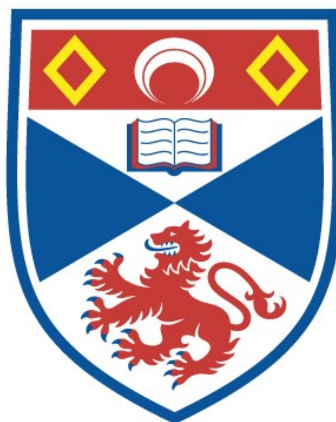
SYNTHESIS OF GANGLIOSIDE ANALOGUES AS BIOLOGICAL PROBES FOR ENZYMES WHICH MODIFY SIALIC ACIDS

William Bruce Turnbull

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A Thesis Submitted for the Degree of Doctor of Philosophy

W Bruce Turnbull

University of St Andrews

September 1998

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Abstract

Ganglioside GM3, a glycosphingolipid found in the outer membrane of all mammalian cells, is expressed at much higher concentrations than normal in cancer cells. It has been reported that GM3 can inhibit signal transduction *via* the EGF receptor kinase, ultimately down regulating cell growth. In contrast to this, de-*N*-acetyl GM3, another ganglioside that has been found in a number of cancer cell lines, is reported to be a potent promoter of cell growth. Evidence suggests that GM3 and de-*N*-acetyl GM3 are inter-converted by enzymes that remove and replace the *N*-acetyl group on sialic acid.

In this thesis, the syntheses of a number of simplified analogues of gangliosides are described, including some in which an inter-glycosidic oxygen atom is replaced by sulfur. Synthesis of *O*-linked ganglioside analogues was achieved by both a totally chemical route and also by a chemo-enzymatic route, involving the use of a recombinant construct of *Trypanosoma cruzi trans*-sialidase for glycosylation with sialic acid. A novel strategy for the synthesis of ganglioside analogues that contain a sialic acid $\alpha(2\rightarrow3)$ galactose thioglycosidic linkage, is also described. These compounds were evaluated as substrates and inhibitors for *Clostridium perfringens* neuraminidase and *Trypanosoma cruzi trans*-sialidase. Some of the ganglioside analogues described herein were used to develop biochemical assays for detecting the putatative de-*N*-acetylase and *N*-acetyltransferase activities that would be required for the inter-conversion of gangliosides GM3 and de-*N*-acetyl GM3.

Declaration

(i) I, William Bruce Turnbull, hereby certify that this thesis, which is approximately 35,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any other previous application for a higher degree.

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Acknowledgements

Thankyous ☺

There are many people who deserve my sincere thanks for help and friendship over the last three years, and for some, over many years before that. I must first thank my supervisor Dr Rob Field for his support and encouragement, for giving me the freedom to make my own way through this project and for presenting me with endless opportunities to see the world of science beyond the Purdie Building, its much appreciated. Also thanks to all my co-workers in the Field group, past and present, for many happy times in the lab, coffee room, pubs and wet afternoons in Caird Park dressed as penguins. There are too many names to mention, but notables are Dr Ravi Kartha for much help, especially in the early days and (soon to be Dr) Jenni Harrison for an endless supply of *trans*-sialidase. I must also thank Prof Ajit Varki of UCSD for welcoming me into his lab and basketball team and Justin Sonnenburg and Dr Andrea Koenig for being exceptional hosts in San Diego.

Outside the lab, I need to thank my long-suffering friends and flatmates who have put up with me day in day out and who have kept my life full of distractions from work. Thanks to Mum and Dad and my brother Graham for supporting me in everything that I've done.

Thanks also to Tom Edwards and Keith Ross for putting roofs over my head whilst semi-homeless and still writing-up.

Financial Support

I must finally give thanks to the Association for International Cancer Research for my studentship and research expenses and also to the School of Chemistry Dr E S Steele Prize Fund for the excellent trip to the Varki lab at UCSD.

Abbreviations Used in the Text

All carbohydrates are named in accord with IUPAC recommendations (*Carbohydr. Res.*, 1997, **243**, 1). Abbreviations for gangliosides (GM1, GM2, GM3, GM4, GD3 etc.) are as suggested by IUPAC and IUBMB (*Pure Appl. Chem.*, 1997, **69**, 2475) and are based on those originally suggested by Svennerholm (*J. Neurochem*, 1963, **10**, 613). The structures of common gangliosides are illustrated in Scheme 1.1.

A. ureafaciens	*Arthrobacter ureafaciens*
Ac	acetyl
ATP	adenosine triphosphate
Bn	benzyl
BSA	bovine serum albumin
Bz	benzoyl
C. perfringens	*Clostridium perfringens*
Cer	ceramide
CMP	cytidine 5'-monophospho
CoA	co-enzyme A
COD	cyclooctadiene
cpm	counts per minute
15-crown-5	1,4,7,10,13-pentaoxacyclopentadecane
18-crown-6	1,4,7,10,13,16-hexaoxacyclohexadecane
DAG	diacylglycerol
deNAc	de-*N*-acetyl
deNAcGM3	de-*N*-acetyl GM3
deNAcGD3	de-*N*-acetyl GD3
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DIG	detergent-insoluble glycolipid-enriched complex
DMF	*N,N*-dimethylformamide
DMTST	dimethyl(methylthio)sulfonium trifluoromethanesulfonate
dpm	disintegrations per minute
EGF	epidermal growth factor
EGF-RK	epidermal growth factor receptor kinase
ES-MS	electrospray mass spectroscopy
FACS	fluorescence activated cell scanning
FAB-MS	fast atom bombardment mass spectroscopy
Gal	galactose
GalNAc	*N*-acetylgalactosamine

GEM	glycosphingolipid-enriched microdomain
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GPI	glycosyl-phosphatidylinositol
GSL	glycosphingolipid
HMPA	hexamethylphosphorustriamide
HPLC	high performance liquid chromatography
IC_{50}	50% inhibition concentration
KDN	2-keto-3-deoxy-D-glycero-D-galacto-2-nonulopyranosylonic acid
kryptofix 21	1,7,10-trioxa-4,13-diazacyclopentadecane
Lac	lactose
LacNAc	*N*-acetyllactosamine
lit.	literature (reference)
ManNAc	*N*-acetylmannosamine
MAPK	mitogen activated protein kinase
Ms	methanesulfonyl
MTC	macrophage-mediated tumour cytotoxicity
Neu5Ac	*N*-acetylneuraminic acid
Neu5Ac2en	2,3-dehydro-*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
$Neu5NH_2$	neuraminic acid
NIS	*N*-iodosuccinimide
NMR	nuclear magnetic resonance
Oct	octyl
PKC	protein kinase C
*p*NP	*p*-nitrophenyl
SAP	sphingolipid activator protein
SE	(2-trimethylsilyl)ethyl
Sia	unspecified sialic acid
sLex	sialyl Lewis x
T. cruzi	*Trypanosoma cruzi*
TBA	tributylammonium
TBDMS	*t*-butyldimethylsilyl
Tf	trifluoromethanesulfonyl (triflyl)
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMSOTf	trimethylsilyltrifluoromethanesulfonate

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Chapter 1

Ganglioside Biology

1.1 Cell Surface Glycosylation

In recent years there has been a growing awareness of the biological importance of carbohydrates in molecular recognition processes and in the control of biological events.[1] In all organisms from bacteria to mammals, the cell surface is covered with a variety of complex carbohydrates (glycans) that form the first line of interaction with other cells, bacteria, bacterial toxins and viruses.[1] The glycans show great diversity in both size and structure despite comprising only a handful of common monosaccharide units. The common cell surface glycans can be classified into the following categories: *N*-linked and *O*-linked glycoproteins, proteoglycans, glycosyl-phosphatidylinositol (GPI) anchored proteins and glycosphingolipids (Figure 1.1). This project concerns the biological properties of a group of glycosphingolipids, the de-*N*-acetyl gangliosides. The structures, biosynthesis and biological properties of gangliosides and glycosphingolipids in general, will now be described in more detail.

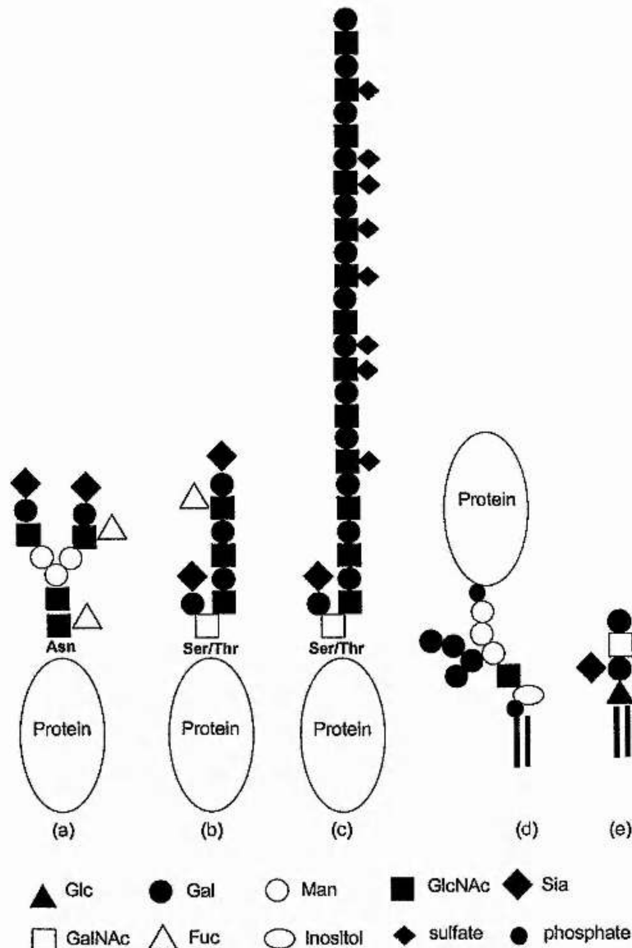


Figure 1.1 *Schematic examples of carbohydrate-containing cell surface molecules: (a) N-linked glycoprotein, (b) O-linked glycoprotein, (c) proteoglycan (keratan sulfate II), (d) GPI anchored protein and (e) glycosphingolipid (GM1).*

1.2 Glycosphingolipids

Glycosphingolipids (GSL) (Figure 1.2) are amphiphilic molecules found in the plasma membrane of all eukaryotic cells. The lipophilic ceramide portion is attached to a diverse range of oligosaccharides which can be divided into seven main classes on the basis of their root oligosaccharide structures: the ganglio-, lacto-, neolacto-, globo-, isoglobo-, mollu- and arthro-series (Appendix 1).[2]

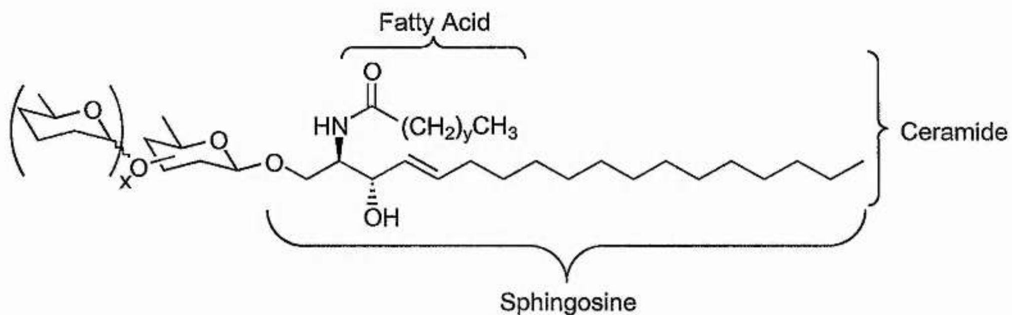


Figure 1.2 Schematic glycosphingolipid. Typically, x=0-8, y=12-22.

The ceramide portion also shows some degree of variation in the length of the fatty acid derived amide chain and 20-carbon sphingosine is also known in some of the more complex GSLs.[3, 4]

1.3 Glycosphingolipid Metabolism (Figure 1.3) [3, 4, 5]

1.3.1 GSL Biosynthesis. GSL biosynthesis begins with the assembly of the ceramide portion by enzymes bound to the cytosolic face of the endoplasmic reticulum. The lipid is then transported to the Golgi apparatus where glucose is first added, also on the cytosolic face. As all subsequent glycosylations occur on the lumenal face of the Golgi, glucosyl ceramide must be translocated to the interior of the Golgi compartment. This is thought to be facilitated by a "flippase" protein. Further elaboration of the glycosphingolipids inside the Golgi occurs under transcriptional control of the necessary glycosyl transferases[6] *i.e.* the relative activities of the various transferases present will determine which GSL core structure will predominate. Even within a particular class of GSL there are certain key biosynthetic branching points which restrict the GSLs to follow specific pathways. One such example is the action of GalNAc transferase I in committing the ganglio-GSLs to follow the A, B, C or O-series pathways (Scheme 1.1).

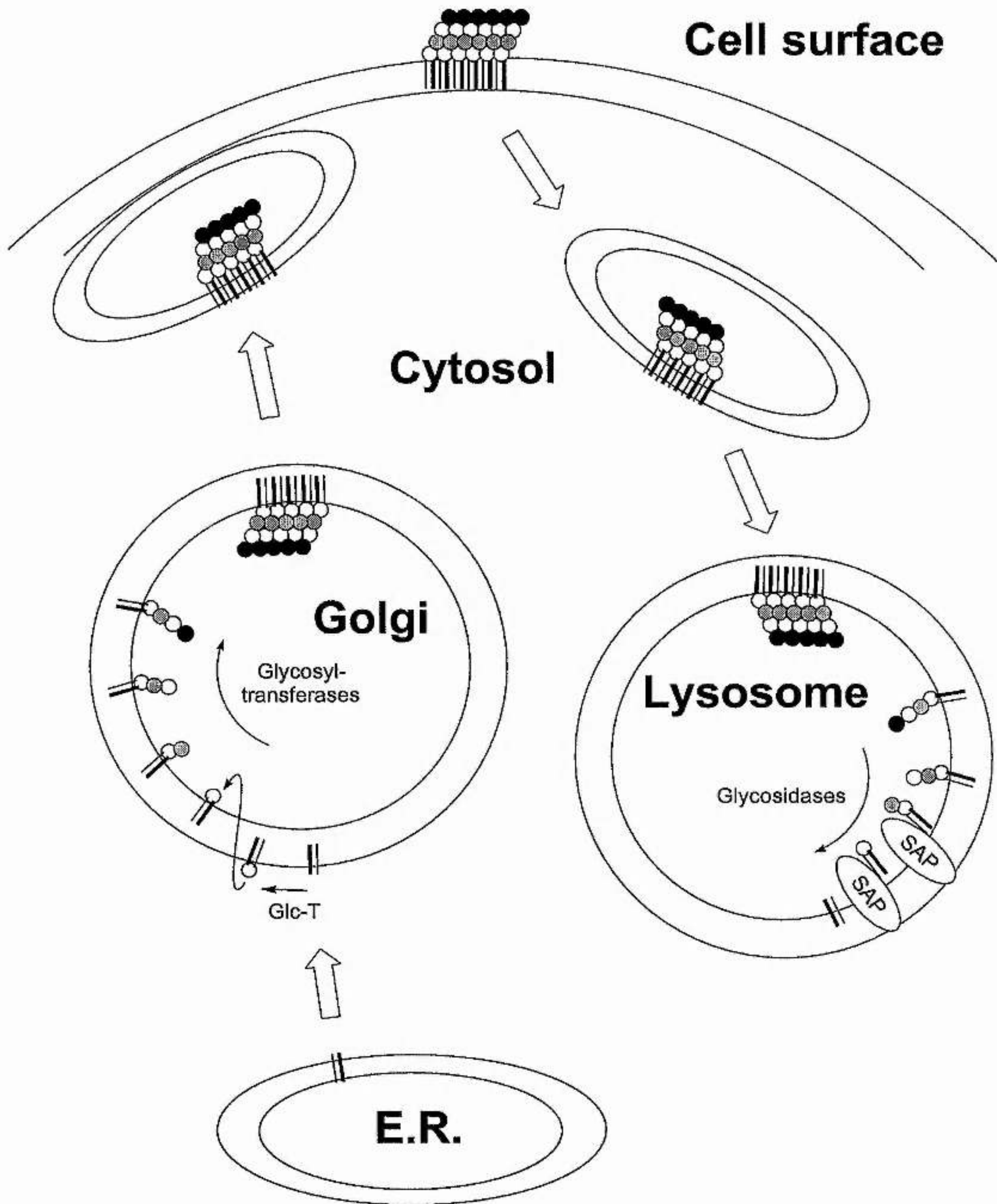
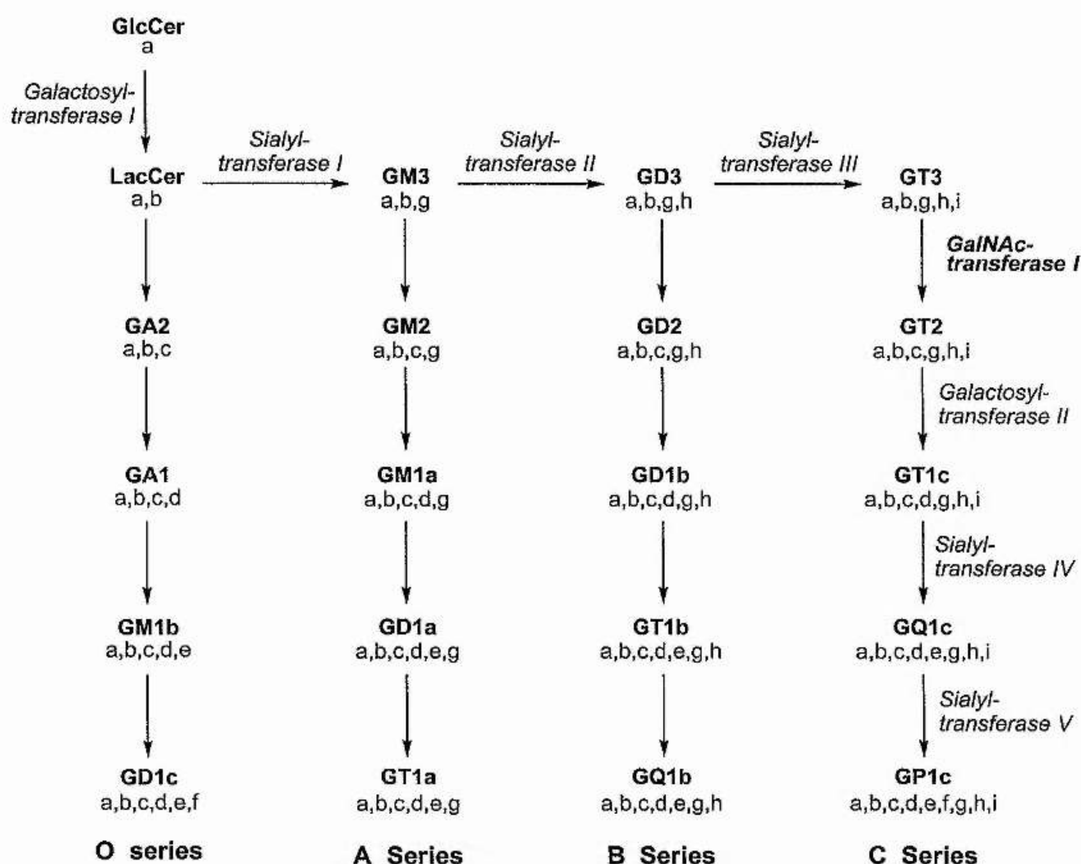
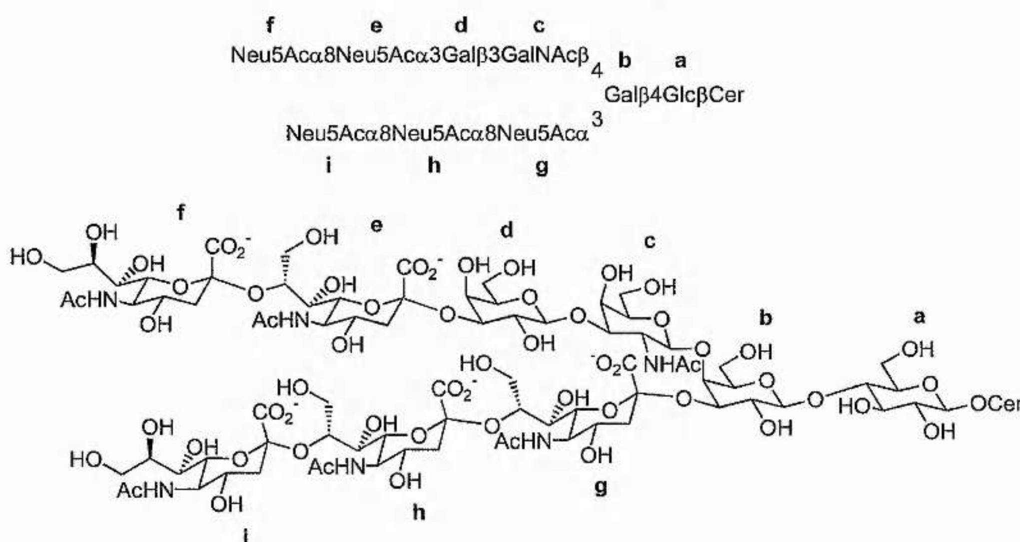


Figure 1.3 *Biosynthesis and degradation of GSLs within a cell.*





Scheme 1.1 *Biosynthesis of A, B, C and O series gangliosides.*

As GSLs travel through the Golgi compartments, they cluster together to form specialised "rafts"[7] or GSL-enriched microdomains (GEMs) which may also be associated with specific membrane bound proteins.[8] On fusing with the plasma membrane, the Golgi compartment is turned inside out, thus delivering the "rafts" of GSLs specifically to the outer leaflet of the plasma membrane (Figure 1.3).

1.3.2 GSL Catabolism. Degradation of GSLs usually requires their reinternalisation and transport to the lysosomes through a series of endosomal compartments (Figure 1.3). Typically, degradation is performed in a similar stepwise manner to biosynthesis, by a series of exoglycosidases[5] but an endoglycosylceramidase which removes the intact oligosaccharide is also known.[9] Removal of the innermost sugar residues requires the assistance of specific sphingolipid activator proteins (SAPs) which lift the GSLs out of the membrane allowing the relevant glycosidases to act more efficiently.[5] These processes ultimately release sphingosine (not produced in its free form during biosynthesis) which may either re-enter the biosynthetic pathway or be further degraded.

1.4 Gangliosides

Gangliosides are GSLs with one or more sialic acid residue(s) attached to the oligosaccharide core.[3]

1.4.1 Structure and Biosynthesis of Sialic Acids.[10, 11] Sialic acid (Sia) is the generic name for a family of 9-carbon monosaccharides based on neuraminic acid, the most common of which is *N*-acetyl neuraminic acid (Neu5Ac), **1** (Figure 1.4).

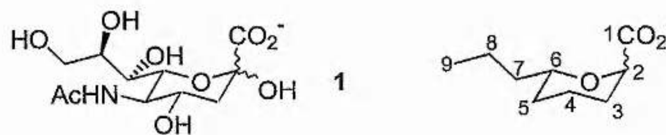
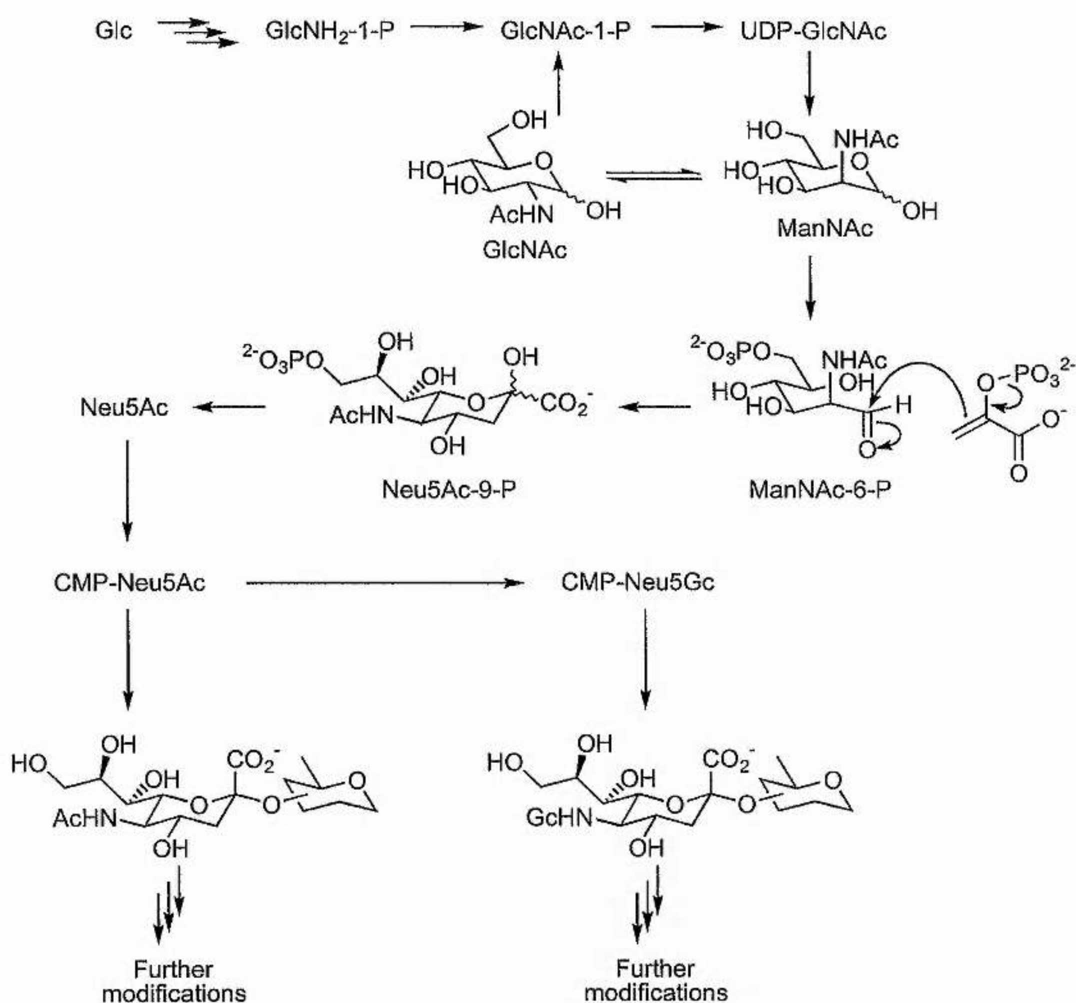


Figure 1.4 *N-acetylneuraminic acid, 1, and its numbering scheme.*

The first key step in the biosynthesis of sialic acids (Scheme 1.2) is the epimerisation of *N*-acetyl glucosamine (GlcNAc; synthesised by *N*-acetylation of glucosamine-1-phosphate) to *N*-acetyl mannosamine (ManNAc).[12] ManNAc is then phosphorylated at the 6-position before condensation with phosphoenolpyruvate to give Neu5Ac-9-phosphate. The phosphate must then be removed and Neu5Ac converted to the nucleotide sugar cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMPNeu5Ac) before it can be incorporated into glycans by a variety of sialyl transferases. CMPNeu5Ac is also the substrate for *N*-acetyl neuraminate monooxygenase,[11] an enzyme which converts the *N*-acetyl group into an *N*-glycolyl (2-hydroxyacetyl) group and leads to the second major series of sialylated glycans, *i.e.* those containing Neu5Gc. Although Neu5Gc is the predominant sialic acid in many mammals (*e.g.* pig, cow), it is not normally detectable in human tissues. However, Neu5Gc has been detected in gangliosides present in both human colon[13] and lung tumours.[14]



Scheme 1.2 *Biosynthesis of sialic acids.*

The CMPSias are the only examples of naturally occurring β-linked sialic acids. All sialic acid *O*-glycosides are α-linked, typically to position 3 or 6 of a galactose residue or position 8 of another sialic acid, although other linkages are known.[11] Sialosides are not usually further glycosylated (apart from Siaα(2→8)Sia which forms the repeat unit of the so-called poly sialic acid), however a wide range of alternative modifications to the basic Neu5Ac or Neu5Gc structures have been reported.[15] These derivatives form the ever growing family of the sialic acids (Figure 1.5).

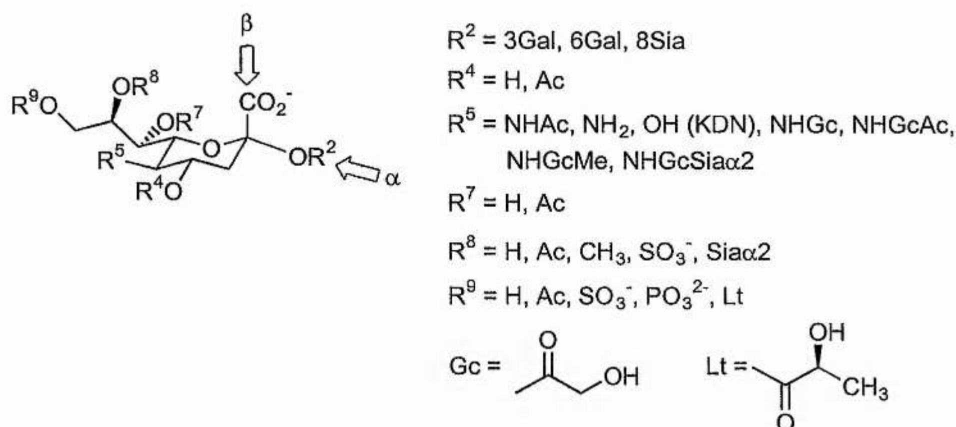


Figure 1.5 *Some modifications of sialic acid found in glycoconjugates.*[10, 15]

The most common modification is acetylation which may be found at any of the 4 Sia hydroxyls (positions 4, 7, 8 and 9). Acetyltransferases and esterases have been extensively studied,[16] although non-enzymatic acetyl migration along the glycerol side-chain can also occur under physiological conditions.[11] Other modifications so far observed include a lactyl ester at *O*-9, methylation at *O*-8, sulfate groups at *O*-8 and *O*-9 and phosphate at *O*-9. The acetamido group at position 5 can be de-acetylated to give the amino sugar [17, 18] and the 2-hydroxyacetyl group of Neu5Gc can be acetylated, methylated or even sialylated.[10] 2,7 and 4,8 anhydro sialic acids have been identified and also lactones formed by cyclisation of the Sia carboxyl group onto a hydroxyl group of the neighbouring sugar residue. Although only few of the enzymes which perform these modifications have been characterised, it is believed that these transferases only act on glycan-bound Sia in the Golgi or other post-Golgi compartments. Although sialic acids are found in some bacteria, viruses, *Drosophila melanogaster* and, phylogenetically, all animals from starfish upwards, many of the modified Sias mentioned above, are both species and tissue specific and may only be expressed at certain points during the development of the organism.[10, 11]

Recently, a new family of sialic acids, which have a hydroxyl group at C-5 (instead of the amino group present in neuraminic acid), was discovered.[19] 2-Keto-3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid (KDN) is biosynthesised in a manner analogous to Neu5Ac, but starting from mannose rather than *N*-acetyl mannosamine. This novel sialic acid, first detected in fish, has been shown to be present in a wide range of organisms from bacteria to mammals including humans.[20] KDN occurs in both glycoproteins and glycolipids, but its discovery and much of the subsequent research concerned its presence as an $\alpha(2\rightarrow8)$ polymer which is expressed temporally during cell development and also in cancer cell lines.[21] KDN

glycosides are resistant to most neuraminidases, but are cleaved by a specific "KDNase", which itself, does not recognise Neu5Ac.[22]

1.5 Biological Functions of Gangliosides

Gangliosides are found at the surface of all mammalian cells, but the composition and distribution of different gangliosides varies according to cell type. There are marked changes in these "ganglioside profiles" during foetal development, cell differentiation and ageing[3] and also during the process of oncogenesis.[23, 24] Indeed, virtually all cancer cells exhibit aberrant ganglioside profiles and the over- or under-expression of various gangliosides is associated with morphological changes of the cell.[25] Gangliosides have been implicated in a wide range of biological activities: cell-cell recognition and adhesion;[24, 26] induction of both an antigenic response[27] and suppression of the immune system;[28, 29] modulation of various membrane-bound enzymes, including those involved in signal transduction, thus controlling both cell proliferation and cell death.[30, 31]

One such biologically important molecule is ganglioside GM3, **2** (Neu5Acα(2$\rightarrow$3)Galβ(1$\rightarrow$4)Glcβ(1$\rightarrow$1)Cer; Figure 1.6), a common glycolipid which is often expressed in high concentrations in cancer cells.

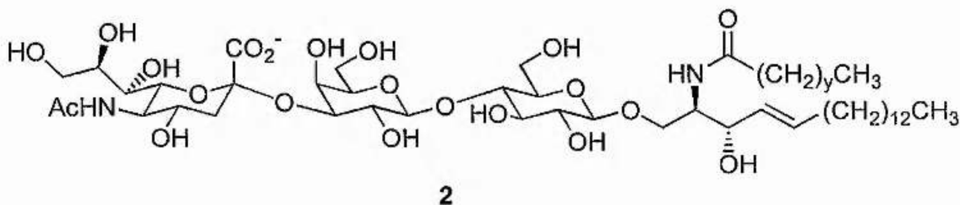


Figure 1.6 *Ganglioside GM3. Typically y=12-22.*

GM3 provides a good illustration of most of the common roles of the gangliosides:

1.5.1 Immunosuppression. Tumour cells often actively shed gangliosides from their membranes, thus surrounding themselves with a high local concentration of glycolipids. This has been implicated in inhibiting the immune response to tumours by inhibiting T-cell proliferation[29, 32] whilst enhancing T-suppressor activity.[28] GM3 is one of the most potent glycolipids involved in these processes.

1.5.2 Macrophage-Mediated Tumour Cytotoxicity. In apparent contradiction to section **1.5.1**, GM3 can activate macrophage-mediated tumour cytotoxicity (MTC).[33] The ganglioside acts by stimulating nitric oxide production by the macrophages. This process is analogous to bacterial lipopolysaccharide stimulation of MTC.

1.5.3 Tumour Associated Carbohydrate Antigens. Although GM3 is present in all mammalian cells, it has been demonstrated to be the antigen recognised by antibodies raised against melanoma cells.[27] However, the immune response to this antigen is density dependent and will only be triggered above a threshold concentration that is only observed in cancer cells. There has been much interest in the use of ganglioside antigens, conjugated to protein carriers, as potential anti-cancer vaccines.[34]

1.5.4 Cellular Recognition and Adhesion. Minimum energy conformational models of gangliosides in membranes indicate that the axis of the oligosaccharide portion is oriented perpendicular to the axis of the ceramide moiety, such that the oligosaccharide lies in the plane of the membrane surface (Figure 1.7).[23] Various biophysical studies have shown that many such molecules come together in large clusters, interspersed with cholesterol molecules (the so-called GSL-enriched microdomains, GEMs) rather than being evenly dispersed across the membrane (Figure 1.7).[7, 8, 23] These clusters may form ideal sites for carbohydrate-protein or carbohydrate-carbohydrate interactions with bacteria,[26] viruses,[35] or with other cells.[24, 36]

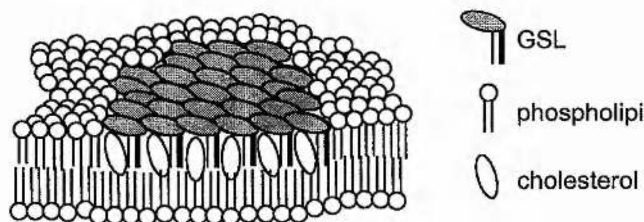


Figure 1.7 *GSL-enriched microdomain in a lipid bilayer.*

1.5.4.1 Bacterial adhesion to Neu5Gc-GM3. Many bacterial adhesion proteins and toxins recognise specific cell surface gangliosides.[37] Ganglioside GM3 with *N*-glycolylated sialic acid has been shown to be the receptor for the enterotoxigenic *E. coli* K99 bacterium.[26] This bacterium causes severe diarrhoea in piglets, lambs and calves, all of which express GM3 principally with Neu5Gc. Older animals mainly carry the inactive Neu5Ac-GM3, and are thus less susceptible to this particular bacterial infection.

1.5.4.2 Viral adhesion to GM3 and CD4/GM3. GM3 is highly expressed on the surface of CD4+ lymphocytes and macrophages. Recent studies have shown that GM3 could act as a cell surface receptor for HIV-1 through its interaction with HIV-1 glycoprotein gp120.[35] Alternatively, the ganglioside may also act as a "fusion co-factor" in association with CD4.[38]

1.5.4.3 Modulation of α5β1 integrin adhesion to fibronectin. It has been known for some time that gangliosides can affect the adhesion of cells to others expressing fibronectin at their surfaces.[39] GM3 has a biphasic effect on adhesion of fibronectin to the α5β1 integrin receptor; at low concentrations of GM3, integrin adhesion is considerably enhanced (relative to no GM3), whereas at higher concentrations, GM3 mildly inhibits the recognition event.[36]

1.5.4.4 GSL-GSL interactions in metastatic deposition. It has been proposed that interactions between GEMs of GM3 on melanoma cells and gangliotriaosylceramide (Gg_3*) **3**, or lactosylceramide **4** (Figure 1.8) on the surfaces of lymphoma or endothelial cells form the basis for initial adhesion prior to metastatic deposition of the cancer cells.[40, 41]

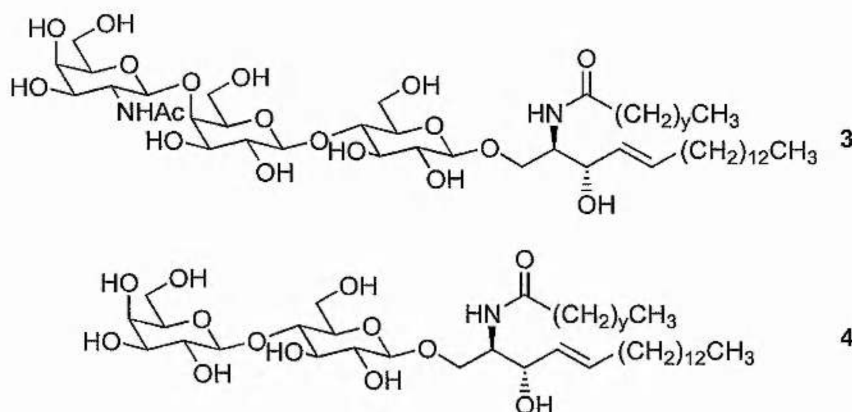


Figure 1.8 *Gangliotriaosylceramide and lactosylceramide. Typically y=12-22.*

GSL-GSL interactions are considerably weaker than those involving adhesion proteins such as integrins. However, under dynamic flow conditions, designed to mimic the microvascular environment under which metastatic depostion of tumour cells occurs, the much more rapid GSL-GSL interactions predominate over the slower acting, but higher affinity integrin based adhesion.[24] It has also been demonstrated that liposomes containing either GM3 or Gg_3 can reduce metastasis *in vivo* and could prove to be an effective anti-adhesion therapy against tumour progression.[42]

* Gangliotriaosylceramide (Gg_3) and lactosyl ceramide (LacCer) may also be thought of as asialo- or O-series gangliosides GA2 and GA3, respectively. However, in the literature, they are more commonly referred to as Gg_3 and LacCer.

1.5.5 Modulation of Membrane Transporters. The flow of ions and metabolites across membranes is often facilitated by specific membrane bound transporter proteins. Incorporation of GM3 into membranes containing either the human erythrocyte glucose transporter (GLUT1)[43] or the sarcoplasmic reticulum Ca^{2+}-ATPase,[44] has been shown to cause conformational changes in each of these proteins. In both cases, the conformational changes are accompanied by an increase in the activity of the transporter.

1.5.6 Signal Transduction. In order to maintain normally functioning healthy tissues all aspects of individual cell function must be tightly controlled. Such control throughout a tissue requires an external stimulus to be recognised by receptors on each cell and the information contained in this recognition event to be amplified and relayed to the nucleus, usually by a complex protein kinase cascade e.g. the mitogen activated protein kinase (MAPK) cascade.[45] Changes in cell function can then be effected by expression of appropriate genes. This process of conveying information from a point outside the cell to the nucleus is called signal transduction.

There have been many reports on the role of gangliosides in controlling signal transduction.[8, 23, 34, 46, 47] Modulation of signal transduction probably forms the basis for GM3 induced inhibition of angiogenesis around tumours (growth of new blood vessels required to supply the growing tumour with nutrients),[47, 48] GM3 promoted cell differentiation[46] and GM3 stimulated apoptosis (programmed cell death) in thymocytes,[49] and probably also several of the biological activities listed above. Gangliosides alone do not appear to activate or inhibit intracellular kinases and phosphatases.[50] The two general mechanisms for the involvement of gangliosides in signal transduction that have been proposed are that (i) they act as *signal transducing* molecules for extracellular messengers,[51] and (ii) they modulate *growth factor induced* activation of growth factor receptor kinases.[46]

1.5.6.1 Gangliosides as signal transduction molecules. Low density detergent-insoluble glycolipid-enriched complexes (DIGs) prepared from B16 melanoma cells contained 90% of cellular GM3 and <2% of total cellular proteins.[8] However, 50-90% of the total amount of several signal transducer proteins (*c-src*, *rho* and *ras*) were found in the DIGs. These transducer proteins were found to be activated when the B16 cells bound to Gg3 covered plates (see section **1.5.4.4**).[8] Similarly, the *src* family kinase *lyn* was found associated with DIGs from rat brain, principally comprising GD3.[51] Anti-GD3 antibodies were able to activate *lyn* and also other substrates including MAPK. Therefore GEMs associated with intracellular protein

kinases may widely act as transduction receptors for various antibodies, lectins and GSL patches on other cells (Figure 1.9).

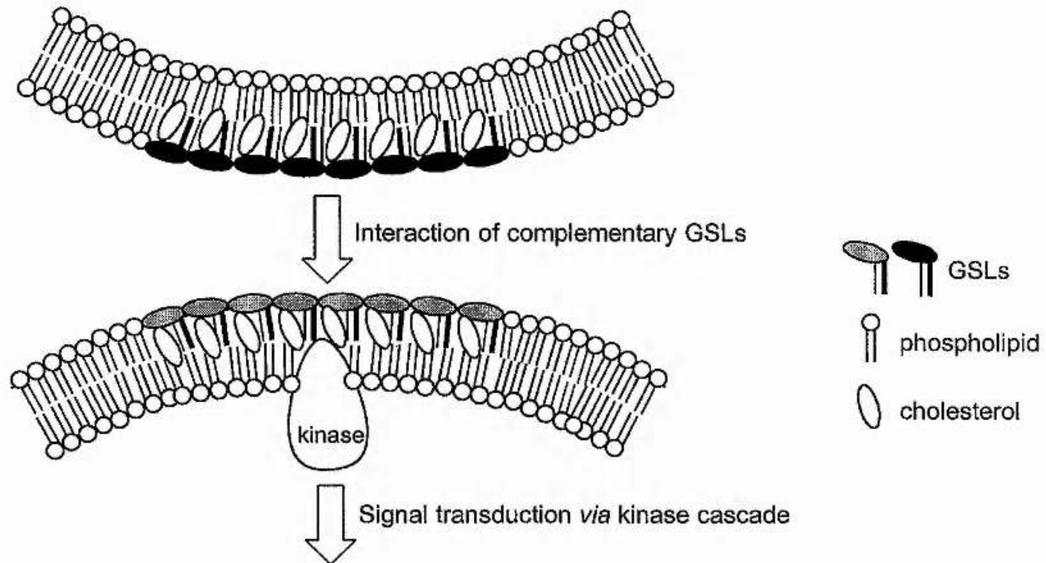


Figure 1.9 *Gangliosides act as signal transducing molecules.*

1.5.6.2 Gangliosides modulate growth factor receptor kinases. Several growth factor receptor kinases are affected by the presence of specific gangliosides.[46] The signal transduction pathway initiated by the interaction of the epidermal growth factor (EGF) peptide and its receptor (EGF-RK), has been shown to be controlled by gangliosides GM3 and de-*N*-acetyl GM3 (deNAcGM3).[17, 30, 52] The roles of EGF-RK, GM3 and deNAcGM3 in this process will now be discussed in more detail.

1.6 Control of the EGF Receptor Kinase by GM3 and DeNAcGM3

1.6.1 EGF Receptor Kinase. The EGF receptor is a 180kDa transmembrane glycoprotein composed of three distinct domains.[53, 54] The extracellular domain has 12 possible sites for asparagine-linked glycosylation and it is expected that most of these sites do have pendant carbohydrate groups.[55] This is the domain that binds the 6kDa EGF peptide and is connected *via* a single membrane spanning region of 23 hydrophobic amino acids to the internal kinase domain which has a *src* type structure. The EGF-RK is over expressed in some tumours and monoclonal antibodies directed against the receptor are in clinical trials as potential anti-cancer drugs.[56]

On binding EGF, a conformational change allows receptor dimerisation to occur and subsequent autophosphorylation of a tyrosine residue on the internal domain activates the kinase (Figure 1.10). The nature of the conformational change is not

fully understood, but there is considerable evidence that dimerisation is normally required for the autophosphorylation,[53] possibly by aligning the kinase domains for an intermolecular reaction (although under certain conditions the monomer can autophosphorylate[52]). Once activated, the EGF receptor kinase acts, *via* a series of transducer molecules, to initiate the MAPK cascade which conveys the message to the nucleus, ultimately enhancing cell growth and division (Figure 1.10).

It has also been shown that EGF-RK is associated with phosphatidylinositol 4-kinase and phosphatidylinositol-4-phosphate 5-kinase.[57] The activities of these two enzymes are enhanced on EGF-RK activation, thus up-regulating the production of inositol 1,4,5-trisphosphate and diacylglycerol which in turn increase cytosolic Ca^{2+} concentration and activate protein kinase C (PKC). PKC is also capable of activating the Raf-1 kinase which leads into the MAPK cascade.[45]

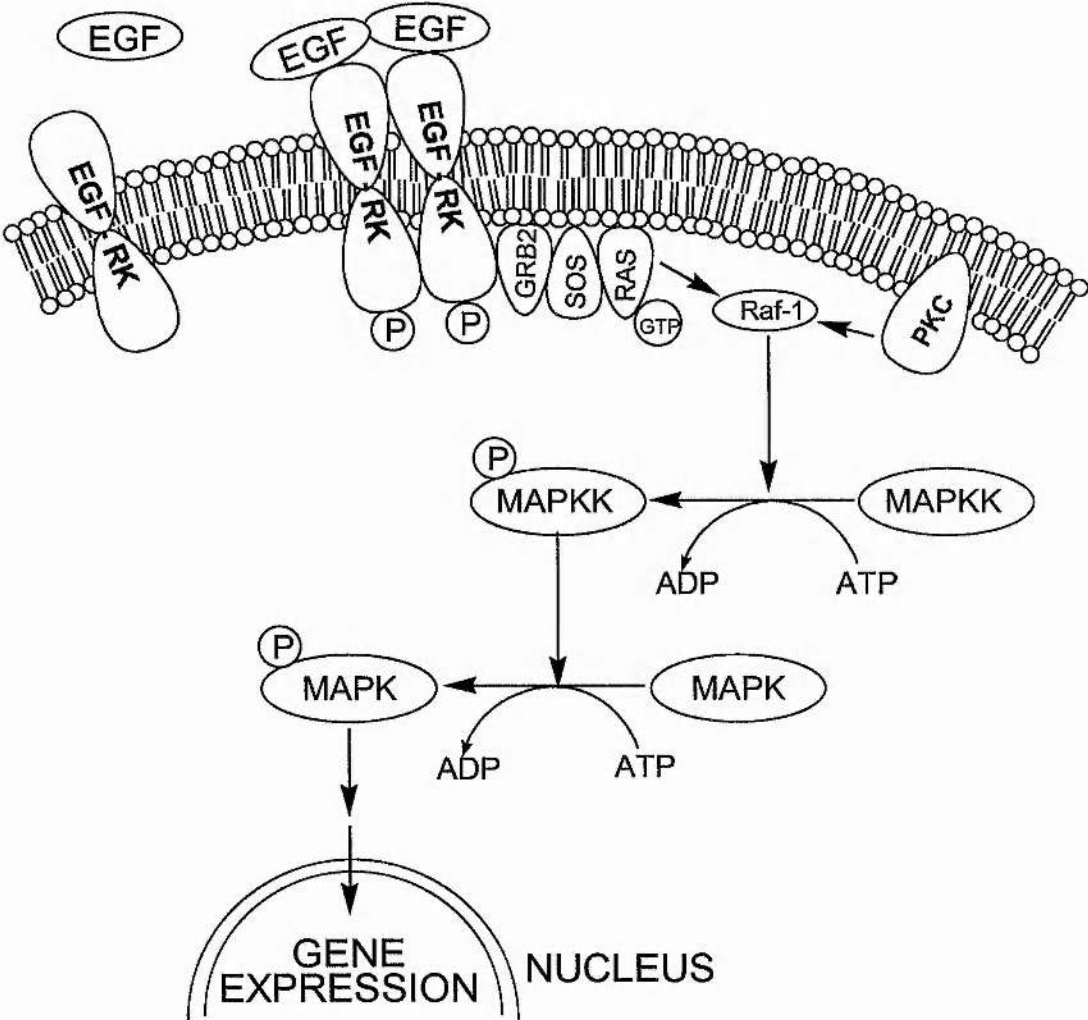


Figure 1.10 *Activation of EGF-RK.*

1.6.2 Modulating Receptor Kinase Activity. There are several lines of evidence that cell proliferation due to growth factor receptor kinases can be regulated by gangliosides.[30] There are a number of ways in which these glycolipids could modulate growth factor receptor kinase activation:

(a) Direct interaction with the extracellular domain could prevent growth factor binding, possibly due to a conformational change of the receptor binding site.

(b) Gangliosides are detergent like molecules and may thus have an effect on the fluidity of the membrane which could alter the ability of the receptors to dimerise.

(c) Either through direct interactions or by changing the membrane environment, gangliosides could prevent kinase activation by inducing a conformational change in protein (see section **1.5.5**).

(d) As the sialic acid residue is negatively charged, it could potentially act as a competitive inhibitor with respect to ATP binding on the intracellular domain.

1.6.3 GM3 and DeNAcGM3. Ganglioside GM3 and the structurally similar compound de-*N*-acetyl-GM3 (deNAcGM3; Figure 1.11) **5** have been observed to modulate the activation of EGF-RK.

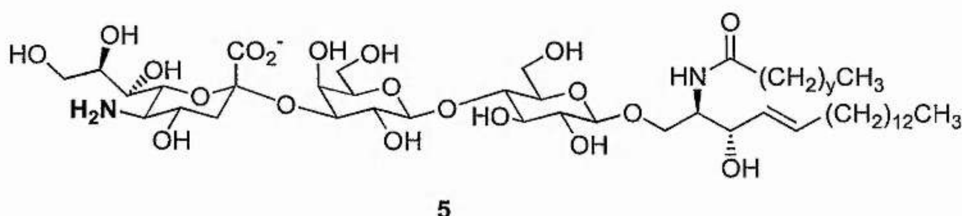


Figure 1.11 *Modified ganglioside deNAcGM3. Typically y=12-22.*

1.6.3.1 GM3 inhibits EGF-RK activation. GM3 has the ability to inhibit EGF stimulated cell growth in a variety of cell lines derived from different tissues.[30, 58, 59] It acts by preventing autophosphorylation of EGF-RK at tyrosine residues.[30] The mechanism of this inhibition is not known, but extensive investigations in this area have been carried out by Hakomori, Bremer and others.[46] Vesicles containing EGF-RK molecules show selective binding to GM3 coated plates.[60] Sialyl lactose conjugated to BSA also has the ability to block the autophosphorylation event.[50] GM3 does not affect intracellular protein phosphatases, which could reduce EGF-RK phosphorylation, nor does it have any direct effect on purified intracellular kinases.[50] EGF binds to its receptor with the same affinity in both the presence and absence of GM3.[61] There are conflicting reports regarding the effect of GM3 on receptor

dimerisation,[46, 52] but GM3 inhibits EGF-RK phosphorylation in both monomeric and dimeric EGF-RK.[52] The inhibitory effect of GM3 may be part of a larger feedback loop control, as addition of EGF to retinal Müller glia has been shown to stimulate ganglioside synthesis two-fold.[62] GM3 constituted over 50% of the gangliosides produced.

Many of the studies mentioned above have relied on the use of membrane fractions prepared in the presence of the detergent Triton X100 and/or the addition of high concentrations of exogenous gangliosides. In the presence of low concentrations of detergent GM3 showed inhibitory properties, but at high detergent concentration, GM3 actually stimulated EGF-RK phosphorylation on addition of EGF.[63] However, experiments using only a freeze-thaw cycle to permeablise the cells confirmed that exogenous GM3 does inhibit EGF-RK activation.[64]

A number of studies have also addressed the question of whether addition of high concentrations of exogenous GM3 simply give rise to a pharmacological effect, or one that is physiologically relevant.[50] Gangliosides added to cell growth medium become incorporated into the cell by a two step mechanism;[61] an initial fast adhesion to cell surface proteins is followed by a much slower insertion into the membrane. Even after 48 hours, much less than 10% of exogenous gangliosides may be incorporated. An elegant study by Weis and Davis demonstrated that endogenous GM3 can have the same effect as exogenously added ganglioside.[65] They transfected the gene for human EGF-RK into a cell line deficient in the epimerase required to synthesise galactose from glucose. Therefore, the cells could not express any GSLs other than glucosyl ceramide in the absence of galactose and GalNAc in the growth medium. On supplementing these two sugars in the growth medium, the cells were able to synthesise GM3 and showed a concomitant reduction in EGF stimulated receptor phosphorylation.

Also, it has been demonstrated that exogenous addition of *Clostridium perfringens* neuraminidase to human skin fibroblasts stimulates cell growth.[66] This has been rationalised as enzymatic degradation of GM3 relieving the inhibitory effect of the ganglioside on EGF-RK. Although, in this case, the sialidase could also be operating by a mechanism as outlined in section **1.5.6.1**, several endogenous extracellular sialidases have been characterised.[66] It may transpire that these have a role in modulating the GM3/EGF-RK interaction.

1.6.3.2 deNAcGM3 enhances EGF-RK activation. DeNAcGM3 **5** is another physiological membrane component which has been identified in a wide variety of cancer cell lines.[52] It was isolated by HPLC of the monosialoganglioside fraction, extracted from B16 melanoma cells (and other cell types) with detection by *N*-acetylation with [^{14}C]acetic anhydride or immunoblotting with a specific monoclonal antibody.[17] DeNAcGM3 occurs only in very low concentrations (~1pmol/5×10^6 B16 cells), but in some tumour cell types it has been observed in higher concentrations than GM3.[52] However, this ganglioside is not observed at the cell surface by immunoblotting techniques. Either deNAcGM3 is present in too low a concentration for detection, or it is simply not expressed at the cell surface, except, perhaps transiently.

The biology of deNAc gangliosides has not been investigated widely, other than the role of deNAcGM3 in modulating EGF-RK activation.[17, 52, 64] Hakomori and co-workers discovered that deNAcGM3 could enhance cell growth when added exogenously to a cell culture.[17] They also reported that this modified ganglioside strongly enhances phosphorylation of EGF-RK in membranes solubilised with the detergent Triton X100. DeNAcGM3 had no effect on the affinity of EGF for its receptor. As mentioned in section **1.6.3.1**, the results of such experiments may be influenced by detergent concentration. Other investigators were able to confirm the effect of deNAcGM3 on EGF-RK in the presence of Triton X100, but not in the absence of detergent.[46, 64] Hakomori subsequently published further convincing evidence in favour of EGF-RK modulation by deNAcGM3.[52] He showed that in the absence or in the presence of minimal amounts of detergent (<0.001%), exogenous deNAcGM3 increased total EGF-RK phosphorylation two fold. This was principally due to increased phosphorylation at serine rather than at tyrosine residues. However, at higher detergent concentration, >0.025% (*i.e.* above the critical micellar concentration for Triton X100), enhanced phosphorylation was mainly of tyrosine residues. Enhanced phosphorylation of serine residues would imply the involvement of a different protein kinase. For example, protein kinase C enhances serine and threonine phosphorylation and inhibits tyrosine phosphorylation of EGF-RK.[67] Thus deNAcGM3 may modulate EGF-RK activation by enhancing an alternative kinase that is serine specific.

The stimulatory effect of deNAcGM3 on EGF-RK activation is in striking contrast to the inhibitory effect of GM3 on the same system; a simple de-N-acetylation creates a potent compound which is strongly antagonistic towards the action of the parent ganglioside.

Curiously, deNAcGM3 inhibited phytohaemagglutinin-induced lymphocyte proliferation by 37% at an exogenous ganglioside concentration of 50 nmol/cm^3.[28] This is in apparent contradiction to the stimulatory effect of deNAcGM3 described above, but the ganglioside literature is not without contradictions.

1.6.3.3 Other sphingolipids that affect signal transduction. Several other degradation products of gangliosides have potent effects on signal transduction. A GM3 analogue which has lost the fatty acyl chain from ceramide (lysoGM3, **6**; Figure 1.12) inhibits EGF-RK activation in the same manner as GM3.[63]

6

Figure 1.12 *Modified ganglioside lyso-GM3.*

However, ceramide and sphingosine both stimulate EGF-RK phosphorylation at serine and threonine residues.[52] Diacylglycerol (DAG), which activates protein kinase C (PKC), is the classic example of a lipid degradation product acting as a secondary messenger. Both lactosylsphingosine and *N,N*-dimethylsphingosine act in contrast to DAG, by down-regulating PKC activity.[9, 17] It was noted in section **1.6.3.1** that, because exogenous GSLs are inefficiently incorporated into the membrane, high concentrations of GSLs (~50μM) are often required to observe biological effects. In the case of ceramide, 1μM or less can can induce changes in signal transduction.[46] Therefore, even slight contamination of GSL preparations with other sphingolipids may give misleading results.

