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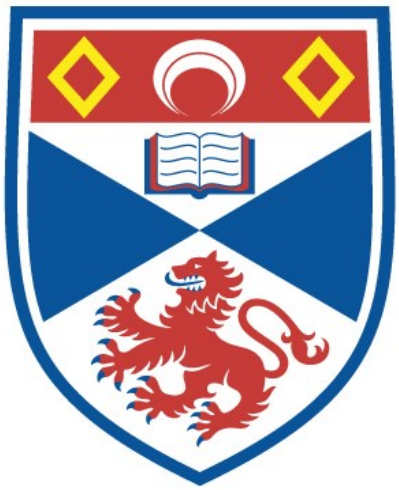

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School of Chemistry

University of St Andrews

Development of Fucosyltransferase Inhibitors

A Thesis Submitted for the degree of Doctor of Philosophy

Robert N. Bowles

September 2001

TK E36

Declaration

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CONTENTS

Abstract

Human α-1,3 fucosyltransferases are a class of enzymes which transfer fucose from the sugar nucleotide donor GDP-fucose onto an oligosaccharide acceptor. These enzymes are widely distributed throughout the tissues of the body and appear to be instrumental in normal development and function. However, excessive or aberrant fucosylation by these enzymes is thought to play a role in a number of disease states; chronic inflammatory conditions such as rheumatoid arthritis and psorias, along with the metastasis of cancer have been implicated here.

Despite the importance of fucosyltransferases, very little is known about their structure and function. In this project we set out to glean information about the active site of α-1,3 fucosyltransferases through the technique of photoaffinity labelling. Our photoaffinity probes were based on LacNAc analogue acceptor substrates of fucosyltransferase and were synthesised by a combination of chemical and enzymatic means. Specifically, we made azide and phenylacetamide derivatives which were converted to the free amine chemically (reduction of azide) or enzymatically (cleavage of phenylacetamide with penicillin G acylase).

Initially we attempted the enzymatic synthesis of the unnatural substrate UDP-3-azido-3-deoxy-galactose from the corresponding azido sugar using the enzymes galactokinase, which we extracted and purified from yeast, and uridyltransferase. The intention was then to couple this to benzyl GlcNAc using galactosyltransferase to produce benzyl 3'-azido-3'-deoxy-LacNAc. Difficulties with this approach led to us adopting a different strategy for producing derivatised LacNAc analogues involving the chemical synthesis of allyl 6-azido-6-deoxy-GlcNAc and subsequent biotransformation to 6-azido-6-deoxy-LacNAc. We also produced an anomerically derived LacNac analogue by way of the phenylacetamido functionality. Photoaffinity probes were made from both LacNAc templates and used to label fucosyltransferase VI.

The photoaffinity labelling technique was further applied to *trans*-sialidase, an enzyme from the protozoan parasite *Trypanosoma cruzi* which is responsible for the

spread of Chagas disease in South and Central America. *Trans*-sialidase transfers sialic acid from host cell oligosaccharides onto the parasite cell surface and is vital for is infectivity. However, it appeared that while the photo-probe was recognised as a substrate by the enzyme, cross-linking was not seen to take place.

Acknowledgements

My first thanks go to my supervisor Rob Field for giving me this opportunity; for all his help and encouragement, and for the good laughs along the way.

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The Field Group

Abbreviations

Ac	Acetyl
ATP	Adenosine 5'-triphosphate
ADP	Adenosine 5'-diphosphate
BSA	Bovine Serum Albumin
$CHCl_3$	Chloroform
CI-MS	Chemical Ionisation Mass Spectrometry
CPM	Counts per minute
DCM	Dichloromethane
DMF	*N,N*-Dimethylformamide
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ES-MS	Electrospray Mass Spectrometry
EtOAc	Ethyl acetate
EtOH	Ethanol
FAB-MS	Fast Atomic Bombardment Mass Spectrometry
FucT	Fucosyltransferase
GalT	β-1,4-galactosyltransferase
GlcNAc	N-Acetylglucosamine
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid])
IPTG	Isopropyl-β-D-thiogalacopyranoside
IR	Infra-Red Spectroscopy
LacNAc	N-Acetyllactosamine
MALDI-TOF	Matrix-Assisted Laser-Desorption-Ionisation Time-Of-Flight
MeOH	Methanol
MES	(2-[N-Morpholino]ethanesulfonic acid)
Neu5Ac	Neuraminic acid, Sialic acid
NMR	Nuclear Magnetic Resonance
PEP	phospho(enol)pyruvate
Pi	Inorganic Phosphate

PK	Pyruvate Kinase
PPi	Pyrophosphate
PSGL-1	P-selectin glycoprotein 1
sLe^x	Sialyl Lewis x
T. cruzi	*Trypanosoma cruzi*
TLC	Thin Layer Chromatography
TsCl	Toluenesulfonyl chloride (*p*)
UDP	Uridine 5'-diphosphate
UDP-Gal	Uridine 5'-diphospho-galactose
UDP-Glc	Uridine 5'-diphospho-glucose
UMP	Uridine 5'-monophosphate
UTP	Uridine 5'-triphosphate
UV	Ultra-violet

Chapter 1

Introduction

1.1. FUCOSYLATED OLIGOSACCHARIDES

Fucosylated oligosaccharides play important functions in numerous cellular processes and have been found in nearly all types of organisms including mammals, insects, molluscs, plants and bacteria.[1] This thesis begins by reviewing the structure, biosynthesis and recognition of fucosylated mammalian glycans. The biological role of such structures will be outlined and current knowledge of fucosyltransferases (FucTs) will be reviewed. For a comprehensive review of the subject see reference 2.

1.2 FUCOSYLATED MAMMALIAN GLYCANS

In the following section the structure and biosynthesis of fucosylated mammalian glycans are discussed.

1.2.1 The Blood Group Antigens

Carbohydrate structures are responsible for organ rejection in xenotransplantation with α-galactosyl epitopes on pig tissue being of great significance.[3] Humans naturally produce large amounts of anti-α-galactosyl antibodies resulting in hyperacute rejection of transplanted pig organs. Likewise, the human blood group antigens are responsible for rejection of incompatible human donor organs and incompatible human blood in transfusion.

The blood group antigens A, B and O (H) represent terminal sugar units based on oligosaccharide precursors which may be of one of four types (type1 – type4).

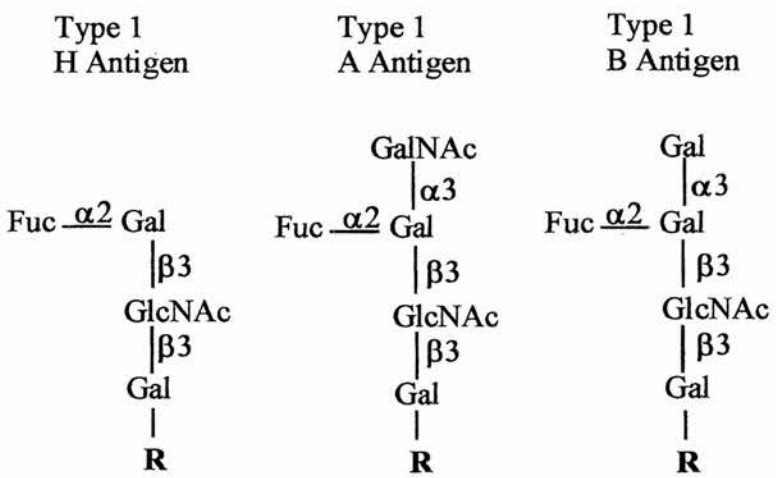

Figure 1. Type 1 A, B and O blood group structures.
(R = N-linked, O-linked or glycolipid glycan precursor)

Figure 1 shows Type 1 H, A and B antigens, which are formed on various precursors by the sequential action of specific glycosyltransferases encoded by three genetic loci (the *ABO*, *H*, and *Secretor* loci). The synthesis of the blood group antigens begins with the modification of the terminal galactose unit on the precursor glycans by α1,2-FucT activity. This produces the blood group H determinant, represented by the disaccharide unit Fucα1,2Gal. The human genome encodes two different α1,2-

FucTs, corresponding to the products of the *H* and the *Secretor* (Se) loci. The *H*-α1,2-FucT is expressed in erythrocytes and utilises type-2 and type-4 precursors to form type-2 and type-4 H antigens on red cells. The *Se* α1,2-FucT is expressed in epithelial cells utilising type-1 and type-3 precursors to form type-1 and type-3 H antigens on, for example, gastrointestinal and respiratory tract epithelia. Figure 2 shows type 1, 2, 3 and 4 H antigens to illustrate the different precursor glycans. The type 1 and type 2 antigen precursors are based on type 1 (Galβ1,3GlcNAc) and type 2 (Galβ1,4GlcNAc) chains; type 3 is an O-linked chain and type 4 a ceramide linked chain.

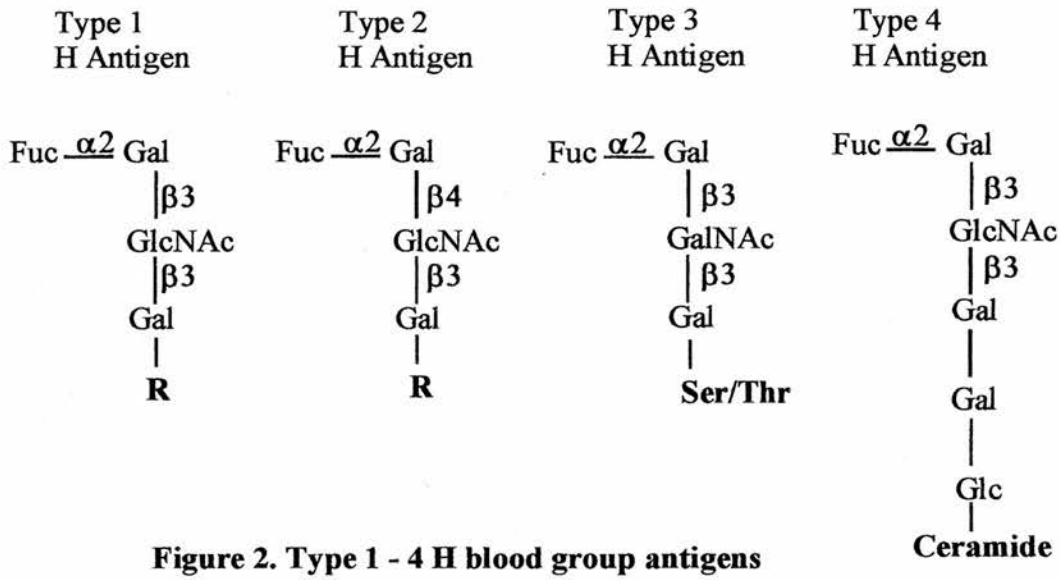

Figure 2. Type 1 - 4 H blood group antigens

(R = N-linked, O-linked or glycolipid glycan precursor)

The next step in the synthesis determines whether the A or B antigen is produced. Expression of the A allele of the ABO locus which encodes α1,3 GalNAcT gives rise to the trisaccharide GalNAcα1,3(Fucα1,2)Gal, the A antigen, whereas expression of the B allele encoding α1,3-galactosyltransferase produces Galα1,3(Fucα1,2)Gal, the B antigen. O alleles encode functionally inert polypeptides that do not further modify the H antigen and therefore represent null alleles at this locus.

Blood group A individuals have genotypes AA or AO, blood group B individuals have genotypes BB or BO and those of blood group AB have the genotype AB.

Blood group O individuals do not express either the A or B antigen thus leaving their H antigens unmodified and are homozygous for the O (null) allele (genotype OO).

Soluble forms of ABH determinants are produced by the epithelia of some exocrine glands, including salivary glands. Most of these tissues express type 1 oligosaccharides (as do all epithelia lining the digestive, respiratory, urinary and reproductive tracts) and therefore soluble antigens are largely represented by type 1 molecules. The α1,2-FucT required to produce the H determinant is encoded by the *Se* locus since the *H* locus encoded α1,2-FucT is not expressed in these tissues. Some individuals are homozygous for null alleles at the *Se* locus and are therefore incapable of synthesizing the H determinant in these tissues. The A and B determinants can also not be produced. These phenotypic individuals are described as nonsecretors since soluble blood group H, A and B antigens cannot be detected in their saliva.

There are important medical implications associated with red blood cell antigen type. The immune system produces IgM antibodies early in life against those blood group determinants which are not present in the individual. This represents a line of defence against oligosaccharide antigens presented by bacterial and fungal organisms which may be similar or identical to the human blood group antigens. A blood type O individual then will carry IgM antibodies (termed isoagglutinins) against A and B determinants. Simlarly, blood group B individuals have IgM isoagglutinins that react with A determinants, but not B (a self antigen) and those of blood group A have isoagglutinins against B determinants but not A. Finally, individuals of blood group type AB do not make either anti-A or anti-B antibodies. Anti-H antibodies are not made in most individuals because a substantial fraction of H structures are not further converted to A or B antigens even in A, B and AB genotypic individuals.

IgM isoagglutinins are the basis for severe reactions seen during incompatible blood transfusions. They circulate in human plasma at levels sufficient to cause complement-dependent lysis of 'foreign' erythrocytes. This acute immunogenic response produces the clinical symptoms which can include hypotension, shock, acute renal failure, and death from circulatory collapse. The problem is avoided by

ensuring compatibility between the transfused cells and the patients ABO blood type. Essentially this means choosing red blood cells that are deficient in the ABO antigens which are also lacking in the recipient, so for example a patient of blood type A may receive blood from a donor typed A or O, but not one typed B or AB. Practically, this is achieved by typing and cross-matching procedures.

The biological purpose of the heterogeneity of blood group determinants are not clear and have been further called into question by the discovery of a rare phenotype where individuals are deficient in red blood cell H, A and B antigens. The so-called Bombay phenotype because the first identified case lived in that city, individuals serum contains isoagglutinins that react with red blood cells from virtually all donors including O blood types. These people are homozygous for null alleles at the *H* α1,2-FucT locus and are also nonsecretors (also homozygous for null alleles at the *Se* α1,2-FucT locus). Incapable of synthesizing A, B or H blood group antigens these individuals consequently have antibodies circulating against these determinants. As a result they are incompatible with blood from all donor types other than Bombay donors. The fact that Bombay individuals exhibit no particular pathological phenotype suggests that no biological advantage is conferred by blood group type heterogeneity. Perhaps these determinants did play a function earlier in evolution but have now been rendered obsolete. Thus, the selective pressures which produced polymorphism at the ABO locus remain unclear.

1.2.2 The Lewis Antigens

The Lewis antigens are a related group of α1,3-fucosylated oligosaccharides that are expressed on the surface of mammalian cells. The term Lewis refers to the family name of people who were suffering from a red blood cell incompatibility problem which lead to the discovery of this group of blood antigens. These antigens are found in several different tissue types in the human body as well as on the surface of red blood cells. There are four known Lewis antigens, Lewis a (Le ^{a}), Lewis b (Le ^{b}), Lewis x (Le ^{x}) and Lewis y (Le ^{y}). The Lewis a and b oligosaccharides have a type 1 core based on the disaccharide unit Gal-ß-1,3-GlcNAc while x and y have a type 2 core derived from Gal-ß-1,4-GlcNAc (N-acetyllactosamine, LacNAc) (Figure 3).

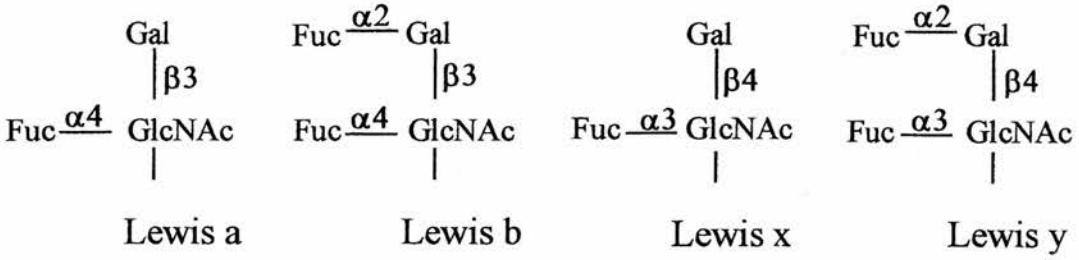

Figure 3. The Lewis antigens

The Lewis a antigen is synthesised by the action of Lewis α1,3/4-FucT on the type 1 precursor. This enzyme is encoded by the Lewis (Le) blood group locus. The Lewis b antigen is synthesised by the action of this same enzyme along with the action of α1,2-FucT encoded by the *Se* blood group locus. The type of Lewis antigens expressed in an individual then depends on the alleles present at the *Le* and *Se* loci. Those individuals who are secretors are able to synthesise the type 1 H determinant (Fucα1,2Galβ1,3GlcNAc) which can then be converted to the Lewis b antigen by the action of the Lewis α1,3/4-FucT (Le^{a-b+} phenotype). Nonsecretors cannot synthesise the H antigen but produce Lewis a by the action of the Lewis enzyme on the unmodified type 1 disaccharide (Le^{a+b-} phenotype). Finally secretor Lewis negative individuals produce only the H antigen (Le^{a-b-} phenotype) while nonsecretor Lewis negative individuals cannot modify at all the unsubstituted precursor chain (also Le^{a-b-} phenotype). Pathways showing the biosynthesis of the H, Le^a and Le^b antigens from the type 1 precursor are outlined in Figure 4.

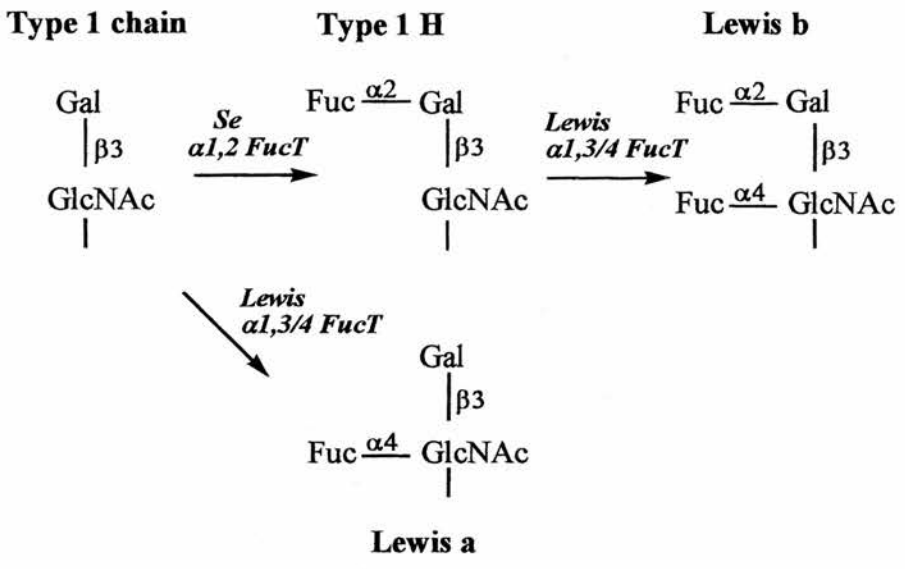

Figure 4. Synthesis of type 1 Lewis antigens

The analogous biosynthesis of the H, Le^x and Le^y antigens from the type 2 precursor are outlined in Figure 5.

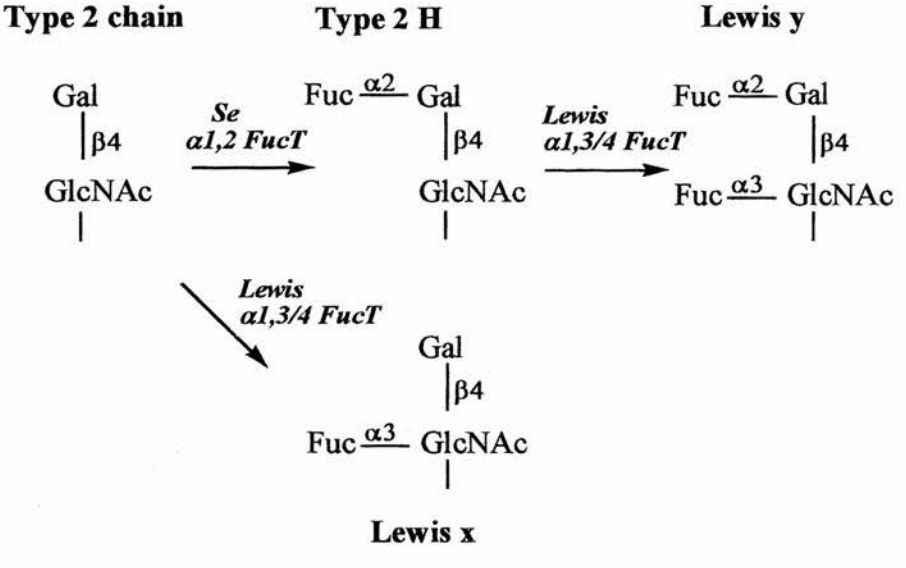

Figure 5. Synthesis of type 2 Lewis antigens

The Lewis a, Lewis b, Lewis x and Lewis y antigens are synthesised for the most part in those epithelial tissues which express the *Se* α1,2-FucT. Soluble forms are also produced which are released into secretions and body fluids. These antigens can then passively adsorb onto red blood cell membranes (Le^a molecules are detected at a

level of between 4500 and 7300 per cell). Immunogenic reactions against these antigens in transfused blood are not associated with any clinical problems unlike with ABO antigens. This is largely because those Lewis antigens present in the donor cells which are not present in the recipient's are rapidly lost after transfusion due to a reversal of the adsorptive process by which they were accumulated in the donor.

Other members of the Lewis blood group antigens include sialyl Lewis x , 3'-sulfo Lewis x , 6'-sulfo-sialyl Lewis x, sialyl Lewis a and 3'-sulfo Lewis a are formed through the actions of one or more α1,3-FucTs and are illustrated in Figure 6 below.

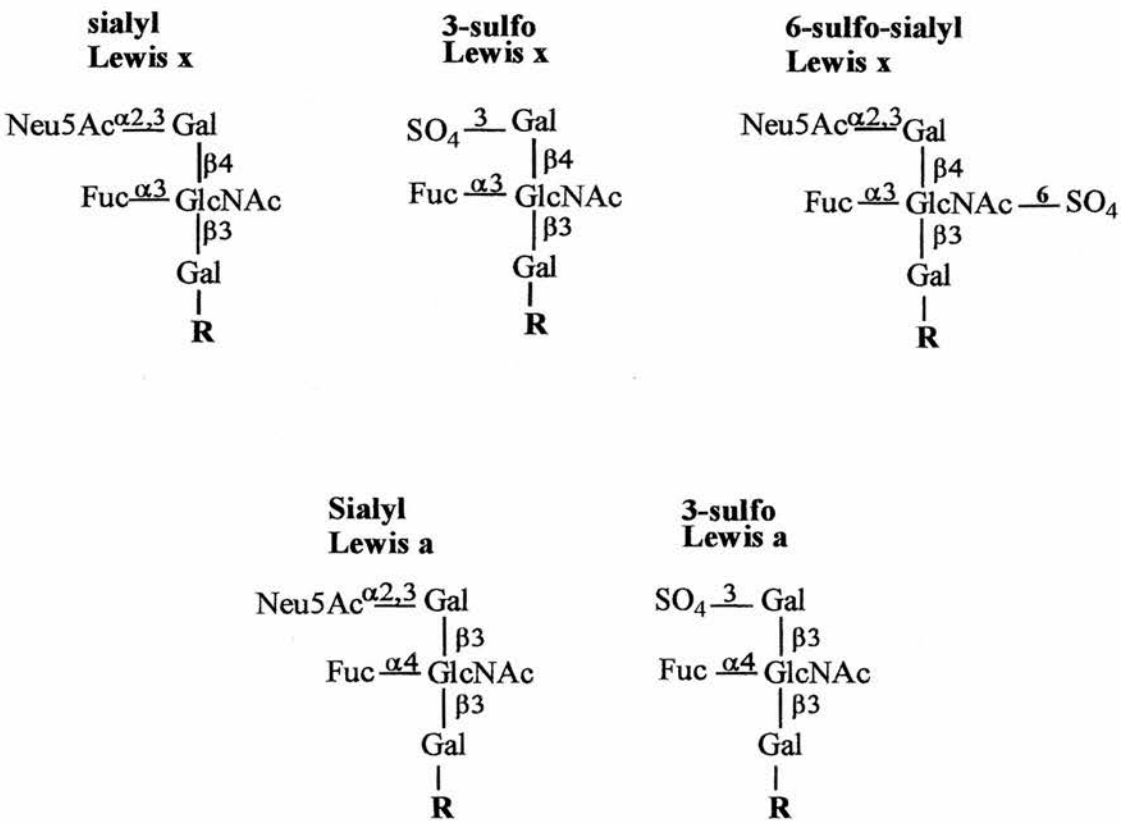

Figure 6. Other Lewis blood group antigens

1.3 SELECTINS

1.3.1 Biological Role of Selectins

The selectins are a family of three membrane-anchored Ca^{2+}-dependent (C-type) lectins that bind to cell surface carbohydrate ligands, in particular fucose-containing Lewis-type antigens.[4] These interactions promote adhesion of leukocytes to platelets, endothelial cells or other leukocytes in response to infection or tissue injury. Selectin-ligand association occurs rapidly but transiently, allowing a free-flowing leukocyte to tether to and then roll on the vessel wall under the shear forces of postcapillary venules. The rolling leukocytes encounter regionally presented chemokines that activate the leukocytes to express integrins. Interaction of these leukocyte integrins with immunoglobulin-like counter receptors strengthens the adhesion and directs the extravasation of the leukocyte into the underlying tissues in response to chemotactic gradients.

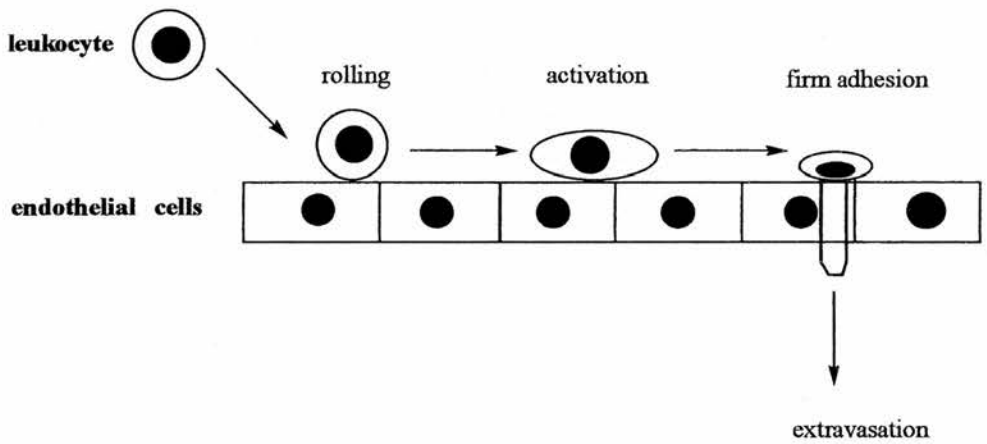

Figure 7. Interaction of leukocytes with endothelial cells

Each selectin has an amino-terminal carbohydrate-recognition domain characteristic of C-type lectins, followed by an epidermal growth factor (EGF)-like module, a series of short consensus repeats (SCRs), a transmembrane domain, and a short cytoplasmic tail (Figure 8).

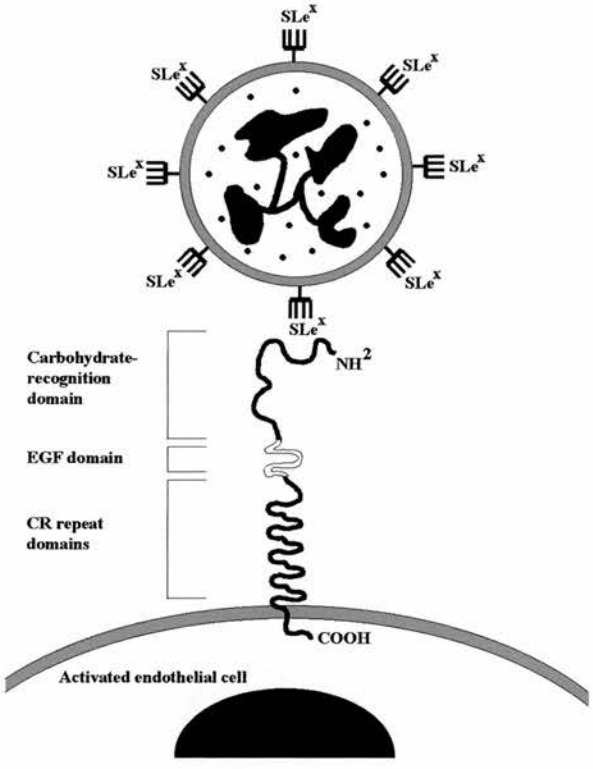

Figure 8. Interaction of E-selectin with leukocyte-expressed SLe^x

Taken from ref 34, p176

Contrasting evidence has been put forward regarding the contribution of these various domains to ligand specificity. Some studies using selectin constructs in which the EGF domain and/or SCRs have been deleted, switched or mutated suggest that these domains do influence ligand specificity, while one group finds no obvious difference in ligand specificity when the EGF domain and SCRs of E- and L-selectin are exchanged. The selectins are linear molecules with little contact between the various domains, and it remains unclear how the EGF domains and SCRs might affect the binding function of the lectin domain.[4]

L-selectin, expressed on most leukocytes binds to constitutively expressed ligands in the endothelium of peripheral lymph nodes, to inducible ligands on endothelium at sites of inflammation, and to ligands on other leukocytes. E-selectin, expressed on activated endothelial cells and P-selectin, expressed on activated platelets and on endothelial cells, bind to ligands on leukocytes. The selectins bind sialylated,

fucosylated oligosaccharides such as sialyl Lewis x, a terminal component of glycoproteins and glycolipids on most leukocytes and some endothelial cells.[5]

1.3.2 Regulation of Selectin Expression

The expression of selectins is normally tightly regulated to ensure that leukocytes tether to, and roll on the blood vessel wall only at appropriate locations. L-selectin is proteolytically shed from leukocytes after cell-cell contact, a mechanism that modulates and then down regulates its function.[6] E- and P-selectin are only expressed on the surface of endothelial cells and/or platelets after the cells are activated. Tumour necrosis factor α (TNF-α), interleukin-1β (IL-1β) or lipopolysaccharide (LPS) transiently induce endothelial cells to transcribe E-selectin mRNA, which leads to synthesis of E-selectin protein. Surface expression of E-selectin peaks within 4 hours of activation and then usually declines over the course of 12-24 hours.[7]

1.3.3 Importance of Selectins

The importance of selectins in humans is underscored by the discovery of a congenital disorder of fucose metabolism, termed leukocyte adhesion deficiency 2 (LAD-2).[8] Because patients with LAD-2 lack fucosylated glycoconjugates, they do not express functional selectin ligands on leukocytes or, presumably on endothelial cells. Leukocytes from these patients do not tether to or roll on P- or E- selectin surfaces. Clinically, the patients have more infectious diseases, supporting the concept that the selectins have an important function in initiating recruitment of leukocytes.

1.3.4 Selectins and Cancer

Metastasis of cancer is a complicated process consisting of many steps. The process starts with the intravasation of cancer cells into the blood stream from the primary tumour lesion. The cancer cells then travel in the blood stream where they interact with various blood cells and finally they adhere to endothelial cells somewhere in the peripheral vessel walls. Adherence to the capillary endothelium then occurs, resulting in extravasation, where the cancer cells enter the connective tissue to form a new metastatic lesion. It is evident that those cancer cells having a higher affinity for

endothelial cells will have a higher chance of metastasising. It has also become evident that this adhesion is mediated by carbohydrate interactions[9].

1.3.4.1 Cancer-Associated Carbohydrate Antigens (CACAs)

Experiments by Kannagi[9] showed that cultured human colon or lung cancer cells can adhere to interleukin-1β (IL-1β) treated human umbilical vein endothelial cells (HUVECs) almost as strongly as leukocytes do. IL-1β activated endothelial cells are known to express three major cell adhesion molecules, ICAM-1, E-selectin and VCAM-1. Inhibition experiments using neutralising monoclonal antibodies specific to these molecules showed that adhesion of cancer cells was significantly inhibited by the anti-E-selectin antibody. The study was expanded to a wide variety of epithelial cancer cells, including those originating in the colon, stomach, pancreas, liver, lung and ovary and demonstrated that the E-selectin-carbohydrate interaction plays an important role in the adhesion of all these cancer cell types to endothelial cells. As sLe^x was expressed on some of these cancer cells, its interaction with E-selectin was assumed to be involved in adhesion. However it was subsequently found[10] that another well known cancer-associated carbohydrate antigen, sLe^a, can also serve as a ligand for E-selectin and is involved in the binding of cancer cells to endothelial cells. Furthermore, it was shown that sLe^a and sLe^x are expressed on different types of cancer cells. Ovary, lung, liver and stomach cancer cells use sLe^x to adhere to endothelial cells whereas some other human cancer cells, including mostly cancers of the digestive organs, such as the colon and pancreas, use sLe^a. These ligands have in fact long been used as serum markers in the diagnosis of cancer in several countries including Japan.[11]

1.3.4.2 CACAs as Prognostic Markers

If E-selectin-carbohydrate ligand interactions do play a significant role in the metastasis of cancer we can reasonably expect that patients with cancer cells strongly expressing such ligands will be at greater risk of metastasis and therefore present with a poorer prognosis. This has indeed been found to be the case with levels of sLe^x expressed on cancers of the lung, ovary, liver, kidney and breast.[12] Likewise, clinical studies show a similar correlation between levels of sLe^a and metastasis of colon, pancreas and biliary tract cancers.[13]

1.3.4.3 E-selectin Expression and Metastasis

E-selectin is not always expressed on every blood vessel wall, but can be induced by inflammatory cytokines such as IL-1β and TNF-α. On the other hand, TGF-β and IL-4, are known to be capable of inhibiting the induction of E-selectin expression. This suggests that the level of E-selectin in patients can fluctuate and implies an additional risk factor in patients, besides the level of CACA expression on the cancer cells, with increased levels of E-selectin facilitating adhesion of cancer cells.

Some cancer cells have an ability to induce expression of E-selectin on endothelial cells. The blood vessels near tumours frequently express E-selectin[14] and many cultured human cancer cell lines produce humoural factors that induce the expression of E-selectin on endothelial cells, including IL-1α and another unknown factor that stimulates leukocytes to secrete IL-1β.[15]

1.3.4.4 Role of Integrins in Adhesion

It has been suggested that after the initial adhesion of cancer cells to endothelium involving carbohydrate-selectin interaction, a second phase of adhesion takes place involving integrins on cancer cells which participate in further binding to the endothelium. During the course of the initial adhesion of cancer cells to endothelia, cancer cells are stimulated by cytokines that are present on the surface of endothelial cells, and this leads to the activation of integrins on the cancer cells. HGF (hepatocyte growth factor), for example, can stimulate the activation of $\alpha_2\beta_1$-integrin on human liver cancer cells, which then show increased adhesion activity to collagen coated plates.[16] Likewise, HB-EGF (heparin-binding epidermal growth factor) can activate $\alpha_2\beta_1$- and $\alpha_3\beta_1$-integrins on human breast and oesophageal cancer cells. It is likely that many other cytokines associated with human endothelial cells will have a similar activating effect on cancer cell integrins. For the cancer cells to respond to cytokines they must possess the appropriate functional receptor, which in the case of HGF is the *c-met* product and EGF-R in the case of HB-EGF.

The HGF-mediated enhancement of integrin expression would be limited to the cancer cells that express the *c-met* oncogene product, which is known to occur mainly on liver cancer cells. Similarly, the HB-EGF-mediated enhancement of

integrin expression would be limited to the cancer cells that express EGF receptor, which is frequently detectable on oesophageal cancer cells. This would indicate that the sets of cytokines and integrins involved in the second stage of cancer cell adhesion to endothelium is highly dependent on the lineage of cancer cells. This is in clear contrast to the molecules involved in the first stage of cancer cell adhesion, where the carbohydrate ligands are expressed on a wide variety of cancer cells, and the selectin-mediated adhesion is observed with cancer cells of various origins.[9]

1.3.4.5 Role of P-Selectin in Metastasis?

P-selectin, first described in activated platelets, is also known to be expressed on endothelial cells and is involved in the recruitment of leukocytes in the inflammatory response.[17] Various cancer cells are known to be bound more frequently and strongly by P-selectin than by E-selectin and so it has been suggested that it plays a more important role in cancer metastasis than E-selectin.[18] However, there are some discrepancies regarding the importance of P-selectin in cancer metastasis with some researchers indicating that cancer cells do not always adhere strongly to these receptors.[19] With regard to leukocytes, it is suggested that P-selectin specifically recognises sLe^x presented by the PSGL-1, (P-selectin-glycoprotein ligand 1), while E-slectin recognises sLe^x but does not require PSGL-1.[20] Most cancer cells fail to express PSGL-1 and do not strongly adhere to CHO cells expressing P-selectin. Transfection of these cancer cells with PSGL-1 cDNA however makes them more adherent to P-selectin-CHO cells.[19] This indicates that cancer cells would need PSGL-like molecules to adhere to P-selectin. The P-selectin-mediated adhesion of platelets to cancer cells, however, has long been implicated in the metastasis of cancer by forming aggregates in the capillaries. This then causes the entrapment of the cancer cell and facilitates its extravasation to the surrounding tissue where the secondary tumour then grows. The mechanistic basis of this reaction remains uncertain.[21]

1.4 FUCOSYLTRANSFERASES

The enzymes responsible for catalysing the attachment of fucose residues to oligosaccharide structures are the fucosyltransferases (FucTs).[22] All known FucTs use the donor sugar nucleotide GDP-fucose and catalyse the transfer of fucose to an appropriate acceptor substrate forming an α linkage. However, the type of α linkage formed depends on the organism, tissue and acceptor substrate. In mammals, FucTs can add fucose in α1,2 linkages to the terminal Gal or Glc, in α1,3 or α1,4 linkages to the internal GlcNAc in LacNAc, and in α1,6 linkages to the asparagine linked GlcNAc residue of N-glycans.

1.4.1 Human α-1,3-Fucosyltransferases

Many of the oligosaccharides found on the surfaces of cells contain fucose residues in α1,3 linkages. Six different types of human α1,3-FucTs have so far been identified and cloned, III, IV, V, VI, VII and IX (see table). They constitute a family of closely related membrane-bound enzymes distinguishable by acceptor specificity, tissue distribution, pH optimum, kinetic properties, cation requirement and sensitivity to inhibitors. As can be seen from table 1 most types of tissue contain more than one type of FucT activity.[22]

Of the FucTs, α1,3-FucT III has the broadest acceptor specificity.[23] Also known as the α1,3/4-FucT it is able to fucosylate type 1 oligosaccharides by forming an α1,4 linkage (to produce Le^a determinants) and also to fucosylate type II oligosaccharides by forming an α1,3 linkage (to produce Le^x determinants). Furthermore, α2,3-sialylated and α1,2-fucosylated type I and II chains can serve as acceptor substrates. FucT III is, in fact, only one of two known glycosyltransferases that is able to form more than one type of linkage.

FucT IV, also called the myeloid enzyme, appears to play an important role in cell-cell recognition during normal embryonic development when it is active in all tissues. In human adults activity is only present in myeloid cells, leukocytes and the brain.[24]

Enzyme	Classification	Tissue Distribution	Substrates	Products
α-1,3-FucT III	Lewis	human milk, gall bladder, kidney, colon	type I, type II sialyl typeI + II fucosyl typeI +II	Le^a, sLe^a, Le^b, Le^x, sLe^x, Le^y
α-1,3-FucT IV	Myeloid	brain, myleoid cells	type II, sialyl type II	Le^x, sLe^x
α-1,3-FucT V		plasma, human milk, liver	type II, sialyl type II	Le^x, sLe^x, sLe^y
α-1,3-FucT VI	Plasma	plasma, kidney, liver, colon	type II, sialyl type II, fucosyl type II	Le^x, sLe^x, sLe^y
α-1,3-FucT VII	Luekocyte	leukocytes	sialyl type II	sLe^x
α-1,3-FucT IX		CNS, stomach, leukocytes	type II	Le^x, Le^y

Table 1. Biochemical Properties of Human α–1,3-FucTs

FucT V is also called plasma type enzyme as this is where most of its activity is found. This enzyme shares a 91% amino acid sequence homology with FucT III and there are some reports that it too can act on type I acceptors.[25]

FucT VI (second plasma type) may be the FucT responsible for the synthesis of the sLe^x on leukocytes, which is involved in the homing of these cells to endothelial tissue in the inflammatory response.[26]

FucT VII may also be responsible for the synthesis of the sLe^x epitope present on the surface of leukocytes. Sequence and functional comparisons with a recently cloned murine α1,3-FucT suggests FucT VII may participate in the generation of fucosylated ligands for L selectin.[27-29]

FucT IX is the latest α1,3-FucT to be cloned. Expressed in the brain, stomach and peripheral blood leukocytes, this gene is highly conserved between the corresponding murine Fuc-T IX suggesting a strong selective pressure for the preservation of the sequence in evolution. FucT IX is thought to be responsible for production of the

developmentally regulated CD15 epitope (determined as the Le^x structure) in neuronal and glial cells and therefore in the development of the CNS.[30,31]

1.4.2 Evolution of FucT Genes

To date, 78 FucT genes have been identified, 48 of them vertebrate genes, 30 invertebrate.[22] Structure and sequence analysis lends strong support to the theory that these numerous and diverse FucTs have arisen through duplication and divergent evolution from one or two ancestral genes.

