UNIVERSITY OF ALBERTA

THE SYNTHESIS OF MELANOMA-ASSOCIATED ANTIGENS GM_3 AND GD_3

BY

JAMES MICHAEL DIAKUR 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**.

IN

PHARMACEUTICAL SCIENCES
(MEDICINAL CHEMISTRY)

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA
FALL 1994

ISBN 0-315-95175-3

Canada

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: James Michael Diakur

TITLE OF THESIS: THE SYNTHESIS OF MELANOMA-ASSOCIATED ANTIGENS GM_3 AND GD_3

DEGREE: DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED: 1994

James Diakur
5512-14 Ave
Edmonton, Alberta, T6L 2B3

Dated June 22 ,1994

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE SYNTHESIS OF MELANOMA-ASSOCIATED ANTIGENS GM_3 AND GD_3 submitted by JAMES MICHAEL DIAKUR in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in PHARMACEUTICAL SCIENCES (MEDICINAL CHEMISTRY).

Dr. A. A. Noujaim (supervisor)

Dr. E. E. Knaus (co-supervisor)

Dr. F. M. Pasutto

Dr. A. J. McEwan

Dr. J. R. Mercer

Dr. L. I. Wiebe

Dr. J. R. Dimmock, External Reader

Dated June 22 ,1994

This thesis is dedicated to Bernadette, Julia, and Jordan who have provided me with their love, support and encouragement throughout my studies.

ABSTRACT

Although the incidence of melanoma is relatively low compared to other cancers, skin cancers in general, are increasing at an alarming rate. As this disease initially appears on the surface of the skin, visual inspection represents preliminary diagnosis, and upon confirmation, wide local excision represents initial therapy. Once melanoma has penetrated the dermis however, it spreads via the blood and lymphatics, resulting in distal metastasis and subsequent poor prognosis. Therefore, methods of locating metastatic colonies and pharmaceutical agents for controlling the spread of melanoma are in demand.

Of the several types of melanoma-associated antigens that have been identified, at present, the gangliosides appear to be the most suited for study on the molecular level. Current literature provides firm biological support for further studying these carbohydrate antigens in this regard. We wish to report herein, the total synthesis of two ganglioside analogs, namely GM_3- and GD_3-4M3P. Our overall design is dependent on only a few synthons, and this strategy allows for future extension to the synthesis of other members of this family of antigens. As well, preparation of unnatural analogs which may display increased biological activity may be realized via this approach.

The title compounds are suited for conjugation to carrier proteins, and the preparation of the corresponding neoglycoconjugates is described. Also, preliminary work on immunization of mice with these artificial antigens in an effort to generate murine monoclonal antibodies (mAbs) is presented. Anti-melanoma antibodies that recognize malignant melanoma are potential delivery vehicles for radionuclides employed in radioimaging, and subsequent therapeutic applications can also be envisaged.

It may be possible to study binding interactions between a mAb and its complementary melanoma-associated ganglioside antigen on the molecular level by current techniques such as nuclear magnetic resonance (NMR) spectroscopy. If so, the GM_3- and GD_3-4M3P derivatives obtained herein display good NMR characteristics in the natural aqueous environment, a trait not displayed by the natural counterparts.

The work reported herein is admittedly only a minute step in the direction of the development of new diagnostic (and possibly therapeutic) agents for melanoma. Hopefully, the title compounds (or generated mAbs) may be useful tools for studying key biological recognition events on the molecular level.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisor, Dr. Tony Noujaim, for his encouragement and support throughout this project. I also wish to thank Dr. Edward Knaus, who as cosupervisor, was alway accessible for both friendly advice and to answer those questions that only chemists dare to ask.

The author is likewise indebted to Dr. Leonard Wiebe, Associate Dean of Pharmacy and Pharmaceutical Sciences for inspiring an atmosphere which merges innovative research and hard work with a pleasant work environment.

Many sincere thanks are extended to G. Bigam, T. Brisbane, L. Kong, G. Aarts and Dr. T.T. Nakashima, Department of Chemistry, University of Alberta, for their valuable NMR spectral services and to Dr. A. Morales for recording the FAB-MS spectra.

I also wish to thank Michelle Kuzma of BRIE for her immunization work in this study.

Finally, the author is grateful to BRIE (Biomira Research Incorporated Edmonton) for its financial support.

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SYMBOLS AND ABBREVIATIONS

Å	Ångstrom unit (10^{-8} cm)
Ac	acetyl group, CH_3CO-
[α]	specific optical activity
Bn	benzyl group, $C_6H_5CH_2$-
n-Bu	*n*-butyl group, $CH_3CH_2CH_2CH_2$-
t-Bu	*t*-butyl group, $(CH_3)_3C$-
CMP	cytidine monophosphate
COSY	correlation spectroscopy
DMF	*N,N*-dimethylformamide, $(CH_3)_2NCHO$
DMSO	dimethyl sulfoxide, $(CH_3)_2SO$
DOPA	dihydroxyphenylalanine
DTPA	diethylenetriaminepentaacetic acid
δ	chemical shift downfield from TMS, given as ppm
E	*entgegen*, opposite sides in the (E,Z) nomenclature of alkenes
ELAM	endothelial leucocyte adhesion molecule
ELISA	enzyme-linked immunosorbent assay
Et	ethyl group, CH_3CH_2-
Fab	antigen binding antibody fragment
FAB	fast atom bombardment
g	gram
GSL	glycosphingolipid
h	hour
HAT	hypoxanthine, aminopterin, thymidine
HPLC	high performance liquid chromatography
HPLPLC	high performance low pressure liquid chromatography
HSA	human serum albumin
Hz	Hertz (sec^{-1} or cycles per second)
IgG	immunoglobulin G
J	coupling constant, in Hz
KLH	keyhole limpet nemocyanin
kD	kilodalton
LBSA	lipid bound sialic acid
M	molar concentration

mAb	monoclonal antibody
Me	methyl group, CH_3-
m/z	mass-to-charge ratio in mass spectrometry
mg	milligram, 10^{-3} gram
MHz	megaHertz = 10^6 Hz
min	minutes
mL	millilitre, 10^{-3} litre
mmol	millimole
mp	melting point
MP	medium pressure
µg	microgram, 10^{-6} gram
µL	microlitre, 10^{-6} litre
4M3P	4-methyl-3-pentenyl group, $(CH_3)C=CHCH_2CH_2$-
NANA	N-acetylneuraminic acid
NGNA	N-glycolylneuraminic acid
nm	nanometer, 10^{-9} meter
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PEG	polyethylene glycol
pH	measure of acidity = -log[H^+]
PNPE	*p*-nitrophenethyl group, *p*-$NO_2C_6H_5CH_2CH_2$-
p-NTFA	*p*-*N*-trifluoracetamidophenethyl group, *p*-$CF_3CONHC_6H_5CH_2CH_2$-
p-TSA	*p*-toluenesulfonic acid
RAID	radioimmunodetection
RAIT	radioimmunotherapy
(R,S)	designation of stereochemical configuration
S_N2	bimolecular nucleophilic substitution mechanism
SPECT	single photon emission computed tomography
THF	tetrahydrofuran
TLC	thin-layer chromatography
TMS	tetramethylsilane
TMU	1,1,3,3-tetramethylurea
UDP	uridine diphosphate
UVR	ultraviolet radiation

1. INTRODUCTION

Although considered to be a relatively low incidence cancer, melanoma is characterized as a high-mortality cancer and continues to display poor prognosis. It is considered in many ways to be a prototypic cancer in that it is; (a) highly aggressive in nature; (b) it is resistant to radiation and drug treatments; and (c) strikes any age group. Originating from pigmented cells on the body surface, melanoma is markedly heterogeneous and is extremely invasive. Coupled with these facts is the fact that skin cancers in general appear to be increasing at an alarming rate, and of the 3200 new cases expected in Canada annually, about 20% will be considered to be fatal. The National Cancer Institute (NCI) predicts 32,000 new melanoma cases in the United States for the year 1993 and 6800 deaths. Amoung women, the incidence of melanoma is rising at a rate second only to that of lung cancer.

Once melanoma has penetrated the dermis, it spreads via the blood and lymphatics, and subsequent rapid progression leads to metastasis to distal tissues and organs. Currently, advanced melanoma is treated by wide local excision, however, once spread beyond the dermis (stage II disease), aggressive chemotherapy generally provides only marginal additional benefit. Commenting on the immediate needs of melanoma research, Dr. Umberto Veronesi, Director of the National Cancer Institute in Milan, Italy summarized the current status of treatment of this disease by the following statements, "... drugs available at present to control the metastases of melanoma, both overt and occult, have limited efficacy, and it is imperative that new agents be produced against melanoma cells. Finally, the new attempts to control melanoma cells by complex immunological means need to be continued with great determination."[1]

1.1 History of Melanoma Diagnosis

Possibly the earliest published report describing melanoma dates back to 1787 when John Hunter excised a recurring lump on the jaw of a 35 year old male patient. Apparently, after removal, the lump reappeared locally over a period of several years until it was struck during a drunken brawl, from which time it began doubling in size in a matter of just weeks. John Hunter's original specimen was preserved at the Hunterian Museum of the Royal College of Surgeons of England as Specimen #219 and he described this specimen as a

"cancerous fungous excrescence". Confirmation that this specimen was indeed melanoma was verified by Bodenham[2] in 1968 upon examination of the preserved specimen.

It appears that the first definite description of melanoma as an actual disease could be credited to Rene Laennec who was already well known for his previous invention, the stethoscope. Laennec applied the term "melanosis"[3] which is derived from the Greek word "melas" meaning black. The earliest in depth studies on melanoma however, can be credited to the British physician William Norris who noted the fact that this disease can be described as similar to cancer.[4] He also described the following epidemiologic features:

(a) a relationship exists between moles and melanoma,

(b) the disease was more common in industrial than rural areas,

(c) patients generally had light colored hair and fair complexions,

(d) possibility for a hereditary predisposition.

Furthermore, Norris noted several pathological features including the following:

(a) although melanoma was generally black, the degree of pigmentation varied,

(b) subcutaneous deposits may develop at other sites,

(c) dissemination could involve bone, liver, heart and lungs.

With respect to treatment of melanoma, Norris advocated wide excision of the tumor and surrounding tissue and further observed that surgical and medical treatments available at that time were totally ineffective when the disease was widely disseminated. During the first half of this century, treatment of melanoma was based primarily on the recommendations of William Handley[5] who suggested wide local excision of the primary lesion in combination with regional lymph node dissection.

1.2 Ozone Alone?

In the latter half of this century, much concern has been raised over the issue of the depletion of the ozone layer which has been associated with a concomitant increase in exposure to solar ultraviolet rays, thus, a possible increase in the incidence of melanoma from recreational exposure to sunlight is anticipated. As a result of these concerns, the Numerical Weather Center in Montreal provides daily forcasts on regional UV levels which are distributed to newspapers as well as radio and television stations across the country. This information is tabulated in the format of a UV index (a number from 1 to 10, where 1 represents minimal UV risk) in an effort to keep Canadians informed of potential health risks associated with daily UV exposure. Since solar energy in the UV-B region (290-320 nm) is typically absorbed by ozone in the earths atmosphere, it is expected that the depletion of the ozone layer will result in an increase in UV-B flux, particularly in the 295-305 nm band.

1.3 DNA Damage and Mutation

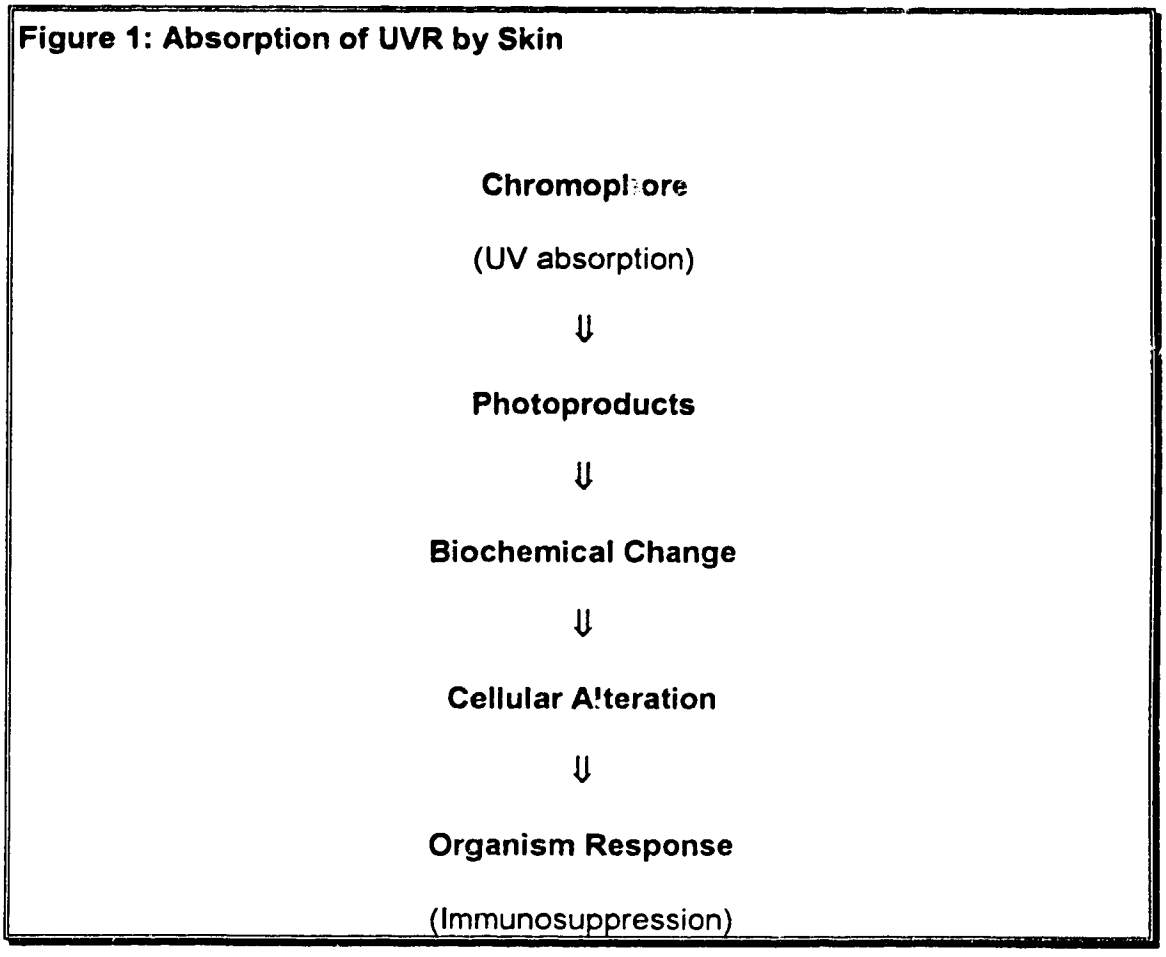
When human skin is exposed to UV radiation, the energy is generally absorbed in the melanocytes by molecules such as melanin, however, absorption of UVR by DNA can result in single and double stranded DNA cleavage along with DNA-protein crosslinking. If the cell is unable to repair the damaged DNA, dire consequences such as the blocking of both RNA transcription and DNA replication may result. As well, inaccurate or untimely repair processes are likely to yield mutations in the DNA structure which can subsequently lead to a variety of undesirable side effects including repressor gene activation or even oncogene activation. Such activation can in turn, translate into the development of transformed phenotypes as outlined in Figure 1.[6]

Data from animal studies has shown that ultraviolet radiation has the ability to alter immune reactions in the skin. For example, mice exposed to suberythemal doses of UVR and then challenged with antigenic chemicals applied directly to the irradiated site failed to display contact allergy.[7] Instead, these mice developed antigen-specific suppressor T lymphocytes which prevented the induction of an immune response upon second challenge. Therefore, what appeared to be only a localized effect (*e.g.*, UV damage to the

skin), was translated to the more serious effect of systemic immune suppression against antigens that enter the body via UVR-exposed skin.

It should be pointed out however, that the etiology of melanoma is likely to be multifactorial involving other factors such as genetic predisposition and exposure to toxic chemicals as well. In any case, cutaneous melanoma arises from malignant transformation of pigment cells in the skin and a deeper understanding of the cellular receptors and mechanisms involved in the growth and spread of melanoma is essential in identifying pharmacological targets for therapy.

Figure 1: Absorption of UVR by Skin



1.3 Melanocytes

Melanocytes are dendritic pigmented cells which are primarily found on the basement membrane of the skin, and their dendrites tend to spread outward towards the surrounding keratinocytes. The pigments, known as the melanins, are highly irregular polymers arrayed in the form of granules and are synthesized in the melanocyte prior to their translocation to the keratinocytes. Melanocytes have also been found in the eye, oropharynx, esophagus, small and large bowel, gallbladder, anus, and vagina. These cells have also been detected in the capsules of lymph nodes leading to the possibility that on rare occasion, melanoma may originate from within the lymph nodes.[8]

Differentiation and growth of melanocytes involves a series of complex events, many of which are not yet clearly understood. The precursors to melanocytes originate in the neural crest (a cluster of cells located in the neural tube) and subsequently migrate from this site to the skin in order to develop into melanocytes. An important observation regarding these precursor cells is that the migration process to the skin involves tissue invasion which is a similar trait to that re-expressed in melanoma cells,[9] although it is not known whether the genes involved are the same.