1.6.4 Biosynthesis of de-*N*-acetyl Gangliosides. The ganglioside deNAcGD3, **7** (Figure 1.13) has been identified in Melur melanoma cells by Varki and co-workers.[68]

7 R^1, R^2 = H, Ac or $R^1 = R^2$ = H

Figure 1.13 *Modified ganglioside deNAcGD3; either, or possibly both sialic acid residues may have a free amino group.*

Whilst studying the turnover of sialic acid *O*-acetyl groups using tritiated acetate or acetyl CoA, they found that the radio-label accumulated in the acetamido group of the sialic acid. As the experiments using [^{3}H]-acetyl CoA were performed using washed Golgi-enriched vesicles, the possibility of [^{3}H]Ac incorporation at the GlcNAc stage of sialic acid biosynthesis (see section **1.4.1**) was considered unlikely in the absence of the required cytosolic enzymes and their associated co-factors. By doubly labeling cells with [1-^{14}C]GlcNAc and [^{3}H]-acetyl CoA, they were able to show that there was a higher rate of *N*-acetyl turnover than Sia turnover for both GM3 and GD3 in Melur cells. However, the expression of deNAc gangliosides appears to be "transient and variable" which has complicated further studies. Genistein, **8**, a tyrosine kinase inhibitor, was able to increase the amount of deNAcGD3 in Melur cells.[18] It has been suggested that genistein may stop the cell cycle at a stage when deNAcGD3 expression is elevated. Immunochemical studies have indicated the presence of deNAcGD3 in lymphocytes and in human melanoma tumours and cell lines.[69] Although GD3 is abundant at the cell surface, deNAcGD3 was only found in an intracellular compartment. However, nocodazole, **9**, an inhibitor of microtubule assembly, caused deNAcGD3 to accumulate at the cell surface,[69] suggesting that it may be transiently expressed outside the cell and then rapidly internalised. No physiological role for deNAcGD3 has been proposed.

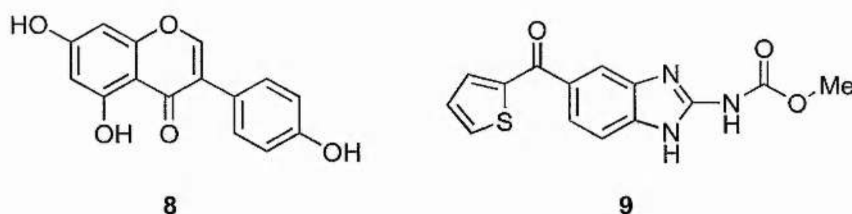
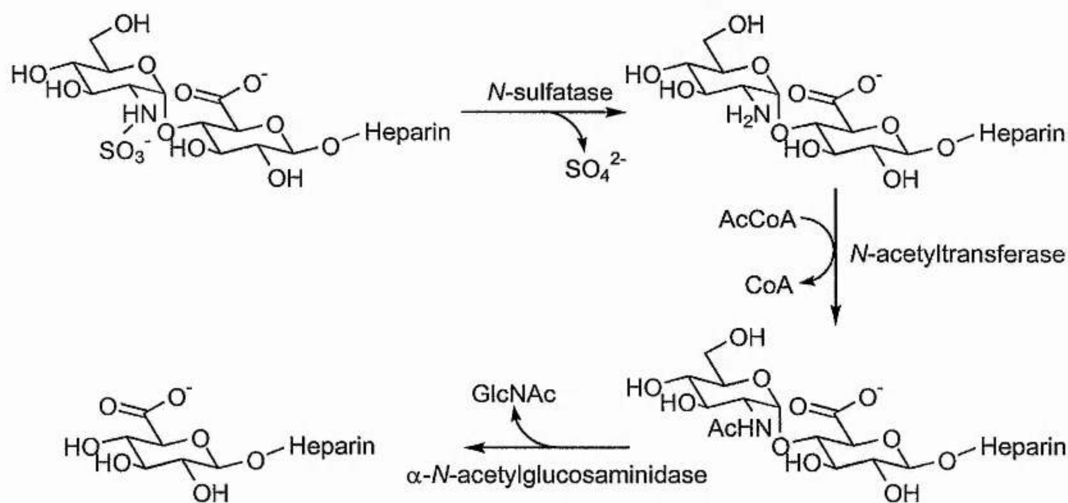


Figure 1.14 *Genistein and nocodazole affect expression of deNAcGD3.*

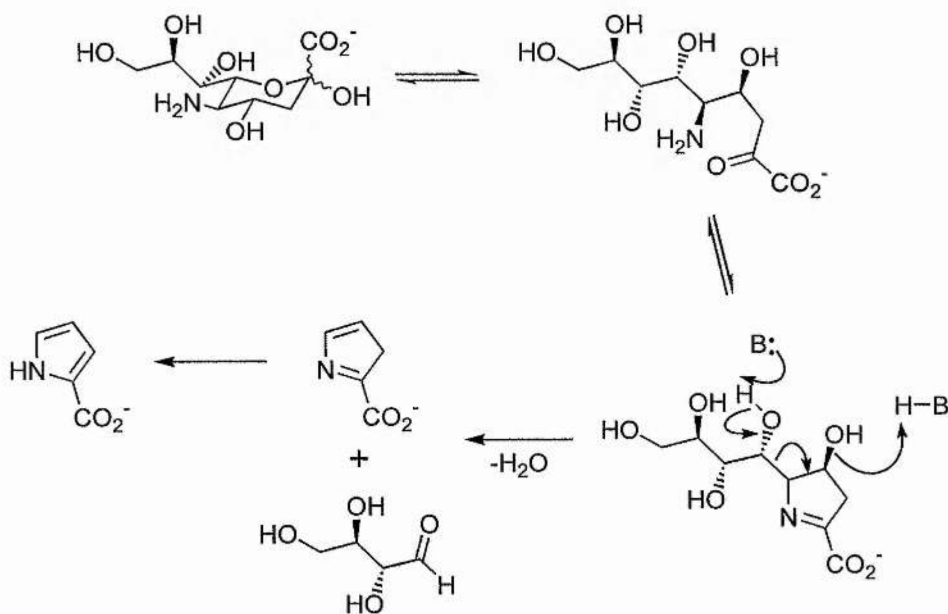
The above studies show that deNAc gangliosides are naturally occurring components of cell membranes and may have potent biological activities. Evidence suggests the presence of specific de-*N*-acetylase and acetyltransferase enzymes which would catalyse the synthesis and degradation of deNAc gangliosides. De-*N*-acetylase enzymes which act on sugars have been reported in the biosynthesis of Nod factors,[70] lipid A,[71] heparin/heparan sulfate (combined *N*-deacetylase/*N*-sulfotransferase)[72] and GPI anchored proteins.[73] *N*-Acetyltransferases are involved in the synthesis of GlcNAc and in the degradation of heparin and heparan sulfate.[74] The latter case is an unusual example of a biosynthetic enzyme involved in a lysosomal catabolic process.

Acetyl-coenzyme A:α-glucosaminide *N*-acetyltransferase is one of at least eight enzymes involved in heparin degradation, the others being sulfatases and exo-glycosidases.[74] As there is no glycosidase capable of hydrolysing either GlcN-sulfate or glucosamine, *N*-sulfated glucosamine residues must be desulfated and then *N*-acetylated prior to further degradation of the heparin chain (Scheme 1.3).



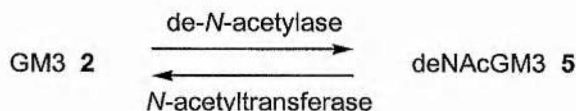
Scheme 1.3 *Degradation of heparin/heparan sulfate involves a N-acetyltransferase.*

Free neuraminic acid is physiologically unstable, degrading by intramolecular condensation to give pyrrole-2-carboxylic acid (Scheme 1.4) .[75] It is thus unlikely that de-*N*-acetylation would occur for free sialic acids, or that deNAc gangliosides should be subject to degradation by a specific neuraminidase which could release Neu5NH$_2$.



Scheme 1.4 *Degradation of neuraminic acid to pyrrole-2-carboxylic acid.*

It is thus more likely that the putative de-*N*-acetylase and *N*-acetyltransferase act on glycan bound sialic acids (Scheme 1.5), with re-*N*-acetylation a requirement prior to hydrolysis of sialic acid *cf.* heparin degradation. Evidence suggested that free amine groups were present on either of the external or internal Sia residues of deNAcGD3.[68] It is perhaps reasonable, therefore, to assume that the same enzyme is capable of synthesising both deNAcGM3 and deNAcGD3.



Scheme 1.5 *Putative enzymes for the metabolism of deNAcGM3.*

Further advances in studying the biological roles of deNAc gangliosides will probably require the identification and isolation of the de-*N*-acetylase and *N*-acetyltransferase. This is the ultimate aim of this project, and to this end we decided to synthesise a number of analogues of GM3 and deNAcGM3 which could be used to set up an assay for the de-*N*-acetylase and *N*-acetyltransferase activities. Such assays could be used to monitor the purification of the relevant enzymes and also to screen potential inhibitors for the enzymes. It should be possible to get a better picture of the biological relevance of deNAc gangliosides by inhibiting the de-*N*-acetylase and/or *N*-acetyltransferase in whole cell systems and then studying any resulting physiological changes.

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Chapter 2

Synthesis and Biological Properties of Thioglycosides

2.1 Thioglycosides in Carbohydrate Chemistry and Glycobiology

Thioglycosides have been extensively studied in both carbohydrate chemistry and glycobiology. Simple alkyl and aryl thioglycosides have proven to be highly versatile glycosyl donors which show excellent stability under a wide range of reaction conditions, but may be selectively activated by a range of "thiophilic" promoters.[1] These include dimethyl(methylthio)sulfonium triflate (DMTST),[1] *N*-iodosuccinimide/triflic acid (NIS/TfOH),[1] and iodine.[2] On the other hand, *S*-linked oligosaccharides have attracted considerable interest as potential inhibitors for glycosidases and lectins, due to their widely reported resistance to enzymatic cleavage.[3, 4] The synthesis and biological activities of thio-glycosides have been reviewed recently.[5] This section is intended as an overview of this topical area of carbohydrate chemistry.

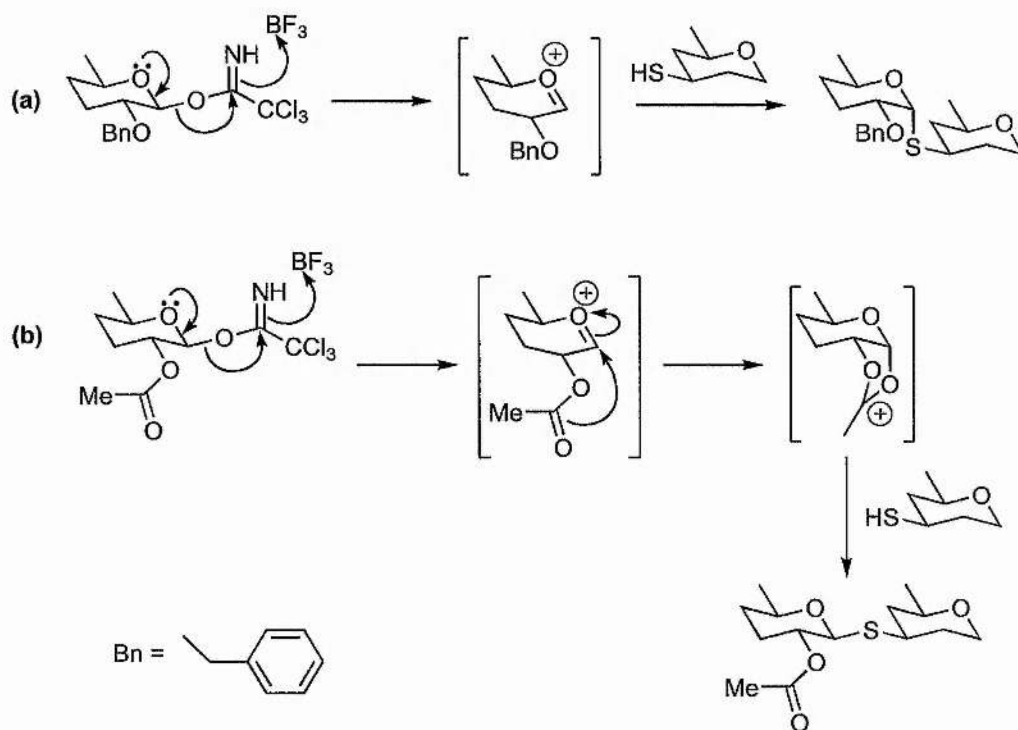
2.2 Synthetic Strategies[5, 6]

There are two basic strategies for the synthesis of thioglycosides, which may be classed by the origin of the inter-glycosidic sulfur atom (Scheme 2.1); *i.e.* the *S*-linkage can either be formed by reaction of a glycosyl donor, bearing a leaving group at its anomeric centre, with a thiol functionality present in the acceptor molecule (thio-glycosylation), or by alkylation of a thiol at the anomeric position of the donor,* using an acceptor with a suitably positioned leaving group (anomeric *S*-alkylation).

Scheme 2.1 *Two strategies for thioglycoside synthesis: thio-glycosylation and anomeric S-alkylation. L = leaving group, e.g. Cl, Br, OTf.*

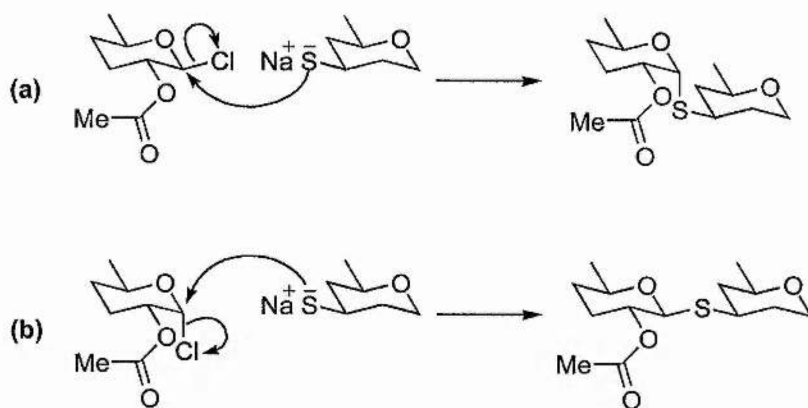
* Strictly speaking, a thioglycose is not a "glycosyl donor" as it does not donate a "glycosyl group" during the coupling reaction. However, this incorrect use of the traditional donor/acceptor terminology is to be found repeatedly in the literature and so it is also used here for simplicity.

2.2.1 Thio-glycosylation. Thio-glycosylation may be sub-divided further into *acid-promoted* and *base-promoted* glycosylation (Scheme 2.2). The former is the traditional approach towards forming both *O*- and *S*-glycosides. This involves the use of Lewis acids to promote the loss of the anomeric leaving group from the donor, which would be a glycosyl halide or trichloroacetimidate or an anhydro-sugar. As this type of glycosylation has a S_N1 type mechanism, stereochemistry must be controlled by the anomeric effect or by neighbouring group participation of an ester adjacent to the anomeric centre.



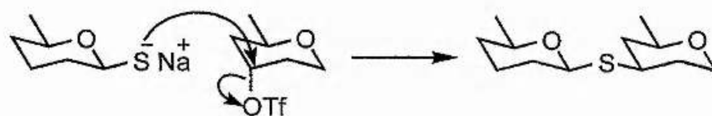
Scheme 2.2 *Acid promoted thio-glycosylation (a) without neighbouring group participation and (b) with neighbouring group participation.*

Base-promoted glycosylation of thiol containing sugars has also been widely employed. The thiol is deprotonated to give, typically, the sodium or caesium thiolate which is used to displace chloride or bromide from the donor. The base-promoted reaction proceeds by a S_N2 type mechanism which allows the stereochemistry of the glycosylation to be determined in advance; *i.e.* a β-halide will give an α-glycoside, regardless of the presence or absence of a participating group at the adjacent carbon atom (Scheme 2.3).



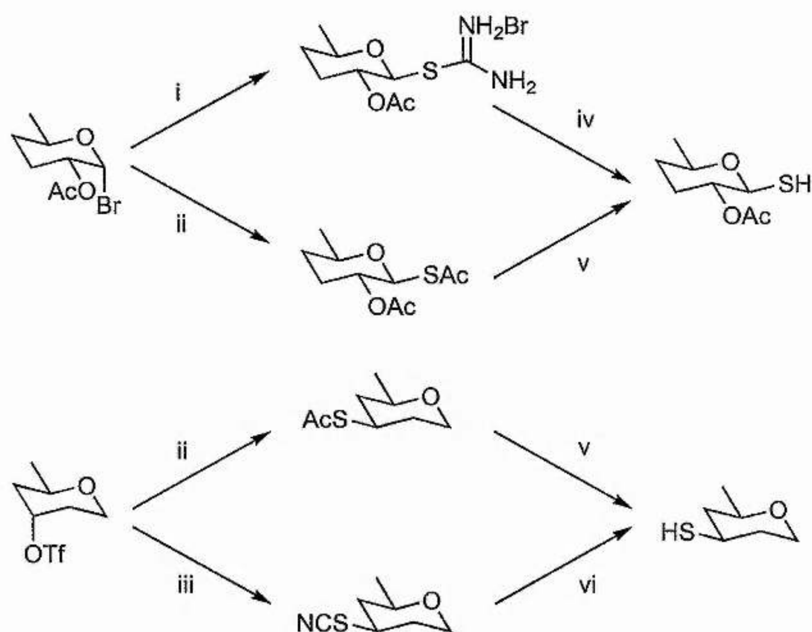
Scheme 2.3 *Base promoted thio-glycosylation to give (a) α-glycosides and (b) β-glycosides.*

2.2.2 Anomeric *S*-Alkylation. Anomeric *S*-alkylation is usually performed under base-promoted conditions with either a halide or sulfonate as the leaving group in the acceptor (Scheme 2.4). For hindered secondary positions, triflate is usually the preferred leaving group. In this approach, the anomeric configuration has previously been set whilst introducing sulfur into the donor, and the S_N2 coupling reaction inverts the configuration of the acceptor at the site of the leaving group.



Scheme 2.4 *Anomeric S-alkylation.*

Initial introduction of sulfur into either the donor or acceptor is usually accomplished by an S_N2 type reaction. Typically potassium thioacetate is the chosen nucleophile, although thiourea and potassium thiocyanate have also been used (Scheme 2.5). It has been reported that thiourea may react by an S_N1 mechanism.[5] Thioacetates are converted to the corresponding thiols by selective deprotection using either sodium methoxide/MeOH at low temperature[7] or hydrazinium acetate in DMF,[8] prior to the coupling reaction. Alternatively, they may be deprotected *in situ* using cysteamine in HMPA[9] or diethylamine in DMF.[10, 11] Pseudothioureas and thiocyanates may be reduced to thiols using potassium metabisulfite and zinc/acetic acid, respectively.[12, 13]



Scheme 2.5 *Introduction of sulfur into donor and acceptor. Typical reagents:* i, thiourea, acetone; ii, KSAc, TBAHSO$_4$, NaOH(aq), CH$_2$Cl$_2$, *or* KSAc, DMF; iii, KSCN, DMF; iv, K$_2$S$_2$O$_5$; v, NaOMe, MeOH *or* N$_2$H$_4$.AcOH, DMF; vi Zn, AcOH.

2.3 Solution Conformations

The conformations of several thioglycosides in aqueous solution have been determined using a combination of NMR spectroscopy, molecular mechanics calculations and X-ray crystallography. The C-S bond is approximately 0.4 Å longer than the C-O bond (1.8 Å *vs.* 1.4 Å) and the C-S-C angle is smaller than the corresponding C-O-C angle (~100° *vs.* ~116°).[14] The net effect is an increase in the distance between adjacent sugar residues of 0.35-0.4 Å (Figure 2.1). The increase in length is accompanied by a decrease in steric interactions between the monosaccharide residues and an increase in the flexibility of the glycosidic linkage.[14]

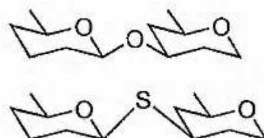


Figure 2.1 comparison of O-glycoside and S-glycoside bond lengths and angles.

2.3.1 Maltose and Galabiose. Methyl 4-thio-α-maltoside **11**[5] and 2-(trimethylsilyl)ethyl 4-thio-β-galabioside **13**[13] both adopt the same overall conformation (albeit with greater flexibility) as the parent *O*-linked disaccharides, **10**

and **12**, respectively (Figure 2.2). However, both compounds show the loss of an interglycosidic hydrogen bond that was present in the *O*-linked compounds. For the thio-galabioside **13**, this has been linked to a 8 $kJmol^{-1}$ decrease in the binding affinity of the disaccharide for the $PapG_{J96}$ adhesin of class I *E. coli*.[13]

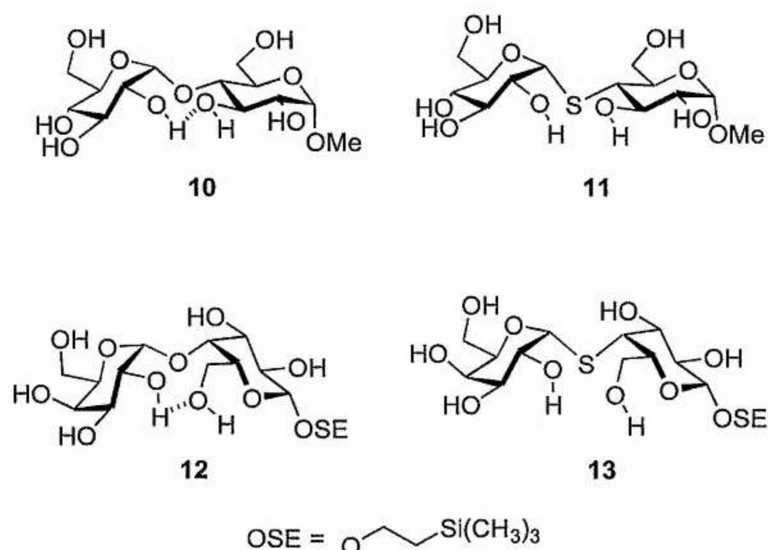


Figure 2.2 *Thio-maltoside 11 and thio-galabioside 13 both show loss of an interglycosidic H-bond*

2.3.2 Sialyl Lewis x. The conformation of a fully *S*-linked analogue of sialyl Lewis x **14** showed considerable deviation from that of *O*-linked sLex **15** (Figure 2.3).[14]

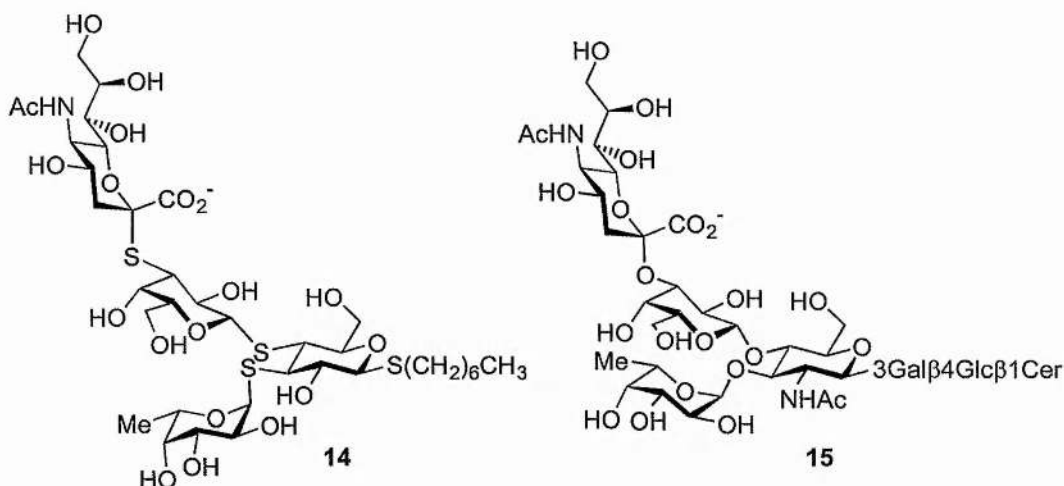


Figure 2.3 *conformations of S-linked and O-Linked sialyl Lewis x.*

The natural compound has a well defined, fairly rigid conformation in which the galactose and fucose residues are stacked 4 Å apart, stabilised by hydrophobic interactions. The same residues in the thio-analogue are over 5 Å apart, and each

apparently adopts several different conformations in solution. No comparative analysis was performed with the Neu5Acα(2→3)Gal linkage, as studies of the *O*-linked compound had shown no well defined conformation.

2.4 Biological Activities

When considering the biological activities of these compounds, it should be noted that there is a distinction between being "resistant to enzymatic hydrolysis" and being an "enzyme inhibitor". Sialic acid *C*-glycoside analogues (*e.g.* 16, Figure 2.4) have been reported to be inhibitors of influenza virus haemagglutinin, and also to be resistant to cleavage by neuraminidase.[15] However, as these compounds do not bind to the neuraminidase, they are not neuraminidase inhibitors.

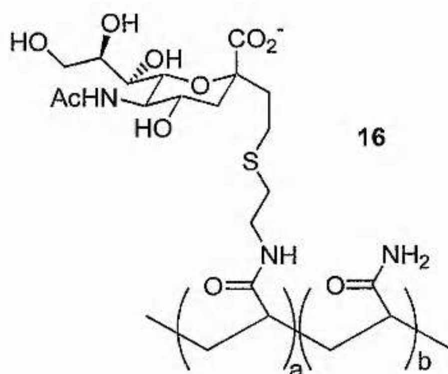


Figure 2.4 *Acrylamide based co-polymer, bearing sialic acid C-glycosides, is an influenza virus haemagglutinin inhibitor.*

Numerous *S*-glycosides have been shown to interact with lectins and glycosidases with K_is and IC_{50}s in the μM-mM range (many glycosidases bind their natural substrates with K_Ms in the low mM range).[5] Thio-oligomers of α- and β-linked glucose have been used to study amylases and cellulases. The fully *S*-linked trisaccharide **17** (Figure 2.5) has been co-crystallised with pancreatic α-amylase and was effective in identifying both the active site of the enzyme and also a second binding site on the surface of the protein.[16] β-Linked trisaccharide **18** is a competitive inhibitor for cellulase from *Trichoderma reesei*. The analogous amino compound was coupled to CH-Sepharose 4B to provide an affinity chromatography column **19** (Figure 2.5) which was used to purify the cellulase from a crude *T. reesei* preparation.[17] As most of the known glycosidases are exo-hydrolases (*i.e.* they

remove monosaccharide residues in a stepwise manner from the non-reducing terminus of oligosaccharides), it is not always necessary to replace all of glycosidic linkages with sulfur. Hemithiocellodextrin **20** with alternating thio- and oxo-linkages is hydrolytically stable in the presence of cellulases, which it inhibits with a K_i in the μM range.[5]

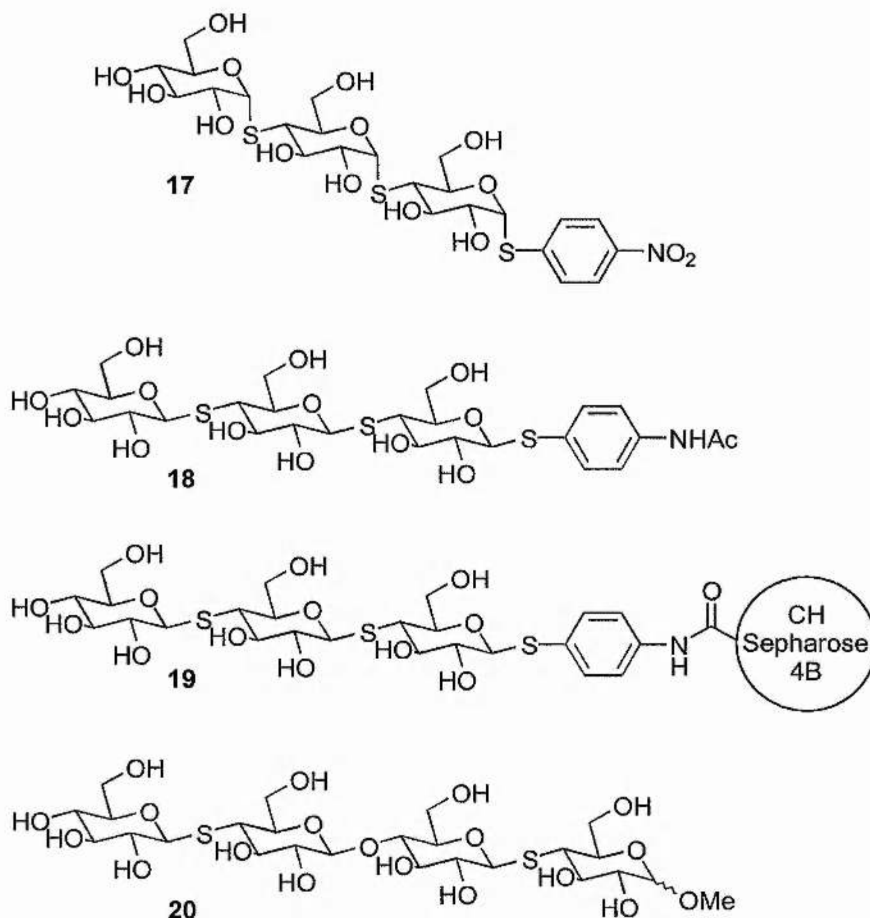


Figure 2.5 *Some S-linked analogues of amylose and cellulose.*

2.5 Thioglycosides of Sialic Acid

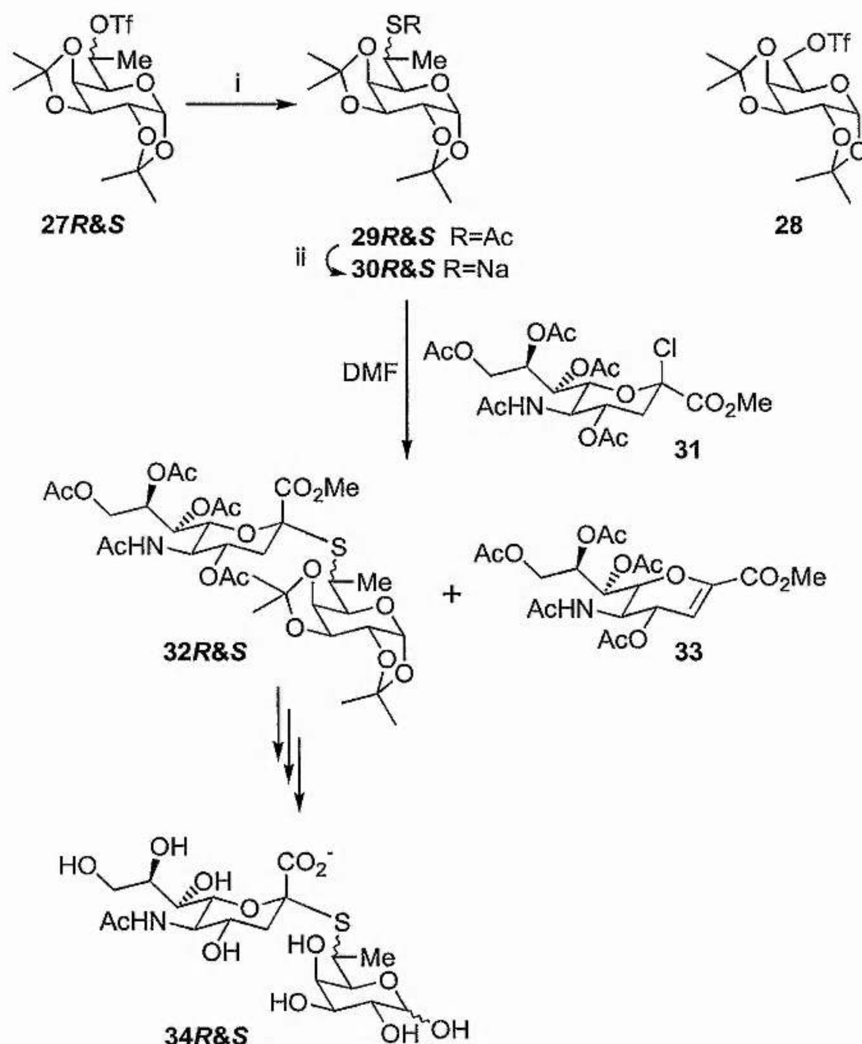
2.5.1 Synthesis. Sialic acid is often found at the non-reducing terminal of cell surface glycans (section 1.4.1) and it forms part of the recognition sites for many viruses, bacteria and bacterial toxins (section 1.5.4). Therefore, thioglycosides of sialic acid have drawn considerable attention, in particular, as potential inhibitors of viral lectins and neuraminidases. Hasegawa and co-workers have synthesised a range of thio-ganglioside analogues *via* the anomeric *S*-alkylation approach (section 2.2.2).[18] This approach gave $\alpha(2\rightarrow6)$ thio-sialosides in good yield but, synthesis of the *S*-linked

ganglioside GM4 analogue **26**, which contains the Neu5Acα(2→3)Gal linkage, proved more difficult.[19] Condensation of sodium thiolate **21** with 3-*O*-triflylgulopyranose acceptors **22** and **24** gave the thio-disaccharides **23** and **25** in 30% and 22% yield, respectively (Scheme 2.6). Although the compound **23** was formed in better yield, considerable problems were then encountered on trying to remove the protecting groups.[19]

Scheme 2.6 *Hasegawa's synthesis of S-linked ganglioside GM4.*

Sabesan and co-workers found it more convenient to prepare their Neu5Acα(2→6)-6-*C*-methyl-6-thio-Gal analogues **34*R*&*S*** (Scheme 2.7) by the base promoted thio-glycosylation approach (section **2.2.1**).[20] The secondary 6-*O*-triflyl compounds **27*R*&*S*** are less reactive than the analogous, primary 6-*O*-triflyl-galactose **28**. Triflate **27*S*** underwent facile displacement with potassium thioacetate to give the 6-*R*-thioacetate **29*R*** in 83% yield, however, the 6-*R*-triflate isomer **27*R*** proved considerably less reactive and more prone to elimination, giving the 6-*S*-thioacetate isomer **29*S*** in only 46% yield. Thio-glycosylation of the corresponding thiolates

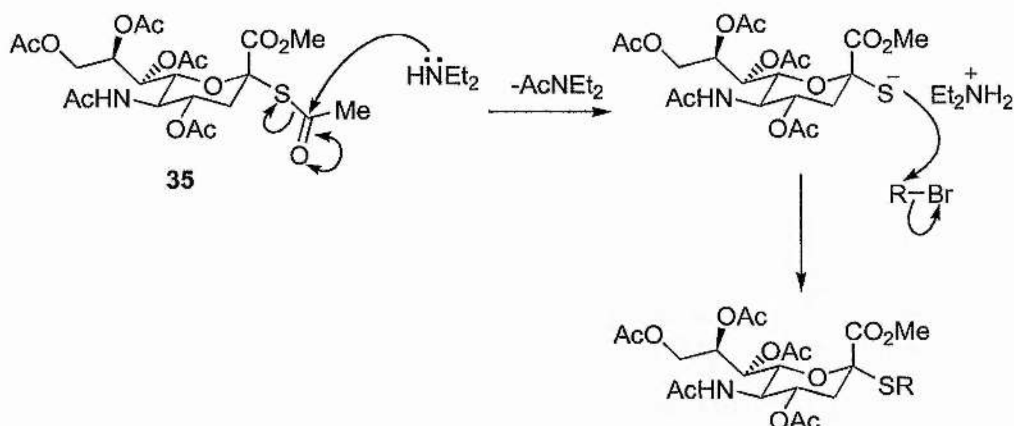
30*R&S*, on the other hand, proceeded in comparable yields (both over 60%). The thio-disaccharide products **32*R&S*** were each contaminated with about 10% of the elimination product **33**, which proved difficult to remove at this stage, but partial deprotecting of the disaccharides then allowed easy purification of the thioglycosides.



Scheme 2.7 *Sabesan's synthesis of Neu5Acα(2→6)-6-C-methyl-6-thio-Gal analogues **34R&S***

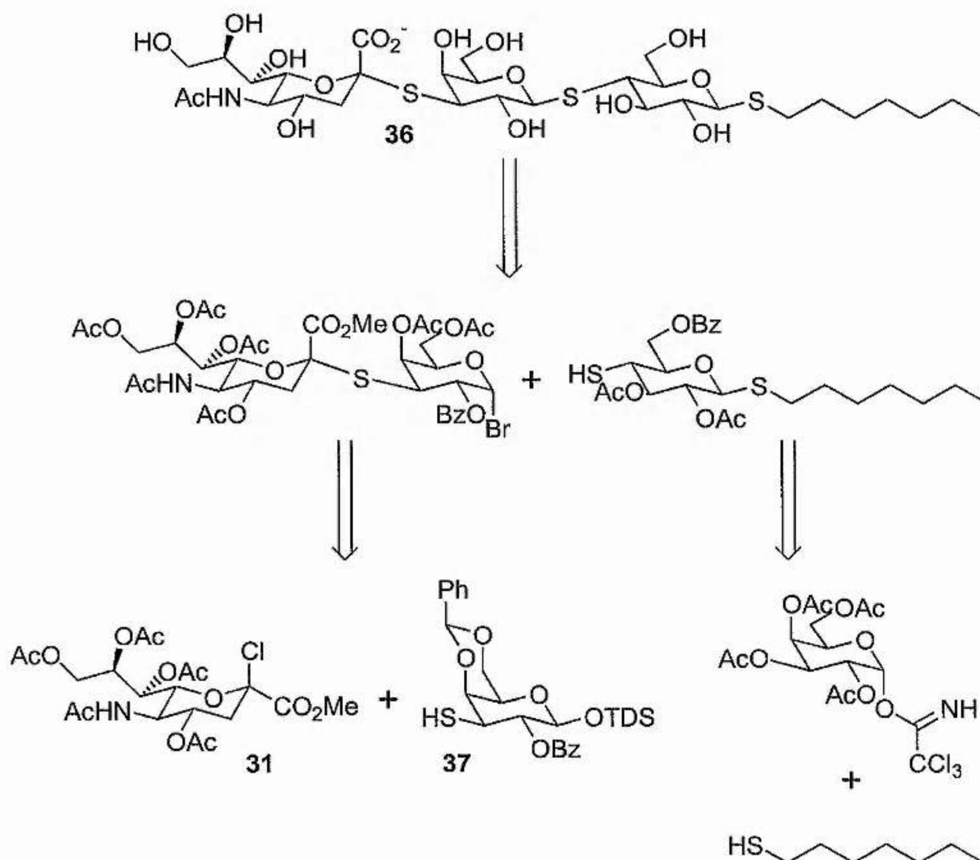
Von Itzstein and co-workers have reported a novel procedure for synthesising sialic acid thio-glycosides from the 2-thioacetate **35** (Scheme 2.8).[10, 11] A combination of DMF and diethylamine as solvent allows *in situ* deprotection of the thioacetate **35**. Whereas the alkylation step works efficiently using simple alkyl bromides and 6-bromo-glucose derivatives, synthesis of sterically hindered disaccharides requires the use of the more reactive triflate derivatives. Using this approach, 3- and 4-

thiosialosides of glucose have been synthesised in 84% and 74% yields, respectively.[11]

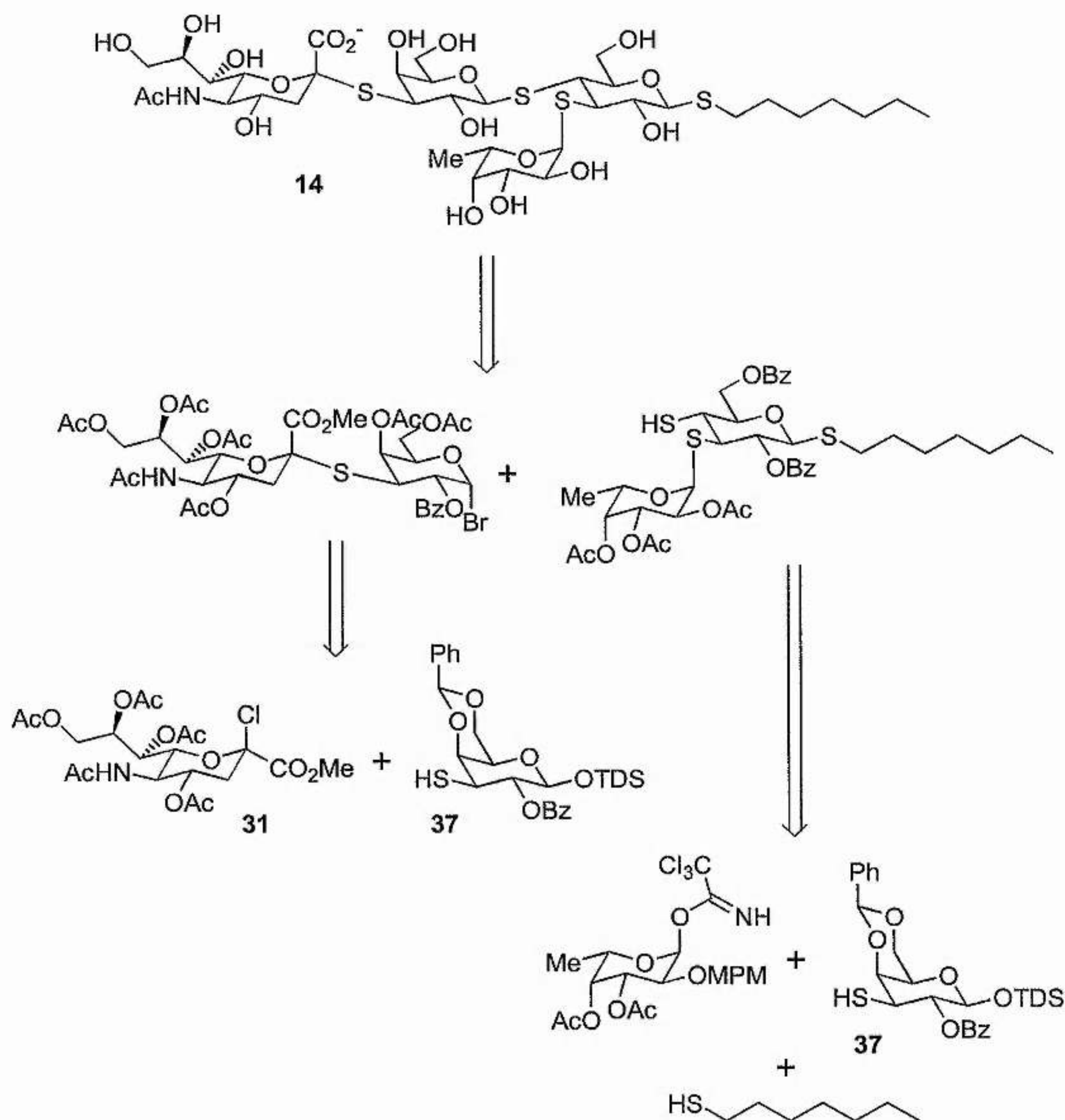


Scheme 2.8 *von Itzstein's procedure for synthesising sialic acid thio-glycosides.*

Schmidt and co-workers have recently described the synthesis of GM3 analogue **36**[21] and the corresponding sialyl Lewis x analogue **14**.[6, 22] These were both synthesised in a block-wise approach from the intermediates indicated in Schemes 2.9 and 2.10.



Scheme 2.9 *Schmidt's retrosynthesis of fully S-linked GM3 analogue* ***36****.*



Scheme 2.10 *Schmidt's retrosynthesis of fully S-linked sLe^x analogue 14.*

They reported that Hasegwa's anomeric *S*-alkylation strategy, using donor **21** and acceptor **38**, gave the key sialylgalactoside **39**, in only 30% yield, whereas the same disaccharide could be made in up to 85% yield by base promoted thioglycosylation of intermediate **37** using kryptofix 21 as co-activator (Scheme 2.11).[21] Attempted anomeric *S*-alkylation under von Itzstein's conditions gave the elimination product **40**.

Scheme 2.11 *Synthesis of Schmidt's key sialylgalactoside **39** by (a) Hasegawa's strategy, (b) Schmidt's startegy and (c) attempted synthesis of **39** by von Itzstein's method.*

2.5.2 Biological Activities. Several sialic acid α(2→6) thio-disaccharides have been tested as neuraminidase inhibitors. Suzuki and co-workers have reported that ganglioside analogues **41**, **42** and **43** (Figure 2.6) are all competitive inhibitors for influenza virus neuraminidases.[23] Galactosyl ceramide based compound **42** was the weakest of these inhibitors (K_i = 100 μM). Inhibition increased on extending the length of the oligosaccharide chain (**43**, K_i = 15 μM), but was greatest for the glucosyl ceramide based compound **41** (K_i = 2.8 μM). This is surprising, as the

natural substrates for the influenza neuraminidases are thought to be glycans containing Neu5Acα(2→3)Gal and Neu5Acα(2→6)Gal epitopes.[20] Compound **41** was a much poorer inhibitor for neuraminidases from the bacteria *Clostridium perfringens* and *Arthrobacter ureafaciens* (*C. perfringens* K_i = 49 μM; *A. ureafaciens* K_i = 400 μM).

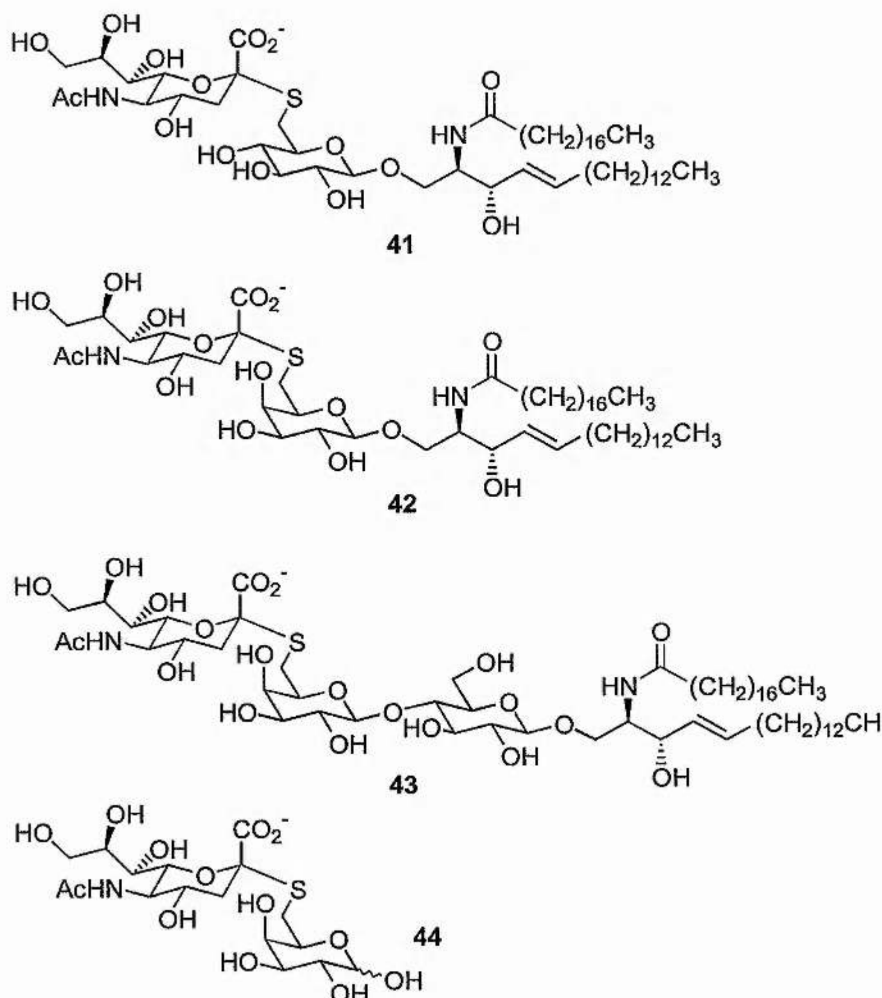


Figure 2.6 *Some sialic acid thio-glycoside neuraminidase inhibitors.*

Sabesan and co-workers found the sialic acid α(2→6)-6-thio-Gal reducing sugar **44** to be a weak inhibitor of influenza virus neuraminidase, even at 8 mM.[20] The conformationally constained 6-*C*-methylgalactose analogue **34*S*** was also a poor inhibitor, but the corresponding *R*-isomer **34*R*** inhibited both influenza virus and *C. perfringens* neuraminidases with a K_i of 300 μM. They proposed that the difference observed for the *R*- and *S*-isomers results from the different conformations which these two compounds adopt in aqueous solution. Although the presence or absence of

a ceramide moiety may make a difference to the recognition of such thioglycosides by the neuraminidases, these two studies can not be directly compared as different substrates were used for the inhibition studies (ganglioside GM3 by Suzuki, Neu5Acα(2→6)Galβ(1→4)GlcNAc by Sabesan).

9-*O*-acetyl derivatives of a number of Siaα(2→6)-6-thio-hexoses (Figure 2.7; **45**, **46**, **47**) described by von Itzstein and co-workers were found to inhibit bovine rotavirus infection in a primate cell line.[11] Both the 6-thioglucose **45** and 6-thiogalactose **47** derivatives had IC_{50} values ~1mM.

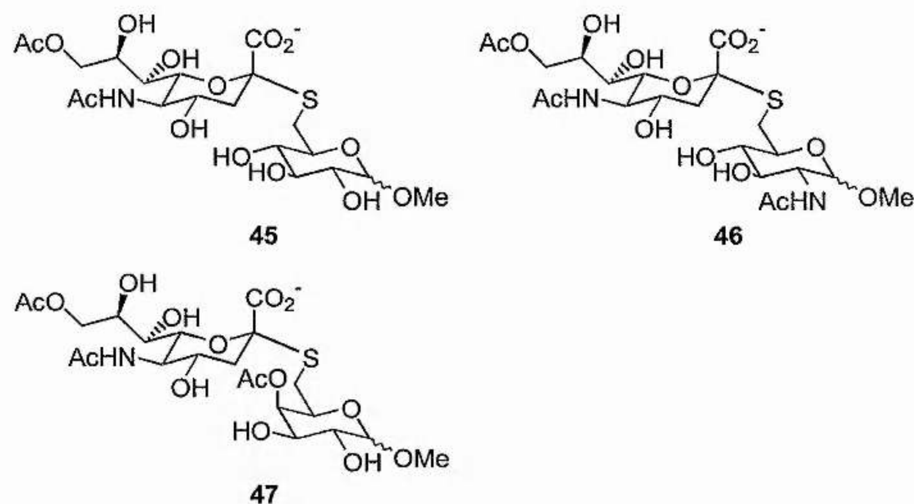


Figure 2.7 *Some sialic acid thio-glycoside rotavirus inhibitors.*

No neuraminidase inhibition data has been reported for Neu5Acα(2→3)-3-thio-Gal compounds, nor for the binding of Schmidt's sLex derivative **14** to Selectins. However, Hasegawa's *S*-linked GM4 analogue **26** (Scheme 2.6) exhibits potent immunosuppresive activity; tetanus toxoid induced lymphocyte proliferation was 95% inhibited by 5μM **26** (determined by [^{3}H]thymidine uptake).[24]

2.6 Concluding Statement

Thioglycosides have proven to be useful, glycosidase-resistant analogues of oligosaccharides and, over recent years, much effort has been invested in the synthesis of such compounds. The subtle change of replacing oxo-linkages with sulfur, increases the flexibility of the glycosidic linkage and, in some cases this may alter the conformation of the saccharide to the detriment of its biological activity. However, there are many examples of thioglycosides that have been used successfully as oligosaccharide mimics and interest in this subject is certain to grow in the future.

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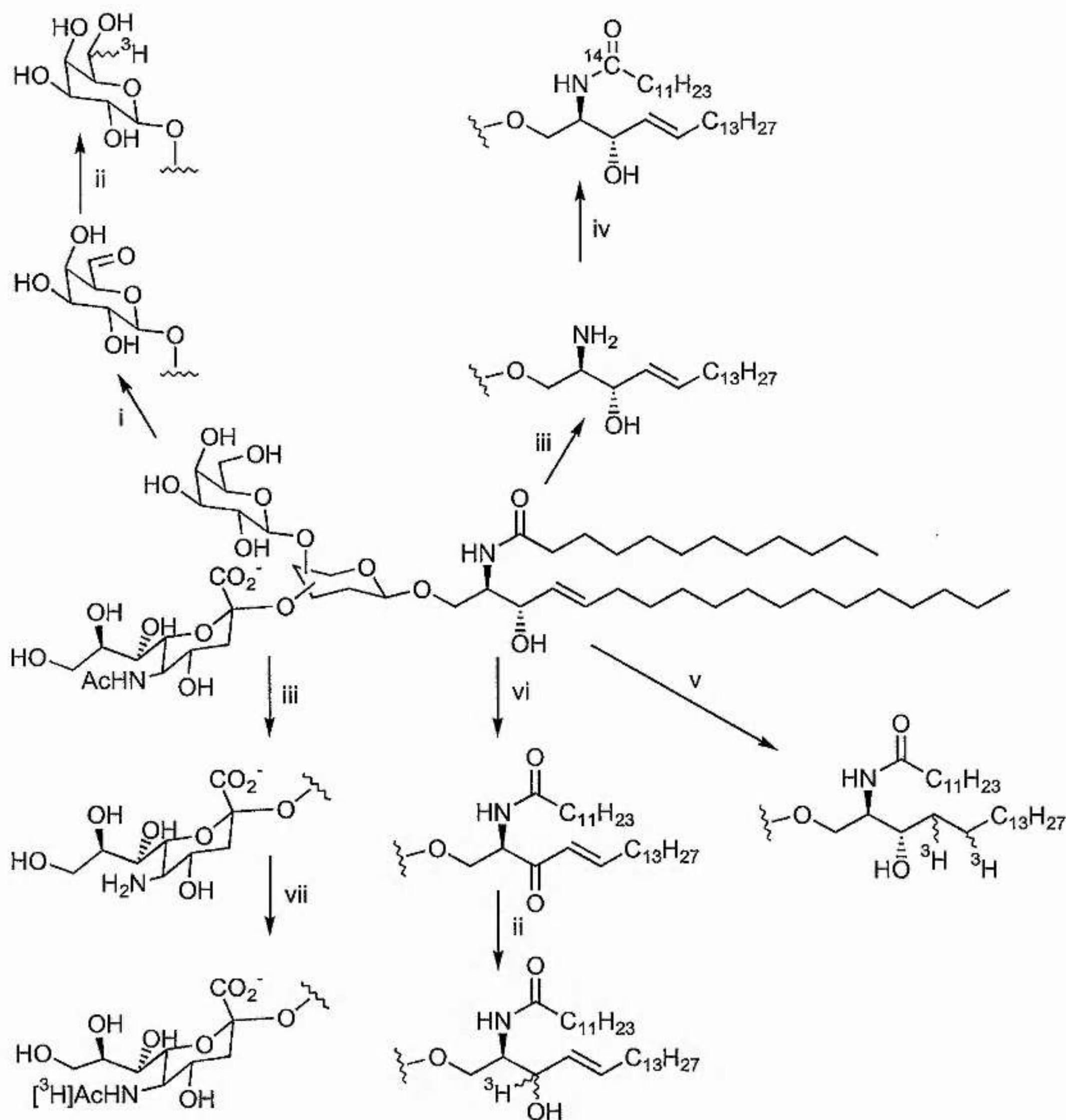
Chapter 3

Design of Substrates for de-*N*-Acetylase and *N*-Acetyltransferase Assays

3.1 Assay Design

Substrate specificity and sensitivity of detection must both be considered when designing an enzyme assay. The substrate to be used in the assay must be sufficiently similar to the enzyme's natural substrate so that it will show a reasonable rate of turn-over with the enzyme. Also, there must be some means of detecting and distinguishing between the substrate and products of the enzyme reaction. The detection method must be as sensitive as possible and preferably, it should not involve a lengthy work-up following the enzyme incubation.

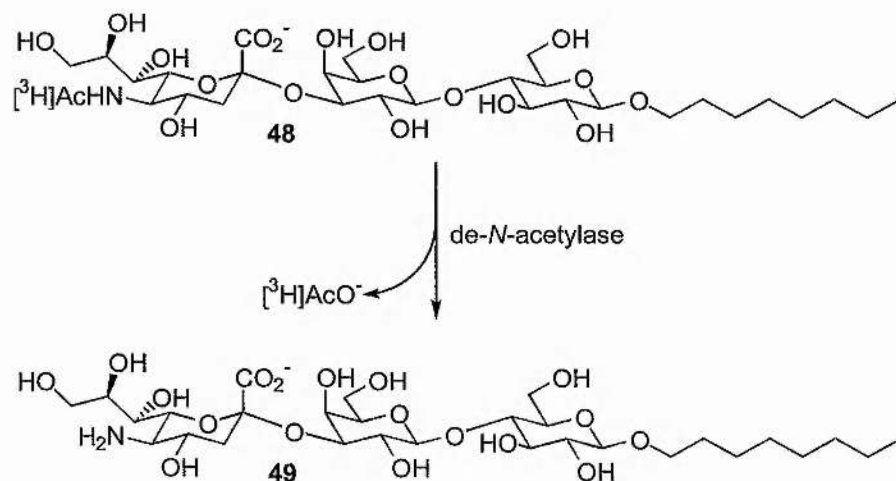
Often, either a radio-label or a flourescent tag are introduced into the assay substrate, as the means of detection. Radio-labeling, although intrinsically more hazardous than using fluorescent tags, has the advantage of not altering the substrate structure, as it involves replacing only individual atoms with unstable isotopes. The synthesis of radio-labeled gangliosides has been reviewed recently.[1] The principal methods that have been used are: enzymatic oxidation (galactose oxidase) of the oligosaccharide portion or oxidation of the sphingosine alcohol with DDQ, each followed by reduction with NaB^3H_4; reduction of the ceramide double bond using NaB^3H_4 and palladium (II) chloride; selective de-*N*-acetylation of sialic acid or de-*N*-acylation of ceramide followed by re-*N*-acetylation/acylation using [^{3}H]acetic anhydride or [^{14}C]stearic acid (Scheme 3.1).