Substantial sequence homology amongst the vertebrate FucT genes immediately points towards a divergent model for their evolution. Additional weight for this hypothesis is provided by the finding of conserved peptide motifs among enzymes within the three distinct FucT gene families, the α-2, α-3 and α-6 families (see Figure 9).

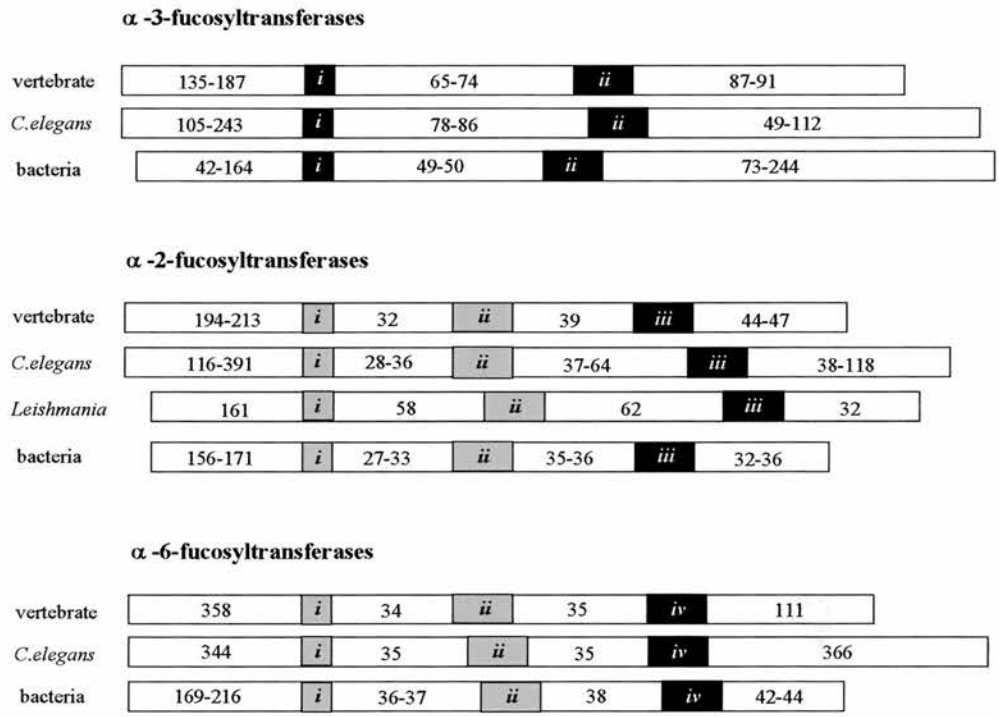

Figure 9. Schematic representation of vertebrate, invertebrate and bacterial FucTs showing the location of the conserved peptide motifs.

The black rectangles i, ii, iii and iv represent different motifs specific to each group of FucT. The grey rectangles i and ii are motifs shared by both α-2 and α-6 FucTs. Figures inside rectangles indicate the range of pre, post and inner-motif peptide lengths.[32]

Two conserved regions are found in the α-3-FucTs and three are found in the α-2 and α-6-FucTs. These characteristic motifs which are present in all vertebrate, invertebrate and bacterial enzymes thus also provide structural signatures for each gene family. The fact that two of the three conserved peptide motifs in the α-2 and α-6 families are actually shared between them suggests these two families of genes have a common ancestor gene. Similarities between the α-2 and α-3 families are not so obvious with less than 20 % sequence similarity and no shared conserved regions. However, hydrophobic cluster analysis does show similarities between motif-I in α-3-FucTs and motif-I in α-2 and α-6-FucTs. Furthermore it has recently been shown that both the recombinant FUT3 enzyme and the purified Lewis α1,3/4-FucT demonstrate a small amount (2-4%) of α1,2-FucT activity. These findings lean

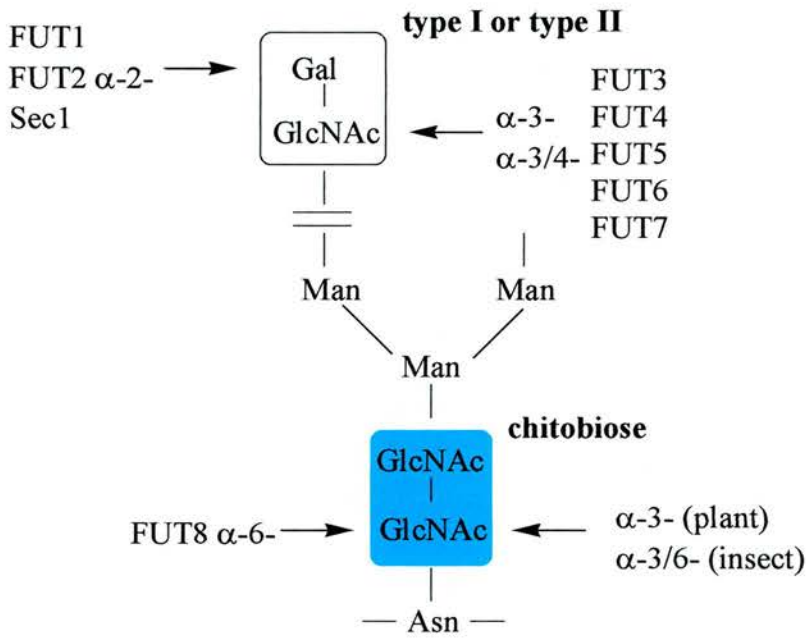

Figure 10. The two main types of acceptor substrate.

Chitobise and type I or type II acceptor and the corresponding fucosyltransferase enzymes able to add fucose in α-2-, α-3- and α-6-linkages. Taken from ref. 32.

towards the possibility of there being a common ancestor gene for the α-2 and α-3 families.

1.4.3 The Ancestor Gene?

In plants and insects there are α-3 FucTs that are capable of fucosylating the asparagine-linked GlcNAc of chitobiose (Figure 10). Since this disaccharide is contained within the first oligomannose oligosaccharide block added to the asparagine of the peptide chain in peptidoglycan synthesis and therefore added before the terminal N-acetyllactosamine (which is the acceptor for the α-2 and α-3-FucTs), this acceptor must have appeared first in evolution. It has therefore been hypothesised that the FucTs using chitobiose as an acceptor might have appeared earlier in evolution than those which act on N-acetyllactosamine and that these enzymes are present forms of the missing link between the α-2/6 and α-3-FucT families. This is illustrated in Figure 11, which depicts the evolutionary relationship of known FucTs. However, further work to establish the presence of conserved peptide motifs shared between these two families is necessary to validate this model.[33]

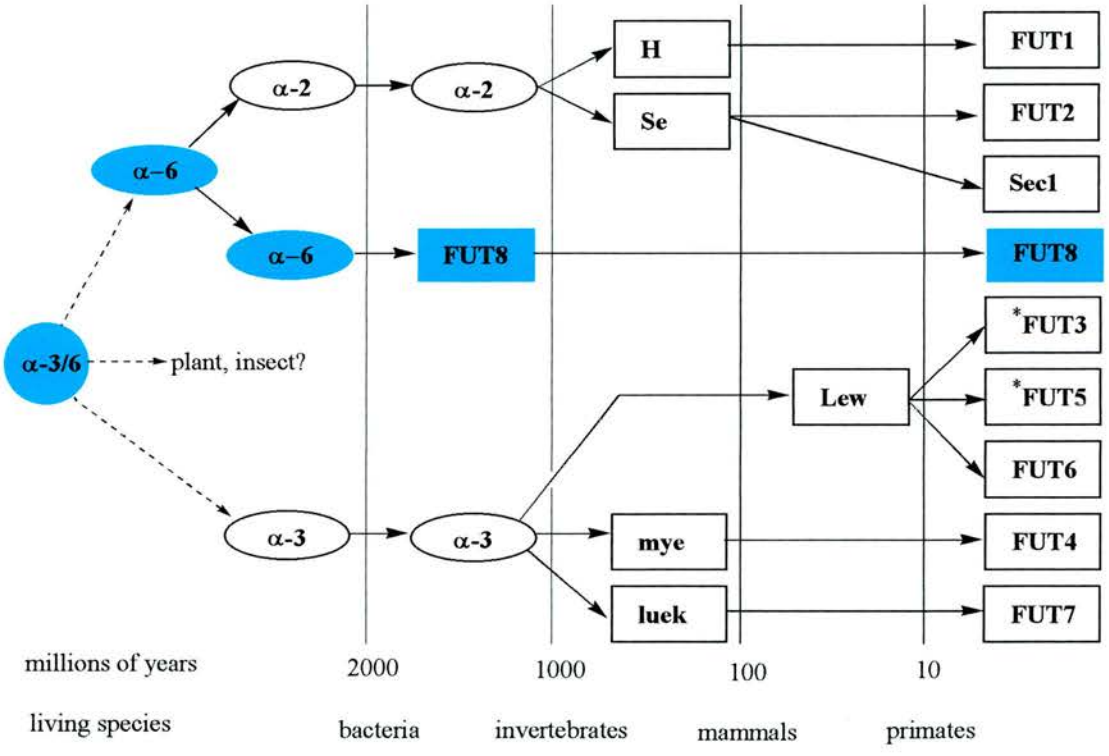

Figure 11. Hypothetical model of divergent evolution of FucT genes.

The rectangles are genes with overall sequence identity >30 % and the ovoids are enzymes with a common peptide motif. The circle is the hypothetical common ancestor of the α2,6- and α1,3-FucT families. The shaded symbols identify enzymes expected to use chitobiose as acceptor substrate, the rest using N-acetyllactosamine. * Can use type 1 and type 2 acceptors. Taken from ref. 32.

1.4.4 Divergent Evolution

It is apparent that not all aspects of FucT evolution have been divergent. For example plants have α1,4-FucTs which can act on type I acceptors to produce Le^a epitopes. In animals the capacity to fucosylate type I acceptors in order to synthesize Le^a epitopes is found only in chimpanzee and man and is therefore a relatively recent occurrence in evolution. It thus appears that α1,4-FucTs have evolved twice, first in plants where they act on type I acceptors and then in primates as an α1,3/4-FucT where they act on both type I and type II acceptors. This suggests that the need to synthesize the Le^a epitope has driven the convergent evolution of this particular FucT activity in plant and primate.

1.4.5 Fucosyltransferases as Therapeutic Targets

The importance of SLe^x in the immune response is underlined by the hereditary disease leukocyte adhesion deficiency type 2a. Due to a defect in the biosynthesis of SLe^x, the neutrophils (phagocytic leukocytes) are not able to adhere to E-selectin on stimulated endothelium and thus migrate into the zone of inflammation. Patients with this condition suffer from repeated severe bacterial infections.[16] However, in many instances too many leukocytes flood to the site of injury which results in normal cells being destroyed. This is the basis of a series of acute and chronic inflammatory diseases such as rheumatoid arthritis, psoriases and dermatitis.[34] Furthermore, high levels of sialyl Lewis x have been found on the surfaces of certain tumour and cancer cells, such as colon and lung cancer cells.[35] This suggests that cancer cells may exploit these ligands to metastasise, with tumour cells released into the blood attaching themselves to platelets by interacting with P-selectin. These tumour aggregates can then be trapped in veins. Cytokines such as interleukin 1 are released from the tumour cell resulting in its extravasation into the tissue where it is then able to grow into a secondary tumour.[9] Thus it can be seen that although sialyl Lewis x is an important ligand in the normal functioning of the body, playing an important role in the inflammatory response, it is also responsible for serious and even fatal disease processes.

In the development of therapeutic agents for these diseases, one strategy being adopted is the interruption of the biosynthetic pathway leading to SLe x formation (Figure 12). The first enzyme involved in SLe^x biosynthesis is N-

acetylglucosaminyltransferase which catalyses the transfer of N-acetylglucosamine (GlcNAc) to the terminus of an N-glycan. The second step is the transfer of a galactose residue from uridine diphosphate galactose (UDP-Gal) to the 4-OH of the GlcNAc residue, catalysed by UDP-Gal:ß-1,4-galactosyltransferase (ß-1,4-GalT). A sialic acid unit is then transferred from cytidine monophosphate sialic acid (CMP-sialic acid) to the 3-OH position of the galactose unit, catalysed by CMP-sialic acid:α-2,3-sialyltransferase (α-2,3-sialylT). The final step is fucosylation. The enzyme catalysing this step is α-1,3-FucT (α-1,3-FucT), transferring a fucose residue from guanidine diphosphate fucose (GDP-fucose) to the 3-OH of the GlcNAc moiety.[36]

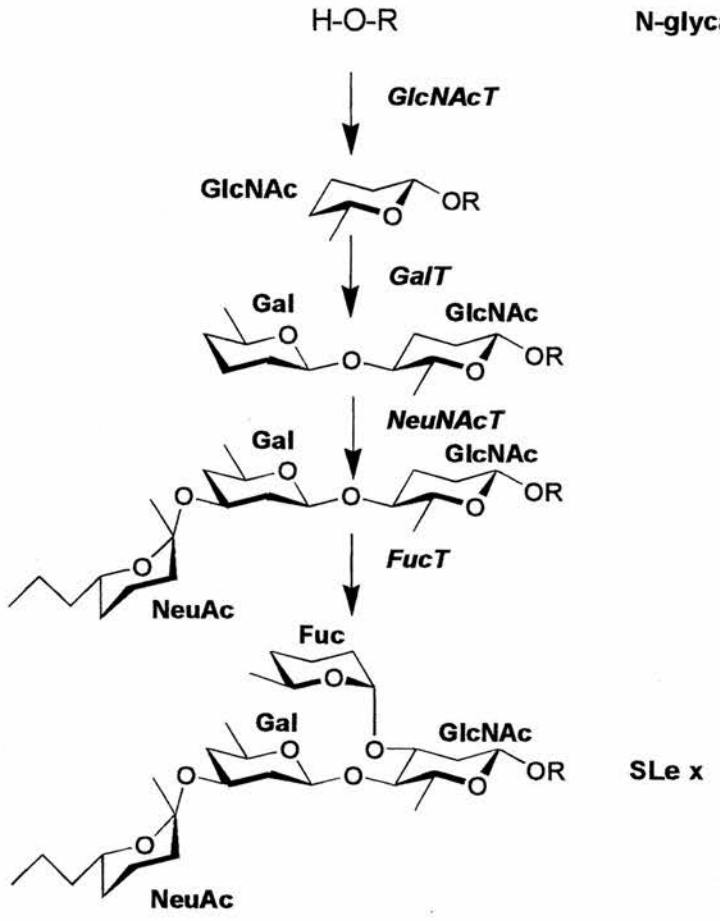

Figure 12. The biosynthetic pathway of sLe x

Of the four enzymes catalysing the biosynthesis of the SLe^x antigen, FucT is the preferred target for inhibition as this would have a minimal effect on the formation of other important carbohydrate antigens.

1.4.6 Mechanism of Fucosyltransferases

Despite the importance of the reactions catalysed by FucTs, very little is known about their detailed mechanism of action. Palcic *et al*[37] have hypothesised that the α-1,2-FucT reaction proceeds through a simple ion-pair transition state which results in the inversion of anomeric configuration of the fucose residue (Figure 13).

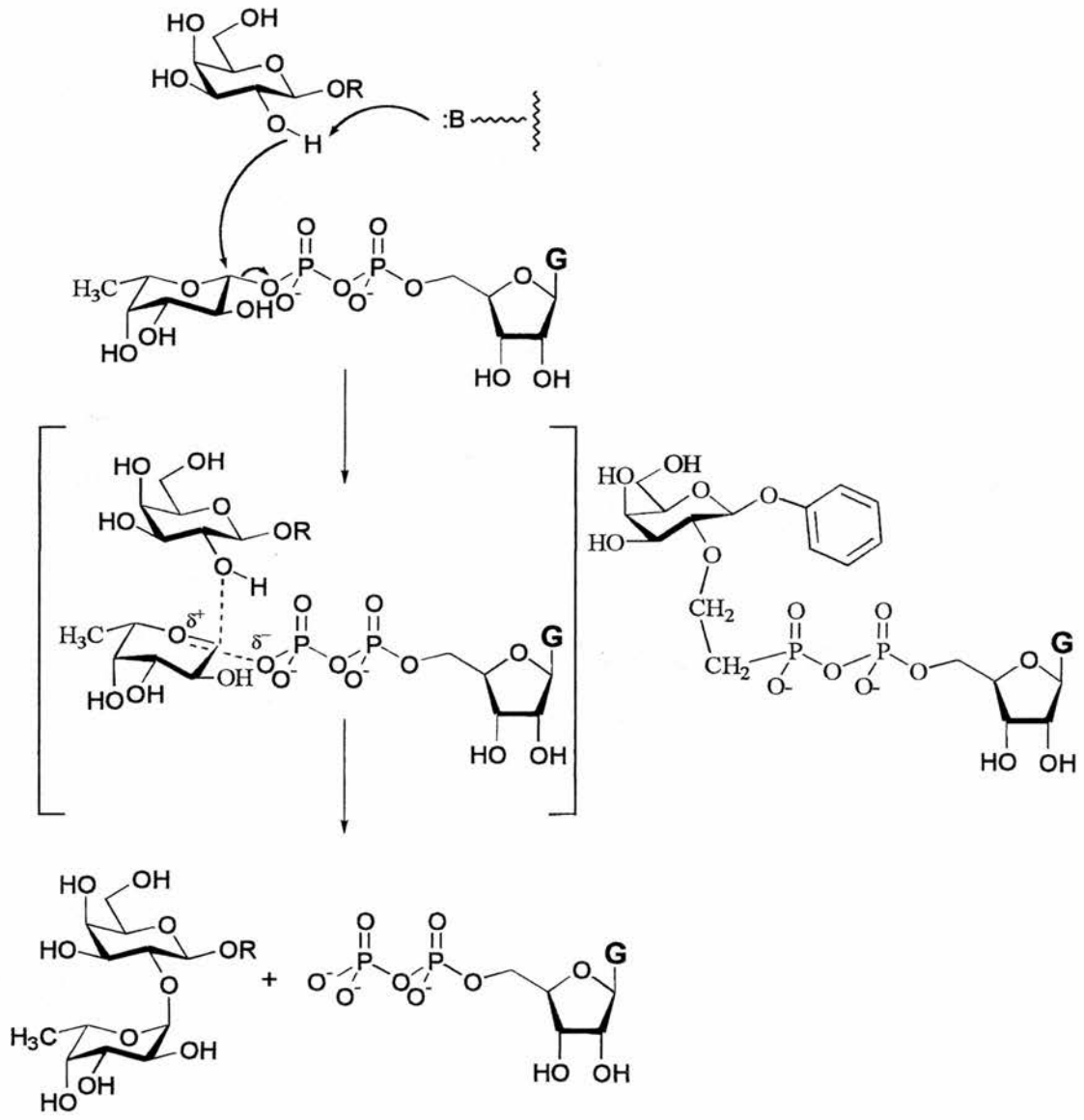

Figure 13. Mechanism and bisubstrate inhibitor for α-1,2 FucT

A bisubstrate inhibitor of an α-1,2-FucT containing elements of the sugar-donor and the acceptor substrate has also been reported by Palcic et al.[37] The enzyme β-galactoside α-1,2-FucT transfers a fucosyl residue from GDP-fucose to the 2-OH

group of β-D-galactopyranosides. The bisubstrate analogue inhibitor is an analogue of the postulated transition state of this reaction, where the O-2 of phenyl galactopyranoside is attached to the terminal phosphorous of GDP by a flexible ethylene bridge (Figure 13). However, the level of inhibition achieved from the above approach was similar or only slightly greater than that of the product inhibitor GDP.

α-1,3-FucTs may operate via a similar mechanism involving a displacement of GDP by the acceptor hydroxyl group, assisted by a base in the enzyme active site (Figure 14).[38]

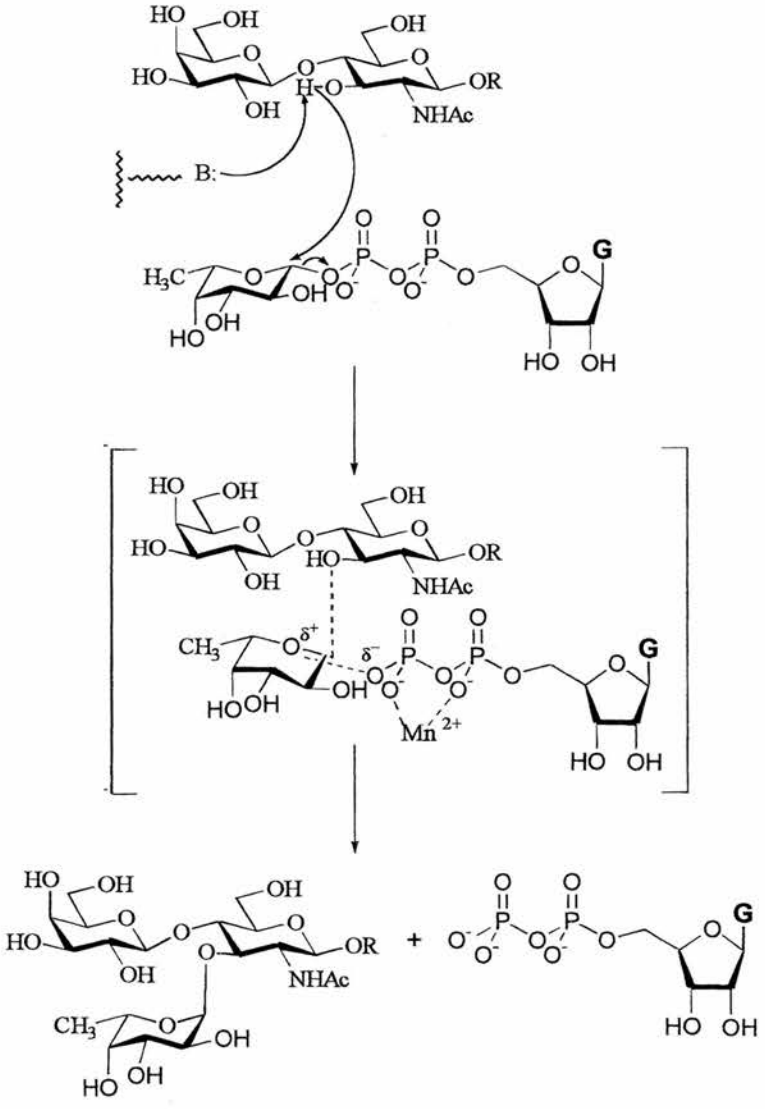

Figure 14. Postulated mechanism for α-1,3-FucT

Glycosyltransferases require metal cofactors, typically manganese (Mn^{2+}).[39] The divalent manganese cofactor of β-1,4-galactosyltransferase has been shown to bind to the enzyme before the donor nucleotide sugar, UDP-galactose,[40] and released from the enzyme in the form of a Mn^{2+}-UDP complex.[41] Thus in galactosyltransferase and possibly FucT VI, the leaving group departure is facilitated by a metal cofactor. Evidence for the requirement of Mn^{2+} as an electrophilic catalyst by FucT is supported by the observation that the non-enzymatic transfer of fucose from GDP-Fuc to the hydroxyl group of water in the presence of $MnCl_2$ is increased by more than a factor of 10.[38]

Studies carried out in the Field group raise doubts about the accepted mechanism for FucT action.[42] Replacement of the 3-OH of LacNAc (i.e. the site for fucosylation) with sulfate or phosphate does not render compounds incapable of acting as substrates for FucTs VI and VII (Table 2). In fact, the V_{max} value for 3-sulfates can be 2-5 fold higher that for the corresponding alcohols, indicating that the chemical transformation step is in fact faster for sulfates than for the alcohols. 3-Phosphates also posses the ability to act as substrates (Table 2).

Compound	***Km*** μm	V_{max} pmol/min/mg	V_{max}***/Km*** relative
Type II			
Unsubstituted	115	2510	100
3-*O*-sulfate	495	5015	46
3-*O*-phosphate	785	1180	7
3,6-di-*O*-sulfate	390	945	11
3,6-di-*O*-phosphate	545	910	8
Sialylated Type II			
Unsubstituted	10	1205	555
3-*O*-sulfate	160	1035	30
3-*O*-phosphate	420	535	6

Table 2. Kinetic data for the turnover of sulfate and phosphate derivatives of LacNAc-Octyl by recombinant human FucT VI

These observations cause one to question the validity of the putative active-site carboxylate-catalysed deprotonation of the acceptor alcohol since it would require a

negatively charged 3-sulfate to sit directly adjacent to the negatively charged active-site carboxylate residue. It is conceivable that the role of the divalent cation in FucT action has been mis-assigned, and that it is actually involved in activation of the acceptor alcohol (c.f. activation of water by Zn^{2+} in carbonic anhydrase).[43] This would result in a scenario where a 3-OH or 3-sulfate would sit in the co-ordination sphere of the metal ion prior to attacking the fucose anomeric centre of GDP-fucose.[42,44] Conceivably, the role of the metal ion has been assigned correctly, but it could also play a role in acceptor activation, as outlined in Figure 15.

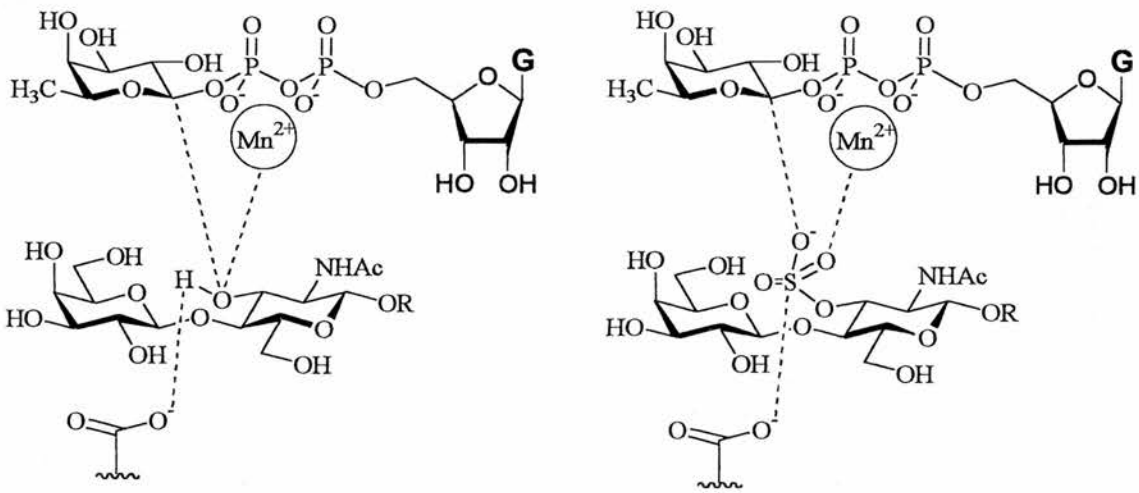

Figure 15. Model for FucT Action on Unsubstituted and 3-Sulfated Acceptor Substrates.

This leaves one with a model where the enzyme recognises the metal ion, and the metal ion recognises the substrate, which may go some way to explaining why traditional approaches to inhibitor design have been largely unsuccessful.

1.4.7 Inhibition of Fucosyltransferases

Seven X-ray crystal structures of sugar nucleotide-dependent glycosyltransferases have been reported to date. The first such structure was that of β-glucosyltransferase from phage T4[45] followed by the crystal structure of spsa, the glycosyltransferase implicated in the synthesis of the spore coat of *Bacillus subtilis*[46] (1.5 Å resolution). Bovine β1,4-galactosyltransferase was next reported[47] (at a resolution of 2.5Å)

followed in rapid succession by MurG from *E. coli*[48], human glucuronyltransferase[49] and rabbit N-acetylglucosaminyltransferase.[50] The latest crystal structure to be reported is the α1,4-galactosyltransferase (LgtC) from *Neisseria meningitidis*[51] (2 Å) which differs from the above enzymes in being a retaining glycosyltransferase whereas the above enzymes all catalyse inverting reactions. This enzyme is also the first to be crystallised with both donor and acceptor analogues demonstrating similarities with many of the inverting enzymes while offering more insights into the structure and mechanism of this class of enzymes. However as yet, no definitive mechanism of action has been elucidated and thus there is still insufficient information on which to base the rational design of α1,3-FucT inhibitors. Only limited success has so far been achieved in the development of inhibitors of glycosyltansferases. Most efforts have concentrated on the production of unreactive structural analogues of GDP-fucose. For example, Cai *et al*[52] synthesised a carba-sugar (pseudo-sugar) analgoue of GDP-fucose where the ring oxygen of the sugar moiety is replaced by a methylene group. The reduced reactivity of the methylene carbon prevents interaction with the acceptor sugar thus preventing glycosyl transfer. This compound therefore behaves as a donor substrate analogue inhibitor (Figure 16).

The approach of Luengo and Gleason[53] to inactivating the fucosyl ring to glycosyl transfer was to synthesise C-glycoside analogues of GDP-fucose. Such an analogue, the phosphonate isotere analogue, where a methylene group replaces the O atom linking the GDP moiety to the fucosyl moiety, is likewise illustrated in Figure 16.

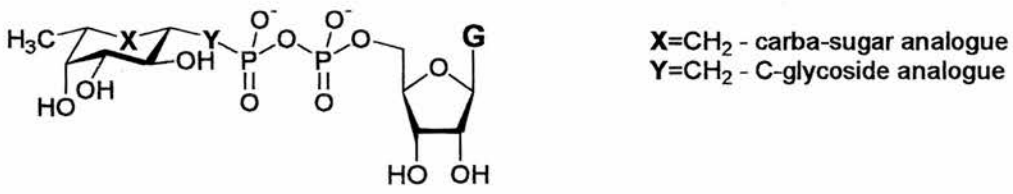

Figure 16. Carba-sugar and C-glycoside analogues of GDP-fucose

The fluorinated fucose analogue of GDP-fucose, GDP 2-Deoxy-2-fluoro-fucose (Figure 17), has also been reported to inhibit α-1,3-FucT with a Ki of similar magnitude to the above GDP-fucose analogues.[54]

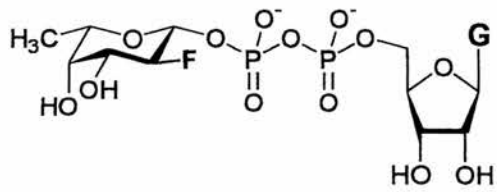

Figure 17. GDP 2-deoxy-2-fluoro-fucose

Certain aza-sugars have been reported to be inhibitors of FucTs and furthermore that this inhibition is synergistically enhanced by GDP.[55] This synergism indicates a possible interaction of aza sugars, GDP and the acceptor sugar in the active site of the enzyme to mimic the transition state of the FucT reaction, as illustrated in Figure 18. Monosaccharide aza-sugars are, however, not specific because they only interfere with the binding of sugar nucleotide to the enzyme, not with the acceptor substrate which determines the specificity of the enzyme. Qiao *et al*[55] have produced an aza-trisaccharide where homofuconojirimycin (a potent inhibitor of α-fucosidase) is linked to LacNAc by the 3-OH group of GlcNAc via an ethylene spacer. This was found to be an effective inhibitor of FucT V in the prescence of GDP. It is presumed the aza-trisaccharide and GDP form a complex which mimics the transition state of the enzymatic reaction. This is also illustrated in Figure 18.

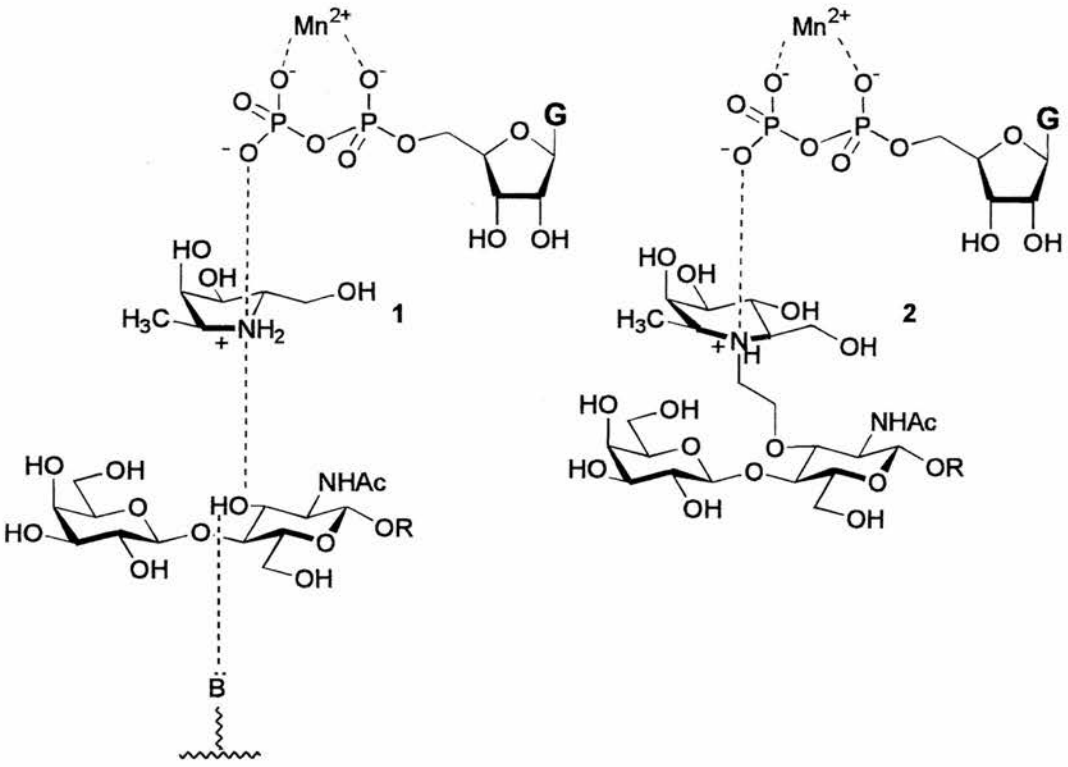

Figure 18. Synergistic inhibition of FucT V by the combination of GDP and an aza sugar (1) or azatrisaccharide (2).

1.4.8 α-1,3-Fucosyltransferase Substrate Specificity

As noted in section 4.1, α-1,3-FucTs are differentially expressed in different tissues. For therapeutic effectiveness, any FucT inhibitor should selectively inhibit the correct FucT isoform. Since most glycosyltransferase inhibitors reported to date are based on substrate analogues, one is drawn to question the substrate specificity of the various α-1,3-FucTs. Whilst numerous efforts have been made to assign *in vivo* function to FucTs based on *in vitro* selectivity studies, there is often poor agreement between publications.[42] The remainder of this section will therefore refer specifically to our own work since this relates directly to the recombinant materials we have access to and which is used in all other studies reported in this thesis.[42] A systematic study of the specificity of recombinant FucTs III-VII using synthetic acceptors has been completed by the Field group in conjunction with Bamford and Britten at GlaxoWellcome.[29] The results obtained are summarised in Table 3.

Compound	FucT III[a]	FucT IV[b]	FucT V[c]	FucT VI[b]	FucT VII[b]
Type I					
Unsubstituted	100	ND	ND	ND	ND
6-*O*-sulfate	46	ND	ND	ND	ND
Type II					
unsubstituted	-	100	-	100	100
6-*O*-sulfate	2	202	84	21260	-
Sialylated Type II					
unsubstituted	-	-	100	555	14
6-*O*-sulfate	-	-	108	1150	888

[a] Relative to Gal-β-1,3-GlcNAc-β-octyl = 100; [b] Relative to Gal-β-1,4-GlcNAc-β-octyl = 100; [c] Relative to NeuNAc-α-2,3-Gal-β-1,4-GlcNAc-β-octyl = 100. ND, not determined, not detectable.

Table 3. Substrate specificity (relative V/Km) of recombinant human α-1,3-FucTs

With a panel of synthetic acceptor substrates, distinct patterns of substrate selectivity emerge for each of the FucTs:

FucT III - only operates effectively on Type I substrates; will tolerate sulfate or phosphate substitution at the GlcNAc 6-position.

FucT IV - operates effectively on Type II substrates, and will tolerate sulfate or phosphate substitution at the GlcNAc 6-position. In addition, it does not accept α-2,3-sialylated substrates.

FucT V - shows a strong preference for α-2,3-sialylated Type II substrates, although addition of sulfate or phosphate to the GlcNAc 6-position negates the need for sialylation.

FucT VI - has a very broad substrate specificity and will operate on many Type II substrates; prefers α-2,3-sialylated to unsubstituted LacNAc-Octyl, but operates extremely effectively on Type II substrates with sulfate substitution at the GlcNAc 6-position.

FucT VII - will operate on unsialylated and α-2,3-sialylated Type II substrates, but shows a marked preference for the latter with sulfate substitution at the GlcNAc 6-position (i.e. doubly charged).

It is clear that FucT VI has the most relaxed specificity towards simple synthetic acceptor substrates (Table 3). Looking more closely at the Michaelis-Menten parameters for substrates for this enzyme (Table 4) shows that for a range of substrates V_{max} varies over 3.5 fold, whereas K_m can vary by 135 fold across the same series of compounds. One can therefore use K_m as a reasonable approximation to K_D (i.e. K_m is a crude measure of affinity). The relatively modest variation in V_{max} (and hence k_{cat}) would appear to suggest that in a number of cases substrate specificity is dominated by substrate recognition, rather than transition state recognition.

Compound	**Km** μm	**V_{max}** pmol/min/mg	**V_{max}/Km** Relative[a]
Type II			
Unsubstituted	115	2510	100
6-*O*- sulfate	0.85	3940	21260
Sialylated Type II			
Unsubstituted	10	1205	555
6-*O*- sulfate	4.4	1100	1150

[a]Relative to Gal-β-14-GlcNac-β-octyl.

Table 4. Kinetic data for the turnover of sulfate and phosphate derivatives of LacNAc-octyl by recombinant human FucT VI

The 6-sulfated Type II substrate has a sub-micromolar K_m value, suggesting a very specific high affinity recognition site for sulfate on FucT VI. The 5 fold selectivity of FucT VI for α-2,3-sialylated Type II LacNAc-Octyl compared to its unsialylated counterpart results largely from a substantial reduction in K_m (11.5 fold), suggesting the specific recognition of sialic acid by this enzyme. Both of these specific recognition sites might be exploited in selective inhibitor design.

1.5. AIMS AND OBJECTIVES

1.5.1 Aims of the Project

When they work well, enzymatic or whole cell biotransformation approaches to oligosaccharide synthesis can be far more efficient than chemical methods.[56,57] This is well illustrated by the chemoenzymatic synthesis of uniformly ^{13}C-enriched sialyl Lewis x tetrasaccharide , which was successfully completed by the Field group in 38 steps (including 10 enzymatic steps).[58] For comparison, the total chemical synthesis of this compound is estimated to require in excess of 60 steps. Whilst much effort has gone into the exploitation of glycosidases and glycosyltransferases for glycoside bond synthesis,[59,60] the chemical synthesis of donor and acceptor substrates for such reactions is still often required. Few studies have investigated or exploited enzymatic methods for the preparation of unnatural oligosaccharides starting from monosaccharide analogues as precursors to donor sugars. Only limited information is available about the substrate specificity of, for instance, galactokinase and galactose-1-phosphate uridyltransferase, which can be used in tandem to prepare UDP-galactose, and hence with the aid of β1,4-galactosyltransferase, LacNAc analogues.[61] Our initial aim was to investigate enzymatic methods for the synthesis of unnatural LacNAc derivatives from modified monosaccharides.

As can be seen from the introductory sections, there are three clear sites that one might look to exploit in the design of selective acceptor analogue inhibitors of α1,3-FucTs (Figure 19), namely:

a) the anomeric position of LacNAc,
b) the 6 position of LacNAc,
c) the 3' position of LacNAc.

