M. Mallet, C. Amatore, P. Sinay, *Synlett*, **1990**, 572-574.

96. L. J. Haynes, F. H. Newth, *Adv. Carb. Chem. Biochem.*, **1955**, *10*, 207-256.

97. R. U. Lemieux, A. R. Morgan, *Can. J. Chem.*, **1965**, *43*, 2199-2204.

98. T. Ogawa, K. Katano, K. Sasajima, M. Matsui, *Tetrahedron*, **1981**, *37*, 2779-2786.

99. K. Jansson, S. Ahlfors, T. Frejd, J. Kihlberg, G. Magnusson, J. Dahmen, G. Noori, K. Stenvall, *J. Org. Chem.*, **1988**, *53*, 5629-5647.

100. W. Koenigs, E. Knorr, *Ber.*, **1901**, *34*, 957-981.

101. G. Wulff, G. Rohle, *Ang. Chem. Int. Ed. Eng.*, **1974**, *13*, 157-169.

102. A.-F. Bochkov, G. E. Zaikov, Chemistry of the O-glycosidic Bond: Formation and Cleavage, Pergamon Press, Oxford, 1979.

103. H. Paulsen, *Ang. Chem. Int. Ed. Eng.*, **1982**, *21*, 155-173.

104. H. Paulsen, *Chem. Soc. Rev.*, **1984**, *13*, 15-45.

105. R. R. Schimdt, *Ang. Chem.Int. Ed. Eng.*, **1986**, *25*, 212-235.

106. H. Kunz, *Ang. Chem. Int. Ed. Eng.*, **1987**, *26*, 294-308

107. R. R. Schimdt, *Pure Appl. Chem.*, **1989**, *61*, 1257-1270.

108. R. R. Schimdt, Comprehensive Organic Synthesis, Pergamon Press, Oxford, 1991.

109. P. Sinay, *Pure Appl. Chem.*, **1991**, *63*, 519-528.

110. K. Toshima, K. Tatsuta, *Chem. Rev.*, **1993**, *93*, 1503-1531.

111. R. U. Lemieux, K. B. Hendriks, R. V. Stich, K. James, *J. Am. Chem. Soc.*, **1975**, *97*, 4056-4062.

112. H. Paulsen, O. Lockhoff, *Chem. Ber.*, **1981**, *114*, 3102-3125.

113. T. Ogawa, M. Matsui, *Carbohydr. Res.*, **1976**, *51*, C13-C18.

114. A. Lubineau, A. Malleron, *Tetrahedron Lett.*, **1985**, *26*, 1713-1716.

115. A. Lubineau, J. Le Gallic, A. Malleron, *Tetrahedron Lett.*, **1987**, *28*, 5041-5044.

116. K. Higashi, K. Nakayama, T. Soga, E. Shioya, K. Uoto, T. Kusama, *Chem. Pharm. Bull.*, **1990**, *38*, 3280-3282.

117. E. Petrakova, U. Spohr, R. U. Lemieux, *Can. J. Chem.*, **1992**, *70*, 233-239.

118. P. S. Vermersch, J. J. G. Termer, F. A. Quiocho, *J. Biol. Chem.* **1992**, *226*, 923-929.

119. U. Spohr, E. Paszkiewicz-Hnatiw, N. Morishima, R. U. Lemieux, *Can. J. Chem.*, **1992**, *70*, 254-271.

120. P. V. Nikrad, H. Beierbeck, R. U. Lemieux, *Can. J. Chem.*, **1992**, *70*, 241-253.

121. O. Hindsgaul, T. Norberg, J. le Pendu, R. U. Lemieux, *Carbohydr. Res.*, **1982**, *109*, 109-142.

122. I. Lindh, O. Hindsgaul, *J. Am. Chem. Soc.*, **1991**, *113*, 216-223.

123. V. Chowdhury, F. H. Westheimer, *Ann. Rev. Biochem.*, **1979**, *48*, 293-325.