The melanins, which are synthesized by the melanocytes and more specifically, by specialized organelles known as melanosomes, are light-absorbing pigments that are found throughout the animal kingdom and are produced via distinct biochemical pathways involving the cellular enzyme tyronase.[10] This enzyme is known to catalyze the oxidation of tyrosine to dihydroxyphenylalanine (DOPA), then to DOPA quinone, and subsequent polymerization reactions lead to melanin formation.[11] Interestingly enough, it has been proposed that melanins may actually function as a sink for potentially damaging free radicals.

1.4 Metastatic Melanoma

After the preliminary diagnosis of melanoma has been made, the next, but most crucial question is whether the cancer has spread to the regional lymph nodes and distal organs. The most fearsome aspect of melanoma is metastasis leading to secondary tumor growth, and despite recent improvements in diagnosis and therapy, the majority of deaths resulting from melanoma are

caused by metastasis.[12] In fact, for many patients with malignant melanoma, the metastasis event has in all likelihood already occurred by the time of diagnosis.[13] Thus, in spite of its relatively low incidence, melanoma remains a high mortality cancer and is generally associated with poor prognosis.

2. MELANOMA-ASSOCIATED ANTIGENS

Relatively few discoveries have rivaled the technology for hybridoma selection, screening, and production first described by Kohler and Milstein,[14] and application of monoclonal antibodies as analytical tools for studying cellular and secreted antigens became apparent soon afterwards. In the field of cancer research, mAbs have become indispensable reagents for the detection of small antigenic differences usually encountered between normal and malignant tissues and have been utilized by tumor immunologists for studying cell differentiation, malignant transformation, and tumor progression. Monoclonal antibodies are now routinely applied in the clinic as reagents for the diagnosis of undifferentiated cancers. A second important contribution of mAb technology has been directed towards the identification of fine morphological features resulting in new and more accurate classification schemes for tumors and their metastases. This clearer understanding has in turn, led to improved tumor prognosis due to a more accurate means of monitoring response to treatment and now, mAbs are playing increased roles as therapeutic agents, primarily as selective delivery vehicles for cytotoxic agents to tumor cells.[15]

2.1 Radiolabeled Monoclonal Antibodies

Radiolabeled monoclonal antibodies directed towards tumor associated antigens have found clinical utility in revealing primary, recurrent, metastatic, and occult carcinomas by utilization of radioimmunodetection (RAID) methodology. This process involves injection of the radiolabeled antibody into the patient followed by subsequent imaging of any abnormal radioactive accumulation that results from the binding of the antibody to the tumor associated antigen. As a direct result of the growing interest in this field, several reviews on the application of radiolabeled antibodies for the detection of cancer have recently appeared.[15,16] It is apparent that the development of monoclonal antibodies specific for tumor associated antigens has lagged behind that of nuclear medicine and radiolabeling methods, nevertheless, mAbs are continually gaining widespread use as valuable tools in the radioimmunodetection of cancer.[17]

Enthusiasm resulting from the ability to image tumors by employing antibodies labeled with an appropriate γ-emitting radionuclide has spurred

interest in the study of mAbs labeled with α- and β-emitting radionuclides for the purpose of cancer therapy.[18] Although fraught with a new set of challenges including limitations in current methodology for chelating the radiolabel to the carrier,[19] the advantage of radioimmunotherapy (RAIT) over strategies employing other drug/toxin-mAb conjugates results from the fact that radiolabeled mAbs can destroy cells distally located from the site of attachment whereas drug and toxin conjugated MAbs must be internalized by the cell. Thus, radiolabeled mAbs can distribute their cytotoxic energy to neighboring tumor cells that may be antigen negative or possess cryptic antigen.

2.2 Factors Affecting RAID & RAIT

The factors governing successful application of RAID and RAIT are many and complex, several of which are not clearly understood at present, however, these factors can be classified into five general categories as shown in Table 1 (adapted from Goldenberg and Larson [20]). Factors **3** - **5** outlined in the table are focused primarily on clinical evaluations while factor **2** is dependant on the properties of the radionuclide chosen. Discussion of these factors is beyond the scope of this thesis and in fact, our primary attention was narrowly centered on factor **1a** regarding the specificity of the monoclonal antibody for the tumor associated antigen.

2.3 Differentiation Antigens

Differentiation antigens are antigens that are regulated during cell differentiation and are useful in; (a) distinguishing one cell lineage from another (*e.g.*, melanocytes from nonmelanocytic cells); and (b) identification of cells at various stages of differentiation (*e.g.*, melanoblast or precursor to melanocyte from mature melanocyte).[21] Analysis of melanoma metastasis has revealed that distinct stages of differentiation exist and that the corresponding melanoma cells have different antigenic properties, a fact that readily avails itself to exploitation for diagnosis and therapy employing mAb technology. Indeed, several melanoma associated antigens have already been detected by monoclonal antibodies and a thorough list has been tabulated by Hersey.[22] Of the more than twenty antigens listed, only three are under current clinical scrutiny as potential targets for therapy; (**1**) melanotransferrin (p97/gp95); (**2**) melanoma chondroitin sulfate; and (**3**) the ganglioside antigens. It should be pointed out that each one of these three antigen groups is expressed on normal tissues as well as on

Table 1: Factors Affecting RAID & RAIT

1. Character of the Antibody;
   - **a.** Specificity for the tumor-associated antigen
   - **b.** Antibody purity
   - **c.** Affinity
   - **d.** Isotype
   - **e.** Whole versus fragment
   - **f.** Dose
   - **g.** Species of origin
   - **h.** Clearance and pharmacokinetics

2. Nature of the Radiolabel;
   - **a.** Physical properties; half-life, mode of decay, and photon or particle energy
   - **b.** Chemical properties; stability of chelate, method of conjugation, mAb-conjugate stability, and immunoreactivity
   - **c.** Specific activity
   - **d.** Dose
   - **e.** Clearance of radiolabel, excretion, critical organ uptake (*ie.* liver, bone marrow)

3. Tumor Properties
   - **a.** Size and location of tumor(s)
   - **b.** Antigen density and modulation
   - **c.** Vascularization and vascular permeability
   - **d.** Target to nontarget ratio
   - **e.** Distribution of tumor associated antigen in other body sites or fluids
   - **f.** Presence (or absence) of circulating antigen and/or complex

4. Imaging Protocol
   - **a.** Planar
   - **b.** Emission tomography (SPECT)
   - **c.** Computer-assisted subtraction or other manipulations
   - **d.** Time interval between administration and measurement

5. Other Factors
   - **a.** Route of administration
   - **b.** Presence or absence of anti-mAb response in host

melanoma cells, however, the level of antigen expression is quantitatively higher on melanoma cells as compared to their normal counterparts. It is this quantitative difference that is being exploited for tumor targeting and selective antitumor effects.

2.3.1 Melanotransferrin

Melanotransferrin (p97/gp95) is a 95-97 kD cell surface sialoglycoprotein[23] related to transferrin and lactoferrin that has been shown to bind ^{59}Fe, implying a functional role in iron metabolism at the cell surface. One qualitative difference between these two protein molecules however, is that p97 is membrane bound and its role in iron transport may be different than the serum iron binding protein transferrin. Most melanomas express increased levels of the p97 antigen as compared to normal tissues which include the uterus, bladder, muscle, and colon. Several distinct epitopes of this glycoprotein have been mapped using mAbs, and of all of the human tumor associated antigens, melanotransferrin has been the most thoroughly characterized on a molecular level. One particular anti-p97 mAb, 96.5, an IgG2a,[24] has been clinically evaluated as an imaging agent both as the ^{111}In-DTPA-96.5 conjugate[25] and as the corresponding chloramine T iodinated (^{131}I) Fab antibody fragment.[26]

The anti-p97 antibody 96.5 has contributed significantly to the development of radiolabeled immunoconjugates directed towards melanoma, however, some drawbacks have continued to frustrate clinical applications with this mAb. Two of the most noteworthy include; (1) variable and heterogeneous expression of p97 among patients and; (2) large uptake of the administered dose by the liver, thereby reducing the amount available for tumor uptake.[27]

2.3.2 Melanoma Chondroitin Sulfate Proteoglycan

This melanoma-associated proteoglycan antigen possesses a core protein of about 240-260 kD which is glycosylated with both N- and O-linked carbohydrates resulting in a proteoglycan with a combined molecular weight of about 450 kD.[28] This antigen is expressed on most (>90%) melanoma tissue specimens, and although its expression appears to be restricted in normal tissues, this antigen is also expressed on small blood vessels, basal cells, skin, and renal collecting tubules. A series of mAbs have been generated against this melanoma-associated proteoglycan, and one in particular, monoclonal antibody

NR-ML-05,[29] an IgG2b, has been incorporated into a melanoma imaging kit. This kit utilizes the diamide dimercaptide ^{99m}Tc chelate conjugated to an NR-ML-05 Fab fragment.[30] It should be mentioned that this diagnostic kit is currently undergoing evaluation for the radioimmunodetection of melanoma in a multicenter phase III clinical trial.[30] The apparent success of this melanoma imaging agent presents a firm foundation for future development of therapeutic isotope, drug, and toxin conjugates based on this murine monoclonal antibody.

2.3.3 Disialogangliosides

Sialic acid-containing glycolipids, or gangliosides, are a group of biologically active cell-surface carbohydrate molecules[31] and melanoma tends to be particularly rich in ganglioside content.[32] One branch of this diverse group of oligosaccharides includes those possessing the characteristic Neu5Acα(2→8)Neu5Ac (di-Neu5Ac) unit such as found in the disialogangliosides GD_2 and GD_3. These sialosides are found in fetal brain,[33] glia, neurons, adrenal medulla, and melanocytes as well as in tumors of neuroectodermal origin.[34] Thus, although these gangliosides are not tumor-specific antigens, it appears that they display a relatively restricted distribution in normal tissues, and it has been suggested that these carbohydrate antigens may serve as potential targets for active specific immunotherapy.[35]

Due to their strategic location on the cell surface, it therefore comes as no surprise, that a relatively large number of murine and human mAbs have been found to be directed against carbohydrate molecules, several of which may be classified as tumor-associated antigens. As it turns out, melanoma cells are abundant in ganglioside content with GM_3 and GD_3 being the most abundant followed by lesser amounts of GM_2 and GD_2.[36] Monoclonal antibodies directed towards naturally occurring gangliosides such as GD_3 have been generated.[37,38,39] Although present on normal melanocytes, expression of these antigens on normal tissues is, as indicated earlier, significantly lower than on melanoma cells. For example, in the case of melanoma, normal melanocytes possess sialyloligosaccharides in the following ratio; GM_3 (>95%), GD_3 (<5%), and only trace amounts of GM_2, while upon neoplastic transformation, the GD_3 content is elevated to 20-80% of the total ganglioside content and Neu5,9Ac_2-GD_3, which has to date been elusive on normal human melanocytes, is found in amounts of up to 10%.[40]

2.3.4 Glycolipid Antigens

Until recently, carbohydrates have been the neglected siblings of pharmaceutical research, due in part to attention captured initially by proteins and then by nucleic acids. Current research efforts in the field of glycotechnology have yielded increased understanding regarding the role that these ubiquitous molecules play *in vivo*, and it is anticipated that carbohydrate based drugs may provide significant contributions to the pharmaceutical industry in the not too distant future.

There are, in fact, far more potential variations created by linking monosaccharides together to form oligosaccharides, than variations which result from linking amino acids to form proteins. It may have been this initial observation of great heterogeneiety that led to the formulation of the viewpoint that these molecules lack special function, thus relegating carbohydrate antigens to low priority targets for fundamental pharmaceutical research. An important ongoing task of the pharmaceutical industry however, is to seek out effective ways to diagnose, treat, and possibly prevent human cancer. In depth studies as to the role cell surface carbohydrates in this regard are essential since virtually all cancer cells are characterized by aberration in structure as well as function, including altered glycosylation patterns of the surface membrane. The resultant carbohydrate epitopes may in turn, serve as potential targets for the detection and possibly even the treatment of human cancer. In fact, this idea has been reduced to practice by application of monoclonal antibody reagents for analysis of tumor associated cell membrane antigens, and interestingly enough, several of these antigens have been identified as carbohydrate in nature.[41] It is possible that aberrant glycosylation contributes to inappropriate cell-cell and cell-matrix interactions leading to properties such as uncontrolled cell division, invasiveness, and metastatic potential typically displayed by certain tumor cells.

The presence of tumor associated lipid antigens is not a recent observation, and the presence of these types of molecules on tumors was suggested more than 60 years ago by Witebsky[42] and by Hirszfeld *et al.*[43] who independently demonstrated that certain rabbit antisera obtained by immunization with tumor tissue homogenate displayed a preferential complement fixation reaction with lipid extracts of the original tumor immunogen. The exact nature of these lipid antigens proved to be elusive at that time.

2.3.5 Ceramide Composition

Glycolipids are amphipathic membrane-bound constituents that are found in mammalian cells predominantly as glycosphingolipids and are composed of the long-chain base sphingosine (see Table 2), a fatty acid, and a sugar residue as shown below:

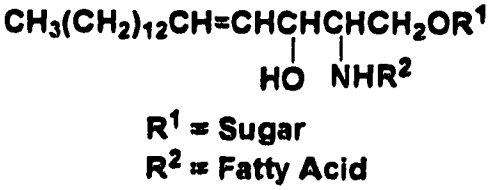


Table 2: Composition Of Ceramide

Sphinganine[a]	Fatty Acid[b]
Sphinganine (d18:0)	Palmitic (16:0)
Sphingosine (d18:1)	Stearic (18:0)
Phytospingosine (t18:0)	2-Hydroxystearic (h18:0)
	Arachidic (20:0)
	Behenic (22:0)
	Lignoceric (24:0)
	Cerebronic (h24:0)
	Nervonic (24:1)

(a) Number in parenthesis denotes number of carbon atoms:double bonds and hydroxyl constituents (d or t).

(b) Number in parenthesis denotes chain length:double bonds and presence or absence of hydroxyl groups (h) at C-2.

The hydrophobic lipid moiety is called ceramide and serves to anchor the glycosphingolipid into the plasma membrane. Ceramide, as shown in Table 2, consists of a sphingoid base derived from the parent (2**S**,3**R**)-2-amino-1,3-octadecanediol, commonly referred to as sphinganine, which is *N*-acetylated with a long chain fatty acid.[44]

2.3.6 Carbohydrate Composition

Of the vast array of pyranose structures available, the repertoire in human cells consists only of the following eight:

Sugar	Abbreviation
D-Galactose	Gal
D-N-Acetylgalactosamine	GalNAc
D-Glucose	Glc
D-N-Acetylglucosamine	GlcNAc
L-Fucose	Fuc
D-Mannose	Man
D-Xylose	Xyl
N-Acetylneuraminic acid	Neu5Ac

The hydrophilic sugar sequence is synthesized by the systematic stepwise transfer of monosaccharides to ceramide via nucleotide sugar donors and the resulting glycosphingolipids can be classified into several distinct series based upon skeletal structure of their carbohydrate chains. The basic skeletal arrangements of six glycolipid series typically found in mammalian cells are shown in Table 3.

In all but the gala series, which originates from galactosylceramide (Gal-Cer), the glycolipid series are built from glucosylceramide (Glc-Cer), which is formed by the action of a glucosyl transferase utilizing uridine 5'-diphosphate (UDP)-Glc and ceramide as substrates. Subsequently, addition of galactose then

leads to the common lactosylceramide (Gal-Glc-Cer) foundation, and from this point, skeletal structure diversification occurs. Thus, addition of galactose in either an α(1→4) or an α(1→3) manner gives rise to the globo series or isoglobo

Table 3: Classification of Glycosphingolipids

Classification	Typical Structure
Ganglio series	Galβ1→3GalNAcβ1→4Galβ1→4Glc-Cer
Globo series	Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer
Isoglobo series	GalNAcβ1→3Galα1→3Galβ1→4Glc-Cer
Lacto series	Galβ1→3GlcNAcβ1→3Galβ1→4Glc-Cer
Neolacto series	Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Cer
Gala series	Galα1→4Gal-Cer

series respectively. On the other hand, addition of N-acetylglucosamine results in the formation of GlcNAcβ(1→3)Galβ(1→4)Glc-Cer, and further elongation by the addition of galactose in a β(1→3), or alternatively, β(1→4) fashion, leads to the formation of the lacto series or the neolactose series glycosphingolipids respectively. It is noteworthy to mention that these last two series are also found in terminal sequences of complex-type N-glycans as well as O-glycans extended from mucin cores.