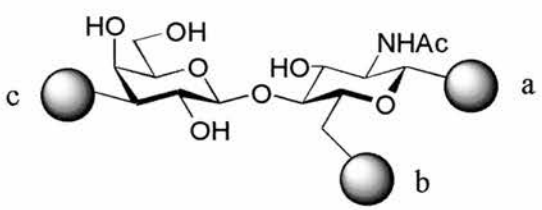

Figure 19. Sites for exploitation in α1,3-FucT inhibitor development

The rational design of oligosaccharide analogues has met with only limited success to date.[62,63] Within the context of an α-1,3-FucT inhibitor development programme, we therefore wished to both incorporate protein structure information and 'play the numbers game' by using combinatorial chemistry methods. The specific aims of this project are therefore to develop methods for the preparation of LacNAc templates that can be functionalised at the anomeric, 6- or 3'-positions, so providing access to, for instance:

a) acceptor-based photoaffinity labels;
b) acceptor-based irreversible inhibitors;
c) libraries of potential competitive acceptor analogue inhibitors.

The introduction of appropriate 'handles' for analytical purposes (e.g. a fluorophore or biotin) presents the requirement for a second derivatisable site in each template. Bifunctional templates are therefore needed that posses two sites that can be orthogonally derivatised. For practical purposes, we chose to use amine-functionalised templates since they can be chemoselectively derivatised on nitrogen without the need for hydroxyl protection. This in turn leads to a need for an orthogonal protection strategy for two nitrogen-based functional groups. Our initial aim was to investigate the use of azide and phenylacetamide. The former can be converted to free amine by reduction with, for instance H_2/Pd-C,[64] PPh_3[65], or propanedithiol.[66] The latter can be hydrolysed with penicillin G acylase,[67] which is commercially available in immobilised, high specific activity form at low cost. The strategy for the exploitation of these orthogonal groups is outlined in Figure 20.

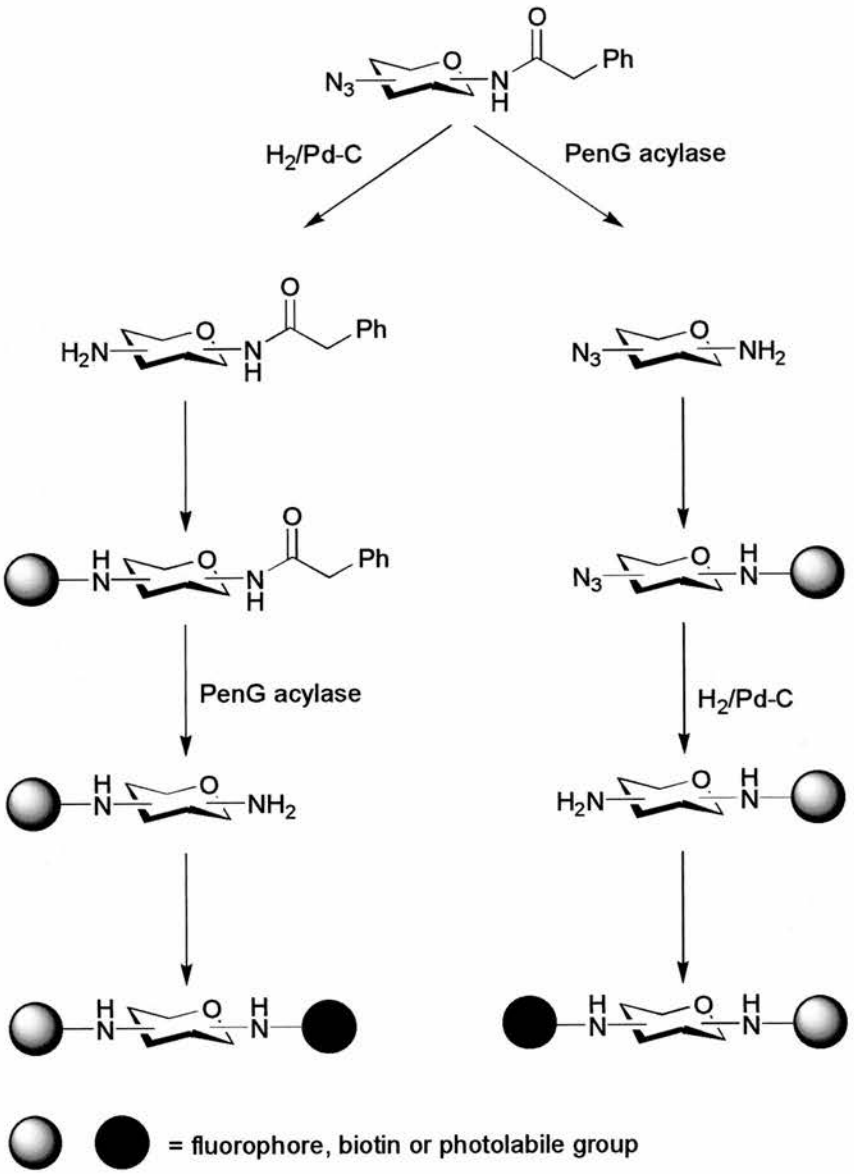

Figure 20. Derivatisation of functional groups

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Chapter 2

Enzymatic Synthesis of Azido LacNAc

2.1 Enzymatic Synthesis of UDP-Galactose

Sugar nucleotides are required in biological systems as glycosyltransferase donor substrates. The enzymatic transfer of galactose by galactosyltransferase utilizes uridine 5'-diphosphogalactose (UDP-Gal) and is therefore a starting point for the enzymatic synthesis of LacNAc analogues. Our intention was to produce UDP-Gal enzymatically using the first two steps of the Leloir pathway which is used by organisms for the interconversion of glucose and galactose.[1] This is illustrated in Figure 21 and has been exploited widely in the literature.[2]

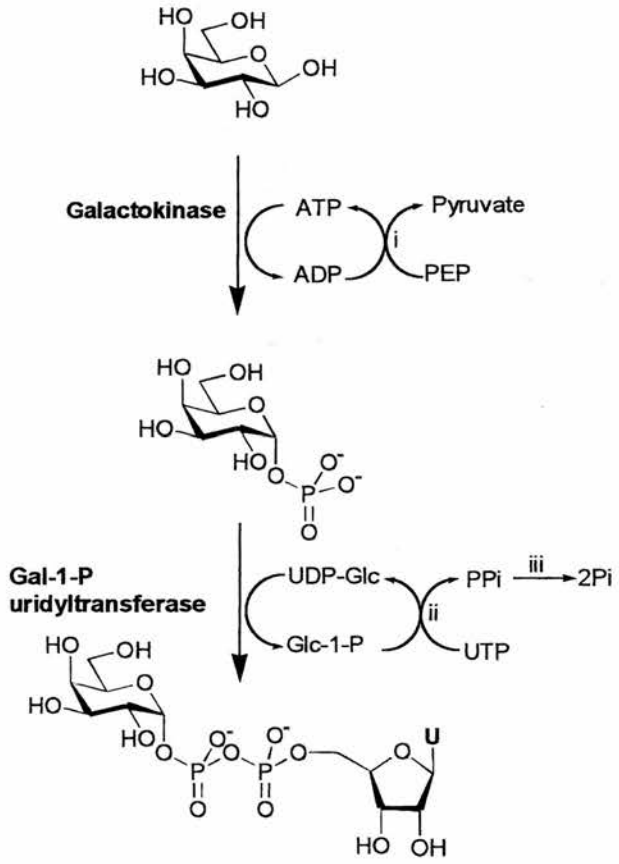

Figure 21. Enzymatic synthesis of UDP-Galactose

(i) Pyruvate kinase, (ii) UDP-Glc pyrophosphorylase, (iii) pyrophosphatase.
PEP = phosphoenolpyruvate.

The first step of this process is the phosphorylation of galactose at the anomeric centre to yield galactose-1-phosphate. This is catalysed by galactokinase, which uses ATP as its phosphate source. The transfer of UMP from UDP-Glc to the sugar

phosphate is then the second step, producing UDP-Gal. This reaction is catalysed by Gal-1-P uridyltransferase. Rather than use stoichiometric amounts of the cofactors ATP and UDP-Glc, these can be regenerated *in situ* to cut down on expense. In addition nucleoside diphosphates have been reported to inhibit galactosyltransferase,[3] which will be present at a later stage in a one pot synthesis of LacNAc and its analogues. ATP is regenerated enzymatically using phosphoenolpyruvate (PEP) and pyruvate kinase (PK), where a phosphoryl group is transferred from PEP to ADP by PK. Regeneration of UDP-Glc involves the condensation of Glc-1-P and UTP catalysed by UDP-Glc pyrophosphorylase. This latter reaction is driven thermodynamically by hydrolysis of the resultant pyrophosphate to phosphate by the action of inorganic pyrophosphatase.

2.2 Production of Galactokinase

The processes described above are all dependent on galactokinase. Whilst this enzyme is commercially available, it is expensive, with a current price of £143 per 20 units. The considerable cost of galactokinase was acknowledged by Whitesides[2] who was able to use crude enzyme extracts from galactose-adapted yeast. We therefore chose to adopt this approach, using a galactokinase purification protocol based on that described by Wilson and Schell.[4]

We looked at two strains of yeast for enzyme production, *Saccharomyces cerevisiae* (Bakers yeast) and *Kluyveromyces fragilis*. Due to the slightly higher biomass attained with *Kluveromyces* (Figure 22) we opted to use this strain for scaled up production.

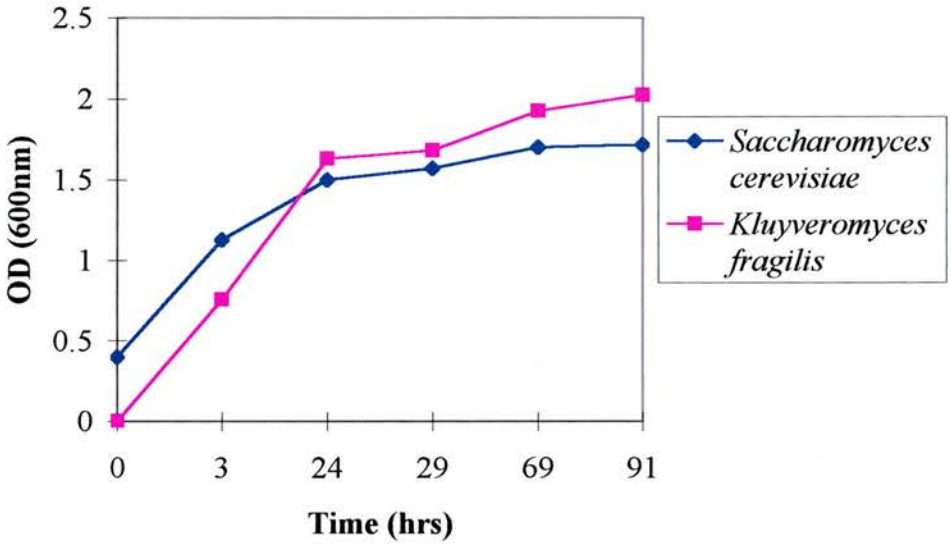

Figure 22. Yeast growth curves

Galactokinase activity was measured at all stages using a radiochemcial assay adapted from a literature procedure,[5] which involves the separation of the tritiated anionic product ([^{3}H]-Gal-1-P) from unreacted starting material ([^{3}H]-Gal) by anion-exchange chromatography. This was performed in one pot, where the ion-exchange resin was added directly to the reaction mixture. The protein purification protocol is summarised in Table 5.

Kluyvermyces fragilis was grown in a galactose medium, to stimulate the production of galactokinase, and cells were harvested during the late log phase of growth. A 10 litre incubator culture typically produced 45 g/litre wet weight cells, which after

Table 5. Purification of Galactokinase from *Kluyveromyces fragilis*.

Purification step	Fraction number	Fraction volume (ml)	[Protein] (mg/ml)	Total protein (mg)	Turnover (%)	Specific activity (nmol/min/mg)	Total activity (nmol/min)	Fold purification	Recovery (%)
Crude lysate		80	8.5	680	49.7	4.87	3312	1	100
Ammonium sulfate precipitate		20	11.5	230	46.8	3.39	778	0.7	23
Streptomycin sulfate precipitate		26	6.0	156	41.9	5.82	908	1.2	27
DEAE cellulose	1	10	1.0	10	42.2	35.2	352	7.2	
	2	10	4.5	45	47.2	8.73	393	1.8	
	3	10	5.0	50	55.4	9.24	462	1.9	
	4	10	1.0	10	51.1	42.6	426	8.7	
	5	10	0.5	5	39.7	66.2	331	13.6	
	total						1964		59
Gel filtration	8	8	0.17	1.36	13.6	66.5	90	13.7	
	9	8	0.15	1.2	32.3	179.3	215	36.8	
	10	8	0.13	1.04	34.6	221.5	230	45.5	
	11	8	0.11	0.88	35.6	270.0	238	55.4	
	12	8	0.10	0.8	22.5	188.0	150	38.6	
	13	8	0.07	0.56	16.4	195.7	110	40.2	
	14	8	0.07	0.56	11.6	138.6	78	28.4	
	15	8	0.07	0.56	6.2	74.3	42	15.3	
	total						1153		35

lyophilisation yielded approximately 100 g of biomass (78 % weight loss). The lyophilised cells were ground with alumina and washed with buffer to give a crude lysate which was subjected to ammonium sulfate precipitation, followed by resuspension and streptomycin sulfate precipitation, to remove nucleic acid. Further purification was then carried out by DEAE-cellulose chromatography at pH 7.4 in buffer A (10 mm Tris-HCl, 1 mM DTT, 1 mM EDTA, 10 % v/v glycerol) containing 7 mM $(NH_4)_2SO_4$. Galactokinase passes straight through the column under these conditions, with most (>80 %) other protein being retained. Concentration of appropriate fractions and further purification by gel filtration (Figure 23) on a Sephadex G-150 column gave active protein samples which possessed six main components, as judged by SDS-PAGE (Figure 24).

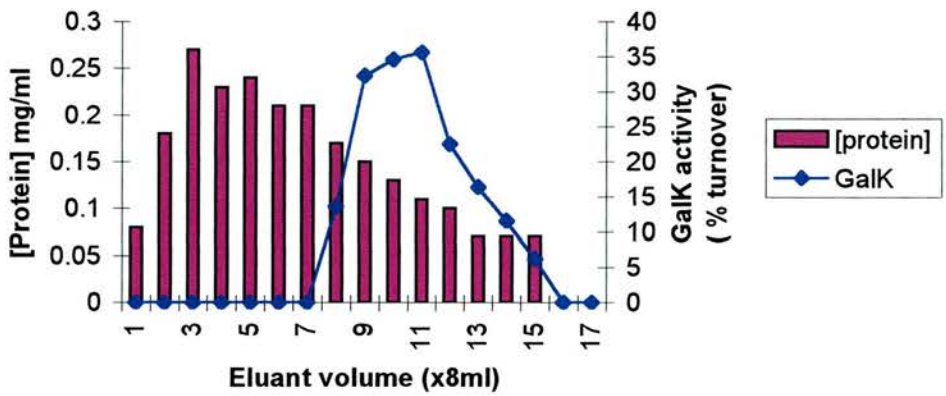

Figure 23. GalK activity in Gel Permeation chromatography fractions

The retention time of the eluted protein corresponded to a molecular weight consistent with that of galactokinase. Similarly, SDS-PAGE reveals a band which corresponds to a molecular weight of 59.2 kDa (Syngene Gene Tools). This approximates to the molecular weight of *K. fragilis* galactokinase as reported by Wilson and Schell[4] to be 58 kDa.

For comparison, the predicted molecular weight of the eluted *Saccharomyces cerevisiae* enzyme, based on its gene sequence obtained from the NCBI data bank (P04385) and analysed by ExPASy Proteomic tools, is 57.9 kDa

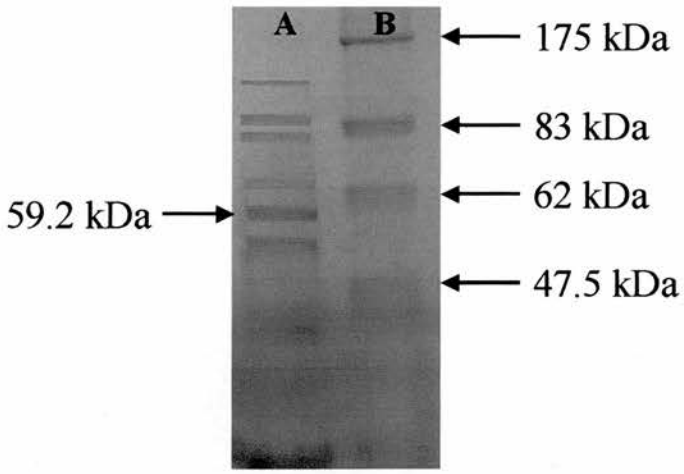

Figure 24. SDS-PAGE Gel (12 % Tris-HCl with 4 % stacking gel)

A = enzyme solution, B = molecular weight markers

2.3 Enzymatic Synthesis of UDP-Galactose Analogues

As it was our intention to synthesise LacNAc analogues containing azido-galactose, we therefore needed to make the donor nucleoside UDP-3-azido-3-deoxy-galactose. Our intention was to attempt an enzymatic synthesis, as outlined in Figure 25.

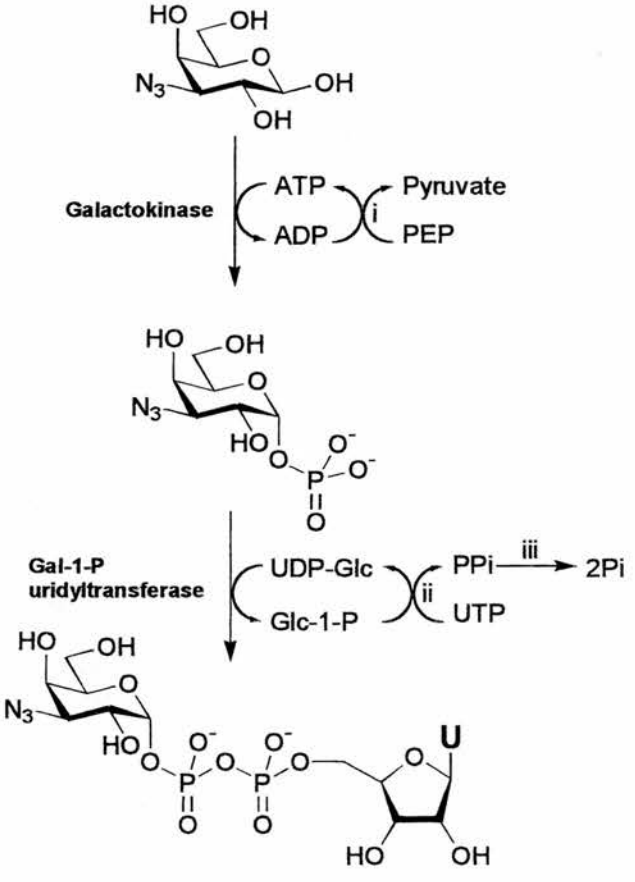

Figure 25. Enzymatic synthesis of UDP-3-N_3-galactose

(i) Pyruvate kinase, (ii) UDP-Glc pyrophosphorylase, (iii) pyrophosphatase.
PEP = phosphoenolpyruvate.

To look at the feasibility of this approach, 3- and 6-N_3-galactose, along with other unnatural substrates were tested for recognition by galactokinase using a radio chemical assay. Galactokinase from gel permeation fraction 4 was used. The assay involved incubating the unnatural substrate and tritiated galactose with enzyme and then passing the reaction mixture through a short column of Sephadex QA25 which retains any phosphorylated sugar. After washing with water to remove any unbound material, the column was eluted with 1 M ammonium acetate and the eluant mixed with scintillation fluid and counted. If the unnatural substrate sugar is recognised by

the galactokinase it will competitively inhibit phosphorylation of the tritiated galactose, resulting in the amount of labelled galactose being reduced with respect to a positive control. The results are tabulated below (Table 6).

Substrates	Conc. (mM)	% Turnover
Galactose	5	8
3-N_3-Galactose	5	22
6-N_3-Galactose	5	32
6-deoxy-Gal	5	45
2-NH_2-Galactose	5	29
*Galactose	1	54

Table 6. Unnatural substrate recognition by galactokinase

The final entry in the table is the positive control containing only labelled galactose, indicating enzyme activity. It can be seen that whilst no appreciable binding of galactokinase to fucose (6-deoxy-galactose) took place, both azido galactose analogues, along with galactosamine, demonstrated a significant degree of inhibition of the enzyme. Although this doesn't prove that the galactose analogues are acting as substrates, the results are nevertheless encouraging. Isolation and characterisation of the products of these reactions would be required to definitely demonstrate that galactokinase is able to phosphorylate these galactose analogues, but this is a laborious and technically demanding exercise. However we were sufficiently encouraged by these preliminary results to continue with our proposed enzymatic synthesis of azido LacNAc. Indeed, the turnover of 2-NH_2-galactose is consistent with the work of Whitesides,[2] with 3-N_3 -galactose apparently binding even better to galactokinase.

2.4 Attempted Enzymatic Synthesis of 3'-LacNAc Derivatives

We set out to prepare benzyl 3'-azido-3'-deoxy-LacNAc according to the reaction scheme described in Figure 26.

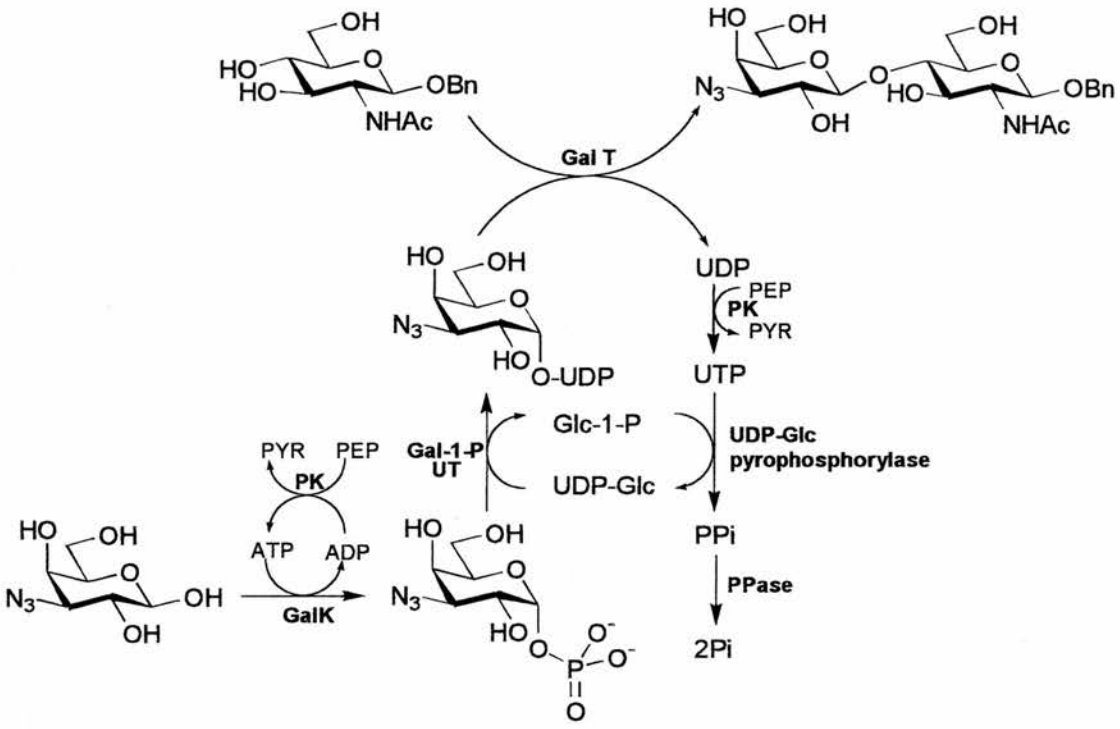

Figure 26. Attempted one pot enzymatic synthesis of 3'-azido-3'-deoxy-LacNAc benzyl glycoside

Benzyl GlcNAc was chosen as the acceptor since it is known to be a very good β-1,4-GalT substrate, with a K_m value two orders of magnitude less than that of the corresponding reducing sugar.[6]

Unpublished and unoptimised preliminary studies by Field with galactose or 3-azido-3-deoxy-galactose (50 mg scale) gave disaccharide products in low yield (<10 % and approx 3 %, respectively). Data from tlc and Bio Gel P-4 gel filtration were consistent with the formation of disaccharides, with material derived from 3-azido-3-deoxy-galactose being notably more hydrophobic (and hence more difficult to separate from benzyl GlcNAc) than the corresponding galactoside, as would be expected. Unfortunately, insufficient material was produced for more complete characterisation by NMR spectroscopy and mass spectrometry.

In the current study, the reaction with galactose was found to proceed to near

completion as can be seen from the TLC in Figure 27.

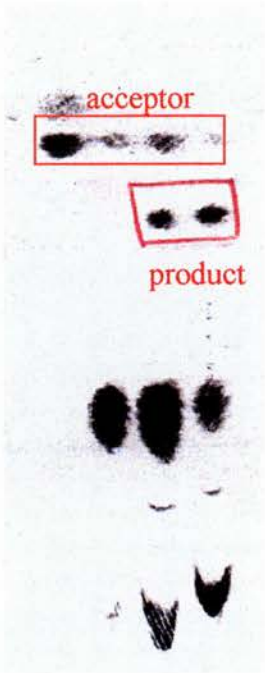

Figure 27. Enzymatic Production of Benzyl LacNAc

Lane 1: starting material (benzyl GlcNAc); Lane 2: reaction mixture before incubation; Lane 3: co-spot of benzyl GlcNAc and reaction mixture after incubation; Lane 4: reaction mixture after incubation.

However attempts to reproduce this earlier work with the corresponding azido sugar met with many difficulties. The poor solubility of benzyl GlcNAc in aqueous solution at even moderate concentrations (2 mg/ml) proved particularly troublesome. It is interesting to note that in general GlcNAc derivatives are substantially less water soluble (>10 fold) than the corresponding Glc, Man or Gal derivatives. This is not immediately apparent from the literature, where often only brief kinetic data are presented that make use of low substrate concentrations (typically « mM). In addition, recent studies from Wong indicate that acceptor substrate inhibition of β-1,4-GalT can be observed at high concentrations with some hydrophobic aromatic GlcNAc glycosides.[7] The Field group have made similar observations on the interaction of octyl and 8-methoxycarbonyloctyl glycosides of LacNAc with FucTs.[8] Since our desired product, benzyl 3'-azido-3'-deoxy-LacNAc, is expected to be no more water soluble than benzyl GlcNAc, there is a real problem being sure that the

product will stay in solution. Together with poor acceptor solubility and precipitation of inorganic salts and protein during the course of the reaction, the precipitation of product renders the initial process investigated untenable. DMSO can be used to aid solubility of hydrophobic acceptor substrates in aqueous solution, however, its effect on other relevant enzymes has not been explored before. Preliminary investigation suggested that at 1% v/v, DMSO had no overall positive effect on this complex multi-enzyme process, although greater acceptor substrate solubility was noted. Numerous attempts were made to carry out the one-pot transformation outlined in Figure 25. On occasions, TLC data were obtained that were consistent with the formation of a small amount of disaccharide material (<5 % conversion). However, results were not reproducible and Rf values for putative disaccharide product were variable. We note from other studies in the Field group that for very poor substrates for this particular multi-enzyme process, formation of disaccharide can result from transfer of glucose from the "catalytic" UDP-Glc used in one of the co-factor recycling reactions (Figure 26) (M. Aloui, T.J. Rutherford and R.A. Field, unpublished observations). It is known that UDP-Glc is a substrate for β-1,4-GalT.[9]

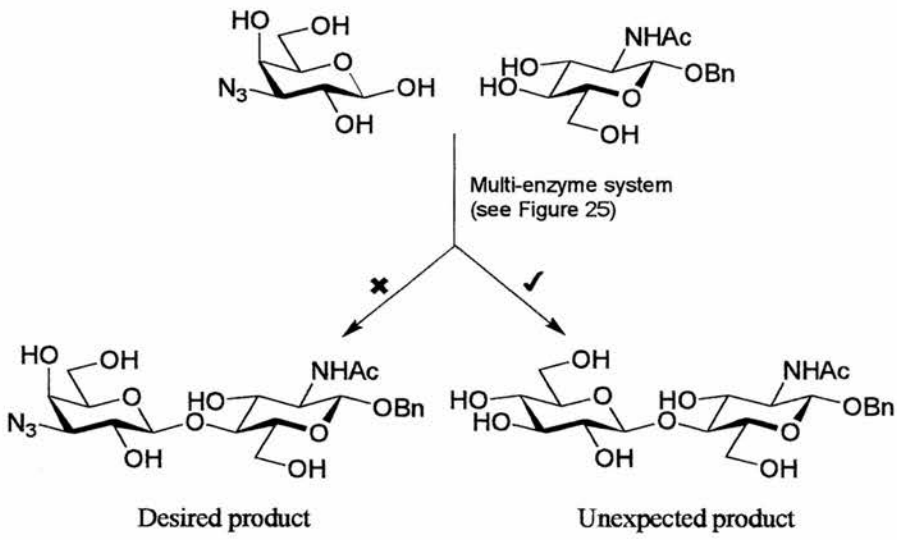

Figure 28. Unexpected enzymatic synthesis of Glc-GlcNAc-Bn

In the current study, it is possible that in some incubations Glc-GlcNAc-Bn was in fact formed, rather than the desired 3-azido-Gal-GlcNAc-Bn. Glc-GlcNAc-Bn was therefore prepared enzymatically from benzyl GlcNAc and UDP-glucose, as outlined

in Figure 28, in an attempt to confirm these observations, and to ascertain whether or not the original preliminary data can be attributed to formation of Glc-GlcNAc-Bn rather than 3-azido-Gal-GlcNAc-Bn. In at least some incubations, this proved to be the case.

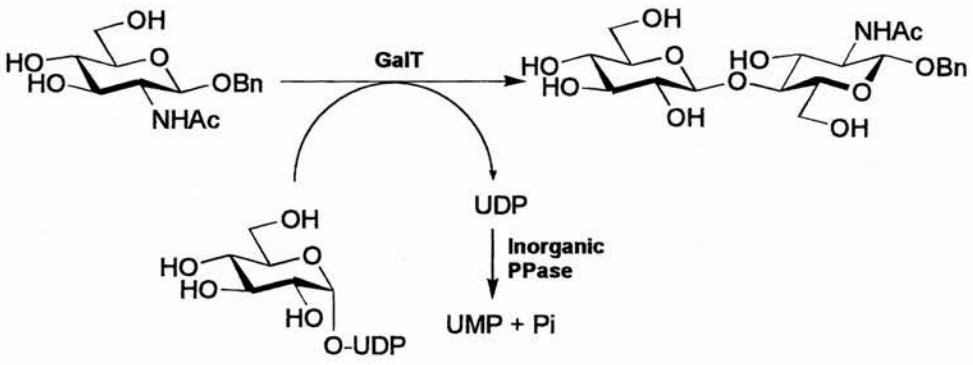

Figure 29. Synthesis of Glc-GlcNAc-Bn

2.5 Summary

In summary it seems likely that small differences in multi-enzyme processes become an issue when one is trying to force poor substrates through many enzymatic steps in one go. Data presented in the previous section support the motion that 3-azido-3-deoxy-galactose does indeed bind to galactokinase (although we have no evidence at this point that it acts as a substrate). We conclude that the a productive way forward might be to investigate either:

a) a 3-pot chemoenzymatic approach - enzymatic synthesis of Gal-1-P analogues with galactokinase, their chemical conversion (using UMP-morpholidate)[10,11] to UDP-Gal analogues, and their subsequent presentation to β-1,4-GalT to effect formation of LacNAc analogues;

b) a two-pot enzymatic approach - enzymatic synthesis of Gal-1-P analogues with galactokinase with *in situ* conversion (galactose-1-phosphate uridyltransferase) to UDP-Gal analogues, followed by separate incubation with β-1,4-GalT to effect formation of LacNAc analogues.

2.6 Conclusion

We conclude that the approach we have adopted may eventually be productive. However, the quantities of galactokinase required are substantial, and further development will likely require the use of over-expressed recombinant enzyme.

2.7 MATERIALS AND METHODS

2.7.1 Materials

Saccharomyces cerevisiae and *Kluyveromyces fragilis* were purchased from Sigma Chemical Co as were all chemicals used other than DTT, (Melford Laboratories), [6-^{3}H] galactose (Amersham) and SDS-PAGE gels (12 % Tris-HCl with 4 % stacking gel) (Bio-Rad). Alumina was type A-5 (Sigma).

2.7.2 SDS-PAGE

All fractions were analysed by SDS polyacrylamide gel electrophoresis. Protein samples (40 μg) were precipitated with trichloroacetic acid (TCA). This was achieved by the addition of a 50 % solution of TCA to make the final concentration 5 % in TCA. The solution was then vortexed and left to sit on ice for 5 minutes. The supernatant was pipetted off and the protein was solubilised in Laemmli buffer (15 μl, Sigma). Samples were heated at 100 °C in a hot block for 10 minutes before loading onto the gel. Pre-stained molecular weight markers (Bio-Rad) used were in the range of 47-205 kDa. Commercial galactokinase (galactose-adapted yeast, Sigma)(2.6 μg) was also loaded on the gel as a reference marker. The gel (10 %) was run under 150 V for 50 minutes in 1 x running buffer (5 x: 25 mM Tris-Base pH 8.3, 195 mM glycine, 0.1 % w/v SDS). Staining with Coomasie blue (0.5 g Coomassie Brilliant Blue in 500 ml destain solution) was achieved in 15 minutes in a water bath (60 °C). Destaining in destain solution (water:methanol:acetic acid, 6:3:1) was also performed in a water bath (60 °C, 1 hour) with a sponge.

2.7.3 Cultivation of Yeast

Conical flasks (250 ml) containing autoclaved growth medium (50 ml) (2 % malt extract, 2 % glucose, 1 % yeast extract) were innoculated with *Saccharomyces cerevisiae* (1 g) or *Kluyveromyces fragilis* (250 mg). The cultures were left to sit at room temperature for 2 h before being incubated overnight in a gyratory shaker at 30 °C and 250 rev./min. The following morning broth flasks containing 500 ml of growth medium (containing 2 % galactose) were innoculated with the overnight cultures and incubated as above. Cell density was monitored by measuring the OD 600. Cells were harvested at late log phase by centrifugation (4000 g, 20 min),

lyophilised and stored at -80 °C. Production of *Kluyveromyces fragilis* was then scaled up by using a 500 ml culture to innoculate 10 litres of growth medium (2% malt extract, 2 % galactose, 2 % yeast extract) in a New Brunswick Microferm 10 L fermentor (30 °C, 200 rpm, air pressure 9). The cells were harvested after 24 hours by centrifugation (4000 g, 20 min, 4 °C).

2.7.4 Galactokinase Assay

This assay measured the phosphorylation of [6-^{3}H]-galactose, which was separated from the assay mixture by the addition of Dowex anion exchange resin (AG 1-X8, 100-200 mesh, acetate form, Bio-Rad) (1 ml suspension), followed by centrifugation. The 50 µl reaction mixture contained 10 µl each of galactose (1 mM, containing approx. 30,000 CPM of [^{3}H]-galactose), assay buffer (50 mM triethanolamine, pH 7), ATP (1 mM), [DTT (1 mM) and $MgCl_2$ (5 mM)] and protein extract. The molarities quoted are the final concentrations in the assay mixture. The reaction mixture was incubated for 1 hour at 37 °C in a water bath after which time the reaction was terminated by quenching with the Dowex suspension (1:4 w/v in water). Scintillation fluid (Fisher High Safe 8) (4 ml) was added to 600 µl of the supernatant and after vortexing was counted for 2 minutes.

Enzyme activity is expressed as a percentage turnover (of substrate), calculated from the proportional reduction in total CPM.

2.7.5 Bradford Assay

Protein concentrations were determined using the Bradford Method.[12]

2.7.6 General Procedure for the Extraction and Purification of Galactokinase from *Kluyveromyces fragilis*

The procedure followed is based on the protocol described by Wilson and Schell.[4]

Extraction: Lyophilised *Kluyveromyces fragilis* (20 g) was ground, using a pestle and mortar, for 2 minutes with alumina (100 g), transferred to a 500 ml conical flask with 200 ml of galactokinase buffer (20 mM triethanolamine, 1 mM EDTA, 1 mM

DTT, pH 7) and stirred at 4°C for 30 minutes. The solution was centrifuged (4000 g x 20 minutes) and the supernatant was decanted (Fraction 1: 150 ml).

Ammonium Sulfate precipitation: Solid ammonium sulfate (38.6 g, 0.266 g/ml) was added to Fraction 1 (150 ml) at 4 °C and the solution was left to stir for a further 15 minutes. After being left to sit for a further 20 minutes, the solution was centrifuged (12,000 g x 20 minutes) and the supernatant was retained. Ammonium sulfate (21.2 g) (0.146 g/ml of original volume) was added as before, the solution was left to stir for 15 minutes and sit for a further 20 minutes. The solution was then centrifuged (12,000 g, 20 minutes) and the supernatant discarded. The pellet was redissolved in galactokinase buffer (40 ml) (Fraction 2).

Streptomycin Sulfate Precipitation: Aqueous streptomycin sulphate solution (10 % w/v, 10 ml) was added slowly with stirring to Fraction 2. The solution was then dialysed against 1 litre of buffer A (10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 10 % v/v glycerol, pH 7.4) over night. The dialysis buffer was changed and the dialysis continued for a further 2 hours. The supernatant was decanted, transferred to tubes and centrifuged (20,000 g x 20 minutes) (Fraction 3).

DEAE-Cellulose Chromatography: A column of DEAE-cellulose (2.5 x 21 cm) was prepared and equilibrated with 400 ml Buffer A containing 7 mM ammonium sulfate. Fraction 3 was made 7 mM in ammonium sulfate by the addition of 0.273 ml of a 1 molar solution. The solution was filtered through a 0.2 μm filter before loading onto the column. Elution was with buffer A containing 7 mM ammonium sulfate at a flow rate of 100 ml/hr. The wash-through elutant was collected with the A_{280} of each 10 ml fraction being measured (using the buffer as a blank). All fractions in the initial peak were pooled i.e. until the A_{280} dropped off to a constant value (about 0.1) (Fraction 4).

Gel Permeation Chromatography: A column of Sephadex G-150 (2.5 x 90 cm) was equilibrated with Buffer A. After combining tubes 11 and 15 from Fraction 4 (20 ml), 10 ml of this was concentrated in an Amicon concentrator under N_2 using a 10 kDa MWCO regenerated cellulose membrane, to a volume of approximately 2 ml and loaded onto the column at a flow rate of 25 ml/hr. Twenty minute (approx. 8 ml) fractions were collected.

2.8 REFERENCES

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Chapter 3

Photoaffinity Labelling

3.1 Photoaffinity Labelling of Glycosyltransferases

In the absence of protein crystal structures, photoaffinity labelling is a potentially powerful tool to help identify the residues which comprise the active site of FucTs. Initial work in the literature on the photolabelling of glycosyltransferases focussed on photoreactive analogues of the donor sugar. One such UDP-galactose analogue, 4-azido-2-nitrophenyluridyl pyrophosphate (ANUP; Figure 30) was synthesised by Lee et al[1] to investigate the binding topography of α-lactalbumin on galactosyltransferase in the lactose synthase complex. This was found to be an effective competitive inhibitor with respect to UDP-galactose in the synthesis of lactose and LacNAc.

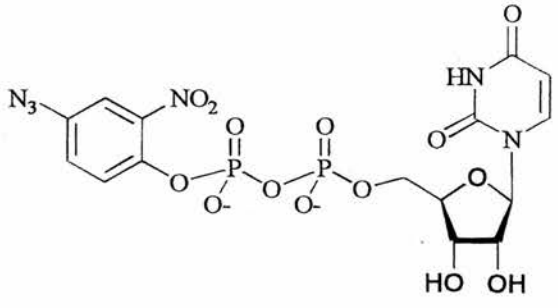


Figure 30. 4-azido-2-nitrophenyluridyl pyrophosphate (ANUP)

However, as different enzymes which transfer the same sugar unit usually use the same donor (eg β-1,4 and α-1,3-galactosyltransferases both use UDP-Gal) this method is non-specific and is therefore of limited use where only crude extracts or semi-purified transferases are available. Photoreactive acceptor substrate analogues were then developed containing diazirine groups, which proved to be acceptor substrates for GalT. Lehmann and Petry[2] linked two GlcNAc molecules by a linear spacer carrying a diazirine group in a variety of positions. One such acceptor substrate is illustrated in Figure 31.