124. D. I. Schuster, W. C. Probst, G. K. Ehrlich, G. Singh, *Photochem. Photobiol.*, **1989**, *49*, 785-804.

125. F. Kotzyba-Hibert, I. Kapfer, M. Goeldner, *Ang. Chem. Int. Ed. Engl.*, **1995**, *34*, 1296-1312.

126. A. Singh, E. R. Thornton, F. H. Westheimer, *J. Biol. Chem.*, **1962**, *237*, 3006-3008.

127. H. Bayley, in Photogenerated Reagents in Biochemistry and Molecular Biology, (Eds. T. S. Works, R. H. Burdon) **1993**, Elsevier, Amsterdam.

128. S. A. Fleming, *Tetrahedron*, **1995**, *51(46)*, 12479-12520.

129. G. D. Prestwich, G. Dorman, J. T. Elliot, D. M. Marecak, A. Chaudhury, *Photochem. Photobiol.*, **1997**, *65(2)*, 222-234.

130. P. J. A. Weber, A. G. Beck-Sickinger, *J. Pep. Res.*, **1997**, *49(5)*, 375-383.

131. M. M. Palcic, L. D. Heerze, O. P. Srivastava, O. Hindsgaul, *J. Biol. Chem.*, **1989**, *264*, 17174-17181.

132. O. P. Srivastava, O. Hindsgaul, M. Shoreibah, M. Pierce, *Carbohydr. Res.*, **1988**, *179*, 137-161.

133. O. Kanie, S. C. Crawley, M. M. Palcic, O. Hindsgaul, *Carbohydr. Res.*, **1993**, *243*, 139-164.

134. S. H. Khan, S. C. Crawley, O. Kanie, O. Hindsgaul, *J. Biol. Chem.*, **1993**, *268*, 2468-2473.

135. U. B. Gokhale, O. Hindsgaul, M. M. Palcic, *Can. J. Chem.*, **1990**, *68*, 1063-1071.

136. O. Hindsgaul, K. J. Kaur, G. Srivastava, M. Blaszczyk-Thurin, S. C. Crawley, L. D. Heerze, M. M. Palcic, *J. Biol. Chem.*, **1991**, *266*, 17858-17862.

137. W. M. Watkins, *Pure Appl. Chem.*, **1991**, *63*, 561-568.

138. M. M. Palcic, L. D. Heerze, M. Pierce, O. Hindsgaul, *Glyconjugate J.*, **1988**, *5*, 49-63.

139. H. Lis, N. Sharon, *Ann. Rev. Biochem.*, **1986**, *55*, 35-67.

140. N. Sharon, H. Lis, *Science*, **1989**, *246*, 227-234.

141. H. B. Boren, G. Ekborg, K. Eklind, P. J. Garegg, A. Pilotti, C. G. Swahn, *Acta Chem. Scand.*, **1973**, *27*, 2639-2644.

142. H. F. Vernay, E. S. Racahman, R. Eby, S. Scheurch, *Carbohydr. Res.*, **1980**, *78*, 267-273.

143. O. Hindsgaul, T. norberg, J. Le Pendu, R. U. Lemieux, *Carbohydr. Res.*, **1982**, *109*, 109-142.

144. S. Hanessian, T. J. Liak, D. M. Dixit, *Carbohydr. Res.*, **1981**, *88*, c14-c19.

145. S. Hanessian, D. M. Dixit, T. J. Liak, *Pure Appl. Chem.*, **1981**, *53*, 129-148.

146. H. Paulsen, M. Paal, *Carbohydr. Res.*, **1984**, *135*, 53-69.

147. U. Spohr, R. U. Lemieux, *Carbohydr. Res.*, **1988**, *174*, 211-238.

148. P. Smid, G. A. de Ruiter, G. A. van der Marel, F. M. Rombouts, J. H. van Boom, *J. Carbohydr. Chem.*, **1991**, *10*, 833-849.