2.3.7 Ganglioside Nomenclature

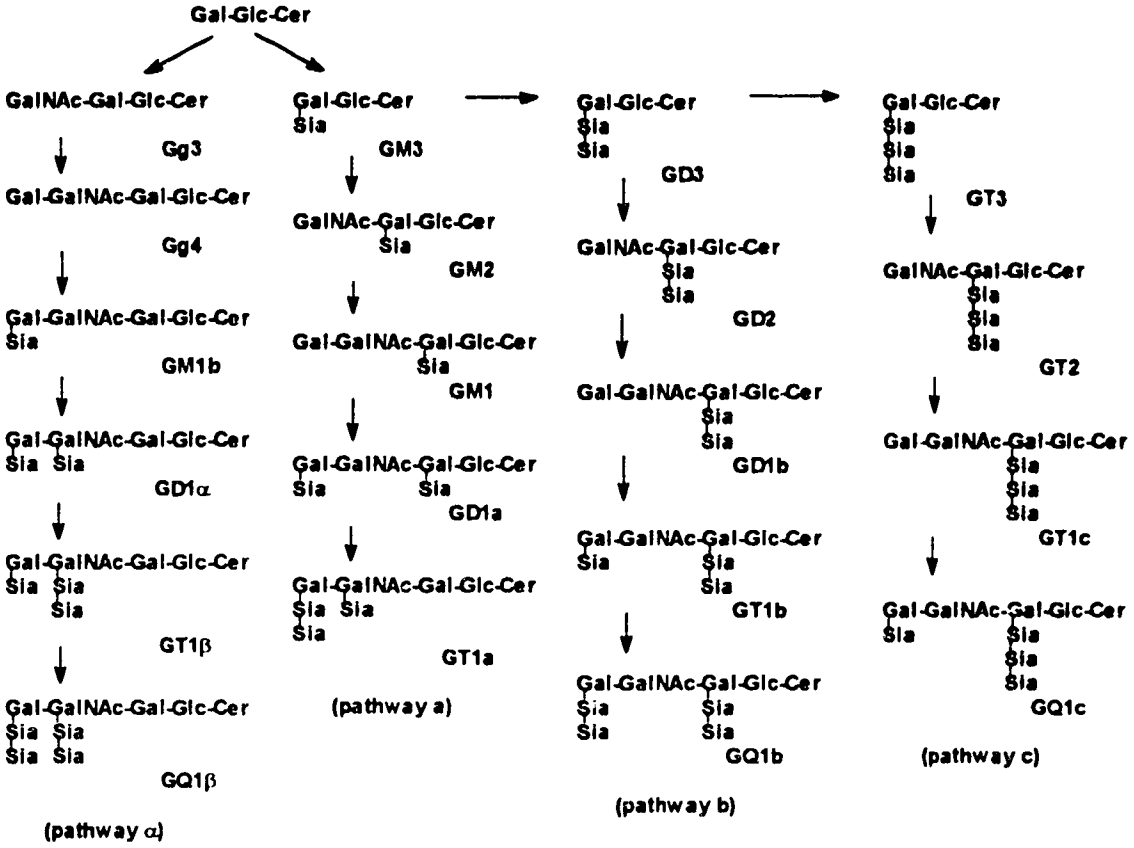
Glycosphingolipids may also occur in nature in their sialylated form, and in the broadest sense, the term ganglioside encompasses all sialic acid containing glycosphingolipids. According to the system of nomenclature put forth by Svennerholm,[45] all of the members of the ganglio series gangliosides receive the designation capital letter G based on the neutral backbone outlined in Table 3. The sialic acid content of the given ganglioside is futher designated by a second letter specifying the number of sialic acid residues: A (asialo-), M (monosialo-), D (disialo-), T (trisialo-), Q (quadrasialo-), *etc.* where the acidic sugar can be either N-acetylneuraminic acid (NANA) or N-glycolylneuraminic

acid (NGNA). Finally, a subscript is added, which is rooted historically on chromatographic behavior,[46] by application of the simple formula 5-n wherein n represents the number of neutral sugars. Alternatively, for asialo gangliosides, instead of the capital letter A, the letter g has also been used to represent the neutral ganglio series backbone followed by a subscript equal to n.

2.4 Ganglioside Biosynthesis

The general biosynthetic pathway leading to the various ganglio series gangliosides has been worked out[47,48] and is shown in Figure 2. A key step in

Figure 2: Biosynthetic Pathway of the Ganglio Series Gangliosides



the overall biosynthesis is the addition of sialic acid in an α(2→3) linkage to yield GM_3, which then serves as the starting point for two series. For entry into

the pathway **a** ganglioside series, enzymatic addition of N-acetylgalactosamine initiates further elaboration by the addition of galactose as shown in Figure 2. Alternatively, addition of a second sialic acid, this time by way of an $\alpha(2\rightarrow8)$ linkage to the sialic acid terminus of GM_3 leads to GD_3. This tetrasaccharide again sits at the crossroad between two series, the pathway **b** series via addition of N-acetylgalactosamine, and pathway **c** series, by the addition of yet another sialic acid to yield GT_3. Pathway α gangliosides are formed by elaboration of the lactose backbone via $\beta(1\rightarrow4)$ addition of N-acetylgalactosamine followed by $\beta(1\rightarrow3)$ addition of galactose followed by sialylation at this terminus.

The enzyme responsible for synthesizing Glc-Cer appears to be unique in its' ability to perform this task[49] however, the subsequent galactosyltransferase is not well defined and current evidence supports a distinct enzyme possessing a relatively restricted acceptor specificity. The N-acetylneuraminic acid transfer enzyme, sialyltransferase I (GM_3 synthase) is responsible for the transformation of Lac-Cer into GM_3 and this enzyme also exhibits a narrow range of specificity. Likewise, sialyltransferase II (GD_3 synthase) involved in the synthesis of GD_3 from GM_3 appears to be highly specific.[50]

In direct contrast to these highly discriminatory enzymes, addition of subsequent sugars along a particular pathway appears to be catalyzed by enzymes displaying relatively broad substrate specificity. Thus, there appears to be a single N-acetylgalactosaminyltransferase that catalyzes the synthesis of Gg_3 (GA_2), GM_2 and GD_2, and a single galactosyltransferase responsible for subsequent elaboration to Gg_4 (GA_1), GM_1 and GD_{1b}.[50] As well, sialylation at the $Gal\beta(1\rightarrow3)GalNAc$ terminus leading to GM_{1b}, GD_{1a} and GT_{1b} appears to be orchestrated by a single enzyme, sialyltransferase IV, and further chain extension is accomplished by sialyltransferase V.

2.4.1 Sialyltransferase Levels in Melanoma

Carbohydrates are not a direct product of DNA in terms of one gene possessing their respective molecular blueprint, however, glycosyltransferase enzymes, which are under direct genetic control, play a key role in the determination of cellular expression of glycolipids. For example, human melanoma cells tend to display relatively high levels of GM_3 and GD_3 whereas normal melanocytes maintain a high level of GM_3 expression while GD_3

expression is minimal in these cells. It is therefore reasonable to suspect that one cause for this apparent difference may be directly linked to increased levels in expression of sialyltransferase II in melanoma, which is responsible for the transfer of N-acetylneuraminic acid from the sugar nucleotide donor CMP-NeuAc to GM_3. Another factor that may play a role in this phenomenon may be the restricted availability of either the sugar donor UDP-N-acetylgalactosamine or the corresponding enzyme, both of which are requisite for further transformation of GM_3 and GD_3.

Although melanomas and neuroblastomas are both considered to be tumors of neural crest origin, one major difference between human melanoma cells and neuroblastoma cells is the relatively abundant expression of GD_3 in the former in contrast to the low levels found in the latter. Recently, Lloyd and coworkers[51] have reported a direct correlation between GD_3 synthase levels and the level of cell surface expression of GD_3 in several melanoma cell lines. In an effort to further elucidate factors governing the expression of gangliosides in malignant melanoma, Ruan and Lloyd[52] have analyzed galactosyl-, N-acetylgalactosyl-, and sialyltransferase levels in crude tumor cell membranes and have found that GD_3 synthase was a dominant enzyme in the majority of the melanoma tumors and cell lines examined whereas activity of this enzyme was minimal in neuroblastoma cell lines. These results served as confirmation that melanoma cells typically display high levels of sialyltransferase II (SAT-II) activity.

Neuroblastoma cells, on the other hand, were found to express elevated levels of GD_2. Surprisingly, even though GD_3 is the direct precursor to GD_2 along the pathway **b** series, neuroblastoma cells express only trace levels of SAT-II! The conclusions drawn from these studies were twofold; (1) melanoma cells characteristically produce increased levels of GD_3 synthase compared to GD_2 synthase allowing for appreciable levels of GD_3 to leave the Golgi apparatus and accumulate at the cell surface; and (2) the high level of expression of GD_2 in neuroblastoma may be directly linked to the efficient action of GD_2 synthase present in these cells on the small amount of GD_3 produced.

The phenotypic ganglioside patterns displayed by these two cell types, both of which originate from tumors of the neural crest, can therefore be explained in terms of the levels of expression of the various glycosyltransferase

enzymes in concert with the availability of the precursor substrates upon which these enzymes act. These results further serve to reinforce the important role that both GM_3 synthase (SAT-1) and GD_3 synthase (SAT-II) play in governing the resultant ganglioside profiles in melanoma and neuroblastoma cells by directing the biosynthesis along either pathway **a** or **b**. In a much broader sense, it is clear that processes governing the aberrant synthesis of carbohydrate antigens are extremely complex and further studies directed at delineation of factors which control transcription and translation of the key enzymes involved in these processes are essential.

2.5 Anti-ganglioside Monoclonal Antibodies As Antigen Probes

There remains little doubt that fields of study pertaining to the analysis of molecules that populate the mammalian cell surface have been direct beneficiaries of monoclonal antibody technology. Owing to the fact that highly specific mAbs directed towards determinants of known sugar sequence and anomeric configuration can be generated, these reagents are valuable aids in probing structure-function traits of various carbohydrate antigens. Thus, by application of these reagents in studies aimed at elucidation of altered glycoprotein as well as glycolipid expession on tumor cells, it may be possible to obtain a deeper understanding on a molecular level, as to the nature of structural changes associated with malignant transformation. Also, since the biosynthesis of these sugars is orchestrated by specific enzymes, it may then be possible to link their level of expression to specific genetic aberrations within the tumor cell.

In this regard, studies employing monoclonal antibodies directed to protein and glycoprotein antigens are greatly complicated by virtue of the fact that glycoproteins often show microheterogeneity and additional information on primary amino acid sequence, protein conformation, and the three dimensional structure is a fundamental prerequisite for such studies. In contrast, studies involving glycolipid antigens are relatively free from these demanding requirements.

Reisfeld and Cheresh have suggested that monoclonal antibodies directed towards gangliosides possess potential utility as reagents for the immunodiagnosis, immunotherapy, and biological characterization of malignant

melanoma cells.[53] Indeed, application of monoclonal antibodies as diagnostic reagents for human melanoma has revealed that cells derived from metastatic lesions display a marked increase in expression of certain gangliosides as compared to cell lines originating from primary melanoma lesions from the same patient.[54] Furthermore, these highly specific anti-ganglioside mAbs display the same degree of precision and specificity as mAbs directed against peptide antigens.

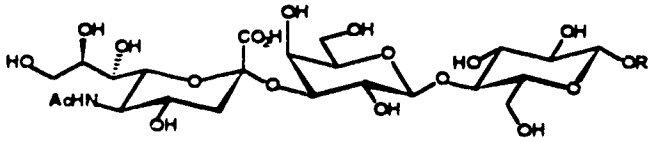
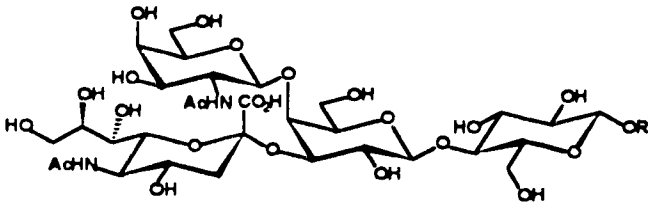
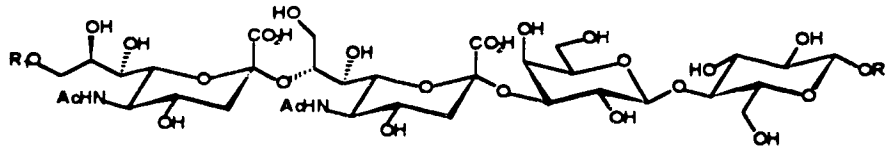
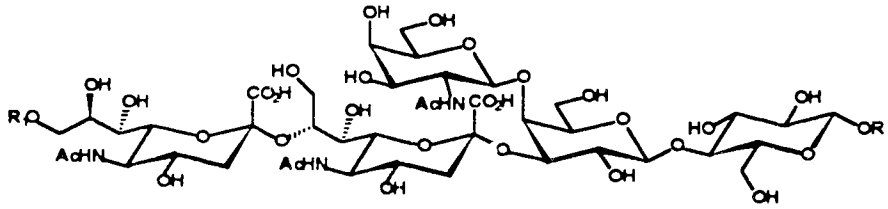
Many initial attempts to elicit anti-ganglioside mAbs involved immunization with complex antigen mixtures including whole cells and tissue extracts due to the fact that gangliosides alone were considered to be poor immunogens. Direct application of immunization protocols originally employed in immunization work with bacterial lipopolysaccharides, namely adsorption of the glycolipid antigen onto *Salmonella minnesota,*[55] to the asialo-ganglioside Gg_3 antigen by Hakomori[56] proved to be an excellent technique for the generation of anti-ganglioside mAbs.

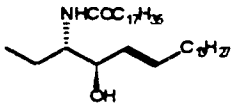
2.5.1 Anti-GM_3 and Anti-GD_3 Monoclonal Antibodies

The chemical structures of six commonly expressed melanoma associated gangliosides are shown in Figure 3 and highly selective monoclonal antibodies have been raised against these stuctures. Owing to the fact that such a topic is beyond the scope of this thesis, only mAbs directed against the pertinent GM_3 and GD_3 epitopes will be highlighted in this brief overview.

Prompted by the initial observation that murine cytotoxic T lymphocytes specific for syngenic melanoma cells killed both human and murine melanoma cells, a stable mouse hybridoma was established by immunization with murine B16 melanoma cells.[57] The resultant mAb, M2590, an IgM, possessed high affinity for the GM_3 trisaccharide which turned out to be a cross-species melanoma antigen. The observation that this mAb was highly specific for melanoma cells even though this antigen is present on normal cells was somewhat perplexing, leading Hakomori and coworkers to speculate that this normal cell component can at a certain threshold density, generate melanoma antigenicity.[58] An IgG3 mAb, DH2,[59] was established by immunization with GM_3-lactone adsorbed onto *Salmonella minnesota*, and was subsequently

Figure 3: Melanoma Associated Gangliosides

Structure	Notation
	GM_3
	GM_2
	R_1 = H, GD_3 R_1 = Ac, Neu5,9Ac_2-GD_3
	R_1 = H, GD_2 R_1 = Ac, Neu5,9Ac_2-GD_2

R = Ceramide = 

determined to be reactive with GM_3. By employing the novel technique of immunization with liposomes containing a mixture of gangliosides extracted from malignant melanoma, Schriever and coworkers were successful in generating an anti-GM_3 mAb MacG1 (IgG2),[60] which along with the anticipated melanoma reactivity, detected tumor-infiltrating macrophages. Recently, Yamaguchi *et al.*[61] successfully established an anti-GM_3 human-mouse hybridoma FCM1 (IgM) by application of a protocol which involved the isolation of human monoclonal antibodies from lymphocytes of melanoma patients, followed by Epstein-Barr virus (EBV) transformation and subsequent fusion with mouse NS-1 myeloma cells.

Several anti-GD_3 mAbs have been established to date, and perhaps the most recognized, R24 (IgG3),[37] has been the subject of ongoing clinical studies in melanoma patients.[62] The striking specificity for melanoma displayed by R24 was firmly established by application of an indirect immunoperoxidase staining technique on cyropreserved unfixed human tissue sections[62] wherein 21 of 21 primary and 37 of 37 malignant tissues displayed intense surface fluorescence. The value of GD_3 as a diagnostic marker was confirmed independently by recent studies aimed at the determination of the frequency of this marker in patients with metastatic malignant melanoma, and in this study,[63] 111 of 119 (93%) tissues were R24 positive.

A comparison of mAb 4.2 (IgM), prepared by Yeh *et al.*,[64] with R24 revealed that although both mAbs reacted with melanoma antigen and GD_3 from brain source in binding assays in plastic plates, only R24 reacted well with brain GD_3 as determined by the TLC immunostaining technique.[65] Since the GD_3 antigen is expressed on the normal cells, it comes as somewhat of a surprise that only the melanoma ganglioside is recognized by mAb 4.2 when compared to the normal brain ganglioside. Also puzzling is the fact that both R24 and 4.2 recognize melanoma but not the same epitope on GD_3. Two possible explanations for this phenomenon have been put forward by Nudelman;[38] (1) GD_3 may be present in unusually high concentrations on melanoma cells and/or; (2) the lipid moiety may be longer in the melanoma associated ganglioside than the C18:0 fatty acid which is the major constituent in normal cells, making this antigen sterically more accesible. Thus, it may be that the longer ceramide tail

affects membrane organization in some way resulting in increased antigenicity as compared to antigens present on normal cells.