Scheme 3.1 *Methods of introducing radio-labels into gangliosides. Reagents:* i, galactose oxidase, catalase; ii, NaB^3H_4; iii, Me_4NOH, aq. *n*-BuOH; iv, $C_6F_5O_2{}^{14}CC_{11}H_{23}$, DMF; v, NaB^3H_4, $PdCl_2$; vi, DDQ, toluene; vii, $[^3H]Ac_2O$, MeOH.

3.1.1 De-*N*-Acetylase Assay. As we were interested in studying the release of the sialic acid *N*-acetyl group, it seemed that introduction of a $[^3H]$acetyl group onto the sialic acid amino function, would be the method of choice. However, preparation of deNAc gangliosides required for radio-labeling (Scheme 3.1) is complicated by hydrolysis of the ceramide *N*-acyl chain to give the "lyso-ganglioside" derivative.[1] Therefore, we decided that it would be advantageous to synthesise ganglioside analogues in which the ceramide portion was replaced by a simple octyl tail. Such compounds have greater water solubility than GSLs and have been used extensively, in conjunction with reverse phase columns, for glycosyl transferase assays.[2, 3] It is

quite possible that deNAcGM3 and deNAcGD3 are synthesised by the same enzyme (see section **1.6.4**). As the Neu5Acα(2→8)Neu5Ac linkage is notoriously difficult to synthesise,[4] it was preferable to make only GM3 analogues. Thus, we chose to make radio-labeled GM3 analogue **48** for the de-*N*-acetylase assay (Scheme 3.2).



Scheme 3.2 Proposed substrate **48** for the de-N-acetylase assay.

Following incubation of the substrate with the de-*N*-acetylase enzyme, it would be necessary to separate free acetate from the ganglioside analogue. This could easily be achieved using a C-18 reverse phase column. Thus, the hydrophobic glycolipid **48** would stick to the C-18 column whereas the acetate could be washed off with water (Figure 3.1).[5] Unaltered **48** could then be recovered by washing the column with aqueous methanol[5] and the fraction of "hot" acetate which has been released, could be determined by liquid scintillation counting of each of the elutions.

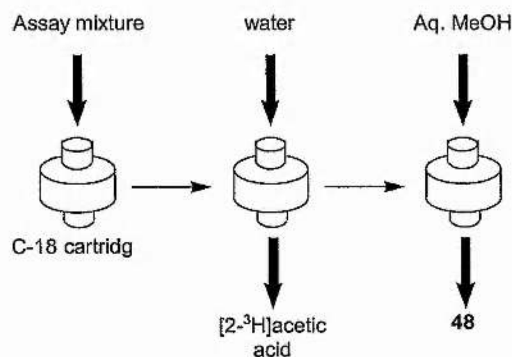
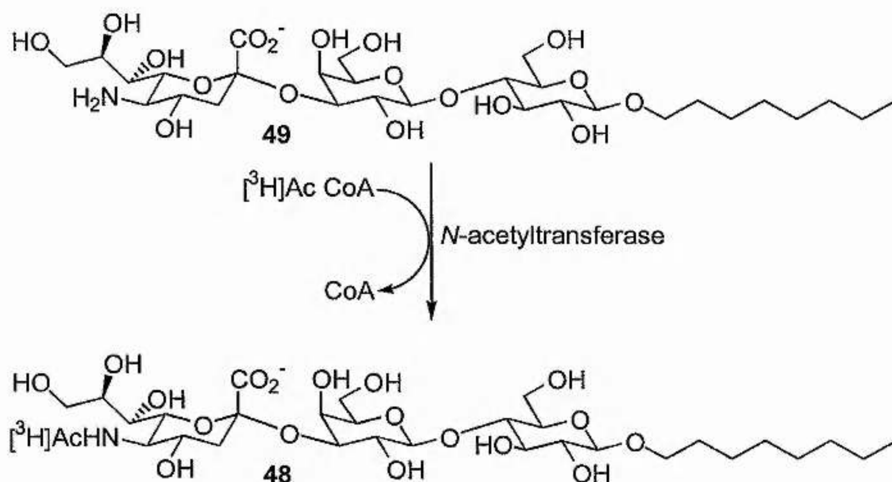


Figure 3.1 *Separation of the de-N-acetylase assay mixture by C-18 reverse phase chromatography.*

3.1.2 *N*-Acetyltransferase Assay. DeNAcGM3 analogue **49** was chosen as the substrate for the *N*-acetyltransferase assay (Scheme 3.3).



Scheme 3.3 *Proposed substrate 49 for the N-acetyltransferase assay.*

This assay would involve incubation of **49** with the enzyme and [^{3}H]-acetyl CoA (acetyl CoA is the putatative acetyl donor for the *N*-acetyltransferase). The product of this reaction would be identical to the synthetic substrate **48** for the de-*N*-acetylase assay. Reverse phase chromatography could be used, as described before, to separate excess [^{3}H]-acetyl CoA from any **48** synthesised by the enzyme (Figure 3.2).

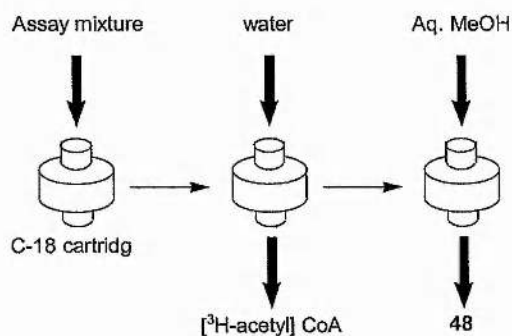


Figure 3.2 *Separation of the N-acetyltransferase assay mixture by C-18 reverse phase chromatography.*

3.1.3 Thioglycoside Analogues. It is possible that these assays could be complicated by enzymatic cleavage of sialic acid from **48,** *i.e.* the substrate for the de-*N*-acetylase assay and the product from the *N*-acetyltransferase assay. Radio-labeled sialic acid, thus produced, could not be distinguished easily from either [^{3}H]acetate or [^{3}H]-

acetyl CoA by means of a reverse phase assay. Similarly, any potential enzyme inhibitors based on these compounds could also be subject to cleavage by neuraminidases. This problem could be avoided by using the analogous thio-glycoside compounds **50** and **51** which should be resistant to enzymatic hydrolysis (Figure 3.3).[6, 7]

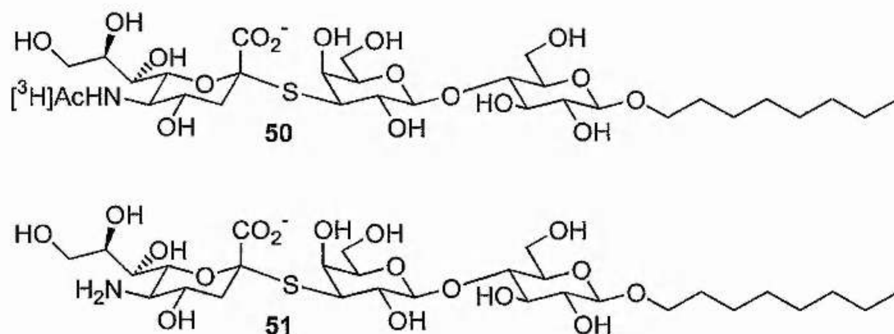


Figure 3.3 *Thioglycoside analogues of GM3 and deNAcGM3.*

As was described in section **2.5.2**, *S*-linked ganglioside analogues can have interesting biological activities. Simple thio-glycoside ganglioside analogues such as compound **51** could be useful scaffolds on which to design neuraminidase inhibitors, and even immunosuppressants, in addition to their intended functions as de-*N*-acetylase substrates and inhibitors. In the next chapter, studies towards the synthesis of the non-radio-labeled GM3 analogues **52** and **53** (Figure 3.4) are described in detail and also a number of thio-sialosides are evaluated as neuraminidase inhibitors.

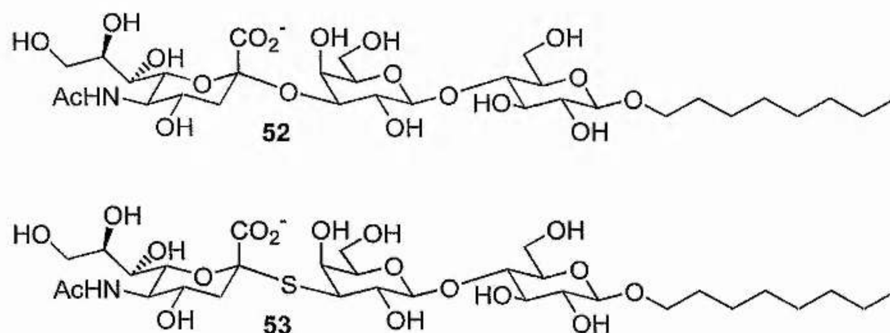


Figure 3.4 *Non-radio-labeled target GM3 analogues.*

3.2 References

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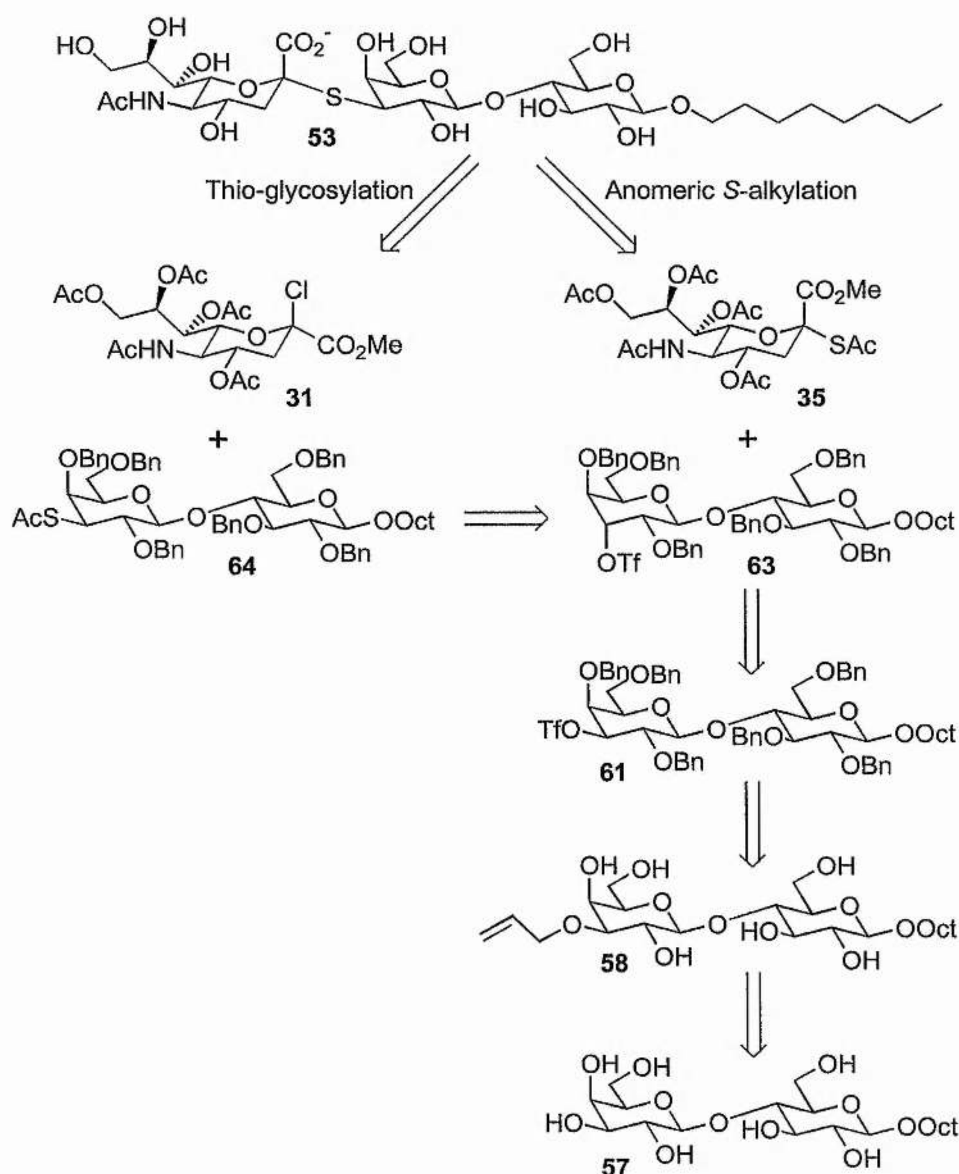
Chapter 4
Results and Discussion

4.1 Studies Towards the Synthesis of Thio-GM3 Analogue 53

At the time of starting this project, the only published procedure for synthesis of the Neu5Acα(2→3)Gal thio-glycosidic linkage was Hasegawa's *S*-linked GM4 synthesis (Scheme 2.6).[1, 2]

4.1.1 Approach 1: the Disaccharide Acceptor

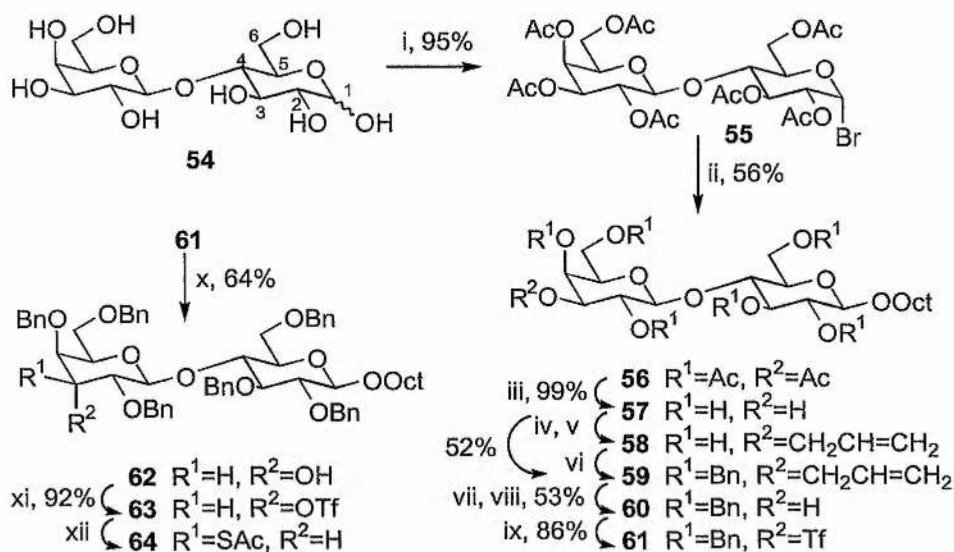


Scheme 4.1 Retrosynthetic analysis of GM3 thioglycoside analogue.

4.1.1.1 Retrosynthesis. The original plan for preparation of the GM3 thioglycoside analogue was to make octyl lactoside and then to selectively protect and then re-expose the galactose 3-OH, leaving it free for introduction of sulfur *via* a double

inversion (Scheme 4.1). Allyl ether formation *via* tin acetal chemistry[3] was chosen for the selective, temporary 3b protection (monosaccharide residues are labeled *a*, *b*, *c* from the reducing terminus for di- and trisaccharides), to be followed by benzylation of the other positions. It was anticipated that inversion of the galactose 3-OH would be achieved using sodium acetate in the nucleophilic displacement of the triflate group in lactoside **61**. Benzyl protection would be compatible with the subsequent deprotection and reactivation of the gulose derivative, thus obtained, to give triflate **63**. Sulfur could then be introduced using either potassium thioacetate (giving **64** for subsequent base promoted thio-glycosylation) or the known sialic acid derivative **35**[4] (by anomeric *S*-alkylation). Although catalytic hydrogenolysis of the benzyl ethers may not have proven compatible with a sulfur linkage in the molecule,[5] there are many alternative cleavage procedures in the literature;[6] *e.g.* reduction by sodium in liquid ammonia has been used to deprotect sulfur-containing carbohydrates.[7]

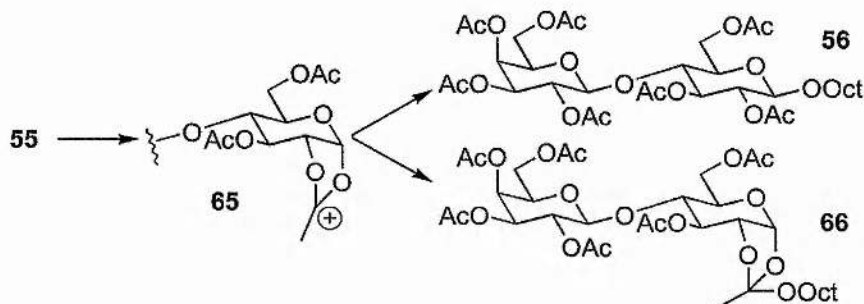
4.1.1.2 Synthesis. Lactose **54**, being inexpensive and readily available, was chosen as the starting material (Scheme 4.2). This was converted in a one pot reaction to the heptaacetyllactosyl bromide **55** in 95% yield with acetic anhydride and 30% HBr/AcOH.



Scheme 4.2 *Synthesis of lactose acceptor. Reagents:* i, HBr/AcOH, Ac_2O; ii, $AgClO_4$, Ag_2CO_3, OctOH, CH_2Cl_2; iii, NaOMe, MeOH; iv, Bu_2SnO, MeOH; v, $H_2C{=}CHCH_2Br$, TBAI, toluene; vi, BnBr, NaH, DMF; vii, $[COD(MePh_2P)Ir]PF_6$, H_2, THF; viii, TFA(aq), CH_2Cl_2; ix, Tf_2O, pyridine, CH_2Cl_2; x, $TBANO_2$, toluene; xi, Tf_2O, pyridine, CH_2Cl_2; xii, KSAc, DMF.

Glycosylation of octanol with compound **55** gave variable results. Removal of bromide by the promoter (silver perchlorate/silver carbonate) gives an initial carbocation which is stabilised by the participation of the acetyl group at position 2

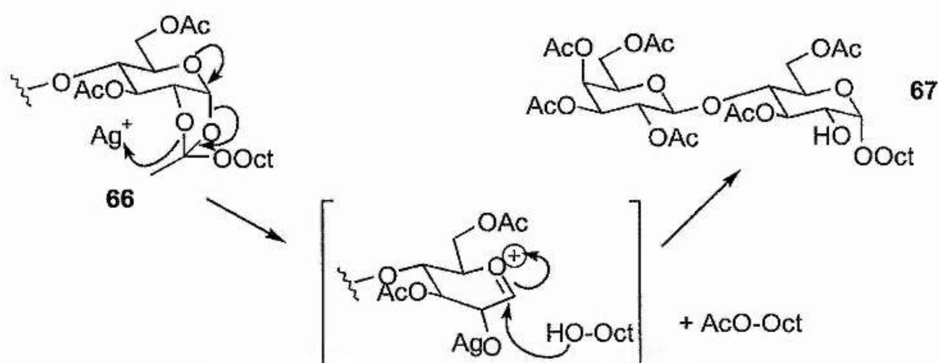
to give cyclic carbocation **65** on the α face of the molecule (Scheme 4.3). Subsequent attack by octanol should regenerate the 2-*O*-acetyl group and form the β-glycoside **56**.



Scheme 4.3 Mechanism for the formation of β-glycoside **56** and orthoester **66**.

However, it was found that a 10% excess of $AgClO_4.H_2O$ and 2.5 equivalents of octanol, dried overnight with 4Å molecular sieves prior to addition of **55** gave a 56% yield of the desired product, **56**, but also a considerable quantity of the orthoester **66** [δ_H ($CDCl_3$) 4.62 (1 H, d, $J_{1b,2b}$ 8.0, 1b-H), 5.64 (1 H, d, $J_{1a,2a}$ 5.2, 1a-H)], formed by trapping **65** with octanol. Although formation of the orthoester creates a new stereogenic centre, only one diastereomer was observed and was tentatively assigned as the *exo*-isomer. The orthoester and glycoside were not easily separated by flash chromatography and so the mixture was treated with 50% aqueous TFA to hydrolyse the orthoester to the hemiacetal prior to chromatography.

It was noted that the $AgClO_4$ suffered some degradation during overnight drying to give a brown solution. This reaction was partially attributed to light sensitivity, but also seemed to be enhanced by the presence of octanol. Repeating the reaction without adding octanol until directly before the bromide, gave no orthoester but a different byproduct identified as *octyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→4)-3,6-di-O-acetyl-α-D-glucopyranoside*, **67** was formed in 27.5% yield along with 43.5% of the desired product, **56**. Compound **67** presumably forms by loss of octyl acetate from the orthoester and attack by octanol to give the thermodynamically favoured α-lactoside, there being no participating groups present on C-2 (Scheme 4.4). Increasing the concentration of $AgClO_4$ to 1.5 equivalents was similarly affected by the timing of the addition of octanol; drying $AgClO_4$ overnight with octanol present gave 34% yield of β-glycoside **56** plus some orthoester **66**, whereas drying in the absence of octanol gave a 24% yield of β-glycoside **56** and 25% yield of α-glycoside **67**.



Scheme 4.4 *Mechanism for the formation of α-glycoside 67*

Following deacetylation of compound **56** in near quantitative yield, selective allyl protection of the 3b position of compound **57** was achieved *via* the stannylene acetal, **68**. In non-polar solvents stannylene acetal **68** dimerises (Figure 4.1) with concomitant stereoelectronic enhancement of the nucleophilicity of the divalent 3b oxygen over the trivalent 4b oxygen.[8] The stannylene acetal is formed exclusively across the 3b and 4b oxygens on refluxing lactoside **57** with dibutyltin oxide in methanol. Iodide promoted reaction of the stannylene acetal with allyl bromide in toluene gave, on work-up, a 2:1 mixture of allyl ether **58** and recovered lactoside **57**. A similar incomplete reaction of methyl lactoside has been reported.[3]

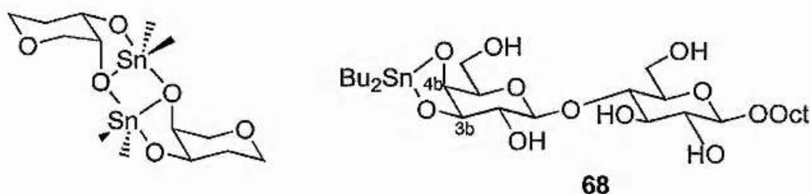
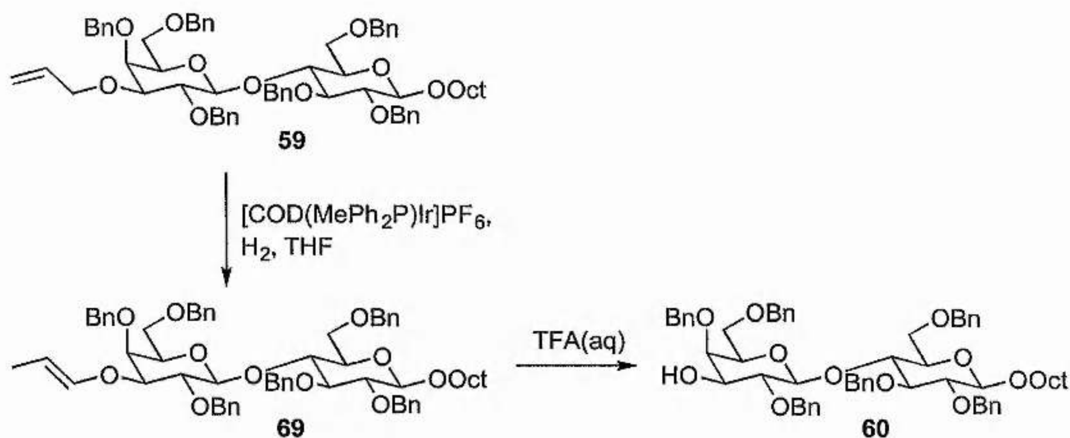


Figure 4.1 *Regioselective stannylene acetal formation and dimerisation.*

Allyl ether **58** was fully characterised as the hexaacetate (*octyl 2,4,6-tri-O-acetyl-3-O-allyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside*). However, for ease of purification, the crude allyl ether was benzylated with NaH/BnBr in DMF before chromatography, giving the fully protected compound **59** in 52% yield from lactoside **57**.

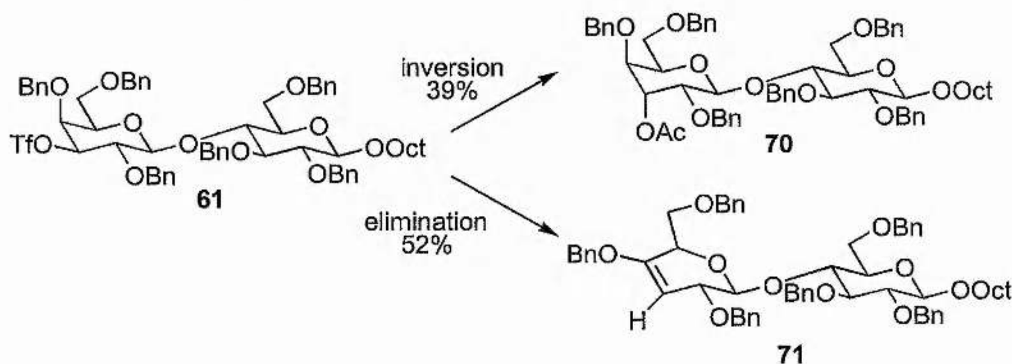
In order to remove the allyl group it was first isomerised to the *trans*-propenyl ether **69** using cyclooctadienebis(methyldiphenylphosphine)iridium (I) hexafluorophosphate {$[COD(MePh_2P)Ir]PF_6$} in THF (Scheme 4.5).[9] It has been reported that this catalyst is less prone to reduce allyl groups to hydrolytically stable propyl ethers than rhodium based catalysts. The reported procedure of 0.1 mol% of the catalyst gave no reaction, but by increasing the amount of catalyst to 0.65 mol%,

the isomerisation occured overnight at room temperature. Subsequent hydrolysis in CH_2Cl_2 with aqueous TFA gave **60** (Scheme 4.5) in a disappointing 53% yield for a reaction which had appeared to have proceeded cleanly by TLC.



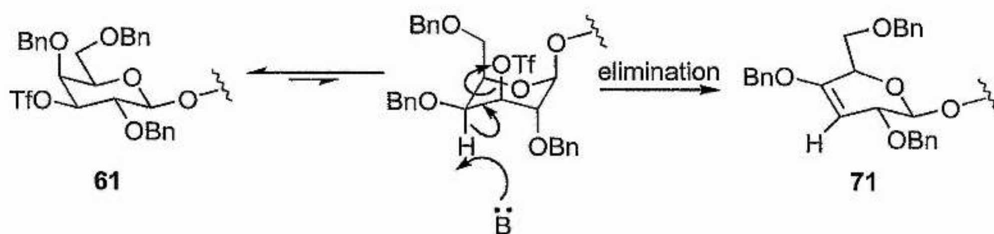
Scheme 4.5 *Two step removal of the allyl ether protecting group.*

Introduction of sulfur in the equatorial configuration, requires position 3b to be converted first to the axial, *gulo*-configuration. It was decided to proceed *via* acetate inversion of triflate **61** with subsequent deacetylation to give mono-alcohol **62**. Although the triflate could be made in good yield and proved sufficiently stable for characterisation, reaction with tetrabutylammonium acetate in DMF, gave the desired axial acetate **70** in only 39% yield as a competing elimination reaction gave compound **71** in 52% yield (Scheme 4.6).



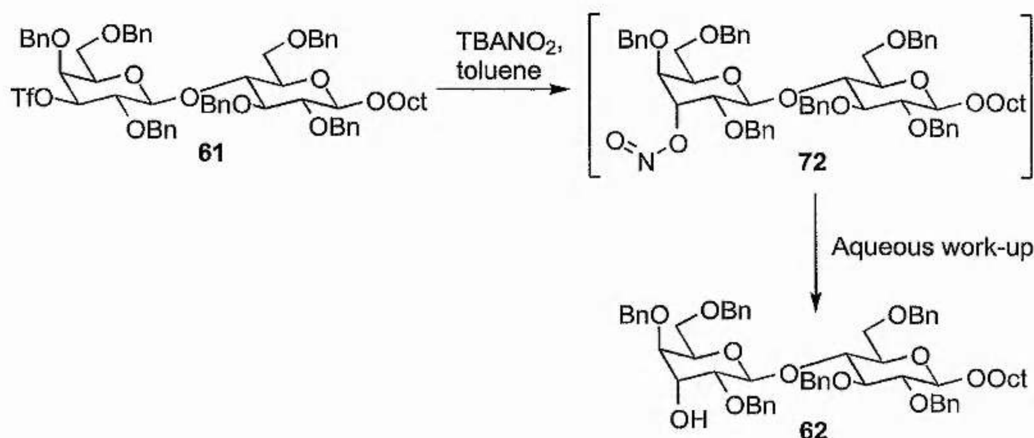
Scheme 4.6 *Competing inversion and elimination reactions of triflate 61.*

Elimination presumably occurs by an E2-type mechanism with acetate acting as base. The required *trans*-diaxial arrangement of proton and leaving group (triflate) can only be achieved following a ring-flip into the $^1C_4$ conformation (Scheme 4.7).



Scheme 4.7 *Mechanism of triflate elimination from compound **61**.*

Reaction of compound **61** with tetrabutylammonium nitrite ($TBANO_2$) was more successful.[5] The initial product is the nitrite ester **72** hydrolyses on aqueous work-up to give mono-alcohol **62** directly in 64% yield (Scheme 4.8).



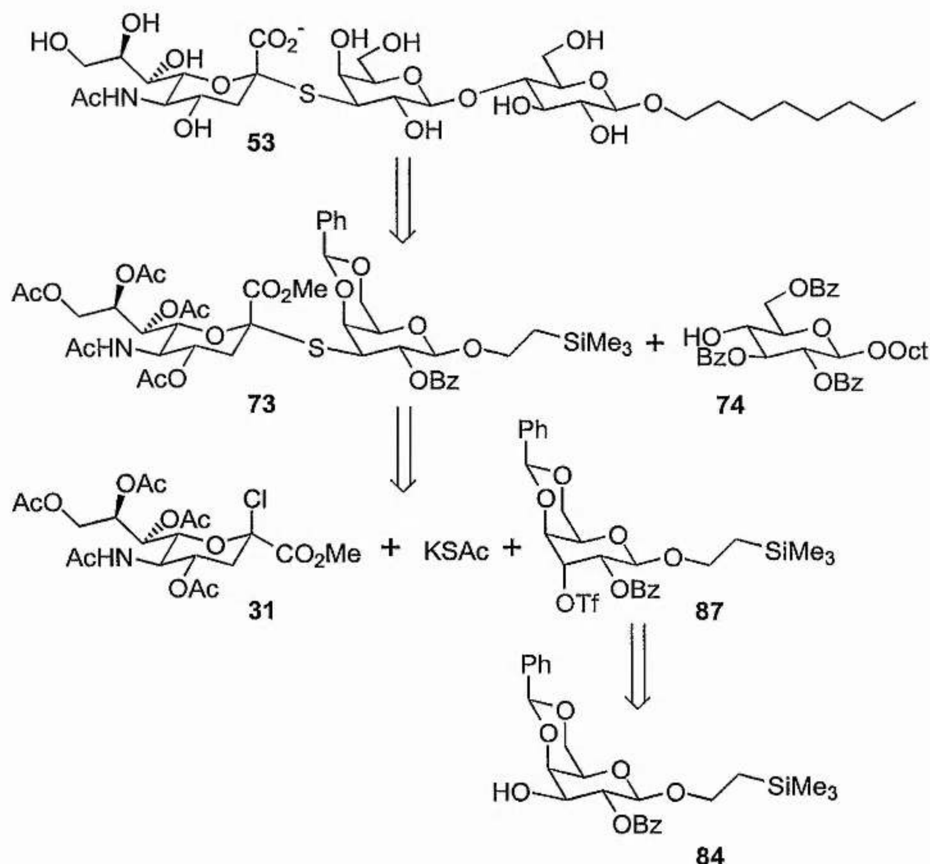
Scheme 4.8 *Nitrite inversion of triflate **61**.*

Compound **62** was easily converted to triflate **63** which again proved surprisingly stable. Problems were however encountered on reaction with potassium thioacetate to give **64**. The two products which formed were found to be inseparable by chromatography. An attempted de-*S*-acetylation of the mixture with NaOMe gave a sticky precipitate with the characteristic smell of thioacetate implying that elimination had occurred. The products of this reaction also proved inseparable.

In consideration of the problems encountered and also the (potentially low yielding) removal of benzyl groups in the presence of the thioglycosidic linkage, a new approach was considered, based on Schmidt's recently published synthesis of a fully thio-linked analogue of sialyl Lewis x (Scheme 2.10).[5]

4.1.2 Approach 2: the Galactopyranosyl/Gulopyranosyl Acceptor

4.1.2.1 Retrosynthesis. The second approach to the synthesis of **53** was to construct the trisaccharide in a stepwise manner from the non-reducing end; *i.e.* to construct the Sia$\alpha(2\rightarrow3)$Gal disaccharide first, and then to activate this as a glycosyl donor for further extension to the trisaccharide (Scheme 4.9).

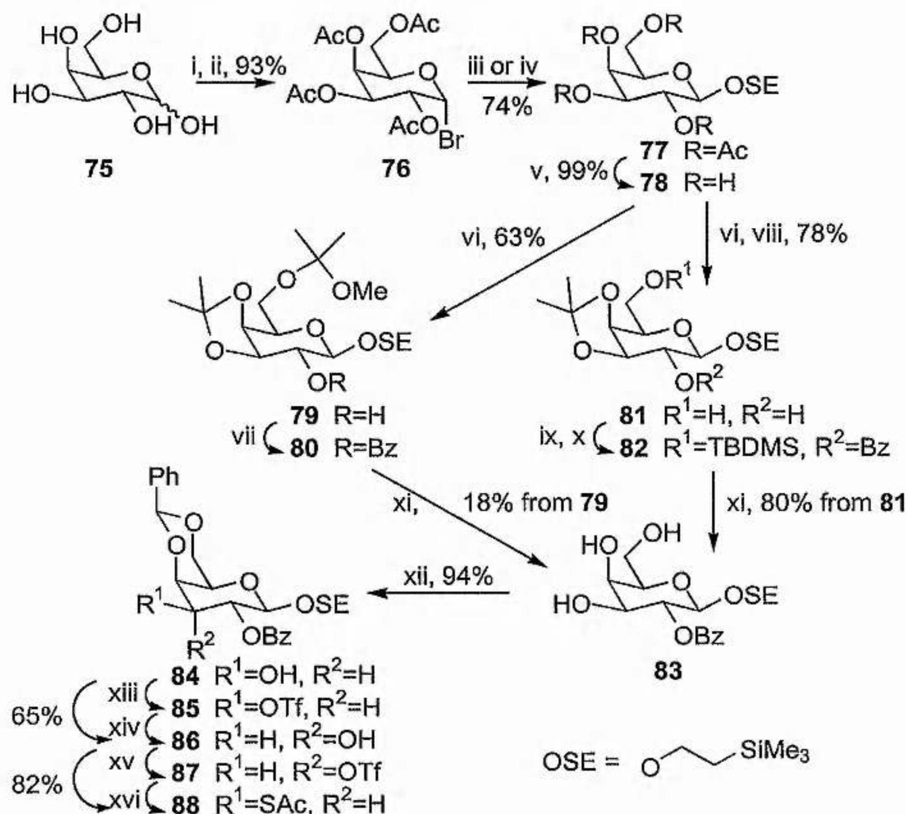


Scheme 4.9 Retrosynthetic analysis of GM3 thioglycoside analogue after Schmidt.

Target disaccharide **73** can be disconnected to the known glycosyl chloride **31**[10] and triflate **87** with thioacetate being introduced into one or other of these electrophiles prior to base promoted coupling. Gulose derivative **87** could be prepared by the triflate/TBANO_2 method[5] from known compound **84**.[11]At the time of starting this synthetic scheme, Schmidt's sLex synthesis[5, 12] had only been published as a preliminary communication, but on paper it appeared to be a considerably more efficient synthesis than that of Hasegawa and co-workers.[2] It was decided, therefore, to use the same protecting groups as Schmidt in both the donor and acceptor (see Scheme 2.10), except for the anomeric blocking group in the galactose derivative. Here, (2-trimethylsilyl)ethanol was chosen as the aglycon rather than Schmidt's thexyldimethylsilyl protecting group. This change would allow the option of

deprotecting at the disaccharide stage to give an analogue of the smaller ganglioside GM4 which may also be suitable for the deacetylase assay. Alternatively, the trimethylsilylethyl group can be easily removed[13] and **73** converted into a suitable donor for glycosylation of an octyl glucoside acceptor (*e.g.* **74**), thus giving trisaccharide **53** on deprotection. This new approach would have the advantage of allowing the synthesis of a wider range of compounds for study, especially in varying the aglycon used.

4.1.2.2 Synthesis of Acceptors. 3-Thio-galactose derivative **88** was prepared as outlined in Scheme 4.10. Acetobromogalactose, **76**, was obtained from galactose, **75**, in two steps by iodine catalysed acetylation[14] followed by bromination with 45% HBr/AcOH. Two procedures for glycosylation of 2-(trimethylsilyl)ethanol with donor **76** were used. The traditional Koenigs-Knorr method, using silver carbonate as promoter, gave galactoside **77** in 74% yield and a new procedure being developed in our research group, using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and iodine as activators[15] gave the same product **77** in 71% yield.



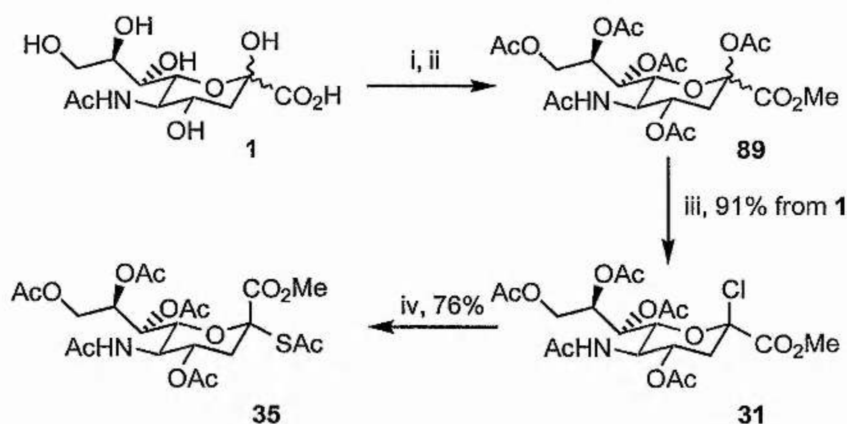
Scheme 4.10 *Synthesis of galactopyranose acceptor. Reagents:* i, Ac_2O, I_2; ii, HBr/AcOH, CH_2Cl_2; iii, $Me_3SiCH_2CH_2OH$, DDQ, I_2, MeCN; iv, $Me_3SiCH_2CH_2OH$, Ag_2CO_3, toluene; v, NaOMe, MeOH; vi, $Pr^i(OMe)_2$, *p*-toluenesulfonic acid; vii, BzCl, pyridine; viii, TFA(aq), CH_2Cl_2; ix, TBDMSCl, pyridine; x, BzCl, pyridine; xi, 80% AcOH(aq); xii, $PhCH(OMe)_2$, camphorsulfonic acid, MeCN; xiii, Tf_2O, pyridine, CH_2Cl_2.

Following deacetylation to give **78,** the 2-*O*-benzoyl compound **83** was prepared according to Lowary *et al.*[11] However, this route gave the target triol in much poorer yield than reported. This problem was largely due to hydrolysis of the unstable 6-O-(2-methoxyisopropyl) group in bis-acetonide **79** prior to, or during the benzoylation step, to give *2-(trimethylsilyl)ethyl 2,6-di-O-benzoyl-3,4-O-isopropylidene-β-D-galactopyranoside* (^{1}H NMR identical to lit.[16]).

An alternative approach was devised and is also outlined in Scheme 4.10. Tetraol **78** was converted to the 3,4-*O*-isopropylidene ketal, **81**, before treating, first with *t*-butyldimethylsilyl chloride (TBDMSCl) and then benzoyl chloride in a one pot reaction in pyridine. The crude mixture was deprotected with 80% aqueous acetic acid before column chromatography to give the 2-*O*-benzoyl compound **83** in 62% from GalOSE **78**. 4,6-Benzylidene derivative **84** was then prepared according to the literature procedure[11] but was found to crystallise on work-up to give the product in 94% yield without need for chromatography.

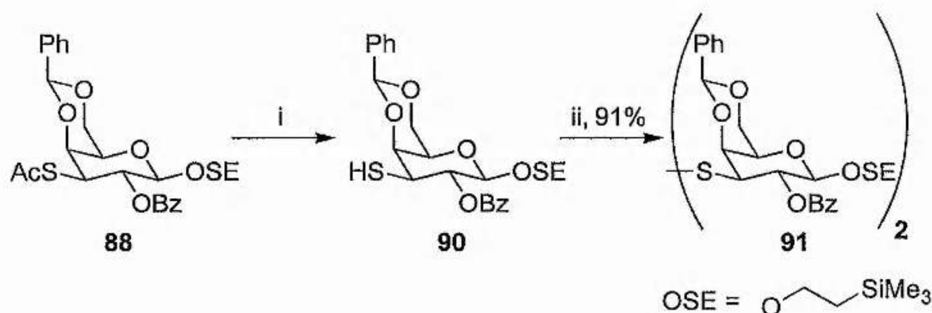
Compound **84** was readily activated as the triflate, **85**, and inversion with tetrabutylammonium nitrite as described above gave the *gulo*-configured sugar **86** in 65% yield. The configuration was confirmed by treating a small sample of **86** with acetic anhydride/pyridine to give the 3-*O*-acetate; ^{1}H NMR, $CDCl_3$; $J_{1,2}$ 8.5 Hz, $J_{2,3}$ 3.6 Hz, $J_{3,4}$ 3.6 Hz. Alcohol **86** was activated as the triflate **87** for inversion back to the *galacto*-configuration. For thio-glycosylation reactions, sulfur was introduced using potassium thioacetate in DMF to give the 3-*S*-acetyl *galacto*-configured compound **88** in 82% yield (^{1}H NMR, $CDCl_3$; $J_{1,2}$ 7.7 Hz, $J_{2,3}$ 11.5 Hz, $J_{3,4}$ 3.3 Hz). Having synthesised the required acceptor compounds, synthesis of the donors was then pursued.

4.1.2.3 Synthesis of Donors. 2-Chloro-sialic acid **31** was made in good yield from *N*-acetylneuraminic acid, **1**, by a modification of the original literature procedure (Scheme 4.11).[10] Briefly, *N*-acetylneuraminic acid was converted to its methyl ester using an acid ion exchange resin in dry methanol and then acetylated in acetic anhydride/pyridine. The peracetate, **89,** was then converted to the thermodynamically more stable β-chloride with HCl in acetyl chloride. The chloride **31** was used without further purification in subsequent reactions, following freeze-drying from dioxan. For anomeric *S*-alkylation reactions, sulfur was introduced in an S_N2 reaction using potassium thioacetate in CH_2Cl_2 to give **35** in 76% yield.[4] Attempting the reaction in DMF, in which the thioacetate was more soluble, led to poor anomeric control, suggesting an S_N1 type reaction in the more polar solvent.



Scheme 4.11 *Synthesis of sialic acid donors 31 and 35. Reagents*: i, Dowex 50WX8-200 (H^+), MeOH; ii, pyridine, Ac_2O; iii, AcCl, HCl; iv, KSAc, CH_2Cl_2.

Selective de-*S*-acetylation of 3-thio-galactoside **88** was achieved using one equivalent of sodium methoxide in MeOH at -40 °C. The starting material and product showed identical mobility on TLC (hexane-EtOAc, 1:1; R_f 0.60), but it was possible to follow the reaction by ^1H NMR spectroscopy. By quenching the reaction with an acidic ion exchange resin after 30 minutes, one hour and two hours, signals for thioacetate **88** were seen to diminish and to be replaced by a new set of signals for the thiol compound **90** (see experimental section for spectral data). Reaction was complete after 2.5 hours. Prolonged reaction, especially in the presence of air, led to a third set of signals appearing, and a new spot on the TLC (hexane-EtOAc, 1:1; R_f 0.52). This product was assigned as the disulfide, **91**. The same product was obtained in high yield by treating thiol **88** with diethylamine in DMF under aerobic conditions (Scheme 4.12). FAB-MS gave a signal at *m/z* 997 corresponding to M+Na for the disulfide.



Scheme 4.12 *Selective deprotection and oxidation of 3-thiogalactose compound 88. Reagents*: i, NaOMe, MeOH, -40°C; ii, Et_2NH, DMF.

4.1.2.4 Attempted reaction of glycosyl chloride 31 and 3-thio-galactoside 92 - base-promoted thioglycosylation [Scheme 4.13 (a)]. Whereas Schmidt and co-workers had achieved de-*S*-acetylation of their 3-thio-galactose derivative using

hydrazinium acetate in DMF to give the thiol,[17] which they then treated with sodium hydride to give the sodium thiolate, it appeared more convenient to use the thiolate **92** obtained directly from the Zemplén reaction in subsequent reactions. However, on attempting to perform the glycosylation reaction with glycosyl chloride **31** under the conditions described by Schmidt (THF, kryptofix 21, room temperature),[5] no reaction was observed. After 48 hours, only a small amount of the disulfide was starting to appear, despite careful attempts to ensure the total absence of oxygen in the flask. It was noted that thiol **90** and thiolate **92** were not very soluble in THF, even in the presence of the aza-crown ether kryptofix 21. Repeating the reaction in the presence of the more common complexing reagent, 15-crown-5, still gave no product. Attempted reaction in DMF was similarly unsuccessful, despite the fact that the reactants were fully in solution.

Scheme 4.13 *Attempted coupling reactions.*

4.1.2.5 Attempted reaction of 2-thio-sialic acid 35 and 3-triflyl-guloside 87 - anomeric *S*-alkylation (Scheme 4.13). Sialic acid thioacetate **35** was selectively de-*S*-acetylated as described by Hasegawa,[4] in a manner analogous to that used for galactoside **88**. Attempted reaction of the sodium thiolate with triflate **87** in DMF was unsuccessful [Scheme 4.13 (b)]; after 5 days at room temperature, the thiolate had partially degraded, and no thio-disaccharide had formed. von Itzstein's procedure for *in situ* de-*S*-acetylation and base-promoted coupling (diethylamine/DMF)[18] was also unsuccessful [Scheme 4.13 (c)], giving only the guloside elimination product, *2-(trimethylsilyl)ethyl 2-O-benzoyl-4,6-O-benzylidene-3-deoxy-β-D*-threo-*hex-2-enopyranoside,* **93**, in 67% yield. A similar result was subsequently reported by Schmidt and co-workers[17] [see section **2.5.1** and Scheme 2.11 (c)] but these eliminations should be compared with von Itzstein's successful synthesis of the Neu5Acα(2→4)-4-thio-Glc linkage from a 4-*O*-triflyl galactose derivative which proceeded in 74% yield.[19]

A similar result was obtained on treating disaccharide triflate **63** and thioacetate **35** with diethylamine in DMF [Scheme 4.13 (d)]. Here, the elimination compound *octyl 2,4,6-tri-O-benzyl-3-deoxy-β-D*-threo-*hex-2-enopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-glucopyranoside* **94** was obtained in 62% yield based on the amount of triflate reacted (15% of compound **63** was recovered unchanged).

Thus, all attempts at forming the sialic acid-galactose thioglycoside, by literature procedures[2, 5] proved fruitless in my hands.

4.1.3 The Gulopyranosyl Problem?

All synthetic approaches to the Neu5Acα(2→3)Gal thio-glycosidic linkage reported so far, have relied on the introduction of sulfur into a suitably activated gulopyranose derivative. As ^{1}H NMR coupling constants indicate (**87**, $CDCl_3$; $J_{1,2}$ 8.5 Hz, $J_{2,3}$ 3.0 Hz, $J_{3,4}$ 3.0 Hz), the gulopyranosyl unit principally adopts the $^{4}C_1$ conformation. As such, it is perfectly set up for elimination of triflate with the *trans*-diaxial arrangement of H-2 and the leaving group [Figure 4.2 (a)]. Overlap of the (C-4)-O bonding orbital with the (C-3)-O antibonding orbital may further facilitate the loss of triflate by weakening the (C-3)-OTf bond. This effect may explain the difference in reactivities observed for the 3-*O*-triflyl gulose derivative and von Itzstein's 4-*O*-triflyl galactose derivative.[19] The 2-chloro-sialic acid derivative **31** is similarly prone to an elimination reaction which may compete with nucleophilic displacement of chloride [Figure 4.2 (b)].[20]

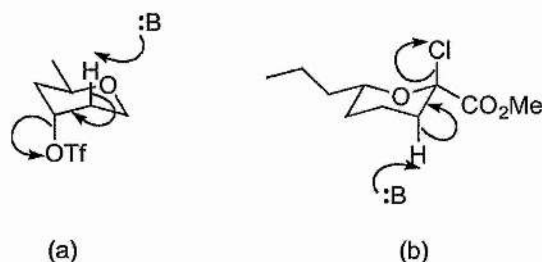
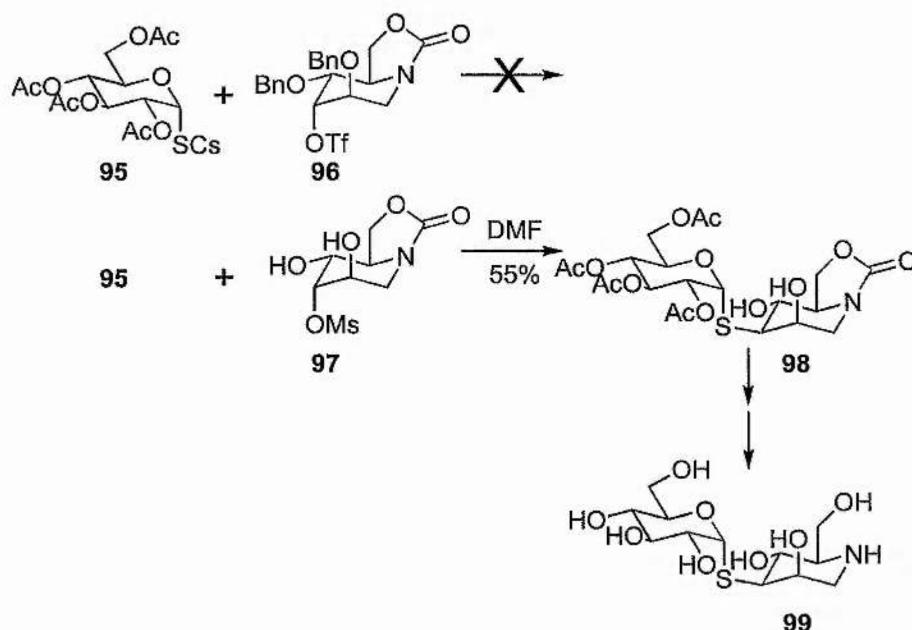


Figure 4.2 *Mechanism of base elimination for 87 and 31.*

The sterically crowded ketose anomeric centre of sialic acid, whether as the chloride or thiolate, will be less reactive to S_N2 reactions than analogous derivatives of aldoses. The problems in the syntheses described so far may be attributed to the relative reactivities of the carbohydrate derivatives to substitution and competing reactions, *i.e.* elimination and/or oxidation of thiolate to disulfide.

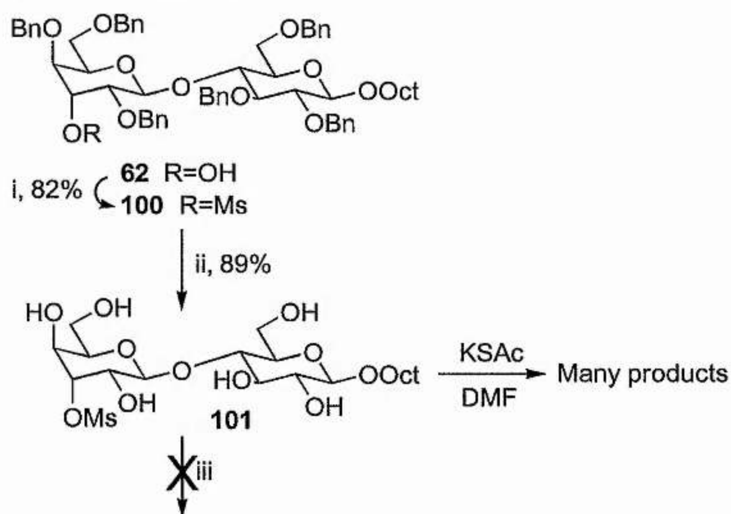
Similar problems were experienced by Hindsgaul and co-workers in their synthesis of the thiogycoside analogue of 1-deoxy-3-*O*-(α-D-glucopyranosyl)-mannojirimycin (Scheme 4.14).[21]



Scheme 4.14 *Synthesis of 1-deoxy-3-O-(α-D-glucopyranosyl)-mannojirimycin 99.*

Here also, the axial triflate leaving group is flanked by an axial and an equatorial sustituent. They concluded that the benzylated compound **96** (*c.f.* **63**) was too sterically hindered to react with 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-glucopyranose. However, they found that the partially deprotected mesyl derivative **97** reacted quite efficiently with glucosyl thiolate **95** giving disaccharide **98** in 55% yield.

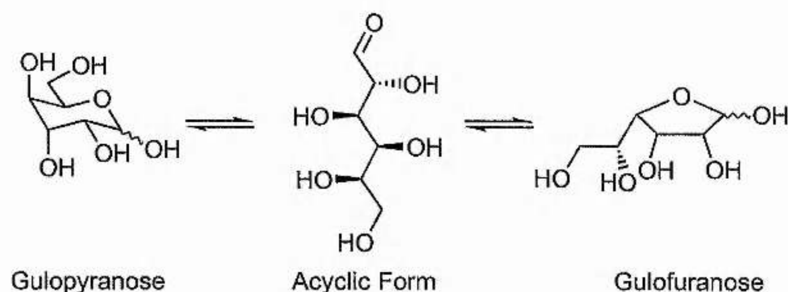
In the same way, guloside **62** was converted to the mesylate, **100**, under standard conditions and then subjected to catalytic hydrogenolysis (Pd on charcoal/H_2/acetic acid) to give the analogous gulosyl glucoside **101** in 73% over two steps (Scheme 4.15). However, an attempt to reproduce the coupling reaction with the sialic acid-gulopyranosyl system proved fruitless. Also, reaction of the mesylate **101** with potassium thioacetate in DMF gave rise to many products, and so this line of investigation was not pursued further.



Scheme 4.15 *Attempted synthesis of 53 after Hindsgaul. Reagents*: i, MsCl, pyridine; ii, H_2, Pd on C, AcOH; iii, **35**, Et_2NH, DMF.

4.1.4 Approach 3: the Galactofuranosyl/Gulofuranosyl Acceptor

Gulose, as with all aldohexoses, can exist in a six-membered (pyranose) ring, an open chain form and a five-membered (furanose) ring (Scheme 4.16). The furanose form of gulose exhibits a number of structural properties that suggest that a 3-*O*-triflyl-D-gulofuranose derivative should be more suitable for S_N2 reactions than a corresponding gulopyranose derivative.

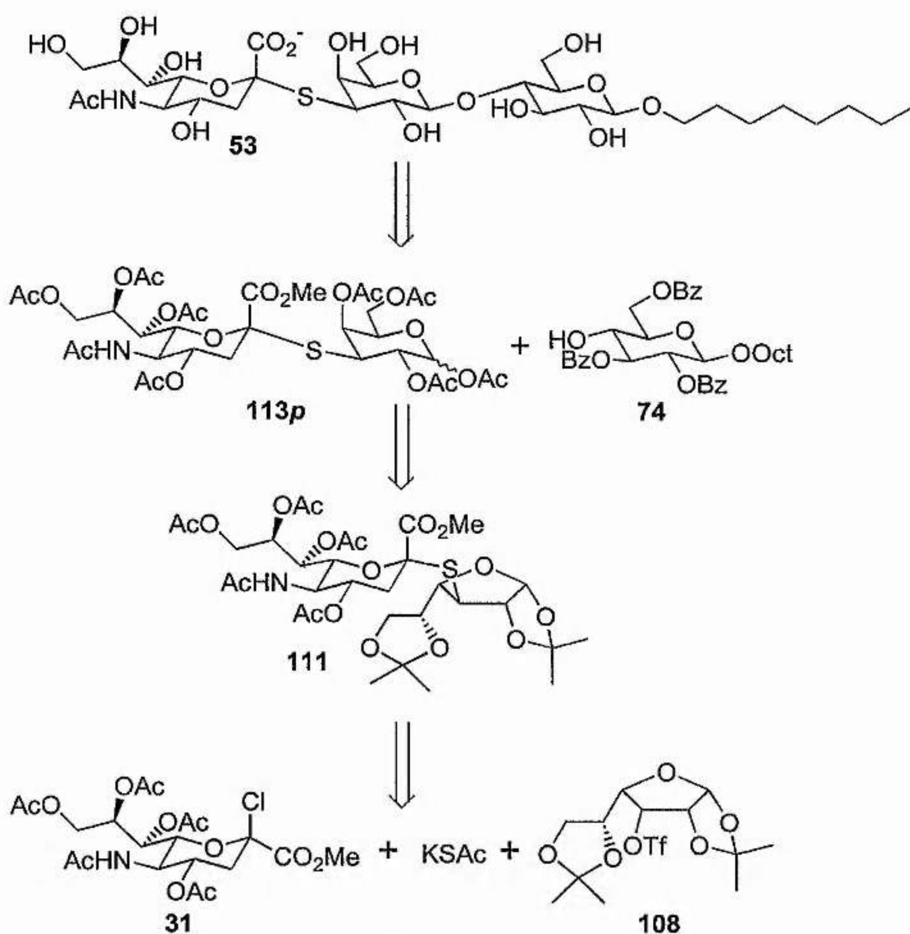


Scheme 4.16 *The structures of gulose.*

For an α-gulopyranose, all ring sustituents are pointing to the lower face of the molecule, which should allow a relatively unhindered line of attack for an incoming sulfur nucleophile. In order to achieve the anti-periplanar arrangement of H-2 and triflate as described above, the furanose ring would have to adopt a less favoured conformation in which other ring sustituents would be eclipsed. Therefore, the furanose ring should be less prone to elimination. In addition, S_N2 reactions are usually more facile in five-membered rings than six-membered rings as the former experiences a reduction of substituent eclipsing in proceeding to the transition state, whereas for the latter, the converse is true.