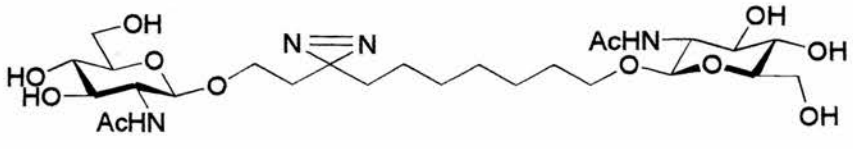


Figure 31. A GalT diazirino-acceptor substrate

As these molecules have a tendency to undergo self cross-linking, their application as photoaffinity labels for glycosyltransferases is limited.[3] Aryl (trifluoromethyl) diazirine groups have been found to be more efficient at cross-linking and produce more stable protein-probe adducts. Hatanaka *et al*[4] have reported the successful labelling of bovine GalT using a biotinylated phenyldiazirine derivative of GlcNAc, illustrated below in Figure 32.

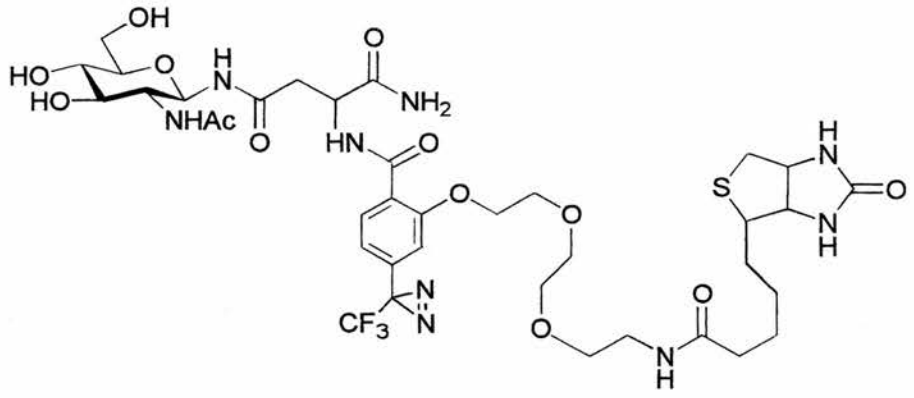


Figure 32. Phenyldiazirine derivative of GlcNAc

The biotin moiety enables the separation of labelled protein from the reaction mixture using biotin-avidin technology and the subsequent luminescent detection of the labelled peptide fragments following proteolytic digestion and chromatographic separation.[5] This procedure is illustrated in Figure 33.

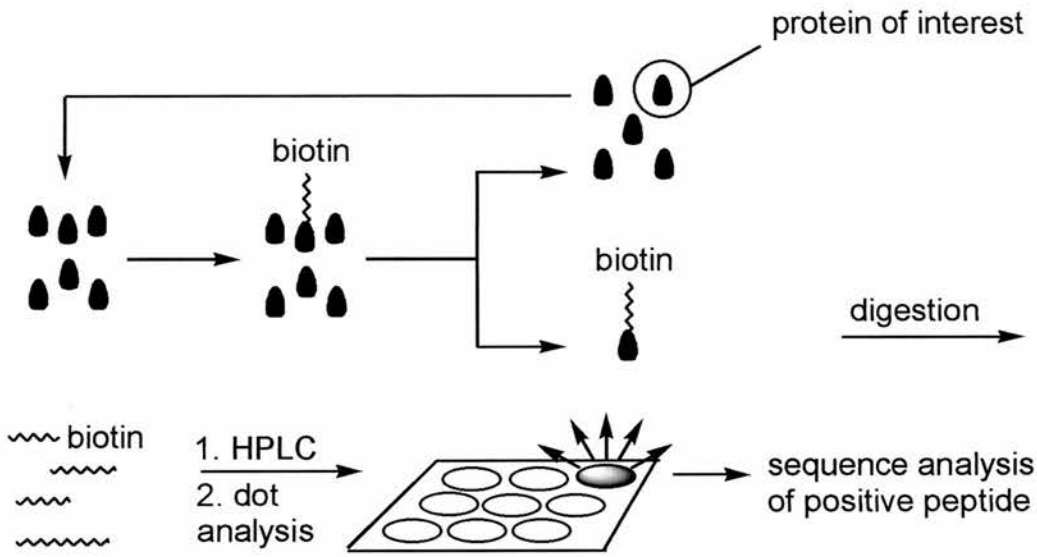


Figure 33. Labelling of GalT acceptor site[5]

This technique therefore has the advantage of obviating the need for radioactive markers to detect the labelled peptides. Furthermore, the phenyldiazirine label is stable in the presence of reducing agents typically used in protein chemistry (e.g. DTT), unlike an arylazide label which is readily reduced. Using an aminophenyl-modified polyvinylidene difluoride (PVDF) membrane the researchers were able to detect by chemiluminesence, biotinylated peptide fragments at the 10 pmol level, using a simple dot blot assay.[5]

More recently, Hatanaka *et al*[6] have used this photoprobe to complement information gained from the recently solved crystal structure of recombinant bovine β 1,4-galactosyltransferase in a UDP-bound complex (2.4 Å). Using details of the photolabelled peptide obtained from the photoaffinity labelling of the enzyme, the authors constructed a molecular docking model (Figure 34) of the photo-probe in the enzyme active site.

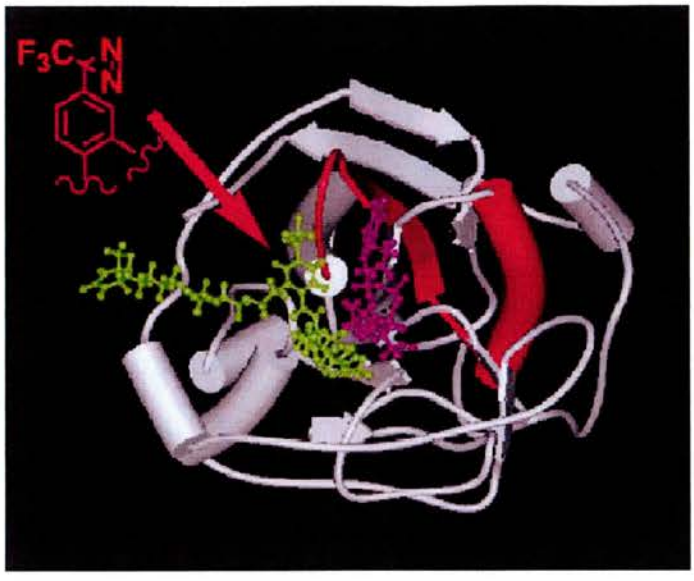


Figure 34. A docking model of β4GalT1.

The photolabelled peptide is coloured in red. The photoprobe (green) and UDP-Gal (pink) are shown as ball and stick drawings.
Taken from reference 6.

This model reveals that the carboxyl group of the supposed catalytically important Asp 318 residue can be located within 2.5 Å distance of the 4-hydroxyl group of GlcNAc (Figure 35). It also shows the other carboxyl residue in the binding region belonging to Glu 317 is beyond the distance required for the formation of a strong hydrogen bond (6.5 Å).

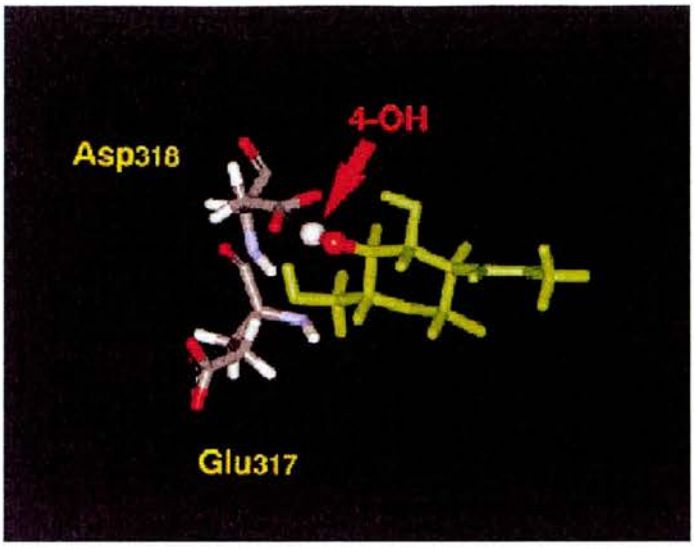


Figure 35. Relative orientation of the 4-OH of GlcNAc to Asp 318

The GlcNAc residue is in green with the 4-hydroxyl group depicted in red and white. Taken from reference 6

Asp 318 is a residue on the well conserved negative cluster, EDDD, identified from the crystal structure and located at the bottom surface of the catalytic domain. Mechanistically, this amino acid is thought to act as an active-site base which abstracts a proton from the 4-hydroxyl group of GlcNAc thus initiating its nucleophilic (S_N2) attack on the C-1 of galactose.

The value of photolabelling of the active-site of the enzyme has therefore been proven from crystallographic studies and has added strong support to the previous assumptions made concerning the mechanism of galactosyl transfer by β4GalT1.

Hashimoto *at al*[7] have successfully used a biotinylated photoreactive bis-glucose derivative (Figure 36) to label erythrocyte glucose transporter (GLUT1). Their choice of photophor in the molecule is a diazirine, stating the disadvantage of aryl azides as being a reduction in labelling specificity due to nucleophilic side reactions. Furthermore, they say that a carbene-introduced linkage is more stable than that introduced by an aryl azide. After labelling, the reaction mixture was resolved by SDS-PAGE and then transferred on to a nitrocellulose sheet where extravidin peroxidase conjugation and chemiluminescent detection revealed the presence of the biotinylated protein band. The researchers further showed that this protein band was

competitively reduced in incubations with D-glucose, stating that this showed the photoprobe to be specific for the binding site of GLUT1.

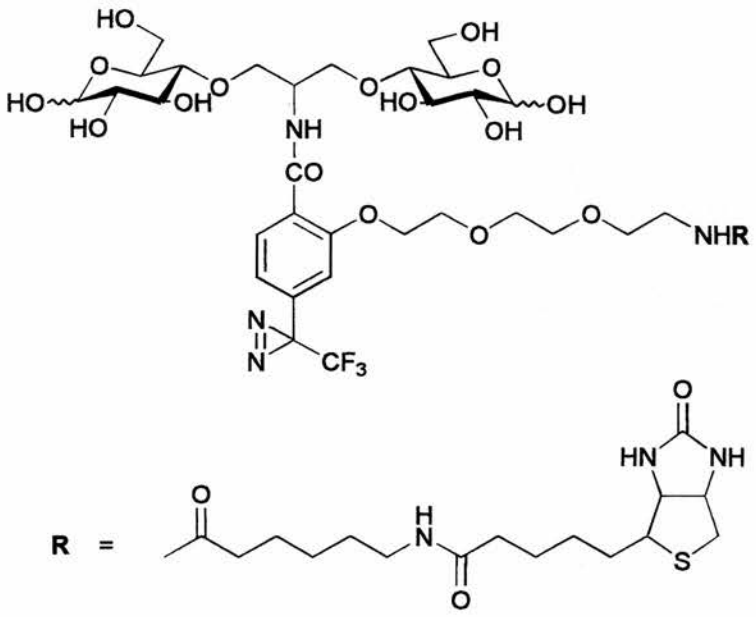


Figure 36. Bis glucose-derived photoprobe

3.2 Anomeric Derivatives

3.2.1 Overview

No fucosyltransferase has been crystallised and its structure solved to date, so there is little structural information available on this class of enzymes. Photoaffinity labelling represents an alternative technology by which structural details can be elucidated.

Our initial strategy for producing a photoaffinity label was to synthesise an anomerically-derived photoprobe from azidopropyl GlcNAc, as illustrated in Figure 37.

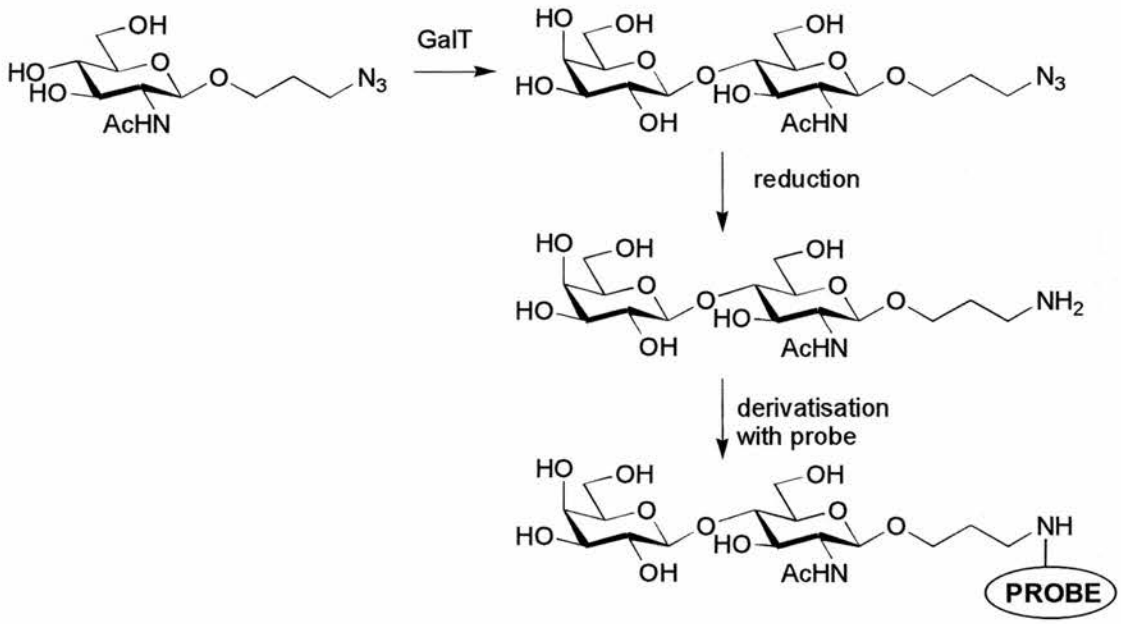


Figure 37. Synthesis of anomeric photoprobe

3.2.2 Azidopropyl GlcNAc

3.2.2.1 Synthesis

Azidopropyl GlcNAc (**1**) can be synthesised using standard literature procedures, as outlined in Figure 38.[8,9,10,11] This synthesis was carried out by Dr. K.P.R. Kartha (University of St. Andrews).

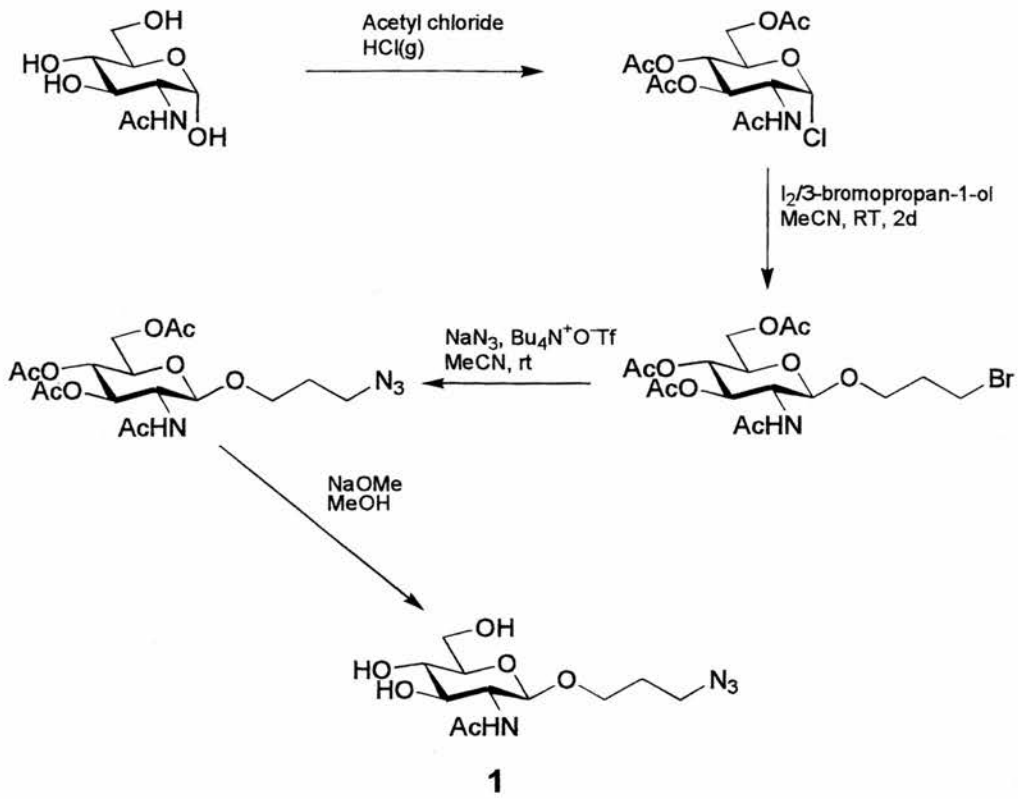


Figure 38. Production of Azidopropyl GlcNAc

3.2.2.2 Biotransformation

The disaccharide (**2**) can be produced enzymatically from azidopropyl GlcNAc (**1**) using UDP-Glc, UDP-Glc 4-epimerase, GalT and alkaline phosphatase (Figure 39).

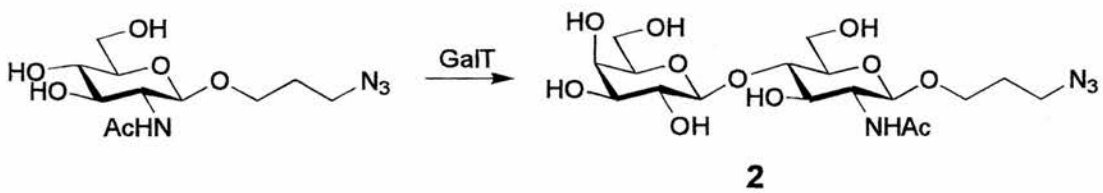


Figure 39. Production of anomerically functionalised disaccharide

Preliminary experiments monitored by TLC showed this to be a reasonably effective process. However, the need to separate hydrophilic donor and acceptor substrates from hydrophilic disaccharide product proved non-trivial. In hindsight, this can realistically be put down to our inexperience at purifying low molecular weight, water-soluble molecules. At that point, an alternative approach was considered that

could make use of straightforward reverse phase chromatographic separation and/or lipophilic sephadex LH-20 gel filtration chromatography. We were drawn to consider replacing the azido group with the more hydrophobic, enzymatically cleavable phenylacetamido group.

3.2.3 Phenylacetamido GlcNAc

Our modified strategy would use the phenylacetamido moiety which can be selectively cleaved using penicillin G acylase to give the free amine functionality; the photoprobe might then be attached chemically.

Waldmann[12] has employed penicillin G acylase to remove N-terminal phenylacetamido protecting groups in the synthesis of phosphopeptides (Figure 40).

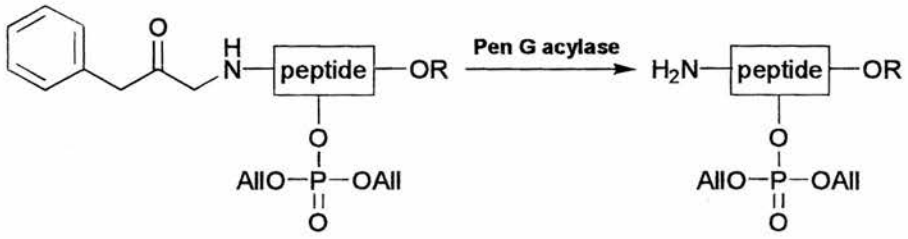


Figure 40. Pen G deprotection of PhAc in phosphopeptide synthesis

Penicillin G acylase mediated deprotection has also been applied to the more complex *p*-phenylacetoxybenzyloxycarbonyl (PhAcOZ) protecting group. This group comprises the enzyme labile phenylacetate esterified to a p-hydroxybenzyl urethane. After Pen G acylase cleavage, the urethane group undergoes spontaneous fragmentation to give the deprotected peptide. This protection group strategy was used by Waldmann[13] in glycopeptide synthesis (Figure 41).

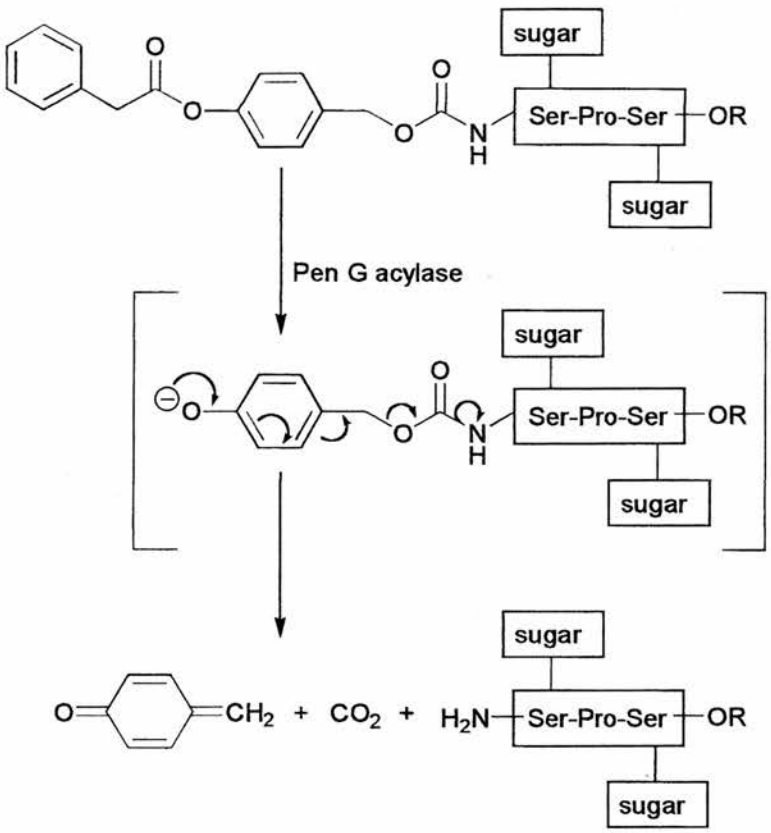


Figure 41. Pen G deprotection of PhAcOz in glycopeptide synthesis

3.2.3.1 Synthesis

3-Phenylacetamidopropyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**3**) can be synthesised from azidopropyl GlcNAc using standard procedures (**1**) as outlined in Figure 42.[10,14] This piece of synthesis was conducted by Dr. K.P.R. Kartha (University of St. Andrews).

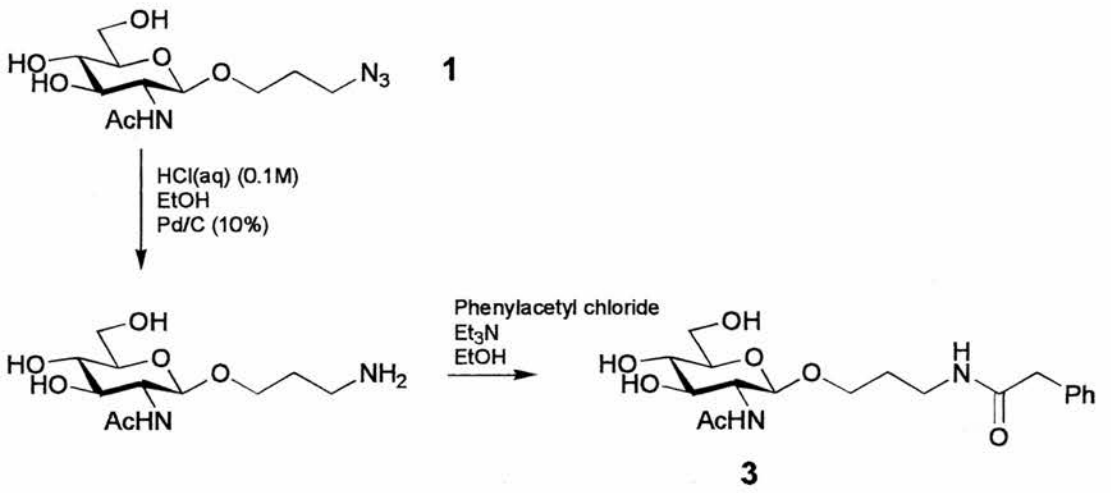


Figure 42. Synthesis of phenylacetamidopropyl GlcNAc

3.2.3.2 Biotransformation

From 3-phenylacetamidopropyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**3**), the disaccharide 3-phenylacetamidopropyl β-D-galactopyranosyl-1,4-(2-acetamido-2-deoxy-β-D-glucopyranoside) (**4**) was produced enzymatically using UDP-Glc, UDP-Glc 4-epimerase, GalT and alkaline phosphatase in 13 % isolated yield. Following purification of the crude reaction mixture by gel filtration (sephadex LH-20; 80 % ethanol in water) and mixed-bed ion-exchange chromatography, the structure of the product, **4**, was confirmed by ^{1}H NMR spectroscopy (δ 4.29, d, $J_{1,2}$ 7.45 Hz; δ 4.34, d, $J_{1',2'}$ 7.65 Hz) and mass spectrometry (FAB-MS: $[M+H^{+}]$ 559). Subsequent cleavage of the phenylacetamido bond of **4** by immobilised penicillin G acylase was easily undertaken to give the free amine (**5**), as outlined in Figure 43, in 57 % yield. ^{1}H and ^{13}C NMR spectroscopy, and mass spectrometry again confirmed the structure of the product.

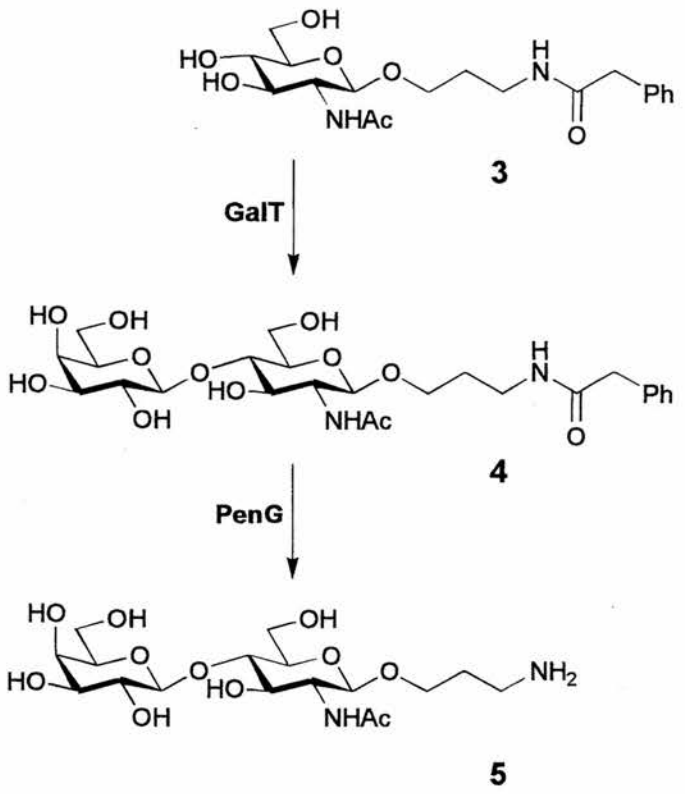
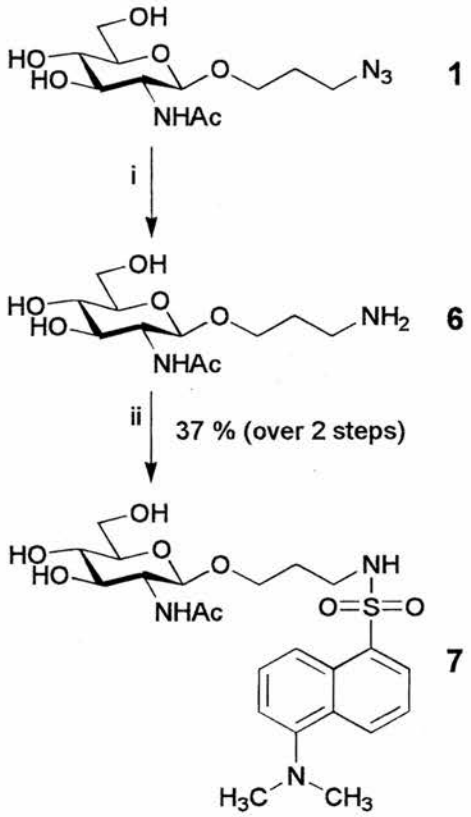


Figure 43. Production of disaccharide with anomeric amine functionality

3.2.4 Photo probes

3.2.4.1 Model study

As a model study, and in order to optimise processes on a milligram scale, the process of attaching the photoaffinity probe to amino compound **5** was investigated by using dansyl chloride as a substitute fluorescent probe and linking this to the functionalised monosaccharide 3-aminopropyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**6**). This compound was obtained by the treatment of the azide (**1**) with acetic acid in the presence of palladium/carbon catalyst which fully reduced the azide group yielding the aminopropyl sugar which was used directly in the next step. Coupling with dansyl chloride under alkaline conditions gave the dansylated glycoside (**7**) in an overall 37 % yield (Figure 44).



Reagents and conditions:

i) AcOH, 10 % Pd/C, 12 hr, RT; ii) EtOH, dansyl chloride, 1 hr, RT

Figure 44. Synthesis of dansylated propylGlcNAc

The above dansylated compound was coupled enzymatically to galactose as outlined in Figure 45 to give the labelled LacNAc derivative (**8**) in 44 % yield. We thus established a protocol for the production of our anomerically derived LacNAc photoaffinity probes, either by attachment of probe to a preformed disaccharide, or by attachment of a probe to a monosaccharide acceptor, followed by enzymatic galactosylation.

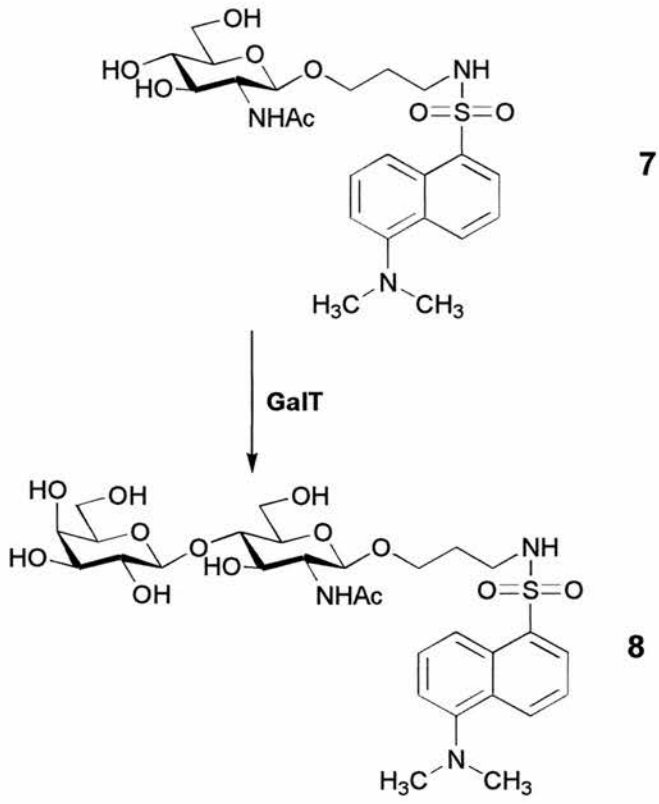


Figure 45. Production of anomerically labelled disaccharide

3.2.4.2 Simple Photo Probe

Having established the feasabilty of producing anomeric derivatives of N-acetyllactosamine by chemoenzymatic methods, we decided to first optimise the coupling conditions with a cheap, simplified photo probe due to the high commercial cost of the biotinylated probe. The simplified disaccharide-photo probe conjugate (**9**) was successfully prepared using the enzymatic route described in Figure 46 with the phenyl acetamido monosaccharide as the starting material.

The key step involving conversion of amino sugar **5** to final probe **9** was achieved in 20 % yield, with the product structure confirmed by ^{1}H NMR spectroscopy and mass spectrometry.

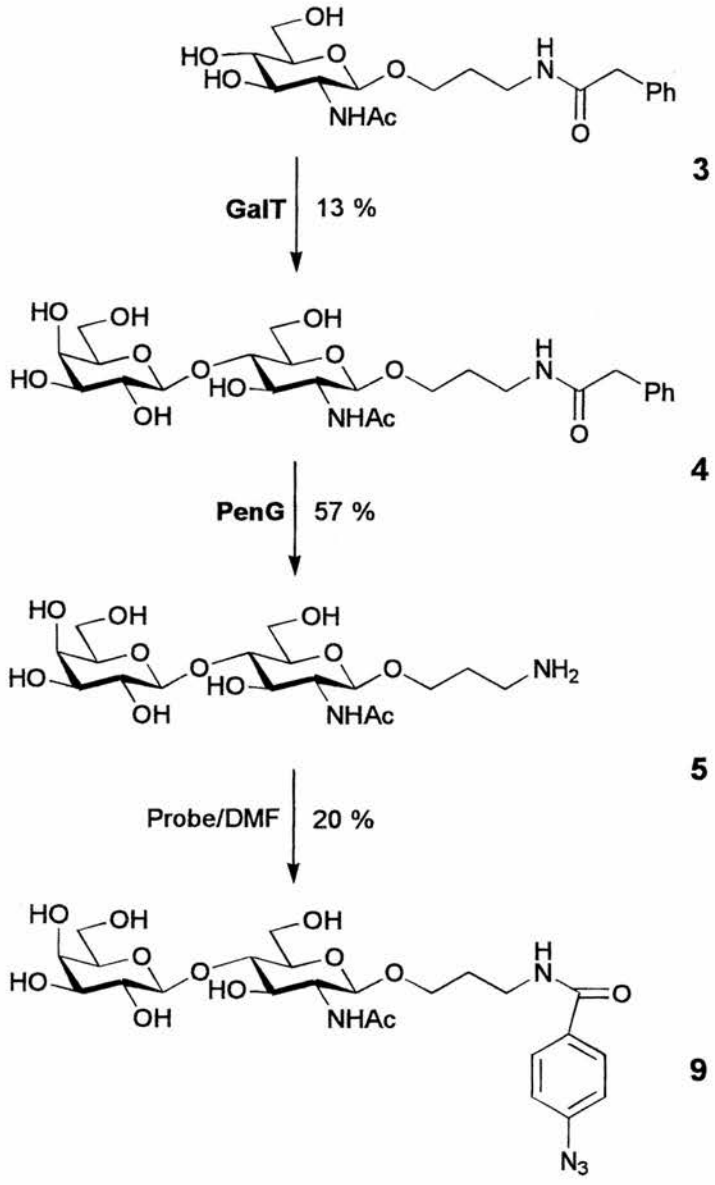


Figure 46. Synthesis of Non-Biotinylated Photo-Probe

3.2.4.3 Biotinylated Photo-Probe

Using the same methodology as for the simple probe, the biotinylated photoprobe (**10**) was successfully synthesised as illustrated in Figure 47.

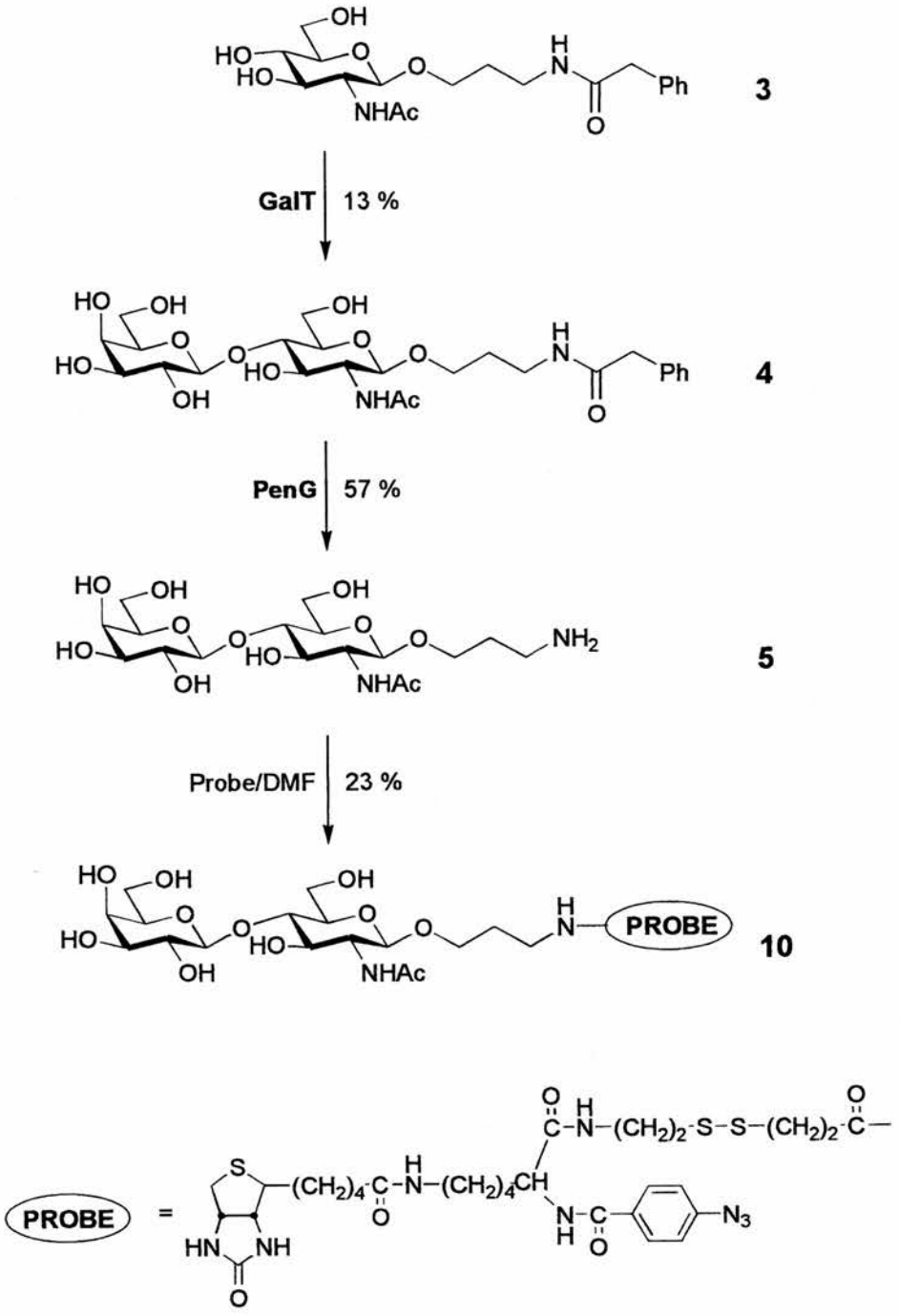


Figure 47. Synthesis of biotinylated photoprobe.

3.3 Photolabelling Studies

3.3.1 Production of FucT VI

Using the methodology described by Britten et al[15] for the production of FucT VII, recombinant fucosyltransferase VI was produced from an insect cell/baculovirus expression system. Firstly the insect cells were grown up to a density of 5 x 10^6/ml (as determined by microscopy) by incubation in growth medium for 24 hours at 28 °C. These cells were then used to produce a high titre working virus stock. After pelleting by centrifugation, the cells were innoculated with the low titre stock suspension of baculovirus and incubated in growth medium for 5 days at 28 °C. The cell culture was then centrifuged and the pellet discarded to leave the working stock virus in the supernatant. A plaque assay[16] was used to determine its titre.

Expression of recombinant enzyme involved infection of insect cells pelleted from a culture of density 5 x 10^6/ml with working virus stock using a higher multiplicity of infection of 1. After incubation for 48 hours the cells were pelleted and washed twice with PBS. Following resuspension in solubilisation buffer, enzyme activity was extracted by sonication using 7 x 15 second bursts on ice. Ultracentrifugation to remove cell debris, followed by filtration of the protein solution through a 0.2 μm syringe filter ensured the sample was prepared for FPLC purification. This was undertaken using a Hi-Trap cation exchange cartridge. Elution was with buffered 1M NaCl solution containing 0.1 % Triton X, with the enzyme being eluted at approximately 30 % of the salt gradient (see elution profile, Figure 48).

Eluted fractions were made up to 10 % in glycerol and then assayed for enzyme activity with the most active being retained (fractions 7 and 8). These fractions were then dialysed against buffer (containing 0.1 % Triton X) to remove the salt. The SDS-PAGE gel of fraction 8 is shown in Figure 49.