Other anti-GD_3 mAbs have recently been reported[66,67] and mAbs cross reacting with both GD_2 and GD_3 have also been generated.[68,69] It should also be pointed out that an anti-GD_3 human-mouse hybridoma HJM1 (IgM) was produced in the same manner from lymph node lymphocytes as was described for GM_3.[70] Due to the fact that regional lymph node resection is a common component of melanoma therapy, the availability of such surgical samples may make this disease ideally suited for studies involving human monoclonal antibodies and anti-idiotypes by fusion and screening of the lymphocytes thus obtained.

The studies briefly highlighted in this section have indicated that the ability for gangliosides to behave as cell surface melanoma-associated antigens depends on several key factors. One of these factors, which is presumably essential for any antigen being considered, is that of cell surface density. In the case of glycolipid antigens, it has also been postulated that ceramide composition and chain length may be factors worthy of consideration, and although the antigenic site is presumed to be the carbohydrate moiety, presentation at the cell surface may be influenced by ceramide composition. As well, the ceramide composition may contribute significantly to glycolipid organization in the cell membrane.

In an effort to shed some light on these issues, a study on the effect of variation of chain length of the fatty acid residue of GM_3-Ceramide on the binding properties of these analogs to anti-GM_3 mAb M2590 was initiated by Itonori and coworkers.[71] Examination of the binding ability by modified enzyme-linked immunosorbent assay (ELISA)[72] as well as by direct binding on thin layer chromatography plates[65] revealed that although GM_3 derivatives with long fatty acid chains reacted well with M2590, those with short chains failed to bind. Inhibition studies confirmed that antigenic activity towards M2590 appeared with the N-hexanoyl derivative and subsequently increased upon further extension of the acyl moiety.

It should be mentioned in passing, that in all studies reported to date, immunizations resulting in the production of mAbs have been carried out on

gangliosides obtained from natural sources (*i.e.* possesing ceramide as the aglycon) and studies have not yet been carried out on synthetic GM_3 and GD_3 materials with less lipophilic aglycons. In any case, the reason that certain cell surface components such as GD_3 behave as cryptic antigens in normal cells while becoming highly exposed in melanoma cells still remains to be clarified. Hopefully, synthetic analogs will be useful in elucidating the key molecular features required for optimum binding.

2.5.2 MAb Based Detection of GM_3 and GD_3 in Tumor Cells

The ability to detect cell surface antigens on biopsied tumor cells has now become a basic requirement in the *in vitro* diagnosis of malignant cells for which monoclonal antibodies are well suited. Success in this area is a prerequisite to subsequent application of the mAb in diagnostic imaging and therapeutic procedures. Since melanoma cells are characteristically noted for quantitative and qualitative changes in ganglioside components, mAbs may be used as probes in determining the distribution patterns of these molecules in normal and malignant tissues.

Studies involving analysis of tissue sections from melanoma patients by employing anti-ganglioside mAbs M2590 (anti-GM_3), R24 (anti-GD_3), and 14.18 (anti-GD_2) revealed that approximately 60% of primary and 75% metastatic lesions expressed GM_3 while GD_2 was found in 25% and 50% of these lesions repectively.[73] On the other hand, virtually all of the primary and metastatic melanomas examined in this study expressed GD_3, however, the percentage of actual primary (data not shown) and metastatic (see Table 4) cells within the lesion that expressed GD_3 was quite variable. These results imply that strategies involving immune attack based on mAbs targeted at this antigen alone could not, in theory, completely eliminate all of the cancer cells within the lesion. In this regard, radiolabelled mAb conjugates may however, possess a distinct advantage over the corresponding toxin conjugates.

On another front, anti-ganglioside mAbs in concert with the highly sensitive immune thin-layer chromatography (ITLC) procedure as adapted by Ritter *et al.*,[74] have been found to be indispensible tools in the detection and quantification of gangliosides obtained directly from human melanoma tissues.[75] Quantification measurements of the tumor extracts were performed by

photodensitometric evaluation at 580 nm and the results indicated an average of 72 μg of GM_3 and 54 μg of GD_3 per gram of melanoma specimen, which according to calculations performed by Hamilton and coworkers, represents approximately 10^8-10^9 molecules per cell. As a result of their efforts in imaging human tumor xenographs using ^{111}In-labeled mAbs, Goldenberg and coworkers have estimated that a minimum of approximately 10^7 target molecules per cell are necessary for efficient antibody-dependant cell-mediated cytotoxicity,[76] thus, in this respect, GD_3 is potentially an effective target.

Table 4: Ganglioside Profile of Metastatic Melanoma[a]

	Percent range of antigen positive cells in section		
	< 5%	5 - 50%	> 50%
Lymph nodes			
GM_3	7[b] (21)[c]	7 (21)	20 (58)
GD_3	0 (0)	5 (15)	29 (85)
GD_2	19 (55)	9 (30)	6 (15)
Subcutaneous melanoma			
GM_3	3 (14)	5 (24)	13 (62)
GD_3	0 (0)	4 (19)	17 (81)
GD_2	10 (48)	4 (19)	7 (33)

(a) adapted from reference 73

(b) number of patients displaying indicated percent range of positive cells

(c) corresponding percentage of total patients in each category

2.5.3 GM_3:GD_3 Ratio

Normal melanocytes possess GM_3 (> 95%) as a major ganglioside, along with minor amounts of GD_3 (< 5%), and sometimes trace quantities of GM_2.[77] Upon neoplastic transformation, the GM_3:GD_3 ratio changes from this relatively constant 19:1 value, to a variable ratio up to 1:15,[36] thus, GM_3 levels positively correlate with favorable prognosis in both biopsy and in cultured melanoma.[78] This increase in GD_3 expression has led to strategies employing anti-GD_3 mAbs in the passive immunotherapy of melanoma.[62,79]

To see if this phenomenon is maintained upon metastasis, Tsuchida *et al.* studied levels of GD_3 expression in tumor biopsy specimens from lymph nodes and subcutaneous tissues.[78] When several tumor specimens were taken from the same patient, only slight deviations in the amount of GD_3 were observed irrespective of both tumor site and date of biopsy. A secondary observation was that the total sialic acid content of biopsy melanotic melanoma was characteristically greater than that of amelanotic melanoma. When taken in conjunction with the fact that the absence of melanin in melanoma cells is typically an indication of undifferentiation,[80] it is possible that tumor cell differentiation may be related to both melanization and ganglioside composition. As well, the higher mortality nodular melanoma characteristically displayed higher levels of sialic acid content than superficially spreading melanoma. This observation led Tsuchida and coworkers to postulate that the resulting increase in negative charge distribution at the cell surface, due to the carboxylate moiety of sialic acid, may be partially responsible for the increased adhesion capacity of tumor cells to other cells thereby influencing metastasis.

Although patients treated for localized thin lesions experienced cure rates in the range of 90-95%,[81] unfortunately, the corresponding rate for patients with lymph node metastasis (Stage II disease) was only 25-35%. The ganglioside ratio may be a clinically significant prognostic indicator for patients with Stage II disease. Thus, patients identified as part of the poor prognostic group based on their tumor GM_3:GD_3 ratio may be more suited for ganglioside based therapy. Ravindranath and coworkers[82] have shown that the GM_3:GD_3 ratio displayed by surgically excised tumors is useful in predicting the probability of survival after surgery and have indicated that this ratio may be valuable in developing therapeutic programs for the management of melanoma.

Forty-two patients with Stage II disease were subdivided into three groups based on tumor biopsy GM_3:GD_3 ratio as a percentage of lipid bound sialic acid (% LBSA) as follows; Group I (15:1 to 1.5:1, 10 patients), Group II (1.4:1 to 1:1.4, 13 patients), and Group III (1:1.5 to 1:5, 19 patients). From group I, 57% of the patients survived five years in contrast to only 14% of the patients in each of the other two groups. It is possible therefore, that the GM_3:GD_3 ratio may be indicative of the degree of tumor differentiation, and although an unanticipated finding, appears to be a well-defined biochemical criterion suitable for the prognosis and therapeutic management of melanoma.[82]

It should be pointed out however, that these results were obtained by densiometric measurement of resorcinol stain intensity under the assumption that signal intensity was directly proportional to the number of sialic acid residues. Some caution in interpretation should therefore be exercised and anti-GM_3 and anti-GD_3 mAbs may assist in improving the reliability of such measurements.

2.6 Gangliosides in Cell Adhesion

Although carbohydrates are present on the surface of both normal cells and tumor cells, in many instances, the exact functional role of these molecules remains to be clearly delineated. It is known that orderly cell-cell or cell-substratum interactions are key processes in the formation of tissues and organs in multicellular organisms, and defects in this basic process result in serious pathological conditions including neoplastic transformation. Cell recognition and adhesion are complex multistep processes, which may be initiated by specific molecular interactions between cells. Information on the involvement of glycosphingolipids is now beginning to emerge. For example, binding of leucocytes at sites of inflammation is now known to be mediated by selectin-carbohydrate interactions between ELAM-1 and sialyl Lewis x.[83,84] In such cases, synthetic derivatives[85] may be of value in studying exact binding requirements of carbohydrates to various protein receptors.

Cell adhesion studies *in vitro* with anti-GD_3 mAb R24, mentioned earlier, and with anti-GD_3 MB3.6[34] have revealed localization of these mAbs in focal adhesion plaques and on human melanoma cells (Melor) as determined by indirect immunofluorescence, and subsequent studies have implicated GD_3 in

melanoma cell attachment mechanisms.[86] Murine antibodies against GD_3 and GD_2 inhibited attachment of human melanoma and neuroblastoma cells respectively to immobilized extracellular matrix components such as fibronectin, vitronectin, collagen, and laminin.[87] Furthermore, these results were in accord with earlier observations by Pukel *et al.*,[37] that mAb R24 induced melanoma cell rounding indicative of modification of cell adhesive properties. This property was not displayed by mAbs directed to several other types of determinants present on the cell surface. Further investigation into this phenomenon has led Reisfeld and Cheresh[53] to postulate that both GD_3 and GD_2 possess the ability to interact directly with melanoma cell surface glycoprotein receptors for fibronectin or vitronectin. Such interactions may in turn, serve to potentiate favorable receptor conformations leading to optimal cell adhesion. Thus, mAbs directed against gangliosides involved in the cell attachment process, would be expected to directly interfere with this essential process involved in tumor cell invasion and distal metastasis.

The fact that a majority of the recognition molecules complementary to carbohydrate molecules are proteins such as lectins and glycosyltransferases is well established. Recently however, some evidence implicating carbohydrate-carbohydrate interactions has been put forth. Cell-cell adhesion processes based on homotypic interactions between the carbohydrate moieties of the stage specific embryonic antigen Lewis x (Le^x) have been noted[88] and this finding has led to the speculation that homotypic Le^x-Le^x interactions may provide the triggering event in the aggregation of murine tetracarcinoma F9 cells.

As mentioned earlier, inhibition of interactions between melanoma cells by anti-ganglioside mAbs suggests that these cell surface components may be involved in the cellular adhesion process of these cells. Compelling evidence for the role of ganglio-series gangliosides in adhesion processes comes from recent studies on specific heterotypic interactions between murine melanoma B16 cells, which express high levels of GM_3, and murine lymphoma L 5178 cells, which express significant levels of Gg_3.[89] This unexpected result arose from the observation that [^{14}C]-cholesterol labeled GM_3 liposomes displayed specific adhesion to Gg_3 coated polystyrene while no such interactions were found for plates coated with several other GSL's including GM_1. Also, GM_3 liposomes did not interact with GM_3 coated solid phase nor did Gg_3 liposomes adhere to Gg_3-

coated plates. Extrapolation to the cellular level indicated specific adhesion of GM_3-expressing B16 melanoma cells to Gg_3-expressing L 5178 (AA12) lymphoma cells but not to the Gg_3 negative L 5178 (AV27) cells. These adhesive interactions required the presence of Ca^{2+} or Mg^{2+} and were inhibited by GM_3, anti-GM_3 mAb DH2, anti-Gg_3 mAb 2D4, and EDTA. In this case, the heterotypic interaction between GM_3 and Gg_3 may in fact, represent the initial cell type-specific recognition event prior to the initiation of the subsequent multi-step cell type-nonspecific adhesion process.

The importance of these findings were further highlighted by the fact that comparison of three murine melanoma cell lines F1, F10, and BL6 for cell adhesion to Gg_3 coated surface revealed that the low metastatic potential F1 cells (lacking cell-surface GM_3 expression) displayed poor binding while the GM_3 rich BL6 cells (highest metastatic potential) displayed the greatest adhesion.[90] In order to rule out the involvement of the integrin receptor in this phenomenon, it was shown that addition of the RGDS sequence, which is the key recognition site for the integrin receptor, inhibits the adhesion of BL6 cells to non-coated plastic surfaces but does not affect the interaction of BL6 cells to Gg_3-coated solid phase. Thus, this GSL-GSL interaction is clearly unrelated to the well-known integrin-based interaction.[91]

Preliminary evidence obtained by culturing B16 melanoma cells on Gg_3 coated culture plates in serum free medium has indicated that the complementary Gg_3-GM_3 interaction enhances both cell spreading and motility.[92] Similar studies to determine GSL-GSL interactions involving GD_3 have not been carried out to date; however, as described earlier, studies with mAb R24 have implicated GD_3 in melanoma cell attachment mechanisms.

2.6.1 Molecular Basis For Gg_3-GM_3 Interactions in Cell Adhesion

Kojima and Hakomori have constructed[89] minimum energy conformation models of both Gg_3 and GM_3 based on hard sphere exoanomeric calculations.[93] These calculations were based on the glycosidic torsion angles shown in Table 5. Interestingly enough, these models revealed that each ganglioside possesses one side that is hydrophilic while the other side of the molecule is hydrophobic. This feature is not uncommon amoung GSLs, and when coupled with the fact that the double bond of the aglyconic moiety confers an L shape bend in these

molecules, it is entirely possible that the overall stabilization of the GSL imbedded in the cell membrane results from the orientation of the hydrophobic face towards the phospholipid bilayer which then allows the hydrophilic face to be exposed to the aqueous extracellular medium. Closer inspection of Corey-Pauling-Koltun (CPK) models of Gg_3 and GM_3 further revealed that orientation of the molecular axes at approximately 40-60° resulted in a remarkable complementary fit between the hydrophobic sides of these two molecules while no corresponding fit could be observed between the hydrophilic sides. The role of the bivalent cation may be to provide additional stabilization in the form of intermolecular chelation of the two hydrophilic surfaces. Much work still needs to be carried out in an effort to totally describe such interactions, and synthetic analogs may be of value in this regard.

Table 5: Glycosidic Torsion Angles For Gg_3-GM_3 Interactions

	φ	ψ
Gg_3		
GalNAcβ(1→4)Gal	54°	10°
Galβ(1→4)Glc	55°	4°
GM_3		
Neu5Acα(2→3)Gal	-170°	-7°
Galβ(1→4)Glc	55°	4°

2.7 Anti-GD_3 Monoclonal Antibodies in Therapy

The fact that GD_3 expressed in melanomas is a clinically relevant antigen

in humans was demonstrated by Houghton *et al.*[62] in a phase 1 clinical study employing anti-GD_3 mAb R24 as an agent for the passive immunotherapy of melanoma. Dippold and Bernhard have determined that the precise epitope recognized by R24 is the terminal trisaccharide sequence of GD_3, namely, Neu5Acα(2$\rightarrow$8)Neu5Acα(2$\rightarrow$3)Gal.[94] Prior studies with this antibody identified 21 of 21 primary melanomas and 37 of 37 melanomas by indirect immunoperoxidase-staining, while melanocytes in the basal layer of normal skin were only weakly stained. These *in vitro* results prompted the phase 1 clinical study, and additional excitement was generated by the observation that along with the absence of serious side effects, this mAb displayed a high specificity for melanoma *in vivo*. This led Houghton and coworkers to speculate that the lack of serious side effects was directly related to the high degree of specificity exhibited by mAb R24. Significant tumor regression was reported for 3 out of 12 patients receiving daily injected doses of 8 mg/m^2 over a period of two weeks and these patients developed rapid local inflammatory responses at the tumor site. Prior *in vitro* studies had demonstrated that anti-GD_3 mAbs readily lyse human melanoma cells in the presence of human complement[95] via the known antibody dependant cell-mediated cytotoxicity (ADCC) mechanism,[96] and that large granular lymphocytes exhibiting natural killer (NK) function were most likely responsible for the ADCC effect on the target melanoma cells.[97]

Analysis of these clinical results led Reisfeld and Cheresh[53] to suggest that glycolipid antigens such as GD_3 may be relevant targets for mAb therapy of human melanomas for the following reasons;

(1) this antigen is expressed at reasonable levels,

(2) the strategic location of glycolipid antigens on the outer layer of the lipid bilayer allows for easy access, and

(3) anti-ganglioside mAbs inhibit melanoma cell-substratum interactions, an effect which may lead to an overall reduction in the rate of tumor cell invasion and subsequent metastasis.

The fact that R24 apparently did not cause antigenic modulations as evidenced by the constant level of cell surface expression of GD_3, even in the presence of substantial antibody levels, was also noteworthy.

2.7.1 Possible Mechanisms For Therapeutic Effects of R24

Additional data from a second phase 1 trial with R24[98] served to further confirm that this mAb can be safely administered to cancer patients and that severe toxicity is uncommon. Anti-mouse antibodies were generally noted 14 days after the first injection, although some patients who displayed a partial response did not develop anti-mouse Ig responses during the first three weeks.