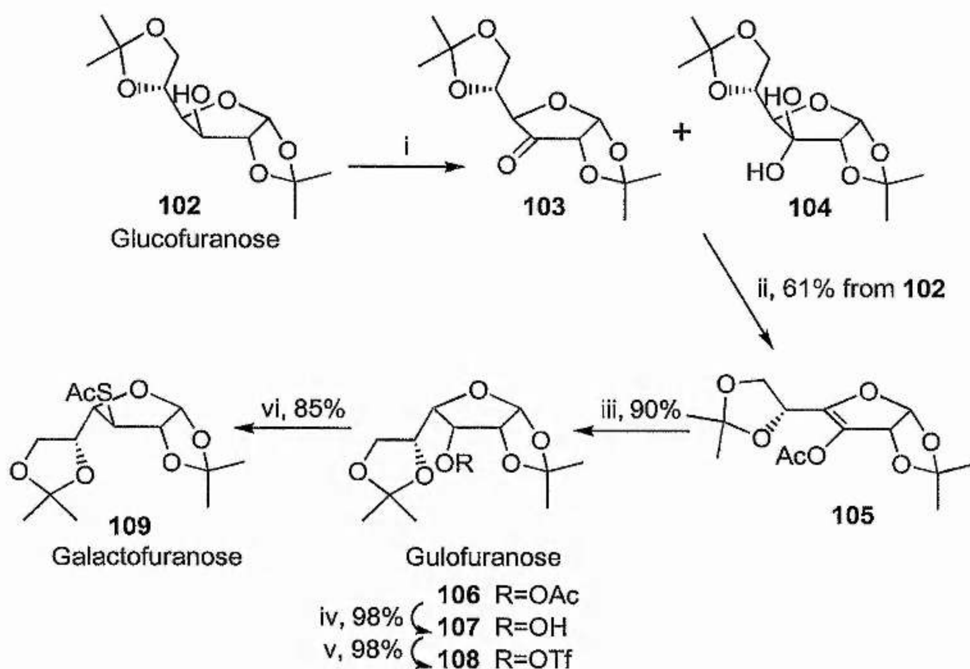
von Itzstein and co-workers have described the synthesis of a number of *S*-linked disaccharides in which sialic acid is connected to a secondary carbon of the other monosaccharide residue.[19] One of these involves the displacement of triflate from an allofuranose derivative. It is worth noting that although von Itzstein and co-workers report the synthesis of a number of *S*-linked analogues of sialic acid-containing disaccharides, they omitted the synthesis of Neu5Acα(2→3)Gal, one of the most commonly occurring sialic acid linkages to be found in nature.

4.1.4.1 Retrosynthesis. Our third approach to the synthesis of compound **53** is thus based on formation of the sialic acid-galactofuranose thioglycosidic bond. Here, reaction of a sialic acid 2-thiolate with an activated gulofuranose would give the Neu5Acα(2→3)Gal*f* thio-glycoside, which would first be transformed back into the galactopyranose form before further elongation to the trisaccharide (Scheme 4.17).



Scheme 4.17 *Retrosynthetic analysis of GM3 thioglycoside analogue* via *gulofuranose 108.*

4.1.4.2 Synthesis. 1,2:5,6-Di-*O*-isopropylidene-α-D-gulofuranose, **107**, was chosen as a suitable gulose derivative. The fused bicyclic ring system, which has C-1 and C-2 as its bridgeheads, should be even less prone to elimination because of the reduced flexibility of the furanose ring and the increased ring strain that would result on formation of an sp^2 centre at C-2. Although gulofuranose **107** is commercially available (retailing at £296 per g), it was preferable to prepare it from diacetone glucose (46p per g) by a modified four step literature procedure (Scheme 4.18).[22] Briefly, the 3-OH of diacetone glucose is oxidised to the ketone, the enol of which is trapped as its acetate. Subsequent stereoselective hydrogenation of the C-3:C-4 double bond from the *exo*-face of the molecule, effects the required inversion of configuration at C-3 and C-4 required to give gulofuranose. Finally, deacetylation gave 1,2:5,6-di-*O*-isopropylidene-α-D-gulofuranose in 54% overall yield.



Scheme 4.18 *Synthesis of gulofuranose and galactofuranose from glucofuranose after Lemieux. Reagents*: i, RuO_2, KIO_4, K_2CO_3, CH_2Cl_2, H_2O; ii, Ac_2O, Et_3N; iii H_2, Pd on C, EtOAc; iv, NaOMe, MeOH; v, Tf_2O, pyridine, CH_2Cl_2; vi, KSAc, DMF.

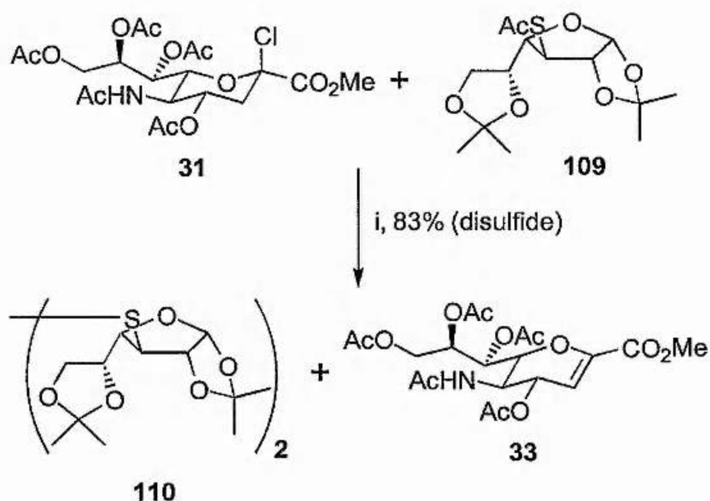
Several reagents were tried for the oxidation of diacetone glucose **102**. No reaction was observed on treating the alcohol with tetrapropylammonium perruthenate and *N*-methylmorpholine-*N*-oxide in either CH_2Cl_2 or acetonitrile.[23] Treatment with pyridine/chromium trioxide/acetic anhydride gave the desired product, but in low yield.[24] The preferred oxidation procedure used potassium periodate/potassium carbonate/hydrated ruthenium (IV) oxide in a biphasic reaction.[25] The colour of the reaction mixture indicates the major ruthenium species present and hence the progress of the reaction: the orange-brown colour present during most of the reaction results from the water soluble perruthenate ion, which may be further oxidised to the CH_2Cl_2 soluble ruthenium tetroxide which has a pale yellow colour. The reaction mixture turning black, indicates that there is no periodate left; the reaction mixture turning pale yellow indicates that there is no substrate left. It has been reported that the lower solubility of potassium periodate over sodium periodate, can give greater control over the rate of reaction, and thus reduce over oxidation.[25]

The initial product of the oxidation reaction, 1,2:5,6-di-*O*-isopropylidene-α-D-*ribo*-hexofuranos-3-ulose **103**, readily hydrates and most publications report the use of the crystalline hydrate **104** in further reactions. However, in my hands, the initially product mixture of ketone and hydrate could not be completely converted into either

component and so it was found to be more convenient to use the partially crystalline, initial product mixture in the next step without purification.

Again a modified procedure for the enolisation/acetylation was to prove more convenient that the published procedure. Rather than acetylation in pyridine/acetic anhydride for 15 hours at 70 °C, the same reaction could be carried out in the more basic mixture of triethylamine/acetic anhydride in 5 hours at room temperature giving a similar yield of enol acetate **105** (~60% from diacetone glucose). Hydrogenation and deacetylation were performed using standard procedures to give gulofuranose **107**.[22]

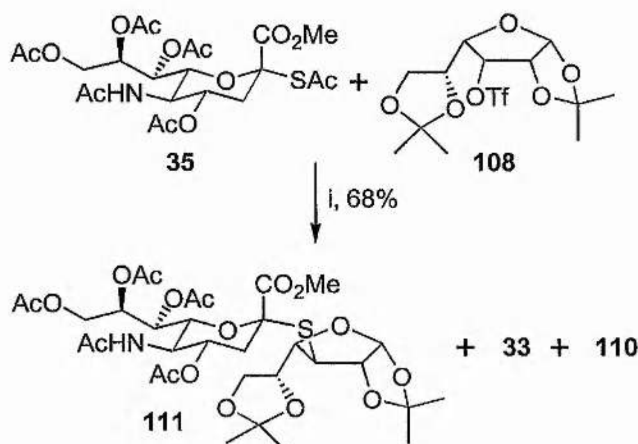
The gulofuranose compound was easily activated as the triflate **108**. Reaction with potassium thioacetate under phase transfer conditions gave the 3-*S*-acetyl galactofuranose compound **109** in good yield. On subjecting a mixture of **109** and 2-chloro-sialic acid **31** to von Itzstein's coupling conditions of diethylamine/DMF,[19] only the disulfide **110** and the sialic acid 2,3-elimination product **33** were recovered (Scheme 4.19).



Scheme 4.19 *Attempted coupling of 31 and 109. Reagents*: i, Et_2NH, DMF.

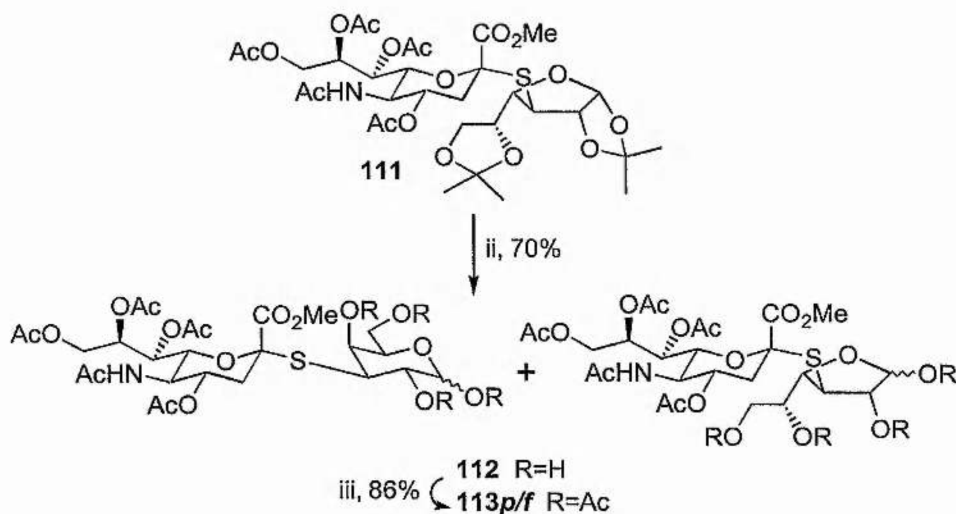
However, treatment of sialic acid thioacetate **35** and 1.3 equivalents of the triflate **108** under the same conditions, gave the desired thio-disaccharide in 68% yield, along with 14% unreacted triflate, and surprisingly, the galactofuranose disulfide **110** in 4% yield (Scheme 4.20). The disaccharide was contaminated with a small amount of the sialic acid elimination product **33** which proved impossible to remove by flash chromatography. An analytical sample was obtained, however, by gel permeation chromatography on lipophilic Sephadex LH20 in methanol. This technique allows separation of components based on molecular size, rather than polarity. As the impurity was easily removed by flash chromatography following partial deprotection

of the disaccharide (removal of either the acetates or acetonides), the product was used in subsequent steps without further purification.



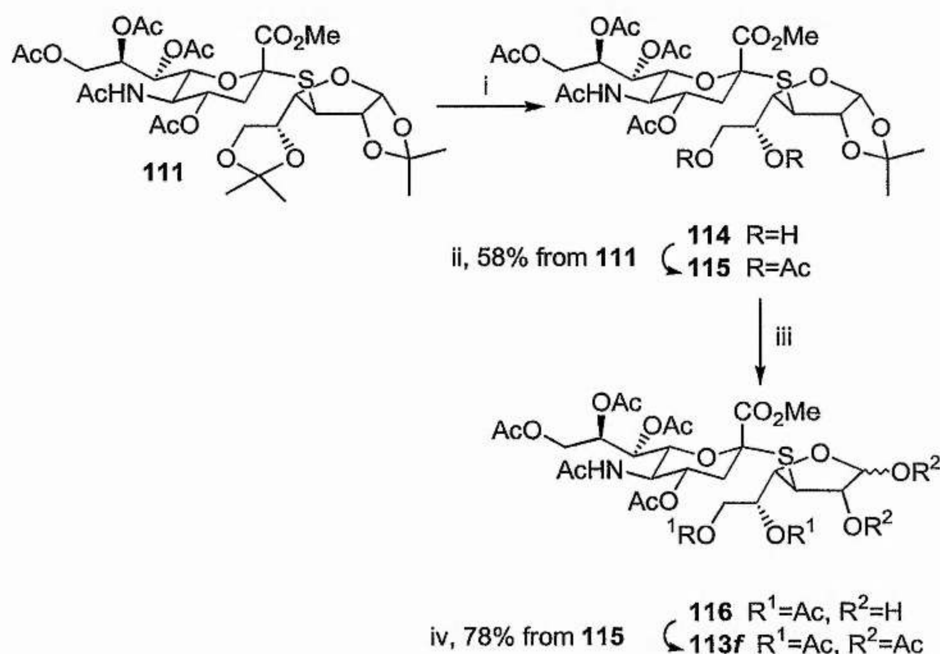
Scheme 4.20 *Synthesis of thio-disaccharide 111.*

Although the 5,6-isopropylidene group of **111** is very acid labile, the 1,2-ketal was only slowly removed on treatment with hot 80% aqueous acetic acid. Treatment with 90% TFA at room temperature for 15-30 minutes readily hydrolysed both ketals in an acceptable 70% yield. The product mixture gave complex ^{1}H NMR spectra in a variety of deuterated solvents. The clearest spectrum was recorded in pyridine-d_5 and showed four anomeric signals corresponding to the α and β anomers of both pyranose and furanose ring forms. A 4:1 pyranose:furanose mixture of peracetates, **113*p/f***, was obtained on acetylation of the crude **112** mixture in pyridine/acetic anhydride (Scheme 4.21).

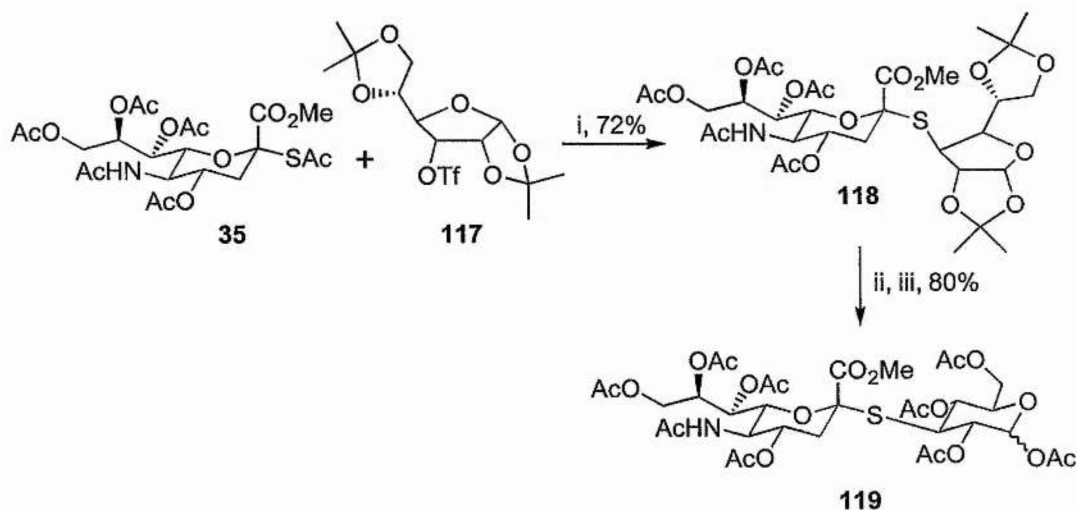


Scheme 4.21 *Attempted conversion of compound 111 to its pyranose ring form. Reagents*: i, Et_2NH, DMF; ii, TFA(aq); iii, Ac_2O, pyridine

In order to confirm which signals were due to furanose and pyranose isomers, the α and β furanose peracetates were synthesised separately (Scheme 4.22). Briefly, the 5,6-isopropylidene group was selectively hydrolysed with aqueous acetic acid and the 5- and 6-hydroxy groups acetylated prior to removal of the 1,2-isopropylidene group with 90% TFA. Acetylation of the 1- and 2-hydroxy groups gave a 1:2 mixture of the α and β anomers **113*f*** in 45% yield from **111** (NMR spectra: Appendix 2).



Scheme 4.22 *Synthesis of α/β galactofuranose peracetates. Reagents*: i, AcOH(aq); ii, Ac_2O, pyridine; iii, TFA(aq); iv, Ac_2O, pyridine.

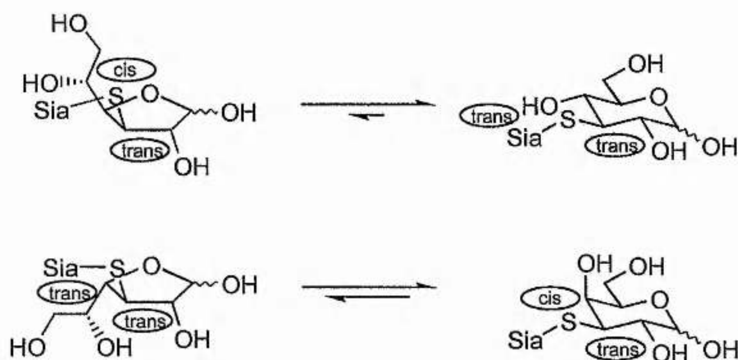


Scheme 4.23 *Synthesis of 118 and 119. Reagents*: i, Et_2NH, DMF; ii, TFA(aq); iii, Ac_2O, pyridine.

S-(Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-1,2:5,6-di-*O*-isopropylidene-3-thio-α-D-glucofuranose, **118**, the *gluco*-isomer of disaccharide **111**, was prepared according to von Itzstein's procedure (Scheme 4.23).[19] Hydrolysis of the acetonides and acetylation under the same conditions used for **111** gave a mixture of only two isomers. Both ^{1}H and ^{13}C NMR ($CDCl_3$) were consistent with the α and β glucopyranose structures **119**; δ_H 5.98 (d, $J_{1,2}$ 8.2, 1a-H β), 6.22 (d, $J_{1,2}$ 3.8, 1a-H α); δ_C 88.7 (1a-C α), 92.5 (1a-C β).

4.1.5 The Galactofuranosyl/Galactopyranosyl Problem?

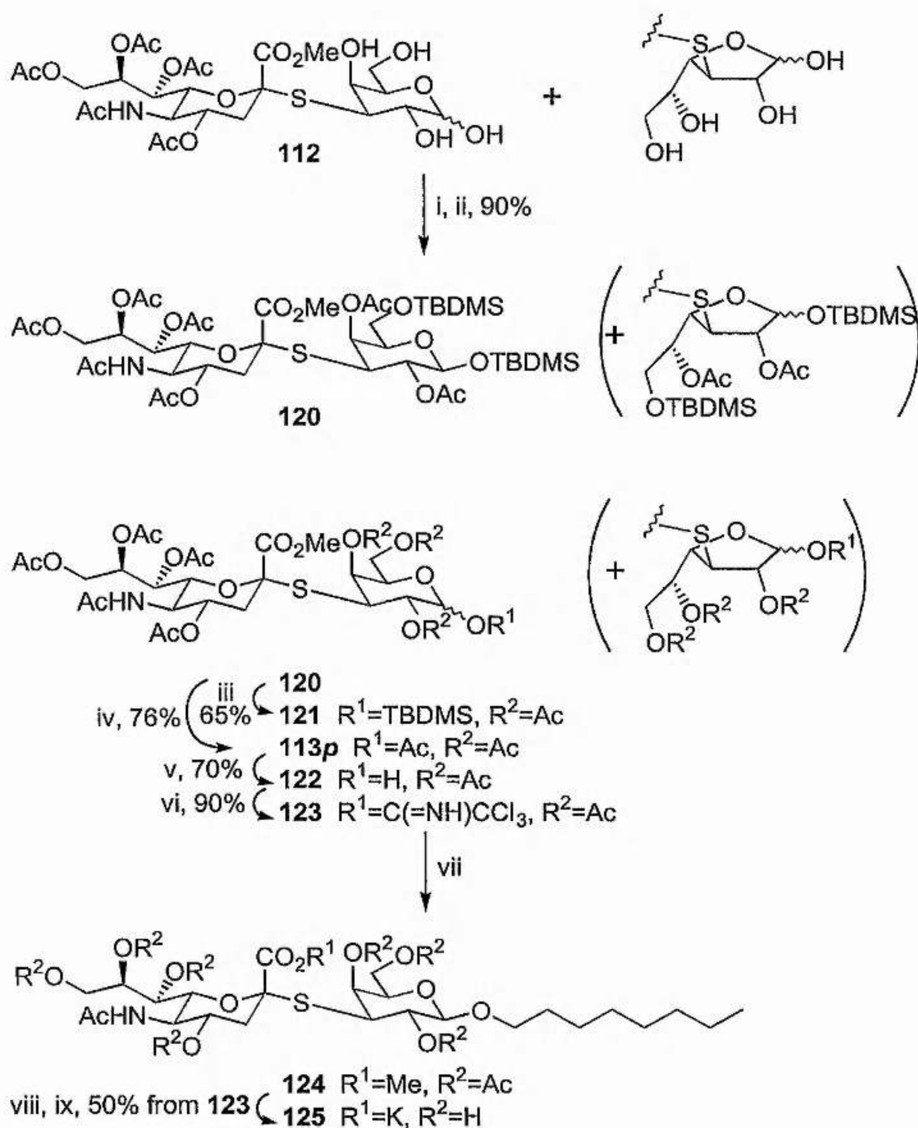
The difference in configuration at C-4, seems to be sufficient to govern whether the compound should adopt either the pyranose or furanose structure. For the *gluco*-configuration, the molecule can avoid the *cis* interaction of the sulfur linkage and C-5, present in the furanose ring, by adopting, exclusively, the pyranose form with its fully equatorial arrangement of sustituents (Scheme 4.24). In other words, there is a large difference in the relative energies of the pyranose and furanose forms, and the equilibrium therefore lies very much in favour of pyranose. However, for the *galacto*-configuration, it is the furanose ring that has the *trans* arrangement of sustituents around the thioglycosidic linkage, and the pyranose ring that has a *cis* arrangement of sulfur and O-4 (Scheme 4.24). The net effect is to bring the relative energies of the two ring forms closer together, resulting in the mixture of isomers observed. The properties of the furanose ring that were advantageous to the coupling reaction, form the basis of the problem encountered in trying to form the pyranose ring.



Scheme 4.24 *The cis and trans arrangement of substituents in the gluco- and galacto-ring forms.*

Many approaches to solving the furanose-pyranose problem were studied; these included acylation (acetylation, benzoylation, pivaloylation) with a variety of reagents at varying temperatures, acetolysis of the acetonides, lactonisation of the sialic acid and stannylene acetal-mediated acylation amongst others, but no

improvement (or, indeed, any clear major product in some cases) was achieved by these methods. The one exception which showed great promise was to treat the **112** mixture with a excess of *t*-butyldimethylsilyl chloride in pyridine (Scheme 4.25). The first silylation occurs at the primary alcohol, as expected, and the second silylation takes place at the anomeric position, but it does so to give, almost exclusively, the β pyranose (^{1}H NMR, $CDCl_3$; $J_{1a,2a}$ 7.4 Hz, $J_{2a,3a}$ 11.8 Hz, $J_{3a,4a}$ 3.3 Hz). The second silylation occurs much more slowly than the first and perhaps, could be thought of as a kinetic resolution of the disaccharide isomers, occurring alongside a re-equilibration of the other components of the mixture.



Scheme 4.25 *Synthesis of ganglioside GM4 analogue 125. Reagents*: i, TBDMSCl, pyridine; ii, Ac_2O, pyridine; iii, TFA(aq) then, Ac_2O, pyridine; iv, H_2SO_4, Ac_2O, AcOH; v, N_2H_4.AcOH, DMF; vi, $NCCCl_3$, DBU, CH_2Cl_2; vii, OctOH, $BF_3.Et_2O$, CH_2Cl_2; viii NaOMe, MeOH; ix, KOH(aq).

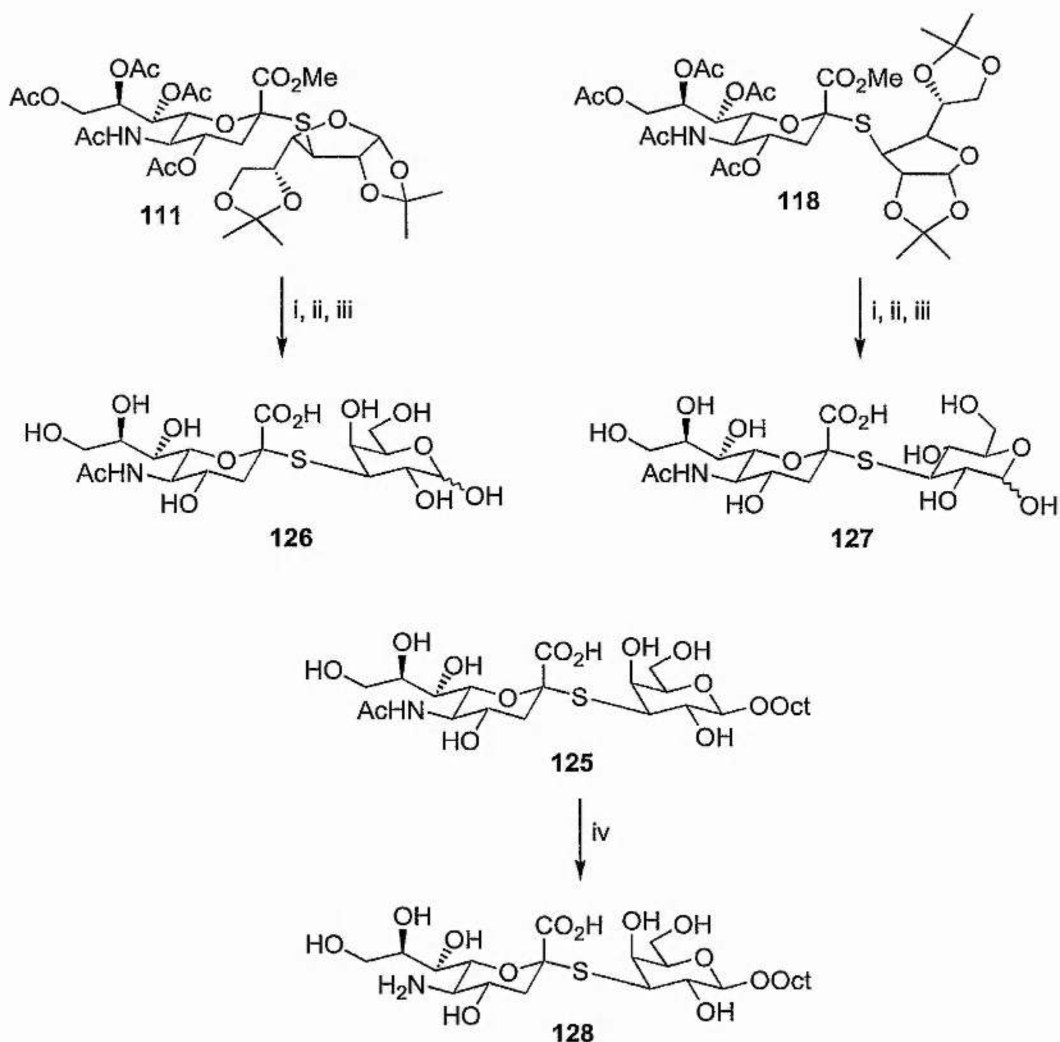
Having tied up the galactose into the pyranose ring, the remaining hydroxyl groups (positions 2 and 4) were acetylated in a one pot reaction to give **120** in 90% yield from **112**. This would prevent re-equilibration of furanose and pyranose on hydrolysis of the TBDMS ethers, as O-4 which would otherwise become the furanose ring oxygen was now blocked as an ester. The minor isomer visible in the ^{1}H NMR spectrum (~5%) could not be clearly identified, but conversion to the peracetate would allow comparison with the mixture resulting from the direct acetylation of **112**.

Several procedures for removing the silyl ethers were investigated. 80% Aqueous acetic acid at 80 °C, 90% aqueous TFA, tetrabutylammonium fluoride in THF and potassium fluoride/18-crown-6 in acetonitrile, all at room temperature readily removed the primary silyl ether [the product of which was acetylated to confirm its structure; **121** ^{1}H NMR ($CDCl_3$) δ_H 5.10 (1 H, d, $J_{1a,2a}$ 7.4, 1a-H)], but none gave complete removal of the anomeric TBDMS group. The harsher conditions of an acetolysis reaction (2.5% H_2SO_4 in acetic acid/acetic anhydride) gave the peracetate **113** in 76% as a mixture of β-*p*:α-*p*:β-*f*, 83:14:3 (NMR spectra: Appendix 2). Comparison of published analytical data with those for this product mixture (and products of subsequent steps in the synthetic sequence) now becomes possible, as the β-pyranose peracetate **113β*p*** is a known compound,[2] used by Hasegawa and co-workers in their synthesis of *S*-linked ganglioside GM4.[1]

The peracetate was selectively deprotected using hydrazinium acetate to give the hemiacetal **122** in 70% yield; $[\alpha]_D$ +30.0 (*c* 0.7 in $CHCl_3$) (lit.,[1] [β anomer] +33.0). The hemiacetal was then converted into an anomeric mixture (β-*p*:α-*p*, 92:6) of trichloroacetimidates **123** in 90% yield using DBU as base (the anomeric ratio of the imidate is unexpected as DBU usually gives principally the α anomer); $[\alpha]_D$ +26.4 (*c* 1.1 in $CHCl_3$) (lit.,[1] [β anomer] +27.5). ^{1}H NMR spectroscopy indicated that this was contaminated by only 2% of a furanose derivative.

Unfortunately, time did not allow for the further extension of this glycosyl donor to the original target trisaccharide. Instead, it was decided to make the octyl glycoside of the disaccharide, as this would be suitable also for the C-18 reverse phase based assay. Thus, the imidate donor **123** was treated with octanol in the presence of boron trifluoride etherate to give the octyl glycoside **124**. Purification of the peracetate proved difficult and so the resulting product mixture was de-acetylated and the methyl ester saponified prior to purification by reverse phase chromatography. ^{1}H NMR spectroscopy showed that the product **125**, obtained in 50% yield from the imidate, was exclusively the β-pyranoside.

4.1.6 Substrates for the Neuraminidase and *trans*-Sialidase Assays. In addition to octyl glycoside **125**, several other thio-sialosides were prepared for the enzyme assays. Portions of sialylated galctofuranose compound **111** and sialylated glucofuranose compound **118** were fully deprotected to give the reducing disaccharides (Scheme 4.26). Briefly, compounds **111** and **118** were de-*O*-acetylated under Zemplén conditions and then the methyl esters were saponified prior to acid hydrolysis of the acetonides to give the reducing disaccharides **126** and **127**, each in ~40% yield from the fully protected disaccharides. Also, a small sample of **125** was de-*N*-acetylated in hot potassium hydroxide solution to give deNAcGM4 thio-analogue **128** in almost quantitative yield (Scheme 4.26).



Scheme 4.26 *Synthesis of thio-sialosides 126, 127 and 128 for neuraminidase assays. Reagents*: i, NaOMe, MeOH; ii, NaOH (aq); TFA (aq); iv, NaOH (aq), Δ.

4.1.7 Conclusions – Thio-Sialoside Synthesis

Attempts to synthesise the Neu5Acα(2→3)Gal thio-disaccharide by literature procedures failed in my hands. However, a thioglycoside analogue of ganglioside GM4 was successfully synthesised *via* a novel route involving a sialic acid-galactofuranose intermediate (**111**). The synthesis was completed in a total of 19 steps from diacetone glucose and sialic acid and in an overall yield of 4%. The longest linear sequence was 15 steps which compares favourably with syntheses already published in the literature.[1, 2, 5] However, the sialic acid-gulofuranose coupling described here, appears to be a more facile reaction than other procedures that have been reported for making the Neu5Acα(2→3)-3-thio-Gal linkage.

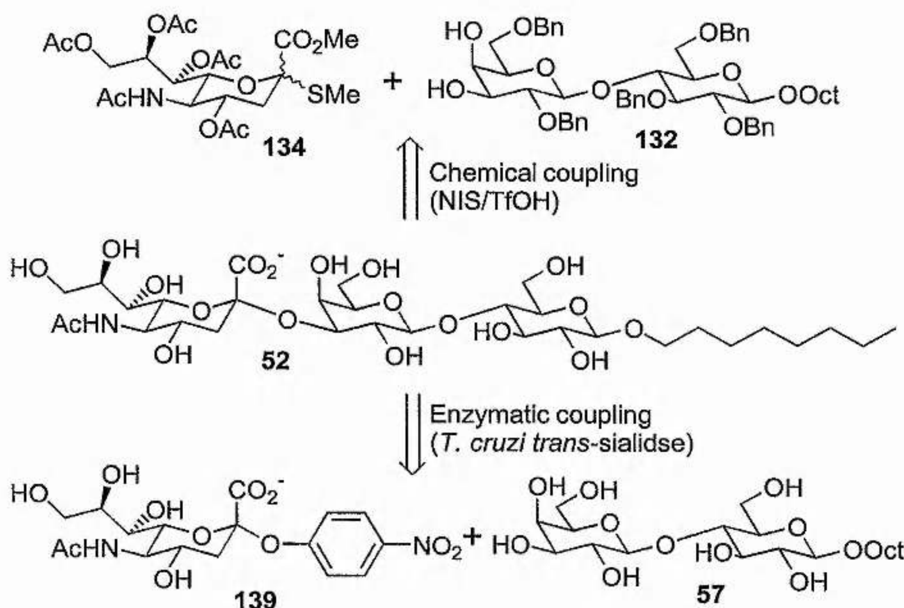
4.2 Synthesis of *O*-Linked GM3 analogue 52

4.2.1 Chemical *vs.* Enzymatic Synthesis. There are two general approaches towards the synthesis of *O*-glycosides: chemical synthesis and enzymatic synthesis. Chemical synthesis of oligosaccharides is a very powerful tool in glycobiology, as has been demonstrated by a number of prominent laboratories around the world in recent years.[26] It presents the opportunity of making well defined single compounds in multi-gram quantities, free from the "micro-heterogeneity" found in the lipid portions of glycolipids isolated from natural sources. Where chemical synthesis truly excels is in the construction of unnatural analogues of compounds, specifically designed as biological probes.[27] The major drawback of the chemical approach is the necessity for extensive (and tedious) protecting group chemistry, which is essential to control both the regio- and stereo-selectivity of glycosylation reactions. This invariably leads to multi-step reaction sequences and often low overall yields.

The alternative is to use the glycosyl transferases and glycosidases, on which Nature relies for constructing and remodeling complex glycans *in vivo*.[28, 29, 30, 31] More and more of these highly precise bio-catalysts are becoming available as synthetic tools, especially the glycosidases which do not rely on expensive nucleotide sugar donors and are often best used in conjunction with organic solvents.[29, 31, 32] The lack of protection and deprotection steps allows much shorter reaction sequences which can be achieved on a much shorter time scale and in yields which can easily rival chemical routes. Where enzymes are used in conjunction with chemical methods, a wide range of non-natural compounds can also be synthesised.[30, 33]

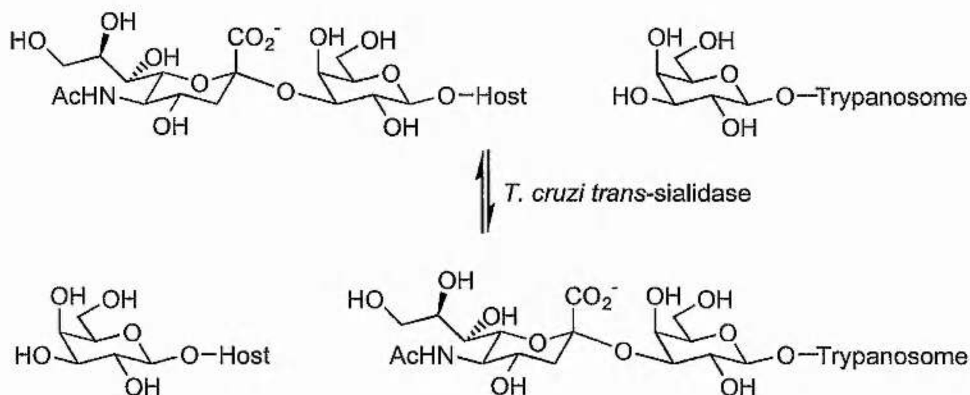
4.2.2 Retrosynthesis. Two routes were considered for the synthesis of the *O*-linked GM3 analogue **52**; one purely chemical and the other using a combination of chemical and enzymatic steps (Scheme 4.27).

There have been several total syntheses of ganglioside GM3,[34, 35, 36] and also many syntheses of the higher gangliosides of which sialyl lactose is the core unit.[37] The usual approach is to glycosylate a suitably protected lactose derivative with a sialic acid donor and then to introduce the ceramide portion as the final step. This convergent approach is in contrast with the biosynthesis of gangliosides which occurs by stepwise glycosylation from the reducing end, starting with glucosyl ceramide.[38] As octyl lactoside had already been made as an intermediate in the original approach to the thioglycoside synthesis, both syntheses described here, involved the sialylation of this acceptor.



Scheme 4.27 *Retrosynthetic analyses for chemical and chemo-enzymatic syntheses of 52.*

The *trans*-sialidase from *Trypanosoma cruzi* was the enzyme of choice for the glycosylation.[39, 40] Most neuraminidases have proven ineffective for use in the "reverse direction" and sialyl transferases require CMP sialoside in either stoichiometric quantities or to be recyled *in situ* using a multi-enzyme system.[41] The *trans*-sialidase is used by the trypanosomatid parasites to steal sialic acid residues from the surface of its host's cells in order to cover its own surface (Scheme 4.28).[39]



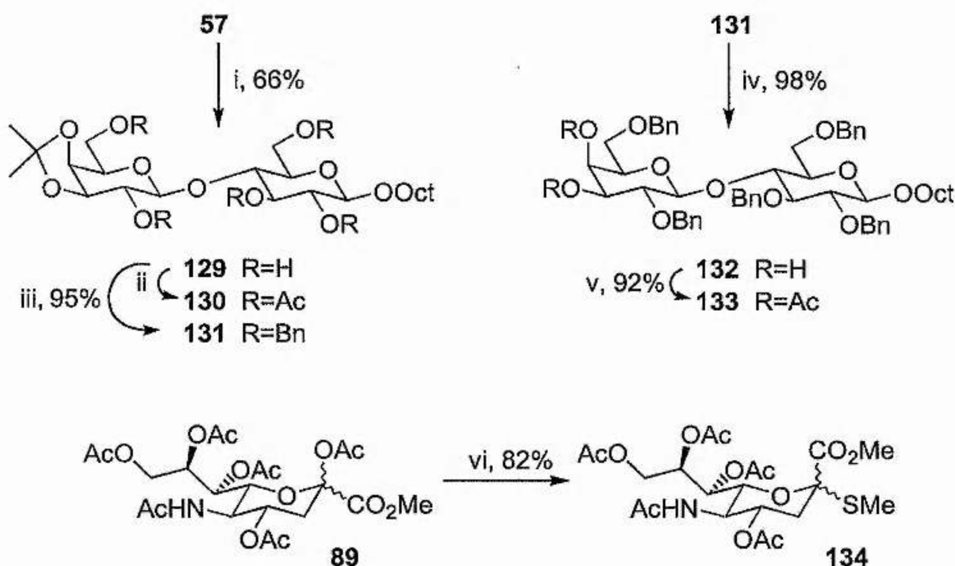
Scheme 4.28 *The Trypanosoma cruzi trans-sialidase transfers sialic acid between the trypanosome and its host.*

Thus, the enzyme uses sialylated oligosaccharides as donors, but can also use simple glycosides such as 4-methylumbelliferyl[42] and *p*-nitrophenyl sialosides There have been several reports of the use of this enzyme in synthesis.[42, 43, 44] A 70 kDa recombinant construct of the *trans*-sialidase[40] has also been used in our laboratory for the sialylation of sulfated and phosphorylated LacNAc compounds,[45] but as these

syntheses were only performed on a very small scale and had involved purification by several different chromatographic steps, the synthesis of the GM3 trisaccharide was first attempted by a chemical route.

4.2.3 A Chemical Synthesis of 52. The approach adopted was based on Hasegawa's synthesis of ganglioside GM2.[46]

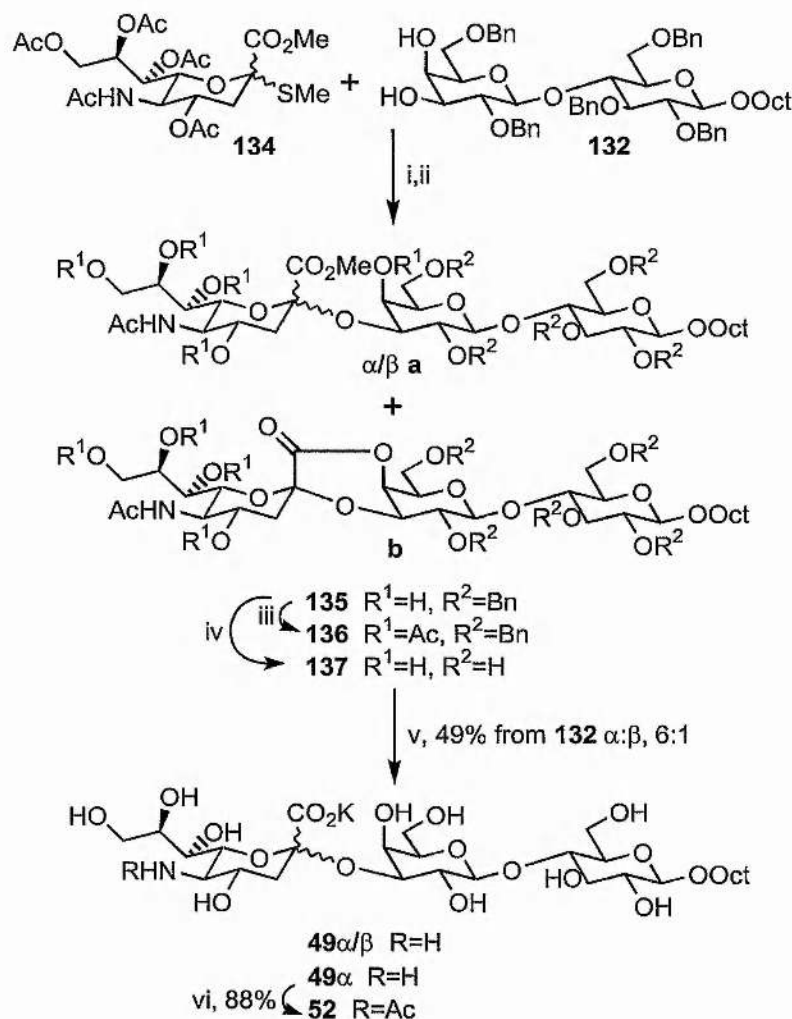
4.2.3.1 Acceptor synthesis. Octyl lactoside **57** was protected with an isopropylidene ketal across the 3b-, 4b-hydroxy groups to give **129** in 66% yield, using an excess of dimethoxypropane and catalytic *p*-toluenesulfonic acid with subsequent selective hydrolysis of any 6a- or 6b-mixed ketals by aqueous TFA. The position of the protecting group was confirmed by acetylation of a small portion of the product. The remaining material was benzylated before hydrolysing the acetonide to give the diol acceptor **132** in 90% yield (Scheme 4.29). Although two hydroxyl groups are still free, glycosylation has shown to be selective for the equatorial 3b position over the less reactive, axial 4b-OH.[46]



Scheme 4.29 *Syntheses of acceptor 132 and donor 134. Reagents*: i, $Pr^i(OMe)_2$, *p*-toluenesulfonic acid; ii, Ac_2O, pyridine; iii, NaH, BnBr, DMF; iv, AcOH(aq); v, Ac_2O, pyridine; vi, Me_3SiSMe, Me_3SiOTf, $ClCH_2CH_2Cl$.

4.2.3.2 Donor synthesis. The donor **134** was prepared in 3 steps by a literature procedure in 74% yield from sialic acid (Scheme 4.29).[47] Briefly, the peracetylated sialic acid methyl ester **89**, prepared as in the synthesis of **31** (Scheme 4.11), was treated with (methylthio)trimethylsilane and TMSOTf to give the methyl thioglycoside **134** as an anomeric mixture (α:β, 1:1).

4.2.3.3 Chemical glycosylation. Glycosylation of diol acceptor **132** was performed with 2.5 equivalents of donor **134** and *N*-iodosuccinimide and catalytic triflic acid as promoter, according to a literature procedure.[46] It proved convenient to deacetylate the product mixture prior to column chromatography, which yielded a mixture of trisaccharides **135a/b** (Scheme 4.30).



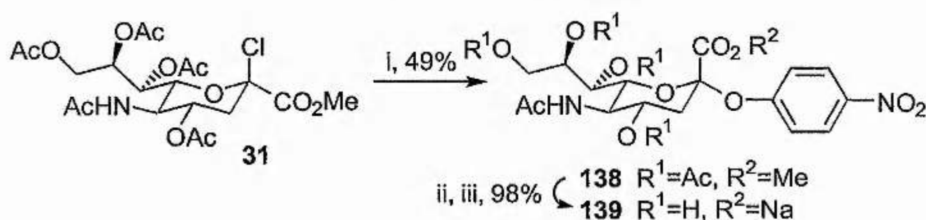
Scheme 4.30 *Synthesis of GM3 analogue 52. Reagents*: i, NIS, TfOH, MeCN; ii, NaOMe, MeOH; iii, Ac_2O, pyridine; iv, H_2, $Pd(OH)_2$, MeOH; v, KOH(aq); vi, Ac_2O, MeOH.

A small sample of the mixture was partially separated by careful chromatography and the fractions reacetylated to give the α-linked 3c→4b lactone **136b** and an α/β mixture of the methyl ester **136a**, identified by comparison of spectral data for analogous literature compounds.[48, 49] It is unclear at what point the lactonisation occurred; acid-promoted lactonisation during the glycosylation reaction is one possibility, but the partial conversion of sialoside methyl esters to lactones under Zemplén deacetylation conditions has also been reported.[49] Hydrogenolysis of each of the deacetylated fractions using palladium hydroxide in MeOH gave a mixture of

lactone **137b** and methyl ester(s) **137a**. The remaining product mixture was debenzylated in a similar fashion and then treated with hot 1 M potassium hydroxide solution to hydrolyse both the ester/lactone and also the sialic acid acetamide, as trial experiments had shown the α and β anomers to be best resolved on TLC as the amino compounds **49α/β**. The deprotected product was obtained in 49% yield from acceptor **132** as a 6:1, α:β mixture, whereas the published procedure had claimed total anomeric control to give the α-sialoside in similar yield.[46] Although the anomers could be separated on a preparative TLC plate, or by repeated flash chromatography, this tedious final purification step gave the desired product in low yield (21%). Reacetylation of the amino group was achieved using 3 equivalents of acetic anhydride in MeOH solution giving **52** in high yield.

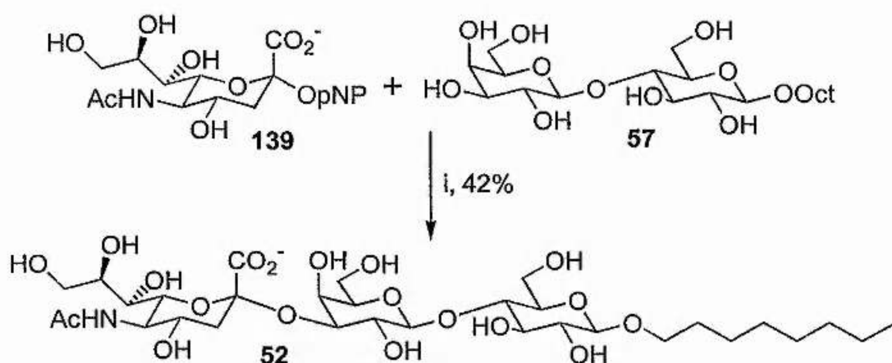
4.2.4 A Chemo-Enzymatic Synthesis of 52. The enzymatic procedure was investigated for comparison.

4.2.4.1 Donor synthesis. The *p-nitrophenyl sialoside* **139** was prepared in 49% yield by nucleophilic displacement of chloride from compound **31** under phase transfer conditions.[50] Deacetylation and saponification of the methyl ester gave the required Neu5AcαpNP *donor* **139** in almost quantitative yield (Scheme 4.31).



Scheme 4.31 *Synthesis of donor 139. Reagents*: i, pNP, $TBAHSO_4$, NaOH(aq), CH_2Cl_2; ii, NaOMe, MeOH; iii, NaOH(aq);

4.2.4.2 Enzymatic glycosylation (Scheme 4.32). The *trans*-glycosylation catalysed by the *trans*-sialidase is fully reversible. Therefore, one would expect the equilibrium product mixture to reflect the relative concentrations of acceptors present and their affinities for the enzyme. After incubating octyl lactoside, **57**, and 1.5 equivalents of the donor **139** with the *trans*-sialidase overnight at 30 °C, TLC indicated that the reaction had gone about half way to completion. Although the reaction can be pushed further towards completion by using higher concentrations of the donor and enzyme, complete consumption of the acceptor was never observed.



Scheme 4.32 *Enzymatic coupling of donor **139** with acceptor **57**. Reagents*: i, *trans*-sialidase, sodium phosphate buffer pH 7.

Therefore, it was more convenient to stop the reaction after a relatively short reaction time and separate the product from the reactants. Reverse phase chromatography on C-18 silica proved to be highly efficient in this regard. Having removed most of the protein by precipitation with ethanol, the supernatant was concentrated and redissolved in water before applying to a C-18 column. The excess Neu5AcαpNP and virtually all of the yellow *p*-nitrophenol eluted off the column with approximately four column volumes of water. The product and acceptor were then eluted using a fairly crude stepwise methanol gradient; the product coming at the end of the 25% MeOH/beginning of the 50% MeOH and the acceptor eluting with 75% MeOH. The column was regenerated by washing with four column volumes each of methanol and then water. By this method 7 mg (42%) of the tri-saccharide **52** was made and purified within 24 hours, from the easily accessible donor and acceptor **139** and **57** (Scheme 4.32).

4.2.5 Conclusions – Chemical *vs.* Enzymatic Synthesis of GM3 Analogue 52. The chemo-enzymatic synthesis was completed in 10 steps from lactose and sialic acid, in an overall yield of 10%. The longest linear sequence was 7 steps from sialic acid. This should be compared with the purely chemical approach, which was four steps longer and gave an overall yield of only 5%. These two yields would have been almost identical had it not been for the poor α-selectivity of the chemical glycosylation, which necessitated a difficult and low yielding chromatographic separation of the α and β anomers. It is also worth noting that whereas the chemo-enzymatic synthesis required only 3 purifications by silica gel or reverse phase chromatography, the chemical synthesis required at least 9 chromatographic steps. The *trans*-sialidase is a useful tool in oligosaccharide synthesis, allowing rapid, easy, stereo- and regioselective synthesis of sialosides in yields which easily rival those attainable by chemical synthesis.

4.3 Neuraminidase Assays

As was discussed in section 2.4, thio-oligosaccharides are resistant towards enzymatic hydrolysis. Also, some sialic acid thio-glycosides have been evaluated as inhibitors for a range of viral and bacterial neuraminidases (section 2.5.2). Of the compounds studied, the best reported K_i for an influenza virus neuraminidase was 2.8 μM for Neu5Acα(2→6)-6-thio-GlcβCeramide, but this compound was a much poorer inhibitor for bacterial neuraminidases.[51] The binding affinity of the best sialic acid thio-glycoside inhibitor is comparable to that of the 2,3-dehydro-sialic acid, Neu5Ac2en **140** (K_i 4 μM)[52] but still very low in comparison to Glaxo's 4-guanidino-Neu5Ac2en compound **141** (K_i 1-0.1 nM)[52] which is in clinical trials as an anti-influenza drug. These sialic acid glycals are thought to mimic the conformation of the oxo-carbonium intermediate **142** which is formed, transiently, during enzymatic hydrolysis of sialosides (Figure 4.3).

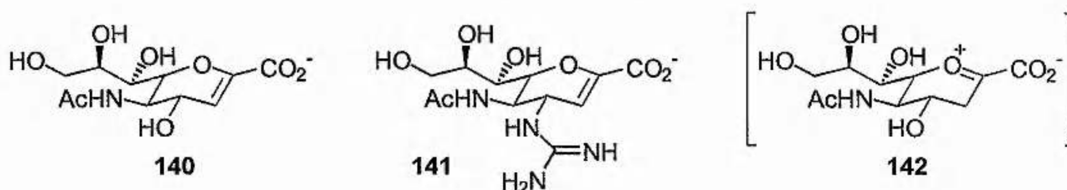


Figure 4.3 *Neu5Ac2en neuraminidase inhibitors mimic oxo-carbonium species 142.*

As yet, no data has been reported on the stability of Neu5Acα(2→3)-3-thio-Gal compounds to enzymatic hydrolysis, nor regarding the properties of such compounds as neuraminidase inhibitors. Although it may be expected that α(2→3)-thioglycosides should prove to be weak inhibitors, like the α(2→6)-compounds, any inhibitory activity would show that at least this class of sialic acid recognising enzyme accepts *S*-linked sialosides. Also, it would be interesting to consider Neu5Acα(2→3)-3-thio-Gal compounds as inhibitors for the *trans*-sialidase from *Trypanosoma cruzi* which is unaffected by Neu5Ac2en based compounds, and thus presumably operates by a different mechanism to bacterial and viral neuraminidases.[53] *T. cruzi* is a parasite which causes Chagas disease, an incurable condition which is endemic in South America. The *trans*-sialidase is vital to the parasites' ability to invade mammalian cells and is thus a key target for developing drugs against Chagas disease.[43]

We thus decided to evaluate a number of the thio-sialosides synthesised (Figure 4.4) for their resistance to enzymatic hydrolysis by, and as inhibitors for *Clostridium perfringens* neuraminidase and *trans*-sialidase from *T. cruzi*. The *O*-linked

deNAcGM3 analogue **49** was also tested to try to confirm the expectation that it would also be resistant to enzymatic hydrolysis. The corresponding *O*-linked GM3 analogue **52** was included as a positive control for both enzymatic hydrolysis and as a competitive inhibitor with respect to the substrates used in the assays.

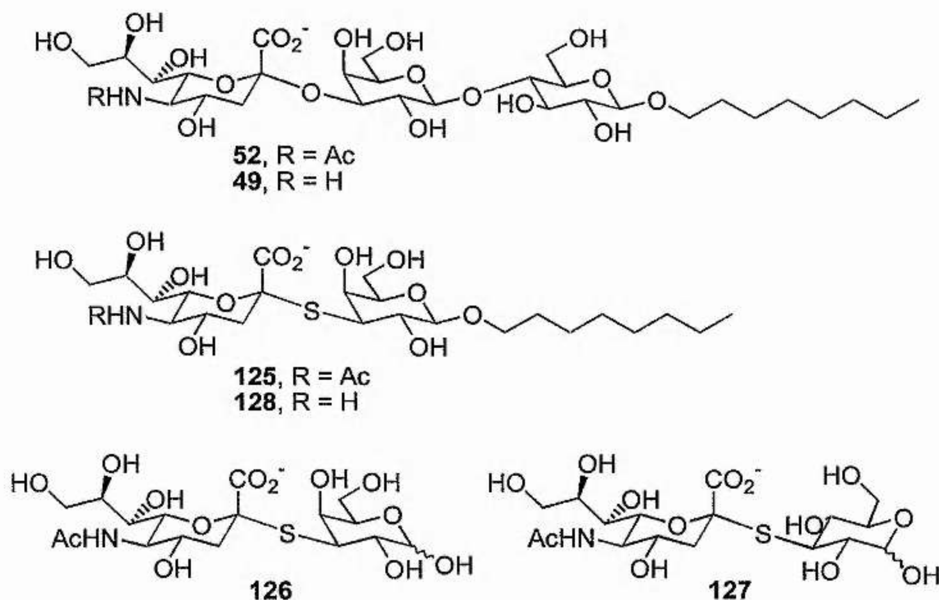
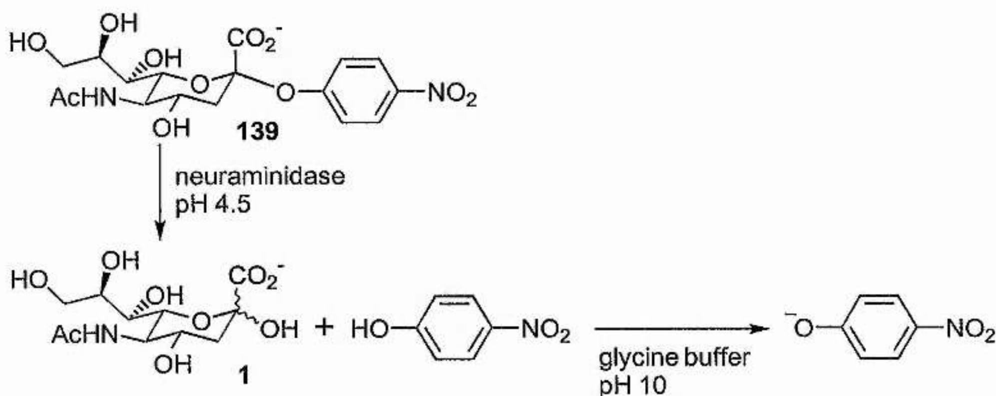


Figure 4.4 *Compounds tested as neuraminidase and trans-sialidase inhibitors.*

4.3.1 *Clostridium perfringens* neuraminidase. A UV absorbance assay, using *p*-nitrophenyl α-sialoside (Neu5AcαpNP) **139** as substrate, was chosen for the *C. perfringens* neuraminidase experiments.[50] The assay was performed as a time-course experiment, withdrawing aliquots from the enzyme incubation at regular intervals and quenching the reaction by addition of glycine buffer at pH 10. At high pH, *p*-nitrophenol released from the sialoside is deprotonated to give the phenolate salt which strongly absorbs light at 400 nm (ε = 18,000) (Scheme 4.33). Measuring changes in absorbance at this wavelength provides a sensitive assay for enzymatic hydrolysis.