149. P. J. Garegg, T. Iversen, S. Oscarson, *Carbohydr. Res.*, **1976**, *50*, c12-c14.

150. M. Nilsson, T. Norberg, *Carbohydr. Res.*, **1988**, *183*, 71-82.

151. D. H. R. Barton, J. Dorchak, J. C. Jaszberenyi, *Tetrahedron Lett.*, **1992**, *48*, 7435-7446.

152. D. H. R. Barton, R. Subramanian, *J. Chem. Soc., Perkin Trans. I*, **1977**, 1718-1723.

153. R. Johansson, B. Samuelsson, *J. Chem. Soc., Perkin Trans. I*, **1984**, 2371-2374.

154. A. Hasegawa, T. Ando, M. Kato, H. Ishida, M. Kiso, *Carbohydr. Res.*, **1994**, *257*, 55-65.

155. A. P. Martinez, W. W. Lee, L. Goodman, *J. Org. Chem.*, **1969**, *34*, 92-97.

156. T. Iversen, D. R. Bundle, *Carbohydr. Res.*, **1982**, *103*, 29-40.

157. H. Schachter, *Trends Glycosci. Glycotechnol.*, **1992**, *4*, 241-250.

158. C. Walsh, *Enzymic Reaction Mechanisms*, W. H. Freeman, New York, **1979**.

159. M. L. Sinnott, in M. I. Page and A. Williams (Eds.) *Enzyme Mechanisms*, The Royal Society of London, London, *259*, **1987**.

160. R. U. Lemieux, K. Bock, L. T. J. Delbaere, S. Koto, V. S. Rao, *Can. J. Chem.*, **1980**, *58*, 631-653.

161. A. Otter, R. U. Lemieux, R. G. Ball, A. P. Venot, O. Hindsgaul, D. R. Bundle, *Eur. J. Biol.*, **1999**, *259*, 295-303.

162. J. B. Lowe, *Cell Biol.*, **1991**, *2*, 289-307.

163. T. L. Lowary, Ph.D. Thesis, University of Alberta, **1993**.

164. M. M. Palcic, unpublished results.

165. B. Wegmann, R. R. Schimdt, *Carbohydr. Res.*, **1988**, *184*, 254-261.

166. D. K. Watt, D. J. Brasch, D. S. Larsen, L. D. Melton, J. Simpson, *Carbohydr. Res.*, **1996**, *285*, 1-16.

167. a) D. K. Fitzgerald, B. Colvin, R. Mawal, K. E. Ebner, *Anal. Biochem.*, **1970**, *36*, 43-61. b) J. E. Sadler, T. A. Beyer, C. L. Oppenheimer, J. C. Paulson, J.-P. Prieels, J. I. Rearick, R. L. Hill, *Methods Enzymol.*, **1982**, *83*, 458-514.

168. K. L. Matta, S. S. Rana, C. F. Piskorz, S. A. Abbas, *Carbohydr. Res.*, **1984**, *131*, 247-255.

169. V. P. Kamath, P. Diedrich, O. Hindsgaul, *Glycoconj. J.*, **1996**, *13*, 315-319.

170. M. Dejter-Juszynski, H. M. Flowers, *Carbohydr. Res.*, **1971**, *18*, 219-226.

171. a) M. Schwyzer, R. L. Hill, *J. Biol. Chem.*, **1977**, *252*, 2338-2345. b) M. Schwyzer, R. L. Hill, *J. Biol. Chem.*, **1977**, *252*, 2346-2355.

172. J. Wang, S. Bauman, R. F. Colman, *Biochemistry*, **1998**, *37*, 15671-15679.

173. M. F. Taylor, A. K. Bhattacharya, K. Rajagopalan, R. Hiipakka, S. Liao, D. C. Collins, *Steroids*, **1996**, *61*, 323-331.