The therapeutic effect of this unconjugated mAb was based on the assumption that the immune system is capable of mediating tumor rejection, either by inducing inflammation or by active immunization. Since the immune system is capable of rejecting large tissues from organ transplantation, it seems possible to invoke immunological destruction of tumor tissue.

The anti-tumor effects of unconjugated mAbs such as R24 may be the result of several factors including:

(1) complement activation resulting in tumor lysis of melanoma cells in the presence of human complement, activation of complement components may be expected to increase vascular permeability,

(2) activation of ADCC involving the direct lysis of target cells by macrophages, NK cells, and neutrophils,

(3) direct interference on cell attachment and growth,

(4) direct activation of GD_3^+ T lymphocytes by the mAb resulting in activated regulatory T cells.

(5) generation of an anti-idiotype response to the monoclonal antibody according to the network theory of Jerne,[99] thereby generating an active immune response. (It should be mentioned that human anti-GD_3 antibodies were not detected in treated patients, however, some patients did develop a specific response to the Fab region of R24 consistent with an anti-idiotype response.[100])

(6) elicitation of an immune response to foreign antigen such as a murine mAb indirectly leading to tumor inflammation and subsequent destruction via the bystander effect.

In regard to these potential mechanisms, antibody characteristics worthy of consideration for the deployment of a particular mAb for therapy include; antibody specificity and avidity, which are a function of the variable region of the immunoglobulin; and, ability to activate effector functions such as complement fixation and ADCC as determined by the Ig constant region.[101]

Above and beyond these effects, arming the antibody with a radionuclide may prove useful for RAID; however, utilization of immunoconjugates for therapy of solid tumors remains complex. Epitope specificity, antibody affinity, and antigen expression are vital to tumor localization. Comparison of several anti-GD_3 antibodies has revealed that complement-mediated lysis was directly related to antibody affinity, and cytotoxicity correlated well with the degree of antibody binding.[94] Typically, the percent of injected dose of mAb that localizes in the tumor is < 0.1% injected dose/gram of tumor, and in many instances, it is less than 0.001% per gram of tumor. Even with an affinity constant of 2×10^7 for R24, biopsies performed one week after injection of ^{131}I-labeled R24 revealed only 0.002-0.003% injected dose/gram of tumor in melanomas. Thus, several other crucial factors such as; tumor size; tumor blood flow; vascular permeability; circulating antigen; anti-mouse response; and tumor necrosis all have a direct impact on targeting. Efforts to bypass unwanted human anti-mouse antibodies have prompted the molecular cloning of an anti-GM_3 human mAb HuMab L612[102] as well as an anti-GD_2 human mAb CAMP-ATH-1H.[103]

In order to fully exploit this type of therapeutic strategy, more details regarding the nature of mAb-antigen interactions are a must. According to Dippold and Bernhard,[94] the abiltiy to compare independent clinical trials and to more completely understand the underlying mechanisms of action of each antibody, rests heavily on detailed analysis of the corresponding anti-ganglioside monoclonal antibodies. Perhaps, with the aid of synthetic analogs and high field NMR spectroscopy, some features of one of these aforementioned considerations, namely epitope specificity, may emerge. In summary, ganglioside antigens have received much recent attention as potential targets for antibody based agents owing to their "increased" expression, immunogenicity, and functional susceptibility to antibody directed cell-mediated cytotoxicity. Further studies are necessary in order to fully exploit these antigens as potential targets for diagnosis and therapy of cancer.

2.7.2 GD_3 Vaccines

Although beyond the scope of our present work, it is noteworthy that gangliosides have been included as major components of tumor vaccines for active specific immunotherapy of melanoma patients.[104] Although it has been recognized for some time that GD_3 is an excellent target for the active specific immunotherapy of melanoma, generation of anti-GD_3 antibodies in cancer patients has proved to be a challenging task.[40] In this regard, application of GD_3-proteosomes[105] appears to be an interesting approach towards augmenting the immunogenicity of this ganglioside antigen. As well, novel synthetic GD_3 antigens with increased immunogenic properties suitable for vaccine preparations may be the targets of future design.

2.8 Probing Structure-Activity Relationships of GM_3 and GD_3

The pKa of the sialic acid residue of gangliosides is in the range of 2.2-2.5, thus, the carboxyl groups are expected to be dissociated at physiological pH. This fact leads to the presence of negative charges, which may be essential for binding cations and interacting with biological molecules.[106] Any attempts to understand the nature of such binding interactions between a mAb and corresponding carbohydrate ligand would be aided by knowledge about the solution conformation of the ganglioside. As a tool, NMR spectroscopy is well suited for conformational analysis of oligosaccharides under the natural environment of aqueous solvation. Unfortunately, due to the fact that gangliosides form micelles under physiological conditions, NMR experiments are typically performed in DMSO-d_6 at slightly elevated (313 °K) temperatures.[107] Out of necessity, it has been assumed that the conformation adopted by the carbohydrate molecules under these conditions directly simulate those experienced in the natural aqueous environment, however, there is no direct way of proving this.

A set of empirical rules greatly assists in correlating ^1H NMR features with structural elements have been developed yielding the structural reporter group concept.[108] These rules were formulated using a variety of oligosaccharides and glycopeptides and apply to D_2O solutions of carbohydrates. So far, in order to directly apply these rules towards the structural characterization of glycolipids, it has been necessary to remove the hydrophobic ceramide portion in order to

achieve good solubility characteristics in D_2O.[109] This can readily be accomplished by employing the ozonolysis-alkaline fragmentation sequence[110] to yield the carbohydrate portion free of ceramide. The resultant product however, is typically a 2:1 β:α anomeric mixture at glucose which may additionally complicate the ^1H NMR spectrum and therefore, homogeneous synthetic derivatives with good ^1H NMR properties in water would be an asset.

Certain key structural reporter groups contain valuable information regarding the stereochemistry of the sialic acid glycosidic linkage. For example, the proton signal for the equatorial proton at C-3 (H-3e) in D_2O consistently resonates at $\delta > 2.5$ ppm (typically 2.7 ppm) for the α-anomer and < 2.5 ppm (usually 2.3 ppm) for the corresponding β-glycoside.[111] Furthermore, this rule holds true for free sialic acid in which 5-8% is in the α-configuration (δ H-3e = 2.75 ppm) and 92-95% in the β-configuration (δ H-3e = 2.25 ppm),[112] and this pattern is also observed in sialic acid trimer $(Neu5Ac)_3$ where there are two α-linkages (δ H-3e = 2.76 and 2.69 ppm) and one β-linkage (δ H-3e = 2.20 ppm).[113]

Application of ^{13}C NMR to this problem is a more challenging task, and along with the need for higher sample concentrations, differences between the spectra of the two anomers are not always obvious. For example, dihedral angles (ϕ) defined by C-1-C-2-C-3-H-3a for the α- and β-anomers were estimated to be 180° and 60° respectively based on theoretical calculations, thus implying a higher $J_{C\text{-}1,H\text{-}3a}$ value for the former. In terms of coupling constants, this represents about 5.5 Hz for the α-anomer as compared to 1.5 Hz for the β-anomer,[114] and these expectations were further confirmed using heteronuclear 2D NMR techniques employing a 180° DANTE pulse.[115] Although these protocols are elegant, information regarding the stereochemistry of the anomeric linkage can be obtained directly from the ^1H NMR spectrum in a relatively straightforward manner when limited amounts of sample are available.

Solution conformations of Neu5Acα(2→3)Galβ→Ceramide (GM_4) and its corresponding reducing sugar have recently been studied using ^1H NMR spectroscopy (nuclear Overhauser enhancements, coupling constants) in conjunction with energy-minimum calculations.[116] These studies were initiated in order to further understand the epitope specificity and binding affinity displayed by a novel murine monoclonal antibody which is highly specific for this

disaccharide sequence.[117] The proton NMR spectra of carbohydrates are complicated due to the fact that a majority of the sugar skeleton protons resonate in the so called "bulk region" (δ 3.5-3.9 ppm). Thus, one-dimensional homonuclear Hartmann-Hann (HOHAHA) spectroscopy was employed in this study in order to obtain separate subspectra of the component sugar residues by selectively exciting one proton in each monosaccharide. In such experiments, the net magnitization is transferred between coupled spins, and therefore relies on J cross-polarization.

Meanwhile, potential energy calculations for this disaccharide revealed three minima in ϕ,ψ conformation space, and interproton distances relevant to conformational analysis by NOE were calculated for each of the three conformers. The largest inter-residue cross-relaxation rates observed for conformer 1 were for the H-3a'/H-3 and H-8'/H-4 proton pairs, while for conformer 2, sialic acid H-8' approaches both H-3 and H-1 of galactose. Conformer 3 displayed rapid cross-relaxation for the H-3a'/H-3 pair and a slower rate for the H-3e'/H-4 and H-3a'/H-4 pairs, thus evidence for all three conformers was obtained. The proton spectrum of GM_4-Ceramide in DMSO-d_6 was in many respects similar to the reducing sugar in D_2O, however, the latter was somewhat complex due to the α,β-anomeric mixture present.

Similar spectral analyses have been carried out recently on the GM_3 trisaccharide and corresponding N-glycolyl derivative[118] in combination with the molecular mechanics MM2 program.[119] In this study, the spatial orientation of GM_3 was investigated using rotating-frame NOE's obtained for C-, O-, and N-linked protons in DMSO-d_6, and the sialic acid-galactose as well as the galactose-glucose linkages were found to be remarkably flexible. Molecular dynamics simulations served to further confirm these results. The sialic acid side chain and acylamino group on the other hand, appeared to be locked in one conformation. Some caution must be exercised in the interpretation of NMR derived information involving fast equilibria since NMR data is based on average values of structural parameters as pointed out by Siebert and coworkers.[118] It was of interest to note that the difference between NOE's obtained in DMSO-d_6 (ceramide derivative) and those obtained in D_2O for both GM_4 and GM_3 were relatively small leading to the possible conclusion that the ceramide aglycon does not appreciably affect the conformation of oligosaccharide moieties.

A novel approach towards the analysis of the solution conformation of GM_3 was to study in detail, intramolecular hydrogen bonds by NMR spectroscopy.[120] Poppe and van Halbeek were successful in bypassing the problem of rapid exchange by observing the hydroxyl protons and their spatial neighbors in pre-steady-state NOE experiments employing careful water suppression techniques. Under these circumstances, a strong intramolecular bond between the sialic acid OH-8 proton and the sialic ring oxygen and/or carboxyl group was clearly manifested. As was pointed out by these authors, this fact may account for the rigidity of the glycerol side chain typically seen in the sialic acid unit of oligosaccharides.

Intrigued by the fact that lack of contact inhibition and regulation of cell proliferation inherent in tumor cells are direct results of altered ganglioside expression and organization, Aubin and coworkers conducted NMR studies on the sialic acid epitope of GM_3 at the surface of a magnetically oriented membrane.[121] Since the accumulation of precursors such as GM_3, GM_2, GD_3 *etc.* along with the concomitant depletion of the more complex gangliosides such as GM_1, GD_{1a}, GT_{1b} *etc.*, is indicative of defective biosynthetic machinery, it comes as no surprise that the precursor GM_3 is involved in a number of events characteristic of malignancy. These biological observations prompted ^{13}C NMR studies in order to study the structure and dynamics of sialic acid within this trisaccharide. Since GM_3 is known to bind to the plant lectin wheat germ agglutinin (WGA) primarily via this terminal sugar, and since the high resolution X-ray crystallographic data on WGA is available,[122] it was possible to elucidate a dominant conformation for this binding using ^{13}C-labeled sialic acid.

To date, the structure of GD_3 has not been investigated to the degree that GM_3 has been. The ^1H NMR spectrum of the GD_3-Ceramide in DMSO-d_6 has been published[123] as has the proton NMR of the reducing sugar in D_2O.[109] A comparison of the detailed ^1H NMR spectrum of GD_3-Ceramide isolated from natural sources along with the structural reporter groups from the reducing sugar in D_2O from this published data is shown in Table 6 (see Figure 4 for assignment of residues). Although only the shifts of the structural reporter groups have been assigned for the reducing sugar, the differences in shift values, and in particular, H-3e of the inner sialic acid (A) are notable, thus the solution conformations in DMSO-d_6 and D_2O may not be identical.

Table 6: Comparison of GD_3-Ceramide[123] and GD_3-OH[109] by NMR

Chemical shifts in ppm from TMS, measured at 40 °C in DMSO-d_6/D_2O

Res	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9
R	3.43 (a)	3.78	3.89	5.36	5.54	1.93		2.03	1.45
	3.97 (b)								
I	4.16	3.05	3.31	3.28	3.28	3.60 (a)			
						3.73 (b)			
II	4.27	3.28	3.63	3.87	3.35	3.45 (a)			
						3.47 (b)			
A			1.69 (a)	3.83	3.57	3.14	3.43	3.43	3.55 (a)
			2.34 (e)						3.81 (b)
C			1.39 (a)	3.46	3.57	3.33	3.21	3.48	3.32 (a)
			2.77 (e)						3.58 (b)

Chemical shifts in ppm (acetone reference), measured at 27 °C in D_2O

Res	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9
I	4.66	3.29							
II	4.52		4.09	3.97					
A			1.74 (a)						
			2.68 (e)						
C			1.74 (a)						
			2.78 (e)						

2.8.1 GD_3 Lactones

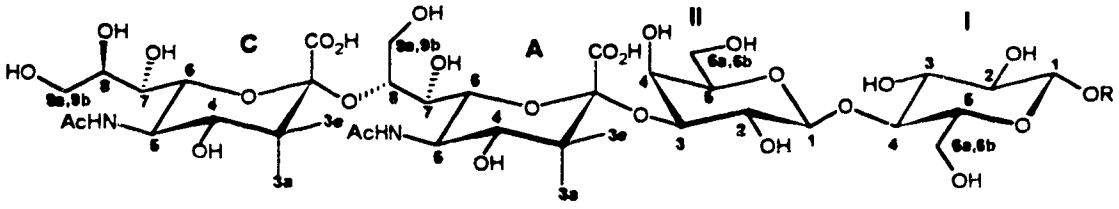
As was briefly mentioned, the sialic acid units possess an ionizable carboxyl group, thereby enabling this residue to interact with cationic cofactors by providing a negative charge. Esterification of the carboxyl group by lactonization would reduce the negative charge at physiological pH potentially leading to modulation of biological functions. As well, lactonization is expected to make the oligosaccharide chain more rigid,[124] which may be functionally significant.

The observance of the presence of an inner ester of GM_3 was first noted by Evans and McCluer.[125] Identical treatment of GD_3 with glacial acetic acid under anhydrous conditions resulted in the formation of the two lactones[123] shown in Figure 4. Lactone 1 behaved as a monosialoganglioside upon anion exchange chromatography, and lactonization had apparently occurred between the carboxyl group of the external sialic acid and the C-9 hydroxyl group of the internal sialic acid as evidenced by the prominent deshielding of the H-9 protons. Lactone II, on the other hand, behaved as a neutral species wherein a second ester linkage was formed between the carboxyl group of the internal sialic acid and the C-2 hydroxyl group, as could be seen by the downfield shift of the corresponding H-2 proton. Lactone formation appears to be quite facile as does hydrolysis to regenerate the original ganglioside.

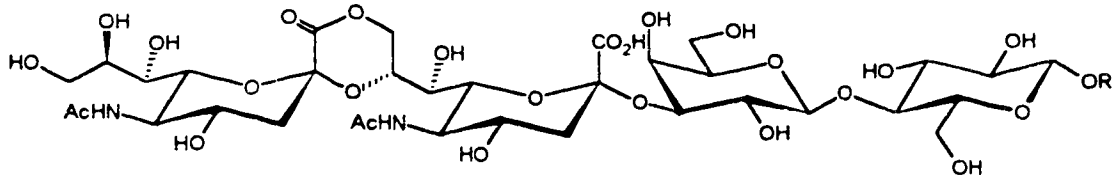
The immunoreactivity of anti-GD_3 mAb R24 with these lactones was investigated,[123] and this antibody was found to weakly bind lactone I, while lactone II was not recognized at all. These results suggested that the reduction of negative charge and/or the resultant overall change in conformation may be responsible for the overall loss of binding. Thus, lactonization results in structural changes in the parent ganglioside thereby providing a new antigenic structure. As a result, receptor activity may be modulated by the lactonization mechanism as exemplified by changes in the immunoreactivity with R24.