Scheme 4.33 Neu5AcαpNP neuraminidase assay.

Acid hydrolysis of Neu5AcαpNP was negligible at pH 4.5 (Figure 4.5), the pH optimum for neuraminidase.[20] However, absorbance measurements needed to be taken shortly after quenching with glycine buffer, as base hydrolysis caused a notable change in absorbance with time (data not shown).

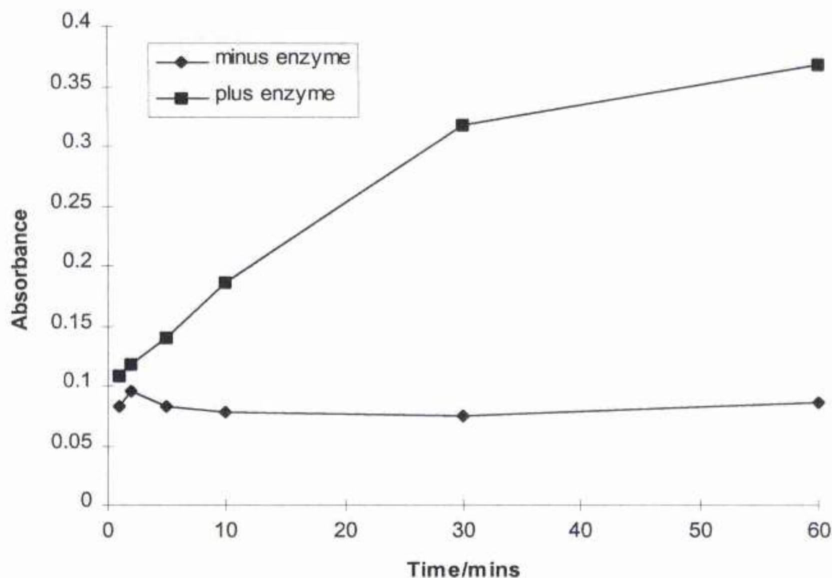


Figure 4.5 *Timecourse showing Neu5AcαpNP hydrolysis in the presence and absence of neuraminidase.*

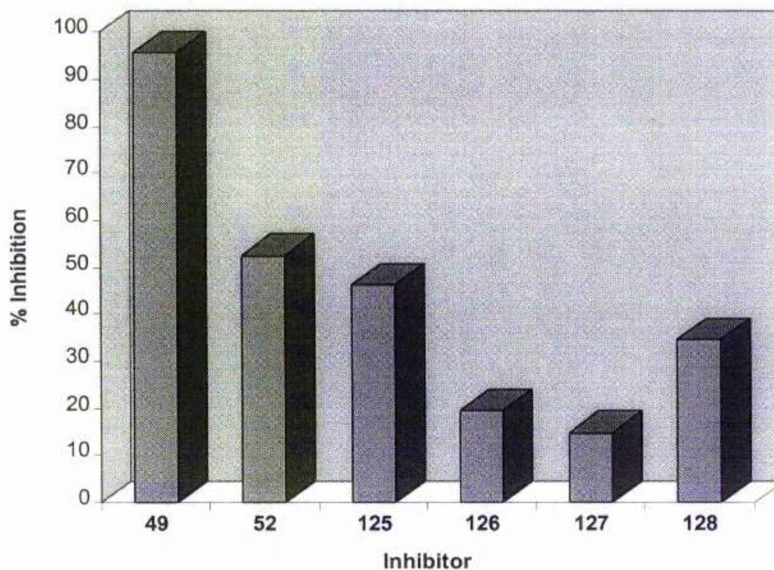


Figure 4.6 *Percentage inhibition of Neu5AcαpNP cleavage by C. perfringens neuraminidase. All inhibitors are 1mM*

4.3.1.1 Inhibition results. Both *O*-linked GM3 analogue **52** and *S*-linked GM4 analogue **125** showed approximately 50% inhibition at 1 mM (Figure 4.6). This would suggest that replacing the interglycosidic oxygen atom with sulfur does not greatly affect the affinity of the *C. perfringens* neuraminidase for $\alpha(2\rightarrow3)$-linked sialosides. As was expected, the deNAc-thiosialoside **128** showed lower inhibition than the *N*-acetylated **125**. Similarly, the reducing disaccharide **126** was a poorer inhibitor than the β-octyl glycoside **125**, but a better inhibitor than the thio-sialylated glucose isomer **127**.

At 1 mM concentration, deNAcGM3 analogue **49** showed over 90% inhibition of the enzyme. However, a series of ten-fold dilutions of the inhibitor all gave inhibition in the region of 15 to 25% (Figure 4.7). Compound **49** is an amphiphilic molecule and, as such, it will show detergent-like properties. It is possible that the critical micellar concentration of the molecule may lie between 0.1 and 1 mM and that the great increase in inhibition that was observed, may result from a detergent effect on the protein. However, the properties of compound **49** were not pursued further.

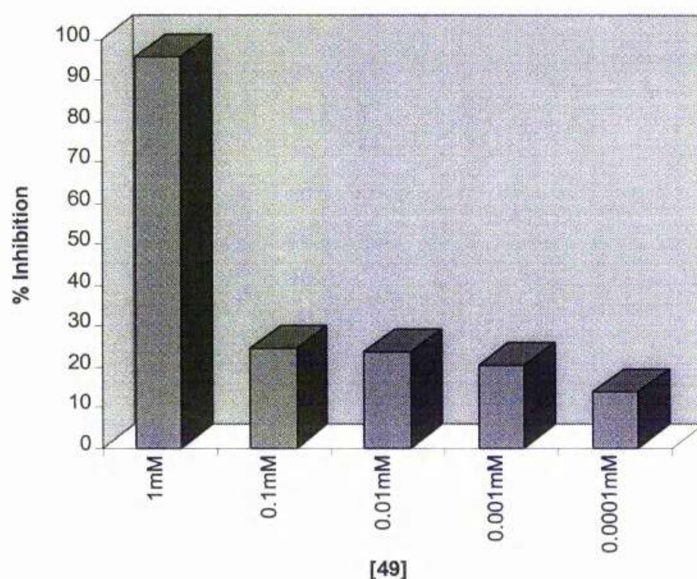


Figure 4.7 *Percentage inhibition of C. perfringens neuraminidase by compound **49** at various concentrations.*

4.3.1.2 Turnover assay results. All of the compounds in Figure 4.4 were tested as substrates for the *C. perfringens* neuraminidase, following the reaction by TLC. Even after 24 hours, only the *O*-linked GM3 analogue **52** had been cleaved by the enzyme. All of the other compounds showed no change.

4.3.2 *Trypanosoma cruzi trans*-sialidase

Although Neu5AcαpNP was used successfully as the donor in the enzymatic synthesis of GM3 analogue **52**, it is a relatively weak substrate for the *trans*-sialidase enzyme.[43] Greater sensitivity can be gained by using a radioactive assay for *trans*-sialidase activity.[43] The assay uses sialyl lactose as the donor and [1-^{14}C]lactose as the acceptor. After incubating a mixture of donor, acceptor, inhibitor and enzyme for 30 minutes, the reaction is quenched by diluting with water and applying the mixture to an anion exchange column. Elution with water gives the neutral, non-sialylated lactose, then elution with 1 M ammonium acetate gives the sialyl lactose (Figure 4.8). The percentage of [1-^{14}C]lactose which has been sialylated can be determined by liquid scintillation counting of the water and ammonium acetate elutions.

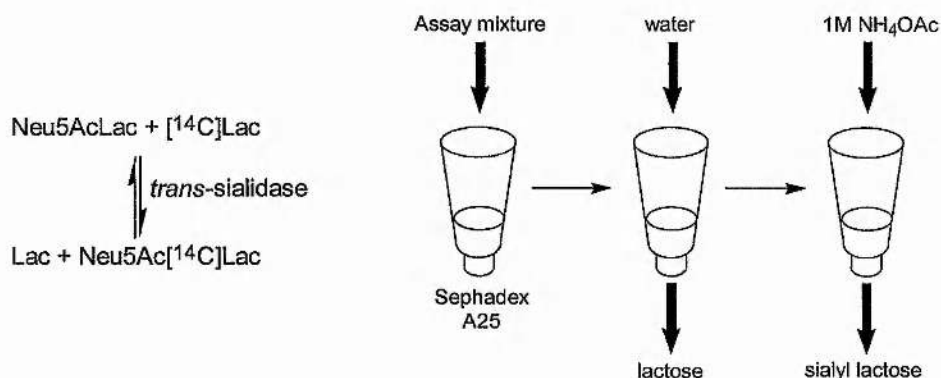


Figure 4.8 *Radioactive assay for trans-sialidase activity.*

In these experiments, the concentration of donor was ~26 times the concentration of the "hot" acceptor, therefore at equilibrium 96% ($^{26}/_{27}$) of the [1-^{14}C]lactose would be sialylated. The control incubation, without any inhibitor present, showed ~40% sialylation in 30 minutes and thus, under the conditions of the assay, the reaction is still far from equilibrium.

4.3.2.1 Inhibition results. None of the thio-glycosides showed inhibition of the *trans*-sialidase at 1 mM concentration (Figure 4.9). The two *O*-linked compounds, **52** and **49** showed an increase in sialic acid incorporation into the radio-labeled lactose. The two-fold increase in sialylation of "hot" lactose in the presence of GM3 analogue **52**, is not surprising as this compound was expected to be a substrate for the enzyme. Thus, the apparent increase in activity is simply due to an increase in the substrate concentration. However, the apparent slight increase in activity with the deNAcGM3 analogue **49**, would imply that this compound is also a substrate for the *trans*-sialidase.

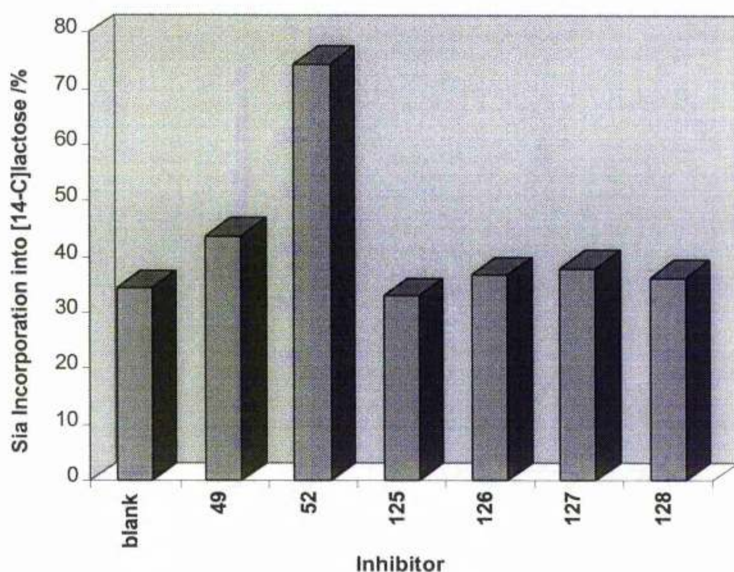


Figure 4.9 *Percentage of radio-labeled lactose which is sialylated in the presence of the various test compounds.*

4.3.2.2 Turnover assay results. The fact that both **52** and **49** are substrates was confirmed on repeating the incubations in the absence of any other sialyl-donor (Figure 4.10). Sialic acid was transferred from both GM3 analogue **52** and also from deNAcGM3 analogue **49**, albeit at approximately a quarter of the rate. As time increased, the percentage of radio-labeled lactose which was sialylated decreased. This was probably due to the residual hydrolytic activity of the enzyme slowly releasing free sialic acid. Increases in radioactivity, above background, in the ammonium acetate elutions from the thio-glycoside assays were negligible. Thus the thio-glycosides showed much greater stability to the *trans*-sialidase enzyme than the *O*-linked sialosides.

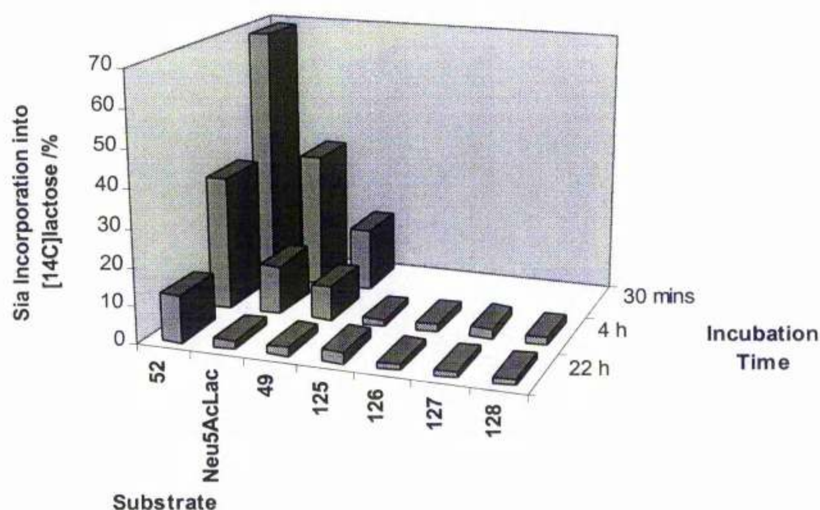


Figure 4.10 *Percentage of radio-labeled lactose which is sialylated in the presence of the test compounds, as a function of time. N.B. all donors are 1 mM except sialyl lactose which is 0.2 mM.*

4.3.3 Conclusions - Neuraminidase and *trans*-Sialidase Assays. Siaα(2$\rightarrow$3)-3-thio-Gal compounds show greater resistance to enzymatic cleavage than the corresponding *O*-linked compounds. The thio-sialosides tested were all weak inhibitors for *C. perfringens* neuraminidase, the best of these being compound **125** which showed approximately 50% inhibition at a concentration of 1 mM. Inhibition was greater for an *N*-acetyl sialoside than for a de-*N*-acetyl sialoside. Also, it was greater for octyl glycosides than for reducing disaccharides and for a sialyl-galactose disaccharide than for a sialyl-glucose disaccharide. None of the compounds tested showed significant inhibition of the *T. cruzi trans*-sialidase. Therefore, Siaα(2$\rightarrow$3)-3-thio-Gal compounds are accepted by a sialoside-recognising enzyme (C. perfringens neuraminidase), but apparently not by another sialoside-recognising enzyme (*T. cruzi trans*-sialidase). DeNAcGM3 analogue **49** was found to be resistant to cleavage by *C. perfringens* neuraminidase, but was a substrate for the *T. cruzi trans*-sialidase.

4.4 References

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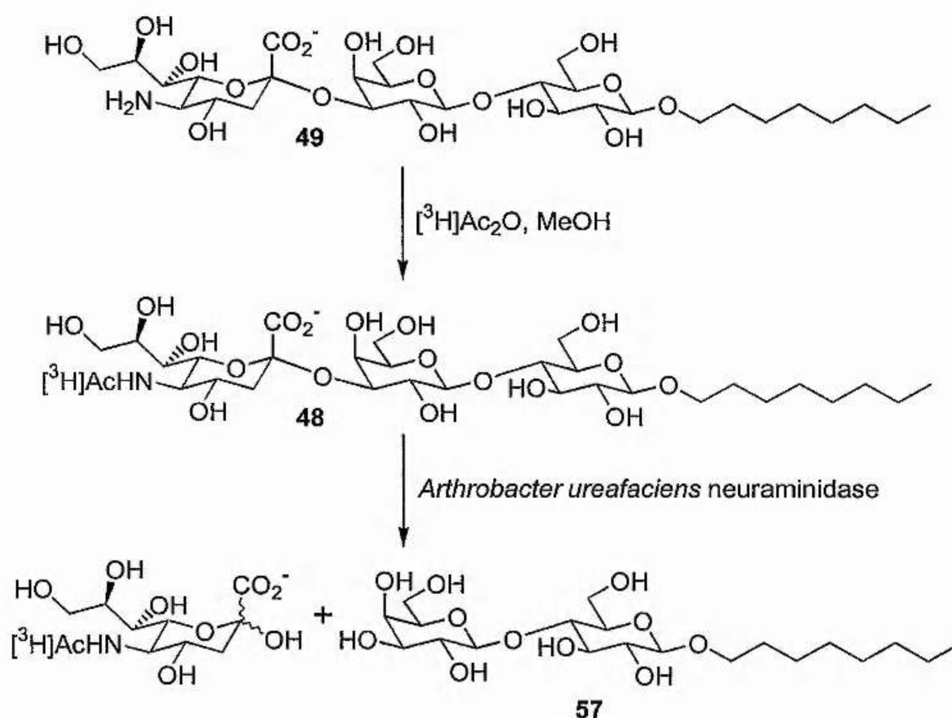
Chapter 5

Development of De-*N*-Acetylase and *N*-Acetyltransferase Assays

Having prepared the target *O*-linked compound **49**, the next steps were to introduce the radio-labeled acetamide and to set up assays, as described in chapter 3, to detect the de-*N*-acetylase and the *N*-acetyltransferase activites.*

5.1 Synthesis of radio-labeled GM3 analogue 48



Scheme 5.1 Synthesis and enzymatic digestion of radio-labeled GM3 analogue **48**.

The amino-compound **49** was treated with two mole equivalents of [^{3}H]acetic anhydride in methanol[1] to yield the required radio-labeled GM3 analogue **48** (163 GBq/mmol, 1.3×10^{10} dpm/mg) (Scheme 5.1). TLC showed ~95% coversion to the acetamide under the conditions used. There was no evidence of *O*-acetylation as **48** was the fastest running spot on the TLC and this co-ran with the corresponding "cold" GM3 analogue **52**. The product was purified twice by treating with aqueous alkali, to destroy excess [^{3}H]acetic anhydride, and reverse phase chromatography on a C-18 "Spice cartridge". Only 1% of the radioactivity was washed off the cartridge with water following re-purification. However, after subjecting a sample of **48** to

* The work described in this chapter was undertaken jointly with Mr Justin Sonnenburg at the UCSD Cancer Center, La Jolla, CA, USA, under the direction of Prof. Ajit Varki.

enzymatic digestion with *Arthrobacter ureafaciens* neuraminidase, 98% of the radioactivity could be eluted from the cartridge with water.

5.2 Assay development. All experiments were conducted using extracts of Melur melanoma cells which have previously been shown to express both gangliosides deNAcGM3 and deNAcGD3.[2] The cells were lysed by nitrogen cavitation and then separated into three fractions (see section **5.5.2**): cytosol, membranes and detergent soluble membranes. Addition of detergent to the membrane fraction opens up intracellular compartments allowing the radio-labeled substrates access to enzymes located within endosomes and lysosomes. As deNAc gangliosides are mainly found inside the cell, appearing on the cell surface only transiently,[3] it was considered that the de-*N*-acetylase and *N*-acetyltransferase would be most likely located within such intracellular compartments. The substrates were incubated with the cell fractions at 37 °C in three different buffers at pH 5, 6 and 7 for 3 hours and then worked-up on a C-18 Spice cartridge. For de-*N*-acetylase assay, divalent metal ions were also included in the assay buffers and the growth medium which contained non-adherent cells was also tested for activity. Negative controls were also included in all experiments. The negative controls for the *N*-acetyltransferase assay were minus cell extract and minus **49** for all conditions. For the de-*N*-acetylase assay the appropriate controls were minus cell extract and minus divalent cations.

5.2.1 *N*-Acetyltransferase assay

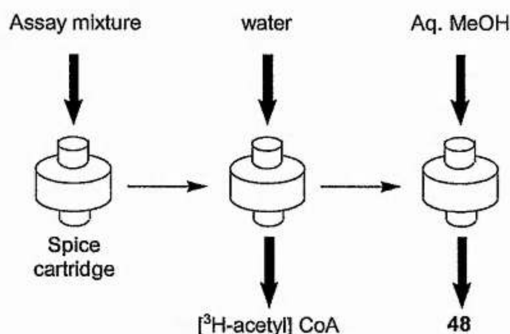


Figure 5.1 *Proposed separation of N-acetyltransferase assay products on C-18 Spice cartridge.*

Difficulties were encountered when trying to separate the products of the *N*-acetyltransferase assay. It had been anticipated that the excess [^{3}H]-acetyl CoA could be washed off the cartridge with water before eluting any products with aqueous methanol (Figure 5.1). However, acetyl CoA proved too hydrophobic to be easily eluted with water, instead it slowly leached off the column. Elution with 3% methanol was more efficient at removing the acetyl CoA, but a control experiment

using chemically synthesised **48**, showed that it was also partially eluted with 3% methanol. It is a normal practice to re-use C-18 cartridges for such assays after regeneration by washing with methanol and then with water.[4] However, this type of assay is typically used for purified enzymes rather than crude cell extracts. We found that the acetyl CoA and the GM3/deNAcGM3 analogues were more easily separated on new than on re-used cartridges suggesting that the cartridges have a much shorter useful life span when used with crude cell extracts. Alternatively, we considered that exposing the cartridges to detergent, present in some of the samples, could result in problems on re-using the cartridges. This was discounted, however, as other investigators have reported re-using C-18 cartridges for glycosyl transferase assays using enzymes solublised with considerably higher concentrations of detergent than we were using.[4] Even when using new cartridges, no clear production of **48** was observed.

Acetyl CoA is the standard acetyl-donor used by many enzymes within the cell. Therefore, there may be many other enzymes which compete with the deNAcGM3:*N*-acetyltransferase for acetyl CoA, and these may give rise to many other [^{3}H]-labeled products. We felt that the assay was still not sensitive enough to detect small amounts of **48** which may have been produced. Further improvements to the assay could probably be gained by increasing the length of the hydrophobic tail in the substrate to allow its easier separation from unreacted [^{3}H]-acetyl CoA. Alternatively, the excess acetyl CoA could perhaps be destroyed either chemically or enzymatically, prior to loading onto the cartridge.

5.2.2 De-*N*-acetylase assay

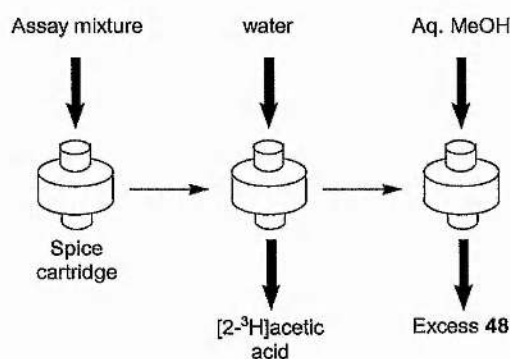


Figure 5.2 *Proposed separation of de-N-acetylase assay products on C-18 Spice cartridge.*

A similar problem was encountered with the de-*N*-acetylase assay. It had been hoped that any [2-^{3}H]acetic acid released during the incubation would be eluted from the cartridge with water and then the remaining **48** could be recovered by washing the

cartridge with aqueous methanol (Figure 5.2). However, the radio-labeled substrate **48** was gradually eluted from the cartridges with water, even after re-purification by reverse phase chromatography (Figure 5.3).

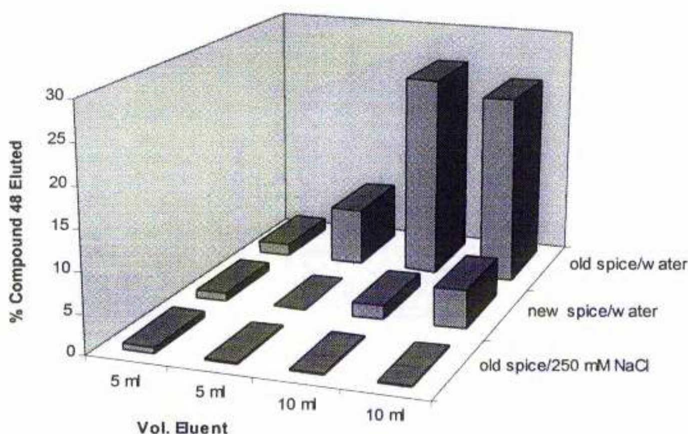


Figure 5.3 *Percentage of 48 eluted from new and reused (old) Spice cartridges by washing with consecutive volumes of water or 250 mM NaCl solution.*

Again, this problem apparently stemmed from a reduced hydrophobicity of the cartridges on re-use. Low background counts could be achieved by using new Spice cartridges for each assay. However, it was found that similar results could be achieved with re-used cartridges when the samples were applied and eluted with 250 mM sodium chloride, rather than with water. The higher ionic strength increased the hydrophobic interaction between the octyl glycoside and the reverse phase silica without greatly affecting the rate of acetate elution (Figure 5.4). Unfortunately, no enzymatic release of acetate was detected whilst using this assay.

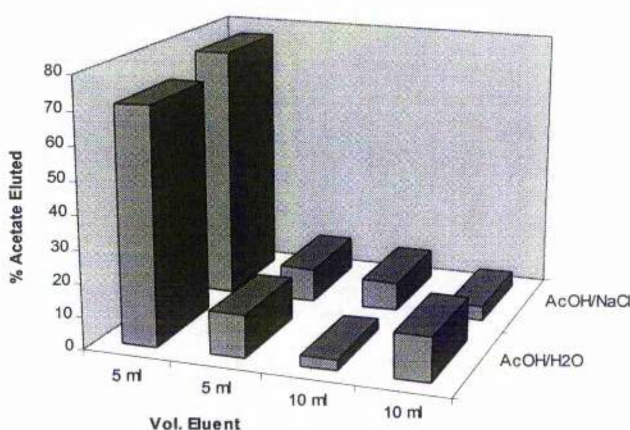


Figure 5.4.. *Percentage of [^{3}H]acetate eluted from Spice cartridges by washing with consecutive volumes of water or 250 mM NaCl solution*

5.2.3 Two phase scintillation counting: the "shake and bake" assay. An alternative assay was also considered for detecting de-*N*-acetylase activity. The so-called "shake and bake" assay was originally developed for monitoring the release of [^{3}H]acetate from acetylcholine.[5] This assay relies on the different solubilities of acetylcholine and acetic acid in organic and aqueous solution. Following the incubation period, the reaction is stopped by addition of a chloroacetic acid buffer at pH 1. This has the dual effect of stopping the reaction and protonating the [2-^{3}H]acetic acid (pK_a 4.8) which can then be extracted efficiently into a toluene/*iso*-amyl alcohol based scintillation mixture. The [^{3}H]-acetyl choline, however, remains in the aqueous phase from which the weak β-particles, emitted by the decaying tritium atoms, can not escape to excite the scintillants in the organic phase. Thus only the released [2-^{3}H]acetic acid is detected by the scintillation counter.

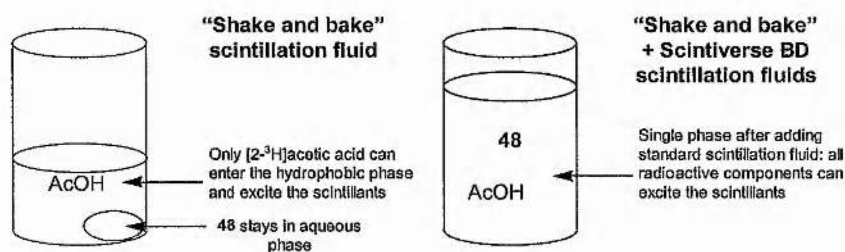


Figure 5.5 *The "shake and bake" de-N-acetylase assay.*

The shake and bake assay could not be used for radio-labeled GM3 because the lipid portion is too large and would pull the compound into the organic phase along with any released acetate.[6] However, octyl glycoside **48** is much more water soluble than GM3 and should thus remain largely in the aqueous phase (Figure 5.5). A trial experiment confirmed that almost all of **48** remained in the aqueous phase and only 4% of the **48** radioactivity could be detected by liquid scintillation counting. At pH 1, a significant portion of the sialic acid will be protonated (pK_a 2.75),[7] increasing the solubility of **48** in the organic phase. By adjusting the pH of the stopping buffer to 3.75, *i.e.* half way between the pK_as of the two acids, it was possible to optimise the desired solubilities of **48** and acetate to give a background reading of less than 1%. Furthermore, it was found that after measuring the amount of free acetate, as described above, addition of a commercial scintillation fluid which was designed for use with aqueous solutions, allowed measurement of the combined radioactivity of both the released and sialic acid-bound [^{3}H]Ac. Even though this assay should have been sufficiently sensitive to detect release of a few percent of the [^{3}H]Ac, no free acetic acid was detected in the cell extract incubations.

5.3 Flow cytometry analysis. A fluorescence activated cell scanning (FACS) analysis[8] using anti-GD3 and anti-deNAcGD3 antibodies was performed on trypsinised Melur cells and on cells that had also been permeabilised with saponin so as to allow the antibodies access inside the cells. This experiment revealed that although GD3 was present on the surface of the cells, no deNAcGD3 could be detected at all (results not shown). DeNAcGD3 had previously been detected inside the cells of this Melur culture by the same assay.[3, 6] However, in the three months since the last positive assay, the cells had been passaged (grown to confluence and then plated on) some thirty times. It is possible that the cells may have changed their phenotype over this period and were no longer expressing deNAc gangliosides. This would explain why no de-*N*-acetylase or *N*-acetyltransferase activities were detected. Further studies will be conducted using a fresh batch of Melur cells or with some of the other cancer cell lines that have been shown to express deNAc gangliosides. This work is being continued in collaboration with the Varki laboratory, UCSD.

5.4 Conclusions. Although no enzyme activities were detected, two assays for the de-*N*-acetylase activity have been developed and these should help in locating the enzyme in the near future. Similar deNAcGM3 analogues to **49**, but with longer hydrophobic tails will be synthesised also. These compounds should help to further improve the *N*-acetyltransferase assay, which should, in turn, also lead to the discovery of this other potentially important enzyme.

5.5 Experimental

5.5.1 General methods. Commercial reagents were used without further purification. [2-^{3}H]Acetic anhydride (8.80 Ci/mmol, 326 GBq/mmol) was from Amersham and was dissolved in dry hexane (~400 mm^3) before use. [^{3}H]-Acetyl CoA (~27 Ci/mmol, ~1 TBq/mmol), which had been prepared by a published enzymatic procedure,[2] was already available in the Varki lab. Conversions between counts per minute (cpm) and Ci/Bq assumed a detection efficiency for ^{3}H disintegrations of 45% by liquid scintillation counting *i.e.* 1 mCi = 37 MBq = 1×10^9 cpm. *Arthrobacter ureafaciens* neuraminidase (EC 3.2.1.18) was purchased from Calbiochem. Analytical TLC was performed on silica gel 60 (Whatman) with detection by charring, following immersion in orcinol dipping reagent, prepared as described in the main experimental section. The assay buffers used for incubations with cell fractions were HOMOPIPES (pH 5), MES (pH 6) and HEPES (pH 7), all at 50 mM concentration. C-18 reverse phase Spice cartridges (Analtech) were washed with MeOH (10 cm^3) and then with water (10 cm^3) before use. The "shake and bake" stopping mixture was a citrate-phosphate buffer (pH 3.75), prepared by mixing solutions of citric acid (32 cm^3; 100 mM) and dibasic sodium phosphate (18 cm^3; 200 mM) and diluting to 100 cm^3 with distilled water. The "shake and bake" scintillation fluid was a solution of *p*-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) (1.2 g) and 2,5-diphenyloxazole (POP) (20 g) in a mixture of toluene (3200 cm^3) and *iso*-amyl alcohol (800 cm^3).[5] Other liquid scintillation counting was performed using Scintiverse BD scintillation fluid (Fisher). The FACS buffer comprised phosphate buffered saline (PBS) containing 1% bovine serum albumin and 0.1% sodium azide.

5.5.2 Cell culture and preparation of cell fractions. Melur cells were grown to confluence in Dulbecco's modified Eagle's (DME) medium containing 2 mM L-glutamine and 10% fetal calf serum in an humidified incubator at 5% CO_2 and 37 °C. The *growth medium* was removed from three 150 mm plates of confluent cells (~4×10^7 cells) and kept for the de-*N*-acetylase assay. The cells were washed with phosphate buffered saline (PBS), then scraped into HEPES buffer (5 cm^3; 10 mM + 100 mM NaCl, pH 7.2) and lysed by nitrogen cavitation by pressurising to 1250 psi for 15 minutes. The resulting white soup was centrifuged at $500 \times g$, 4°C for 15 minutes in order to pellet nuclei and intact cells. The post-nuclear supernatant, thus obtained, was centrifuged at $100{,}000 \times g$, 4°C for 30 minutes to give the *cytosol fraction* as the supernatant and the *membrane fraction* as a pellet which was resuspended in HEPES buffer (2 cm^3). Triton-X 100 was added to half of the membrane fraction to a final detergent concentration of 0.2% and this was

centrifuged again at 100,000 × *g*, 4°C for 30 minutes. The pellet of detergent insoluble material was discarded and the supernatant was used as the *detergent soluble membrane fraction*. The cell fractions were kept at -20 °C until used.

5.5.3 Synthesis of octyl (sodium 5-[^{3}H]acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside, 48

[2-^{3}H]Acetic anhydride in hexane (125 mm^3, 850 nmol; 7.5 mCi, 277.5 MBq) was added to a solution of **49** (240 μg, 340 nmol) in MeOH (250 mm^3). After 2.5 h at room temperature, TLC ($CHCl_3$-MeOH-H_2O, 10:10:3) showed >90% conversion of **49** to a compound that co-ran with *octyl (potassium 5-acetamido-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosylonate)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside*, **52**. After a further 1.5 h at room temperature, the solution was warmed to 30 °C and the solution was concentrated to a small volume under a stream of nitrogen. The residue was re-dissolved in water (300 mm^3), and sodium bicarbonate solution (300 mm^3; 1 M) was added. After 30 mins at room temperature, the mixture was transferred to a C-18 Spice cartridge and eluted with water (15 cm^3), then with MeOH-H_2O, 1:1 (5 cm^3), and finally with MeOH (5 cm^3). An aliquot of each of these fractions (10 mm^3) was diluted with water (1 cm^3) and a small portion of the diluted material (10 mm^3) was subjected to liquid scintillation counting. The results showed that 80% of the radioactivity (6 mCi, 222 MBq) eluted with water and 20% of the radioactivity (1.5 mCi, 55 MBq) eluted in the MeOH-containing fractions. The MeOH containing fractions were combined and then concentrated to dryness under reduced pressure to give *GM3 analogue* **48** (1.5 mCi, 4.4 Ci/mmol; 55MBq, 163 GBq/mmol). The product was divided equally between 10 vials, each containing ~25 μg, 1.5 × 10^8 cpm. The compound was stored at -20 °C until required.

A sample of the radio-labeled compound (50 ng, 67 pmol, ~300,000 cpm) was subjected to enzymatic digestion by *A. ureafaciens* neuraminidase (4 mU) in MES buffer (pH 6, 10 mm^3) for 30 mins at 37 °C. The reaction was quenched by addition of water (1 cm^3) and the mixture was loaded onto a C-18 Spice cartridge. The cartridge was eluted with water (10 cm^3) and then with MeOH (5 cm^3). Scintillation fluid (9.5 cm^3) was added to an aliquot (0.5 cm^3) of each of the elutions and after mixing, the radioactivity was measured using a scintillation counter. 98% of the radioactivity appeared in the water elutate, whereas a control experiment in which **48** had been similarly incubated but in the absence of neuraminidase, showed 97% of

the radioactivity to be in the MeOH elution. Following a second purification by treating with bicarbonate and work-up by Spice cartridge as described above, 99% of the radioactivity was found in the MeOH elution.

5.5.4 Typical assay procedures

5.5.4.1 *N*-Acetyltransferase assay. [^{3}H-acetyl] CoA (~90,000 cpm) was incubated with each of the three cell fractions at pH 5, 6 and 7, with and without amino-compound **49** (10 μg). Incubations were conducted for 3 h at 37 °C in a total reaction volume of 50 mm^3. The reaction was quenched by addition of water (1 cm^3) and the mixture was loaded onto a C-18 Spice cartridge. The cartridge was eluted with water (4 cm^3) and then with MeOH-H_2O, 3:97 (5 cm^3), 1:1 (5 cm^3) and finally with MeOH (5 cm^3). Scintillation fluid (18 cm^3) was added to an aliquot (2 cm^3) of each of the last two elutions and after mixing, the radioactivity was measured using a scintillation counter.

5.5.4.2 De-*N*-acetylase assay - C-18 cartridge method. *N*-[^{3}H-Acetyl] compound **48** (~70,000 cpm) was incubated with each of the three cell fractions or with growth medium, each at pH 5, 6 and 7, with or without 1 mM Mg^{2+}, Ca^{2+} and Zn^{2+} ions added to the assay buffers. Incubations were conducted for 3 h at 37 °C in a total reaction volume of 50 mm^3. The reaction was quenched by addition of water (1 cm^3) and the mixture was loaded onto a C-18 Spice cartridge. The cartridge was eluted with NaCl solution (10 cm^3; 250 mM) and then with water (5 cm^3) and finally with either MeOH or acetonitrile (5 cm^3). Scintillation fluid (18 cm^3) was added to an aliquot (2 cm^3) of each of the elutions and after mixing, the radioactivity was measured using a scintillation counter.

5.5.4.3 De-*N*-acetylase assay - "shake and bake" method. *N*-[^{3}H-Acetyl]-compound **48** (~70,000 cpm) was incubated with each of the three cell fractions or with growth medium as for the C-18 cartridge method described above. The reaction was quenched by addition of citrate-phosphate buffer pH 3.75 (150 mm^3) and transferred to a 20 cm^3 scintillation vial. "Shake and bake" scintillation fluid (10 cm^3) was added and the samples were shaken well. After allowing the samples to settle for 15 mins at room temperature, the amount of free [2-^{3}H]acetic acid was measured by scintillation counting. Scintiverse BD (10 cm^3) was then added to each scintillation vial and after mixing, the total radioactivity was also measured by scintillation counting.

5.5.5 FACS analysis. The FACS analysis was performed by a published procedure.[8] Briefly, ~1 × 10^6 trypsinised cells were stained with the primary antibody (R24 for GD3 and SGR37 for deNAcGD3), and then washed twice with FACS buffer. The cells were then stained with the secondary antibody (fluorescene isothiocyanate-conjugated goat anti-mouse IgG). Following washing with FACS buffer, as before, the cells were fixed with FACS buffer containing 1% formaldehyde. These cells were analysed by flow cytometry as described previously.[8]

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Chapter 6

Experimental

6.1 General Methods

6.1.1 Synthetic Procedures. 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 or Whatman) with detection by fluorescence and/or by charring following immersion in a dilute ethanolic solution of sulfuric acid. Orcinol dipping reagent, 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), was used for deprotected compounds. Flash chromatography was performed with silica gel 60 (Fluka). Reverse phase chromatography was performed on 15 g C-18 silica gel 100 (Fluka) eluting with H_2O (80 cm^3) and then MeOH-H_2O, 1:3 (20 cm^3), 1:1 (20 cm^3), 3:1 (20 cm^3) and finally with MeOH (20 cm^3). For smaller scale work C-18 Sep-pak cartridges (Waters) were used and eluted with 2 cm^3 of each of the above eluents.

During work-up, organic solutions were washed two or three times with equal volumes of each of the aqueous solutions listed. Standard work-up **A** involved washing organic solutions successively with water, saturated $NaHCO_3$ solution and water; standard work-up **B** involved washing organic solutions successively with 1M HCl solution, saturated $NaHCO_3$ solution and water. All such organic solutions were then dried over anhydrous Na_2SO_4 and concentrated. All concentrations were performed *in vacuo*.

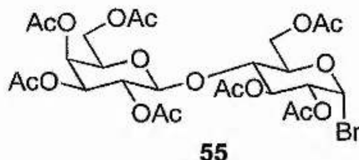
Optical rotations were measured at the sodium D-line and at ambient temperature, with an Optical Activity AA-1000 polarimeter. $[\alpha]_D$ Values are given in units of 10^{-1} deg cm^2 g^{-1}. Melting points were measured using a Gallenkamp melting point apparatus and are uncorrected. IR spectra were recorded as thin films on NaCl plates using a Perkin-Elmer 1710 IRFT spectrometer. Fast atom bombardment (FAB) mass spectra were recorded on a Fisons VG Autospec spectrometer using a 3-nitrobenzyl alcohol matrix. Electrospray mass spectra (ES-MS) were recorded on a Fisons VG Biotech electrospray mass spectrometer. Unless stated otherwise, ^1H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 spectrometer at 300 MHz and 75 MHz, respectively. ^1H NMR spectra were referenced to the following internal standards: $CHCl_3$, δ_H 7.26 in $CDCl_3$; CD_2HOD, δ_H 3.31 in CD_3OD; CH_3OH, δ_H 3.43 in D_2O. ^{13}C NMR spectra were referenced to the following internal standards: $CDCl_3$, δ_C 76.9 in $CDCl_3$; CD_3OD, δ_C 49.15 in CD_3OD; CH_3OH δ_C 49.9 in D_2O. *J*-values are given in Hz. For di- and tri-saccharides, the monosaccharide residues are labeled *a*, *b*, *c* from the reducing terminus. Only partial NMR data are given for some

compounds; other spectral features were in accord with the proposed structures. Signals for CF_3 were not observed in any of the ^{13}C NMR spectra of compounds containing the trifluoromethanesulfonyl group.

6.1.2 Enzymatic Procedures. *trans*-Sialidase experiments were conducted using a crude *E. coli* extract (~6 mg/cm^3) of a 70 kDa recombinant construct of the *Trypanosoma cruzi* enzyme.[2] *Clostridium perfringens* α2-3,6-neuraminidase (EC 3.2.1.18) was purchased from Sigma. [D-Glucose-1-^{14}C]lactose (53 mCi/mmol, 200 μCi/cm^3; 1.96 GBq/mmol; 7.4 MBq/cm^3) was from Amersham and was diluted 100-fold before use. Liquid scintillation counting was performed using Opti Phase 'Hisafe' 3 scintillation fluid (Fisher). The "glycine stopping buffer" (pH 10) was a solution of glycine (4 g), sodium chloride (1.4 g) and sodium carbonate decahydrate (1.76 g) in distilled water (200 cm^3).

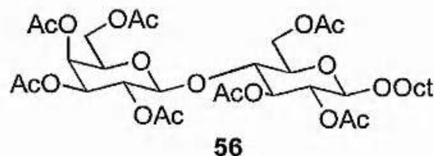
6.2 Synthetic Procedures

2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl bromide, 55[3, 4]



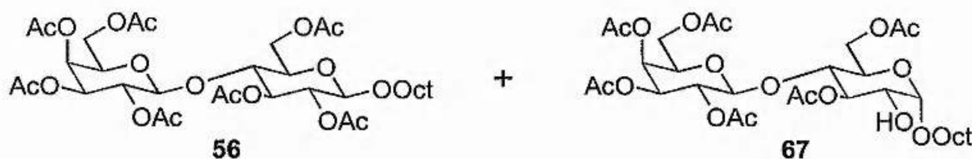
To a suspension of lactose **54** (15.00 g, 43.8 mmol) in acetic anhydride (75 cm^3) at 0 °C under N_2 was added hydrogen bromide in acetic acid (15 cm^3; 33% w/v solution) and the mixture was allowed to warm to room temperature. After stirring for 1 h, TLC (hexane-EtOAc, 1:1) indicated that the lactose had been fully acetylated. The solution was cooled to 0 °C and more hydrogen bromide in acetic acid (75 cm^3; 33% w/v solution) was added. After a further 2 h stirring at room temperature, the mixture was diluted with CH_2Cl_2 (150 cm^3) and subjected to standard work-up A. Concentration gave a syrup which crystallised on addition of EtOAc to give the *bromide* **55** (28.60 g, 95%), mp 137-138 °C (lit.,[3] 138 °C, lit.,[4] 145 °C decomp.); $[\alpha]_D$ +109.6 (*c* 1.0 in $CHCl_3$) (lit.,[3] 108.2, lit.,[4] 108.7); δ_H($CDCl_3$) 1.95-2.20 (7 × 3 H, 7 s, 7 × AcO), 3.8 (1 H, d, $J_{3a,4a}$ 9.9, 4a-H), 4.50 (1 H, d, $J_{1b,2b}$ 7.7, 1b-H), 4.75 (1 H, dd, $J_{1a,2a}$ 3.8, $J_{2a,3a}$ 9.9, 2a-H), 4.95 (1 H, dd, $J_{2b,3b}$ 10.2, $J_{3b,4b}$ 3.3, 3b-H), 5.15 (1 H, dd, $J_{1b,2b}$, $J_{2b,3b}$, 2b-H), 5.45 (1 H, d, $J_{3b,4b}$, 4b-H), 5.55 (1 H, t, $J_{2a,3a}$, $J_{3a,4a}$, 3a-H), 6.50 (1 H, d, $J_{1a,2a}$ 4.1, 1a-H).

Octyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside, 56



Method one. A suspension of silver perchlorate (4.96 g, 22 mmol), 4Å molecular sieves (10 g), octanol (4.56 cm^3, 50 mmol) and dry CH_2Cl_2 (110cm^3) was stirred overnight at room temperature in a tin foil covered flask in order to exclude light. Silver carbonate (8.27 g, 30 mmol) was added, followed by compound **55** (8.27 g, 30mmol) and the mixture was stirred at room temperature for 24 h. The mixture was then filtered through Celite and aqueous TFA (1cm^3; 50% v/v solution) was added to

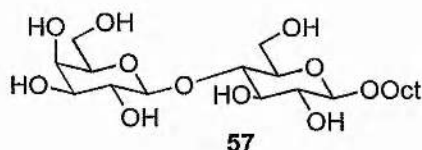
the filtrate. After stirring for 2 h, the solution was subjected to standard work-up A. Concentration gave a syrup. Flash chromatography (silica gel; hexane-EtOAc, 3:1→1:1) gave the desired *acetylated octyl lactoside* **56** as an amorphous mass (8.45 g, 56%), (Found: C, 54.77; H, 7.26. $C_{32}H_{50}O_{17}$ requires C, 54.54; H, 7.00%); $\delta_H(CDCl_3)$ 0.86 (3 H, t, J 6.9, $C_7H_{14}CH_3$), 1.25 (10 H, m, $(CH_2)_5$ CH_3), 1.55 (2 H, m, OCH_2CH_2), 1.94-2.14 (7 × 3 H, 7 s, 7 × AcO), 3.45 (1 H, m, OCH_2), 4.40, 4.50 (2 × 1 H, 2 d, $J_{1a,2a}$, $J_{1b,2b}$ 7.7, 8.0, 1a-H, 1b-H), 4.86 (1 H, dd, $J_{1a,2a}$, $J_{2a,3a}$ 9.3, 2a-H), 4.94 (1 H, dd, $J_{2b,3b}$ 10.2, $J_{3b,4b}$ 3.2, 3b-H), 5.09 (1 H, dd, $J_{1b,2b}$, $J_{2b,3b}$, 2b-H), 5.17 (1 H, t, $J_{2a,3a}$, $J_{3a,4a}$ 9.3, 3a-H), 5.33 (1H, d, $J_{3b,4b}$ 3.3, 4b-H); $\delta_C(CDCl_3)$ 14.1, 20.5, 20.7 (4), 20.9, 22.7, 25.85, 29.3 (2), 29.45 (2), 31.8, 60.9, 62.2, 66.8, 69.3, 70.3, 70.8, 71.1, 71.9, 72.7, 73.0, 76.5, 100.8, 101.2, 169.35, 169.85, 170.1, 170.3, 170.4, 170.6, 170.7.



Method two. A suspension of silver perchlorate (4.96 g, 22 mmol), 4Å molecular sieves (10 g), and dry CH_2Cl_2 (125cm^3) was stirred overnight at room temperature in a tin foil covered flask in order to exclude light. Octanol was added (4.56 cm^3, 50 mmol) followed by silver carbonate (8.27 g, 30 mmol) and compound **55** (8.27 g, 30mmol) and the mixture was stirred at room temperature for 24 h. The mixture was then filtered through Celite and gave a syrup on concentration. Flash chromatography (silica gel; hexane-EtOAc, 3:1→1:1) gave the desired *acetylated octyl lactoside* **56** as an amorphous mass (6.51 g, 43.5%) which gave identical analytical data to the above. Further elution (hexane-EtOAc, 1:2) gave *octyl 2,3,4,6-tetra-O-acetyl-β-*D-*galactopyranosyl-(1→4)-3,6-di-O-acetyl-α-*D-*glucopyranoside* **67** as a glassy solid (3.90 g, 27.5%), (Found: C, 54.16; H, 7.09. $C_{32}H_{50}O_{17}$ requires C, 54.18; H, 7.13%); $[\alpha]_D$ +64.4 (c 1 in $CHCl_3$); $\delta_H(CDCl_3)$ 0.86 (3 H, m, $C_7H_{14}CH_3$), 1.26 (10 H, m, $(CH_2)_5$ CH_3), 1.55 (2 H, m, OCH_2CH_2) , 1.94-2.14 (6 × 3 H, 6 s, 6 × AcO), 4.49 (1 H, d, $J_{1b,2b}$ 8.0, 1b-H), 4.81 (1 H, dd, $J_{1a,2a}$ 3.8, 1a-H), 4.93 (1 H, dd, $J_{2b,3b}$ 10.4, $J_{3b,4b}$ 3.5, 3b-H), 5.09 (1 H, dd, $J_{1b,2b}$, $J_{2b,3b}$, 2b-H), 5.20 (1 H, t, $J_{2a,3a} = J_{3a,4a}$ 9.5, 3a-H), 5.33 (1H, dd, $J_{3b,4b}$, $J_{4b,5b}$ 1.0, 4b-H). A sample was acetylated using pyridine-acetic anhydride and purified by flash chromatography (silica gel; hexane-EtOAc 1:1) to give *octyl 2,3,4,6-tetra-O-acetyl-β-*D-*galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-α-*D-*glucopyranoside*. The following ^{1}H NMR data are consistent with

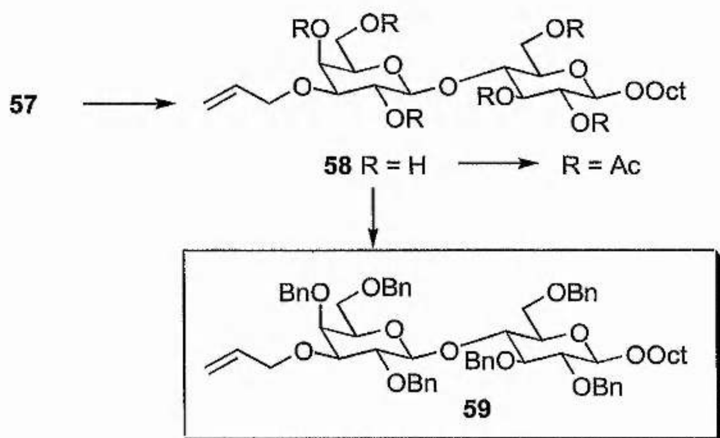
the *peracetylated α lactoside*: δ_H($CDCl_3$) 0.85 (3 H, m, $C_7H_{14}CH_3$), 1.28 (10 H, m, $(CH_2)_5$ CH_3), 1.55 (2 H, m, OCH_2CH_2) , 1.94-2.20 (7 × 3 H, 7 s, 7 × AcO), 4.46 (1 H, d, $J_{1b,2b}$ 8.0, 1b-H), 4.74 (1 H, dd, $J_{1a,2a}$ 3.6, $J_{2a,3a}$ 10.1, 2a-H), 4.86 (1 H, dd, $J_{2b,3b}$ 10.4, $J_{3b,4b}$ 3.6, 3b-H), 4.94 (1 H, d, $J_{1a,2a}$, 1a-H), 5.09 (1 H, dd, $J_{1b,2b}$, $J_{2b,3b}$, 2b-H), 5.32 (1H, br d, $J_{3b,4b}$, 4b-H), 5.44 (1 H, t, $J_{2a,3a}$, $J_{3a,4a}$ 10.1, 3a-H).

Octyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside, 57



A solution of both compound **56** (8.35 g, 11.2 mmol) and sodium metal (100 mg, 4.3 mmol) in dry MeOH (80 cm^3) was stirred for 1 h at room temperature. More MeOH (120 cm^3) was added to dissolve the resulting precipitate and the solution was neutralised with Amberlite IRC-50 (H^+) resin (1 g). Filtration and concentration gave the fully deprotected compound **57** as an amorphous white solid (5.02 g, 99%); $[\alpha]_D$ -9.2 (*c* 1 in MeOH); δ_H[CD_3OD-D_2O (1:1)] 0.86 (3 H, t, *J* 6.9, $C_7H_{14}CH_3$), 1.22-1.40 (10 H, m, $(CH_2)_5$ CH_3), 1.62 (2 H, m, OCH_2CH_2) , 4.40, 4.41 (2 × 1 H, 2 d, $J_{1a,2a,}$ $J_{1b,2b}$ 7.4, 8.0, 1a-H, 1b-H); δ_C[CD_3OD-D_2O (1:1)] 13.7, 23.0, 26.4, 29.7, 29.9, 30.1, 32.0, 61.3, 61.8, 69.7, 70.3, 71.9, 74.15, 74.2, 75.9, 75.9, 76.5, 80.1, 103.6, 104.5; ES-MS (-ve): *m/z* 453 $(M-H)^-$, 489.5 $(M+Cl)^-$ ($C_{20}H_{38}O_{11}$ requires *m/z* 454).

Octyl 3-*O*-allyl-2,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside, 59



A suspension of dibutyl tin oxide (1.18 g, 4.7 mmol) and compound **57** (2.10 g, 4.6 mmol) in MeOH (200cm^3) was refluxed for 3.5 h at which point all the suspended

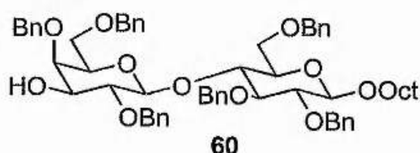
material had dissolved. The mixture was concentrated to a white amorphous solid which was dried *in vacuo* overnight. The solid was dissolved in dry toluene ($20 cm^3$) and 4Å molecular sieves (2 g), allyl bromide (6.0 ml, 70.0 mmol) and tetrabutylammonium iodide (1.71 g, 4.7 mmol) were added. The mixture was stirred at 75 °C for 7 h, cooled to room temperature and concentrated to a yellow solid. The solid was suspended in CH_2Cl_2 ($10 cm^3$) and filtered through Celite to remove by-products. The residue was washed exhaustively with MeOH and the combined filtrates were concentrated to a glassy yellow solid (2.64 g) which was used in the next step without further purification.

A small sample (25 mg) of this crude product was purified by flash chromatography (silica gel; MeOH-CH_2Cl_2, 1:4) to give *octyl 3-O-allyl-β-*D*-galactopyranosyl-(1→4)-β-*D*-glucopyranoside* **58** as an amorphous solid; $\delta_H(CDCl_3)$ 0.80 (3 H, t, J 6.9, $C_7H_{14}Me$), 1.20 (10 H, m, $(CH_2)_5$ CH_3), 1.55 (2 H, m, OCH_2CH_2) 4.25, 4.40 (2 × 1 H, 2 d, $J_{1a,2a,}$ $J_{1b,2b}$ 7.4, 7.7, 1a-H, 1b-H), 5.15 (1 H, d, J_{cis} 10.4, 3-H allyl), 5.25 (1 H, dd, J_{gem} 1.4, J_{trans} 17.3, 3′-H allyl), 5.85 (1 H, m, 2-H allyl); $\delta_C(CDCl_3)$ 13.8, 22.4, 25.65, 26.0, 29.2, 29.4, 31.6, 61.4, 61.8, 66.6, 69.7, 70.3, 70.9, 73.0, 74.4, 74.6, 74.95, 79.8, 80.3, 102.6, 103.45, 118.0, 134.4. The sample was acetylated using pyridine-acetic anhydride and purified by flash chromatography (silica gel; hexane-EtOAc 1:1) to give *octyl 2,4,6-tri-O-acetyl-3-O-allyl-β-*D*-galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-*D*-glucopyranoside* as a syrup, (Found: C, 56.56; H, 7.33. $C_{35}H_{54}O_{17}$ requires C, 56.29; H, 7.29%); $[\alpha]_D$ +5.1 (*c* 1.0 in $CHCl_3$); ν_{max}/cm^{-1} 3060 (C=*C-H*), 2930, 2855 (CH_2, CH_3), 1750 (C=O), 1225, 1055 (C-O); $\delta_H(CDCl_3)$ 0.86 (3 H, t, J 6.9, $C_7H_{14}CH_3$), 1.22-1.32 (10 H, m, $(CH_2)_5$ CH_3), 1.54 (2 H, m, OCH_2CH_2), 2.00-2.13 (6 × 3 H, 6 s, 6 × AcO), 4.38, 4.44 (2 × 1 H, 2 d, $J_{1a,2a,}$ $J_{1b,2b}$ 8.0, 7.7, 1a-H, 1b-H) 4.87 (1 H, dd, $J_{1a,2a}$, $J_{2a,3a}$ 9.6, 2a-H), 4.98 (1 H, dd, $J_{1b,2b}$, $J_{2b,3b}$ 9.9, 2b-H) 5.15 (1 H, d, J_{gem} 1.6, J_{cis} 10.7, 3-H allyl), 5.18 (1 H, br t, $J_{2a,3a}$, $J_{3a,4a}$ 9.3, 3a-H), 5.21 (1 H, dd, J_{gem} 1.6, J_{trans} 17.3, 3′-H allyl), 5.36 (1H, d, $J_{3b,4b}$ 3.3, 4b-H), 5.66-5.81 (1 H, m, 2-H allyl).