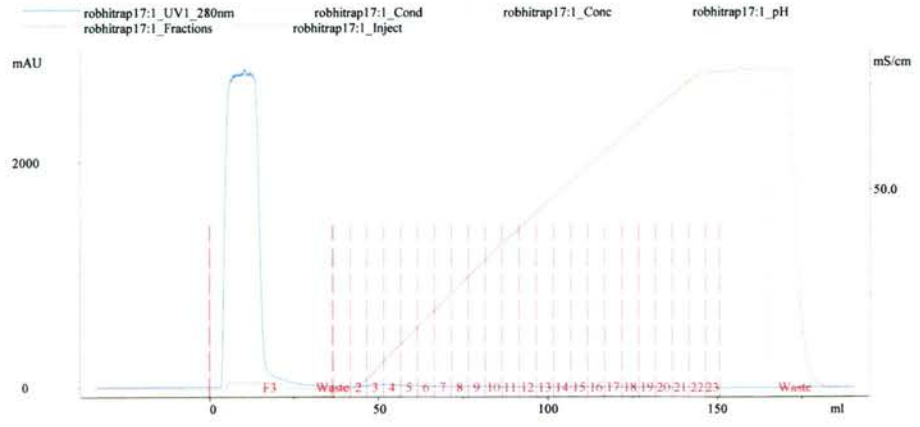


Figure 48. FPLC elution gradient. Fraction 8 is the most active

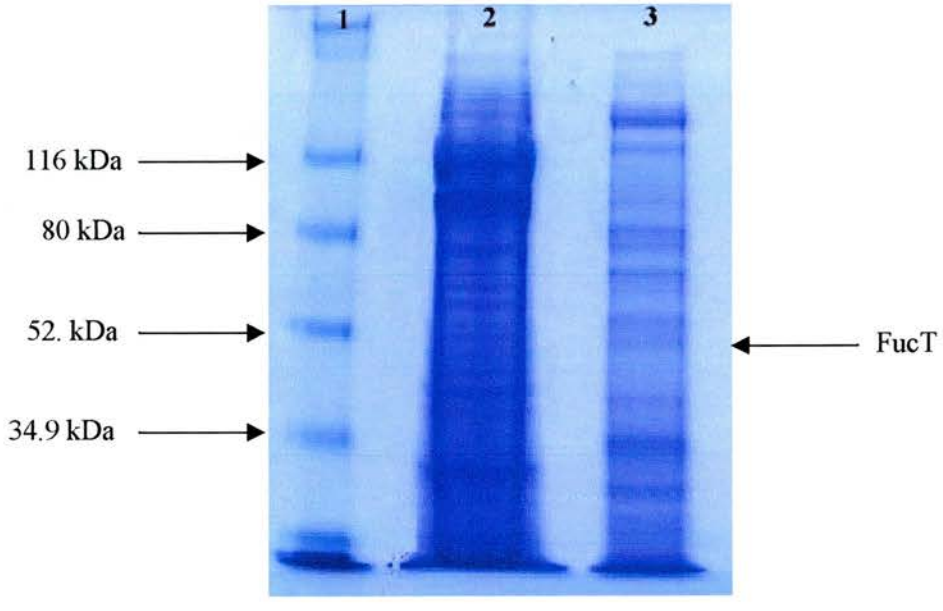


Figure 49. SDS-PAGE gel of FPLC fraction 8.

Lane 1-markers; lane 2-crude lysate; lane 3-fraction 8.

Under the above conditions the protein was found to be stable with good activity being retained. Table 7 summarises the purification.

Table 7. Purification of FucT VI from baculovirus transformed *S. frugiperda*

Fractions 1 and 2 correspond to column fractions 7 and 8.

Purification step	Fraction number	Fraction volume (ml)	[Protein] (mg/ml)	Total protein (mg)	Turnover (%) *	Specific activity (pmol/min/mg)	Total activity (pmol/min)	Fold purification	Recovery (%)
Crude lysate		40	37.4	1496	20	176	263,296	1	100
FPLC	1	10	4.76	47.6	18	1260	59,976	7.2	23
	2	10	3.0	30	16	1787	53,610	10.1	20
	total						113,586		43

* assayed with 1mM GDP-Fuc

3.3.2 Cross-Linking of FucT VI with Photoaffinity Label

Cross-linking of FucT VI with our photoaffinity label was achieved by incubating the enzyme solution with biotinylated probe (**10**) at room temperature before performing photolysis through UV irradiation. Photolysis of the photolabile azide group on the probe results in the emission of N_2 and the generation of a highly reactive nitrene which is then able to covalently bond to amino acid residues of the protein.

Before performing this cross-linking procedure however, it was first important to establish that our photoprobe did indeed act as a substrate for the enzyme. This was achieved by running the FucT assay with the photoaffinity label replacing LacNAc as the acceptor substrate (used at a final concentration of 0.25 mM). As can be seen from Table 8 the substrate is indeed being turned over to an appreciable degree.

Table 8. Turnover of Photoaffinity Label by FucT VI

Fraction	CPM	% Turnover
Blank	2,767	
Photoaffinity Label	17,605	24
Total Counts	88,344	

Since there is a significant degree of recognition of the photoaffinity label by the enzyme, then photolysis by U.V. illumination should result in significant inhibition of that enzyme activity. This was examined by cross-linking the enzyme and label by incubating the two together and then photolysing with U.V. light as described above. The photoaffinity label was used at a concentration of approximately 1mM. The activity of the labelled enzyme was then measured. The results of the assay in Table 3 demonstrate a significant loss of activity in photo-inactivated enzyme preparation.

Table 9. Inactivation of Enzyme by Photolabelling

Fraction	CPM	% Turnover
Blank	2,420	
Control	21,421	30
Probe	5,974	6
Total Counts	88,340	

3.3.3 Chemiluminescent Detection of Labelled Protein

Attempts were made to use a chemiluminescent coupled enzyme system to photographically detect photolabelled enzyme on a western blot. The procedure involved incubating photoprobe (0.006 mg or 0.03 mg) in 50 µl enzyme solution (3.0 mg protein/ml) before photolysing with UV as before. After addition of SDS sample buffer and heating in a hot block, the sample was then run on an SDS-PAGE gel with 'rainbow' markers. Western blotting onto a nitrocellulose film was then performed and the film 'blocked' using a BSA solution (blocking buffer). Streptavidin-linked horseradish peroxidase (streptavidin-HRP) was dissolved in blocking buffer in a specimen tube into which the western blot was placed. After allowing to incubate on a shaker at room temperature for 30 minutes, the blot was then rinsed thoroughly with buffer to ensure all unbound streptavidin-HRP was removed. Chemiluminescent signalling solution was poured over the surface of the blot and left for one minute. The strip was then dried with a tissue, wrapped in plastic film and exposed to a photographic plate. Development of the plate reveals protein bands labelled by the photo-probe.

The conditions were varied in terms of amount of protein and probe used, the concentration of streptavidin-HRP used and the length of time of exposure to the photographic plate, to try and optimise visualisation (and enable subsequent purification) of labelled enzyme. However, rather than seeing one labelled protein band on the photograph, it was evident that a lot of non-specific binding of probe to other proteins in the sample was taking place, resulting in a number of labelled bands (Figure 50). Indeed, as can be seen from the SDS-PAGE gel, fraction 8 from FPLC purification still contains many proteins. The proteins in this sample are essentially hydrophobic having been solubilised in buffer containing Triton-X 100 detergent. It

would appear this hydrophobicity may be responsible for binding to hydrophobic areas on the probe thus allowing covalent attachment after photolysis. This situation could be partly ameliorated by using a purer enzyme sample for labelling, but even more so by using a non-membrane associated enzyme for the studies i.e. a freely soluble FucT extracted from, for example, human milk.

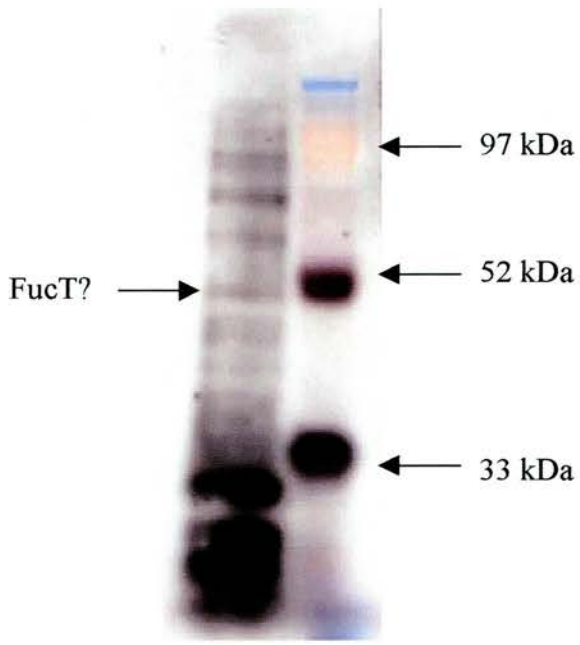


Figure 50. Photograph of chemiluminescently enhanced western blot

Lane 1 is the blot; lane 2 molecular weight markers

3.4 MATERIALS AND METHODS - BIOLOGICAL WORK

Fucosyltransferase VI was produced from an insect cell expression system involving the infection of *Spodoptera frugiperda* (Sf9) cells by recombinant baculovirus. As the enzyme is not freely water soluble and not excreted into the medium, extraction involved solubilising the Sf9 cells in detergent containing buffer.

3.4.1 Preparation of Working Virus Stock

A 200 ml suspension of Sf9 (*Spodoptera frugiperda*) cells having a density of 2×10^5 cells/ml was pelleted by centrifugation (800 g, 10 mins, 20 °C) and the supernatant removed by aspiration. The pellet was then resuspended in 400 ml of fresh, sterile medium (TC 100 + 10 % heat inactivated FBS, + 2 mM glutamine) in a stirrer flask and incubated at 28 °C for 24 hours in a water bath. The cell density was then established to be, by microscopy, approximately 5×10^6 cells/ml. The cells were pelleted as above and then innoculated with 20 ml of a baculovirus suspension of titre 1×10^7 pfu (plaque forming units)/ml giving a desired multiplicity of infection (M.O.I) of 0.1. Innoculation was achieved by pouring the innoculum onto the cell pellet over which it was allowed to rock gently for 1 hour at room temperature. The innoculum was then removed and fresh medium (400 ml) added. Incubation for 5 days as above then rendered the cells (as determined by microscopy) well infected. Harvesting of the growth medium by centrifugation and discarding of pellet produced the working stock virus, the titre of which was determined by plaque assay.

3.4.2 Baculovirus Plaque Assay

To each well of a Costar six-well (35 mm diameter) sterile plate was added 1.5×10^6 Sf9 cells from a suspension culture in a volume of 2 ml. The plates were left at room temperature on a level surface for 2 hours to allow the cells to adhere to the plastic after which time the media was removed by aspiration. Ten fold dilutions of the working virus stock from 10^{-3} to 10^{-8} (0.5 ml) were added to each well and left at room temperature, again on a level surface, for 1 hour, with periodic rotation. After removal of the virus innoculum by aspiration, the plates were then overlayed with 2 ml of a 3 % agarose solution mixed with complete medium (1:1), ensuring the overlay was not too hot when adding to the cells. The plate was then left for some 15 minutes until the overlay had set. Growth media (1 ml) was then placed on the solid

overlay in each well and the plate incubated in a humid box at 27.5 °C for 5 days. The growth medium was removed from each well by aspiration followed by the addition of 2 ml of 5 % Neutral Red Stain in PBS. This was left to incubate for a further 2 hours in the humid box at 27.5 °C. The stain solution was then decanted from the wells and the plate stored in an inverted position in the dark overnight enabling the stain to develop. The plaques, which appear as clearly visible holes in the stained cell monolayer, were then counted, a single plaque being caused by one single virus (or pfu). The titre of the working virus stock can then be established. This was found to be 2×10^7 pfu/ml.

3.4.3 Expression and Production of FucT VI

A cell suspension of density 5×10^6 cells/ml was produced as before and the cells pelleted. Working virus stock innoculum (100 ml) was used to infect the cells but with a higher M.O.I. of 1. After incubation while in a stirrer flask (28 °C, 72 hrs), the cells were pelleted by centrifugation (800 g, 10 mins). After removal of the supernatant the cells were resuspended in phosphate saline buffer (PBS) and centrifuged as above. This step was repeated after which the cell pellet was removed and stored at -20 °C.

To the cell pellet was added approximately 10X the volume of solubilisation buffer (1 % TritonX-100, 20 mM $MgCl_2$, 10 % glycerol, 50 mM MES, 1 mM DTT, 0.02 % NaN_3, pH 6.3) and then stirred vigorously on ice until the pellet was completely dispersed. The mixture, still on ice, was then sonicated (MSE Soniprep 150) (seven 15 second blasts with one minute cooling period in between). Ultracentrifugation (100,00 g, 1 hr, 4 °C) to remove the cell debris followed by filtration of the supernatant through a 0.2 μm syringe filter ensured the resulting protein solution was suitable to subject to FPLC.

3.4.4 Purification of FucT VI

FPLC was performed under refrigeration using a 5 ml HiTrap column on an Akta Explorer (Pharmacia). The eluant buffer was running buffer (50 mM MES, 0.1 % TritonX-100, 0.02 % NaN_3, pH 6.3) containing 1M NaCl. The flow rate was 5 ml/min and 5 ml fractions were collected. Runs were doubled up to give 10 ml fractions The most active fractions were made up to 10 % in glycerol and then

dialysed in Pierce Slide-A-Lyzer cassettes overnight The dialysis solution (50 mM MES, 20 mM $MgCl_2$, 0.1 % TX-100, 10 % glycerol, 1 mM DTT, pH 6.3) was then changed for fresh solution and dialysis continued for another 4 hours. The fractions were assayed before being stored in the freezer at -20 °C.

3.4.5 FucT Assay

This one pot assay measured the enzymatic transfer of tritiated fucose from GDP-[6-^{3}H]-Fuc to LacNAc. Tritiated product (Galβ1,4([^{3}H]-Fuc α1,3)GlcNAc) remains in the supernatant while unreacted anionic starting material (GDP-[^{3}H]-Fuc) is sedimented with Dowex anion exchange resin (AG 1-X8, 100-200 mesh, acetate form, Bio-Rad) (1 ml suspension). The 50 µl reaction mixture contained 5 µl each of GDP-Fuc (1 mM), GDP-[6-^{3}H]-Fuc (0.1 µCi, approx. 90,000 cpm), assay buffer (500 mM HEPES-NaOH, pH 7.2), $MnCl_2$ (75 mM) and ATP (10mM). To this was added 12.5 µl of LacNAc (40 mM) and 12.5 µl of fucosyltransferase solution. The molarities quoted are as added ie before dilution. LacNAc was replaced by probe when assaying this for substrate recognition. The reaction mixture was incubated for 1 hour at 37 °C in a water bath after which time the reaction was terminated by quenching with the Dowex suspension (1:4 w/v in water). After vortexing briefly, the mixture was centrifuged and 600 µl of supernatant removed. This was mixed with scintillation fluid (Fisher High Safe 8) (4 ml) and counted for 2 minutes.

Enzyme activity is expressed as a percentage turnover of substrate, calculated from the proportion of measured counts to the total number of counts in the assay.

3.4.6 Measurement of Protein Concentration

Protein concentration was measured using the Bio-Rad (non Dc) protein assay. The procedure involved diluting the protein assay dye 5 X in purified water. To 900 µl of this was added 100 µl of protein sample. After mixing, absorbance was measured at 595 nm. The BSA standard curve samples were made up using FPLC running buffer (50 mM MES, 0.1 % TX-100).

3.4.7 SDS-PAGE

SDS polyacrylamide gel electrophoresis was performed using a NuPAGE system. Protein samples (30-180 μg) were concentrated using a 30 K centricon filter and precipitated using trichloroacetic acid. A 72 % solution was added to the protein sample to give a final concentration of 7.2 % in TCA. The solution was then vortexed and left to sit on ice for 5 minutes. After pipetting off the supernatant the protein was solubilised in 4 X SDS sample buffer plus mercaptoethanol added to 1/10 final sample volume. Samples were heated at 100 °C in a hot block for 10 minutes before loading onto the NuPAGE 10 % Bis-Tris gel. Pre-stained molecular weight markers (Novex) used were in the range of 2.5-200 kDa. The gel was run under 150 V for 50 minutes in MOPS running buffer containing NuPAGE antioxidant

3.4.8 Western Blotting

The SDS-PAGE gel was sandwiched onto a nitrocellulose film in a NuPAGE tank. To 160 ml of stock transfer buffer (28.8 g glycine and 6 g Tris made up to 200 ml in distilled water) was added 400 ml of methanol and the solution made up to 2000 ml in distilled water. This was used to fill the tank. Transference of protein to the nitrocellulose film was achieved by the application of 100 V for 60 minutes.

3.4.9 Chemiluminescent Detection of Labelled Protein

The nitrocellulose blot was 'blocked' by shaking in phosphate buffer saline containing 0.1 % Tween 20 (PBST) and 5 % BSA (blocking buffer) overnight at room temperature. Streptavidin-HRP was diluted in blocking buffer in a specimen tube to concentrations of between 0.05 and 25 μg of streptavidin-HRP per ml. The nitrocellulose blot was placed in the streptavidin-HRP solution and allowed to shake at room temperature for 30 minutes. The blot was then rinsed several times with PBST to ensure all unbound streptavidin-HRP was washed away. The blot was then placed on a tray, protein surface uppermost, and SuperSignal West Pico Chemiluminescent Substrate solution (Pierce) (2 + 2 ml), which was mixed immediately before use, poured over the surface and left for 1 minute. The blot was then removed from the tray and dabbed gently with a tissue to remove any excess liquid. It was then wrapped in clingfilm and placed face down on photographic paper in a Kodak cassette. Times of exposure varied from 1 minute to 2 hours. Overnight

exposures were also carried out. Development of film was in an automated RGII X-ray film processor (Fuji).

A blot could be re-probed with streptavidin-HRP after first treating with stripping buffer (100 mM 2-mercaptoethanol, 2 % SDS, 62.5 mM Tris-HCl, pH 6.7). Incubating the blot in stripping bufferfor an hour at room temperature would suffice to remove bound streptavidin-HRP. This was checked by addition of chemiluminescent substrate and exposing to photographic film as before. The non re-appearance of previous bands showed that stripping was complete. After rinsing with PBST, the blot was then ready for another round of probing with streptavidin-HRP after re-blocking of non-specific sites.

3.5 EXPERIMENTAL - CHEMICAL WORK

General Methods

All reagents and solvents were dried prior to use according to standard methods[1]. Commercial reagents were otherwise used without further purification.
Analytical TLC was performed on silica gel 60-F_{254} (Merck) with detection by fluorescence and/or by charring following immersion in a dilute ethanolic solution of sulphuric acid or spraying with orcinol [which was prepared by the careful addition of conc. sulfuric acid (20 cm^3) to an ice cold solution of 3,5-dihydroxytoluene (360 mg) in EtOH (150 cm^3) and water (10 cm^3)]. Column chromatography was performed with silica gel 60 (Fluka), 70-230 mesh.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 spectrometer at 300 MHz and 75 MHz respectively. ^{1}H NMR spectra were referenced to the following internal standards: δ_H 7.26 in $CDCl_3$, 3.35 in CD_3OD, 4.75 in D_2O. ^{13}C NMR spectra were referenced to the following internal standards: δ_C 76.9 in $CDCl_3$; δ_C 49.0 in CD_3OD. *J*-values are given in Hz. Only partial (diagnostic) NMR data are given for some compounds; other spectral features were in accord with the proposed structures.

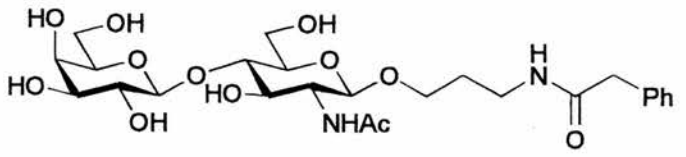
UDP-Galactose 4-Epimerase and β1,4-Galactosyltransferase were purchased from Calbiochem as was the GDP-Fucose. All other commercial enzymes and nucleoside sugars used were purchased from Sigma. Penicillin G acylase was used immobilised on macroporous oxirane acrylic beads.

Galactokinase and Galactose-1-phosphate uridyltransferase were from galactose adapted yeast. UDP-Glucose pyrophosphorylase and inorganic pyrophosphatase were from Bakers Yeast. Pyruvate Kinase was from rabbit muscle; galactosyltransferase was from bovine milk; epimerase was recombinant from *E. coli* and alkaline phosphatase was from bovine intestinal mucosa.

3-Azidopropyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**) and 3-

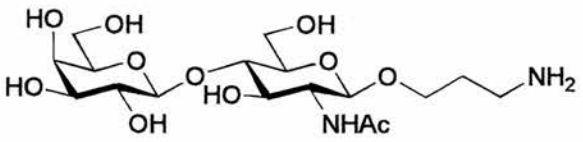
phenylacetamidopropyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**3**) and were kindly provided by Dr K. P. R. Kartha.

3-Phenylacetamidopropyl β-D-galactopyranosyl-1,4-(2-acetamido-2-deoxy-β-D-glucopyranoside) (4).



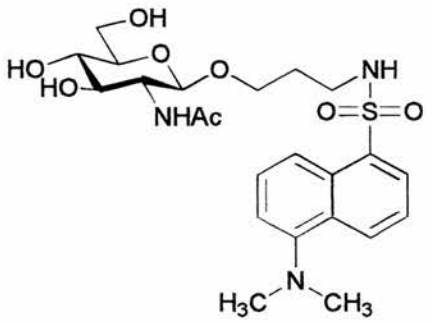
To a buffered solution (3 ml of HEPES 100 mM, KCl 20 mM, $MgCl_2$ 10 mM, $MnCl_2$ 5 mM, 0.02 % NaN_3, pH 7.4) of 3-phenylacetamidopropyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**3**) (200 mg, 0.50 mmol) was added UDP-glucose (450 mg, 1.5 mol equiv), UDP-galactose 4'-epimerase (2 U), β1,4-galactosyltransferase (1 U) and alkaline phosphatase (16 U). Nitrogen was passed over the solution and the reaction mixture was incubated in a shaker-incubator for 36 hours (150 rpm, 37 °C). Precipitate was removed by centrifugation and the supernatant was loaded onto a Sephadex LH20 gel filtration column (2.8 x 73 cm). Elution with 80 % ethanol at a rate of 12 ml/hour gave fractions which were monitored by T.L.C. ($CHCl_3$/MeOH/H_2O, 10:10:3). After lyophilisation of appropriate fractions, the resulting material was dissolved in 1ml of water, loaded on a column of TMD8 mixed bed ion exchange resin (1.4 x 10.5 cm) and eluted with water (30 ml). Lyophilisation of the eluant yielded disaccharide (**4**) (35 mg, 13 %). δ_H (D_2O) 1.64 (2H, t, CH_2), 1.90 (3H, s, $CH_3CO.N$), 2.98 (1H, m, O.C*H*$_2$$CH_2CH_2$.N), 3.01 (1H, m, O.C*H*$_2$$CH_2CH_2$.N), 4.29 (1H, d, *J* 7.5, H-1), 4.34 (1H, d, *J* 7.7, H-1'), 7.21-7.35 (5H, m, Ar). δ_C (D_2O) 21.8 (*C*H_3CO.N), 28.0 (OCH$_2$*C*H$_2$CH$_2$N), 36.1 (OCH$_2$CH$_2$*C*H$_2$N), 42.3 (Ph*C*H$_2$), 54.8 (*C*-6'), 59.8 and 60.8 (*C*-2 and *C*-6), 67.5, 68.4, 70.8, 72.3, 74.5, 75.2 and 78.3 (*C*-3, *C*-4, *C*-5, *C*-3', *C*-4', *C*-5' and O*C*H$_2$), 101.0 and 102.7 (*C*-1 and *C*-1'), 127.2 and 129.0 (5*C*s, Ar), 135.1 (Ar quat), 174.5 and 174.6 (2 x *C*O). FAB-MS (+ve): *m/z* 559 ($M+H^+$) and 581 ($M+Na^+$) ($C_{25}H_{38}N_2O_{12}$ requires *m/z* 558).

3-Aminopropyl β-D-galactopyranosyl-1,4-(2-acetamido-2-deoxy-β-D-glucopyranoside) (5).



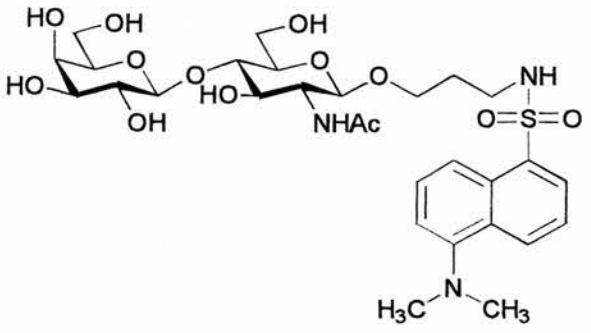
To a solution of phenylacetamide (**4**) (20 mg, 35.8 μmol) in ammonium bicarbonate (50mM, pH 7.9, 1ml) was added immobilised penicillin G acylase (19 U) and the reaction mixture was placed in a shaker-incubator (150 rpm) for 12 hours at 37 °C. Completion of the reaction was checked by T.L.C. (MeCN/IPA/50mM KCl, 10:67:23). The solution was then loaded on to a column of TMD8 mixed bed ion-exchange resin (3 ml). After washing with water (15 ml), HCl (1M, 15 ml) was used as the elutant and T.L.C. (IPA/NH_3/H_2O, 6:3:2) used to confirm the presence of product. Lyophilisation of the eluant gave aminopropyl glycoside (**5**) (9 mg, 57 %). δ_H (D_20) 2.10 (3H, s, $CH_3CO.N$), 4.49 (1H, d, *J* 7.9 *H*-1), 4.57 (1H, d, *J* 8.1 *H*-1'), 7.31-7.40 (2H, m, NH_2). δ_C (D_20) 24.7 ($NHCOCH_3$), 35.7 ($OCH_2CH_2CH_2NH_2$), 53.1 and 53.6 (C-6' and $OCH_2CH_2CH_2NH_2$), 58.7 and 59.1 (C-2 and C-6), 66.0, 66.6, 69.0, 70.3, 70.5, 72.8, 73.4, and 76.5 (remaining 7 ring Cs and $OCH_2CH_2CH_2NH_2$), 99.8, 100.9 (*C*-1 and *C*-1'), 172.8 (*C*O). ES-MS (+ve): *m/z* 441 ($M+H^+$) and 463 ($M+Na^+$) ($C_{17}H_{32}N_2O_{11}$ requires 440).

3-(5-Dimethylamino-1-naphthalenesulfonyl)propyl 2-acetamido-2-deoxy-β-D-glucopyranoside (7).



3-Azidopropyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**) (63 mg, 0.21 mmol), palladium (63 mg of 10 % Pd-C), water (1.5 ml) and acetic acid (1.5 ml) were stirred together under hydrogen for 12 hours at room temperature after which time T.L.C. (DCM/MeOH, 8:2) indicated complete reduction of the azide. The reaction mixture was passed through a bed of Celite, which was subsequently washed with methanol, and solvent was removed *in vacuo*. Addition of water (25 ml) was followed by lyophilisation to give 70 mg of solid which was dissolved in ethanol (2 ml) containing dansyl chloride (112 mg, 2 mol equiv) and the solution was made alkaline by the dropwise addition of triethylamine. The reaction mixture was left to stir in darkness at room temperature for 1 hour. T.L.C. (DCM/MeOH, 8:2) showed product formation. The reaction mixture was evaporated to dryness, redissolved in a minimal volume of methanol and loaded on to a Sephadex LH20 gel filtration column (2.8 x 73 cm). Elution with methanol at a rate of 12ml/hour gave fractions which were monitored by T.L.C. (MeCN/IPA/50mM KCl, 10:67:23). Evaporation of relevant fractions to dryness gave dansylated glycoside (**7**) (41 mg, 37 %). δ_H (CD_3OD) 1.59 (2H, p, $OCH_2CH_2CH_2N$), 1.87 (3H, s, $CH_3CO.N$) 2.88 (6H, s, 2 x CH_3), 2.92 (2H, t, $OCH_2CH_2CH_2N$), 3.20-3.32 (2H, m, H-3 and H-4), 3.34-3.46 (2H, m, H-5 and 1H of OCH_2), 3.54-3.68 (2H, m, H-6a and H-2), 3.74-3.90 (2H, m, H-6b and 1H of OCH_2), 4.27 (1H, d, *J* 8.51, *H*-1), 7.26 (1H, d, Ar), 7.59 (2H, m, Ar), 8.17 (1H, d, Ar), 8.34 (1H, d, Ar), 8.55 (1H, d, Ar). δ_C (CD_3OD) 22.9 ($CH_3CO.N$), 30.8 ($OCH_2CH_2CH_2N$), 41.0 ($OCH_2CH_2CH_2N$), 45.8 (2 x NCH_3), 57.2 (*C*-2), 62.8 (*C*-6), 67.6, 72.1, 76.1 and 77.9 (*C*-3, *C*-4, *C*-5 and $OCH_2CH_2CH_2N$), 102.7 (*C*-1), 116.5,120.7, 124.4, 129.2, 130.2 and 131.0 (6 x Ar), 131.2, 131.3, 137.2 and 153.3 (4 x Ar quat), 173.9 (*C*O). ES-MS (+ve): 534 ($M+Na^+$) ($C_{23}H_{33}N_3O_8S$ requires *m/z* 511).

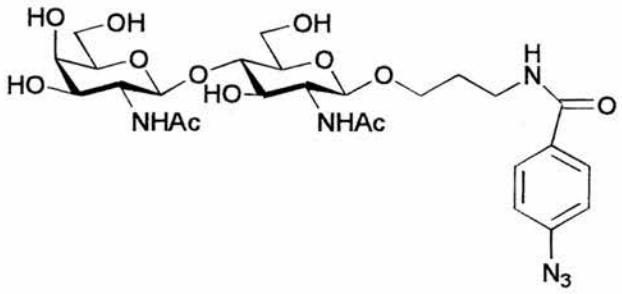
3-(5-Dimethylamino-1-naphthalenesulfonyl)propyl β-D-galactopyranosyl-1,4-(2-acetamido-2-deoxy-β-D-glucopyranoside) (8).



To 1.35 ml of buffer (HEPES 100 mM, KCl 20 mM, $MgCl_2$ 10 mM, $MnCl_2$ 5 mM, 0.02 % NaN_3, pH 7.4) was added 3-(5-Dimethylamino-1-naphthalenesulfonyl)propyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**7**) (41 mg, 0.08 mmol) in 150 μl methanol along with UDP-glucose (144 mg, 3 mol equiv), UDP-galactose 4'-epimerase (2.8 U), β1,4-galactosyltransferase (5 U) and alkaline phosphatase (30 U). Nitrogen was passed over the solution and the reaction mixture was incubated in a shaker-incubator for 36 hours (150 rpm, 37 °C) after which time TLC showed the complete consumption of monosaccharide starting material. The reaction mixture was then loaded onto a Sephadex LH20 gel filtration column (2.8 x 73 cm) and eluted with 100 % methanol at a rate of 12 ml/hour giving fractions which were monitored by T.L.C. ($CHCl_3$/MeOH/H_2O, 10:10:3). Appropriate fractions were pooled and lyophilised to yield disaccharide (25 mg, 44 %). δ_H (CD_3OD) 1.50 (2H, p, $OCH_2CH_2CH_2N$), 1.76 (3H, s, $CH_3CO.N$), 2.78 (6H, s, 2 x CH_3), 2.82 (2H, t, $OCH_2CH_2CH_2N$), 3.18-3.82 (13H, m, H-2, H-3, H-4, H-5, H-6a, H-6b, H-2', H-3', H-4', H-5', H-6a', H-6b' and $OCH_2CH_2CH_2N$), 4.19 (1H, d, *J* 8.0, H-1), 4.26 (1H, d, *J* 7.1, H-1'), 7.17 (1H, d, Ar), 7.49 (2H, m, Ar), 8.08 (1H, d, Ar), 8.25 (1H, d, Ar), 8.46 (1H, d, Ar). δ_C (CD_3OD) 22.3 ($CH_3CO.N$), 30.3 ($OCH_2CH_2CH_2N$), 40.5 ($OCH_2CH_2CH_2N$), 45.2 (2 x NCH_3), 56.0 (*C*-6'), 61.4 and 62.0 (*C*-2 and *C*-6), 67.1, 69.8, 72.1, 73.6, 74.3, 76.0, 76.6 and 80.5 (*C*-3, *C*-4, *C*-5, *C*-2', *C*-3', *C*-4', *C*-5' and OCH_2), 102.2 and 104.6 (*C*-1 and *C*-1'), 116.0, 120.1, 123.9, 128.7, 129.7 and 130.7 (6 x *C*H Ar), 130.5, 130.8 and 152.8 (3 x Quat Ar), 173.2 (*C*O). ES-MS (+ve): 696

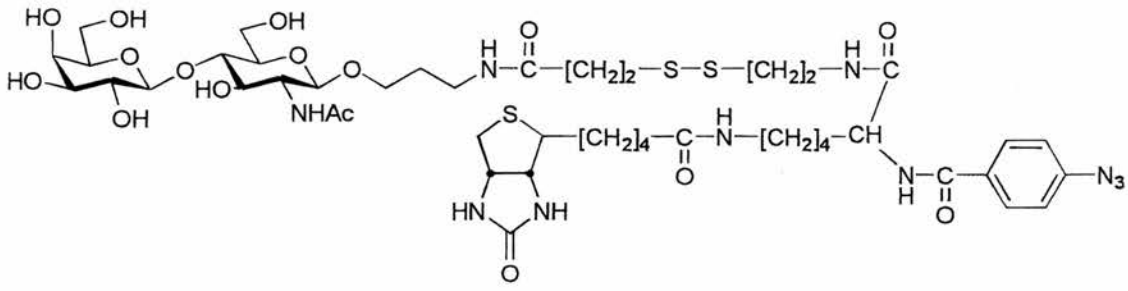
($M+Na^+$), ES-MS (-ve): 672 ($M-H^+$) ($C_{29}H_{43}N_3O_{13}S$ requires *m/z* 673). HRMS: Found 674.2595. $C_{29}H_{43}N_3O_{13}S$ ($M+H^+$) requires 674.2597.

3-*p*-Azidophenylpropyl β-D-galactopyranosyl-1,4-(2-acetamido-2-deoxy-β-D-glucopyranoside) (9).



To a solution of phenylacetamide (**4**) (4 mg, 0.007 mmol) in ammonium bicarbonate (50 mM, pH 7.9, 150 μl) was added immobilised penicillin G acylase (15 U) and the reaction mixture was placed in a shaker-incubator (150 rpm) for 12 hours at 37 °C. Completion of the reaction was checked by T.L.C. (MeCN/IPA/50mM KCl, 10:67:23). The reaction mixture was then vortexed and the supernatant pipetted into another eppendorf. To this was added Sulfo-HSAB (N-Hydroxysulfosuccinimydyl-4-azidobenzoate) (2.4 mol equiv, 6.32 mg in 38 μl DMF) and incubated at 37 °C for 3 hours after which time T.L.C. (MeCN/IPA/50mM KCl, 10:67:23) indicated the almost complete disappearance of starting material. The reaction mixture was then purified by loading onto a Varian preparative silica column (500 mg, 2.8 ml) and washing with ethyl acetate. Elution with methanol and evaporation to dryness gave the title compound (**9**) (0.84 mg, 20 %). δ_H (D_2O) 1.84 (2H, t, CH_2), 2.01 (3H, s, $CH_3CO.N$), 4.49 (1H, d, $J_{1,2}$ 7.8, *H*-1) and 4.57 (1H, d, $J_{1,2}$ 7.8, *H*-1), 7.21 (2H, d, Ar), 7.79 (2H, d, Ar). MS-ES (+ve): *m/z* 586 ($M+H^+$). MS-ES (-ve): *m/z* 584 ($M-H^+$) ($C_{24}H_{35}N_5O_{12}$ requires *m/z* 585).

Biotinylated Probe (10).



To a solution of phenylacetamide (**4**) (9 mg, 0.016 mmol) in ammonium bicarbonate (50 mM, pH 7.9, 150 μl) was added immobilised penicillin G acylase (20 U) and the reaction mixture was placed in a shaker-incubator (150 rpm) for 12 hours at 37 °C. Completion of the reaction was checked by T.L.C. (MeCN/IPA/50mM KCl, 10:67:23). The reaction mixture was then vortexed and the supernatant pipetted into another eppendorf and lyophilised. The lyophilate was redissolved in 100 μl DMF and to this was added Sulfo-SBED (sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido)-hexanoamido]ethyl-1,3'-dithioproprionate (20 mg in minimal vol. of DMF, 1.4 equiv). After incubating at 37 °C for 1 hour T.L.C. (MeCN/IPA/50 mM KCl, 10:67:23) showed the reaction had gone to completion. Purification of product was by preparative reverse-phase HPLC using a Supelco Supelcosil ABZ+, 5 μm, 10 cm x 21.2 mm column. Eluant was MeCN with 0.05 % TFA and 0.1 % aqueous TFA, using a gradient 20-60 % over 20 minutes and a flow rate of 4 ml/min. Detection was with U.V. at 270 nm. Pooling of the relevant fractions and drying under vacuum gave the biotinylated probe (**10**) (4.1 mg, 23 %). δ_H (D_2O) 1.84 (3H, s, $CH_3CO.N$), 2.44 (2H, t, CH_2), 7.02 (2H, d, Ar), 7.64 (2H, d, Ar). MS-ES (+ve): *m/z* 1103 ($M+H^+$) and 1125 ($M+Na^+$) ($C_{45}H_{70}N_{10}O_{16}S_3$ requires *m/z* 1102).

3.6 REFERENCES

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Chapter 4

Chemoenzymatic Synthesis of Azido-LacNAc Derivatives

4.1 Benzyl 6-azido-6-deoxy-LacNAc

Problems encountered with the enzymatic coupling of 3'-azido-3'-deoxy-D-galactose to benzyl GlcNAc prompted us to try an alternative approach to producing an azido–functionalised LacNAc derivative. This was to chemically synthesise benzyl 6-azido-6-deoxy GlcNAc and enzymatically couple to galactose to give the disaccharide benzyl 6-azido-6-deoxy LacNAc, as illustrated in Figure 51.