Maggio and coworkers proposed the idea that the modulatory effects exhibited by gangliosides on the function of the cell membrane result from their ability to induce dramatic modifications on membrane organization and topology.[126] Such effects include modification of molecular packing, cell curvature, interfacial micropolarity, electrostatics and free energy at the

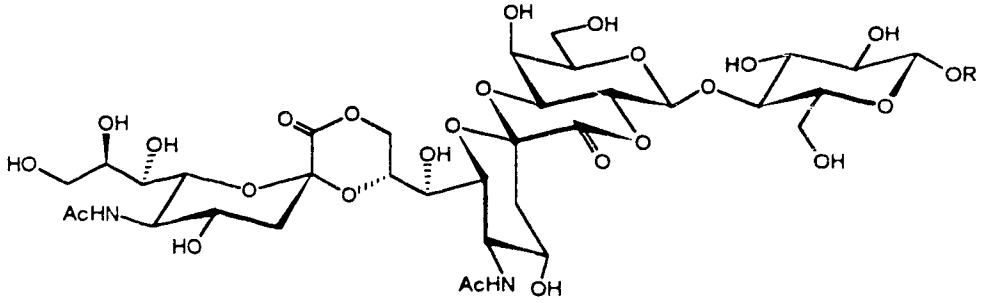
Figure 4: Structure of GD_3 and its Lactones



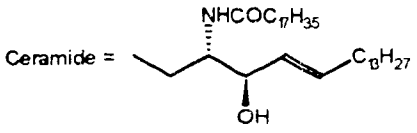
R = Ceramide (GD_3)
R = H (reducing sugar)



Lactone I



Lactone II



membrane surface, and these effects may rely on the structure, number, and conformation of the carbohydrate moieties in the polar head group. The precise nature of membrane organization of gangliosides and the significance of chemical alterations on the overall organization are mysteries which remain to be solved. Efforts to gain insight into these characteristics on the molecular level have been initiated by Maggio *et al.* who devised experiments to compare the intermolecular packing and surface potential of GD_3 and its lactone derivatives in monolayers.[127] In this study, space filling models of possible orientations of GD_3 and its corresponding lactones at the monolayer interface were constructed based on ^1H NMR data. The space-filled contours revealed that the oligosaccharide chain of GD_3 extended outward, perpendicular to the interface. Upon lactonization to lactone I, the spacial orientation of the lactone ring was found to be perpendicular to the plane of the terminal sialic acid. With respect to rigidity, free rotation of this lactone unit was possible and the oligosaccharide chain was still able to adopt an overall straight conformation while spatial perturbation was minimal. Upon formation of the second lactone however, the two fused rings imposed a barrier to rotation around the innermost sialic acid, yielding a rigid structure. As well, the oligosacharide chain was now found to be distorted resulting in displacement from the vertical position displayed in the two precursors. Such territorial encroachment leads to increased spacing requirements thereby affecting molecular packing. Conceivably, identical changes in the cell membrane would be expected to affect intermolecular and intermembrane recognition reactions.

2.8.2 Protein-Carbohydrate Interactions; Can They Be Seen By NMR?

Traditionally, X-ray crystallography has been employed to study the phenomenon of molecular recognition. Recently however, it has become possible to estimate binding affinities for such interactions by NMR spectroscopy. For example, interactions between influenza virus hemagglutinin and various sialic acid derivatives were probed by ^1H NMR spectroscopy,[128] and studies using various sialic acid analogs in this way, have yielded information on the three-dimensional structure of the protein-carbohydrate complex.[129] This information is valuable in designing synthetic analogs which display higher affinity binding.

Application of NMR spectroscopy towards understanding molecular interactions between antibodies and their peptide antigens has been instrumental in designing better synthetic peptide vaccines.[130] Studies on the molecular interactions between a monoclonal antibody Fab fragment and a fifteen amino acid unit corresponding to residues 50-64 of the β-subunit of cholera toxin were performed with the aid of 2-D TRNOE (transferred NOE) difference spectroscopy. It may be possible to employ such techniques towards the study of molecular interactions between GD_3 and anti-GD_3 Fab fragments which recognize melanoma. The fact that some of the cabohydrate protons resonances may be clearly separated from the peptide proton resonances may make this approach an attractive one in the case of gangliosides.

It is well known that enzymes catalyze reactions by lowering the activation energy of the transition state. In 1946, Linus Pauling suggested that enzymes perform this task by binding to the transition state rather than the substrate molecule[131] thereby utilizing binding energy to reduce the chemical activation energy. Although both enzymes and monoclonal antibodies are proteins, a likely difference in mode of action of these two classes of molecules stems from the fact that enzymes bind readily to high-energy structures while antibodies bind low-energy structures. Antibodies can however, induce structural changes in their binding ligands, and it is conceivable that mAbs can recognize conformations other than the minimum energy conformation. In this regard, application of NMR spectroscopy to the study of the binding reaction between synthetic carbohydrate antigens and their corresponding Fab fragments in the natural aqueous environment may lead to a deeper understanding of such immunological events on the molecular level.

2.8.3 Other Variations In Ganglioside Structure

Although beyond the scope of this present work, it should be pointed out that gangliosides, such as GD_3, are also found to be *O*-acetylated at various sites of the terminal sialic acid (see Figure 3) and the *N*-acetyl moiety is sometimes replaced by the *N*-glycolyl ($NHCOCH_2OH$) group. Carbohydrates already have a larger capacity to carry information than amino acids, due to the larger number of inherent chiral centers, and when combined with the possible variations of lactonization, *O*-acetylation, variations in *N*-acetylation, and combinations thereof, the potential for storage of biological information in just

one ganglioside such as GD_3, is indeed remarkable. Furthermore, the well documented observation that mAbs preferentially bind to tumor cells even though the same structure is present on normal cells, albeit in much lower concentrations, is in itself puzzling, and further investigation as to the nature of this phenomenon on the molecular level is warranted.

3. SYNTHETIC ASPECTS

3.1 Glycosylation Of Sialic Acid

Stereoselective synthesis using Neu5Ac donors is of current interest in order to provide tools for the detailed study of biological functions such as those highlighted in the previous chapter. Glycosylation with sialic acid donors has proved to be a challenging task for the carbohydrate chemist for the following three reasons:[132]

(1) The C-2 carbon of sialic acid, to which the new glycosidic linkage is to be formed, is quarternary and possesses an electron withdrawing carboxylate moiety. Thus, substitution at this center is disfavored sterically as well as electronically.

(2) The combination of a carboxyl substituent at C-2 in concert with a deoxy structure at C-3 guarantees that the formation of the 2,3-dehydro derivative upon activation of the anomeric center will be a competitive side reaction.

(3) While the vast majority of sialosides found in nature are α-glycosides, the fact that the C-3 carbon is deoxy precludes the deployment of traditional stereocontrolling strategies such as neighboring group participation or *in situ* anomerization.

Nevertheless, improved procedures which provide reasonable yields of sialyl glycosides have been worked out. Owing to the fact that an excellent review article on this topic is currently available,[133] such methods will not be covered in detail herein.

Along with the problems typically encountered in the synthesis of these molecules is the additional difficulty in determination of the anomeric configuration of the newly formed sialic acid linkage. Enzymatic hydrolysis using sialidase[134] and ^{1}H NMR spectroscopy have been found to be particularly useful in elucidation of the stereochemistry of the newly formed anomeric bond. In combination with the previously mentioned observation that for deprotected sialosides, the chemical shift of the α-(H-3e) is downfield from that of the

corresponding β-(H-3e) (in D_2O), some trends in the chemical shifts of acetyl protected Neu5Ac have also emerged including:

(1) chemical shift of α-H-4 < β-H-4[135]

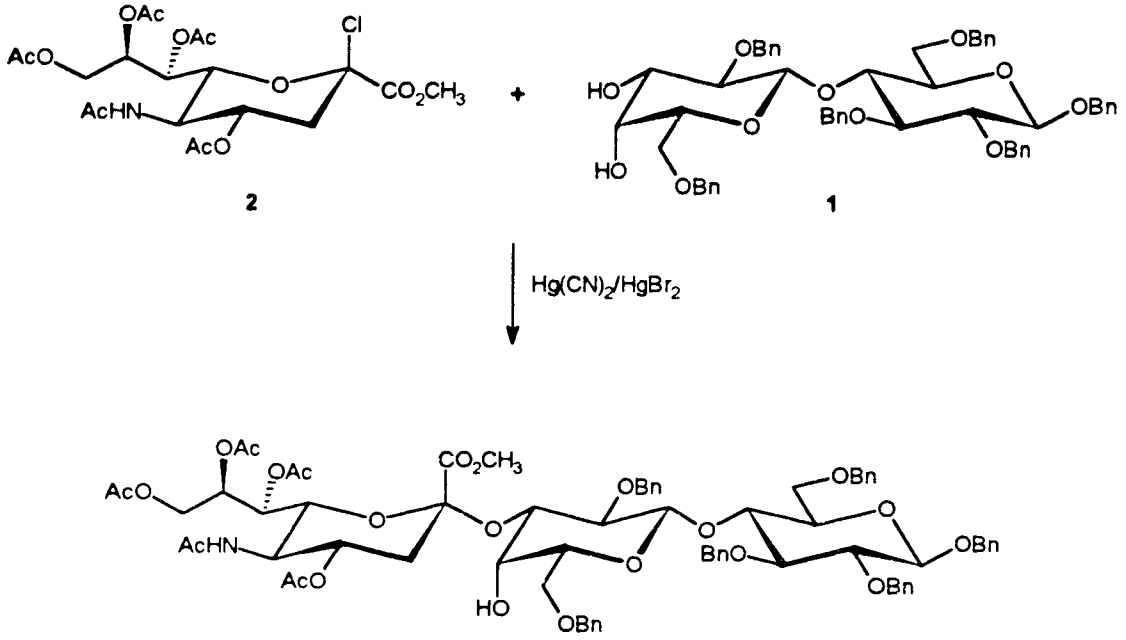
(2) chemical shift of α-H-8 > β-H-8[136]

(3) coupling constants $\alpha\text{-}J_{7,8} > \beta\text{-}J_{7,8}$[137]

(4) chemical shift difference α-|H-9a - H-9b| < β-|H-9a - H-9b|[137]

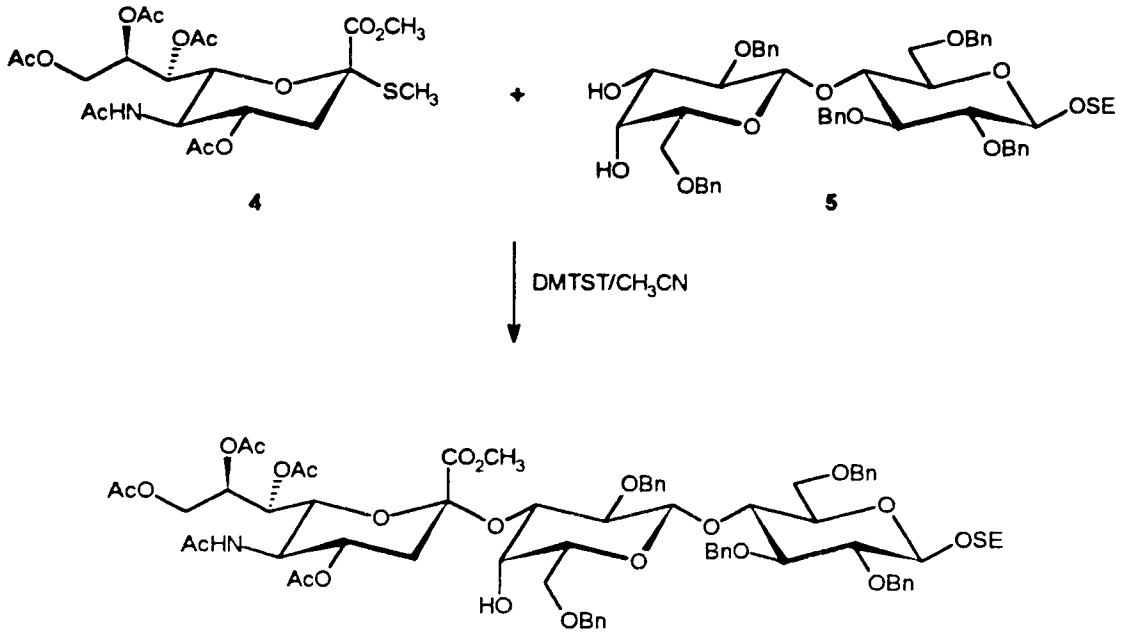
3.1.1 Synthesis of GM_3

The first chemical synthesis of GM_3 with clear evidence for the configurational assignment at C-2 was published by Sugimoto and Ogawa,[138] who introduced the lactose acceptor **1** to sialic acid donor **2**. The 3',4'-lactose diol **1**, in turn, was prepared in three steps from lactose; (1) H^+, $(CH_3)_2C(OCH_3)_2$; (2) BnBr, NaH; and (3) HOAc, H_2O; while chloride **2** was already known.[139] Thus, mercuric cyanide-mercuric bromide-promoted glycosylation of diol **1** with sialic acid derivative **2** in dichloroethane containing



powdered molecular sieves 4Å, gave 20-25% of a mixture of sialosides ($\beta/\alpha \approx$ 2.5/1) from which the blocked GM_3 **3** with the required α-stereochemistry was retrieved in about 7% isolated yield.

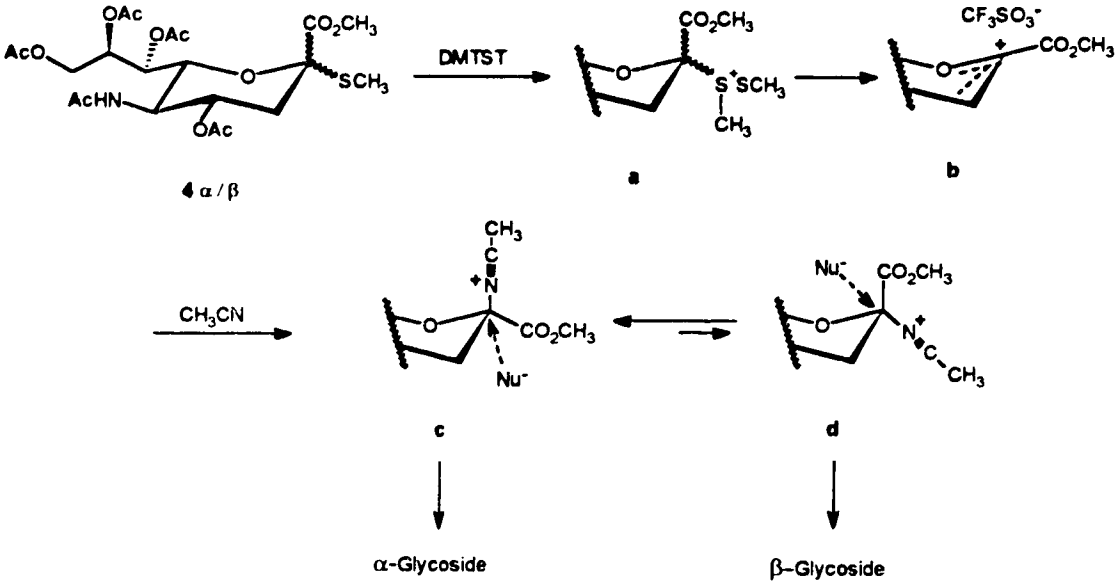
Subsequently, improved yields have been achieved by application of the thioglycoside methodology in oligosaccharide synthesis. Promotion of the glycosylation reaction is invoked by the use of dimethyl(methylthio)sulfonium triflate (DMTST),[140] and selective activation is possible due to the high thiophilicity of this reagent. Thus, reaction of methyl α-2-thioglycoside **4** (2 equiv) with 2-(trimethylsilyl)ethyl lactose acceptor **5**, in acetonitrile under DMTST (4 equiv) activation at -30 °C, yielded the blocked GM_3 **6** in 47% yield.[141]



6 SE = $CH_2CH_2Si(CH_3)_3$

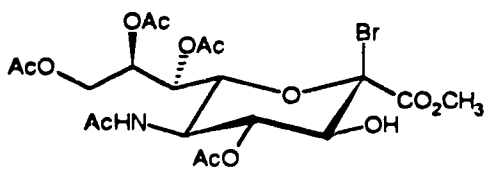
The fact that no β-isomer was detected was attributed to solvent participation as shown in Figure 5. The possible mechanism for the formation of the therrnodynamically less favored α-glycoside involves the formation of acetonitrium ions **c** and **d** from intermediates **a** and **b** at low temperature. Presumably, the equilibrium favours the β-acetonitrium ion **c** which then undergoes S_N2 displacement to provide the α-glycoside of sialic acid.[156]

Figure 5: Possible Mechanism For Stereoselective Glycosylation In CH_3CN



3.1.2 Synthesis of GD_3

The first stereocontrolled synthesis of the ganglioside GD_3 was reported by Ogawa *et al.*[142] The strategy employed in this synthesis was adapted from earlier work by Kondo and coworkers,[143] who utilized the 2β-bromo-3-hydroxy derivative **7** armed with a C-3 substituent capable of neighboring group assistance for the stereocontrolled synthesis of the α-Neu5Ac(2→8)Neu5Ac unit. Thus, modifications were made to incorporate either a phenylseleno **8** or phenylthio **9** auxiliary at the C-3 position of the sialic acid donor. In this way, stereocontrol is achieved via the episelenonium or episulfonium ion respectively. Preparation of **9** from the precursor 2,3-dehydro **10** derivative was accomplished in four steps; (1) NaOMe/MeOH; (2) $PhCH_2Br$, KOH, BaO, n-Bu_4NI; (3) CH_2N_2/Et_2O-MeOH; and (4) CBr_4, $(Me_2N)_3P$/THF; in approximately 65% overall yield. Next, the synthesis of sialyl acceptor **11** was carried out in four steps from **10**; (1) NaOMe/MeOH; (2) $PhCH(OMe)_2$, CSA/DMF; (3) $PhCH_2Br$, KOH, CaH_2,