174. A. T. Zhao, R. Bessalle, A. Bisello, C. Nakamoto, M. Rosenblatt, L. J. Suva, M. Chorev, *Proc. Natl. Acad. Sci. USA*, **1997**, *94*, 3644-3649.

175. a) R. Adar, N. Sharon, *Eur. J. Biochem.*, **1996**, *239*, 668-674. b) N. Sharon, H. Lis, *Essays in Biochem.*, **1995**, *30*, 59-75. c) N. Sharon, *Trends in Biochemical Sciences*, **1993**, *18*, 221-226.

176. K. Yamamoto, Y. Konami, T. Osawa, T. Irimura, *J. Biochem.*, **1992**, *111*, 436-439.

177. B. J. Howlett, A. E. Clarke, *Biochem. J.*, **1981**, *197*, 695-706.

178. S. Bassarab, R. B. Mellor, D. Werner, *Lectins: Biol. Biochem., Clin. Biochem.*, **1986**, *5*, 565-571.

179. L. Yan, P. P. Wilkins, G. Alvarez-Manilla, S.-I. Do, D. F. Smith, R. D. Cummings, *Glycoconj. J.*, **1997**, *14*, 45-55.

180. U. K. Laemmeli, *Nature*, **1970**, *227*, 680-685.

181. H. Towbin, T, Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. USA*, **1979**, *76*, 4350-4354.

182. L. Bhattacharya, J. Fant, H. Lonn, F. C. Brewer, *Biochemistry*, **1990**, *29*, 7523-7530.

183. R. H. Aebersold, J. Leavitt, R. A. Saavedra, L. E. Hood, *Proc. Natl. Acad. Sci. USA*, **1987**, *84*, 6970-6974.

184. D. Savage, G. Mattson, S. Desai, G. Nielander, S. Morgensen, E. Conklin, in Avidin-Biotin Chemistry: A Handbook, Pierce Chemical Company, USA, 1994.

185. Y. Zhao, T. W. Muir, S. B. H. Kent, E. Tischer, J. M. Scardina, B. T. Chait, *Proc. Natl. Acad. Sci. USA*, **1996**, *93*, 4020-4024.