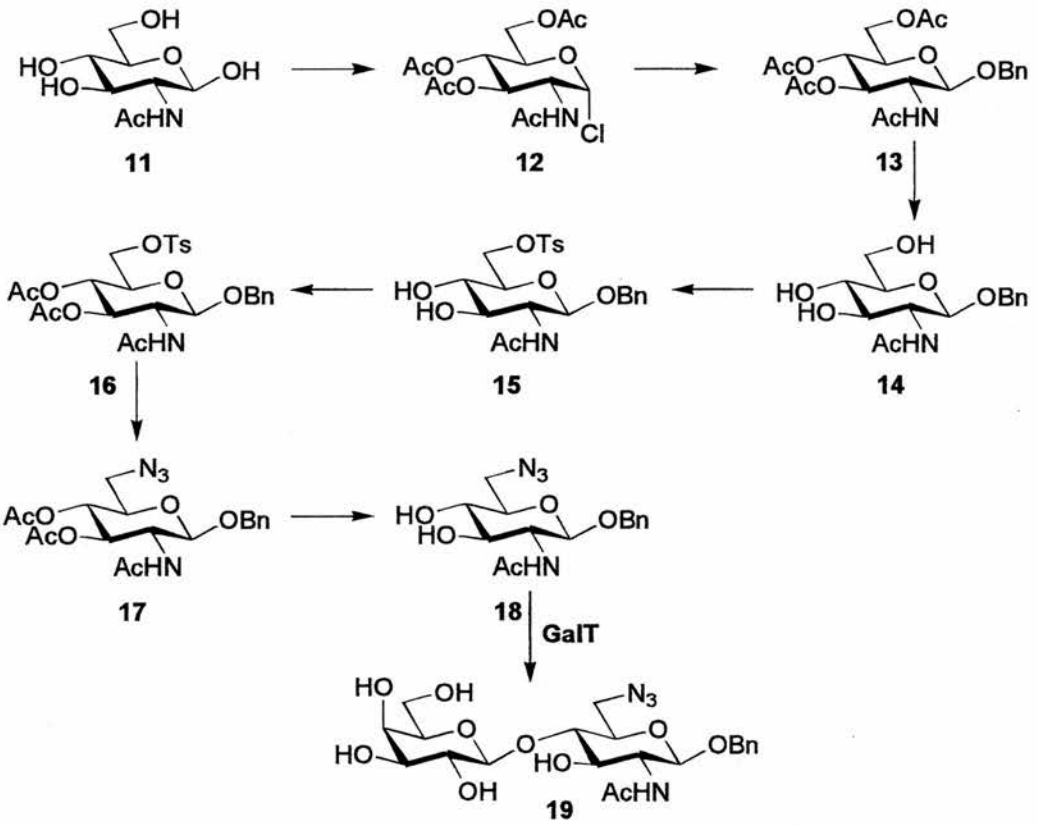


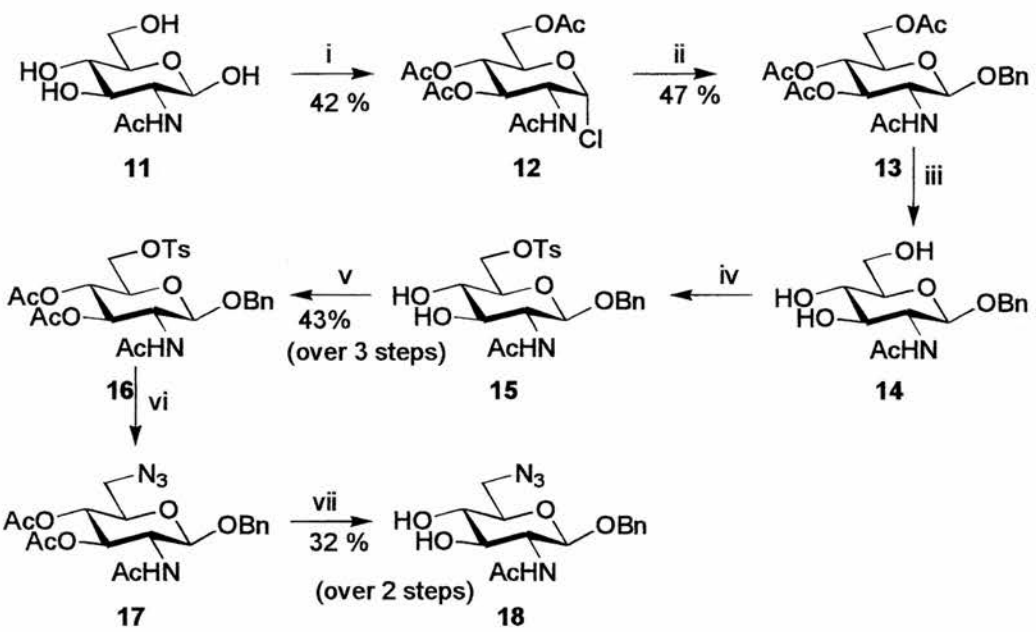
Figure 51. Chemoenzymatic synthesis of benzyl 6-azido-6-deoxy-LacNAc

The chemical synthesis of benzyl 6-azido-6-deoxy GlcNAc (**18**) is outlined in Section 4.1.1, and its enzymatic coupling to galactose to produce benzyl 6-azido-6-deoxy LacNAc (**19**) shown in Section 4.1.2.

4.1.1 Chemical Synthesis of Benzyl 6-azido-6-deoxy GlcNAc (18)

This synthesis followed that previously outlined by Field and co-workers,[1] starting from N-acetylglucosamine (**11**) (Figure 52). Reaction with acetyl chloride overnight gave the per-*O*-acetylated GlcNAc chloride (**12**) which was worked up and

crystallised from diethyl ether. The known benzyl glycoside (**13**)[2] was synthesised by reaction of glycosyl chloride (**12**) with benzyl alcohol and iodine in acetonitrile,[3] with the β-configuration of the product being confirmed by ^{1}H NMR spectroscopy ($J_{1,2}$ 8.2 Hz). Subsequent deprotection using sodium metal in methanol gave benzyl GlcNAc (**14**).



Reagents and conditions:

i) Acetyl chloride, o/n, RT; ii) BnOH, I_2, MeCN, 72 hr; iii) NaOMe, MeOH; iv) TsCl, pyridine, -35 °C → RT, 8 hr; v) Ac_2O, o/n; vi) NaN_3, DMF, 120-140 °C o/n; vii) NaOMe, MeOH.

Figure 52. Chemical synthesis of benzyl 6-azido-6-deoxy-GlcNAc

Selective tosylation of the primary alcohol of benzyl GlcNAc (**14**) was performed with the reaction mixture initially maintained at a temperature of -35 °C in an acetonitrile/dry ice bath. A solution of tosyl chloride in DCM was added dropwise over a period of approximately 30 minutes and the reaction mixture was allowed to warm to room temperature. After 12 hours, T.L.C. indicated that the formation of tosylate (**15**) had proceeded to around 75 %, at which stage the mixture was cooled again and 5 mole equivalents of acetic anhydride were added dropwise. The reaction was left overnight when T.L.C. showed only a trace of unprotected compound left. After working the reaction up, the resulting solid was loaded onto a silica gel column

and eluted with toluene:ethyl acetate, 2:1-0:1 This gradient was chosen after running a number of test T.L.C.s and appeared to be appropriate. However, no defined band of compound was eluted from the column; rather it appeared to leach out from solid material at the top of the column over a large volume of solvent. As with a number of GlcNAc-based compounds explored in this study, even protected forms of this sugar had unpredictable solubility in organic solvents.

The eluant was concentrated to dryness and the resulting solid was dissolved in a minimal volume of hot isopropanol and allowed to cool on the bench. The resulting crystals were combined with those obtained from a second round of crystallisation and compound (**16**) was found to be >95 % pure by ^{1}H N.M.R. A further round of crystallisation produced clean per-acetate (**13**).

The tosyl compound (**16**) was heated with 10 equivalents of sodium azide in DMF at 120 °C overnight. This appeared to result in only a partial azide displacement so the temperature was increased to 140 °C and the reaction allowed to continue for another 3 hours after which time the reaction was complete. After work up, attempts were made to crystallise the azido sugar (**17**) from the resulting brown syrup, but this was unsuccessful. The syrup was then subject to silica gel chromatography, eluting with a gradient of ethyl acetate: toluene from 2:1 to 1:0. Two different compounds, as judged by T.L.C. and NMR, were pooled separately. They were dissolved in anhydrous methanol and stored in the fridge overnight after which time T.L.C. showed no difference between the two. The polarity of both compounds were found to be the same. It thus appeared that di-acetylated and mono-acetylated sugars were eluted from the column, both of which subsequently spontaneously deacetylated to form the di-hydroxy sugar (**18**) (Figure 53). Presumably a trace of base in the methanol was sufficient to effect de-O-acetylation.

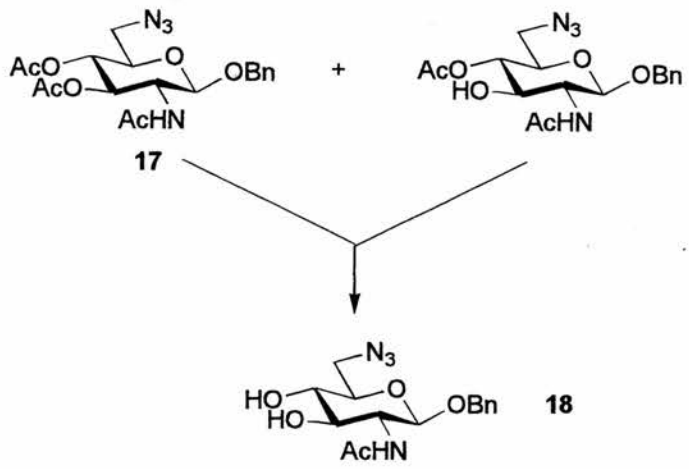


Figure 53. Spontaneous deacetylation

The methanolic solution was indeed found to be basic so it was neutralised with Dowex H^+ ion-exchange resin. After removal of the resin by filtration, the filtrate was evaporated to dryness. Due to the presence of contaminating spots on the T.L.C., the free azido sugar (**18**) was crystallised from methanol, giving an overall yield of 32 % from (**16**). The structure was confirmed by ^{1}H NMR spectroscopy (δ 4.50, d, $J_{1,2}$ 8.7 Hz; δ 4.59, d, J 12.3 Hz; OCH_2Ph; δ 4.82, d, J 12.3 Hz; OCH_2Ph), ^{13}C NMR spectroscopy (δ 102.4, C-1; δ 138.9, Ar quat), elemental analysis and IR spectroscopy (ν_{max}/cm^{-1} 2086 [N_3]).

4.1.2 Enzymatic Synthesis of Benzyl 6-azido-6-deoxy-LacNAc (19)

Compound **18** was enzymatically attached to galactose, as outlined in Figure 54. The reaction mixture, buffered at pH 7.4, was incubated for 72 hours at 37 °C in a shaker-incubator. Because of the significant difference in commercial cost between the two sugar nucleotides, the donor nucleotide UDP-Gal was formed *in situ* from UDP-Glc by the action of UDP-Gal 1,4-epimerase, as described by Wong.[4] Commercial bovine β-1,4 galactosyltransferase then transferred galactose from UDP-Gal to the benzyl glycoside acceptor (**18**) giving the disaccharide (**19**). Because nuclotide diphosphates have been reported to inhibit GalT,[4] alkaline phosphatase was added to the reaction mixture to cleave the UDP by-product.

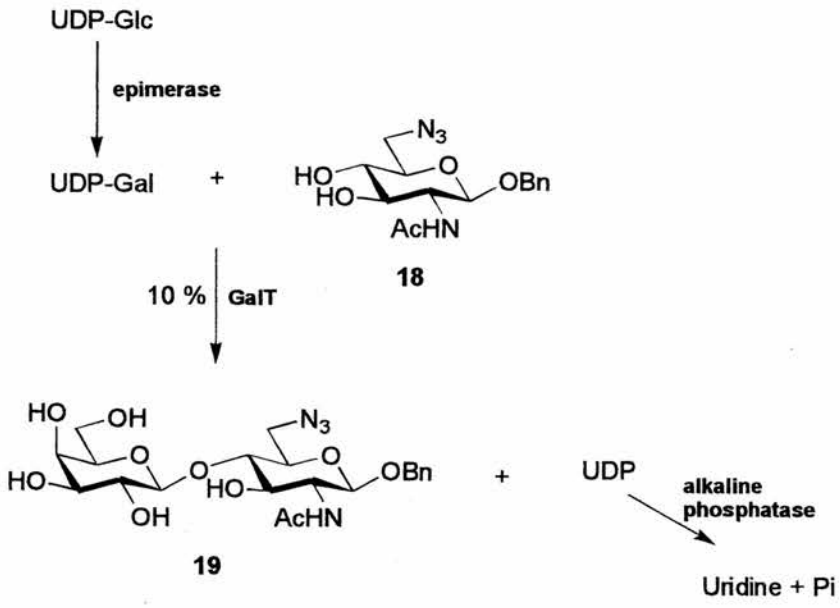


Figure 54. Enzymatic coupling of galactose to benzyl 6-N_3-GlcNAc

Following incubation, the crude reaction mixture was subject to mixed-bed ion-exchange chromatography and then further purified by gel filtration through a Sephadex LH-20 column in 80% aqueous ethanol to give benzylated disaccharide (**19**) in 10 % yield. The structure of the product was then confirmed by ^{1}H NMR spectroscopy (δ 4.46 d, $J_{1,2}$ 8.1 Hz; δ 4.60, d, $J_{1',2'}$ 8.7 Hz), mass spectrometry (ES-MS: $[M + H]^+$ 499) and IR spectrometry (ν_{max}/cm^{-1} 2086 [N_3]).

As with the 3-azido-3-deoxy-galactose-based biotransformations reported in Chapter 2, problems were encountered with the solubility of the azido monosaccharide in aqueous solution necessitating its dissolution in an organic solvent prior to its addition to the enzyme buffer.

A study was undertaken to establish the tolerance of epimerase and GalT to organic solvents. The above reaction (Figure 54) was attempted in buffer containing 10 % of a number of different solvents and the reaction monitored by TLC (Figure 55). The solvents tested were: **1** DMSO; **2** DMF; **3** Acetone; **4** MeOH; **5** THF. Product can be seen to be formed with all of these solvents (along with the concomitant loss of UDP-Gal). **C** designates the control using aqueous buffer only and with no enzymes present. No product is formed here and no UDP-Gal consumed.

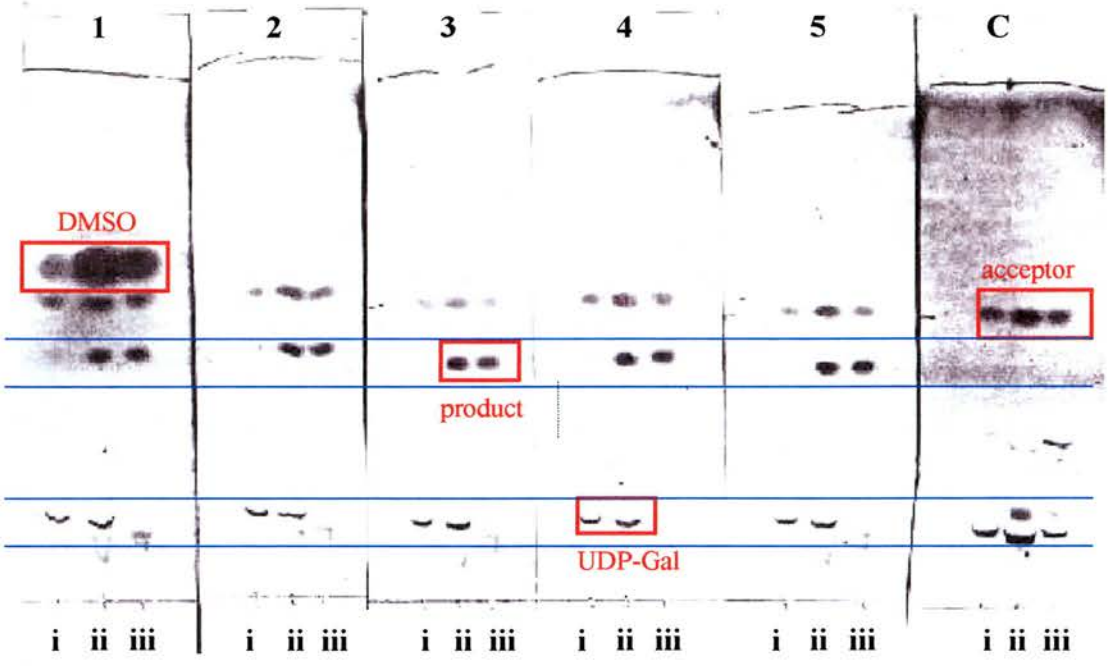


Figure 55. Epimerase and GalT function in different organic solvents

Solvents used: 1 DMSO; 2 DMF; 3 Acetone; 4 MeOH; 5 THF. 6 was the control in absence of Enzyme. Lanes on each plate are: i sample from reaction mixture before incubation; ii co-spot; iii sample from reaction mixture after incubation.

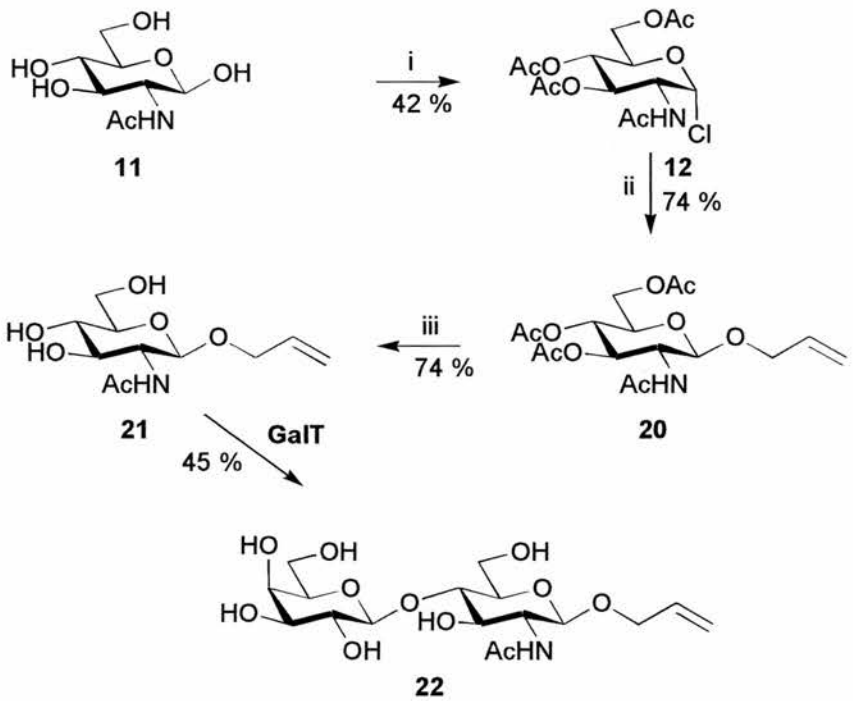
As the reaction appeared to proceed equally well in each of the solvents examined we decided to use methanol for convenience. To dissolve 100 mg of the acceptor azido sugar, 5 ml of methanol was required and therefore a final volume of 50 ml of buffer. Although product was isolated from the reaction, the total yield amounted to only 10 %. It was felt this yield might be improved if solubility could be further increased and hence buffer volumes further reduced. Wong[4] reported the effective use of allyl GlcNAc as an acceptor substrate for bovine β-1,4-GalT so the decision was made to synthesise the 6-azido derivative of allyl GlcNAc to use instead as our acceptor sugar.

4.2 Allyl LacNAc Compounds

Before attempting to synthesise allyl 6-azido-6-deoxy-GlcNAc and enzymatically attach galactose, we decided first to synthesise the underivatised sugar allyl GlcNAc to get a feel for its solubility and suitability for subsequent biotransformation. Should that be successful then no problems would be envisaged with the azido sugar, albeit it being somewhat less polar.

4.2.1 Synthesis of Allyl LacNAc

Allyl LacNAc was prepared by a combination of chemical and enzymatic transformations, as outlined in Figure 56.



Reagents and conditions:

i) Acetyl chloride, o/n, RT ii) $HgBr_2$, $Hg(CN)_2$, AllOH, DCM, $CaSO_4$, 2 d, RT. iii) NaOMe, MeOH.

Figure 56. Chemoenzymatic synthesis of allyl LacNAc

The per-acetylated chloride (**12**) was made from GlcNAc (**11**) as before. Reaction of **12** with allyl alcohol using mercury bromide and mercury cyanide as a promoter[5] was seen to go to completion by T.L.C. After removal of the heavy metal salts by filtration and removal of solvent by evaporation, the resulting solid was redissolved

in ethyl acetate, loaded onto a silica column and eluted with ethyl acetate to give pure per-acetylated allyl GlcNAc (**20**). Deprotection with sodium methoxide in methanol gave allyl GlcNAc (**21**). The structure was confirmed by ^{1}H NMR spectroscopy (δ 4.42, d, $J_{1,2}$ 8.2 Hz; δ 5.82-5.95, m, OCH_2CHCH_2), ^{13}C NMR spectroscopy (δ 135.9 [OCH_2CHCH_2]) and mass spectrometry (CI-MS: $[M+H]^+$ 262).

The enzymatic biotransformation of allyl GlcNAc (**21**) to allyl LacNAc (**22**) was performed on a 150 mg acceptor scale. Solubility of the allyl sugar over the benzyl sugar was found to be much improved, with a total volume of buffer of only 2 ml required for this reaction (Figure 57). Four units of epimerase, 4 units of GalT, 30 units of alkaline phosphatase and 3 equivalents of UDP-Glc were used in the reaction, which was buffered at a pH of 7.4. After incubation at 37 °C for 72 hours T.L.C. showed that the reaction had gone to approximately 70 % completion.

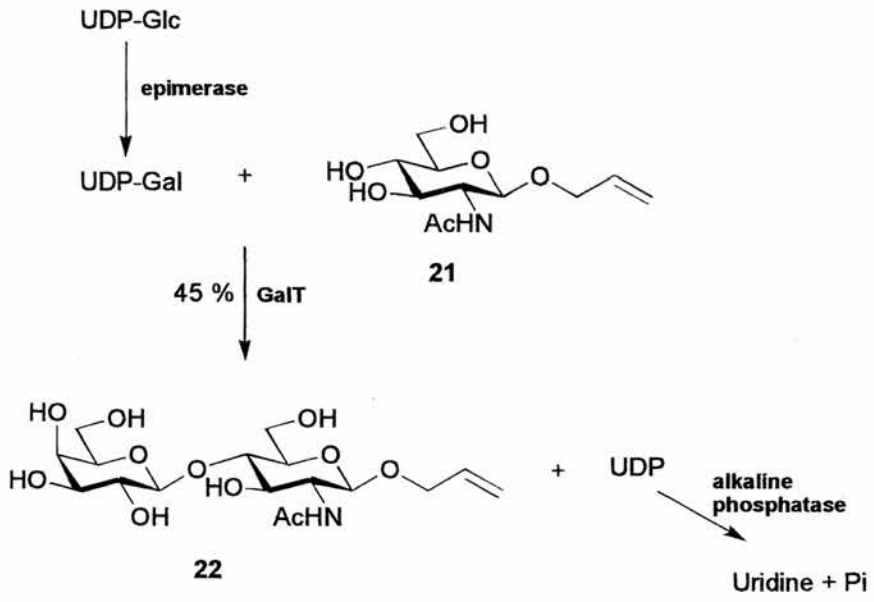


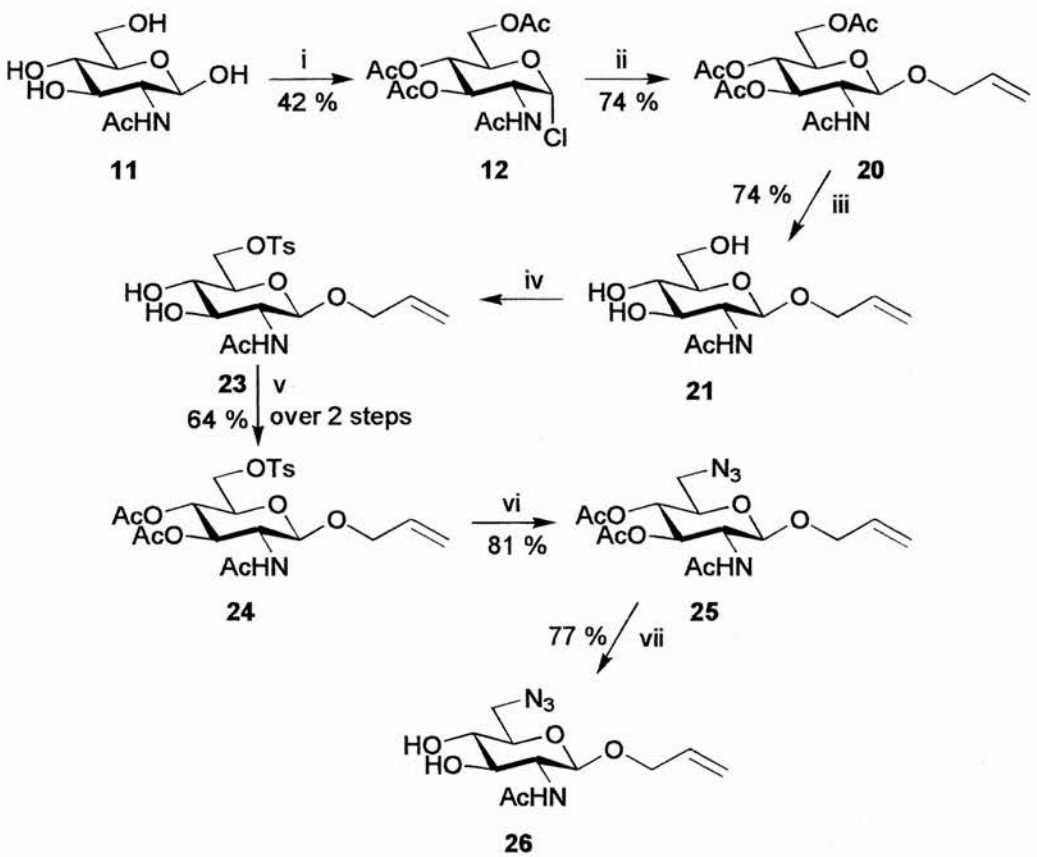
Figure 57. Enzymatic coupling of galactose to allyl GlcNAc

The reaction mixture (total volume 2 ml) was then loaded directly onto a Sephadex LH-20 column in water and the collected fractions (1.2 ml) were checked by TLC. Relevant fractions were pooled and then left to stir overnight at room temperature with added mixed bed ion exchange resin. After this time, T.L.C. showed that all contaminants had been removed. The resin was then removed by filtration and the filtrate lyophilised. This gave allyl LacNAc in 45 % yield. The structure was

confirmed by ^{1}H NMR spectroscopy (δ 4.37, d, $J_{1,2}$ 8.0 Hz; δ 4.48, d, $J_{1',2'}$ 7.4 Hz), ^{13}C NMR spectroscopy (δ100.1 and δ103.0 [C-1 and C-1']) and mass spectrometry (MALDI-TOF $[M+Na]^{+}$ 446).

4.2.2 Synthesis of Allyl 6-azido-6-deoxy LacNAc

Having successfully produced allyl LacNAc by chemoenzymatic synthesis, we then synthesised allyl 6-azido-6-deoxy-GlcNAc, confident that the ensuing enzymatic galactosylation would present no difficulty. The synthetic route to allyl 6-azido-6-deoxy GlcNAc (**26**), outlined in Figure 58, essentially follows the route outlined previously for the corresponding benzyl glycoside.



Reagents and conditions:

i) Acetyl chloride, o/n, RT ii) $HgBr_2$, $Hg(CN)_2$, AllOH, DCM, $CaSO_4$, 2 d, RT. iii) NaOMe, MeOH; iv) TsCl, pyridine, -35 °C → RT, 8 hr; v) Ac_2O, o/n; vi) NaN_3, DMF, reflux 60-70 °C, o/n; vii) NaOMe, MeOH.

Figure 58. Synthesis of allyl 6-N_3-6-deoxy GlcNAc

The selective mono-tosylation of allyl GlcNAc (**21**) to give **23** was by the same procedure as used in the synthesis of benzyl 6-azido-6-deoxy GlcNAc (Figure 44), again the reaction going to approximately 75 % completion. Dry methanol was then added to the reaction mixture on ice to quench excess tosyl chloride, and the mixture was allowed to warm to room temperature with stirring. The methanol was then removed under vacuum and the pyridine was co-evaporated with toluene. The resulting brown solid was stored under vacuum prior to acetylation (pyridine/acetic anhydride) to produce **24**.

Tosylate (**24**) in DMF was heated with sodium azide at a temperature of between 60 and 70 °C. This temperature was a lot lower than that used in previous attempts at this reaction due to problems with degradation of the compound. This lower temperature proved to be adequate as after 2.5 hours the reaction had proceeded approximately 85 % as judged by TLC Following continuation overnight, TLC showed only a trace of UV- active starting material left, the reaction having virtually gone to completion. After work up crystals were obtained which were dissolved in ethyl acetate and treated with activated charcoal to decolourise. Removal of charcoal and solvent gave pure sugar (**25**) in 81 % yield. Deprotection gave the free azido sugar (**26**) in 77 % yield, the structure of which was confirmed by ^{1}H spectroscopy (δ 4.48, d, $J_{1,2}$ 8.5 Hz; δ 5.82-5.95, m, OCH_2CHCH_2), ^{13}C NMR spectroscopy (δ 101.9 [C-1]; δ 135.7 [OCH_2CHCH_2]) and mass spectrometry (CI-MS $[M+H]^+$ 287). Compound (**26**) was then presented as an acceptor for enzymatic galactosylation by β 1,4-galactosyltransferase (Figure 59).

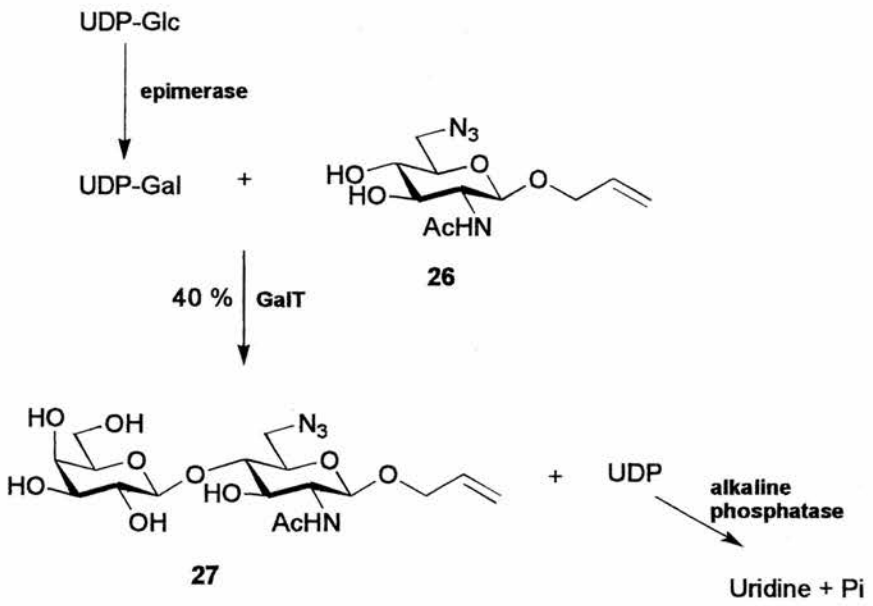


Figure 59. Enzymatic coupling of galactose to allyl 6-N_3- GlcNAc

The 70 mg scale reaction was incubated under nitrogen over the weekend at 37 °C in a total volume of 1.5 ml of buffer (pH 7.4). A total of 5.4 U of epimerase, 4 U of GalT, 30 U of alkaline phosphatase and 3 equivalents of UDP-Glc were used in the reaction. After 6 hours the reaction had proceeded by 50 % as judged by TLC Following the weekend of incubation the reaction appeared to have gone to completion. Treatment with mixed bed resin was followed by purification through a Sephadex LH20 column. Lyophilisation of pooled fractions gave pure disaccharide (**27**) in 40 % yield. This was confirmed by ^{1}H NMR (δ 4.36 d, $J_{1,2}$ 7.7 Hz; δ 4.53, d, $J_{1',2'}$ 8.0 Hz), ^{13}C NMR (δ 100.0 and 101.1 [C-1 and C-1']) and mass spectrometry (MALDI-TOF-MS: $[M+Na]^+$ 471).

Comparison of the allyl LacNAc (**22**) and allyl 6-N_3-LacNAc (**27**) ^{1}H NMR spectra reveals a characteristic change of 2 double-doublets in the latter spectrum. These were concluded to be the two H-6 protons, visible in the allyl LacNAc spectrum, but which have been shielded by the azido group and consequently shifted up field and are hidden amongst other signals in **27**. These two signals have therefore been thus assigned H-6_a and H-6_b (δ 3.73 [1H, dd, $J_{6a,6b}$, 12.1, H-6_a]; δ 3.89 [1H, dd, $J_{6a,6b}$, 12.1, H-6_b]) in allyl LacNAc (**22**).

The double doublet at δ 3.43 has been assigned H-2 based on the coupling constant $J_{1,2}$ = 8.0 Hz. Furthermore, H-2 is attached to an N-linked carbon and would therefore be expected to be shifted further up-field than H-2'.

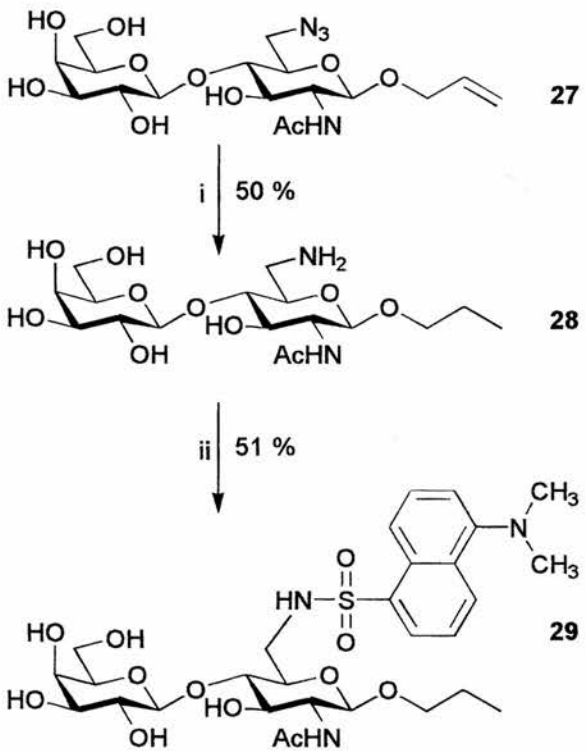
4.3 Azido-LacNAc Derivatives

4.3.1 Photoprobe

Having synthesised an anomerically-derived photoprobe (**10**) we were now in a position to synthesise a C-6 derived photoprobe from allyl 6-azido-6-deoxy LacNAc (**27**). This presents the potential of being able to label a second distant area within the active site of the enzyme of interest and hence lead to a more full understanding of its mechanism of action.

4.3.1.1 Synthesis of Fluorescent Substitute Probe

To test the feasibility of attaching a probe to the C-6 of the LacNAc ring in terms of possible stearic hindrance, we first tried coupling to dansyl chloride as a substitute fluorescent probe as outlined in Figure 60.



Reagents and conditions:

i)$Pd(OH)_2/C$, EtOH, H_2O, 2 hr, RT; ii)Dansyl chloride, I hr, RT

Figure 60. Synthesis of 6-dansylamino-6-deoxy LacNAc derivative 29

This involved having to reduce the azide functionality to a free amine for this coupling which was achieved by dissolving the azido sugar (**27**) in a mixture of

ethanol and water and hydrogenated by stirring under H_2 in the presence of palladium hydroxide catalyst. After 3 hours TLC showed this reaction to have gone to completion. Reduction of the allyl chain was also expected to have occurred and this was subsequently confirmed by ^{1}H NMR. As the allyl chain was originally incorporated into the molecule to improve solubility over a benzyl substituent, its reduction to a propyl chain was seen as being inconsequential. The catalyst was removed by filtration through Celite which was then washed thoroughly with methanol. After removal of the methanol under vacuum, the amine was then dissolved in methanol containing dansyl chloride and the reaction mixture made basic by the dropwise addition of triethylamine. After leaving the reaction to stir overnight in the dark, TLC showed the reaction to be complete. Purification of the sugar was by preparative TLC run in $CHCl_3$:MeOH: H_2O, 10:10:3. The dansylated sugar was identified by fluoresence under long wave UV, marked with pencil and scraped off the glass backing plate. The sugar was then extracted into MeOH which after removal by evaporation then gave the dansylated disaccharide (**29**) in 30 % yield, the structure of which was confirmed by ^{1}H NMR (δ 4.10, d, $J_{1,2}$ 8.2 Hz; δ 4.24, d, $J_{1',2'}$ 7.7 Hz).

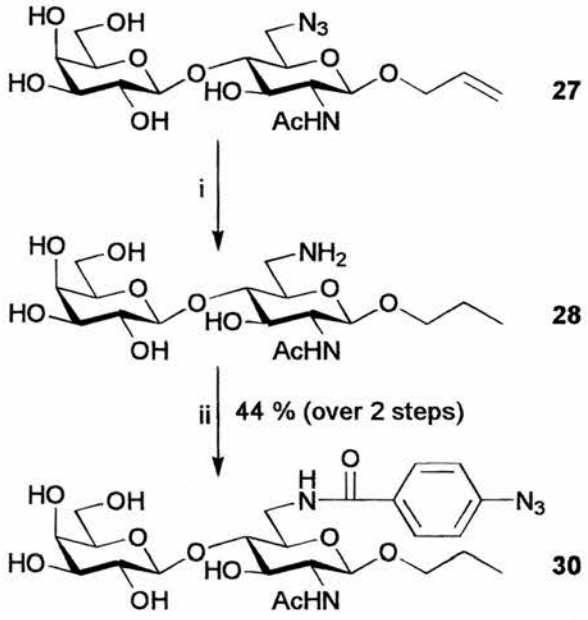
To establish recognition of a C-6 substituted analogue by FucT VI, the dansylated sugar (**29**) was used as the acceptor substrate in an assay of fucosyltransferase activity (at a final concentration of 2 mM). The result tabulated below indeed demonstrates fucosylation of this compound.

Table 10. Turnover of dansylated substrate by FucT VI

Fraction	CPM	% Turnover
Blank	2,011	
Dansyl sugar (29)	8,296	39
Total Counts	39,702	

4.3.1.2 Synthesis of Non-Biotinylated Probe

Having shown that **29** is recognised as an acceptor substrate by FucT VI, we then proceeded with synthesising a C-6 derived photo-active probe. We initially synthesised a non-biotinylated probe using the Sulfo-HSAB reagent used previously in the synthesis of (**9**) (Figure 61). This would allow us to carry out cross-linking studies before considering use of the biotinylated probe.



Reagents and conditions:

i) $Pd(OH)_2/C$, EtOH, H_2O, 2 hr, RT; ii) Probe/DMF

Figure 61. Synthesis of C-6 derived photoprobe

The amine (**28**) was produced as above but redissolved in DMF and Sulfo-HSAB added (in DMF). Stirring at room temperature resulted in the reaction going to completion as indicated by TLC. The reaction mixture was then streaked on a TLC plate and, after allowing the DMF to dry off, run in DCM/MeOH/H_2O, 8:2:0.1. The relevant band of compound was identified under UV illumination, marked with pencil and scraped off the glass plate. Methanol was used to extract the compound from the silica. Evaporation of the methanol then gave the photoprobe (**30**) in 44 % yield, the structure of which was confirmed by ^1H NMR (δ 4.39, d, $J_{1,2}$ 8.0 Hz; δ 4.41, d, $J_{1',2'}$ 7.8 Hz). Comparison of this spectrum with that of allyl-6-azido-LacNAc (**27**) demonstrates the reduction of the allyl chain. This is evident from the

disappearance of the multiplet at δ 5.74 - 5.90 (the allylic C*H*), and the triplet at δ 5.14 - 5.26 (the terminal CH_2 of the allylic group) and the appearance of the multiplet due to $OCH_2CH_2CH_3$ at δ 1.32 - 1.41 and the appearance of the triplet at δ 0.66 due to the methyl group of the propyl chain.

Substrate recognition of the photoprobe was confirmed by assay. The probe was tested at two concentrations, 2 mM and 12.5 mM (Table 11). The turnover at both concentrations of probe results in a similar degree of turnover as that seen with the control (containing 10 mM LacNAc as the acceptor). The enzyme would appear to be saturated therefore at a 2mM acceptor substrate concentration.

Table 11. Turnover of Photoprobe (30) by FucT VI

Fraction	CPM	% Turnover
Blank	2623	
Control	10,428	44
Probe (2 mM)	10,321	44
Probe (12.5 mM)	10,177	43
Total Counts	40,296	

4.3.1.2.1 Cross-Linking of FuvT VI with Photo-probe

Probe (**30**) was added to the protein solution at a final concentration of 2 mM and the mixture vortexed and incubated at 37 °C in a water bath for 10 minutes to ensure interaction of enzyme and probe. The sample was then irradiated under short wave UV for 15 minutes to activate the photo-labile probe azide, and the protein assayed for activity. The results tabulated below (Table 12), clearly demonstrate effective inhibition of activity and hence cross-linking of probe and enzyme. The positive control, with water replacing probe, was incubated and irradiated along with the sample containing the probe.

Table 12. Inhibition of FucT VI by Photoprobe

Fraction	CPM	% Turnover
Blank	2,307	
Positive Control	10,754	28
Labelled Protein	3,011	6
Total Counts	69,740	

4.4 Photoaffinity Labelling of *Trans*-sialidase

4.4.1 Introduction

Chagas disease (American trypanosomiasis) is a debilitating and often fatal condition caused by the flagellate protozoan *Trypanasoma cruzi,* discovered by Carlos Chagas in 1909. This parasite is transmitted to human and other mammalian hosts by blood sucking Reduviid bugs such as *Triatoma infestans* and *Rhodnius prolixus.*[6] During a blood meal these insect vectors defecate thus releasing infective stages of the parasite near the bite wound. Scratching of the bite wound then results in the infective forms reaching the blood stream. The multistage life cycle of the parasite, which exists in three morphological forms, is depicted in Figure 62.