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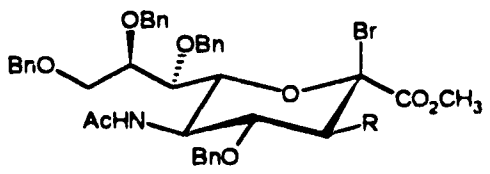
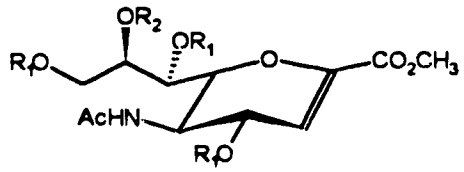
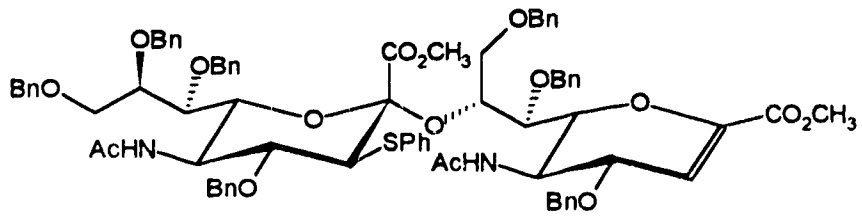


8 R = SePh
9 R = SPh



	R_1	R_2
10	Ac	Ac
11	Bn	H

n-Bu_4NI then CH_2N_2/Et_2O-MeOH; and (4) $BH_3 \cdot NMe_3$, $AlCl_3$/THF; in 50% yield from **11**. Introduction of bromide **9** to **11** under $Hg(CN)_2/HgBr_2$ catalyzed promotion gave **12** in 64% yield, and subsequent conversion to the desired



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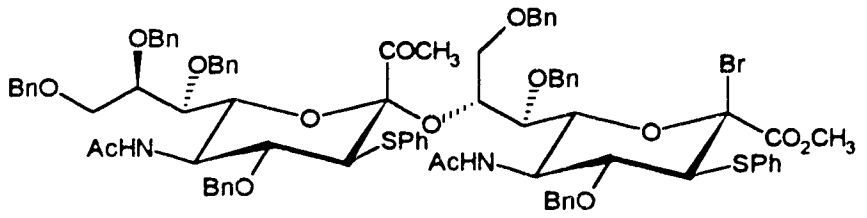
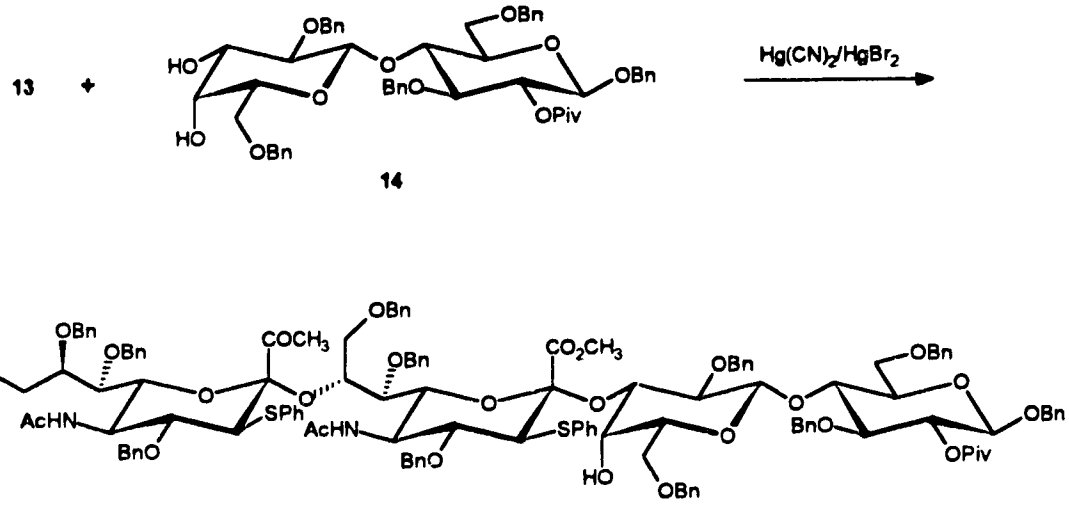


13

di-Neu5Ac donor **13** was finally achieved in 80% yield after three additional steps; (1) NBS/aqueous MeCN; (2) PhSK/t-BuOH-THF; and (3) DBU/toluene. Glycosidation of lactose acceptor **14** with donor **13**, again under the influence of $Hg(CN)_2/HgBr_2$ catalysis in carbon tetrachloride, produced α-glycoside **15** containing only a trace of the β-isomer (α/β ≈ 60/1) in 48% combined yield. Finally, prior to any subsequent deprotection steps, this tetrasaccharide required

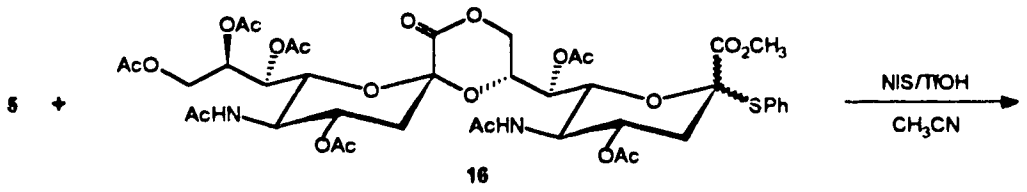


15 Piv = Pivaloyl

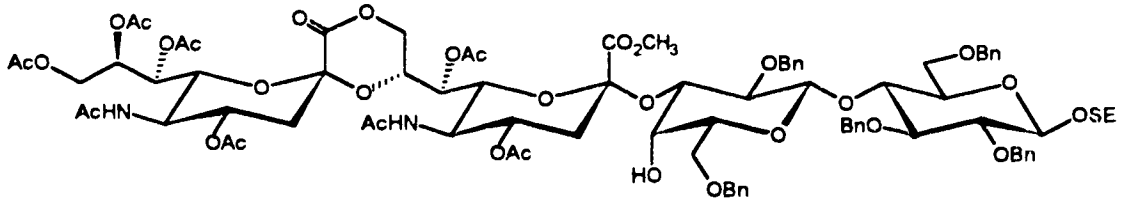
acetylation followed by removal of the phenylthio groups (Ph_3SnH, AIBN/benzene, 78% yield). Although particularly elegant, the production of the di-Neu5Ac donor involves a multistep synthesis, and post glycosylation, removal of the stereocontrolling auxillary represents an additional step under conditions in which certain groups (*i.e.* alkenes) are not compatible.

In view of the fact that sialic acid dimer O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid) - (2→8) - 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid (di-Neu5Ac) is now readily available via the procedure of Roy and Pon,[144] Abbas and coworkers[145] were first to capitalize on this process to prepare 2β-chloro di-Neu5Ac synthons analogous to monosialyl donor **1**. Subsequently, Diakur and Roy successfully utilized one of these synthons for the regio- and stereoselective glycosylation of the 3-OH position of a galactose derivative.[146] Although the yield of the sialylation reaction was low, these authors suggested that use of the

corresponding thioglycoside analogous to the monosialyl thioglycoside **4** previously utilized by Kanie *et al.*,[147] may lead to improved yields in the synthesis of di-Neu5Ac containing compounds such as GD_3. At the time of the completion of the work described in this thesis, a report of the use of such a donor for the synthesis of GD_3 has appeared. Thus, Hasegawa and coworkers[148] reacted lactose-SE derivative **5** (2 equiv) with sialyl donor **16** (1 equiv) in the presence of NIS (2 equiv)/TfOH (0.2 equiv) in acetonitrile in order to obtain the tetrasaccharide **17** (30%), and have recently applied this reaction to the galactose derivative **18**.[149]

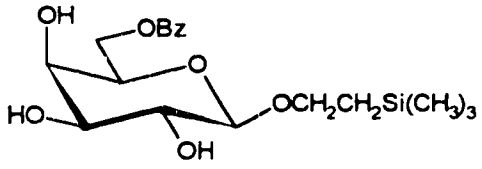


16



17 SE = $CH_2CH_2Si(CH_3)_3$

We report, in a subsequent section, a summary of our efforts in this area.



18

Although gangliosides can be extracted from natural sources, the full potential inherent in chemical synthesis will be realized in the preparation of

various analogs, some of which will hopefully display increased biological activity. Thus, chemical synthesis has the advantage of yielding novel derivatives of a target oligosaccharide (*i.e.* deoxyfluoro- or azido-analogs) with potentially novel biological properties.

3.2 Artificial Antigens

Haptens are defined as "small molecules" which are not capable of generating an immune response, but are capable of reacting with antibodies. Oligosaccharides generally fall into this category and must be associated with an immunogenic protein molecule in order to elicit the production of immunoglobulins. The resulting artificial antigens can be thought of as an immunogenic molecule armed with a selected target epitope to which at least some of the newly generated antibodies may react. In order to achieve this association in a covalent fashion, the carbohydrate moiety should be functionalized with a linking arm suitable for conjugation to carrier proteins. Several types of linking strategies are currently available, and the reader is pointed towards several excellent review articles dedicated to this subject,[150,151] therefore, only methodologies pertinent to this work are briefly highlighted in this section.

Karl Landsteiner (1868 - 1943) was the first to study the reaction of antibodies towards chemically defined haptens. Historically, Landsteiner studied anti-hapten antibodies generated against artificial antigens which were prepared by coupling *p*-diazophenyl glycosides to various carrier proteins.[152] Thus, an adaptation of this original method has been to sacrifice the reducing sugar terminus of an oligosaccharide to reductive amination in the presence of a *p*-aminobenzyl-containing linking arm as is shown in Figure 6.[153] As well, the utility of incorporation of such an aglycon in the synthesis of complex carbohydrates has been demonstrated on several occasions.[154]

A general method for the coupling of glycosides to proteins has been described by Bernstein and Hall,[155] and these authors have outlined several criteria for selecting a spacer arm for the preparation of semi-synthetic glycoconjugates. Such a linking arm should display the following characteristics:

(1) compatibility with reagents and protecting groups employed in the total synthesis,

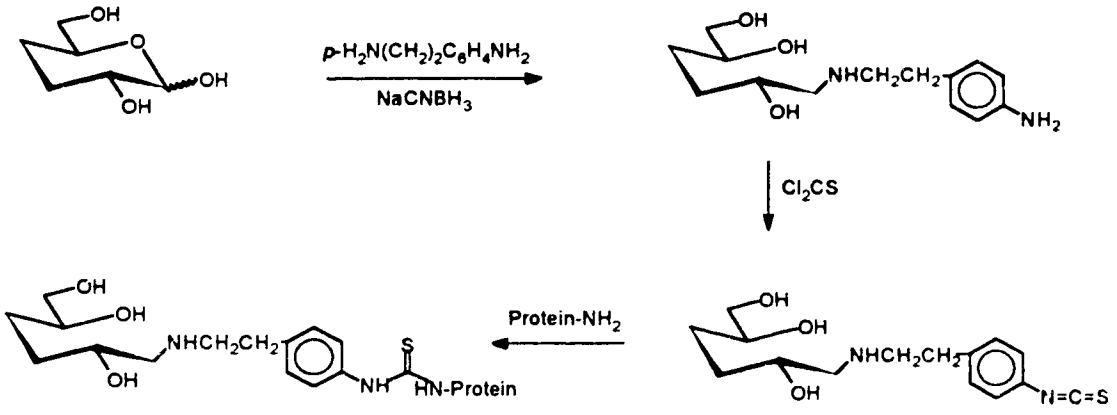
(2) required flexibility in terms of hydrophobicity and antigenic characteristics,

(3) selective activation for conjugation leading to good conjugation yields,

(4) formation of a hydrolytically stable covalent linkage to an amino acid of the carrier,

(5) minimal alteration of the surface charge of the protein.

Figure 6: Conjugation Strategy For Reducing Sugars



The alkenic moiety described by these authors can be viewed as a masked aldehyde which can be exploited for conjugation to molecules possessing free amino groups. The aldehyde functionality can be revealed under the relatively mild conditions of ozonolysis, and subsequent covalent attachment is formalized by borohydride reduction of the Schiff base formed by reaction of this newly generated carbonyl center and the ε-amino moiety of lysine residues present on proteins. Potentially, such a hydrocarbon moiety can mimic the hydrophobic ceramide aglycon to some extent. To date, reports dealing with the incorporation of artificial linking arms to the gangliosides GM_3 and GD_3 are sparse.

3.3 Objectives

Enthusiasm for studying in detail tumor associated antigen-antibody complexes, is driven in part by the hope that an increased understanding of such interactions will prove useful in the diagnosis and management of cancer. Development of sensitive and specific serum tests, as well as labeled antibody imaging tests, would greatly facilitate diagnosis. Furthermore, subsequent management would be enhanced if these reagents were found to be valuable tools in staging, prognosis, monitoring response to therapy, and early detection in relapse.

The objective of this project was to chemically synthesize and conjugate the GD_3 melanoma-associated antigen and the related precursor GM_3 structure to carrier proteins in order to provide artificial antigens bearing these chemically defined epitopes. In turn, these structures are found in increased density on the cancer cell surface as well as in the serum of patients. These antigens are then to be applied towards generating and screening for mAbs which potentially display immunoreactivity towards certain types of cancer, specifically melanoma.

Definition of the antigenic site as well as the binding site of the corresponding monoclonal antibody on a molecular level is crucial to understanding the specificity of the mAb for the hosts tumor as well as to the design of clinically important mAb-based imaging and therapeutic agents. This study however, focuses only on the preliminary stages of antigen preparation with possible extension into generation of the complementary murine mAbs. The resulting chemically synthesized antigens would be of subsequent benefit in probing the nature of binding interactions by X-ray crystallography of the combined antigen-monoclonal antibody complex. More interestingly, since the naturally occuring gangliosides readily form liposomes in aqueous media resulting in poor ^1H NMR specta in D_2O, these synthetic analogues, which are not expected to form liposomes in aqueous media, may prove valuable in studying interactions with monoclonal antibody Fab fragments directly in aqueous solution by ^1H NMR spectroscopy. The precise goals for this project were:

(1) to synthesize the related GM_3 and GD_3 antigens bearing a suitable linking arm,

(2) to prepare GM_3- and GD_3-KLH antigens for immunization studies,

(3) to prepare GM_3- and GD_3-HSA for screening mAbs,

(4) if time permits, depending on the difficulty of synthesis, attempt to generate a murine anti-GD_3 mAb.

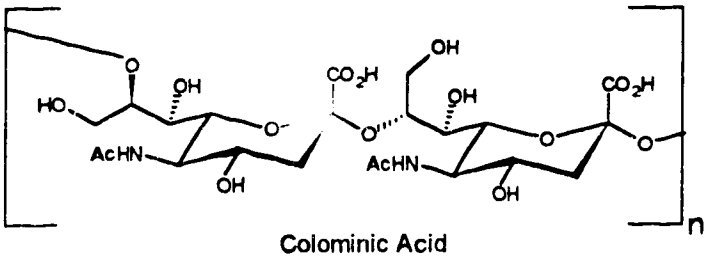
The results of our efforts towards these goals are outlined in the following section.

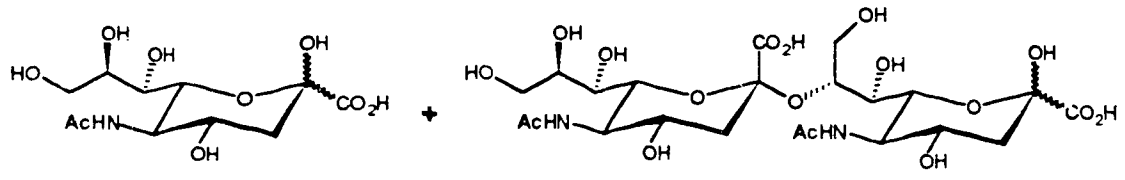
4. RESULTS & DISCUSSION

4.1 Synthesis of Neu5Ac Donors

4.1.1 Hydrolysis of Colominic Acid

Based on the fact that the most common Neu5Ac glycosyl donor to date has been the 2β-chloro derivative **4**,[139] it was anticipated that Neu5Acα(2→8) Neu5Ac donors **15** and **18**, which can be directly prepared from the dimer **12**, would provide convenient entry into the melanoma associated ganglioside series. Indeed, the starting α-(2→8) di-sialyl glycoside **12** can be prepared in gram quantities from naturally occuring poly-α-(2→8) sialic acid (colominic acid from *E.coli*) via graded acid hydrolysis. Following the procedure of Jennings *et al.*,[157] an aqueous solution of colominic acid (2 g) adjusted to pH 2 with 0.1 M HCl was heated to 80 °C for 75 min,[144] then neutralized with 0.1M aqueous sodium hydroxide, and the resulting solution was evaporated *in vacuo*. Ion exchange chromatography of this concentrate over Sephadex DEAE® resin using a 0.1M-0.3M aqueous sodium chloride gradient separated the complex mixture into three distinct fractions (*e.g.*, mono, di-sialyl, and oligomers).