186. K. P. Williams, S. E. Shoelson, *J. Biol. Chem.*, **1993**, *268*, 5361-5364.

187. E. H. Holmes, *J. Biol. Chem.*, **1990**, *265*, 13150-13156.

188. A. Vielink, W. Ruger, H. P. C. Driessen, P. S. Freemont, *EMBO J.*, **1994**, *13*, 3413-3422.

189. S. J. Charnock, G. J. Davies, *Biochem.*, **1999**, *38*, 6380-6385.

Chapter VIII

APPENDIX

8.1 *Kinetic plot*

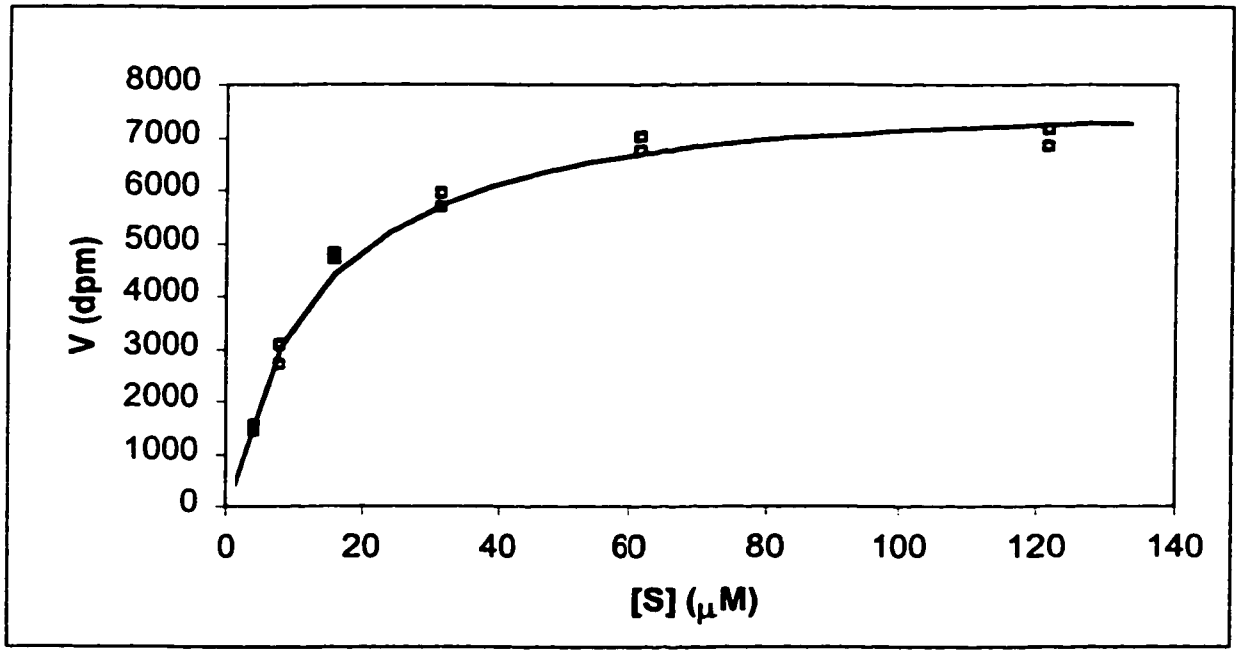


Figure 54. **Non-Linear Regression plot of the native disaccharide (1) with A2 enzyme.**