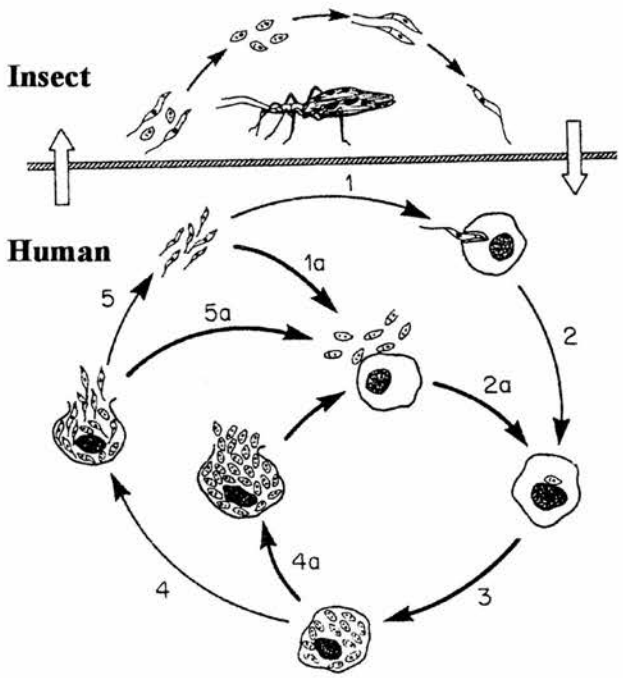


Figure 62. Life cycle of *Trypanosoma cruzi.*

The upper part of the diagram depicts the ingestion of the trypomastigote by the insect vector and its subsequent differentiation through the epimastigote form to the infective metacyclic trypomastigote. Arrows 1 to 5 illustrate invasion of the human host cell by the trypomastigote (1), its transformation into the amastigote form (2), multiplication in the cytoplasm (3), rupture of host cell and differentiation back into trypomastigotes (4) and release of trypomastigotes into the blood stream (5). A subcycle can also occur when amastigotes, derived from either premature rupture of host cells (4a, 5a) or through extracellular differentiation of trypomastigotes (1a), are ingested by macrophages, where they can survive and complete the intracellular cycle. Taken from ref 7.

Trypomastigotes are the non-dividing forms of the parasite which exist in the blood stream of the mammalian host where they can infect new host cells or be ingested by the insect vectors. In the insect's midgut lumen, the trypomastigotes differentiate to epimastigotes, which are the forms that multiply in invertebrates. In the insect rectum, the epimastigotes then differentiate again into the non-dividing metacyclic trypomastigotes which are the infective forms released in the excreta while the insect feeds. Once in the blood stream of the vertebrate host, the metacyclic trypomastigotes invade cells through the formation of a membrane-bound vacuole, thus initiating the intracellular cycle, which lasts an average of 4-5 days. Disruption of the vacuolar membrane is the first step of this cycle, occuring within 1-2 hours after invasion. This results in the parasites reaching the cytoplasm whereupon they differentiate into amastigotes, the intracellular replicative stages. After a lag period of approximately 20 hours, the amastigotes start dividing by binary fission. With a doubling time of around 12 hours, this means that approximately 500 parasites are generated from each one originally invading the cell by the end of the intacellular cycle. At this point, the amastigotes differentiate into trypomastigotes and the host cell ruptures, releasing the parasites into the blood stream. They can now re-invade other host cells or be ingested by an insect vector to complete the life cycle.[7]

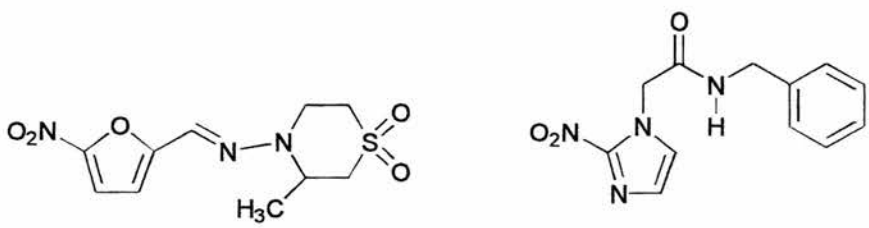
According to the World Health Organisation, between 16 and 18 million people are estimated to be infected with *T. cruzi* in Central and South America,[8] with 50,000 deaths annually and 100,000 new cases being observed every year. It is estimated 100 million people are at risk.[6] There is also some concern that the disease may become established in the United States where between 50,000 and 100,000 infected persons, mainly immigrants from Central America, may now be living.[9]

Chagas disease is a complex process with a poorly understood pathophysiology. In its acute phase, which is characterised by active infection, the disease is seldom fatal and spontaneously resolves within 3-4 months in the majority of cases. The majority of infected persons remain asymptomatic. An inflammatory lesion, known as a chagoma may develop at the site of the infection. Swelling of the eye, known as Romana's sign is a common symptom. Other acute clinical symptoms may include fever, malaise, lymphadenopathy, hepatosplenomegaly, vomiting and diarrhoea. In a

small minority of infected individuals, however, an acute myocarditis, sometimes resulting in death may occur.[10,11]

In the chronic form the patient may not develop any symptoms for many years with the parasite remaining dormant. However, up to 30 % of infected individuals will, many years after acquiring the infection, become ill. In most cases the heart is affected resulting in death from dysrhythmias or congestive heart failure. The gut may also be affected leading to fatal megaaoesaphagus or megacolon. Very little is known about the mechanisms underlying this pathology, although autoimmune processes resulting in tissue destruction by the patient's lymphocytes have been implicated.[9,10,11]

At present there is no effective treatment for Chagas disease and the absence of new drugs makes the search for active chemotherapeutic agents an urgent priority for treating this illness.[6] Current trypanocidal compounds in use produce toxic side effects. These are the nitro-heterocycles Nifurtimox and Benznidazole[6,9] (Figure 63).



Nifurtimox Benznidazole

Figure 63. Structure of Nifurtimox and Benznidazole

Both of these drugs can only be used in the early stages of the disease (which is often undetectable anyway), being practically useless in the advanced stages.[9] They are thought to work by interfering with the trypanosomes redox balance, to which they are known to be very sensitive.[6]

Trypanosomes, like their mammalian hosts, use reduced glutathione (GSH) as their first line of defence against peroxidative damage.[12] Unlike mammals, however, they lack the enzyme glutathione reductase (GR), instead keeping their GSH in the reduced form by non-enzymatic reaction with the reduced thiol compound

trypanothione ($T[SH]_2$) (18-bis-glutathionylspermidine). This in turn is kept in its reduced form by trypanothione reductase (TR), an enzyme specific to trypanosomes[12] (Figure 64).

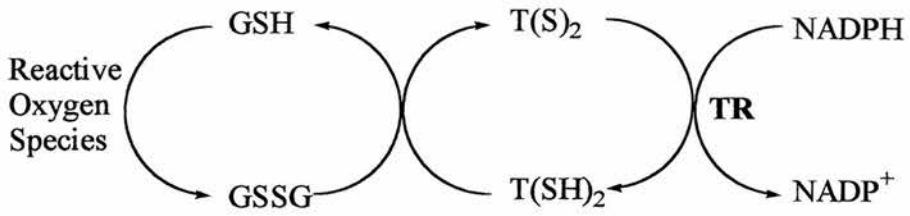


Figure 64. Reaction catalysed by trypanothione reductase

Trypanosomal TR is unable to process GSSG and conversely the mammalian GR cannot reduce $T(S)_2$ thus providing an exquisite biochemical difference between host and parasite for possible chemotherapeutic exploitation. The trypanocidal properties of Nifurtimox and Benznidazol are due to their ability to inhibit the parasites TR. However, Nifurtimox appears to be a better inhibitor of GR than TR thus perhaps explaining its almost intolerable side effects.[13] Furthermore it also behaves as a turncoat inhibitor by promoting the formation of radical oxygen species by GR associated NADPH oxidase.[14]

Another current drug employed in Chagas disease chemotherapy is gentian violet, a cationic triphenylmethane drug (Figure 65). This is used in blood banks in some endemic regions to try and eradicate blood-stream transmission of Chagas' disease.[12]

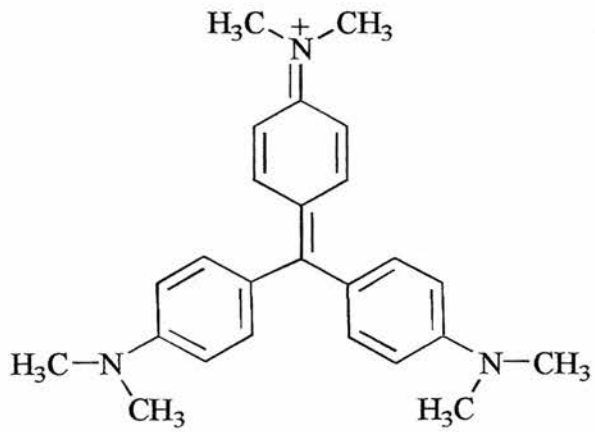


Figure 65. Gentian violet used to cleanse blood banks of *T. cruzi*.

Although this compound is able to kill trypomastigotes (primarily due to mitochondrial damage), its use has several disadvantages in that it gives blood an unacceptable deep violet colouring, it stains patients' tissues and has some safety impications such as potential mutagenicity, carcinogenicity and toxic effects on erythrocytes.[6,8]

4.4.2 Sialic Acid

It has become increasingly evident in recent years that sialic acid (neuraminic acid, Nea5Ac) is of great physiological significance.[15] Indeed, the biological importance of the sialylated ligand sialyl Lewis x is discussed in the introduction to this thesis. Moreover, the clinical significance of sialoconjugates and associated neuraminidases, with respect to pathological infection, is stimulating much interest in this sugar. The role of neuraminidase in viral infection is well documented.[16] In September 1999 (in the UK), Glaxo-Wellcome launched the neuraminidase inhibitor Relenza (Zanamavir) as an anti-influenza drug. The correlation between neuraminidase production and the pathogenicity of enterobacteria is also being established.[17] This corrlation also pertains to *Helicobacter pylori* infection which is strongly associated with duodenal ulcer, duodenitis and antral gastritis.[17]

4.4.2.1 *T. cruzi* and Trans-sialidase

The pathogenicity of *T. cruzi* is also dependent on sialic acid. Before invading, the parasite must first be able to adhere to the surface of the mammalian host cell and to do this it requires to have a negatively charged glycopeptide coat. This is attained by scavenging (negatively charged) sialic acid from host cell glycoconjugates, since the parasite does not produce sialic acid itself, and transferring them onto oligosaccharides on the parasite cell surface. The sialic acid is linked α-2,3 to a terminal galactose of either N- or O-linked oligosaccharides. This process of trans-glycosylation is achieved enzymatically by a cell surface *trans*-sialidase.[18] Activity of this enzyme can be correlated with the life-cycle stages of *T. cruzi*, being greatest in the trypomastigate (infectious) form but completely absent in the non-infectious amastigote.[19] As this enzyme differs from mammalian sialyltransferases, which use CMP-sialic acid as the sugar donor,[20] it represents an alternative target for therapeutic intervention.

4.4.2.2 Acceptor Specificity of *Trans*-sialidase

A study on the substrate specificity of *trans*-sialidase by Jennifer Harrison[21] using a library of Galβ(1,4)GlcNAcβ-O-Octyl analogues was carried out. These analogues were modified at the C-2', 3', 4', 6' and 3 and 6 positions. Four modifications were made at each position, namely the hydroxyl group being replaced with an amine, carboxylic acid, amide or guanidino group. This compound library is illustrated in Figure 66.

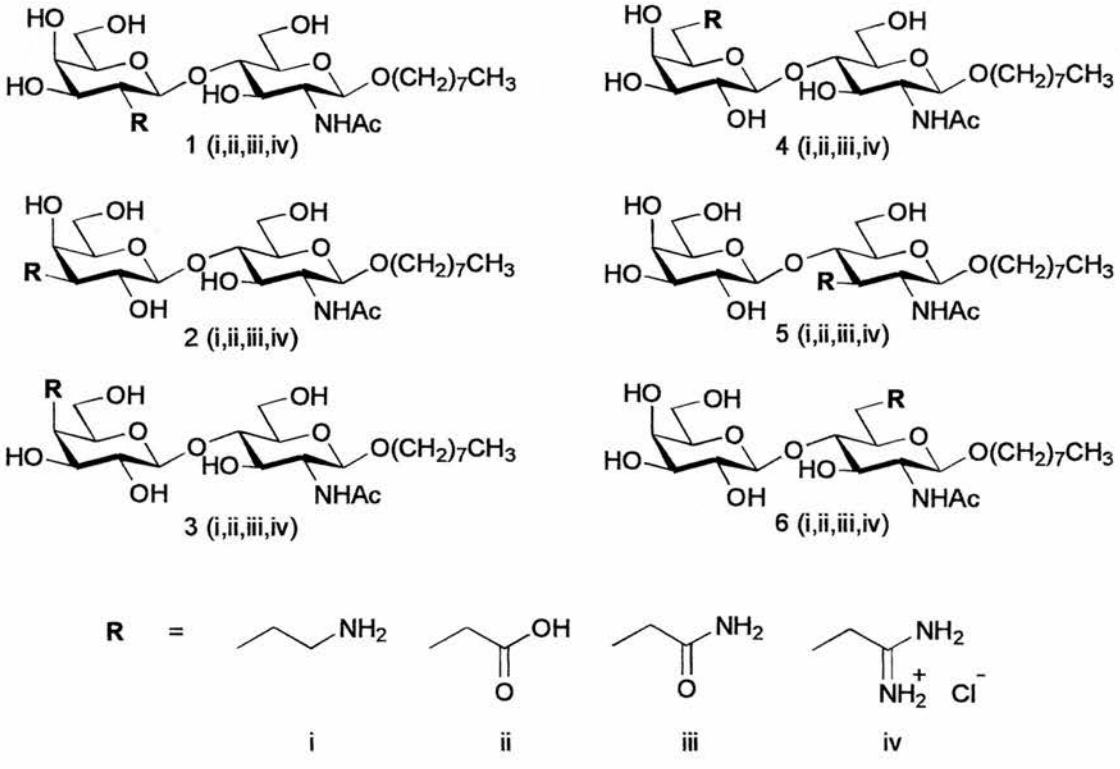


Figure 66. Galβ(1,4)GlcNAcβ-O-Octyl analogue library

The above 24 compounds were assayed as acceptor substrates for *trans*-sialidase. The results of these assays showed that the type of modification in a given position (ie. i, ii, iii or iv) made little or no difference to how the substrate was tolerated but that the position of modification was the important factor. Compound 2 derivatives were non-sialylatable as would be expected as C-3' is the position of attachment of sialic acid and compound 1 and 3 derivatives (modifications at C-2' and C-4') demonstrated only minimal turnover. However, modifications at the other 3 positions ie C-6', C-3 and C-6 (derivatives of compounds 4, 5 and 6 respectively) made no significant difference to the turnover of the substrate as compared to the unmodified

compound (where **R**=OH). The hydroxyl groups therefore at positions 2', 3' and 4' of Galβ(1,4)GlcNAcβ-O-Octyl are required for attachment of sialic acid by *trans*-sialidase whereas the hydroxyl groups at the 6' position and the 3 and 6 positions play little or no role in the binding of the enzyme and subsequent sialylation. The results of this study are summarised in Figure 67.

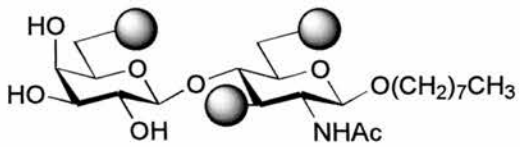


Figure 67. Derivatisable hydroxyl groups in *trans*-sialidase substrate

4.4.3 Photolabelling of *Trans*-sialidase

Whilst a number of sialidases have been crystallised and their structures elucidated by X-Ray diffraction, no such information exists for *trans*-sialidase. Photoaffinity labelling represents an alternative technique for gaining structural information on enzyme active sites. We set out to perform some preliminary photolabelling studies on *trans*-sialidase. As this enzyme recognises the same minimum acceptor substrate as FucT ie LacNAc derivatives, the possibility exists that we can use the same probes to label both enzymes. Using the information in the above section (4.4.2.2) on the substrate specificity of *trans*-sialidase, we know this enzyme will tolerate substitutions at the C-6 position. We therefore decided to use the previously made photoprobe **30** (Figure 68) in initial substrate and inhibition studies.

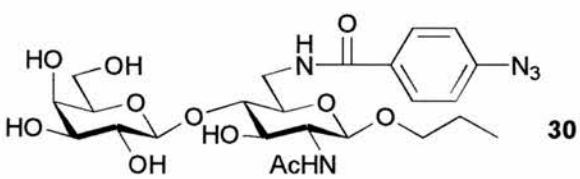


Figure 68. Possibe *trans*-sialidase photoprobe.

4.4.3.1 Production of *Trans*-sialidase

A recombinant *trans*-sialidase clone was grown up in culture and the protein over-expressed. The basis of the subsequent purification is the 'His-tag' on the protein. This is a histidine tag of 6 residues at the C-terminus of the protein. The high affinity

of histidine for nickel cations (Ni^{2+}) enables an efficient purification on a nickel resin (NiNTA) column. The chelated protein can then be eluted with imidazole.

4.4.3.2 Purification of *Trans*-sialidase

The bacterial clone is positive for ampicillin resistance and was therefore cultured on an agar plate containing ampicillin. A single colony of the clone was picked and grown up in a 10 ml culture which in turn was used to innoculate a 1 litre culture. At the appropriate cell density, *trans*-sialidase expression was induced by the addition of IPTG. The cells were harvested by centrifugation and then lysed by sonication. After removal of the cell debris by centrifugation, the supernatant was passed through a syringe filter before purification on the nickel resin column. Collected fractions were pooled and dialysed against buffer containing glycerol.

4.4.3.3 Protein Concentration

This was determined to be 0.3 mg/ml.

4.4.3.4 SDS-PAGE

The purified protein was analysed by SDS polyacrylamide gel electrophorsis (Figure 69). *Trans*-sialidase can be seen as the major band with an apparent molecular weight of approximately 80 kDa.

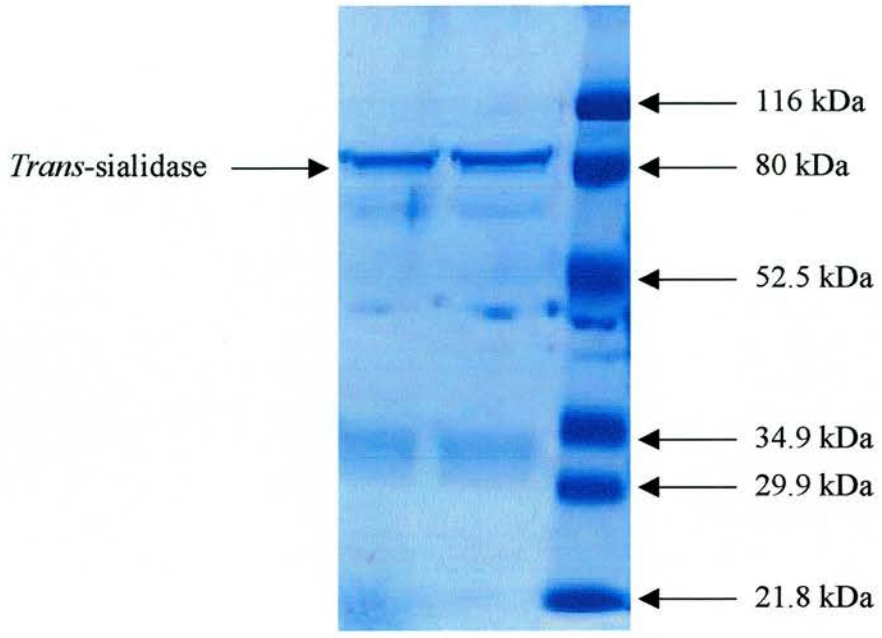


Figure 69. SDS-PAGE gel of purified *trans*-sialidase

Land A and B - protein sample, Lane C - MWt markers

4.4.3.5 *Trans*-sialidase Assay

This radiochemical assay measures the transfer of sialic acid, by *trans*-sialidase, from sialyl lactose to [^{14}C] lactose. This is represented in Figure 70.

Figure 70. Basis of *trans*-sialidase assay.

* denotes radio-label

The charged material (containing sialic acid) can be separated from uncharged material by anion exchange chromatography, using a column of Sephadex AG-25 resin. After washing with water, the charged material is then eluted from the resin using NH_4OAc. The wash and eluant are mixed with scintillation fluid and counted in a scintillation counter. Higher counts in the eluant corresponds to greater enzyme activity.

The assay was later modified so it could be performed in one pot for convenience ('quick'assay). Rather than using a column on which to carry out the anion exchange step, anionic exchange resin was added directly to the reaction mixture, vortexed and then sedimented by microfuging. The supernatant was then counted, lower counts corresponding to greater substrate turnover.

4.4.3.6 Cross-linking of *Trans*-sialidase with Photoaffinity Label

Again, before attempting to cross-link probe and enzyme we first ascertained whether the probe was an acceptor substrate for *trans*-sialidase. This was achieved by adding the probe to the *trans*-sialidase assay mixture (where it replaced water) as a potential competitive substrate to the radio-labelled lactose. Should **30** behave as an acceptor substrate, the corresponding reduction in sialylation of [^{14}C] lactose would be reflected by an increase in CPM in the supernatant (in 'quick' assay) as compared to the control (no competitive substrate). This indeed was shown to be the case, with the probe added at a final concentration of 5 mM. This result detailed in Table 13 shows a marked decrease in sialylation of *Lactose from 29 % turnover to 14 % turnover in the presence of the probe. The blank contains no enzyme giving maximal CPM in the supernatant.

Table 13. Recognition of Probe by *Trans*-sialidase

Fraction	CPM	% Turnover of *Lactose
Blank	8,561	
Control	6,040	29
Probe	7,338	14
Total Counts	26,820	

Cross-linking of *trans*-sialidase was attempted by incubating the enzyme solution with compound **30** at 37 °C before performing photolysis through UV irradiation. The protein was then subsequently assayed for *trans*-sialidase activity. However, no reduction in activity was observed. The immediate conclusion drawn from this was that the active site does not extend to the C-6 position on the molecule and therefore the probe is unable to interact with it.

4.5 MATERIALS AND METHODS

4.5.1 Fucosyltransferase Assay

This assay was modified from that described previously in section 3.4.5 to accommodate counting in a 96 well microplate (TopSeal, Packard). Quenching of the reaction was with 200 µl of Dowex AG 1-X8 anion exchange resin (1:2 v/v in water). After vortexing and microfuging, 75 µl of supernatant was mixed with 225 µl of scintillant (Microscint 20, Packard) and counted for 1 minute (TopCount NXT, Packard).

4.5.2 *Trans*-sialidase Clone

The *E. coli* (XL1-Blue) expressing recombinant *trans*-sialidase was obtained from Sergio Schenkman's laboratory in the Department of Cell Biology, Escola Paulista de Medicina, Sao Paulo, Brazil.

4.5.3 Cell Culture

A glycerol stock of the *trans*-sialidase clone was streaked out on an agar plate containing 100 µg/ml ampicillin and left overnight at 37 °C. A single colony was then picked and transferred to 10 ml of sterile LB Miller broth containing 100 µg/ml ampicillin and incubated in a shaker-incubator overnight (37 °C, 200 rpm). The culture was then transferred into 1 litre of sterile LB Miller broth (containing 100 µg/ml ampicillin) and incubated with agitation (37 °C, 200 rpm) until the optical density (OD_{600}) reached between 0.6 and 0.8. IPTG was then added to a final concentration of 1 mM to induce protein expression, and the culture incubated for a further 6 hours (30 °C, 200 rpm). At this point the cells were harvested by centrifugation (Beckman, 8,000 g, 15 mins, 4 °C).

The cell pellet was re-suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8.5) and sonicated (5 x 30 second bursts with 1 min cooling in between). The crude lysate was then centrifuged (12,000 g, 20 min, 4 °C) and the supernatant decanted before filtering through a 0.4 µm sterile syringe filter. The filtrate was loaded onto a NiNTA column which was pre-equilibrated with lysis buffer and pumped through at 2 ml/min. Eluent was monitored using a Biocad HPLC UV detector at 280 nm. The column was washed with wash buffer (50 mM

NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, pH 8.5) at 2 ml/min until the UV absorbance fell below 0.01. Elution of protein was then with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, pH 8.5) at the same flow rate. Elution was continued until the UV absorbance fell below 0.01. The fractions were pooled and dialysed against dialysis buffer (50 mM TRIS, 2mM DTT, 10 % glycerol, pH 8.5) and stored at -20 °C.

4.5.4 Bradford Assay

Protein concentration was determined using an adaption of the Bradford method,[24] using commercial Bradford reagent (Sigma). To 500 µl of dye was added 499 µl of water and 1µl of protein sample. Absorbance was measured at 595 nm and protein concentration determined from a BSA standard curve.

4.5.5 SDS-PAGE

The dialysed protein was analysed by SDS polyacrylamide gel electrophoresis. The *trans*-sialidase sample (6 µg) was solubilised in Laemmli buffer (15 µl, Sigma) and heated at 100 °C in a hot block for 10 minutes before loading onto the gel (10 %). Pre-stained molecular weight markers (Bio-Rad) used were in the range of 21.8-116 kDa. The gel was run under 150 V for 50 minutes in 1 x running buffer (5 x: 25 mM Tris-Base pH 8.3, 195 mM glycine, 0.1 % w/v SDS). Staining with Coomasie blue (0.5 g Coomassie Brilliant Blue in 500 ml destain solution) was achieved in 15 minutes in a water bath (60 °C). Destaining in destain solution (water:methanol:acetic acid, 6:3:1) was also performed in a water bath (60 °C, 1 hour) with a sponge.

4.5.6 *Trans*-sialidase Assay

(i) 'Standard' assay

This assay measured the sialylation of [^{14}C] lactose, which was separated from the assay mixture by passing through a column of QAE Sephadex A-25 (0.5 ml, Sigma). The column was washed with 1 ml water to remove all uncharged material. Sialylated material was then eluted from the column by flushing with NH_4OAc (1 M, 2 ml). Radioactivity was measured in the wash and eluant by taking 75 µl of sample and mixing with 225 µl of scintillation fluid (Microscint 20, Packard) in a microplate

(TopSeal, 96-Well, Packard). Each sample was counted for 5 minutes (TopCount NXT, Packard). The 50 µl reaction mixture contained 10 µl each of buffer (100 mM HEPES, pH 7.5), sialyl lactose (1 mM), [^{14}C] lactose (0.02 µCi/10 µl, approx. 30,000 CPM), *trans*-sialidase solution and H_2O. The molarities quoted are the final concentrations in the assay mixture. H_2O was replaced by probe (**30**, 5 mM) when assaying for substrate recognition. The reaction mixture was incubated for 1 hour in a water bath at 37 °C after which time it was terminated by quenching with the addition of water (1 ml).

(ii) 'Quick' Assay

Latter assays were performed in one pot. The reaction mixture was incubated as above and then quenched with a 200 µl suspension of Dowex AG 1-X8 anion exchange resin (1:2 v/v in water). After vortexing briefly and microfuging, 75 µl of the supernatant was transferred to the 96 well microplate, mixed with scintillant and counted as above.

Enzyme activity is expressed as a percentage turnover of substrate, calculated from the proportion of counts in the eluent/ supernatant, to total counts in the assay.

4.5.7 Attempted Cross-Linking of *Trans*-sialidase

The photoprobe (**30**) was added to *trans*-sialidase enzyme solution at a concentration of 5 mM and allowed to incubate in a water bath in the dark at 37 °C for 30 minutes. The mixture was then irradiated under a short wave UV lamp at a distance of 5 cm for 15 minutes. The protein was then assayed as detailed above in section 4.5.4.

4.6 FUTURE WORK

4.6.1 FucT VI Inhibitors

We hope to develop inhibitors of FucT VI based on those already developed by Hindsgaul[25] for GlcNAcT V, an important enzyme controlling the branching pattern of *N*-linked oligosaccharides. These are illustrated in Figure 71.

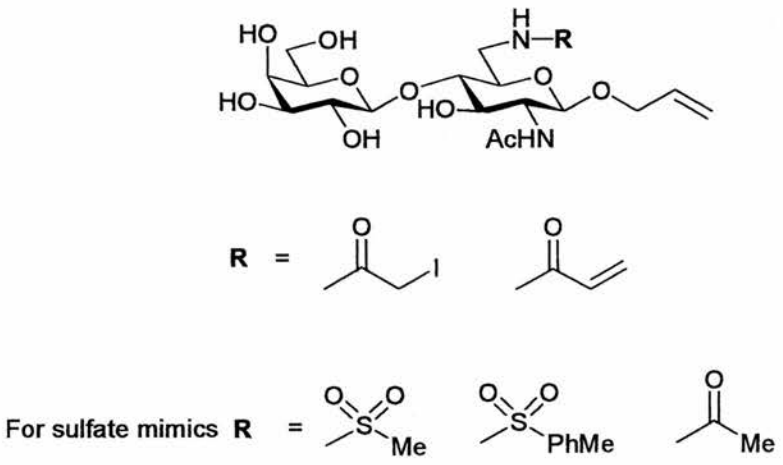


Figure 71. Potential FucT VI mimics

4.6.2 Photoaffinity Labelling

4.6.2.1 FucT VI

Having obtained positive cross-linking results with the probe (**30**), we know the enzyme will tolerate bulky substituents at the C-6 position of the LacNAc analogue and that the active site interacts at this position, hence enabling alkylation by **30**. This therefore invites the possibility of labelling FuvT VI with a C-6 derived biotinylated probe (Figure 72) which would allow subsequent isolation of the labelled peptide. This would complement information obtained from labelling the active site using the anomeric biotinylated probe (**10**). We therefore foresee the possibility of gaining sequence information on two distinct (or possibly over-lapping) areas of the FucT VI active site.

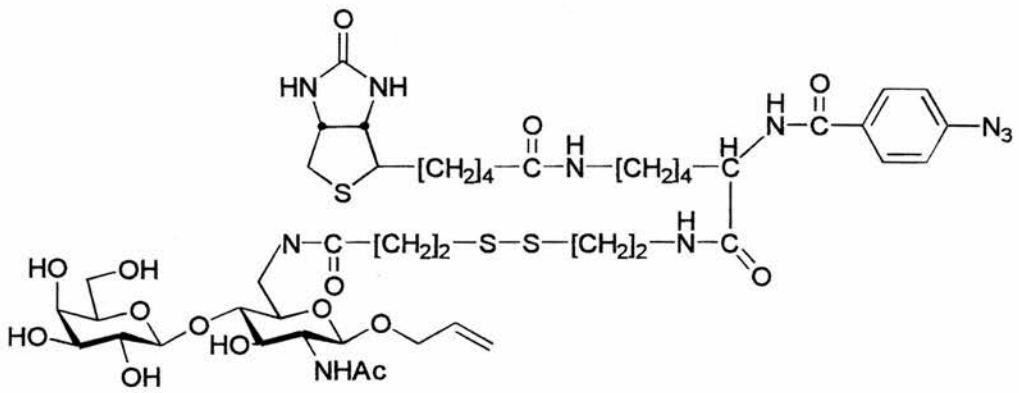


Figure 72. C-6 derived Biotinylated Photoprobe

4.6.2.2 *Trans*-sialidase

Although the C-6 derived photoprobe was recognised as an acceptor substrate for *trans*-sialidase, we were unable to achieve cross-linking with it. It thus appears the photo-labile moiety at this position cannot interact with the enzyme active site, but is instead projecting freely into solution. It would also be worth attempting to cross-link *trans*-sialidase with the C-6 derived biotinylated probe illustrated in Figure 72 above as there may be a possibility that this probe may wrap backwards over the top of the sugar and interact with the enzyme active site. The anomeric biotinylated probe **10** may interact with *trans*-sialidase as it did with FucT VI. Failing these approaches, one would be forced to consider synthesising a photoprobe derived at the 3' position of the LacNAc analogue as illustrated below (Figure 73). As discussed previously in section 1.5, we know 3' hydroxyl group modifications can be tolerated by the enzyme.

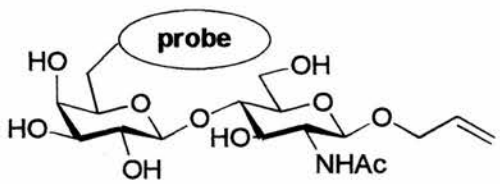


Figure 73. Potential *Trans*-sialidase photoprobe

Further investigation into interaction of the active site of *trans*-sialidase with the C-6 position on our LacNAc analogue may be worthwhile. The use of azido-analogue substrates of acyltransferase have been reported as irreversible inhibitors after

photolysis with UV light. The photoreactive phospholipid analogues 1-acyl-2-(12-azidooleoyl)glycero-3-phosphocholine (N_3-PC) and 1-acyl-2-(12-azidooleoyl)glycero-3-phosphoethanolamine (N_3-PC) have been shown to cross-link acyltransferase.[26] Likewise, the photoreactive donor substrate analogues 12-azidooleoyl-CoA and 12-[(azidosayicyl) amino] dodecanoyl CoA were also shown to cross link acyltransferase after photolysis with UV.[27] It may therefore be possible to use the underived azido sugar (**27**) to label *trans*-sialidase, this smaller group perhaps facilitating active-site interaction. Should this be the case then biotinylation at the anomeric position of **27** would allow recovery of labelled peptide. This potential photo-probe is illustrated below (Figure **74**).

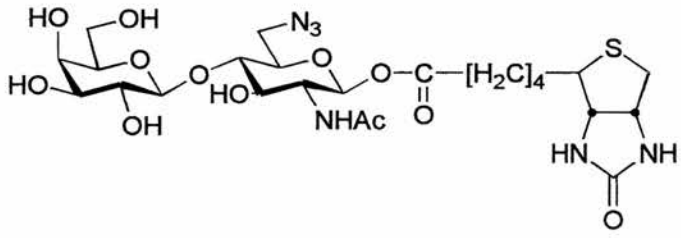


Figure 74. Potential *Trans*-sialidase Probe

4.6.3 Kinetic Studies

4.6.3.1 'Natural' Oligosaccharide Acceptor

Preliminary kinetic studies with the heptasaccharide Galβ1,4GlcNAcα1,2Manα1,6(Galβ1,4GlcNAcα1,2Manα1,3)ManOMe (Figure 75) show this to have a higher affinity for FucT ie lower Km than LacNAc.

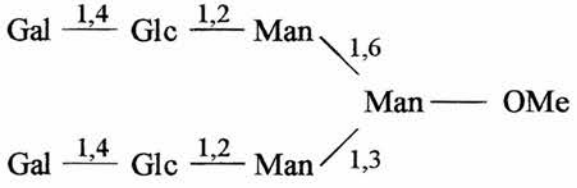


Figure 75. Heptasaccharide Acceptor

This would suggest that recognition of the acceptor substrate by this enzyme extends beyond the immediate hydroxyl groups on LacNAc as has been discussed in section

1.5. This has implications for enzyme inhibitor design programmes, where perhaps the 'minimalist' approach often taken, in terms of the substrate analogues examined, is missing more distal interactions that may be taking place between the enzyme and the natural acceptor substrate.

To further examine recognition of FucT with respect to this heptasaccharide, it would be worth investigating whether there is any selectivity for one terminal LacNAc residue over the other.

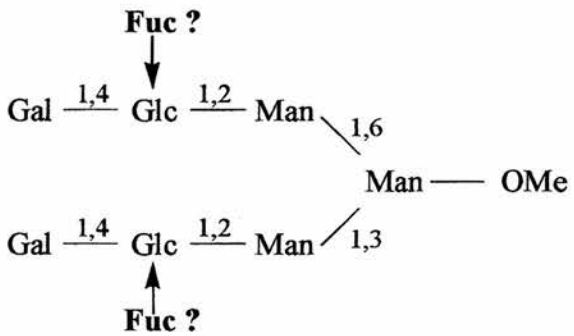


Figure 76. Branch Selectivity of FucT VI?

4.4 Experimental

General Methods

All reagents and solvents were dried prior to use according to standard methods. Commercial reagents were otherwise used without further purification.

Analytical TLC was performed on silica gel 60-F_{254} (Merck) with detection by fluorescence and/or by charring following immersion in a dilute ethanolic solution of sulphuric acid or spraying with orcinol.

An orcinol dip was prepared by the careful addition of conc. sulfuric acid (20 cm^3) to an ice cold solution of 3,5-dihydroxytoluene (360 mg) in EtOH (150 cm^3) and water (10 cm^3).

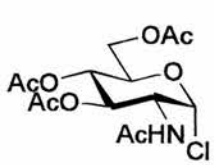
Column chromatography was performed with silica gel 60 (Fluka), 70-230 mesh.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 spectrometer at 300 MHz and 75 MHz, respectively. ^{1}H NMR spectra were referenced to the following internal standards: δ_H 7.26 in $CDCl_3$, 3.35 in CD_3OD, 4.75 in D_2O. ^{13}C NMR spectra were referenced to the following internal standards: δ_C 76.9 in $CDCl_3$; δ_C 49.0 in CD_3OD. *J* values are given in Hz. Only partial (diagnostic) NMR data are given for some compounds; other spectral features were in accord with the proposed structures.

UDP-Galactose 4-epimerase (recombinant from *E. coli*) and β1,4-Galactosyltransferase (bovine milk) were purchased from Calbiochem and alkaline phosphatase (bovine intestinal mucosa) from Sigma. UDP-Glucose was from Sigma.

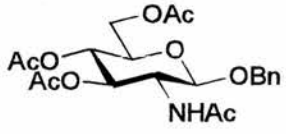
2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (12).

The title compound was synthesised essentially as described by Horton.[28]



2-Acetamido-2-deoxy-D-glucopyranose (5 g, 22.6 mmol) was added to acetyl chloride (75 ml) and the mixture stirred at room temperature. The reaction underwent spontaneous reflux. After stirring overnight the mixture had become an amber solution. DCM (75 ml) was added and the solution was then poured onto ice (200 ml) and stirred vigorously. Once the fizzing had stopped, the mixture was transferred to a separating funnel and the DCM was separated and run directly onto more ice (250 ml) in sat. $NaHCO_3$ solution (75 ml). The mixture was stirred vigorously and then transferred once again to a separating funnel where the DCM was separated, dried ($MgSO_4$) and concentrated *in vacuo* to a volume of about 15 ml. Diethyl ether (50 ml) was then added and crystallisation occurred. The pink crystals obtained were recrystallised (hexane-ethyl acetate, 1:1) to give the title compound (**12**) as white crystals (3.78 g, 42 %), m.p. 125-127 °C (lit;[28] 127-128 °C), $[\alpha]_D$ +108 (*c* 1, chloroform) [lit;[7] +110 (chloroform)], δ_H (300 MHz, $CDCl_3$): 1.96 (3H, s, NHCOC*H*$_3$), 2.02 (6H, s, 2 x OCOC*H*$_3$), 2.08 (3H, s, OCOC*H*$_3$), 4.0-4.3 (3H, m, 5,6 and 6'-H), 4.5-4.55 (1H, m, 2-H), 5.19 (1H, t, *J* 9.6 Hz, 4-H), 5.30 (1H, t, *J* 9.9 Hz, 3-H), 5.88 (1H, d, *J* 8.5 Hz, N*H*), 6.17 (1H, d, *J* 3.8 Hz, 1-H); δ_C (75 MHz, $CDCl_3$): 20.4, 20.5, 20.6 and 23.0 (4 x CO*C*H$_3$), 53.5 (C2), 61.2 (C6), 67.0, 70.1 and 70.9 (C3-C5), 93.7 (C1), 169.3, 170.3, 170.7 and 171.6 (4 x *C*OCH$_3$).

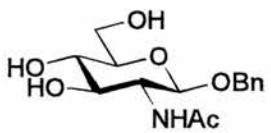
Benzyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (13).



To dry acetonitrile (4 ml) were added 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy α-D-glucopyranosyl chloride (**12**) (366 mg, 1 mmol), iodine crystals (380 mg, 1.5 mol equiv) and benzyl alcohol (0.17 ml, 2.5 mol equiv). The reaction mixture was stirred under nitrogen at room temperature for 72 hours after which time TLC (DCM/MeOH, 9.6:0.4) showed the reaction to be complete. The product was extracted into DCM and iodine was removed by washing with a 10 % aqueous sodium thiosulphate solution. The resulting organic extract was washed with H_2O, dried over anhydrous sodium sulphate and evaporated to dryness. Chromatography (silica gel: ethyl acetate) produced the title compound (**13**) (205 mg, 47 %), m.p.