To a solution of the crude compound **58** in DMF ($50 cm^3$) at 0 °C was added sodium hydride (60% dispersion in oil; 1.80 g, 45 mmol) in portions and the mixture stirred at 0 °C for 1.5 h. Benzyl bromide ($3.53 cm^3$, 30 mmol) was added dropwise and the mixture was stirred for a further 2 h. After careful addition of MeOH ($5 cm^3$) and concentration, the residue was partitioned between diethyl ether and water, the aqueous phase being extracted twice with diethyl ether before washing the combined organic extracts with saturated NaCl solution, drying and concentration to an orange

oil. Flash chromatography (silica gel; hexane-EtOAc, 6:1→4:1) gave compound **59** as a colourless syrup (2.50 g, 52%); $[\alpha]_D$ +4.0 (*c* 0.75 in $CHCl_3$); ν_{max}/cm^{-1} 2920, 2840 (CH_2, CH_3), 1090 (C-O), 730, 695 (Ar-H); $\delta_H(CDCl_3)$ 0.87 (3 H, m, $C_7H_{14}CH_3$), 1.22-1.42 (10 H, m, $(CH_2)_5$ CH_3), 1.62 (2 H, m, OCH_2CH_2) 4.15 (2 H, m, 1-H allyl), 4.20-5.02 (12× 1 H, 12 AB d, 6 × OCH_2Ph), 4.36, 4.43 (2 × 1 H, 2 d, $J_{1a,2a}$, $J_{1b,2b}$ 8.0, 7.4, 1a-H, 1b-H) 5.17 (1 H, m, 3-H allyl), 5.31 (1 H, m, 3′-H allyl), 5.85-5.99 (1 H, m, 2-H allyl), 7.10-7.40 (30 H, m, Ar-H); $\delta_C(CDCl_3)$ 13.9, 22.5, 26.0, 29.1, 29.2, 29.6, 31.65, 68.0, 68.3, 69.9, 71.4, 72.85, 72.9, 73.3 (2), 73.4, 74.5, 74.8, 75.1 (2), 75.2, 79.8, 81.7, 82.3, 82.9, 127.0-128.31 (Ar C), 102.7, 103.55, 116.3, 135.0, 138.1, 138.5, 138.8, 138.9, 139.1, 139.2.

Octyl 2,4,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside, 60



A suspension of compound **59** (1.90 g, 1.8 mmol) and cyclooctadiene-bis(methyldiphenylphosphine)iridium (I) hexafluorophosphate in freshly distilled oxygen-free THF, was placed under dry N_2 in a flask sealed with a septum. The red mixture was degassed by evacuating the flask and refilling it with dry N_2 three times. The flask was evacuated once more and this time filled with H_2. Within 3 seconds the red suspension had become a colourless solution which was stirred for a further 5 minutes before degassing as before and leaving the mixture to stir at room temperature overnight under N_2. ^1H NMR of a small portion of the reaction mixture showed the isomerisation to be complete [$\delta_H(CDCl_3)$ 6.15 (1H, m, $H_3CCH{=}CH$-O-)]. The mixture was concentrated to a syrup and taken up in CH_2Cl_2 (10 cm^3), to which was added aqueous TFA (2 cm^3; 50% v/v solution) and the mixture was stirred at room temperature for 1 h. The organic layer was subjected to standard work-up **A**, before concentration to a syrup. Flash chromatography (silica gel; hexane-EtOAc, 4:1→3:1) gave the desired *mono alcohol* **60** as a colourless syrup (1.06 g, 53%), (Found: C, 74.92; H, 7.98. $C_{62}H_{74}O_{11}$ requires C, 74.82; H, 7.49%); $[\alpha]_D$ +1.1 (*c* 0.47 in $CHCl_3$); ν_{max}/cm^{-1} 3090 (O-H), 2925, 2860 (CH_2, CH_3), 1090 (C-O), 730, 700 (Ar-H); $\delta_H(CDCl_3)$ 0.88 (3 H, m, $C_7H_{14}CH_3$), 1.20-1.40 (10 H, m, $(CH_2)_5$ CH_3), 1.65 (2 H, m, OCH_2CH_2), 4.26-5.06 (12× 1 H, 12 AB d, 6 × OCH_2Ph), 4.39, 4.44 (2 × 1 H, 2 d, $J_{1a,2a}$, $J_{1b,2b}$ ~7.7, ~9, 1a-H, 1b-H), 7.00-7.30 (30 H, m, Ar-H).

Octyl 2,4,6-tri-*O*-benzyl-3-*O*-trifluoromethanesulfonyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside, 61

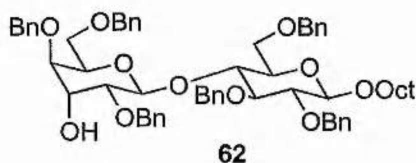
To a solution of compound **60** (492 mg, 494 μmol) and pyridine (68 mm^3, 840 μmol) in CH_2Cl_2 (5 cm^3) at 0 °C was added dropwise a dichloromethane solution (0.5 cm^3) of trifluoromethanesulfonic anhydride (116 mm^3, 692 μmol). The mixture was allowed to warm to room temperature, and after stirring for 30 min it was diluted with CH_2Cl_2 (25 cm^3) and subjected to standard work-up **A**, before concentration to a syrup. Flash chromatography (silica gel; hexane-EtOAc, 6:1) gave the desired *triflate* **61** as a colourless syrup (476 mg, 86%), (Found: C, 67.61; H, 6.86. $C_{63}H_{73}F_3O_{13}S$ requires C, 67.12; H, 6.53%); $[\alpha]_D$ +0.9 (*c* 1.25 in $CHCl_3$); ν_{max}/cm^{-1} 2930, 2865 (CH_2, CH_3), 1415, 1210 (SO_2-O), 1155 (-SO_2-), 1090 (C-O), 735, 700 (Ar-H); $\delta_H(CDCl_3)$ (assignments by COSY) 0.90 (3 H, m, $C_7H_{14}CH_3$), 1.20-1.45 (10 H, m, $(CH_2)_5$ CH_3), 1.64 (2 H, m, OCH_2CH_2) , 3.40 (1 H, dd, $J_{1a,2a}$ 7.6 $J_{2a,3a}$ 9.4, 2a-H), 3.50 (1 H, t, $J_{2a,3a}$, $J_{3a,4a}$ 9.4, 3a-H), 3.80 (1 H, dd, $J_{1b,2b}$ 7.6, $J_{2b,3b}$ 10.0, 2b-H), 4.04 (1H, br d, $J_{3b,4b}$ 2.9, 4b-H) 4.27-4.95 (12× 1 H, 12 AB d, 6 × OCH_2Ph), 4.35 (1 H, d, $J_{1a,2a}$, 1a-H), 4.36 (1 H, d, $J_{1b,2b}$, 1b-H), 4.66 (1 H, m, 3b-H), 7.05-7.40 (30 H, m, Ar-H).

Octyl 3-*O*-acetyl-2,4,6-tri-*O*-benzyl-β-D-gulopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside, 70

A mixture of compound **61** (33 mg, 29.3 μmol), tetrabutylammonium acetate (44 mg 146 μmol), 4Å molecular sieves (10 mg) and DMF (0.3 cm^3) were stirred at room temperature for 24 h. The mixture was diluted with diethyl ether (50 cm^3), filtered through Celite and the filtrate was washed with water before drying and concentration to give a syrup. Flash chromatography (silica gel; hexane-EtOAc, 4:1) gave two products: the first compound eluted was the 3b,4b elimination product,

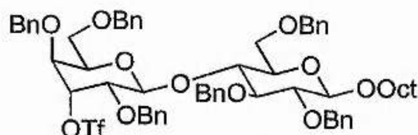
*octyl 2,4,6-tri-O-benzyl-3-deoxy-β-*D-erythro-*hex-3-enopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-glucopyranoside*, **71** (R_f 0.36) as a waxy white solid (15 mg, 52%); ν_{max}/cm^{-1} 2925, 2857 (CH_2, CH_3), 1664 (*C*=*C*-OR), 1091 (C-O), 797 (C=*C-H*) 731, 696 (Ar-H); $\delta_C(CDCl_3)$ 13.9 (C_7H_{14}*Me*), 95.3 (3b-C), 101.8, 103.5 (1a-C, 1b-C), 154.4 (4b-C); the second compound eluted was the inversion product, *octyl 3-O-acetyl-2,4,6-tri-O-benzyl-β-D-gulopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-glucopyranoside*, **70** (R_f 0.23) as a colourless syrup (12 mg, 39%); $\delta_H(CDCl_3)$ 4.20-5.02 (12× 1 H, 12 AB d, 6 × OCH_2Ph), 4.38 (1 H, d, $J_{1a,2a,}$ 7.4, 1a-H), 4.96 (1 H, d, $J_{1b,2b}$ 7.5, 1b-H), 5.47 (1 H, m, 3b-H), 7.00-7.45 (30 H, m, Ar-H). Compound **70** was deacetylated in a manner analogous to the preparation of compound **57** and gave an identical ^{1}H NMR spectrum to compound **62** $\delta_H(CDCl_3)$ 4.20-5.02 (12× 1 H, 12 AB d, 6 × OCH_2Ph), 4.39 (1 H, d, $J_{1a,2a,}$ 7.9, 1a-H), 4.85 (1 H, d, $J_{1b,2b}$ 8.0, 1b-H)

Octyl 2,4,6-tri-*O*-benzyl-β-D-gulopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside, 62



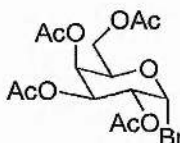
A mixture of compound **61** (0.441 g, 0.4 mmol), 4Å molecular sieves (0.10 g), tetrabutylammonium nitrite (2.26 g, 7.8 mmol) and toluene (4.5 cm^3) were stirred for 24 h at room temperature. The mixture was diluted with diethyl ether (150 cm^3), filtered and washed with water before drying and evaporation to a syrup. Flash chromatography (silica gel; hexane-EtOAc, 3:1) gave the *inverted alcohol* **62** as a colourless syrup (0.25 g, 64%); $[\alpha]_D$ -10.4 (*c* 0.85 in $CHCl_3$); ν_{max}/cm^{-1} 3470 (O-H), 2925, 2865 (CH_2, CH_3), 1100 (C-O), 740, 705 (Ar-H); $\delta_H(CDCl_3)$ 4.20-5.02 (12× 1 H, 12 AB d, 6 × OCH_2Ph), 4.39 (1 H, d, $J_{1a,2a,}$ 7.9, 1a-H), 4.85 (1 H, d, $J_{1b,2b}$ 8.0, 1b-H), 7.10-7.45 (30 H, m, Ar-H); $\delta_C(CDCl_3)$ 13.9, 22.5, 26.0, 29.1, 29.3, 29.6, 31.7, 65.7, 67.75, 67.95, 68.7, 70.0, 71.5, 72.8, 73.0, 73.1, 73.2, 74.8, 75.1 (2), 75.4, 76.9, 81.8, 82.9, 99.7, 103.5, 127.1-139.2 (Ar C).

Octyl 2,4,6-tri-*O*-benzyl-3-*O*-trifluoromethanesulfonyl-β-D-gulopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside, 63



Compound **62** (28 mg, 28 μmol) was treated in a manner analogous to the preparation of compound **61**. TLC (hexane-EtOAc, 4:1) showed the reaction to be complete after 1 h at room temperature. The reaction was worked-up as described previously to give the *triflate* **63** as a colourless syrup (29 mg, 92%), (Found: C, 67.33; H, 6.95. $C_{63}H_{73}F_3O_{13}S$ requires C, 67.12; H, 6.53%); $[\alpha]_D$ -11.0 (*c* 1.20 in $CHCl_3$); ν_{max}/cm^{-1} 2935, 2865 (CH_2, CH_3), 1410, 1205 (SO_2-O), 1135 (-SO_2-), 1090 (C-O), 735, 700 (Ar-H); $\delta_H(CDCl_3)$ (assignments by COSY) 0.90 (3 H, m, $C_7H_{14}CH_3$), 1.20-1.45 (10 H, m, $(CH_2)_5$ CH_3), 1.64 (2 H, m, OCH_2CH_2), 3.40 (1 H, dd, $J_{1a,2a}$ 7.8 $J_{2a,3a}$ 9.2, 2a-H), 3.50 (1 H, m, OCH_2) 3.53 (1 H, t, $J_{2a,3a}$, $J_{3a,4a}$ 9.2, 3a-H), 3.64 (1 H, dd, $J_{1b,2b}$ 8.0, $J_{2b,3b}$ 3.3, 2b-H), 3.94 (1 H, m, OCH_2) 4.23-4.93 (12 × 1 H, 12 AB d, 6 × OCH_2Ph), 4.36 (1 H, d, $J_{1a,2a}$, 1a-H), 4.86 (1 H, d, $J_{1b,2b}$, 1b-H), 5.01 (1 H, t, $J_{2b,3b}$, $J_{3b,4b}$ 3.3, 3b-H) 7.10-7.40 (30 H, m, Ar-H).

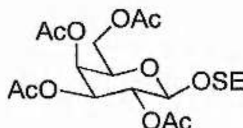
2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide, 76[5]



Iodine (0.25 g, 0.98 mmol) was added to a suspension of D-galactose, **75** (4.90 g, 27.2 mmol) in acetic anhydride (25 cm^3) and the mixture was stirred at room temperature overnight at which point all of the suspended material had dissolved. The solution was diluted with CH_2Cl_2 (150 cm^3) and washed with saturated $Na_2S_2O_4$ solution and with saturated Na_2CO_3 solution before drying and concentration to a slightly brown syrup. The syrup was dissolved in CH_2Cl_2 (200 cm^3), cooled to 0 °C and hydrogen bromide in acetic acid (45 cm^3; 45% w/v solution) was added dropwise. After stirring for 3 h the solution was poured into iced NaCl solution, the organic layer was separated and subjected to standard work-up A. Concentration gave a syrup which was crystallised from diethyl ether-hexane to give the *bromide*

76 as needles (10.40 g, 93%), mp 82-83 °C (lit.,[5] 84-85 °C); $\delta_H(CDCl_3)$ 1.99, 2.04, 2.09, 2.13 (4 × 3 H, 4 s, 4 × AcO), 4.12 (2 H, m, 6-H, 6′-H), 4.47 (1 H, br t, $J_{5,6} = J_{5,6'}$ 6.3, 5-H), 5.02 (1 H, dd, $J_{1,2}$ 3.8, $J_{2,3}$ 10.4, 2-H), 5.39 (1 H, dd, $J_{2,3}$, $J_{3,4}$ 3.3, 3-H), 5.51 (1 H, dd, $J_{3,4}$, $J_{4,5}$ 1.4, 4-H), 6.68 (1 H, d, $J_{1,2}$, 1-H).

2-(Trimethylsilyl)ethyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside, 77[6]

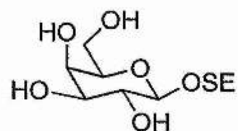


77

Method one. A mixture of compound **76** (10.40 g, 25.3 mmol), 2-(trimethylsilyl)ethanol (9.06 cm^3, 63.2 mmol), 4Å molecular sieves (26 g), and dry toluene (170 cm^3) was stirred for 30 min at room temperature in a tin foil covered flask in order to exclude light. Silver carbonate (6.98 g, 25.3 mmol) was added and the mixture stirred as before overnight. TLC (CH_2Cl_2-acetone, 97:3) showed there was some donor remaining, so more silver carbonate, (1.75 g, 6.3 mmol) was added and the mixture was stirred for a further 3 h. The mixture was filtered through Celite and concentrated to a slightly coloured oil. Flash chromatography (silica gel; CH_2Cl_2-acetone, 97:3) gave the *acetylated galactoside* **77** as colourless syrup (8.38 g, 74%); $\delta_H(CDCl_3$, lit.,[6]) 0.01 (9 H, s, Si*Me*₃), 0.96 (2 H, m, OCH_2CH_2Si), 1.98, 2.05, 2.05, 2.15 (4 × 3 H, 4 s, 4 × AcO), 3.56 (1 H, m, OCH_2CH_2Si), 3.90 (1 H, td, $J_{4,5}$ 1.0, $J_{5,6} = J_{5,6'}$ 6.8, 5-H), 3.99 (1 H, m, OCH_2CH_2Si), 4.12, 4.20 (2 × 1 H, 2 dd, $J_{5,6}$ 6.9, $J_{5,6'}$ 6.6, $J_{6,6'}$ 11.1, 6-H, 6′-H), 4.48 (1 H, d, $J_{1,2}$ 8.0, 1-H), 5.01 (1 H, dd, $J_{2,3}$ 10.4, $J_{3,4}$ 3.6, 3-H), 5.20 (1 H, dd, $J_{1,2}$ 8.0, $J_{2,3}$ 10.4, 2-H), 5.38 (1 H, dd, $J_{3,4}$ 3.3, $J_{4,5}$ 1.1, 4-H).

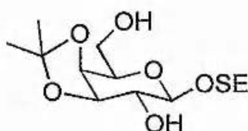
Method two. A mixture of DDQ (4.15 g, 18.3 mmol), 2-(trimethylsilyl)ethanol (4.36 cm^3, 30.4 mmol), 4Å molecular sieves (10 g), and dry acetonitrile (50 cm^3) were stirred for 1.5 h at room temperature before adding compound **76** (5.00 g, 12.2 mmol) and stirring as before for 30 min. Iodine (4.64 g, 18.3 mmol) was added and the mixture stirred for 50 min. The mixture was diluted with CH_2Cl_2 (400 cm^3) and washed with dilute $Na_2S_2O_4$ solution, before drying and concentration to an orange syrup. Flash chromatography (silica gel; CH_2Cl_2-acetone, 97:3) gave the *acetylated galactoside* **77** as colourless syrup 93.88 g, 71%). Analytical data were identical to the above.

2-(Trimethylsilyl)ethyl β-D-galactopyranoside, 78[6]



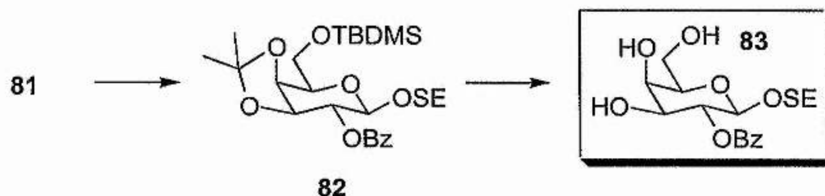
A solution of both compound **77** (8.38 g, 18.7 mmol) and sodium metal (100 mg, 4.3 mmol) in dry MeOH (100 cm^3) was stirred for 1 h at room temperature. The solution was neutralised with Amberlite IRC-50 (H^+) resin (1 g). Filtration and concentration gave the fully deprotected compound **78** as an amorphous white solid (5.19 g, 99%); δ_H(D_2O, lit.,[6]) 0.01 (9 H, s, Si*Me*$_3$), 1.00 (2 H, m, OCH_2CH_2Si), 3.46 (1 H, dd, $J_{1,2}$ 8.0, $J_{2,3}$ 9.9, 2-H), 3.61 (1 H, dd, $J_{2,3}$, $J_{3,4}$ 3.3, 3-H), 3.90 (1 H, br d, $J_{3,4}$, 4-H) 4.02 (1 H, m, OCH_2CH_2Si), 4.38 (1 H, d, $J_{1,2}$, 1-H); δ_C(D_2O) -1.8 (3), 18.4, 61.7, 69.2, 69.5, 71.6, 73.8, 75.9, 103.1.

2-(Trimethylsilyl)ethyl 3,4-*O*-isopropylidene-β-D-galactopyranoside, 81[7]



A solution of compound **78** (5.19 g, 18.5 mmol) and *p*-toluenesulfonic acid (80 mg, 0.4 mmol) in 2,2-dimethoxypropane (100 cm^3) was stirred at room temperature for 22 h. The reaction was quenched with triethylamine (0.5 cm^3) and concentrated to a syrup which was dissolved in CH_2Cl_2 (100 cm^3). Aqueous TFA (0.75 cm^3; 50% v/v) was added and after stirring for 30 min, the reaction was again quenched with triethylamine and washed with NaCl solution before drying and evaporation to a crystalline solid. This was recrystallised (EtOAc-hexane) to give compound **81** as needles (4.63 g, 78%), mp 89-90 °C (lit.,[7] 88-89.5 °C); δ_H($CDCl_3$) 0.01 (9 H, s, Si*Me*$_3$), 1.00 (2 H, m, OCH_2CH_2Si), 1.34, 1.50 (2 × 3 H, 2 s, CMe_2), 4.07 (1 H, dd, $J_{2,3}$ 7.1, $J_{3,4}$ 5.8, 3-H), 4.14 (1 H, dd, $J_{3,4}$, $J_{4,5}$ 2.2, 4-H) 4.18 (1 H, d, $J_{1,2}$ 8.2, 1-H).

2-(Trimethylsilyl)ethyl 2-*O*-benzoyl-β-D-galactopyranoside, 83[8]

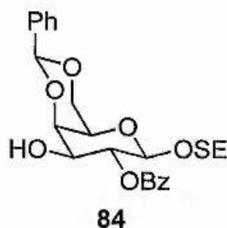


t-Butyldimethylsilylchloride (2.64 g, 17.6 mmol) was added to a solution of compound **81** (4.33 g, 13.5 mmol) in pyridine (50 cm^3) at 0 °C. The solution was stirred at room temperature for 2 h before cooling again to 0 °C. A dichloromethane solution (50% v/v) of benzoyl chloride (2.04 cm^3, 17.6 mmol) was added dropwise and the mixture stirred at 0 °C for 1 h, before concentration and coevaporation with toluene. The residue was taken up in CH_2Cl_2 (200 cm^3), subjected to standard work-up **B** and concentrated to give a syrup.

A small sample of the syrup (100 mg) was purified by flash chromatography (silica gel; hexane-EtOAc 9:1) to give *2-(trimethylsilyl)ethyl 2-O-benzoyl-6-O-t-butyldimethylsilyl-3,4-O-isopropylidene-β-D-galactopyranoside* **82** as needles, mp 84-85 °C (Found: C, 60.60; H, 8.69. $C_{27}H_{46}O_7Si_2$ requires C, 60.2; H, 8.6%); $[\alpha]_D$ +12.6 (*c* 1.40 in $CHCl_3$); ν_{max}/cm^{-1} 2930, 2855 (CH_2, CH_3), 1730 (C=O), 1385, 1365 (Pr^i, Bu^t C-H), 1270, 835, shoulder ~865 (Si-C), 1255, 1065 (C-O), 1110 (Si-O), 775, 715 (Ar-H); δ_H($CDCl_3$) -0.09 (9 H, s, Si*Me*$_3$), 0.10 (6 H, s, Si*Me*$_2$$Bu^t$), 0.87 (2 H, m, OCH_2CH_2Si), 0.91 (9 H, s, $SiMe_2Bu^t$) 1.34, 1.62 (2 × 3 H, 2 s, CMe_2), 3.50 (1 H, m, OCH_2CH_2Si), 4.26 (1 H, dd, $J_{3,4}$ 5.5, $J_{4,5}$ 1.6, 4-H) 4.31 (1 H, dd, $J_{2,3}$ 7.1, $J_{3,4}$, 3-H), 4.48 (1 H, d, $J_{1,2}$ 8.2, 1-H), 5.21 (1 H, dd, $J_{1,2}$, $J_{2,3}$, 2-H), 7.39-8.08 (5 H, m, ArH).

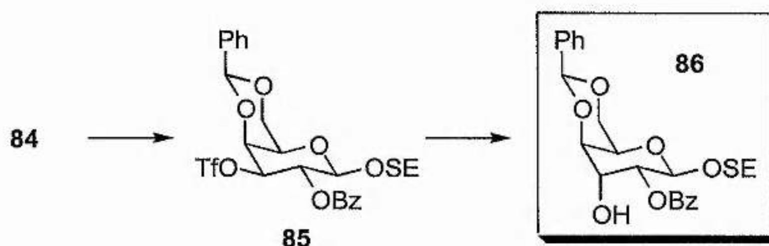
The crude syrup was dissolved in aqueous acetic acid (250 cm^3; 80% v/v) and stirred at 75-80 °C for 1 h. After cooling, the solution was concentrated and co-evaporated several times with toluene. Flash chromatography (silica gel; EtOAc) gave compound **83** as an amorphous white solid (4.18 g, 80%); δ_H($CDCl_3$, lit.,[8]) -0.10 (9 H, s, Si*Me*$_3$), 0.86 (2 H, m, OCH_2CH_2Si), 3.81 (1 H, dd, $J_{2,3}$ 9.9, $J_{3,4}$ 3.0, 3-H), 4.14 (1 H, d, $J_{3,4}$, 4-H), 4.56 (1 H, d, $J_{1,2}$ 8.0, 1-H), 5.23 (1 H, dd, $J_{1,2}$, $J_{2,3}$, 2-H), 7.39-8.10 (5 H, m, ArH).

2-(Trimethylsilyl)ethyl 2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-galactopyranoside, 84[8]



A solution of compound **83** (4.03 g, 10.5 mmol), benzaldehyde dimethylacetal (2.36 cm^3, 15.75 mmol) and camphorsulfonic acid (80 mg) in acetonitrile (40 cm^3) was stirred at room temperature for 20 h. The reaction was quenched with triethylamine (0.5 cm^3) and concentrated to a syrup which was dissolved in CH_2Cl_2 (250 cm^3) and washed with NaCl solution before drying and concentration to a syrup which crystallised on standing. The crystals were filtered, washed with hexane and dried in vacuo, giving compound **84** as needles (4.65 g, 94%), mp 144-146 °C; δ_H($CDCl_3$, lit.,[8]) -0.10 (9 H, s, SiMe_3), 0.90 (2 H, m, OCH_2CH_2Si), 2.65 (1 H, d, $J_{3,OH}$ 11.3, OH), 3.54 (1 H, br s, 5-H), 3.58 (1 H, m, OCH_2CH_2Si), 3.89 (1 H, ddd, $J_{2,3}$ 9.9, $J_{3,4}$ 3.8 $J_{3,OH}$, 3-H), 4.11 (1 H, dd, $J_{5,6}$ 1.9, $J_{6,6'}$ 12.4, 6-H), 4.26 (1 H, dd, $J_{3,4}$, $J_{4,5}$ 1.1, 4-H), 4.38 (1 H, dd, $J_{5,6'}$ 1.4, $J_{6,6'}$, 6′-H), 4.62 (1 H, d, $J_{1,2}$ 8.0, 1-H), 5.36 (1 H, dd, $J_{1,2}$, $J_{2,3}$, 2-H), 5.58 (1 H, s, PhCH), 7.30-8.10 (10 H, m, Ar-H).

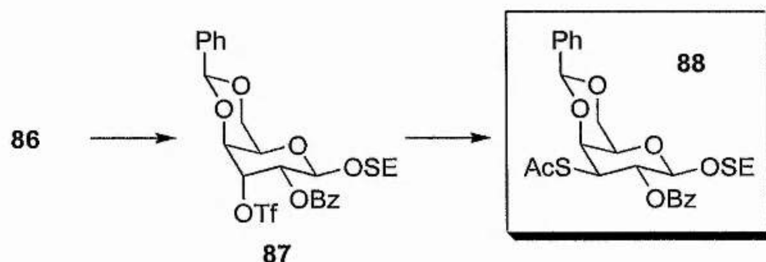
2-(Trimethylsilyl)ethyl 2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-gulopyranoside, 86



To a solution of compound **84** (1.00 g, 2.11 mmol) and pyridine (0.29 cm^3, 3.59 mmol) in CH_2Cl_2 (10 cm^3) at 0 °C was added dropwise a dichloromethane solution (1.0 cm^3) of trifluoromethanesulfonic anhydride (0.5 cm^3, 2.95 mmol). After stirring for 30 min at this temperature, the mixture was diluted with CH_2Cl_2 (90 cm^3) and subjected to standard work-up **A**. Concentration and co-evaporation with toluene gave *2-(trimethylsilyl)ethyl 2-O-benzoyl-4,6-O-benzylidene-3-O-trifluoromethanesulfonyl-β-D-galactopyranoside* **85** as a crystalline solid which was

used in the next step without further purification; δ_H($CDCl_3$) -0.10 (9 H, s, Si*Me*$_3$), 0.90 (2 H, m, OCH$_2$C*H*$_2$Si), 3.55 (1 H, m, OC*H*$_2$CH$_2$Si), 3.57 (1 H, m, 5-H), 4.01 (1 H, m, OC*H*$_2$CH$_2$Si), 4.13 (1 H, dd, $J_{5,6}$ 1.6, $J_{6,6'}$ 12.6, 6-H), 4.42 (1 H, dd, $J_{5,6'}$ 1.4, $J_{6,6'}$, 6′-H), 4.52 (1 H, dd, $J_{3,4}$ 3.8, $J_{4,5}$ 0.8, 4-H), 4.67 (1 H, d, $J_{1,2}$ 8.0, 1-H), 5.10 (1 H, dd, $J_{2,3}$ 10.2, $J_{3,4}$, 3-H), 5.61 (1 H, s, PhCH), 5.72 (1 H, dd, $J_{1,2}$, $J_{2,3}$, 2-H), 7.30-8.10 (10 H, m, Ar-H). The crude product and tetrabutylammonium nitrite (1.83 g, 6.33 mmol) were dissolved in DMF (4.5 cm^3) and stirred under nitrogen for 24 h at room temperature. The mixture was diluted with CH_2Cl_2 (300 cm^3) and washed with water before drying and evaporation on to silica (4 g). Flash chromatography (silica gel, 40 g; toluene-EtOAc, 6:1) gave the *inverted alcohol* **86** as white needles (0.65 g, 65%), mp 145-146 °C (Found: C, 63.40; H, 6.78. $C_{25}H_{32}O_7$ requires C, 63.54; H, 6.82%); $[\alpha]_D$ -40.2 (*c* 1 in $CHCl_3$); ν_{max}/cm^{-1} 2930, 2900 (CH_2, CH_3), 1725 (C=O), 1270, 840 (Si-C), 1065 (C-O), 1120 (Si-O), 740, 715 (Ar-H); δ_H($CDCl_3$) -0.05 (9 H, s, Si*Me*$_3$), 0.91 (2 H, m, OCH$_2$C*H*$_2$Si), 2.35 (1 H, br s, OH), 3.59 (1 H, m, OC*H*$_2$CH$_2$Si), 3.90 (1 H, m, 5-H), 4.06 (1 H, m, OC*H*$_2$CH$_2$Si), 4.08-4.14 (2 H, m, 4-H, 6-H), 4.35-4.42 (2 H, m, 3-H, 6′-H), 5.07 (1 H, d, $J_{1,2}$ 8.5, 1-H), 5.36 (1 H, dd, $J_{1,2}$, $J_{2,3}$ 3.3, 2-H), 5.58 (1 H, s, PhCH), 7.30-8.10 (10 H, m, Ar-H). A small sample sample was acetylated using pyridine-acetic anhydride and purified by flash chromatography (silica gel; hexane-EtOAc 3:2) to give *2-(trimethylsilyl)ethyl 3-O-acetyl-2-O-benzoyl-4,6-O-benzylidene-β-D-gulopyranoside* as a colourless syrup; δ_H($CDCl_3$) -0.05 (9 H, s, Si*Me*$_3$), 0.90 (2 H, m, OCH$_2$C*H*$_2$Si), 2.15 (3 H, s, AcO), 3.60 (1 H, m, OC*H*$_2$CH$_2$Si), 3.80 (1 H, m, 5-H), 4.02-4.13 (3 H, m, OC*H*$_2$CH$_2$Si, 4-H, 6-H), 4.39 (1 H, dd, $J_{5,6'}$ 1.4, $J_{6,6'}$ 12.4, 6′-H), 5.00 (1 H, d, $J_{1,2}$ 8.5, 1-H), 5.43 (1 H, dd, $J_{1,2}$, $J_{2,3}$ 3.6, 2-H), 5.56 (1 H, s, PhCH), 5.60 (1 H, t, $J_{2,3}$, $J_{3,4}$ 3.6, 3-H), 7.30-8.10 (10 H, m, Ar-H).

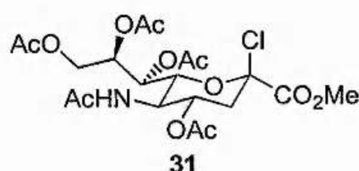
2-(Trimethylsilyl)ethyl 3-*S*-acetyl-2-*O*-benzoyl-4,6-*O*-benzylidene-3-thio-β-D-galactopyranoside, 88



Compound **86** (486 mg, 1.03 mmol) was treated in a manner analogous to the preparation of compound **85**. TLC (hexane-EtOAc, 3:2) showed the reaction to be

complete after 4.5 h at 0 °C. The reaction was worked-up as described previously to give *2-(trimethylsilyl)ethyl 2-O-benzoyl-4,6-O-benzylidene-3-O-trifluoromethanesulfonyl-β-D-gulopyranoside* **87** as a syrup which was used in subsequent reactions without further purification; $\delta_H(CDCl_3)$ -0.05 (9 H, s, Si*Me*$_3$), 0.90 (2 H, m, OCH_2CH_2Si), 3.61 (1 H, m, OCH_2CH_2Si), 3.88 (1 H, m, 5-H), 4.02 (1 H, m, OCH_2CH_2Si), 4.16 (1 H, dd, $J_{5,6}$ 1.6, $J_{6,6'}$ 12.6, 6-H), 4.30 (1 H, m, 4-H), 4.42 (1 H, dd, $J_{5,6'}$ 1.4, $J_{6,6'}$, 6′-H), 5.01 (1 H, d, $J_{1,2}$ 8.5, 1-H), 5.40 (1 H, t, $J_{2,3} = J_{3,4}$ 3.0, 3-H), 5.50 (1 H, dd, $J_{1,2}$, $J_{2,3}$, 2-H), 5.62 (1 H, s, PhCH), 7.30-8.10 (10 H, m, Ar-H). Potassium thioacetate (588 mg, 5.15 mmol) was added to a soultion of the crude *triflate* in DMF (2.5 cm^3) and the mixture was stirred for 20 h at room temperature. The mixture was diluted with CH_2Cl_2 (50 cm^3) and washed with water before drying and evaporation. Flash chromatography (silica gel; hexane-EtOAc, 7:3) gave the *thioacetate* **88** as a glassy solid (433 mg, 82%), mp 124-126 °C; ν_{max}/cm^{-1} 2955, 2860 (CH_2, CH_3), 1735 (C=O, ester), 1710 (C=O, thiol ester), 1250, 840 (Si-C), 1030 (C-O), 1110 (Si-O), 760, 710 (Ar-H); $\delta_H(CDCl_3)$ -0.01 (9 H, s, Si*Me*$_3$), 0.86 (2 H, m, OCH_2CH_2Si), 2.20 (3 H, s, AcS), 3.55 (1 H, m, OCH_2CH_2Si), 3.70 (1 H, m, 5-H), 4.02 (1 H, m, OCH_2CH_2Si), 4.09 (1 H, dd, $J_{5,6}$ 1.6, $J_{6,6'}$ 12.4, 6-H), 4.14 (1 H, dd, $J_{3,4}$ 3.3, $J_{4,5}$ 0.8, 4-H), 4.24 (1 H, dd, $J_{2,3}$11.5, $J_{3,4}$, 3-H), 4.38 (1 H, dd, $J_{5,6'}$ 1.4, $J_{6,6'}$, 6′-H), 4.73 (1 H, d, $J_{1,2}$ 7.7, 1-H), 5.44 (1 H, dd, $J_{1,2}$, $J_{2,3}$, 2-H), 5.50 (1 H, s, PhCH), 7.30-8.00 (10 H, m, Ar-H); FAB-MS: *m/z* 553 (M+Na)$^+$. (Found: [M+Na]$^+$ 553.1655. $C_{27}H_{34}O_6SSiNa$ requires *m/z* 553.1692).

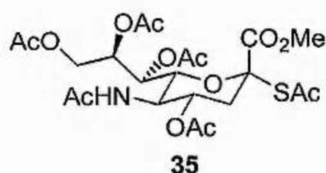
Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-chloro-2,3,5-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosonate, 31[9]



N-Acetyl neuraminic acid, **1** (5.00 g, 16.2 mmol) and Dowex 50WX8-200 (H$^+$) resin (17 g) were stirred in dry methanol (500 cm^3) at room temperature for 3 h. The mixture was filtered and the resin was washed with methanol (5 × 100 cm^3). The combined extracts were concentrated to an amorphous white solid which was suspended in acetic anhydride (40 cm^3) and cooled to 0 °C. Pyridine (35 cm^3) was added dropwise and the mixture was stirred at room temperature for 4 days at which point all of the suspended material had dissolved. The mixture was concentrated and co-evaporated with toluene before being taken-up in CH_2Cl_2 (250 cm^3) and subjected

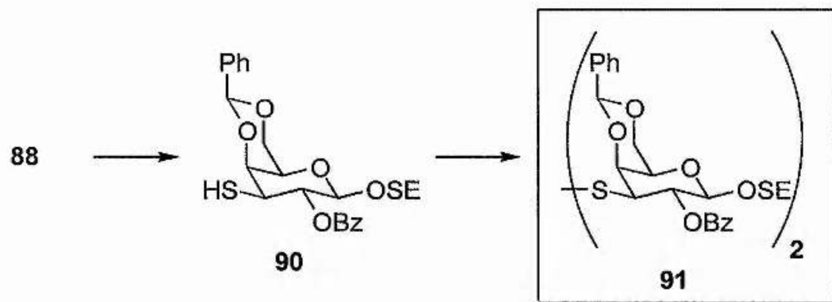
to standard work-up B. Concentration gave *peracetate* **89** as a glassy solid which was freeze-dried from dioxane to give a white powder. The powder was dissolved in acetyl chloride (150 cm^3), cooled to -20 °C and hydrogen chloride gas was bubbled through the solution for 5 min. The flask was stoppered and allowed to gradually warm to room temperature without stirring. After 24 h, TLC (elution with EtOAc 3 times) indicated that the reaction was not complete and so the mixture was cooled as before and hydrogen chloride gas was bubbled through the solution for a further 10 min before allowing the solution to return to room temperature. After 24 h the mixture was concentrated and co-evaporated with toluene several times to give a glassy solid which was freeze-dried from dioxane to give the *chloride* **31** as a white powder (7.48 g, 91%); $[\alpha]_D$ -59.2 (*c* 1.0 in $CHCl_3$) (lit.,[9] -63); $\delta_H(CDCl_3)$ 1.89 (3 H, s, AcN), 2.04-2.11 (4 × 3 H, 4 s, 4 × AcO), 2.26 (1 H, dd, $J_{3ax,3eq}$ 14.0, $J_{3ax,4}$ 11.5, 3ax-H), 2.77 (1 H, dd, $J_{3ax,3eq}$, $J_{3eq,4}$ 4.7, 3eq-H), 3.87 (3 H, s, CO_2Me) 4.06 (1 H, dd, $J_{8,9}$ 6.0, $J_{9,9'}$ 12.4, 9-H), 4.20 (1 H, q, $J_{4,5} = J_{5,6} = J_{5,NH}$ 10.4, 5-H), 4.35 (1 H, dd, $J_{5,6}$, $J_{6,7}$ 1.9, 6-H), 4.43 (1 H, dd, $J_{8,9'}$ 2.5, $J_{9,9'}$, 9′-H), 5.16 (1 H, td, $J_{7,8} = J_{8,9}$ 6.6, $J_{8,9'}$, 8-H), 5.39 (1 H, m, 4-H), 5.46 (1 H, dd, $J_{6,7}$, $J_{7,8}$, 7-H), 5.66 (1 H, d, $J_{5,NH}$, NH).

Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate, 35[10]



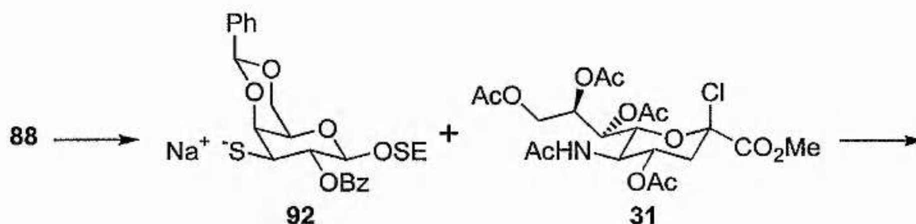
A mixture of potassium thioacetate (4.48 g, 39.2 mmol) and **31** (4.00 g, 7.84 mmol) in CH_2Cl_2 (40 cm^3) was stirred for 24 h at room temperature. The mixture was diluted with CH_2Cl_2 (350 cm^3) and washed with water before drying and decolourising with charcoal. Flash chromatography (silica gel; CH_2Cl_2-MeOH, 100:1→100:3) gave the *thioacetate* **35** as a glassy solid which was freeze-dried from dioxane to give a white powder (3.28 g, 76%),$[\alpha]_D$ -12.5 (*c* 1 in $CHCl_3$) (lit.,[10] -15.6); $\delta_H(CDCl_3)$ 1.87 (4 H, m, AcN, 3_{ax}-H), 2.01-2.13 (4 × 3 H, 4 s, 4 × AcO), 2.27 (3 H, s, AcS), 2.61 (1 H, dd, $J_{3ax,3eq}$ 12.9, $J_{3eq,4}$ 4.7, 3_{eq}-H), 3.78 (3 H, s, CO_2Me) 4.02 (1 H, dd, $J_{8,9}$ 6.0, $J_{9,9'}$ 12.4, 9-H), 4.09 (1 H, q, $J_{4,5} = J_{5,6} = J_{5,NH}$ 10.4, 5-H), 4.40 (1 H, dd, $J_{8,9'}$ 2.5, $J_{9,9'}$, 9′-H), 4.65 (1 H, dd, $J_{5,6}$, $J_{6,7}$ 2.5, 6-H), 4.90 (1 H, m, 4-H), 5.21 (1 H, td, $J_{7,8}$, $J_{8,9}$ 6.0, $J_{8,9'}$, 8-H), 5.35 (1 H, dd, $J_{6,7}$, $J_{7,8}$, 7-H), 5.65 (1 H, d, $J_{5,NH}$, NH).

Bis[2-(trimethylsilyl)ethyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-deoxy-β-D-galactopyranosid-3-yl] disulfide, 91



A solution of compound **88** (30 mg, 58.3 μmol) in dry MeOH (1.2 cm^3) was degassed by evacuating the flask and refilling it with dry N_2 three times. The flask was cooled to -40 °C and a solution of sodium methoxide in MeOH (12 mm^3, 55.4 μmol) was added and stirred for 2.5 h at this temperature. The solution was neutralised with Amberlite IRC-50 (H^+) resin (50 mg). Filtration and concentration gave the crude *thiol* **90**; $\delta_H(CD_3OD)$ -0.01 (9 H, s, Si*Me*$_3$), 0.80 (2 H, m, OCH_2CH_2Si), 3.35 (1 H, dd, $J_{2,3}$ 11.0, $J_{3,4}$ 3.3, 3-H), 3.59 (1 H, m, OCH_2CH_2Si), 3.74 (1 H, m, 5-H), 4.02 (1 H, m, OCH_2CH_2Si), 4.17 (1 H, dd, $J_{5,6}$ 1.6, $J_{6,6'}$ 12.4, 6-H), 4.24 (1 H, dd, $J_{3,4}$, $J_{4,5}$ 0.8, 4-H), 4.25 (1 H,dd, $J_{5,6'}$ 1.4, $J_{6,6'}$, 6′-H), 4.70 (1 H, d, $J_{1,2}$ 8.0, 1-H), 5.20 (1 H, dd, $J_{1,2}$, $J_{2,3}$ 11.0, 2-H), 5.66 (1 H, s, PhCH), 7.30-8.20 (10 H, m, Ar-H). A solution of the crude thiol in DMF (300 mm^3) and diethylamine (150 mm^3) was stirred overnight at room temperature, in a flask which was open to the air. The mixture was concentrated and redissolved in CH_2Cl_2 (10 cm^3) and subjected to work-up B. Flash chromatography (silica gel; toluene-EtOAc, 6:1) gave the *disulfide* **91** as a glassy solid (26 mg, 91%), $[\alpha]_D$ +72.0 (*c* 0.5 in $CHCl_3$); ν_{max}/cm^{-1} 2950 (CH_2, CH_3), 1730 (C=O), 1260, 835 (Si-C), 1080 (C-O), 710 (Ar-H); $\delta_H(CDCl_3)$ -0.01 (9 H, s, Si*Me*$_3$), 0.80 (2 H, m, OCH_2CH_2Si), 2.89 (1 H, br s, 5-H), 3.10 (1 H, dd, $J_{2,3}$11.5, $J_{3,4}$ 3.3, 3-H), 3.45 (1 H, m, OCH_2CH_2Si), 3.88 (2 H, m, OCH_2CH_2Si, 6-H), 4.17 (1 H, d, $J_{3,4}$, 4-H), 4.20 (1 H, m, 6′-H), 4.28 (1 H, d, $J_{1,2}$ 7.7, 1-H), 5.29 (1 H, dd, $J_{1,2}$, $J_{2,3}$, 2-H), 5.47 (1 H, s, PhCH), 7.30-8.20 (10 H, m, Ar-H); $\delta_C(CDCl_3)$ -1.7, 17.7, 58.3, 66.5, 68.3, 68.7, 70.1, 74.5, 101.0, 101.4, 126.2 (2), 128.0 (2), 128.5 (2), 128.8, 129.9 (2), 130.2, 133.3, 137.4, 165.0; FAB-MS: *m/z* 997 $(M+Na)^+$ ($C_{50}H_{62}O_{12}S_2Si_2$ requires *m/z* 974).

Attempted reaction of 2-(trimethylsilyl)ethyl 3-*S*-acetyl-2-*O*-benzoyl-4,6-*O*-benzylidene-3-thio-β-D-galactopyranoside, 88 and methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-chloro-2,3,5-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosonate, 31

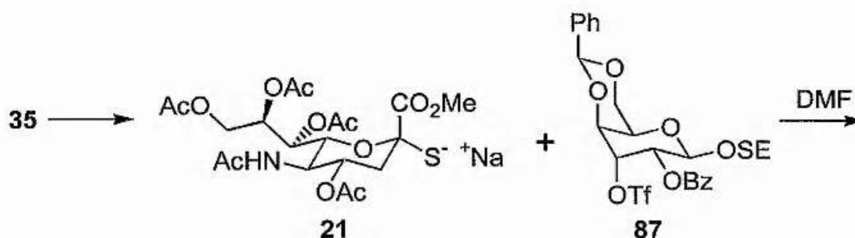


Compound **88** (30 mg, 58.3 μmol) was de-*S*-acetylated in a manner analogous to the preparation of **91**. However, instead of neutralising the reaction mixture as before, the solution was concentrated to dryness at 0 °C to give the *sodium thiolate* **92** as an amorphous solid. This was used in subsequent steps without further purification.

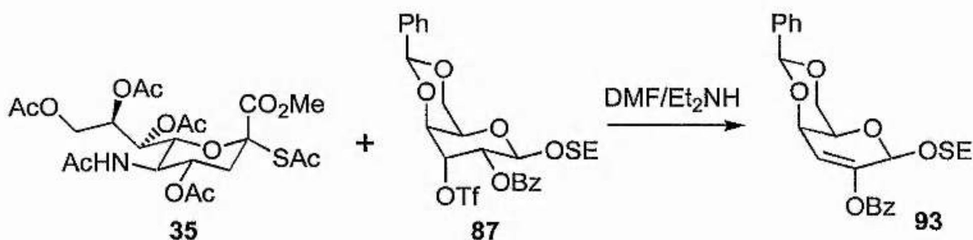
Method one. Compound **31** (30 mg, 58.8 μmol) was added to a solution of *sodium thiolate* **92** in DMF (0.5 cm^3). After stirring the mixture under nitrogen for 2 days at room temperature, no formation of the desired *thio-disaccharide* was observed. However, TLC (hexane-EtOAc, 1:1) indicated that some of the *thiolate* had oxidised to the *disulfide*.

Method two. A solution of 1,7,10-trioxa-4,13-diazacyclopentadecane (kryptofix 21) (4 mg, 12.6 μmol) in THF (50 mm^3) was added to a suspension of compound **31** (30 mg, 58.8 μmol) and the *thiolate* **92** (58.3 μmol) in freshly distilled oxygen-free THF (550 mm^3). The mixture was stirred for two days at room temperature under dry N_2. TLC (hexane-EtOAc, 1:1) indicated that some of the *thiolate* had oxidised to the *disulfide*, however, no formation of the desired *thio-disaccharide* was observed.

Attempted reaction of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate, 35 and 2-(trimethylsilyl)ethyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-trifluoromethanesulfonyl-β-D-gulopyranoside, 87



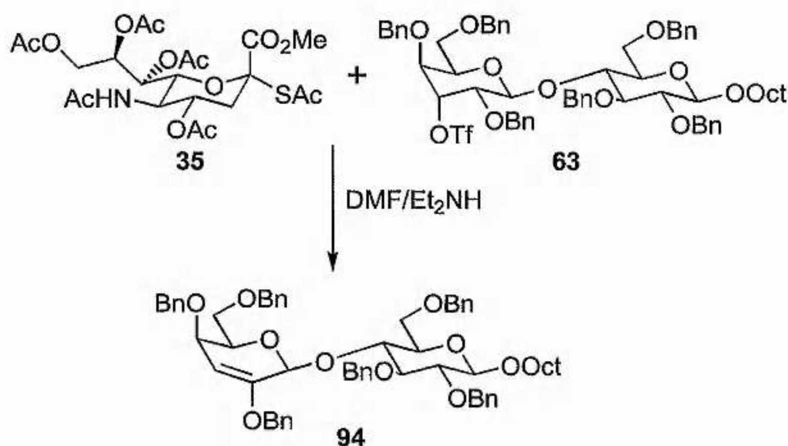
Method one. A solution of compound **35** (50 mg, 91 μmol) in dry MeOH (2 cm^3) was degassed by evacuating the flask and refilling it with dry N_2 three times. The flask was cooled to -40 °C and a solution of sodium methoxide in MeOH (20 mm^3, 87 μmol) was added and stirred for 1 h at this temperature. The solution was concentrated to dryness at 0 °C to give the *sodium thiolate* **21** as an amorphous solid. A solution of 2-(trimethylsilyl)ethyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-trifluoromethanesulfonyl-β-D-gulopyranoside, **87**, (58 mg, 100 μmol), prepared as described in the synthesis of **88**, and the *thiolate* in DMF (0.5 cm^3) was stirred for 5 days at room temperature under N_2. No formation of the desired *thio-disaccharide* was observed, however, TLC (EtOAc) indicated that the *thiolate* had degraded.



Method two. Diethylamine (0.1 cm^3) was added dropwise to a stirred solution of *2-(trimethylsilyl)ethyl 2-O-benzoyl-4,6-O-benzylidene-3-O-trifluoromethanesulfonyl-β-D-gulopyranoside*, **87** (50 mg, 83 μmol, prepared as described in the synthesis of **88**) and compound **35** (30 mg, 55 μmol), in DMF (0.2 cm^3) at 0 °C. The reaction mixture was allowed to warm to room temperature overnight. The mixture was concentrated to a syrup, re-dissolved in EtOAc (10 cm^3) and subjected to standard work-up **B**. No formation of the desired *thio-disaccharide* was observed. However, flash chromatography (silica gel; hexane-EtOAc, 4:1) gave *2-(trimethylsilyl)ethyl 2-O-benzoyl-4,6-O-benzylidene-3-deoxy-β-D-*threo-*hex-2-enopyranoside,* **93** as a glassy

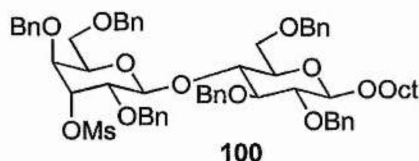
solid (24 mg, 67%), $[\alpha]_D$ -31.2 (*c* 0.25 in $CHCl_3$); ν_{max}/cm^{-1} 2925 (CH_2, CH_3), 1735 (C=O), 1250, 835 (Si-C), 1065 (C-O), 1110 (Si-O), 760, 700 (Ar-H); $\delta_H(CDCl_3)$ -0.04 (9 H, s, Si*Me*$_3$), 0.89 (2 H, m, OCH_2CH_2Si), 3.71 (1 H, br s, 5-H), 3.78 (1 H, m, OCH_2CH_2Si), 3.94 (1 H, m, OCH_2CH_2Si), 4.21 (1 H, dd, $J_{5,6}$ 2.5, $J_{6,6'}$ 12.9, 6-H), 4.42-4.49 (2 H, m, 4-H, 6′-H), 5.60 (1 H, s, PhCH), 5.64 (1 H, m, 1-H), 5.96 (1 H, d, $J_{3,4}$ 5.5, 3-H), 7.33-8.11 (10 H, m, Ar-H); $\delta_C(CDCl_3)$ -1.7, 18.0, 65.0, 66.25, 69.4, 69.7, 95.6, 101.0, 114.0, 126.4 (2), 128.2 (2), 128.5 (2), 128.8, 129.0, 130.2 (2), 133.75, 137.9, 151.2, 164.3.

Attempted reaction of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate, 35 and octyl 2,4,6-tri-*O*-benzyl-3-*O*-trifluoromethanesulfonyl-β-D-gulopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside, 63



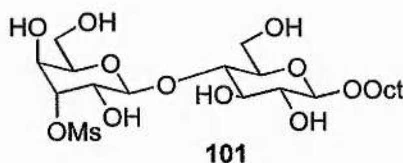
Diethylamine (50 mm^3) was added dropwise to a stirred solution of *triflate* **63** (46 mg, 41 μmol), and *thioacetate* **35** (15 mg, 27 μmol), in DMF (100 mm^3) at 0 °C. The reaction mixture was allowed to warm to room temperature overnight. The mixture was concentrated to a syrup, re-dissolved in EtOAc (10 cm^3) and subjected to standard work-up **B**. No formation of the desired *thio-trisaccharide* was observed. However, flash chromatography (silica gel; hexane-EtOAc, 6:1) gave firstly unreacted *triflate* (7 mg, 15%) and then *octyl 2,4,6-tri-O-benzyl-3-deoxy-β-D-*threo-*hex-2-enopyranosyl-(1→4)-236-tri-O-benzyl-β-D-glucopyranoside* **94** as a colourless syrup (21 mg, 62% based on *triflate* consumed); $\delta_H(CDCl_3)$ 4.39-5.14 (12× 1 H, 12 AB d, 6 × OC*H*$_2$Ph), 4.36 (1 H, d, $J_{1a,2a}$ 8.0, 1a-H), 5.09 (1 H, d, $J_{1b,2b}$ 8.0, 1b-H), 5.35 (1 H, m, 3b-H), 7.00-7.45 (30 H, m, Ar-H); FAB-MS: *m/z* 999 $(M+Na)^+$ ($C_{62}H_{72}O_{10}$ requires *m/z* 976).

Octyl 2,4,6-tri-*O*-benzyl-3-*O*-methanesulfonyl-β-D-gulopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside, 100



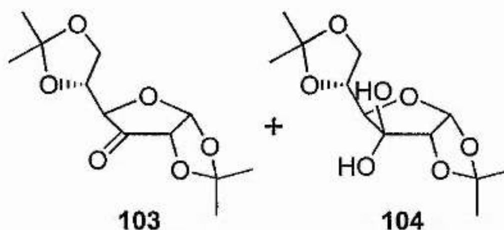
A mixture of methanesulfonyl chloride (95 mg, 830 μmol), **62** (80 mg, 83 μmol) and pyridine (1 cm^3) was stirred at room temperature over night. The mixture was concentrated, redissolved in CH_2Cl_2 (10 cm^3) and subjected to standard work-up B. Flash chromatography (silica gel; hexane-EtOAc, 5:1) gave the *mesylate* **100** as a colourless syrup, (74 mg, 82%), (Found: C, 70.56; H, 7.31%. $C_{63}H_{76}O_{13}S$ requires C, 70.50; H, 7.14%); $[\alpha]_D$ -8.7 (*c* 1.1 in $CHCl_3$); $\delta_H(CDCl_3)$ 0.90 (3 H, t, *J* 6.8, $C_7H_{14}CH_3$), 1.24-1.46 (10 H, m, $(CH_2)_5$ CH_3), 1.68 (2 H, m, OCH_2CH_2), 2.82 (3 H, s, SO_2Me), 4.24-4.99 (12 × 1 H, 12 AB d, 6 × OCH_2Ph), 4.41 (1 H, d, $J_{1a,2a}$ 8.0, 1a-H), 4.92 (1 H, d, $J_{1b,2b}$ 8.0, 1b-H), 5.00 (1 H, m, 3b-H) 7.16-7.40 (30 H, m, Ar-H); $\delta_C(CDCl_3)$ 13.9, 22.4, 26.0, 29.0, 29.2, 29.6, 31.6, 38.3, 67.4, 68.4, 69.9, 71.5, 73.0, 73.15, 73.25, 73.5, 74.5, 74.8, 74.9, 75.1, 77.0, 77.2, 81.7, 82.65, 99.6, 103.55, 127.1, 127.3, 127.4, 127.5, 127.6, 127.9, 127.95, 128.0, 128.1, 128.2, 128.4, 128.45, 137.4, 137.6, 138.1, 138.3, 138.6, 139.0.