8.2 *^{1}H NMR spectra*

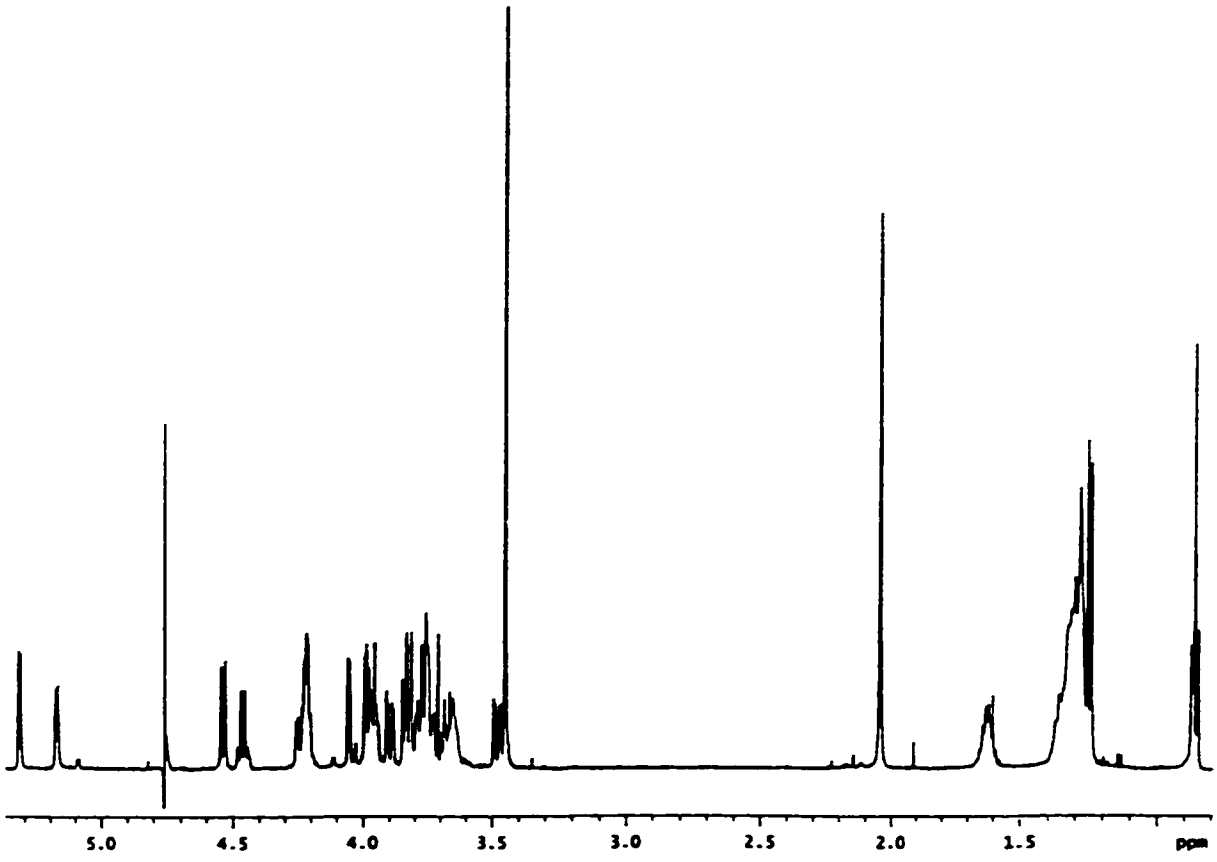


Figure 55. **Spectrum of the 4'-OMe trisaccharide (43).**

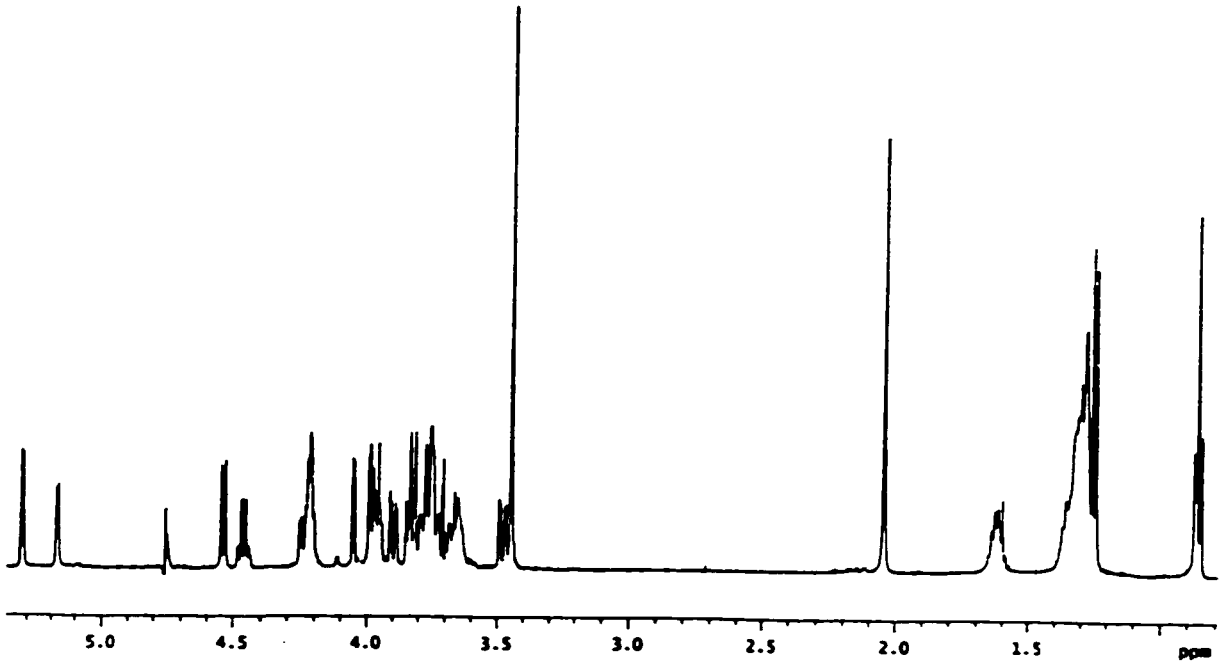