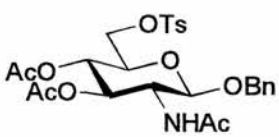
162-165 °C (lit[2]; 164-165 °C), $[\alpha]_D$ -51 (*c* 1, chloroform) [lit[2]; -54 (chloroform)], δ_H ($CDCl_3$) 1.88 (3H, s, $CH_3CO.N$), 1.99, 2.08 (9H, 2s, 3 x $CH_3CO.O$), 3.67 (1H, m, H-5), 3.95 (1H, m, H-2), 4.21-4.60 (2H, m, 6-H_a and 6-H_b), 4.56 (1H, d, *J* 12.4, CH_2Ph), 4.62 (1H, d, $J_{1,2}$ 8.2, H-1), 4.85 (1H, d, *J* 12.4, CH_2Ph), 5.07 (1H, t, H-3/H-4), 5.20 (1H, t, H-3/H-4), 5.67 (1H, d, NH), 7.30 (5H, m, Ar).

Benzy 2-acetamido-2-deoxy-β-D-glucopyranoside (14)



To 35 ml of anhydrous sodium methoxide solution was added (**13**) (2.8 g, 6.4 mmol). This was left to stir at room temperature for 30 minutes after which time T.L.C. (DCM/MeOH, 9.6:0.4) showed the total absence of starting material. The reaction mixture was neutralised by the addition of Amberlite 120 cation exchange resin which was then removed by filtration. Evaporation of the filtrate to dryness gave the deprotected sugar (**14**) in near quantitative yield. This material was used directly in the next step without further purification.

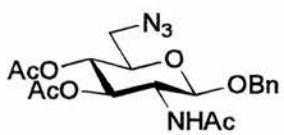
Benzyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside (16).



A solution of tosyl chloride (1.53 g, 1.25 mol equiv) in DCM (20 ml) was added dropwise to a solution of benzy 2-acetamido-2-deoxy-β-D-glucopyranoside (**14**) (2 g, 6.43 mmol) in pyridine (30 ml), maintained at a temperature of –35 °C in an acetonitrile/dry ice bath. The reaction mixture was allowed to warm to room temperature and stirring was continued for 8 hours at which point TLC (DCM:MeOH, 6:1) indicated the reaction was complete. Acetic anhydride (3.0 ml, 5 mol equiv) was then added to the reaction mixture which was allowed to stir

overnight. TLC (DCM/MeOH, 9.7:0.3) indicated completion of reaction. The reaction mixture was diluted with DCM and washed successively with dilute HCl, saturated aqueous $NaHCO_3$ solution, H_2O, and then dried over anhydrous sodium sulphate and evaporated to dryness. Chromatography (silica gel: toluene-ethyl acetate 2:1-0:1) gave compound (**16**) as a solid (1.5 g, 43 %), m.p.172-174 °C. δ_H ($CDCl_3$) 1.84 (3H, s, C*H*$_3$CO.N), 1.98 (6H, s, 2 x C*H*$_3$CO.O), 2.41 (3H, s, ArC*H*$_3$), 3.65-3.75 (1H, m, *H*-5), 4.03-4.13 (3H, m, *H*-2, 6_a and 6_b), 4.48 and 4.75 (2H, 2d, *J* 12 .1, C*H*$_2$Ar), 4.60 (1H, d, $J_{1,2}$ 8.2, *H*-1), 4.90 (1H, t, *J* 9.9 and 9.3, *H*-4/3), 5.18 (1H, t, *J* 9.3 and 10.4, *H*-3/4)), 5.71 (1H, br d, *J* 8.8, N*H*), 7.20-7.40 (7H, m, Ar*H*), 7.76 (2H, d, Ar*H*). δ_C ($CDCl_3$) 20.5, 20.6, 21.6 (2 x *C*H$_3$CO.O and Ts-*C*H$_3$) and 23.2 (*C*H$_3$CO.N), 54.3 (*C*-2), 68.2, 69.0, 70.7, 71.7, 72.2 (*C*3-*C*6 and *C*H$_2$Ph), 99.4 (*C*-1), 128.0, 128.2, 128.6, 130.0 (Ar), 132.6 (Bn quat), 137.0 (Ts quat), 145.3 (Ts quat), 169.7, 170.4, 171.0 (3x*C*O). FAB-MS (+ve): ***m/z*** 550 ($M+H^+$) and 572 ($M+Na^+$) ($C_{26}H_{31}NO_{10}S$ requires ***m/z*** 549). HRMS: Found 550.1742. $C_{26}H_{31}NO_{10}S$ ($M+H^+$) requires 550.1747.

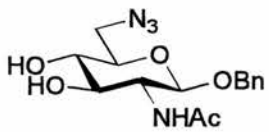
Benzyl 2-acetamido-3,4-di-*O*-acetyl-6-azido-2,6-di-deoxy-β-D-glucopyranoside (17).



To a solution of (**16**) (1.2 g, 2.18 mmol) in DMF (10 ml) was added sodium azide (1.15 g, 10 mol equiv) and the reaction mixture was refluxed over night at 140 °C after which time TLC (ethyl acetate:toluene, 2:1) indicated complete consumption of tosylate. Excess sodium azide was removed by filtration and the filtrate was concentrated to a brown syrup. Chromatography (silica gel: ethyl acetate-toluene, 2:1-1:0) gave two compounds, one of which after drying under vacuum was benzyl 2-acetamido-3,4-di-*O*-acetyl-6-azido-2,6-di-deoxy-β-D-glucopyranoside (**17**) (192 mg, 21 %). δ_H ($CDCl_3$) 1.86 (3H, s, C*H*$_3$CO.N), 1.98 (6H, s, 2 x C*H*$_3$CO.O), 3.14 - 3.19 and 3.37 - 3.46 (2H, 2m, *H*-6a and *H*-6b), 3.66 - 3.73 (1H, m, *H*-5), 3.93 - 4.02 (1H, m, *H*-2), 4.59 (1H, d, *J* 12.1, C*H*$_2$Ar), 4.71 (1H, d, $J_{1,2}$ 8.51, *H*-1), 4.87 (1H, d, *J* 12.1, C*H*$_2$Ar), 4.96 and 5.24 (2H, 2t, *H*-3 and *H*-4), 6.01 (1H, d, *J* 8.8, N*H*), 7.28

(5H, s, Ar*H*). δ_C ($CDCl_3$) 20.6 (2 x $CH_3CO.O$), 23.3 ($CH_3CO.N$), 51.5 (*C*-2), 54.2 (*C*-6), 70.0, 70.5, 72.0 and 73.8 (*C*-3, *C*-4, *C*-5 and CH_2Ph), 99.8 (*C*-1), 128.0 (Ar), 128.7 (Ar), 137.4 (Ar quat), 169.8, 171.0 and 171.2 (3 x *C*O). ν_{max}/cm^{-1} 2100 (N_3)
The second compound was presumed to be one or both of the corresponding mono-O-acetates since it was readily converted to **18** on treatment with basic methanol.

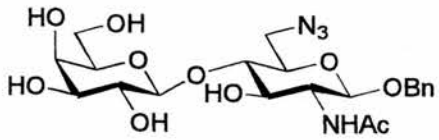
Benzyl 2-acetamido-6-azido-2,6-di-deoxy-β-D-glucopyranoside (18).



To a solution of (**16**) (1.2 g, 2.18 mmol) in DMF (10 ml) was added sodium azide (1.15 g, 10 mol equiv) and the reaction mixture was refluxed over night at 140 °C after which time T.L.C. (ethyl acetate:toluene, 2:1) indicated complete consumption of tosylate. Excess sodium azide was removed by filtration and the filtrate was concentrated to a brown syrup. Chromatography (silica gel: ethyl acetate-toluene, 2:1-1:0) gave two compounds, the faster moving di-*O*-acetate (benzyl 2-acetamido-3,4-di-*O*-acetyl-6-azido-2,6-di-deoxy-β-D-glucopyranoside) (**17**) and a slower moving, presumably mono-*O*-acetate (benzyl 2-acetamido-3/4-*O*-acetyl-6-azido-2,6-di-deoxy-β-D-glucopyranoside). Both of these compounds on standing in the refrigerator in anhydrous MeOH gave a single product, as shown by TLC (ethyl acetate/toluene, 2:1), owing presumably to spontaneous de-*O*-acetylation. The methanolic solution at this stage was found to be basic (pH~8) and hence was neutralised by treatment with Dowex AG50 H^+ ion exchange resin. The resin was removed by filtration and the filtrate was evaporated to dryness. Crystallisation from methanol gave azido sugar (**18**) (231 mg, 32 %), m.p. 197-199 °C (EtOH, Et_2O) (Found: C, 53.55; H, 5.95; N, 16.72. $C_{15}H_{20}N_4O_5$ requires C, 53.56; H, 5.99; N, 16.66%); δ_H (CD_3OD) 1.95 (3H, s, $CH_3CO.N$), 3.24-3.36 and 3.40-3.56 (2H and 3H respectively, 2m, *H*-3-*H*-$_{6a,b}$), 3.74 (1H, t, *H*-2), 4.50 (1H, d, *J*= 8.7 Hz, *H*-1), 4.59 (1H, d, *J* 12.3, OCH_2Ph), 4.82 (1H, d, *J* 12.3, OCH_2Ph), 7.20-7.36 (5H, m, Ar*H*). δ_C (CD_3OD) 23.2 (CH_3), 53.7 (*C*-6), 58.0 (*C*-2), 72.0 (CH_2Ph), 73.9, 76.0, 80.0 (*C*-3 to C-5), 102.4 (*C*-1), 129.8, 129.9, 130.0 (Ar), 138.9 (Ar quat), 174.8 (*C*O). ν_{max}/cm^{-1}

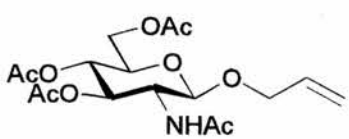
2086 (N_3). ES-MS (+ve): *m/z* 337 ($M+H^+$) ($C_{15}H_{20}N_4O_5$ requires *m/z* 336). HRMS: Found 337.1509. $C_{15}H_{20}N_4O_5$ ($M+H^+$) requires 337.1512.

Benzyl β-D-galactopyranosyl-1,4-(2-acetamido-6-azido-2,6-dideoxy-β-D-glucopyranoside) (19).



To 50ml of buffer (HEPES 100 mM, KCl 20 mM, $MgCl_2$ 10 mM, $MnCl_2$ 5 mM, 0.02 % NaN_3, pH 7.4) were added **18** (100 mg, 0.30 mmoles in 5 ml MeOH), UDP-glucose (273 mg, 1.5 mol equiv), UDP-galactose 4'-epimerase (2 U), β1,4-galactosyltransferase (2 U) and alkaline phosphatase (16 U). Nitrogen was passed over the solution and the reaction mixture was incubated in a shaker-incubator (150 rpm) for 72 hours at 37 °C. The mixture was then loaded on to a column of TMD8 mixed bed ion exchange resin (1.4 cm diam. x 10.5 cm) and was eluted with 10 % aqueous methanol solution (100 ml) and evaporated to dryness. The presence of product was confirmed by TLC ($CHCl_3$/MeOH/H_2O, 10:10:3). The resulting solid was dissolved in 80 % aqueous ethanol solution (3.5 ml) and loaded onto a Sephadex LH20 gel filtration column (2.8 x 73 cm). Elution was with 80 % aqueous ethanol at a rate of 12 ml/hour which followed by lyophilisation gave (**5**) as a white solid (15.3 mg, 10 %). δ_H (D_2O) 1.95 (3H, s, $CH_3CO.N$), 3.73 (1H, t, *J* 8.0, H-2), 4.46 (1H, d, $J_{1,2}$ 8.1, *H*-1), 4.60 (1H, d, $J_{1,2}$ 8.7, *H*-1), 7.39-7.66 (5H, m, Ar). ν_{max}/cm^{-1} 2086 (N_3). ES-MS (+ve): *m/z* 499 ($M+H^+$) and 521 ($M+Na^+$) ($C_{21}H_{30}N_4O_{10}$ requires 498).

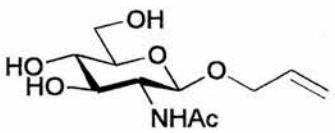
Allyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (20).



To dry dichloromethane (34.6 ml) were added mercury bromide (0.34 g, 0.1 mol equiv), mercury cyanide (2.86 g, 1.2 mol equiv), powdered calcium sulphate (3.46 g)

and allyl alcohol (1.6 ml, 2.5 mol equiv) and allowed to stir for some minutes before adding 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**12**) (3.46 g, 9.5 mmol). The reaction mixture was stirred under nitrogen at room temperature for 72 hours after which time TLC (DCM/MeOH, 9.6:0.4) showed the complete consumption of starting material. The reaction mixture was passed through a bed of Celite, which was subsequently washed with dichloromethane and methanol and solvent was removed *in vacuo*. Chromatography (silica gel: ethyl acetate) produced the title compound (**20**) (2.70 g, 74 %), m.p. 154-156 °C (lit[29]; 160 °C) $[\alpha]_D$ –17, (*c* 1, chloroform) (lit[30]; -17.5). δ_H ($CDCl_3$) 1.96 (3H, s, C*H*$_3$CO.N), 2.03 (6H, s, 2 x C*H*$_3$CO.O), 2.09 (3H, s, C*H*$_3$CO.O), 3.67-3.72 (1H, m, H-5), 3.83-3.92 (1H, dd, $J_{1,2}$ 8.4, $J_{2,3}$ 8.7, H-2), 4.06-4.13 (2H, m, OC*H*$_2$CHCH$_2$), 4.14 (1H, dd, $6_a/6_b$), 4.27 (1H,dd, $6_a/6_b$), 4.32-4.38 (2H, m, OC*H*$_2$CHCH$_2$), 4.71 (1H, d, $J_{1,2}$ 8.4, H-1), 5.08 (1H, t, *J* 9.3 and 9.9, H-4/3), 5.19-5.25 (2H, m, OCH$_2$CHC*H*$_2$), 5.28 (1H, t, *J* 8.7 and 9.0, H-3/4), 5.48 (1H, d, *J* 9.9, N*H*), 5.81-5.94 (1H, m, OCH$_2$C*H*CH$_2$). δ_C ($CDCl_3$) 20.5 and 20.6 (3 x *C*H$_3$CO.O), 23.3 (*C*H$_3$CO.N), 54.7 (*C*-2), 62.2 (*C*-6), (68.7, 70.0, 71.8, and 72.4 (C3-6 and O*C*H$_2$CHCH$_2$), 99.7 (C-1), 118.0 (OCH$_2$CH*C*H$_2$), 133.7 (OCH$_2$*C*HCH$_2$), 169.6, 170.6, 170.9 and 171.1 (4 x *C*O). CI-MS (+ve): 388 ($M+H^+$) ($C_{17}H_{25}NO_9$ requires *m/z* 387). HRMS: Found 388.1612. $C_{17}H_{25}NO_9$ ($M+H^+$) requires 388.1608.

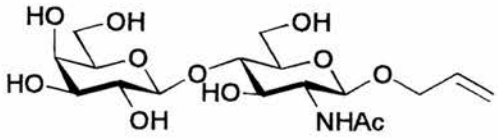
Allyl 2-acetamido-2-deoxy-β-D-glucopyranoside (21).



To 28 ml of anhydrous sodium methoxide solution was added (**20**) (2 g, 5 mmol). This was left to stir at room temperature for 30 minutes after which time TLC (DCM/MeOH, 9.6:0.4) showed the total absence of starting material. The reaction mixture was neutralised by the addition of Amberlite 120 cation exchange resin which was then removed by filtration. Evaporation of the filtrate to dryness gave the deprotected sugar (**21**) (1.0 g, 74 %); mp 173-175 °C (EtOH:Et_2O, 1:1) (lit,[31] 172-174 °C), $[\alpha]_D$ –33 (*c* 0.75, water) [lit;[31] -33.9]. δ_H (CD_3OD) 1.96 (3H, s, C*H*$_3$CO.N),

3.22-3.37 and 3.63-3.70 (4H, 2m, H-3,4,5 and H-6_a), 3.44 (1H, dd, $J_{1,2}$ 8.2, $J_{2,3}$ 10.2, H-2), 3.87 (1H, dd, 6_b), 4.02-4.10 and 4.29-4.36 (2H, 2m, OCH_2CHCH_2), 4.42 (1H, d, $J_{1,2}$ 8.2, H-1), 5.10-5.30 (2H, m, OCH_2CHCH_2), 5.82-5.95 (1H, m, OCH_2CHCH_2). δ_C (CD_3OD) 23.0 ($CH_3CO.N$), 57.5 (C-2), 63.0 (C-6), 70.9, 72.4, 76.3, 78.2 (C-3, C-4, C-5 and OCH_2), 102.1 (C-1), 117.2 (OCH_2CHCH_2), 135.9 (OCH_2CHCH_2), 174.1 (CO). CI-MS (+ve): 262 ($M+H^+$) ($C_{11}H_{19}NO_6$ requires *m/z* 261). HRMS: Found 262.1296. $C_{11}H_{19}NO_6$ ($M+H^+$) requires 262.1291.

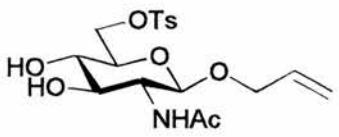
Allyl β-D-galactopyranosyl-1,4-(2-acetamido-2-deoxy-β-D-glucopyranoside) (22)



To 2ml of buffer (HEPES 100 mM, KCl 20 mM, $MgCl_2$10 mM, $MnCl_2$ 15 mM, 0.02 % NaN_3, pH 7.4) were added allyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**21**) (150mg), UDP-Glucose (1080 mg, 3 equiv), UDP-galactose 4'-epimerase (4.2 U), β1,4-galactosyltransferase (4 U) and alkaline phosphatase (30 U). Nitrogen was passed over the solution and the reaction mixture was incubated in a shaker-incubator for 72 hours (150 rpm, 37 °C). The solution was then loaded onto a Sephadex LH20 gel filtration column (2.8 x 73 cm). Elution with water at a rate of 12 ml/hour gave fractions which were monitored by TLC ($CHCl_3$/MeOH/H_2O, 10:10:3). After pooling appropriate fractions, mixed Supelco ion exchange resin was added and allowed to stir for a number of hours. Purification of the solution was monitored by TLC ($CHCl_3$/MeOH/H_2O, 10:10:3) until contaminants were removed. The resin was then removed by filtration and the filtrate lyophilised to give the disaccharide (**22**) (110 mg, 45 %). δ_H (D_2O) 1.93 (3H, s, $CH_3CO.N$), 3.44 (1H, dd $J_{1,2}$ 8.0, $J_{2,3}$ 10.2, H-2), 3.57 (1H, dd, $J_{2',3'}$ 9.9, $J_{3',4'}$ 3.3, H-3'), 3.60-3.69 (7H, m, H-3, H-4, H-5, H-2', H-5', H-6a' and H-6b'), 3.73 (1H, dd, $J_{6a,6b}$ 12.1, H-6a), 3.83 (1H, d, $J_{3',4'}$ 3.3, H-4'), 3.89 (1H, dd, $J_{6a,6b}$ 12.1, H-6b), 4.01-4.12 and 4.19-4.29 (2H, 2m, OCH_2CHCH_2), 4.37 (1H, d, $J_{1,2}$ 8.0, H-1), 4.48 (1H, d, $J_{1,2}$ 7.4, H-1), 5.12-5.26 (2H, m, OCH_2CHCH_2), 5.74-5.88 (1H, m, OCH_2CHCH_2). δ_C (D_2O) 22.1 ($CH_3CO.N$), 55.1 (C-2), 60.1 and 61.0 (C-6 and C-6'), 68.6, 70.5, 71.0, 72.5 (2Cs), 74.8, 75.4 and

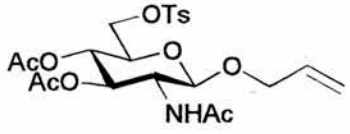
78.6 (remaining 8 ring Cs) 100.1 and 103.0 (C-1 and C-1'), 118.3 (OCH_2CHCH_2), 133.5 (OCH_2CHCH_2), 174.8 ($CH_3CO.N$). MALDI-TOF MS: *m/z* 446 ($M+Na^{+}$) and 462 ($M+K^{+}$) ($C_{17}H_{29}NO_{11}$ requires *m/z* 423).HRMS: Found 424.1816. $C_{17}H_{29}NO_{11}$ ($M+H^{+}$) requires 424.1819.

Allyl 2-acetamido-2-deoxy-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside (23).



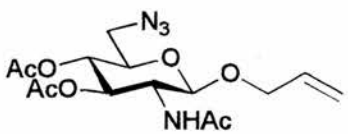
A solution of tosyl chloride (0.45 g, 1.25 mol equiv) in DCM (12 ml) was added dropwise to a solution of allyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**21**) (0.5 g, 1.91 mmol) in pyridine (15 ml), maintained at a temperature of –35 °C in an acetonitrile/dry ice bath. The reaction mixture was allowed to warm to room temperature and stirring was continued for 8 hours at which point TLC (DCM/MeOH, 6:1) indicated the reaction was complete. After coevaporation with toluene the reaction mixture was diluted with DCM and washed successively with dilute HCl, saturated aqueous $NaHCO_3$ solution, H_2O, and then dried over anhydrous sodium sulphate and evaporated to dryness. Chromatography (silica gel: DCM : MeOH, 6:1) gave the title compound (**11**) as an oil (76 mg, 10 %), $[\alpha]_D$ –18 (*c* 1, chloroform). δ_H (CD_3OD) 1.94 (3H, s, $CH_3CO.N$), 2.44 (3H, s, $ArCH_3$), 3.19-3.30 and 3.55-3.61 (4H, 2m, H-3,4,5 and H-6_a), 3.36-3.43 (1H, dd, $J_{1,2}$ 8.5, $J_{2,3}$ 10.4, H-2), 3.94 (1H, dd, 6_b), 4.12-4.20 and 4.33-4.37 (2H, 2m, OCH_2CHCH_2), 4.35 (1H, d, $J_{1,2}$ 8.5), 5.10-5.26 (2H, m, OCH_2CHCH_2), 5.78-5.90 (1H, m, OCH_2CHCH_2), 7.41 (2H, d, Ar*H*), 7.79 (2H, d, Ar*H*). δ_C (CD_3OD) 21.6 and 22.9 ($CH_3CO.N$ and Ts-CH_3), 57.1 (*C*-2), 70.7 and 71.7 (C-6 and OCH_2CHCH_2), 70.8, 75.0 and 75.8 (*C*-3, *C*-4 and *C*-5), 101.7 (*C*-1), 117.2 (OCH_2CHCH_2), 129.2 and 131.2 (4 x Ar), 134.5 (Ts quat), 135.6 (OCH_2CHCH_2), 146.6 (Ts quat), 173.9 (*C*O). MALDI-TOF MS 438 ($M+Na^{+}$) ($C_{18}H_{25}NO_8S$ requires 415). HRMS: Found 416.1383. $C_{18}H_{25}NO_8S$ ($M+H^{+}$) requires 416.1379.

Allyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-6-*O*-*p*-toluenesulfonyl-β-D-glucopyranoside (24).



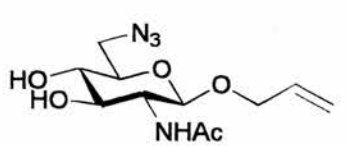
A solution of tosyl chloride (0.45 g, 1.25 mol equiv) in DCM (12 ml) was added dropwise to a solution of allyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**21**) (0.5 g, 1.91 mmol) in pyridine (15 ml), maintained at a temperature of –35 °C in an acetonitrile/dry ice bath. The reaction mixture was allowed to warm to room temperature and stirring was continued for 8 hours at which point TLC (DCM:MeOH, 6:1) indicated the reaction was complete. Acetic anhydride (1.23 ml, 5 mol equiv) was then added to the reaction mixture which was allowed to stir overnight. T.L.C. (DCM/MeOH, 9.5:0.5) indicated completion of reaction. The reaction mixture was diluted with DCM and washed successively with dilute HCl, saturated aqueous $NaHCO_3$ solution, H_2O, and then dried over anhydrous sodium sulphate and evaporated to dryness. Crystallisation from isopropanol produced the title compound (**24**) (612 mg, 64 %), m.p. 158-160 °C. (Found: C, 52.91; H, 5.94; N, 2.75. $C_{22}H_{29}NO_{10}S$ Requires C, 52.90; H, 5.85; N, 2.80 %); $[\alpha]_D$ 0 (*c* 1, chloroform). δ_H ($CDCl_3$) 1.92 (3H, s, $CH_3CO.N$), 1.98 and 2.00 (6H, 2s, 2 x $CH_3CO.O$), 2.44 (3H, s, ArCH_3), 3.71-3.86 (2H, m, *H*-5 and *H*-2), 3.97-4.15 and 4.20-4.27 (3H, 2m, OCH_2CHCH_2, *H*-6_a and *H*-6_b), 4.63 (1H, d, $J_{1,2}$ 8.2, *H*-1), 4.85-4.92 and 5.15-5.28 (4H, 2m, H-3, H-4 and OCH_2CHCH_2), 5.64 (1H, d, *J* 8.8, N*H*), 5.75-5.88 (1H, m, OCH_2CHCH_2), 7.26 (2H, d, Ar*H*), 7.76 (2H, d, Ar*H*). δ_C ($CDCl_3$) 20.4, 20.5, 21.5 (2 x $CH_3CO.O$ and Ts-CH_3) and 23.1 ($CH_3CO.N$), 54.3 (*C*-2), 68.0 and 69.7 (C-6 and OCH_2CHCH_2), 68.8, 71.4 and 72.0 (*C*-3, *C*-4 and *C*-5), 99.3 (C-1), 117.6 (OCH_2CHCH_2), 127.9 and 129.7 (4 x Ar), 132.2 (Ts quat), 133.2 (OCH_2CHCH_2), 145.0 (Ts quat), 169.4, 170.2 and 170.7 (3 x *C*O). CI-MS (+ve): 500 ($M+H^+$) ($C_{22}H_{29}NO_{10}S$ requires *m/z* 499). HRMS: Found 500.1600. $C_{22}H_{29}NO_{10}S$ ($M+H^+$) requires 500.1590.

Allyl 2-acetamido-3,4-di-*O*-acetyl-6-azido-2,6-di-deoxy-β-D-glucopyranoside (25).



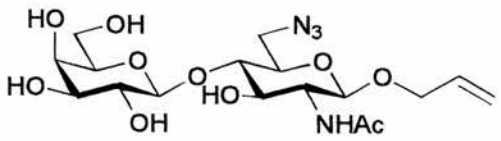
To a solution of (**24**) (116 mg, 0.23 mmol) in anhydrous DMF (1.1 ml) was added sodium azide (151 mg, 10 mol equiv) and the reaction mixture was refluxed at between 60-70 °C overnight after which time TLC (ethyl acetate:toluene, 2:1) indicated complete consumption of tosylate. The DMF was then removed under vacuum and the resulting solid transferred to a separating funnel in DCM and water. After vigorous shaking the organic layer was washed a second time and then dried over anhydrous sodium sulphate. The solution was then stirred with activated charcoal and then filtered through Celite and silica. Concentration of the filtrate to dryness gave the azido sugar (**25**) (70 mg, 81 %), $[\alpha]_D$ –12.2 (*c* 1, chloroform : methanol, 1:1), ν_{max}/cm^{-1} 2100 (N_3). δ_H ($CDCl_3$) 1.94 (3H, s, C*H*$_3$CO.N), 2.02 (6H, s, 2 x C*H*$_3$CO.O), 3.17 and 3.41 (2H, 2dd, 6_a and 6_b), 3.66-3.72 (1H, m, H-5), 3.83-3.93 (1H, m, H-2), 4.07-4.14 and 4.32-4.38 (2H, 2m, OC*H*$_2$CHCH$_2$), 4.74 (1H, *J* 8.2, H-1), 4.91-4.97 and 5.18-5.33 (3H, 2m, H-3, H-4 and OCH$_2$CHC*H*$_2$), 5.65 (1H, d, *J* 8.8, NH), 5.79-5.92 (1H, m, OCH$_2$C*H*CH$_2$). δ_C ($CDCl_3$) 20.5 and 20.6 (2 x *C*H$_3$CO.O) and 23.2 (*C*H$_3$CO.N), 51.2 and 54.8 (C-2 and C-6), 69.8, 69.9, 72.1 and 73.6 (C-3, C-4, C-5 and O*C*H$_2$CHCH$_2$), 99.4 (C-1), 118.0 (OCH$_2$CH*C*H$_2$), 133.5 (OCH$_2$*C*HCH$_2$), 169.7, 170.4 and 171.0 (3 x CO). CI-MS (+ve): 371 ($M+H^+$) ($C_{15}H_{22}N_4O_7$ requires *m/z* 370). HRMS: Found 371.1571. $C_{15}H_{22}N_4O_7$ ($M+H^+$) requires 371.1567.

Allyl 2-acetamido-6-azido-2,6-di-deoxy-β-D-glucopyranoside (26).



To 1.5 ml of anhydrous sodium methoxide solution was added (**25**) (150 mg, 0.40 mmol). This was left to stir at room temperature for 30 minutes after which time T.L.C. (DCM/MeOH, 9.6:0.4) showed the total absence of starting material. The reaction mixture was neutralised by the addition of Amberlite 120 cation exchange resin which was then removed by filtration. Evaporation of the filtrate to dryness gave the deprotected sugar (**26**) (90 mg, 77 %), $[\alpha]_D$ –41.6 (*c* 0.2, methanol)..δ_H (CD_3OD) 1.98 (3H, s, $CH_3CO.N$), 3.23-3.32 and 3.38-3.51 (5H, 2m, H-3, 4, 5, 6a and H-6b), 3.68 (1H, dd, $J_{1,2}$ 8.5 $J_{2,3}$ 10.2, H-2), 4.02-4.11 and 4.26-4.34 (2H, 2m, OCH_2CHCH_2), 4.48 (1H, d, $J_{1,2}$ 8.5, H-1), 5.11-5.17 and 5.22-5.31 (2H, 2m, OCH_2CHCH_2), 5.82-5.95 (1H, m, OCH_2CHCH_2). δ_C (CD_3OD) 22.9 ($CH_3CO.N$), 52.9 (C-2), 57.5 (C-6), 70.9, 73.2, 75.9, 77.3 (C-3, C-4, C-5 and OCH_2), 101.9 (C-1), 117.5 (OCH_2CHCH_2), 135.7 (OCH_2CHCH_2), 173.9 (CO). CI-MS (+ve): 287 ($M+H^+$) ($C_{11}H_{18}N_4O_5$ requires *m/z* 286). HRMS: Found 287.1362. $C_{11}H_{18}N_4O_5$ ($M+H^+$) requires 287.1355.

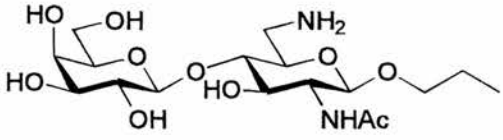
Allyl β-D-galactopyranosyl-1,4-(2-acetamido-6-azido-2,6-di-deoxy-β-D-glucopyranoside) (27)



To 1.25 ml of buffer (HEPES 100 mM, KCl 20 mM, $MgCl_2$ 10 mM, $MnCl_2$ 15 mM, 0.02 % NaN_3, pH 7.4) were added allyl 2-acetamido-6-azido-2,6-di-deoxy-β-D-glucopyranoside (**26**) (67 mg, 0.23 mmol), UDP-Glucose (428 mg, 3 equiv), UDP-galactose 4'-epimerase (5.4 U), β1,4-galactosyltransferase (4 U) and alkaline phosphatase (30 U). Nitrogen was passed over the solution and the reaction mixture was incubated in a shaker-incubator for 72 hours (150 rpm, 37 °C). TLC ($CHCl_3$/MeOH/H_2O, 10:10:3) showed the reaction to have gone to completion. The solution was then loaded onto a Sephadex LH20 gel filtration column (2.8 x 73 cm) in two separate and equal lots. Elution with water at a rate of 12 ml/hour gave fractions which were monitored by TLC ($CHCl_3$/MeOH/H_2O, 10:10:3). After pooling appropriate fractions, mixed Supelco ion exchange resin was added and

allowed to stir for a number of hours. Purification of the solution was monitored by TLC ($CHCl_3$/MeOH/H_2O, 10:10:3) until all contaminants were removed. The resin was then removed by filtration and the filtrate lyophilised to give the disaccharide (**27**) (42 mg, 40 %). δ_H (D_2O) 1.93 (3H, s, CH_3CO.N), 3.43 (1H, dd $J_{1,2}$ 8.0, $J_{2,3}$ 10.2, H-2), 3.57 (1H, dd, $J_{2',3'}$ 9.9, $J_{3',4'}$ 3.0, H-3'), 3.60-3.71 (9H, m, H-3, H-4, H-5, H-6a, H-6b, H-2', H-5', H-6a' and H-6b'), 3.83 (1H, d, $J_{3',4'}$ 3.0, H-4'), 4.02-4.12 and 4.20-4.30 (2H, 2m, OCH_2CHCH$_2$), 4.36 (1H, d, $J_{1,2}$ 7.7, H-1), 4.53 (1H, d, $J_{1,2}$ 8.0, H-1), 5.14-5.26 (2H, m, OCH$_2$CHCH_2), 5.74-5.90 (1H, m, OCH$_2$CHCH$_2$). δ_C (D_2O) 20.2 (*C*H$_3$CO.N), 48.4 (C-6'), 53.1 and 59.1 (C-2 and C-6), 66.6, 68.6, 69.0, 70.3, 70.5, 71.7, 73.5, 77.4 (remaining 7 ring Cs and O*C*H$_2$CHCH$_2$), 100.0 and 101.1 (C-1 and C-1'), 116.4 (OCH$_2$CH*C*H$_2$), 131.3 (OCH$_2$*C*HCH$_2$), 172.6 (CH$_3$*C*O.N). MALDI-TOF MS 471 (M+Na$^+$) ($C_{17}H_{28}N_4O_{10}$ requires 448). HRMS: Found 449.1884. $C_{17}H_{28}N_4O_{10}$ (M+H$^+$) requires 449.1884.

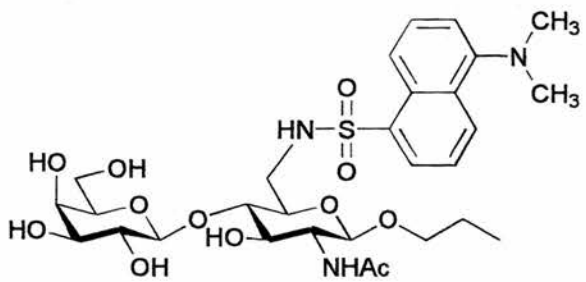
Propyl β-D-galactopyranosyl-1,4-(2-acetamido-6-amino-2,6-di-deoxy-β-D-glucopyranoside) (28)



To a mixture of ethanol (400 µl) and water (200 µl) were added palladium hydroxide on carbon (5 mg) and azido sugar (**27**) (9 mg, 20.1 µmol). This was allowed to stir at room temperature for 3 hours after which time TLC ($CHCl_3$:MeOH:H_2O, 10:10:3) indicated the reaction had gone to completion. The reaction mixture was passed through a bed of Celite, which was subsequently washed with methanol. Removal of solvent *in vacuo* gave the title compound (5.0 mg, 59 %). δ_H (D_2O) 0.95 (3H, t, OCH$_2$CH$_2$CH_3), 1.58-1.68 (2H, m, OCH$_2$CH_2CH$_3$), 2.00 (3H, s, CH_3CO.N), 3.41-3.96 (H-2, H-3, H-4, H-5, H-6$_a$, H-6$_b$, H-2', H-3', H-4', H-5', H-6a', H-6b' and OCH$_2$), 4.39 (1H, d, $J_{1,2}$ 7.6, H-1/H-1'), 4.46 (1H, d, $J_{1,2}$ 8.1, H-1/H-1'). δ_C (D_2O) 10.7 (OCH$_2$CH$_2$*C*H$_3$), 22.9, 23.8 and 30.7 (*C*H$_3$CO.N, OCH$_2$*C*H$_2$CH$_3$ and *C*-6), 56.8 (*C*-2), 62.5 (*C*-6'), 70.3, 72.4, 72.5, 74.3, 74.9 and 77.3 (*C*-3, *C*-4, *C*-5, *C*-2', *C*-3', *C*-4', *C*-5' and OCH$_2$CH$_2$CH$_3$), 102.8 and 105.4 (C-1 and C-1'), 173.7 (CH$_3$*C*O.N).

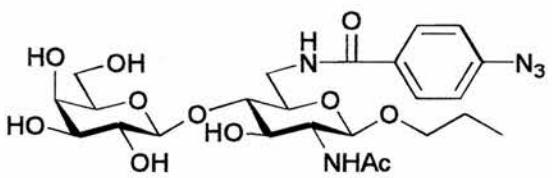
ES-MS (+ve): *m/z* 425 ($M+H^+$) and 447 ($M+Na^+$) ($C_{17}H_{32}N_2O_{10}$ requires *m/z* 424). HRMS: Found 425.2134. $C_{17}H_{32}N_2O_{10}$ ($M+H^+$) requires 425.2135.

Propyl β-D-galactopyranosyl-(1,4)-2-acetamido-2,6-dideoxy-6-(5-dimethylamino-1-naphthalenesulfonyl)amido-β-D-glucopyranoside (29)



In 200 µl of MeOH was dissolved amino sugar (**28**) (5 mg, 0.01 mmol) and dansyl chloride (6.3 mg, 2 mol equiv) and the solution made basic by the addition of triethylamine. The reaction mixture was left to stir at room temperature, overnight, in the dark, after which time TLC (DCM:MeOH, 8:2) indicated the reaction had gone to completion. The mixture was then loaded onto a preparative TLC plate which was subsequently placed in a solvent tank containing $CHCl_3$:MeOH:H_2O, 10:10:3. After an appropriate time interval, the plate was removed and dried and the relevant compound was identified by viewing under a long wave UV lamp. This was marked with pencil and scraped off the glass plate. The compound was then extracted from the silica into methanol. Removal of solvent *in vacuo* gave dansylated disaccharide (**29**) (3.9 mg, 51 %).δ_H (CD_3OD) 0.92 (3H, t, $OCH_2CH_2CH_3$), 1.45-1.57 (2H, m, $OCH_2CH_2CH_3$), 1.95 (3H, s, $CH_3CO.N$), 2.93 (6H, s, 2 x CH_3), 3.11-3.85 (13H, m, *H*-2, *H*-3, *H*-4, *H*-5, *H*-6_a, *H*-6_b, *H*-2', *H*-3', *H*-4', *H*-5', *H*-6_a', *H*-6_b', and $OCH_2CH_2CH_3$), 4.10 (1H, d, *J* 8.2, *H*-1/*H*-1'), 4.24 (1H, d, *J* 7.7, *H*-1/*H*-1'), 7.34 (1H, d, Ar), 7.64 (2H, m, Ar), 8.29 (1H, d, Ar), 8.44 (1H, d, Ar), 8.61 (1H, d, Ar).

Propyl β-D-galactopyranosyl-(1,4)-2-acetamido-6-[3-*p*-azidophenylamido]-2-dideoxy-β-D-glucopyranoside (30)



To a mixture of ethanol (400 μl) and water (200 μl) were added palladium hydroxide on carbon (5 mg) and azido sugar (**27**) (6.4 mg, 14.3 μmol). This was allowed to stir at room temperature for 3 hours after which time TLC (MeCN/IPA/50 mM KCl, 10:67:23) indicated the reaction had proceeded in excess of 90 %. The reaction mixture was passed through a bed of Celite, which was subsequently washed with methanol. After removal of solvent *in vacuo* the resulting solid was redisolved in 300 μl of DMF to which Sulfo-HSAB (N-hydroxysulfosuccinimydyl-4-azidobenzoate) (6.12 mg, 1.2 mol eq) in DMF was added. This mixture was allowed to stir at room temperature overnight when TLC showed all the free amine had been consumed. The mixture was then separated by preparative TLC (DCM/MeOH/H_2O, 8:2:0.1) and the relevant compound scraped from the plate and extracted into methanol. Removal of the methanol *in vacuo* gave the photoprobe (3.6 mg, 44 %). δ_H (D_2O) 0.66 (3H, t, $OCH_2CH_2CH_3$), 1.32-1.41 (2H, m, $OCH_2CH_2CH_3$), 1.91 (3H, s, $CH_3CO.N$), 3.33-3.85 (H-2, H-3, H-4, H-5, H-6_a, H-6_b, H-2', H-3', H-4', H-5', H-6a',H-6b' and OCH_2), 4.39 (1H, d, *J* 8.0, H-1/H-1'), 4.41 (1H, d, *J* 7.8, H-1/H-1'), 7.65 (2H, d, Ar), 8.04 (2H, d, Ar). ES-MS (+ve): *m/z* 592 ($M+Na^+$) ($C_{24}H_{35}N_5O_{11}$ requires 569). HRMS: Found 592.2223. $C_{24}H_{35}N_5O_{11}$ ($M+Na^+$) requires 592.2231.

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