Octyl 3-*O*-methanesulfonyl-β-D-gulopyranosyl-(1→4)-β-D-glucopyranoside, 101



A mixture of **100** (70 mg, 65.2 μmol) and palladium on charcoal (280 mg; 10% w/w) in acetic acid (7 cm^3) was stirred under an atmosphere of hydrogen for 6 h at room temperature. The mixture was filtered through Celite and the filtrate was concentrated onto silica (0.5 g). Flash chromatography (silica gel, 2.5g; CH_2Cl_2-MeOH, 4:1) gave the *title compound* **101** as an amorphous solid (31 mg, 89%), $\delta_H(CD_3OD)$ 0.90 (3 H, t, *J* 6.8, $C_7H_{14}CH_3$), 1.23-1.42 (10 H, m, $(CH_2)_5CH_3$), 1.62 (2 H, m, OCH_2CH_2), 3.18 (3 H, s, SO_2Me), 4.28 (1 H, d, $J_{1a,2a}$ 8.0, 1a-H), 4.68 (1 H, d, $J_{1b,2b}$ 8.2, 1b-H), 4.81 (1 H, m, 3b-H); $\delta_C(CD_3OD)$ 14.5, 23.8, 27.2, 30.5, 30.7, 30.9, 33.1, 38.5, 62.0, 62.2, 68.0, 69.45, 71.1, 74.9, 75.8, 76.5, 76.6, 81.1, 83.1, 102.8, 104.4.

1,2:5,6-Di-*O*-isopropylidene-α-D-*ribo*-hexofuranos-3-ulose (hydrate), 103 & 104[11]



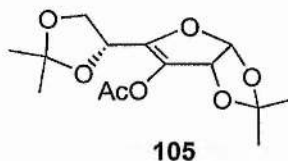
Ruthenium (IV) oxide hydrate (200 mg, ~1 mmol) was added to a cooled (0 °C), vigorously stirred mixture of *1,2:5,6-di-O-isopropylidene-α-D-glucofuranose* **102** (5.20 g, 20.0 mmol), potassium carbonate (0.66 g, 4.78 mmol) and potassium periodate (5.98 g, 26.0 mmol) in CH_2Cl_2 (35 cm^3) and H_2O (35 cm^3). The orange-brown mixture was stirred for 20 minutes at 0 °C and then allowed to warm to room temperature. After 5.5 h, the mixture had turned black, indicating that there was no more oxidant remaining. More potassium carbonate (0.36 g, 2.60 mmol) and potassium periodate (3.22 g, 14.0 mmol) were added and the mixture became orange-brown, as before. After a further 1.5 h, the mixture became a pale yellow colour, indicating that there was no substrate remaining. The reaction was quenched with *iso*-propyl alcohol (3 cm^3), turning it black. The mixture was filtered and the organic and aqueous phases separated. The aqueous extract was washed several times with CH_2Cl_2 and the combined organic extracts were dried and concentrated to a syrup which partially crystallised on standing as a mixture of the *ketone* **103** and its *hydrate* **104** (4.58g, ~80%). The mixture was used in subsequent reactions without further purification. The ratio of *hydrate* to *ketone* could be varied by dissolving the mixture in either aqueous ether or dry acetone to give predominantly the *hydrate* or the *ketone*, respectively.

Ketone **103**: δ_H($CDCl_3$, lit.,[12]) 1.33, (6 H, s, CMe_2), 1.43, 1.45 (2 × 3 H, 2 s, CMe_2), 4.03 (2 H, m, 4-H, 6-H), 4.34 (2 H, m, 5-H, 6′-H), 4.39 (1 H, d, $J_{1,2}$ 4.4, 2-H), 6.14 (1 H, d, $J_{1,2}$, 1-H); δ_C($CDCl_3$) 25.1, 25.8, 26.9, 27.3, 64.1, 76.2, 77.1, 78.8, 103.0, 110.3, 114.2, 209.0.

Hydrate **104**: $[\alpha]_D$ +36.4 (*c* 1.0 in H_2O) (lit.,[11] +40.2, *c* 1 in H_2O); δ_H($CDCl_3$, lit.,[12]) 1.35, 1.37, 1.48, 1.57 (4 × 3 H, 4 s, 2 × CMe_2), 3.77 (1 H, s, OH), 3.89 (1 H, dd, $J_{4,5}$ 6.6, 4-H) 4.08 (1 H, dd, $J_{5,6}$ 6.0, $J_{6,6'}$ 8.8, 6-H), 4.14 (1 H, dd, $J_{5,6'}$ 6.3, $J_{6,6'}$, 6′-H), 4.20 (1 H, s, OH), 4.26 (1 H, d, $J_{1,2}$ 3.8, 2-H), 4.45 (1 H, m, 5-H), 5.84 (1 H, d, $J_{1,2}$,

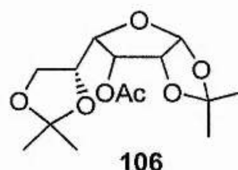
1-H); $\delta_C(CDCl_3)$ 24.9, 26.3, 26.4, 26.7, 66.6, 73.9, 78.5, 83.6, 101.0, 104.2, 110.0, 113.2.

3-*O*-Acetyl-1,2:5,6-di-*O*-isopropylidene-α-D-*erythro*-hex-3-enofuranose, 105[13]



Triethylamine (45 cm^3) was added dropwise to a cooled (0 °C), stirred solution of **103/104** (4.58 g) in acetic anhydride (45 cm^3). The mixture was warmed to room temperature and stirred for 5.5 h before concentrating to a syrup. The product mixture was redissolved in CH_2Cl_2 and subjected to standard work-up B. Decolourising with charcoal and then flash chromatography (silica gel; toluene-EtOAc, 9:1) gave the *enol acetate* **105** as a syrup which became a waxy white solid in the freezer over several days (3.64 g, 61% from *1,2:5,6-di-O-isopropylidene-α-D-glucofuranose*), $[\alpha]_D$ -29.1 (*c* 1.0 in $CHCl_3$) (lit.,[13] -33.0, *c* 1 in $CHCl_3$); $\delta_H(CDCl_3)$ 1.35, 1.42, 1.44, 1.50 (4 × 3 H, 4 s, 2 × CMe_2), 2.18 (3 H, s, AcO), 4.04 (2 H, m, 6-H, 6′-H), 4.65 (1 H, m, 5-H), 5.36 (1 H, d, $J_{1,2}$ 5.5, 2-H), 6.00 (1 H, d, $J_{1,2}$, 1-H); $\delta_C(CDCl_3)$ 20.3, 25.4, 25.5, 27.6, 27.65, 65.7, 68.4, 80.7, 103.9, 110.3, 113.3, 128.9, 145.2, 169.0.

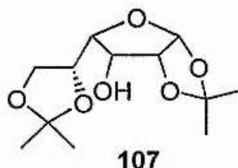
3-*O*-Acetyl-1,2:5,6-di-*O*-isopropylidene-α-D-gulofuranose, 106[14]



A mixture of **105** (3.45 g, 11.5 mmol) and palladium on charcoal (340 mg; 5% w/w) in EtOAc (35 cm^3) was stirred under an atmosphere of hydrogen for 12 h at room temperature. The mixture was filtered through Celite and the filtrate concentrated to give a crystalline solid. Recrystallisation (hexane), gave needles (2.77 g). Flash chromatography of the filtrate (silica gel; toluene-EtOAc, 5:1→4:1) gave a further portion (360 mg) of the *title compound* **106** (total yield: 3.13 g, 90%), mp 74 °C (lit.,[14] 73-74 °C); δ_H($CDCl_3$, lit.,[12]) 1.34, 1.38, 1.43, 1.57 (4 × 3 H, 4 s, 2 × CMe_2), 2.12 (3 H, s, AcO), 3.52 (1 H, dd, $J_{5,6'}$ 7.1, $J_{6,6'}$ 8.2, 6′-H), 4.09 (2 H, m, 4-H, 6-H),

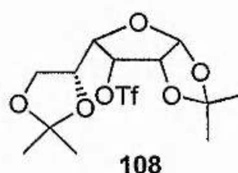
4.61 (1 H, m, 5-H), 4.81 (1 H, dd, $J_{1,2}$ 3.8, $J_{2,3}$ 5.5, 2-H), 5.06 (1 H, dd, $J_{2,3}$, $J_{3,4}$ 6.6, 3-H), 5.81 (1 H, d, $J_{1,2}$, 1-H).

1,2:5,6-Di-*O*-isopropylidene-α-D-gulofuranose, 107[13]



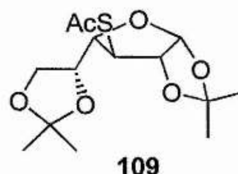
A solution of both compound **106** (2.33 g, 7.68 mmol) and sodium methoxide (40 mg, 0.74 mmol) in dry MeOH (25 cm^3) was stirred for 1 h at room temperature. The solution was neutralised with Amberlite IRC-50 (H^+) resin (0.25 g). Filtration and concentration gave compound **107** as a white powder (1.95 g, 98%); $[\alpha]_D$ +8.8 (*c* 1.0 in $CHCl_3$) (lit.,[13] +7.5, *c* 1 in $CHCl_3$); $\delta_H(CDCl_3)$ 1.33, 1.37, 1.40, 1.58 (4 × 3 H, 4 s, 2 × CMe_2), 2.72 (1 H, d, $J_{3,OH}$ 6.2, OH), 3.66 (1 H, dd, $J_{5,6'}$ 7.3, $J_{6,6'}$ 8.8, 6′-H), 3.86 (1 H, dd, $J_{3,4}$ 5.6, $J_{4,5}$ 8.8, 4-H) 4.18 (1 H, dd, $J_{5,6}$ 6.8, $J_{6,6'}$, 6-H), 4.21 (1 H, m, 3-H), 4.45 (1 H, m, 5-H), 4.62 (1 H, dd, $J_{1,2}$ 4.0, $J_{2,3}$ 6.2, 2-H), 5.74 (1 H, d, $J_{1,2}$, 1-H).

1,2:5,6-Di-*O*-isopropylidene-3-*O*-trifluoromethanesulfonyl-α-D-gulofuranose, 108



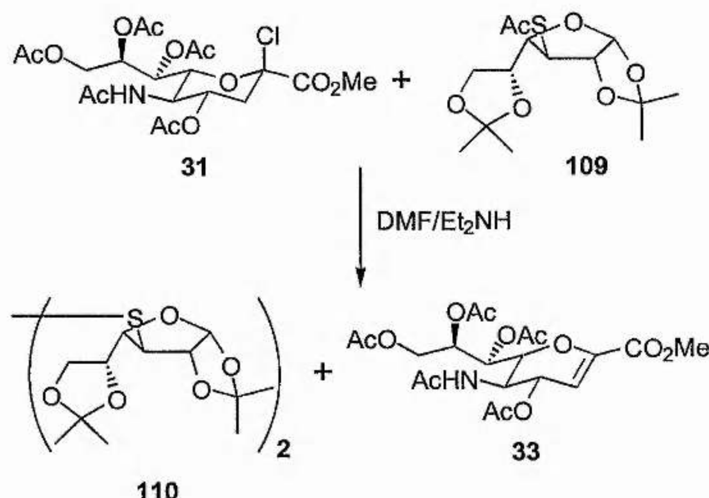
Compound **107** (2.00 g, 7.68 mmol) was treated in a manner analogous to the preparation of compound **85**. TLC (hexane-EtOAc, 3:2) showed the reaction to be complete after 1 h at 0 °C. The reaction was worked-up as described previously to give the *triflate* **108** as a crystalline solid which was used in subsequent reactions without further purification (2.95 g, 98%); $\delta_H(CDCl_3)$ 1.37, 1.43, 1.45, 1.61 (4 × 3 H, 4 s, 2 × CMe_2), 3.65 (1 H, dd, $J_{5,6'}$ 6.3, $J_{6,6'}$ 8.8, 6′-H), 4.05 (1 H, dd, $J_{3,4}$ 5.8, $J_{4,5}$ 8.8, 4-H) 4.15 (1 H, dd, $J_{5,6}$ 6.6, $J_{6,6'}$, 6-H), 4.56 (1 H, m, 5-H), 4.80 (1 H, dd, $J_{1,2}$ 4.1, $J_{2,3}$ 5.8, 2-H), 5.09 (1 H, t, $J_{2,3}$, $J_{3,4}$, 3-H), 5.83 (1 H, d, $J_{1,2}$, 1-H).

3-*S*-Acetyl-1,2:5,6-di-*O*-isopropylidene-3-thio-α-D-galactofuranose, 109



A mixture of **108** (760 mg, 1.94 mmol), potassium thioacetate (434 mg, 3.80 mmol), potassium carbonate (1.31 g, 9.5 mmol) and tetrabutylammonium hydrogensulfate (645 mg, 1.90 mmol) in a mixture of EtOAc (8 cm^3) and H_2O (8 cm^3) was vigorously stirred for 2 h at room temperature. The mixture was diluted with EtOAc (50 cm^3) and washed with water before drying and concentration to give a syrup. Flash chromatography (silica gel; hexane-EtOAc, 8:2) gave the *thioacetate* **109** as a glassy solid which was freeze-dried from dioxane to give a white powder (524 mg, 85%), $[\alpha]_D$ -3.4 (*c* 1 in $CHCl_3$); ν_{max}/cm^{-1} 2985, 2935 (CH_2, CH_3), 1700 (C=O), 1375 (Pr^i, C-H), 1065 (C-O); $\delta_H(CDCl_3)$ 1.33, 1.38, 1.43, 1.60 (4 × 3 H, 4 s, 2 × CMe_2), 2.36, (3 H, s, AcS), 3.69 (1 H, dd, $J_{2,3}$ 1.6, $J_{3,4}$ 4.7, 3-H), 3.84 (1 H, dd, $J_{5,6'}$ 6.6, $J_{6,6'}$ 8.5, 6′-H), 3.98 (1 H, dd, $J_{3,4}$, $J_{4,5}$ 7.1, 4-H) 4.07 (1 H, dd, $J_{5,6}$ 6.6, $J_{6,6'}$, 6-H), 4.41 (1 H, m, 5-H), 4.61 (1 H, dd, $J_{1,2}$ 3.8, $J_{2,3}$, 2-H), 5.91 (1 H, d, $J_{1,2}$, 1-H); FAB-MS: *m/z* 341 $(M+Na)^+$ ($C_{14}H_{22}O_6S$ requires *m/z* 318).

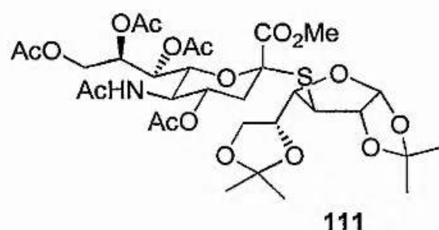
Attempted reaction of 3-*S*-acetyl-1,2:5,6-di-*O*-isopropylidene-3-thio-α-D-galactofuranose, 109 and methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-chloro-2,3,5-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosonate, 31



Diethylamine (0.3 cm^3) was added dropwise to a stirred solution of *glycosyl chloride* **31** (86 mg, 0.17 mmol) and *thioacetate* **109** (70 mg, 0.22 mmol), in DMF (0.6 cm^3) at 0 °C. The reaction mixture was allowed to warm to room temperature overnight. The mixture was concentrated to a syrup, re-dissolved in EtOAc (10 cm^3) and subjected to standard work-up **B**. No formation of the desired *thio-disaccharide* was observed. However, flash chromatography (silica gel; hexane-EtOAc, 7:3) gave *bis[3-deoxy-1,2:5,6-di-O-isopropylidene-α-D-galactofuranos-3-yl] disulfide*, **110** as a glassy solid (50 mg, 83%), (Found: C, 52.86; H, 7.14, $C_{24}H_{38}O_{10}S_2$ requires C, 52.35; H, 6.96%); $[\alpha]_D$ -96.4 (*c* 1.025 in $CHCl_3$); $\delta_H(CDCl_3)$ 1.34, 1.36, 1.44, 1.56 (4 × 6 H, 4 s, 4 × CMe_2), 3.34 (2 H, dd, $J_{2,3}$ 2.2, $J_{3,4}$ 5.8, 3-H), 3.87 (4 H, m, 4-H, 6′-H) 4.06 (2 H, dd, $J_{5,6}$ 6.9, $J_{6,6'}$ 8.5, 6-H), 4.40 (2 H, m, 5-H), 4.77 (2 H, dd, $J_{1,2}$ 3.8, $J_{2,3}$ 2.2, 2-H), 5.84 (2 H, d, $J_{1,2}$, 1-H); $\delta_C(CDCl_3)$ 25.1, 26.25, 26.6, 27.25, 54.1, 65.6, 75.4, 83.9, 86.7, 105.3, 110.1, 114.0; FAB-MS: *m/z* 573 $(M+Na)^+$.

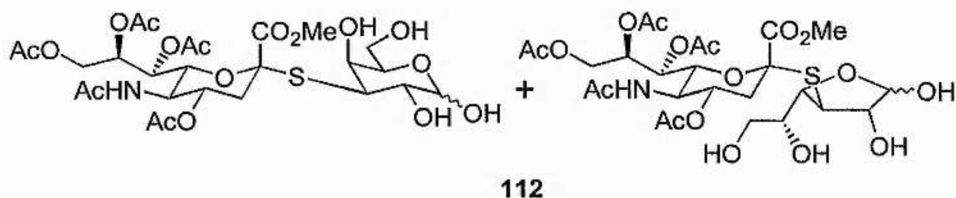
Further elution (EtOAc) gave *methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-2,6-anhydro-3,5-dideoxy-D-*glycero-*α-D-*galacto-*non-2-enopyranosonate* **33**; $\delta_H(CDCl_3)$ 1.93 (3 H, s, AcN), 2.05, 2.06, 2.07, 2.12 (4 × 3 H, 4 s, 4 × AcO), 3.80 (3 H, s, CO_2Me) 4.19 (1 H, dd, $J_{8,9}$ 7.1, $J_{9,9'}$ 12.3, 9-H), 4.38 (2 H, m, 5-H, 6-H), 4.58 (1 H, dd, $J_{8,9'}$ 3.2, $J_{9,9'}$, 9′-H), 5.21 (1 H, dd, $J_{7,8}$ 4.4, $J_{8,9}$, $J_{8,9'}$, 8-H), 5.50 (2 H, m, 4-H, 7-H), 5.81 (1 H, d, $J_{5,NH}$ 8.3, NH), 6.01 (1 H, d, $J_{3,4}$ 3.3, 3-H).

S-(Methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-1,2:5,6-di-O-isopropylidene-3-thio-α-D-galactofuranose, 111



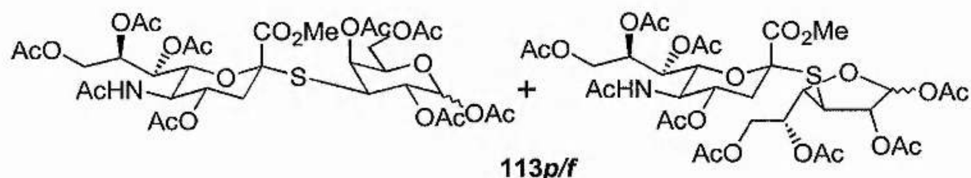
Diethylamine (12.5 cm^3) was added dropwise to a stirred solution of *triflate* **108** (2.27 g, 5.80 mmol) and *thioacetate* **35** (2.44 g, 4.44 mmol), in DMF (25 cm^3) at 0 °C. The reaction mixture was allowed to warm to room temperature overnight. The mixture was concentrated to a syrup, re-dissolved in EtOAc (250 cm^3) and subjected to standard work-up **B** before evaporating the residue onto silica (5 g). Flash chromatography (silica gel, 50 g; hexane-EtOAc, 7:3) gave firstly *bis[3-deoxy-1,2:5,6-di-O-isopropylidene-α-D-galactofuranos-3-yl] disulfide* **110** (63 mg) and then the unreacted *triflate* **108** (313 mg). Further elution (EtOAc) gave the desired *thio-disaccharide* **111** as an amorphous solid (2.26 g, 68%) contaminated with the *2,3-dehydro-sialic acid* **33** (~5% by ^{1}H NMR). The product was used in subsequent reactions without further purification, but an analytical sample was obtained by gel permeation chromatography (Sephadex LH-20; MeOH), (Found: C, 50.92; H, 6.25, N, 1.74. $C_{32}H_{47}NO_{17}S$ requires C, 51.26; H, 6.32, N, 1.87%); $[\alpha]_D$ -5.0 (*c* 1 in $CHCl_3$); ν_{max}/cm^{-1} 2990 (CH_2, CH_3), 1740 (C=O, ester), 1660, 1540 (C=O, amide), 1065 (C-O); $\delta_H(CDCl_3)$ 1.30, 1.34, 1.40, 1.56 (4 × 3 H, 4 s, 2 × CMe_2), 1.87 (3 H, s, AcN), 1.99, 2.00, 2.05, 2.10 (4 × 3 H, 4 s, 4 × AcO), 2.76 (1 H, dd, $J_{3bax,3beq}$ 12.5, $J_{3beq,4b}$ 4.7, $3b_{eq}$-H), 3.56 (1 H, m, 3a-H), 3.59 (1 H, dd, $J_{5a,6a'}$ 6.6, $J_{6a,6a'}$ 8.5, 6a′-H), 3.74-3.83 (5 H, m, 4a-H, 6b-H, CO_2Me), 3.94 (1 H, q, $J_{4b,5b} = J_{5b,6b} = J_{5b,NH}$ 10.2, 5b-H), 4.06-4.14 (2 H, m, 6a′-H, 9b-H), 4.23 (1 H, dd, $J_{8b,9b'}$ 2.5, $J_{9b,9b'}$ 12.6, 9b′-H), 4.43 (1 H, m, 5a-H), 4.76 (1 H, dd, $J_{1a,2a}$ 3.6, $J_{2a,3a}$ 0.8, 2a-H), 4.91 (1 H, m, 4b-H), 5.29 (1 H, dd, $J_{6b,7b}$ 1.6, $J_{7b,8b}$ 9.3, 7b-H), 5.34 (1 H, d, $J_{5b,NH}$, NH), 5.58 (1 H, m, 8b-H), 5.82 (1 H, d, $J_{1a,2a}$, 1a-H); FAB-MS: *m/z* 772 $(M+Na)^+$ ($C_{32}H_{47}NO_{17}S$ requires *m/z* 749).

S-(Methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-3-thio-D-galactose, 112



Compound **111** (400 mg, 533 μmol) was dissolved in aqueous trifluoroacetic acid (4 cm^3; 90% v/v) and stirred at room temperature for 15 mins. The solution was concentrated and co-evaporated several times with toluene. Flash chromatography (silica gel; EtOAc then MeOH) gave compound **112**, which was freeze dried from water as an α/β mixture of both furanose and pyranose isomers (251 mg, 70%); $[\alpha]_D$ +51.0 (*c* 1 eqm. in MeOH); ν_{max}/cm^{-1} 3375 (OH, NH br), 2925, 2853 (CH_2, CH_3), 1735 (C=O, ester), 1660, 1560 (C=O, amide), 1065 (C-O); δ_H(pyridine-d_5) 5.64 (d, $J_{1,2}$ 4.4, 1a-H α-pyr/fur), 5.69 (d, $J_{1,2}$ 7.4, 1a-H β-pyr), 5.88 (d, $J_{1,2}$ 4.4, 1a-H α-pyr/fur), 5.90 (s, 1a-H β-fur); FAB-MS: *m/z* 692 $(M+Na)^+$. (Found: $[M+Na]^+$ 692.1841. $C_{26}H_{39}O_{17}SNa$ requires *m/z* 692.1836).

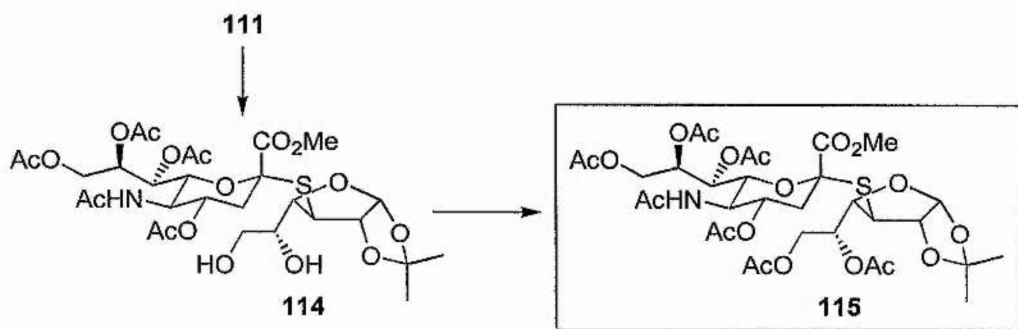
S-(Methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-1,2,4,6-tetra-O-acetyl-3-thio-α/β-D-galactopyranose, -(2→3)-1,2,5,6-tetra-O-acetyl-3-thio-α/β-D-galactofuranose, 113*p/f*



112 (100 mg, 149 μmol) was dissolved in a mixture of pyridine (1.0 cm^3) and acetic anhydride (1.0 cm^3) and stirred at room temperature overnight. The mixture was concentrated to an oil and flash chromatography (silica gel; CH_2Cl_2-acetone, 9:1→3:1) gave the *peracetate* **113*p/f*** (107 mg, 86%) as an α/β mixture in both pyranose and furanose ring forms (α-*p*:β-*p*:α-*f*:β-*f*, 20:60:10:10), (Found: C, 48.65; H, 5.74, N, 1.62. $C_{34}H_{47}NO_{21}S$ requires C, 48.74; H, 5.65, N, 1.67%); $[\alpha]_D$ +35.4 (*c* 1 in $CHCl_3$); ν_{max}/cm^{-1} 2965 (CH_2, CH_3), 1745 (C=O, ester), 1670, 1540 (C=O, amide), 1060 (C-O); $\delta_H(CDCl_3)$ 2.60-2.76 (1 H, m, $3b_{eq}$-H), 3.83 (3 H, s, CO_2Me),

5.64 (ddd, $J_{7b,8b}$ 9.6, $J_{8b,9b}$ 2.5, $J_{8b,9b'}$ 6.9, 8b-H β-pyr), 6.03 (d, $J_{1,2}$ 8.2, 1a-H β-pyr), 6.17 (s, 1a-H β-fur), 6.23 (d, $J_{1,2}$ 4.1, 1a-H α-pyr), 6.25 (d, $J_{1,2}$ 4.1, 1a-H α-fur); δ_C($CDCl_3$) 36.8 (3b-C β-pyr), 37.3 (3b-C α-pyr), 37.4 (3b-C β-fur), 38.0 (3b-C α-fur), 80.45 (2b-C α-pyr), 80.8 (2b-C β-pyr), 82.6 (2b-C α-fur), 83.25 (2b-C β-fur), 89.1 (1a-C α-pyr), 92.7 (1a-C β-pyr), 92.8 (1a-C α-fur), 99.4 (1a-C β-fur).

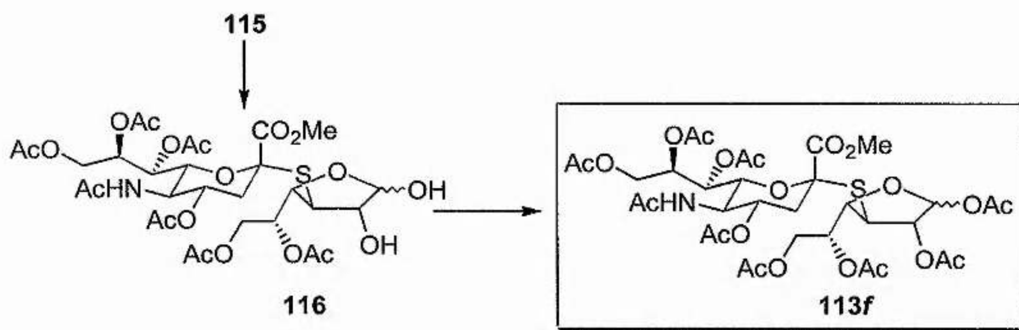
S-(Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-5,6-di-*O*-acetyl-1,2-*O*-isopropylidene-3-thio-α-D-galactofuranose, 115



Compound **111** (200 mg, 267 μmol) was dissolved in aqueous acetic acid (4 cm^3; 50% v/v) and stirred for 15 mins at 75 °C. After cooling, the solution was concentrated and co-evaporated several times with toluene. Flash chromatography (silica gel; CH_2Cl_2 then CH_2Cl_2-MeOH, 98:2→95:5) gave *S-(methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-1,2-O-isopropylidene-3-thio-α-D-galactofuranose* **114** as a solid foam (114 mg); δ_H($CDCl_3$) 1.30, 1.55 (2 × 3 H, 2 s, CMe_2), 1.86 (3 H, s, AcN), 1.99, 2.00, 2.08, 2.12 (4 × 3 H, 4 s, 4 × AcO), 2.75 (1 H, dd, $J_{3bax,3beq}$ 12.6, $J_{3beq,4b}$ 4.4, $3b_{eq}$-H), 2.90 (2 H, brs OH), 3.59 (1 H, dd, $J_{5a,6a'}$ 5.2, $J_{6a,6a'}$ 11.8, 6a′-H), 3.68 (1 H, dd, $J_{5a,6a}$ 3.0, $J_{6a,6a'}$, 6a-H), 3.79 (3 H, s, CO_2Me), 4.26 (1 H, dd, $J_{8b,9b'}$ 2.6, $J_{9b,9b'}$ 12.5, 9b′-H), 4.80 (1 H, d, $J_{1a,2a,}$ 3.85, 2a-H), 4.88 (1 H, m, 4b-H), 5.28 (1 H, dd, $J_{6b,7b}$ 1.6, $J_{7b,8b}$ 9.0, 7b-H), 5.55 (2 H, m, 8b-H, NH), 5.81 (1 H, d, $J_{1a,2a}$, 1a-H). The *diol* was dissolved in a mixture of pyridine (2cm^3) and acetic anhydride (1.5 cm^3) and stirred overnight at room temperature. The mixture was concentrated, redissolved in EtOAc and subjected to standard work-up B. Flash chromatography (silica gel; CH_2Cl_2-acetone, 9:1→2:1) gave the *title compound* **115** as a glassy solid (124 mg, 58% from **111**), (Found: C, 50.28; H, 6.13, N, 1.70. $C_{33}H_{47}NO_{19}S$ requires C, 49.93; H, 5.97, N, 1.76%);$[\alpha]_D$ +13.8 (*c* 1 in $CHCl_3$); δ_H($CDCl_3$) 1.33, 1.63 (2 × 3 H, 2 s, CMe_2), 1.88 (3 H, s, AcN), 1.99-2.16 (6 × 3 H, 6 s, 6 × AcO), 2.76 (1 H, dd, $J_{3bax,3beq}$ 12.6,

$J_{3beq,4b}$ 4.7, $3b_{eq}$-H), 3.60 (1 H, m, 3a-H), 3.74-3.80 (5 H, m, 4a-H, 6b-H, CO_2Me) 3.89 (1 H, m, 5b-H), 4.12-4.19 (2 H, m, 6a′-H, 9b-H), 4.25 (1 H, dd, $J_{8b,9b'}$ 2.5, $J_{9b,9b'}$ 12.6, 9b′-H), 4.32 (1 H, dd, $J_{5a,6a}$ 3.8, $J_{6a,6a'}$ 12.1, 6a-H), 4.76 (1 H, dd, $J_{1a,2a}$ 3.6, $J_{2a,3a}$ 1.1, 2a-H), 4.95 (1 H, m, 4b-H), 5.21-5.30 (2 H, m, 5a-H, 7b-H), 5.32 (1 H, d, $J_{5b,NH}$ 9.6, NH), 5.64 (1 H, m, 8b-H), 5.73 (1 H, d, $J_{1a,2a}$, 1a-H); $\delta_C(CDCl_3)$ 20.44, 20.47, 20.51, 20.56, 20.8, 23.0, 26.75, 27.0, 38.0, 46.0, 49.7, 61.2, 62.95, 53.2, 66.5, 68.3, 69.1, 69.3, 73.9, 80.6, 82.6, 89.8, 105.3, 114.4, 168.4, 169.3, 169.7, 170.2, 170.4, 170.5, 170.7.

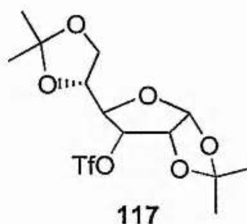
S-(Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-1,2,5,6-tetra-*O*-acetyl-3-thio-α/β-D-galactofuranose, 113*f*



Compound **115** (60 mg, 75 μmol) was dissolved in aqueous trifluoroacetic acid (1 cm^3; 90% v/v) and stirred for 15 mins at room temperature. The solution was concentrated and co-evaporated several times with toluene. Flash chromatography (silica gel; CH_2Cl_2 then CH_2Cl_2-acetone, 2:1) gave *S-(methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero-*α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-5,6-di-O-acetyl-3-thio-α/β-D-galactofuranose* **116** as a glassy solid (45 mg). The *diol* was dissolved in a mixture of pyridine (2cm^3) and acetic anhydride (1.5 cm^3) and stirred overnight at room temperature. The mixture was concentrated, redissolved in EtOAc and subjected to standard work-up B. Flash chromatography (silica gel; CH_2Cl_2-acetone, 9:1→3:1) gave the *title compound* **113*f*** as a glassy solid (50 mg, 78% from **115**; α:β, 1:2), (Found: C, 48.69; H, 5.49, N, 1.54. $C_{34}H_{47}NO_{21}S$ requires C, 48.74; H, 5.65, N, 1.67%); $[\alpha]_D$ -5.6 (*c* 1 in $CHCl_3$); $\delta_H(CDCl_3)$ 1.87 (3 H, s, AcN), 1.99-2.18 (24 H, overlapping signals, AcO), 2.68-2.76 (1 H, m, $3b_{eq}$-H), 3.82 (s, CO_2Me α), 3.83 (s, CO_2Me β), 6.17 (s, 1a-H β), 6.25 (d, $J_{1,2}$ 4.1, 1a-H α); $\delta_C(CDCl_3)$ 20.25, 20.5, 20.6, 20.65, 20.75, 20.8, 23.0, 30.7, 37.4, 38.0, 41.7, 44.1, 49.4, 53.3, 53.4, 61.4, 62.0, 62.6, 66.3, 66.75, 67.2, 67.4, 68.6, 69.1, 69.3, 73.7, 74.0,

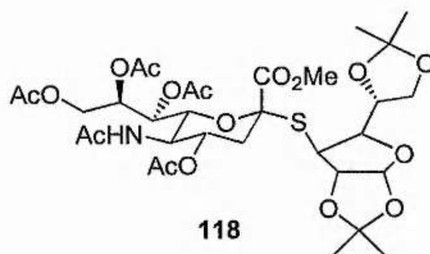
79.8, 82.2, 82.6, 83.25, 85.6, 92.8, 99.4, 168.6, 168.8, 169.2, 169.75, 170.1, 170.2, 170.3, 170.4, 170.6, 170.75.

1,2:5,6-Di-*O*-isopropylidene-3-*O*-trifluoromethanesulfonyl-α-D-allofuranose, 117[15]



1,2:5,6-Di-*O*-isopropylidene-α-D-allofuranose (100 mg, 0.38 mmol) was treated in a manner analogous to the preparation of compound **85**. TLC (hexane-EtOAc, 3:2) showed the reaction to be complete after 1 h at 0 °C. The reaction was worked-up as described previously to give the *triflate* **117** as a syrup which was used in subsequent reactions without further purification (147 mg, 97%); δ_H($CDCl_3$, lit.,[15]) 1.34, 1.38, 1.44, 1.58 (4 × 3 H, 4 s, 2 × CMe_2), 3.92 (1 H, dd, $J_{5,6}$ 4.4, $J_{5,6'}$ 8.5, 5-H), 4.08-4.26 (3 H, m, 4-H, 6-H, 6′-H), 4.77 (1 H, dd, $J_{1,2}$ 3.8, $J_{2,3}$ 5.2, 2-H), 4.91 (1 H, dd, $J_{2,3}$, $J_{3,4}$ 6.9, 3-H), 5.84 (1 H, d, $J_{1,2}$, 1-H); δ_C($CDCl_3$) 24.6, 26.0, 26.3, 26.7, 66.1, 75.0, 77.6, 77.7, 82.8, 104.1, 110.3, 114.4.

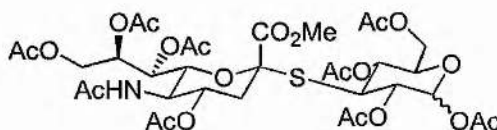
***S*-(Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-1,2:5,6-di-*O*-isopropylidene-3-thio-α-D-glucofuranose, 118**[15]



Diethylamine (0.225 cm^3) was added dropwise to a stirred solution of **117** (71 mg, 0.18 mmol) and **35** (65 mg, 0.12 mmol) in DMF (0.45 cm^3) at 0 °C. The mixture was allowed to warm to room temperature overnight and then concentrated to a syrup. The syrup was dissolved in EtOAc (10 cm^3) and subjected to standard work-up **B**. Flash chromatography (silica gel; Et_2O then EtOAc) gave the *thio-disaccharide* **118** as an amorphous solid (65 mg, 72%), but contaminated with the *2,3-dehydro-sialic*

acid **33** (~5% by ^{1}H NMR). The product was used in subsequent reactions without further purification, but an analytical sample was obtained by gel permeation chromatography (Sephadex LH-20; MeOH), $[\alpha]_D$ -12.8 (*c* 1.0 in $CHCl_3$) (lit.,[15] -10.6, *c* 0.66 in $CHCl_3$); $\delta_H(CDCl_3)$ 1.27, 1.29, 1.38, 1.48 (4 × 3 H, 4 s, 2 × CMe_2), 1.85 (3 H, s, AcN), 2.00, 2.01, 2.12, 2.13 (4 × 3 H, 4 s, 4 × AcO), 2.79 (1 H, dd, $J_{3bax,3beq}$ 12.6, $J_{3beq,4b}$ 4.7, $3b_{eq}$-H), 3.57 (1 H, d, $J_{3a,4a}$ 3.6, 3a-H), 3.80 (3 H, s, CO_2Me), 3.83 (1 H, m, 6b-H), 3.92 (1 H, dd, $J_{5a,6a}$ 4.1 $J_{5a,6a'}$ 8.5, 5a-H), 3.94-4.05 (2 H, m, 6a-H, 5b-H), 4.13-4.24 (3 H, m, 4a-H, 6a′-H, 9b-H), 4.32 (1 H, dd, $J_{8b,9b'}$ 2.5, $J_{9b,9b'}$ 12.6, 9b′-H), 4.87 (1 H, d, $J_{1a,2a}$ 3.3, 2a-H), 4.90 (1 H, m, 4b-H), 5.25-5.32 (2 H, m, NH, 7b-H), 5.43 (1 H, m, 8b-H), 5.80 (1 H, d, $J_{1a,2a}$ 3.3, 1a-H); $\delta_C(CDCl_3)$ 20.5, 20.6 (2), 20.8, 23.0, 25.2, 25.8, 26.3, 26.9, 38.1, 49.4, 49.7, 53.0, 61.5, 67.3, 67.4, 69.2, 69.5, 74.2, 74.5, 79.0, 82.7, 87.5, 104.7, 109.2, 112.1, 168.1, 169.9, 170.2, 170.6. 170.9.

***S*-(Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-1,2,4,6-tetra-*O*-acetyl-3-thio-α/β-D-glucopyranose, 119**

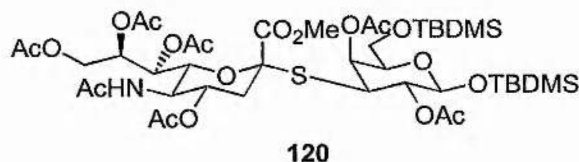


119

Compound **118** (116 mg, 155 μmol) was dissolved in aqueous trifluoroacetic acid (1.0 cm^3; 90% v/v) and stirred at room temperature for 15 mins. The solution was concentrated and co-evaporated several times with toluene. Flash chromatography (silica gel; EtOAc then EtOAc-MeOH, 2:3) gave a glassy solid (84 mg) which was dissolved in a mixture of pyridine (1.0 cm^3) and acetic anhydride (1.0 cm^3) and stirred at room temperature overnight. The mixture was concentrated to an oil, redissolved in EtOAc (10 cm^3) and subjected to standard work-up B. Flash chromatography (silica gel; CH_2Cl_2-acetone, 9:1→3:1) gave the *peracetylated α/β pyranose* **119** (104 mg, 80%; α-*p*:β-*p*, 42:58) as an amorphous solid, (Found: C, 48.73; H, 5.96, N, 1.61. $C_{34}H_{47}NO_{21}S$ requires C, 48.74; H, 5.65, N, 1.67%); $[\alpha]_D$ +23.2 (*c* 1 in $CHCl_3$); $\delta_H(CDCl_3)$ $\delta_H(CDCl_3)$ 1.83-2.25 (28 H, m, AcN, AcO, $3b_{ax}$-H), 2.69, 2.75 (1 H, 2 dd, $3b_{eq}$-H α, β), 3.82 (3 H, s, CO_2Me), 5.98 (d, $J_{1,2}$ 8.2, 1a-H β), 6.22 (d, $J_{1,2}$ 3.8, 1a-H α); $\delta_C(CDCl_3)$ 20.4, 20.5, 20.55, 20.6, 21.0, 21.2, 22.8, 22.9, 37.6, 38.3, 43.9, 45.9, 48.8, 49.3, 52.8, 61.3, 61.7, 61.8, 62.4, 66.9, 67.4, 67.5,

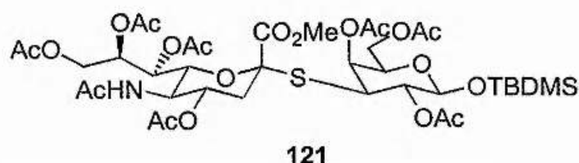
68.0, 68.7, 69.0, 69.2, 69.3, 71.0, 74.15, 74.3, 74.7, 83.1, 83.3, 88.7, 92.5, 168.5, 169.0, 169.2, 169.4, 169.5, 169.6, 169.8, 169.85, 170.4, 170.5, 170.8.

tert-Butyldimethylsilyl _S_-(methyl 5-acetamido-4,7,8,9-tetra-_O_-acetyl-3,5-dideoxy-D-_glycero_-α-D-_galacto_-2-nonulopyranosylonate)-(2→3)-2,4-di-_O_-acetyl-6-_O_-(_tert_-butyldimethylsilyl)-3-thio-β-D-galactopyranoside, 120



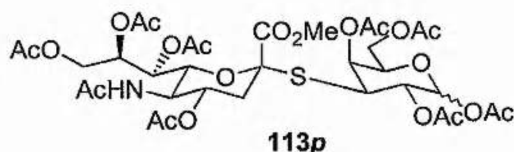
t-Butyldimethylsilylchloride (281 mg, 1.87 mmol) was added to a solution of compound **112** (250 mg, .0373 mmol) in pyridine (2.5 cm^3) at 0 °C. After stirring for 2 h at 0 °C more *t*-butyldimethylsilylchloride (281 mg, 1.87 mmol) was added and the mixture was allowed to warm slowly to room temperature overnight. The reaction mixture was quenched with MeOH (1 cm^3) and concentrated. The residue was redissolved in pyridine (2 cm^3) and acetic anhydride (1.5 cm^3) and stirred at room temperature overnight. The mixture was concentrated again, and the residue was taken up in CH_2Cl_2 (20 cm^3), subjected to standard work-up **B** and concentrated to give a syrup. Flash chromatography (silica gel; CH_2Cl_2 then CH_2Cl_2-MeOH, 100:3) gave the *bis-silylated compound* **120** (331 mg, 90%) predominantly in the β-pyranose form, but contaminated with a small quantity (~5%) of a furanose derivative. (Found: C, 51.11; H, 7.46, N, 1.36. $C_{42}H_{71}NO_{19}SSi_2$ requires C, 51.36; H, 7.28, N, 1.43%); $[\alpha]_D$ +9.9 (*c* 0.75 in $CHCl_3$); ν_{max}/cm^{-1} 2960, 2860 (CH_2, CH_3), 1750 (C=O, ester), 1660, 1540 (C=O, amide), 1370 (Bu^t C-H), 1225, 840 (Si-C), 1055 (C-O); δ_H($CDCl_3$) -0.01, 0.03 (2 × 3 H, 2 s, $SiMe_2$), 0.11 (6 H, s, $SiMe_2$), 0.85, 0.87 (2 × 9 H, 2 s, 2 × $SiCMe_3$), 1.85 (4 H, m, AcN, $3b_{ax}$-H), 2.00, 2.03, 2.04, 2.05 (6 H), 2.14, 2.18 (18 H, 5 s, 6 × AcO), 2.64 (1 H, dd, $J_{3bax,3beq}$ 12.6, $J_{3beq,4b}$ 4.4, $3b_{eq}$-H), 3.46 (1 H, dd, $J_{5a,6a'}$ 7.7, $J_{6a,6a'}$, 6a′-H), 3.56 (1 H, dd, $J_{5a,6a}$ 5.8, $J_{6a,6a'}$ 9.9, 6a-H), 3.58 (1 H, dd, $J_{2a,3a}$ 11.8, $J_{3a,4a}$ 3.3, 3a-H), 3.69 (1 H, dd, $J_{5b,6b}$ 11.0, $J_{6b,7b}$ 2.2, 6b-H), 3.78-3.86 (4 H, m, 5a-H, CO_2Me) 4.05 (1 H, m, 5b-H), 4.08 (1 H, dd, $J_{8b,9b}$ 4.1, $J_{9b,9b'}$ 12.6, 9b-H), 4.18 (1 H, dd, $J_{8b,9b'}$ 3.0, $J_{9b,9b'}$, 9b′-H), 4.77 (1 H, dd, $J_{1a,2a}$ 7.4, $J_{2a,3a}$, 2a-H), 4.82 (1 H, m, 4b-H), 5.01 (1 H, dd, $J_{3a,4a}$, $J_{4a,5a}$ 0.8, 4a-H), 5.08 (1 H, d, $J_{1a,2a}$, 1a-H), 5.16 (1 H, d, $J_{5b,NH}$ 10.2, NH), 5.35 (1 H, dd, $J_{6b,7b}$, $J_{7b,8b}$ 10.2, 7b-H), 5.56 (1 H, ddd, $J_{7b,8b}$, $J_{8b,9b}$, $J_{8b,9b'}$, 8b-H).

tert-Butyldimethylsilyl _S_-(methyl 5-acetamido-4,7,8,9-tetra-_O_-acetyl-3,5-dideoxy-D-_glycero_-α-D-_galacto_-2-nonulopyranosylonate)-(2→3)-2,4,6-tri-_O_-acetyl-3-thio-β-D-galactopyranoside, 121



Trifluoroacetic acid (0.5 cm^3) was added, with stirring, to a solution of **120** (50 mg, 50.9 μmol) in CH_2Cl_2 (0.5 cm^3) at room temperature. After 30 mins, the mixture was diluted with EtOAc (10 cm^3) and subjected to standard work-up A. The resulting crude product was treated with a mixture of pyridine (1.0 cm^3) and acetic anhydride (1.0 cm^3) overnight at room temperature and, following concentration, flash chromatography (silica gel; CH_2Cl_2 then EtOAc) gave a mono-silylated derivative as a glassy solid (30 mg, 65%). The following ^{1}H NMR spectral data are consistent with the structure of the tert-_butyldimethylsilyl β-D-galactopyranoside_, **121**: δ_H($CDCl_3$) 0.11 (6 H, s, $SiMe_2$), 0.87 (9 H, s, $SiCMe_3$), 1.86 (4 H, m, AcN, $3b_{ax}$-H), 2.00-2.20 (21 H, 6 s, 7 × AcO), 2.63 (1 H, dd, $J_{3bax,3beq}$ 12.6, $J_{3beq,4b}$ 4.7, $3b_{eq}$-H), 3.61 (1 H, dd, $J_{2a,3a}$ 11.8, $J_{3a,4a}$ 3.3, 3a-H), 3.70 (1 H, dd, $J_{5b,6b}$ 11.0, $J_{6b,7b}$ 2.2, 6b-H), 3.82 (4 H, m, 5a-H, CO_2Me) 3.97-4.11 (4 H, m, 6a-H, 6a′-H, 5b-H, 9b-H), 4.19 (1 H, dd, $J_{8b,9b'}$ 3.0, $J_{9b,9b'}$ 12.4, 9b′-H), 4.80 (2 H, m, 2a-H, 4b-H), 4.86 (1 H, d, $J_{3a,4a}$ 3.3, 4a-H), 5.10 (1 H, d, $J_{1a,2a}$ 7.4, 1a-H), 5.18 (1 H, d, $J_{5b,NH}$ 10.2, NH), 5.34 (1 H, dd, $J_{6b,7b}$ 2.2, $J_{7b,8b}$ 10.2, 7b-H), 5.56 (1 H, ddd, $J_{7b,8b}$, $J_{8b,9b}$ 3.9, $J_{8b,9b'}$, 8b-H).

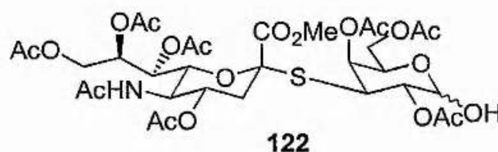
_S_-(Methyl 5-acetamido-4,7,8,9-tetra-_O_-acetyl-3,5-dideoxy-D-_glycero_-α-D-_galacto_-2-nonulopyranosylonate)-(2→3)-1,2,4,6-tetra-_O_-acetyl-3-thio-α/β-D-galactopyranose, 113_p_[16]



A stirred solution of **120** (100 mg, 101 μmol) in a mixture of acetic anhydride (0.5 cm^3) and acetic acid (0.25 cm^3) was cooled to 0 °C. Dilute sulfuric acid (0.25 cm^3; 10% v/v in acetic acid) was added and the mixture stirred for 10 mins at 0 °C and for 1 h at room temperature. The mixture was diluted with CH_2Cl_2(20 cm^3) and subjected to standard work-up A. Flash chromatography (silica gel; CH_2Cl_2 then

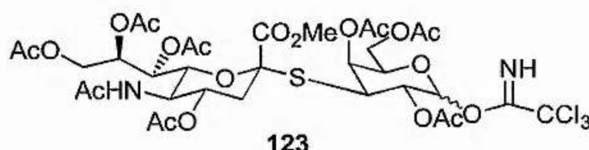
CH_2Cl_2-MeOH, 99:1→95:5) gave the *peracetate* **113*p*** as a glassy solid (65 mg, 76%; β:α, 83:14), but contaminated by a small quantity (~3%) of the β furanose derivative. ^{1}H NMR for β pyranose; δ_H($CDCl_3$, lit.,[16]) 1.85 (3 H, s, AcN), 1.86 (1 H, m, $3b_{ax}$-H), 1.99-2.20 (8 × 3 H, 8 s, 8 × AcO), 2.63 (1 H, dd, $J_{3bax,3beq}$ 12.6, $J_{3beq,4b}$ 4.4, $3b_{eq}$-H), 3.69 (1 H, dd, $J_{5b,6b}$ 10.7, $J_{6b,7b}$ 2.2, 6b-H), 3.79 (1 H, dd, $J_{2a,3a}$ 11.5, $J_{3a,4a}$ 3.6, 3a-H), 3.83 (3 H, s, CO_2Me) 3.89 (1 H, dd, $J_{8b,9b}$ 6.6, $J_{9b,9b'}$ 12.0, 9b-H), 3.95 (1 H, dd, $J_{5a,6a}$ 6.9, $J_{6a,6a'}$, 11.5, 6a-H), 4.05 (1 H, dd, $J_{5a,6a'}$ 3.3, $J_{6a,6a'}$, 6a′-H), 4.07 (1 H, m, 5b-H), 4.30 (1 H, m, 5b-H), 4.33 (1 H, dd, $J_{8b,9b'}$ 2.2, $J_{9b,9b'}$, 9b′-H), 4.80 (1 H, m, 4b-H), 4.91 (1 H, m, 4a-H), 4.95 (1 H, dd, $J_{1a,2a}$ 8.0, $J_{2a,3a}$, 2a-H), 5.18 (1 H, d, $J_{5b,NH}$ 10.4, NH), 5.24 (1 H, dd, $J_{6b,7b}$ 2.2, $J_{7b,8b}$ 10.2, 7b-H), 5.63 (1 H, m, 8b-H), 6.02 (1 H, d, $J_{1a,2a}$, 1a-H).

***S*-(Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-2,4,6-tri-*O*-acetyl-3-thio-β-D-galactopyranose, 122**[17]



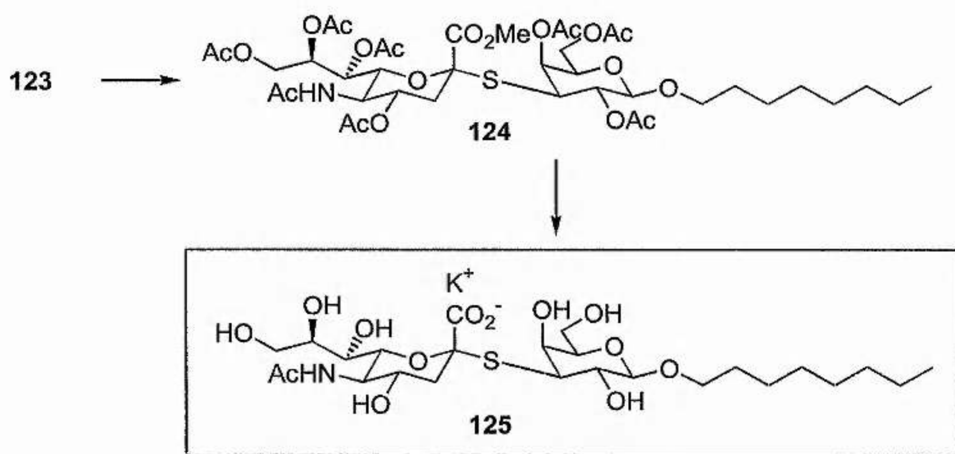
A solution of both **113*p*** (210 mg, 0.25 mmol) and hydrazinium acetate (25 mg, 0.275 mmol) in DMF (3.5 cm^3) was heated at 50 °C for 7 h. The mixture was cooled to room temperature and concentrated to a syrup which was redissolved in CH_2Cl_2 and subjected to standard work-up B. Flash chromatography (silica gel; toluene-MeOH, 9:1) gave the *title compound* **122** as a colourless syrup (140 mg, 70%) contaminated with a small amount of a furanose isomer which proved difficult to remove despite repeated chromatography. $[\alpha]_D$ +30.0 (*c* 0.7 in $CHCl_3$) (lit.,[17] +33.0); δ_H($CDCl_3$) 1.86 (3 H, s, AcN), 1.88 (1 H, dd, $J_{3ax,3eq}$ 12.6 $J_{3ax,4}$ 11.8, $3b_{ax}$-H), 2.01, 2.03, 2.04, 2.06, 2.07, 2.19, 2.22 (7 × 3 H, 7 s, 7 × AcO), 2.64 (1 H, dd, $J_{3bax,3beq}$ 12.6, $J_{3beq,4b}$ 4.7, $3b_{eq}$-H), 3.66-3.74 (2 H, m, 3a-H, 6b-H), 3.83 (3 H, s, CO_2Me) 3.93-4.19 (5 H, m, 5a-H, 6a-H, 6a′-H, 5b-H, 9b-H), 4.29 (1 H, dd, $J_{8b,9b'}$ 2.7, $J_{9b,9b'}$ 12.4, 9b′-H), 4.70 (1 H, dd, $J_{1a,2a}$ 7.7, $J_{2a,3a}$ 11.8, 2a-H), 4.83 (1 H, m, 4b-H), 4.91 (1 H, dd, $J_{3a,4a}$ 3.6, $J_{4a,5a}$ 1.1, 4a-H), 5.04 (1 H, d, $J_{1a,2a}$, 1a-H), 5.22 (1 H, d, $J_{5b,NH}$ 10.2, NH), 5.31 (1 H, dd, $J_{6b,7b}$ 2.2, $J_{7b,8b}$ 10.2, 7b-H), 5.59 (1 H, m, 8b-H).

S-(Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-*O*-2,4,6-tri-*O*-acetyl-3-thio-α/β-D-galactopyranosyl trichloroacetimidate, 123[17]



1,8-Diazabicyclo[5.4.0]undec-7-ene (20 mm^3) was added to a stirred solution of **122** (63 mg, 67 μmol) in CH_2Cl_2 (2.5 cm^3) and trichloroacetonitrile (0.5 cm^3). After 2 h at room temperature the dark brown solution was concentrated to a syrup and passed down a short column (silica gel; CH_2Cl_2 then acetone) to remove most of the colour. Careful column chromatography (silica gel; CH_2Cl_2-MeOH, 100:3→95:5) gave the desired *imidate* **123** as a syrup (67 mg, 90%; β:α, 92:6) contaminated with ~2% of a furanose isomer (as judged by ^{1}H NMR). $[\alpha]_D$ +26.4 (*c* 1.1 in $CHCl_3$) (lit.,[17] [β anomer] +27.5); $\delta_H(CDCl_3)$ 1.85 (3 H, s, AcN), 1.88 (1 H, m, $3b_{ax}$-H), 1.99, 2.00, 2.02, 2.05, 2.09, 2.14, 2.21 (7 × 3 H, 7 s, 7 × AcO), 2.64 (1 H, dd, $J_{3bax,3beq}$ 12.6, $J_{3beq,4b}$ 4.4, $3b_{eq}$-H), 3.70 (1 H, dd, $J_{5b,6b}$ 10.7, $J_{6b,7b}$ 2.2, 6b-H), 3.81 (1 H, dd, $J_{2a,3a}$ 11.5, $J_{3a,4a}$ 3.6, 3a-H), 3.84 (3 H, s, CO_2Me) 3.93 (1 H, dd, $J_{8b,9b}$ 6.0, $J_{9b,9b'}$ 12.4, 9b-H), 4.01 (1 H, dd, $J_{5a,6a}$ 6.9, $J_{6a,6a'}$ 11.3, 6a-H), 4.02-4.12 (2 H, m, 6a′-H, 5b-H), 4.30 (1 H, dd, $J_{8b,9b'}$ 2.2, $J_{9b,9b'}$, 9b′-H), 4.33 (1 H, m, 5a-H), 4.82 (1 H, m, 4b-H), 4.95 (1 H, d, $J_{3a,4a}$, 4a-H), 5.10 (1 H, dd, $J_{1a,2a}$ 8.0, $J_{2a,3a}$, 2a-H), 5.16 (1 H, d, $J_{5b,NH}$ 10.2, NH), 5.28 (1 H, dd, $J_{6b,7b}$ 2.2, $J_{7b,8b}$ 10.2, 7b-H), 5.62 (1 H, ddd, $J_{7b,8b}$, $J_{8b,9b}$ 2.2, $J_{8b,9b'}$, 8b-H), 6.15 (1 H, d, $J_{1a,2a}$, 1a-H), 8.43 (1 H, s, C=NH).