Figure 56. **Spectrum of the 3'-OMe trisaccharide (44).**

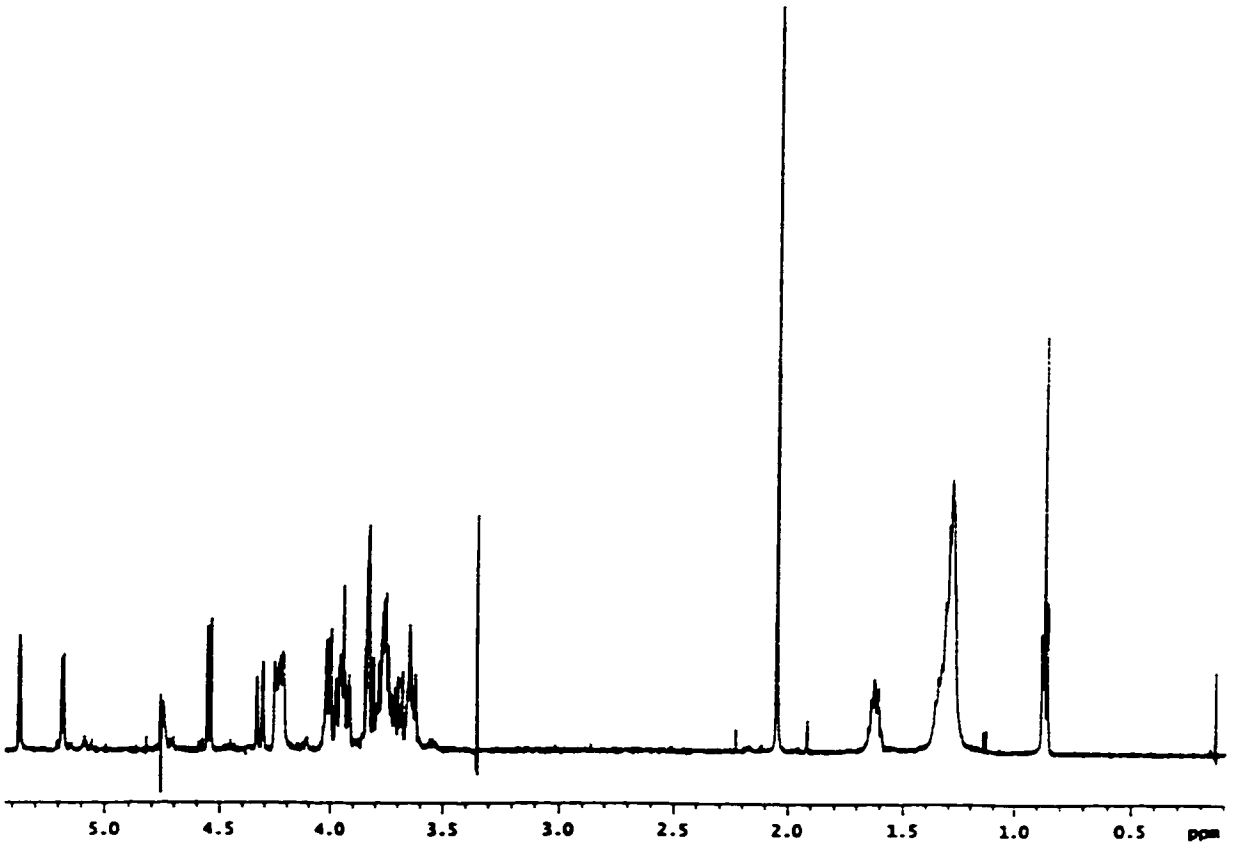


Figure 57. Spectrum of the *arabino* trisaccharide (45).