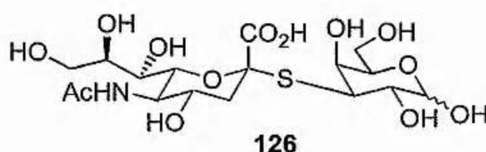
Octyl S-(potassium 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-3-thio-β-D-galactopyranoside, 125



A suspension of **123** (50 mg 53 μmol) and 4Å molecular sieves (200 mg) in dry CH_2Cl_2 (2 cm^3) was stirred for 3 h at room temperature. The mixture was cooled to 0 °C and octanol (21 mm^3, 130 μmol) was added, followed by boron trifluoride etherate (7.5 mm^3, 58.5 μmol) and the mixture was stirred at this temperature for 3 h. The mixture was then diluted with CH_2Cl_2 (8 cm^3), filtered through Celite and subjected to standard work-up B. Repeated column chromatography (silica gel; CH_2Cl_2-MeOH, 97:3) failed to purify the *disaccharide* fully and so the crude product was used in subsequent deprotection steps. Crude *octyl S-(methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosylonate)-(2→3)-2,4,6-tri-O-acetyl-3-thio-β-D-galactopyranoside* **124** (37 mg), $\delta_H(CDCl_3)$ 0.86 (3 H, t, J 6.9, $C_7H_{14}CH_3$), 1.26-1.44 (10 H, m, $(CH_2)_5CH_3$), 1.56 (2 H, m, OCH_2CH_2), 1.86 (4 H, m, AcN, $3b_{ax}$-H), 2.00-2.20 (21 H, 6 s, 7 × AcO), 2.63 (1 H, dd, $J_{3bax,3beq}$ 12.6, $J_{3beq,4b}$ 4.7, $3b_{eq}$-H), 3.51 (1 H, m, OCH_2), 3.63 (1 H, dd, $J_{2a,3a}$ 10.7, $J_{3a,4a}$ 3.3, 3a-H), 3.69 (1 H, dd, $J_{5b,6b}$ 10.7, $J_{6b,7b}$ 2.2, 6b-H), 3.81-3.89 (4 H, m, CO_2Me, OCH_2), 3.96-4.12 (5 H, m, 5a-H, 6a-H, 6a′-H, 5b-H, 9b-H), 4.29 (1 H, dd, $J_{8b,9b'}$ 2.7, $J_{9b,9b'}$ 12.4, 9b′-H), 4.75-4.86 (3 H, m, 1a-H, 2a-H, 4b-H), 4.88 (1 H, d, $J_{3a,4a}$ 3.0, 4a-H), 5.16 (1 H, d, $J_{5b,NH}$ 10.2, NH), 5.31 (1 H, dd, $J_{6b,7b}$ 2.2, $J_{7b,8b}$ 10.2, 7b-H), 5.59 (1 H, ddd, $J_{7b,8b}$ 10.2, $J_{8b,9b}$ 2.7, $J_{8b,9b'}$ 5.2, 8b-H); $\delta_C(CDCl_3)$ 13.8, 20.4-20.5 (6), 21.2, 22.4, 22.9, 25.6, 29.0, 29.1, 29.3, 31.6, 36.8, 45.4, 49.0, 53.0, 62.2, 62.25, 66.9, 67.2, 68.4, 69.1, 69.35, 70.0, 72.0, 73.5, 80.8, 101.6, 169.0, 169.7, 170.0, 170.2, 170.3, 170.4 (3), 170.9.

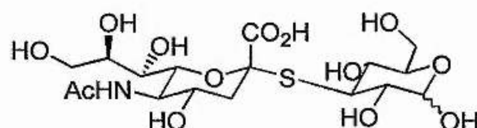
A solution of both the crude *octyl glycoside* **124** (37 mg) and sodium methoxide (2 mg) in dry MeOH (3 cm^3) was stirred for 2 h at room temperature. The solution was neutralised with acetic acid and concentrated to a solid. The mixture was redissolved in potassium hydroxide solution (3 cm^3; 1 M). After standing for 3 h at room temperature, the solution was desalted on a reverse phase column (C-18 silica gel; $H_2O \rightarrow MeOH$) to give the fully deprotected compound **125** as a glassy solid (17 mg, 50% from **123**), $[\alpha]_D$ +42.9 (*c* 0.9 in MeOH); $\delta_H(CD_3OD$; 500 MHz) 0.90 (3 H, t, *J* 6.8, $C_7H_{14}CH_3$), 1.26-1.40 (10 H, m, $(CH_2)_5CH_3$), 1.62 (2 H, m, OCH_2CH_2), 1.71 (1 H, m, $3b_{ax}$-H), 2.00 (3 H, s, AcN), 2.91 (1 H, dd, $J_{3bax,3beq}$ 12.6 $J_{3beq,4b}$ 4.2, $3b_{eq}$-H), 4.28 (1 H, d, $J_{1a,2a}$ 7.4, 1a-H); $\delta_C(CD_3OD)$ 14.5, 22.7, 23.8, 27.3, 30.5, 30.7, 30.9, 33.1, 43.15, 52.9, 54.0, 63.2, 64.7, 69.4, 70.2, 70.3, 70.4, 70.7, 73.2, 77.1, 79.2, 85.9, 106.4, 175.5, 175.75; ES-MS (-ve): *m/z* 598 $(M-K)^-$ ($C_{25}H_{44}NO_{13}KS$ requires *m/z* 637).

S-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-3-thio-D-galactose, 126



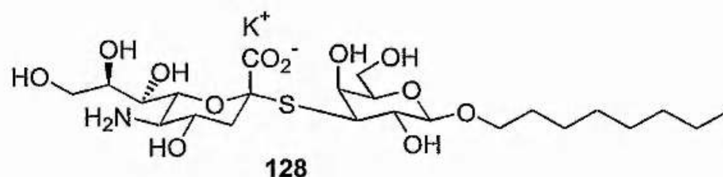
A solution of both **111** (38 mg) and sodium methoxide (2 mg) in dry MeOH (400 mm^3) was stirred for 2 h at room temperature. The solution was neutralised with acetic acid and concentrated onto silica. Flash chromatography (silica gel; CH_2Cl_2-MeOH, 9:1) gave a white solid which was redissolved in sodium hydroxide solution (1 cm^3; 0.1 M). After standing for 24 h at room temperature, the solution was neutralised with acetic acid and concentrated. The crude product was dissolved in aqueous trifluoroacetic acid (1 cm^3; 90% v/v) and stirred at room temperature for 30 mins. The solution was concentrated and co-evaporated several times with toluene. Purification of the residue by gel permeation chromatography (Biogel P2; H_2O) gave the title compound **126** as a white powder after freeze-drying (10 mg, 40%), $[\alpha]_D$ +77.8 (*c* 0.65 in H_2O); $\delta_H(D_2O)$ 1.91 (1 H, m, $3b_{ax}$-H), 2.11 (3 H, s, AcN), 2.89 (1 H, m, $3b_{eq}$-H), 4.75, 5.36 (1 H, m, 1a-H); $\delta_C(D_2O)$ 23.0, 41.7, 41.8, 47.8, 48.5, 51.9, 52.6, 55.6, 62.3, 62.4, 63.6, 63.9, 67.1, 69.2, 69.5, 69.9, 70.4, 71.5, 72.3, 72.6, 72.9, 75.9, 78.5, 83.5, 85.0, 85.2, 92.9, 98.9, 103.2, 175.8, 176.2; ES-MS (-ve): *m/z* 486 $(M-H)^-$ ($C_{17}H_{29}NO_{13}S$ requires *m/z* 487).

S-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-3-thio-D-glucose, 127



A solution of both **118** (90 mg, 120 μmol) and sodium methoxide (3 mg, 55 μmol) in dry MeOH (0.5 cm^3) was stirred for 2 h at room temperature. The solution was neutralised with acetic acid and concentrated on to silica. Flash chromatography (silica gel; CH_2Cl_2-MeOH, 9:1) gave a white solid which was redissolved in sodium hydroxide solution (1.5 cm^3; 0.1 M). After standing for 24 h at room temperature, the solution was neutralised with acetic acid and concentrated. The crude product was dissolved in aqueous trifluoroacetic acid (1 cm^3; 90% v/v) and stirred at room temperature for 30 mins. The solution was concentrated and co-evaporated several times with toluene. Purification of the residue by gel permeation chromatography (Biogel P-2; H_2O) gave the title compound **127** as a white powder after freeze-drying (23 mg, 39%), $[\alpha]_D$ +26.4 (*c* 1 in H_2O); $\delta_H(D_2O)$ 1.92-2.04 (1 H, m, $3b_{ax}$-H), 2.10 (3 H, s, AcN), 2.86-2.96 (1 H, m, $3b_{eq}$-H), 4.76, 5.33 (1 H, m, 1a-H); $\delta_C(D_2O)$ 23.0 (6), 42.2, 42.3, 51.7, 52.6, 55.3, 62.1, 62.2, 63.7, 68.8, 68.9, 69.2, 69.6, 70.75, 72.5, 72.7, 73.3, 73.5, 76.15, 76.2, 79.5, 92.5, 98.3, 176.25 (2); ES-MS (-ve): *m/z* 486 $(M-H)^-$ ($C_{17}H_{29}NO_{13}S$ requires *m/z* 487).

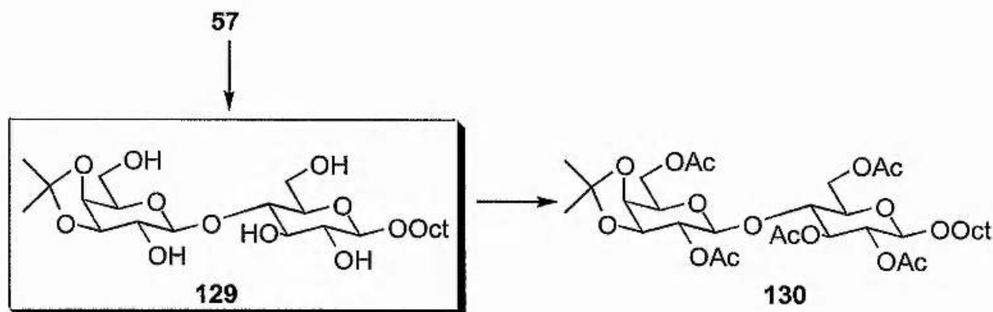
Octyl S-(potassium 5-amino-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-3-thio-β-D-galactopyranoside, 128



A solution of **125** (2 mg) in potassium hydroxide solution (0.5 cm^3;1 M) was heated for 20 h at 90 °C. After cooling to room temperature, the solution was neutralised with acetic acid and desalted on a reverse phase column (Sep-pak; H_2O→MeOH) to give the *de-N-acetyl* compound **128** as a glassy solid (~2 mg, ~100%); $\delta_H(CD_3OD)$ 0.90 (3 H, t, *J* 6.9, $C_7H_{14}CH_3$), 1.26-1.42 (10 H, m, $(CH_2)_5CH_3$), 1.57-1.72 (3 H, m, OCH_2CH_2, $3c_{ax}$-H), 2.90 (1 H, dd, $J_{3bax,3beq}$ 12.6 $J_{3beq,4b}$ 4.4, $3b_{eq}$-H), 3.06 (1 H, t,

$J_{4b,5b}$ = $J_{5b,6b}$ 10.0, 5b-H), 4.27 (1 H, d, $J_{1a,2a}$ 6.6, 1a-H); ES-MS (+ve): *m/z* 596 $(M+H)^+$, 558 $(M-K+2H)^+$ ($C_{23}H_{42}NO_{12}SK$ requires *m/z* 595).

Octyl 3,4-*O*-isopropylidene-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside, 129

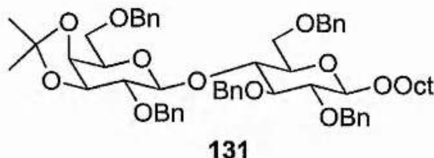


A solution of *octyl lactoside* **57** (2.00 g, 4.40 mmol) and *p*-toluenesulfonic acid (80 mg, 0.4 mmol) in 2,2-dimethoxypropane (40 cm^3) was stirred at room temperature for 60 h. The reaction was quenched with triethylamine (0.5 cm^3) and concentrated to a solid foam which was suspended in EtOAc (30 cm^3). Aqueous TFA (200 mm^3; 50% v/v) was added and after stirring for 1 h, the reaction was again quenched with triethylamine and evaporated onto silica (10 g). Flash chromatography (silica gel, 100 g; EtOAc then EtOAc-MeOH, 19:1) gave the *title compound* **129** as an amorphous white solid (1.44 g, 66%), $[\alpha]_D$ +9.9 (*c* 0.865 in MeOH); $\delta_H(CD_3OD)$ 0.90 (3 H, t, *J* 6.9, $C_7H_{14}Me$), 1.26-1.40 (13 H, m, $(CH_2)_5CH_3$, CMe_2), 1.48 (3 H, s, CMe_2), 1.62 (2 H, m, OCH_2CH_2), 4.28, 4.37 (2 × 1 H, 2 d, $J_{1a,2a,}$ $J_{1b,2b}$ 7.4, 7.7, 1a-H, 1b-H); $\delta_C(CD_3OD)$ 14.5, 23.8, 26.6, 27.2, 28.5, 30.5, 30.7, 30.9, 33.1, 62.1, 62.6, 71.1, 74.6, 75.0, 75.2, 75.5, 76.5, 76.6, 81.0, 81.2, 104.4 (2), 111.3.

A small sample (80 mg) of the above was acetylated using pyridine-acetic anhydride and purified by flash chromatography (silica gel; toluene-EtOAc 2:1) to give *octyl 2,6-di-O-acetyl-3,4-O-isopropylidene-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside* **130** as a colourless syrup, (Found: C, 56.48; H, 7.72, $C_{33}H_{52}O_{16}$ requires C, 56.25; H, 7.44%); $[\alpha]_D$ +6.4 (*c* 1 in $CHCl_3$); ν_{max}/cm^{-1} 2930 (CH_2, CH_3), 1750 (C=O), 1370 (Pr^i, C-H), 1042 (C-O); $\delta_H(CDCl_3)$ 0.84 (3 H, t, *J* 6.9, $C_7H_{14}CH_3$), 1.20-1.30 (13 H, m, $(CH_2)_5CH_3$, CMe_2), 1.50 (5 H, m, OCH_2CH_2, CMe_2), 2.00, 2.01, 2.05, 2.08, 3.00 (5 × 3 H, 5 s, 5 × AcO), 3.42 (1 H, m, OCH_2), 3.58 (1 H, ddd, $J_{4a,5a}$ 9.9, $J_{5a,6a}$ 4.9, $J_{5a,6a'}$ 1.9, 5a-H), 3.72 (1 H, m, 4a-H), 3.80 (1 H, m, OCH_2), 3.90 (1 H, m, 5b-H), 4.09-4.17 (3 H, m, 6a-H, 3b-H, 4b-H), 4.25 (1 H, dd, $J_{5b,6b}$ 7.1, $J_{6b,6b'}$ 11.5, 6b-H), 4.30 (1 H, dd, $J_{5b,6b'}$ 5.2, $J_{6b,6b'}$, 6b′-H), 4.31 (1 H, d,

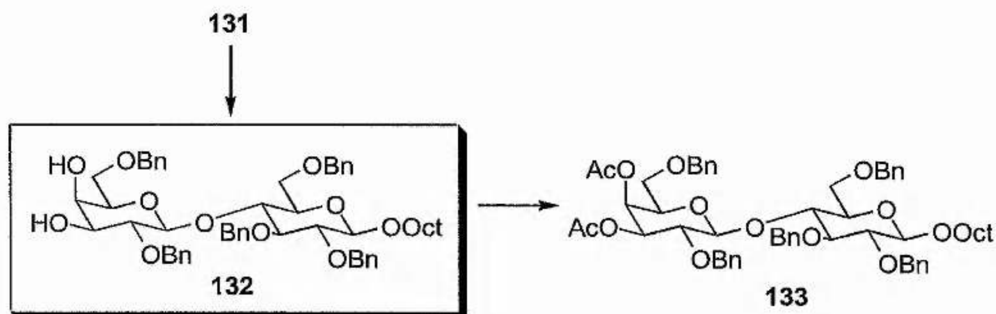
$J_{1b,2b}$ 7.7, 1b-H), 4.42 (2 H, m, 1a-H, 6a′-H), 4.82 (1 H, m, 2b-H), 4.87 (1 H, dd, $J_{1a,2a}$ 8.0, $J_{2a,3a}$ 9.6, 2a-H), 5.16 (1 H, m, 3a-H).

Octyl 2,6-di-*O*-benzyl-3,4-*O*-isopropylidene-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside, 131



Sodium hydride (60% dispersion in oil; 350 mg, 8.6 mmol) was added in portions to a cooled (0 °C), stirred solution of compound **129** (660 mg, 1.33 mmol) in DMF (13 cm^3) and the mixture was stirred at 0 °C for 30 mins. Benzyl bromide (0.87 cm^3, 7.32 mmol) was added dropwise and the mixture was allowed to warm to room temperature and stirred for a further hour. After careful addition of MeOH (1 cm^3) and concentration, the residue was partitioned between diethyl ether and water, the aqueous phase being extracted twice with diethyl ether before washing the combined organic extracts with saturated NaCl solution, drying and concentration to a colourless oil. Flash chromatography (silica gel; hexane-EtOAc, 6:1) gave compound **131** as a colourless syrup (1.20 g, 95%), (Found: C, 73.89; H, 7.87, $C_{58}H_{72}O_{11}$ requires C, 73.70; H, 7.68%); $[\alpha]_D$ +15.8 (*c* 1.05 in $CHCl_3$); ν_{max}/cm^{-1} 3030 (Ar-H), 2930, 2860 (CH_2, CH_3), 1370 (Pr^i C-H), 735, 700 (Ar-H); $\delta_H(CDCl_3)$ 0.90 (3 H, t, *J* 6.9, $C_7H_{14}CH_3$), 1.27-1.46 (16 H, m, $(CH_2)_5CH_3$, CMe_2), 1.68 (2 H, m, OCH_2CH_2), 4.32-4.99 (10× 1 H, 10 AB d, 5 × OCH_2Ph), 4.41, 4.46 (2 × 1 H, 2 d, $J_{1a,2a,}$ $J_{1b,2b}$ 8.0, 7.7, 1a-H, 1b-H), 7.20-7.45 (25 H, m, Ar-H); $\delta_C(CDCl_3)$ 13.9, 22.4, 25.9, 26.2, 27.7, 29.0, 29.2, 29.5, 31.6, 68.2, 68.7, 69.9, 71.8, 73.0 (2), 73.2, 73.5, 74.8, 74.9, 75.2, 76.3, 79.2, 80.5, 81.7, 82.8, 101.7, 103.6, 109.6, 127.2-128.2 (Ar C), 138.3, 138.4, 138.5, 138.7, 139.0.

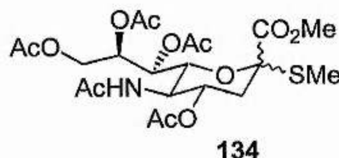
Octyl 2,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside, 132



Compound **131** (1.02 g, 1.08 mmol) was dissolved in aqueous acetic acid (50 cm^3; 80% v/v) and stirred at 80 °C for 2 h. After cooling, the solution was concentrated and co-evaporated several times with toluene. Flash chromatography (silica gel; hexane-EtOAc, 3:2) gave compound **132** as a colourless syrup (0.96 g, 98%), (Found: C, 73.25; H, 7.61, $C_{55}H_{68}O_{11}$ requires C, 72.98; H, 7.57%); $[\alpha]_D$ +18.8 (*c* 1 in $CHCl_3$); ν_{max}/cm^{-1} 3450 (OH br), 3030 (Ar-H), 2925, 2860 (CH_2, CH_3), 735, 700 (Ar-H); $\delta_H(CDCl_3)$ 0.90 (3 H, t, *J* 6.9, $C_7H_{14}CH_3$), 1.25-1.46 (10 H, m, $(CH_2)_5CH_3$), 1.67 (2 H, m, OCH_2CH_2), 2.40 (2 H, brs, OH), 4.38-5.02 (10× 1 H, 10 AB d, 5 × OCH_2Ph), 4.40, 4.60 (2 × 1 H, 2 d, $J_{1a,2a}$, $J_{1b,2b}$ 8.0, 8.0, 1a-H, 1b-H), 7.20-7.40 (25 H, m, Ar-H); $\delta_C(CDCl_3)$ 13.9, 22.4, 25.95, 29.0, 29.2, 29.5, 31.6, 68.2, 68.5, 68.6, 69.9, 72.7, 73.0, 73.3, 73.4, 74.7 (2), 75.0, 75.05, 76.7, 79.9, 81.7, 82.7, 102.5, 103.6, 127.2-128.4 (Ar C), 137.95, 138.25, 138.3, 138.6, 139.2.

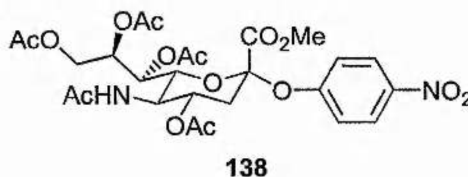
A small sample of the *diol* (100 mg) was acetylated using pyridine-acetic anhydride and purified by flash chromatography (silica gel; hexane-EtOAc 4:1) to give *octyl 3,4-di-O-acetyl-2,6-di-O-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-glucopyranoside* **133** as a colourless syrup (100 mg, 92%), $\delta_H(CDCl_3)$ 0.89 (3 H, t, *J* 6.9, $C_7H_{14}CH_3$), 1.26-1.44 (10 H, m, $(CH_2)_5CH_3$), 1.67 (2 H, m, OCH_2CH_2), 1.95, 1.99 (2 × 3 H, 2 s, 2 × AcO), 3.71 (1 H, dd, $J_{5a,6a}$ 1.6, $J_{6a,6a'}$ 11.0, 6a-H), 3.79 (1 H, dd, $J_{5a,6a'}$ 4.1, $J_{6a,6a'}$, 6a′-H), 3.91-4.03 (2 H, m, OCH_2, 4a-H), 4.19-4.98 (10× 1 H, 10 AB d, 5 × OCH_2Ph), 4.38 (1 H, d, $J_{1a,2a}$ 7.7, 1a-H), 4.53 (1 H, d, $J_{1b,2b}$ 7.7, 1b-H), 4.87 (1 H, dd, $J_{2b,3b}$ 10.2, $J_{3b,4b}$ 3.3, 3b-H), 5.39 (1 H, d, $J_{3b,4b}$, 4b-H), 7.15-7.40 (25 H, m, Ar-H).

Methyl (methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α/β-D-*galacto*-2-nonulopyranosid)onate, 134[18]



A suspension of peracetylated sialic acid methyl ester **89** (2.0 g, 3.75 mmol), 4Å molecular sieves (500 mg), (methylthio)trimethylsilane (1.96 cm^3, 13.8 mmol), and trimethylsilyltrifluoromethanesulfonate (0.68 cm^3, 3.75 mmol) in dry 1,2-dichloroethane (30 cm^3) was stirred under nitrogen for 7 h at 50 °C. The mixture was cooled to 0 °C and sodium carbonate solution (25 cm^3;1 M) was added. The mixture was separated and the organic layer was washed with water before drying and concentration to an oil. Flash chromatography (silica gel; CH_2Cl_2 then CH_2Cl_2-MeOH, 200:1→50:1) gave the *methyl thioglycoside* **134** as a foam which was freeze-dried from dioxan (1.61 g, 82%; α:β, 1:1); $[\alpha]_D$ -32.2 (*c* 1.05 in $CHCl_3$) (lit.,[7] [α anomer] +17.8); δ_H($CDCl_3$, lit.,[7] [α anomer]) 1.84, 1.85 (2 × 3 H, 2 s, αAcN, βAcN), 2.49 (1 H, dd, $J_{3ax,3eq}$ 14.0, $J_{3eq,b}$ 4.9, β3_{eq}-H), 2.69 (1 H, dd, $J_{3ax,3eq}$ 12.9, $J_{3eq,b}$ 4.7, α3_{eq}-H), 4.85 (1 H, m, α4-H), 5.23 (1 H, m, β4-H); δ_C($CDCl_3$) 11.7, 20.5, 20.6, 20.7, 20.9, 22.8, 22.9, 36.7, 37.6, 49.1, 49.2, 52.6, 52.7, 62.05, 62.2, 66.9, 67.2, 68.3, 68.55, 69.2, 69.6, 71.95, 72.4, 73.9, 82.8, 84.5, 167.9, 170.15, 170.2, 170.3, 170.5, 170.65, 170.9, 171.0.

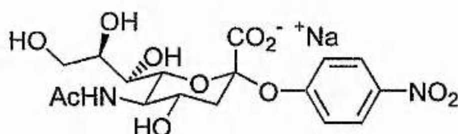
4-Nitrophenyl *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate), 138[19]



4-Nitrophenol (210 mg, 1.51 mmol) was added to a vigorously stirred mixture of **31** (500 mg, 0.98 mmol) and tetrabutylammonium hydrogensulfate (410 mg, 1.21 mmol) in CH_2Cl_2 (25 cm^3) and 1 M NaOH solution (25 cm^3). After 15 mins, the mixture was diluted with CH_2Cl_2 (200 cm^3) and subjected to standard work-up A. Flash chromatography (silica gel; EtOAc) gave the *acetylated pNP glycoside* **138** as a colourless syrup which crystallised on standing (293 mg, 49%), mp 110 °C (lit.,[19] 107-112 °C, lit.,[20] 104-108 °C); δ_H($CDCl_3$, lit.,[19]) 1.91 (3 H, s, AcN), 2.03-2.17 (4 ×

3 H, 4 s, 4 × AcO), 2.28 (1 H, t, $J_{3ax,3eq}$ = $J_{3ax,4}$ 12.9, 3_{ax}-H), 2.73 (1 H, dd, $J_{3ax,3eq}$ 12.9, $J_{3eq,4}$ 4.4, 3_{eq}-H), 3.64 (3 H, s, CO_2Me) 4.10 (2 H, m, 5-H, 9′-H), 4.21 (1 H, dd, $J_{8,9}$ 2.2, $J_{9,9'}$ 12.9, 9-H), 4.60 (1 H, dd, $J_{5,6}$ 10.7, $J_{6,7}$ 1.4, 6-H), 4.96 (1 H, m, 4-H), 5.35 (2 H, m, 7-H, 8-H), 5.46 (1 H, d, $J_{5,NH}$ 10.2, NH), 7.13 (2 H, m, Ar-H), 8.17 (2 H, m, Ar-H).

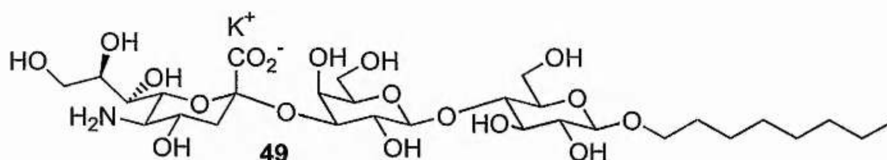
4-Nitrophenyl *O*-(sodium 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate), 139[20]

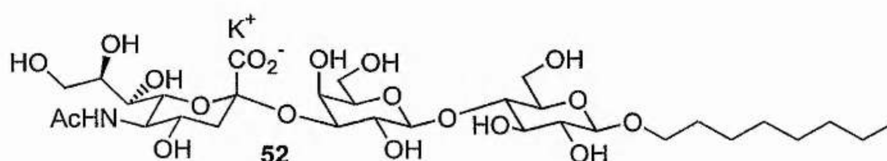


139

A solution of **138** and sodium methoxide (4 mg, 74 μmol) in dry MeOH (2 cm^3) was stirred for 2 h at room temperature. The solution was neutralised with acetic acid and concentrated to give a pale yellow solid which was redissolved in sodium hydroxide solution (1.63 cm^3; 0.1 M). After standing for 24 h at room temperature, the solution was freeze dried to give the *pNP sialoside* **139** as a yellow solid (72 mg, 98%), $[\alpha]_D$ +45.2 (*c* 0.5 in H_2O) (lit.,[20] [acid form] +48.5° *c* 1.3 in MeOH); δ_H(D_2O, lit.,[19] [acid form]) 2.04 (4 H, m, AcN, 3_{ax}-H), 2.83 (1 H, dd, $J_{3ax,3eq}$ 12.9, $J_{3eq,4}$ 4.4, 3_{eq}-H), 7.28, 8.21 (2 × 2 H, 2 d, *J* 8.9, Ar-H).

Octyl (potassium 5-amino-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside, 49 and octyl (sodium/potassium 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside, 52





Method one: chemical glycosylation. A suspension of **132** (200 mg 220 μmol), **134** (288 mg 550 μmol) and 3Å molecular sieves (600 mg) in dry acetonitrile (2 cm^3) was stirred for 2 h at room temperature under nitrogen. The mixture was cooled to -35 °C and *N*-iodosuccinimide (247 mg, 1.10 mmol) was added, followed by trifluoromethanesulfonic acid (10 mm^3, 110 μmol) and the mixture was stirred at this temperature for 2 h. The mixture was diluted with CH_2Cl_2 (50 cm^3), filtered through Celite and the filtrate was washed successively with 1 M solutions of sodium carbonate, sodium thiosulfate and sodium chloride, before drying and concentration to a syrup. In order to facilitate separation of products from starting materials, the mixture was deacetylated prior to chromatography: a solution of the crude product mixture and sodium methoxide (6 mg, 110 μmol) in MeOH (10 cm^3) was stirred for 2 h at room temperature. The mixture was neutralised with acetic acid and concentrated on to silica (2 g). Flash chromatography (silica gel; CH_2Cl_2-MeOH, 99:1→97:3) gave a mixture of tri-saccharides **135a/b** (150 mg).

A small portion (20 mg) of this mixture was partially separated by careful column chromatography (silica gel; CH_2Cl_2-MeOH, 99:1→97:3) to give first, the lactone, *octyl (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyloyl-1c→4b-lactone)-(2→3)-2,6-di-O-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-glucopyranoside* **135b**; δ_H(CD_3OD; *cf.* compound *14* in Ref. 21) 1.69 (1 H, dd, $J_{3cax,3ceq}$ 13.8 $J_{3cax,4c}$ 11.3, $3c_{ax}$-H), 2.01 (3 H, s, AcN), 2.26 (1 H, dd,

$J_{3cax,3ceq}$, $J_{3ceq,4c}$ 5.5, 3c$_{eq}$-H), 3.25 (1 H, dd, $J_{1a,2a}$ 8.0, $J_{2a,3a}$ 9.3, 2a-H), 4.10 (1 H, dd, $J_{2b,3b}$ 9.3, $J_{3b,4b}$ 3.9, 3b-H), 4.42 (1 H, d, $J_{1b,2b}$ 7.7, 1b-H), 4.45 (1 H, d, $J_{1a,2a}$, 1a-H), and then *octyl (methyl 5-acetamido-3,5-dideoxy-D-*glycero-*α/β-D-*galacto-*2-nonulopyranosylonate)-(2→3)-2,6-di-O-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-glucopyranoside* **135a** as an anomeric mixture (α:β, 7:3); δ_H(CD_3OD) 1.73 (m, $J_{3cax,3ceq}$ 12.9 $J_{3cax,4c}$ 11.8, β3c$_{ax}$-H), 2.01 (m, α/βAcN, α3c$_{ax}$-H), 2.68 (m, α/β3c$_{eq}$-H), 3.58 (s, βCO_2Me), 3.80 (s, αCO_2Me). Both samples were acetylated with pyridine and acetic anhydride to verify their structures:

lactone **136b**; δ_H($CDCl_3$) 1.88 (3 H, s, AcN), 1.92, 2.00, 2.03, 2.15, (4 × 3 H, 4 s, 4 × AcO), 4.60 (1 H, dd, $J_{2b,3b}$ 9.3, $J_{3b,4b}$ 3.9, 3b-H), 4.18 (1 H, m, 5c-H), 4.37 (1 H, d, $J_{1a,2a}$ 7.7 1a-H), 5.05 (1 H, m, 8c-H), 5.25 (1 H, dd, $J_{6c,7c}$ 1.9, $J_{7c,8c}$ 6.3, 7c-H), 5.30 (1 H, d, $J_{5c,NH}$ 10.2, NH), 5.49 (1 H, m, 4c-H); FAB-MS: *m/z* 1369 $(M+Na)^+$ ($C_{74}H_{91}NO_{22}$ requires *m/z* 1346).

α/β methyl esters **136a**; δ_H($CDCl_3$, *cf.* compounds *25* and *26* in Ref. 22) 1.75, 1.96, 1.99, 2.00, 2.08 (5 × 3 H, 5 s, 5 × βAcO), 1.85 (3 H, s, α/βAcN), 2.60 (m, α/β3c$_{eq}$-H), 3.43 (s, βCO_2Me), 3.83 (s, αCO_2Me), 5.05 (d, $J_{3b,4b}$ 3.3, α4b-H), 5.32 (dd, $J_{6c,7c}$ 2.2, $J_{7c,8c}$ 8.5, α7c-H), 5.39 (t, $J_{6c,7c} = J_{7c,8c}$ 2.0, β7c-H), 5.59 (1 H, m, α8c-H).

A suspension of the remaining tri-saccharide mixture (130 mg) and palladium hydroxide on charcoal (130 mg; 10% w/w) in MeOH (30 cm^3) was stirred for 24 h under an atmosphere of hydrogen. The mixture was filtered through Celite and concentrated. A solution of the residue in potassium hydroxide solution (10 cm^3;1 M) was heated for 20 h at 90 °C. After cooling to room temperature, the solution was neutralised with acetic acid and desalted on a reverse phase column (C-18 silica gel; H_2O→MeOH) to give *octyl (potassium 5-amino-3,5-dideoxy-D-*glycero-*α/β-D-*galacto-*2-nonulopyranosylonate)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside* **49**α/β as a glassy solid (80 mg, 49%; α:β, 6:1), δ_H(CD_3OD) β anomer- 2.34 (dd, $J_{3cax,3ceq}$ 12.6 $J_{3ceq,4c}$ 4.4, 3c$_{eq}$-H), 2.90 (t, $J_{4c,5c} = J_{5c,6c}$ 9.6, 5c-H), α anomer- 2.73 (t, $J_{4c,5c} = J_{5c,6c}$ 9.9, 5c-H), 2.79 (dd, $J_{3cax,3ceq}$ 12.4 $J_{3ceq,4c}$ 4.7, 3c$_{eq}$-H).

Repeated flash chromatography (silica gel; CH_2Cl_2-MeOH-H_2O, 6:5:1) gave **49**α as a glassy solid (35 mg, 21%), $[\alpha]_D$ -24.8 (*c* 0.5 in MeOH); δ_H(CD_3OD) 0.90 (3 H, t, *J* 6.9, $C_7H_{14}CH_3$), 1.26-1.42 (10 H, m, $(CH_2)_5CH_3$), 1.47-1.70 (3 H, m, OCH_2CH_2,

$3c_{ax}$-H), 2.73 (1 H, t, $J_{4c,5c} = J_{5c,6c}$ 9.9, 5c-H), 2.79 (1 H, dd, $J_{3cax,3ceq}$ 12.4 $J_{3ceq,4c}$ 4.7, $3c_{eq}$-H), 4.28 (1 H, d, $J_{1a,2a}$ 8.0, 1a-H), 4.43 (1 H, d, $J_{1b,2b}$ 8.0, 1b-H); δ_C(CD_3OD) 14.5, 23.8, 27.2, 30.5, 30.7, 30.9, 33.1, 42.1, 54.6, 62.3, 62.9, 64.9, 68.9, 70.1, 71.1 (2), 72.25, 73.7, 74.9, 76.6 (2), 77.2, 77.5, 81.3, 101.3, 104.5, 105.3, 175.75; ES-MS (+ve): *m/z* 704 $(M-K+2H)^+$ ($C_{29}H_{52}NO_{18}K$ requires *m/z* 741).

Re-*N*-acetylation of 49. Acetic anhydride (64 mm^3, 64 μmol; 1 M solution in MeOH) was added to a stirred solution of **49**α (15 mg, 21.3 μmol) in MeOH. After 18 h the solution was concentrated and the residue was redissolved in water (2 cm^3). Reverse phase chromatography (C-18 silica gel; $H_2O \rightarrow$MeOH) gave *octyl (potassium 5-acetamido-3,5-dideoxy-D-*glycero-*α-D-*galacto-*2-nonulopyranosylonate)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside,* **52** as a glassy solid (14 mg, 88%), $[\alpha]_D$ -7.2 (*c* 1 in MeOH); δ_H(CD_3OD) 0.90 (3 H, t, *J* 6.9, $C_7H_{14}CH_3$), 1.26-1.42 (10 H, m, $(CH_2)_5CH_3$), 1.62 (2 H, m, OCH_2CH_2), 1.73 (1 H, m, $3c_{ax}$-H), 2.00 (3 H, s, AcN), 2.86 (1 H, dd, $J_{3cax,3ceq}$ 12.4 $J_{3ceq,4c}$ 4.4, $3c_{eq}$-H), 4.28 (1 H, d, $J_{1a,2a}$ 7.7, 1a-H), 4.43 (1 H, d, $J_{1b,2b}$ 7.7, 1b-H); δ_C(CD_3OD) 14.5, 22.7, 23.8, 27.2, 30.5, 30.7, 30.9, 33.1, 42.25, 54.1, 62.2, 62.9, 64.8, 69.2, 69.5, 70.3, 71.1, 73.2, 75.0, 75.1, 75.15, 76.6, 77.2, 77.9, 81.2, 101.3, 104.5, 105.3, 175.3, 175.8; ES-MS (-ve): *m/z* 744 $(M-K)^-$ ($C_{31}H_{54}NO_{19}K$ requires *m/z* 783).

Method two: *trans*-sialidase glycosylation. *pNP sialoside* **139** (3 mg, 6.7 μmol) was added to a solution of *octyl lactoside* **57** (2 mg, 4.4 μmol) and *Trypanosoma cruzi trans*-sialidase (~1 mg) (see general methods, **7.1**) in 50 mM phosphate buffer (pH 7, 1 cm^3). The reaction mixture was incubated for 18 h at 30 °C. TLC ($CHCl_3$-MeOH-H_2O, 6:4:1) indicated ~50% turnover of the *octyl lactoside*. The reaction was quenched by addition of EtOH (0.5 cm^3) to precipitate the protein and the resulting mixture was centrifuged at 13,000 × *g* for 3 mins. The supernatants of five such incubations were combined and concentrated. The residue was redissolved in water (2 cm^3) and subjected to reverse phase chromatography (C-18 silica gel; $H_2O \rightarrow$MeOH). 50% aqueous methanol gave the *title compound* **52** as a white powder following freeze-drying from water (7 mg, 42%). Analytical data were identical to those obtained for the chemically synthesised compound.

A solution of enzymatically synthesised **52** (5 mg) and potassium hydroxide (1 cm^3;1 M) was heated for 20 h at 90 °C. After cooling to room temperature, the solution was neutralised with acetic acid and desalted on a reverse phase column (C-18 silica gel; $H_2O \rightarrow$MeOH) to give the *de-N-acetyl* compound **49** as a glassy solid (4.5 mg, 95%) Analytical data were identical to those for the chemically synthesised compound.

6.3 Enzyme Assays

6.3.1 *Clostridium perfringens* Neuraminidase Inhibition Assay.[19] pNP-sialoside **139** (0.35 μmol) and the inhibitor (0.35 μmol) in 50 mM sodium acetate buffer (pH 4.5, 0.35 cm^3) were incubated at 37 °C. Aliquots (50 mm^3) were withdrawn after 5, 10 and 15 minutes and immediately quenched with glycine stopping buffer (pH 10, 0.95 cm^3; see general methods, **7.1**) and their UV absorbance was read at 400 nm against stopping buffer as blank. Twenty minutes after starting the incubation, *C. perfringens* neuraminidase (8 mU) was added, and the mixture was incubated at 37 °C, as before. After a further 5, 10 and 15 minutes, aliquots (50 mm^3) were withdrawn, diluted with stopping buffer and their UV absorbances were read as above. The rate of enzymatic hydrolysis of the substrate was determined by plotting a graph of absorbance *vs.* time for each inhibitor in the presence and absence of neuraminidase. Enzyme inhibition was calculated as a percentage of the rate of substrate turnover in the absence of inhibitors.

6.3.2 *Clostridium perfringens* Neuraminidase Turnover Assay. The potential substrates (0.2 μmol) were incubated with *C. perfringens* neuraminidase (2 mU) in 50 mM sodium acetate buffer (pH 4.5, 100 mm^3) at 37 °C. TLC ($CHCl_3$-MeOH-H_2O, 6:4:1) after 24 h showed that all of *GM3 analogue* **52** had been cleaved to sialic acid and octyl lactoside, but all of the other compounds which had been tested remained unchanged.

6.3.3 *Trypanosoma cruzi trans*-Sialidase Inhibition Assay.[23] *trans*-Sialidase enzyme (10 mm^3) (see general methods, **7.1**) was added to 50 mM phosphate buffer (pH 7, 40 mm^3) containing 2,3-sialyllactose (10 nmol), [^{14}C]lactose (~35,000 cpm, 0.38 nmol) and the inhibitor (50 nmol). Following incubation for 30 mins at 30 °C, the reaction mixture was quenched by diluting with water (0.95 cm^3) an applied to a column of Sephadex A25 (0.5 cm^3) which had been pre-equilibrated with water. [^{14}C]Lactose was eluted from the column by washing with water (1 cm^3) and then sialyl-[^{14}C]lactose was eluted with ammonium acetate solution (2 cm^3; 1 M). Scintillation fluid (8 cm^3) was added to each of the elutions and after mixing, the radioactivity was measured using a scintillation counter.

6.3.4 *Trypanosoma cruzi trans*-Sialidase Turnover Assay. *trans*-Sialidase enzyme (10 mm^3) (see general methods, **7.1**) was added to 50 mM phosphate buffer (pH 7, 40 mm^3) containing [^{14}C]lactose (~35,000 cpm, 0.38 nmol) and the potential sialyl-donor (50 nmol). The mixture was incubated for 30 mins, 4 h or 22 h at 30 °C and then worked-up as described for the inhibition assay **7.3.3**.

6.4 References

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Chapter 7

Conclusions and Future Work

7.1 Conclusions

De-*N*-acetyl gangliosides are naturally occurring glycosphingolipids that have been detected in several different cancer cell lines (section 1.6.3.2). It has been suggested that one such compound, deNAcGM3, has a potent effect on signal transduction, ultimately up-regulating cell growth (section 1.6.3.2). However, deNAc gangliosides are only expressed transiently and little is known about their biology. The aims of this project were to synthesise a number of analogues of gangliosides GM3 and deNAcGM3 and to use these compounds to develop assays for the de-*N*-acetylase and *N*-acetyltransferase activities which allow the inter-conversion of GM3 and deNAcGM3. Such assays could then be used to monitor the purification of the relevant enzymes and also to screen potential inhibitors for the enzymes.

To this end, several *S*-linked and *O*-linked sialosides were synthesised (sections 4.1 and 4.2). Both chemical and enzymatic glycosylations were investigated for the synthesis of *O*-linked GM3 analogue **52** (Figure 7.1) (section 4.2). The enzymatic procedure using recombinant *Trypanosoma cruzi trans*-sialidase was found to be the method of choice allowing rapid, easy, stereo- and regio-selective synthesis of the target sialoside in good yield.

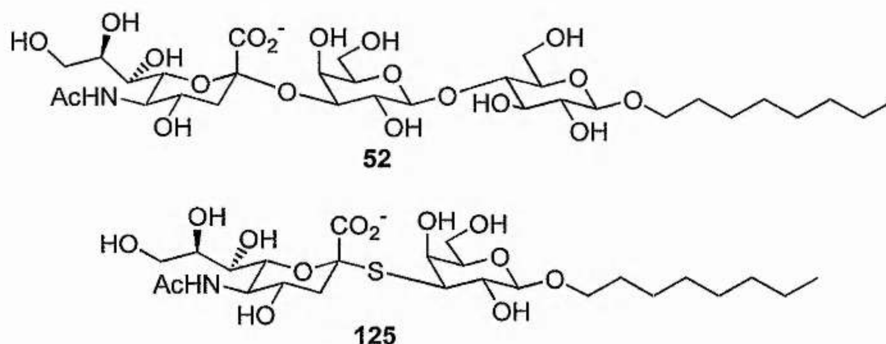


Figure 7.1 *GM3 analogue 52 and S-linked GM4 analogue 125.*

Difficulties were encountered in trying to synthesise the corresponding thioglycoside analogues (sections 4.1.1 and 4.1.2). Literature procedures failed in my hands, but a novel strategy for the synthesis of Neu5Acα(2→3)-3-thio-Gal containing compounds was developed and successfully used to synthesise GM4 analogue **125** (Figure 7.1) (sections 4.1.4 and 4.1.5). The thioglycosides synthesised in this project were found to be resistant to enzymatic hydrolysis by *Clostridium perfringens* neuraminidase and *T. cruzi trans*-sialidase (section 4.3). GM4 analogue **125** was found to inhibit *C. perfringens* neuraminidase with an IC_{50} of ~1 mM (section 4.3.1).

Assays for the de-*N*-acetylase and *N*-acetyltransferase activities were developed in collaboration with Prof. Ajit Varki's group at the UCSD Cancer Center (chapter 5). However, initial studies failed to locate the target enzymes. Further experiments suggested that the particular cell line which had been used in these studies had changed phenotypically and was no longer expressing deNAc gangliosides (section 5.3). Studies towards the identification and characterisation of the de-*N*-acetylase and *N*-acetyltransferase enzymes are continuing in collaboration with the Varki group.

7.2 Future Work

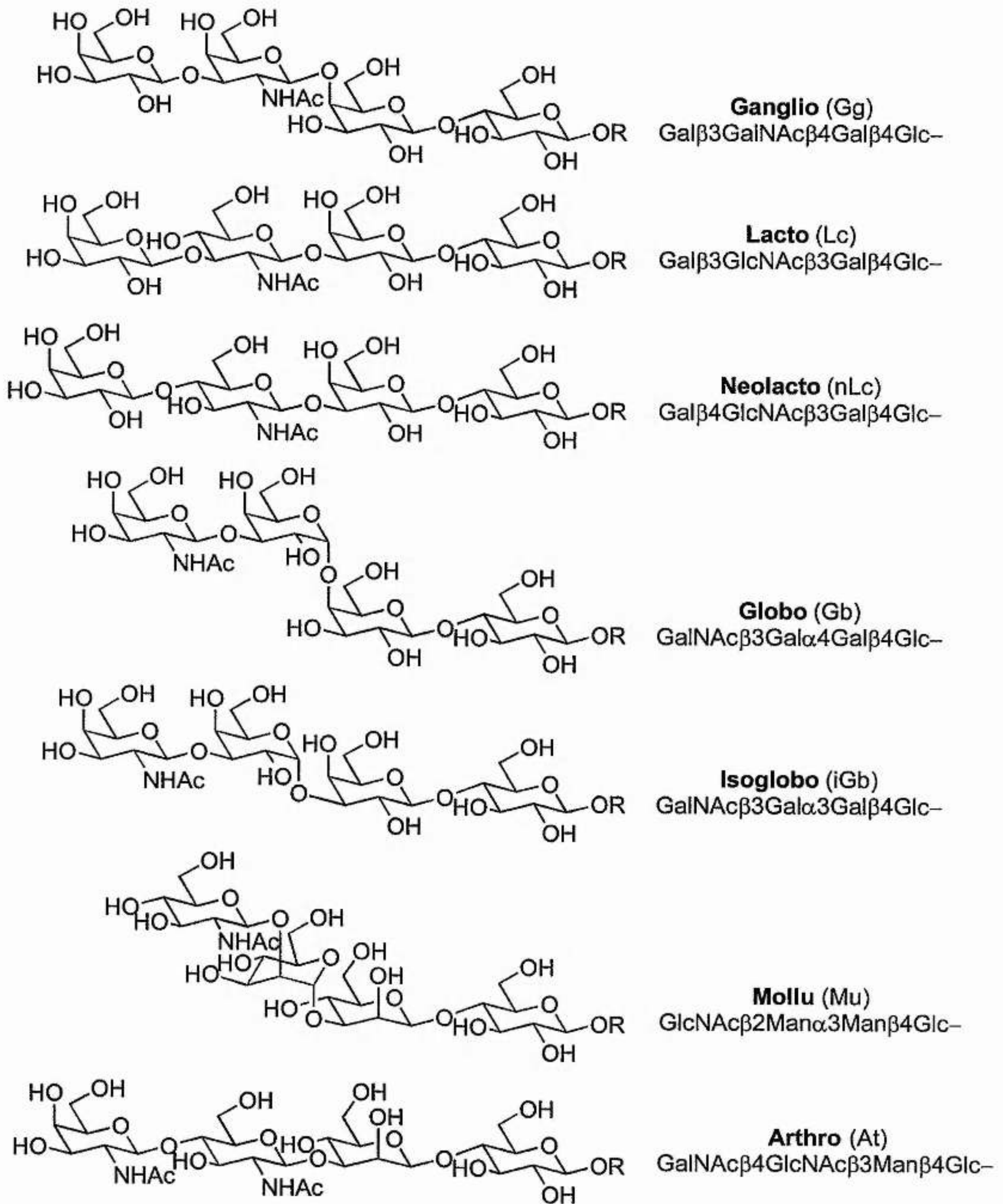
Clearly, the first priority of future work is to identify and purify the de-*N*-acetylase and *N*-acetyltransferase enzymes. DeNAcGM3 analogues with longer hydrophobic tails need to be synthesised to improve the sensitivity of the *N*-acetyltransferase assay (section 5.2.1) and a panel of different cancer cell lines will need to be screened for the target enzymes. After the enzymes have been located and preferably purified, a range of GM3/deNAcGM3 analogues bearing modified sialic acids could be synthesised for structure-activity studies.

Potential inhibitors for the enzymes could be based on either the *S*-linked or *O*-linked analogues that have been synthesised in this study. The de-*N*-acetylation reaction presumably proceeds *via* a tetrahedral transition state. Transition state analogues could be prepared as potential inhibitors, by replacing the sialic acid acetamido group with a tetrahedral group *e.g.* a sulfonamide.

Inhibitors based on thioglycosides have been shown to be resistant to degradation by glycosidases. If such inhibitors were attached to a resin, they could potentially be used to purify the de-*N*-acetylase by affinity chromatography, in a manner analogous to the purification of cellulase described in section 2.4. It is also possible that inhibiting the synthesis or degradation of deNAc gangliosides in whole cells, could shed further light on the biological roles of these novel and illusive membrane components.

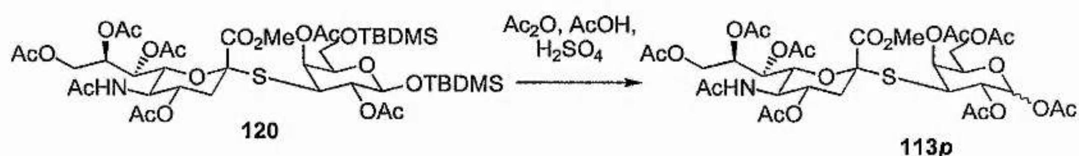
Appendices

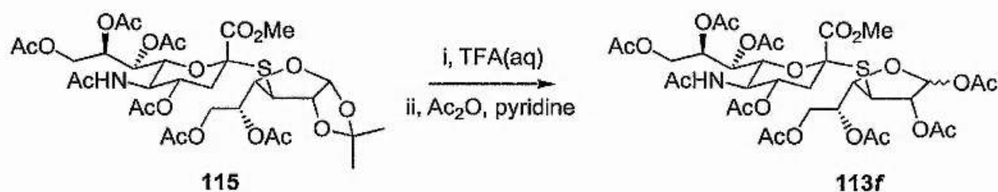
Appendix 1. Core Structures of Glycosphingolipids

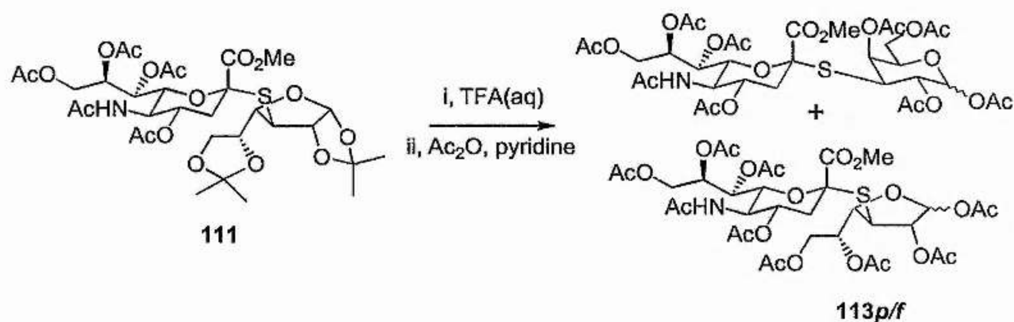


Appendix 2. ^1H and ^{13}C NMR Spectra of Compound 113

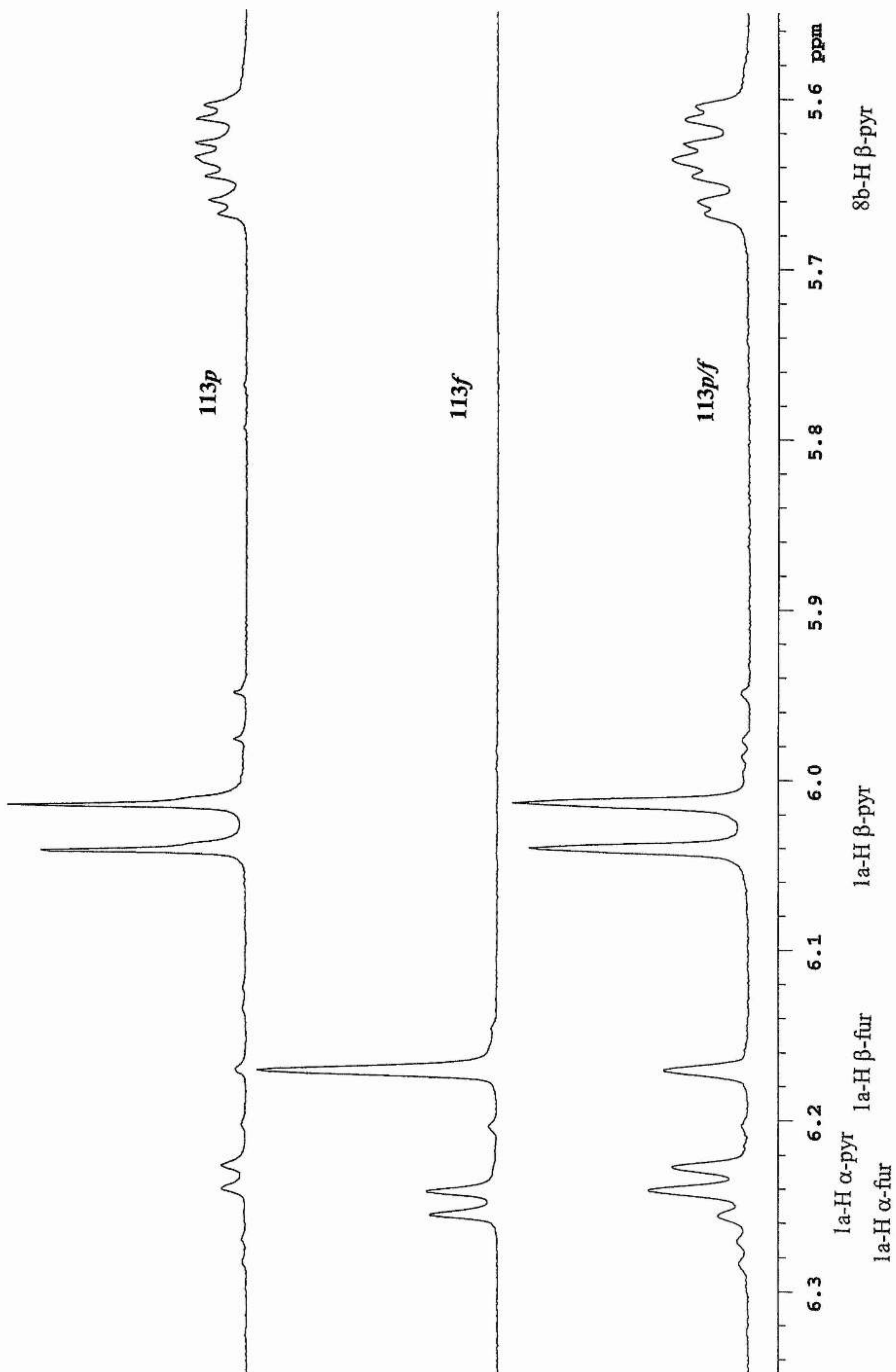
Fully acetylated sialyl galactose thioglycoside **113** was synthesised in three different reactions, each giving different mixtures of pyranose and furanose isomers. ^1H and ^{13}C NMR spectra of the product mixtures arising from each of the reactions shown below, are reproduced on the next two pages.







^{1}H NMR Spectra of compound 113



^{13}C NMR Spectra of compound 113