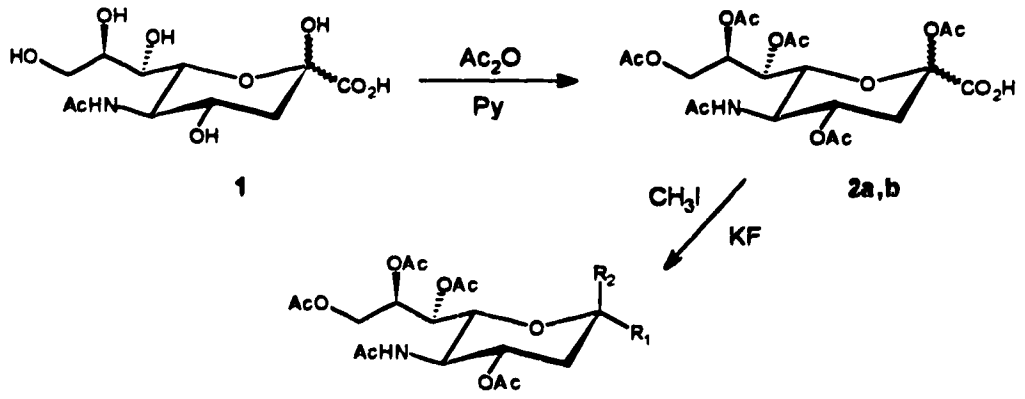
1 + **12**

After five runs on this scale, the identical fractions were pooled and the combined mono-sialyl fractions provided N-acetylneuraminic acid **1** which was obtained in approximately 43% overall yield. The di-sialyl fractions were pooled and further purification by column chromatography over silica gel using 8:1:1 isopropanol-ammonia-water as eluant provided Neu5Acα(2→8)Neu5Ac **12** in 12% yield. The slower eluting oligomer containing fractions could be combined and the resulting mixture could in theory, be recycled again through the acidic hydrolysis process.

4.1.2 Model Studies With Neu5Ac

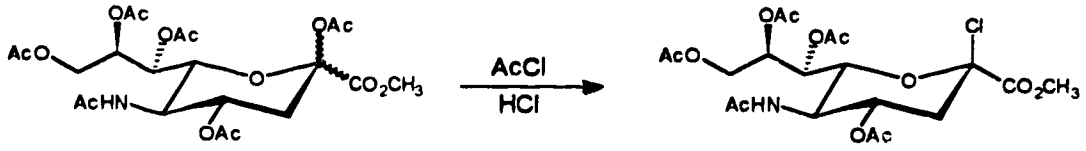
In an effort to provide ^{1}H NMR standards to assist in future structural assignments and to simultaneously provide model compounds for conjugation studies, the *N*-acetylneuraminic acid **1** obtained from the acid hydrolysis of colominic acid was first activated as the known chloride **4** for deployment in the sequential preparation of the model α- and β-linked sialic acid derivatives. This chloride could then act as a sialic acid donor upon application of the classical Koenigs-Knorr glycosidation methodology. Thus, following the method of Ogura *et al.*,[158] sialic acid **1** was *O*-acetylated using 2:1 pyridine-acetic anhydride to provide the per-*O*-acetylated acid **2**, presumably as an α/β mixture **2a/2b** at C-2, however, the crude product was not purified at this stage. Instead, the crude product was directly esterified by reaction of the corresponding potassium salt of the anomeric mixture **2a,b** with methyl iodide to give the fully blocked methyl ester as an α/β mixture (**3a/3b** = 1/10, after separation by chromatography over silica gel) in 78% overall yield from **1**. The anomeric mixture of acetates **3a,b** could be employed directly in the halogenation step without the need for separation, therefore, a mixture of acetates **3a,b** in chloroform was treated with acetyl chloride containing a trace of hydrochloric acid to provide chloride **4** (89% yield). Analysis of this chloride by ^{1}H NMR spectroscopy revealed the product to be >80% pure, therefore, the chloride was used directly in the subsequent Koenigs-Knorr condensation without prior purification. Reaction of the β-chloride **4** with 4-penten-1-ol (3 equiv) in the presence of silver trifluoromethanesulfonate as promoter, yielded an α/β mixture of pentenyl glycosides **5a,b** in 66% yield (**5a/5b** = 5.7/1) after separation by chromatography over silica gel. An attempt to react **5a** with the *p*-nitrophenethyl 6-*O*-t-butyldiphenylsilyl-β-**D**-glucopyranoside derivative as an acceptor by utilizing *N*-iodosuccinimide as an iodine source in accordance with the procedure of Fraser-Reid and coworkers[159] was

unsuccessful at providing the desired sialoside and this approach was abandoned.



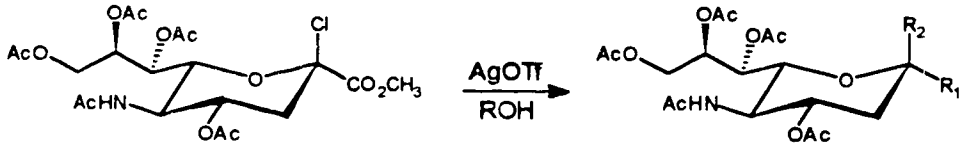
1 **2a,b**

3a R_1 = OAc, R_2 = CO_2CH_3
3b R_1 = CO_2CH_3, R_2 = OAc



3a,b **4**

De-*O*-esterification of the α-pentenyl sialoside **5a** with methanol-0.5 M aqueous potassium hydroxide provided sialoside **6a** (quantitative) and this compound is armed with suitable functionality on the aglycon moiety for subsequent activation and conjugation to carrier proteins. Likewise, de-*O*-esterification of the β-pentenyl sialoside **5b** under the same conditions provided



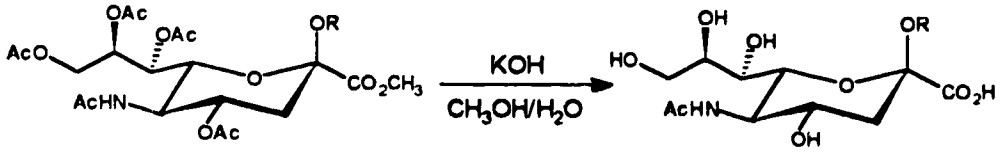
4

5a R_1 = OR, R_2 = CO_2CH_3
5b R_1 = CO_2CH_3, R_2 = OR

R = $CH_2CH_2CH_2CH{=}CH_2$

6b, also in quantitative yield after chromatography over silica gel. A comparison of the ^1H NMR spectra of the two anomeric sialosides **6a** and **6b** showed the expected downfield shift of H-3e to δ 2.74 ppm for the α-sialoside **6a** when compared to δ 2.35 ppm for the β-sialoside **6b**.[111] For a comparison of ^{13}C shifts of **6a** and **6b**, see Table 7.

5a **6a**



5b **6b**

R = $CH_2CH_2CH_2CH{=}CH_2$

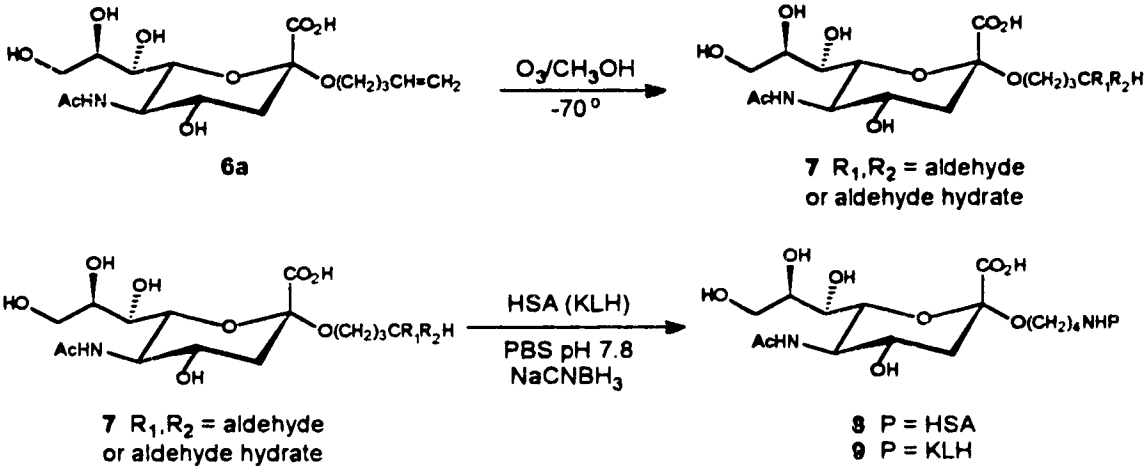
4.1.3 Conjugation of 7 to Carrier Proteins

In order to prepare sialoconjugate antigens of **6a**, the alkenic moiety present on the aglycon was exploited as a masked aldehyde. This procedure utilizes reductive ozonolysis to unveil the masked carbonyl functionality as the initial step. Covalent conjugation is achieved by subsequent reductive amination of the Schiff base formed between this newly generated aldehyde and the carrier protein, presumably via reaction with the ε-amino moiety of the lysines on the protein molecule. The reduction of this imine to a stable secondary amine was accomplished under mild reaction conditions with the aqueous stable sodium cyanoborohydride reducing agent.[155,160] Thus, treatment of a methanolic solution of **6a** at -70 °C, first with ozone (typically 3-5 min), then with methyl sulfide[161] to complete the two stage reductive ozonolysis process yielded the aldehyde **7** (or equivalent). The proton NMR spectrum of **7** (D_2O) was devoid of alkenic proton resonances, however, compound **7** did not display the expected

Table 7: Comparison of ^{13}C NMR Shift Values for 6a and 6b.

Carbon	Shift (δ) ppm	
	α-Sialoside 6a	β-Sialoside 6b
Carbonyl	175.33	176.30
	174.36	175.66
$H\underline{C}=CH_2$	139.61	140.06
$HC=\underline{C}H_2$	115.48	115.33
C-2	101.41	100.93
C-4,6,7,8	73.30	71.22
	72.49	71.08
	68.99 (2X)	69.03
		67.78
C-9, OCH_2	65.10	64.24
	63.32	63.59
C-5	52.66	52.97
C-3	41.16	40.79
CH_2	30.13	30.61
	28.97	29.04
$CH_3C=O$	22.75	22.96

downfield shifted aldehyde proton, nor did the ^{13}C NMR spectrum reveal the corresponding aldehydic carbonyl carbon, therefore it was presumed that the aldehyde was in the hydrated form as has previously been noted by others.[160] This fact was further supported by the appearance of a new single proton bearing carbon peak (belonging to the aliphatic side chain) in the region of δ 100-110 ppm in the carbon NMR spectrum. Conjugation of sialic acid derivative **7** to the carrier proteins human serum albumin (HSA) and keyhole limpet hemocyanin (KLH) in phosphate buffered saline (PBS) at pH 7.8 and room temperature, provided the corresponding HSA- and KLH-sialoglycoconjugates **8**

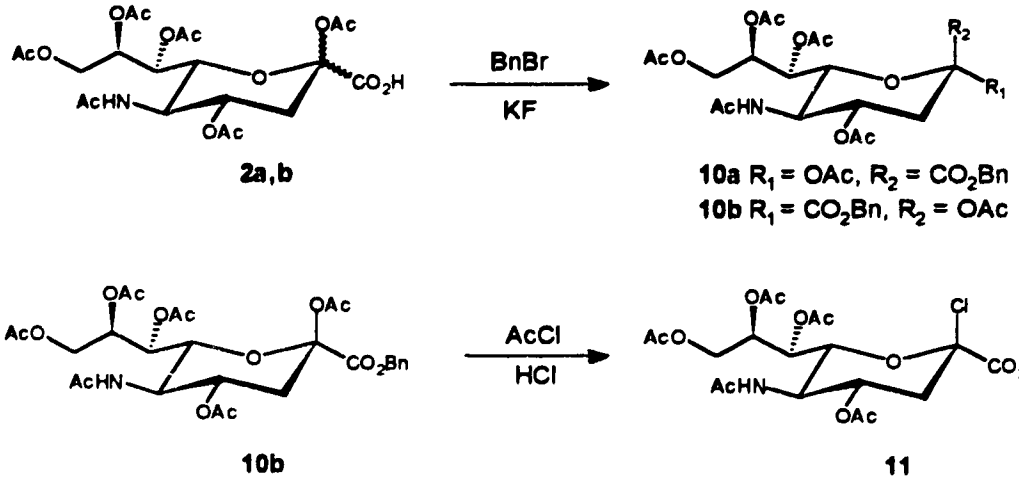


and **9** respectively after exhaustive dialysis and lyophilization. The sialic acid content of these semisynthetic glycoconjugates was estimated by the procedure of Svennerholm[162] as modified by Miettinen[163] and the results of the resorcinol hydrochloric acid assays are shown in Graphs 1 and 2 (Appendix 1, see page 202). From these curves, it was found that the corresponding N values (N = # moles sialic acid/# moles protein) were 20 and 740 for **8** and **9** respectively.

4.1.4 Synthesis of the Sialyl Chloride Donor as its Benzyl Ester

In order to prepare the sialyl chloride donor as the benzyl ester, the α/β mixture **2a/2b** was esterified at the carboxyl moiety by reaction of the corresponding potassium salt of this anomeric C-2 mixture, this time with benzyl bromide, to give the fully blocked benzyl ester as the α/β mixture **10a/10b**. Separation of the resulting C-2 anomeric mixture by chromatography over silica gel using 15:10:1, then 10:10:1 hexane-ethyl acetate-ethanol as the eluting

solvents, gave a small amount of **10a** (as an impure mixture) followed by pure **10b** in 83% yield. An analytical sample of **10a** could be obtained by rechromatography over silica gel using 60:1 chloroform-methanol as eluant. Treatment of a solution of **10b** in 1:1 chloroform-acetyl chloride containing a trace of hydrochloric acid provided chloride **11** (quantitative) of sufficient purity (>85% as determined by ^{1}H NMR) for subsequent reactions. In fact, benzyl ester chloride derivative **11** was found to be more stable, and its ^{1}H NMR spectrum



2a,b

10a R_1 = OAc, R_2 = CO_2Bn
10b R_1 = CO_2Bn, R_2 = OAc

10b **11**

visibly cleaner than the corresponding methyl ester chloride **4**, an observation that was worth keeping in mind for the future design of a di-Neu5Ac synthon. Also, compounds **4** and **11** displayed a significant downfield shift for H-3a ($\delta \approx$ 2.3 ppm) as compared to their precursor acetates ($\delta \approx$ 2.1 ppm).

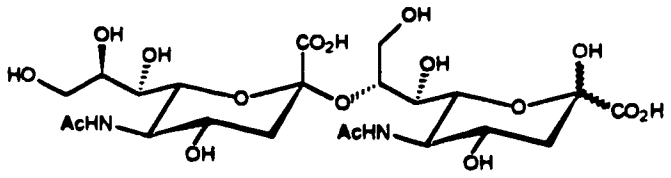
4.2 Synthesis of Di-sialyl Donors

4.2.1 Synthesis of a Di-sialyl Chloride Donor as the Lactone Ester 15

Neu5Acα(2→8)Neu5Ac dimer **12**, obtained previously from polysaccharide degradation, was first acetylated using 2:1 pyridine-acetic anhydride to provide a mixture of per-*O*-acetylated acid lactones **13a/13b**, again presumably as an α/β mixture at C-2. This crude mixture was then directly esterified by reaction of the corresponding potassium salt of the anomeric mixture **13a,b** with benzyl bromide to furnish a mixture of fully blocked benzyl ester-lactones **14a** and **14b** (11% and 55% yield, respectively). The structural assignments for lactones **14a** and **14b** were based primarily on their ^{1}H NMR

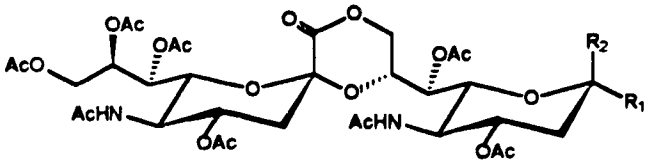
portraits, which, contrary to expectations, displayed only 5 protons in the aromatic region along with 9 rather than 10 acetate signals anticipated for a di-benzyl ester structure. Furthermore, lactone formation has been previously observed in the group B meningococcal polysaccharide[164] and in the G_{D1b} ganglioside,[165] indicating a common feature of the α-(2→8) linkage.

Although some of the spectral assignments for the proton resonances of compound **14b** remain tentative, several proton shifts were verified by decoupling experiments. Decoupling of the acetamido-NH doublet centered at δ 5.88 ppm caused the collapse of the H-5 multiplet at δ 4.20 while irradiation of the other acetamido-NH doublet at δ 5.52 ppm (NH') likewise affected the H-5' quartet at δ 4.17. To assign signals on the reducing terminal sialic acid moiety, decoupling of the doublet of doublets at δ 5.17 ppm (H-7) resulted in the collapse of the obscure multiplet at δ 4.35, presumably attributed to the H-8 resonance, while irradiation of the doublet of doublets at δ 4.45 ppm (H-9a) revealed that the overlapping resonances located at δ 4.37-4.32 were due to the H-9b and H-8 signals. With respect to the terminal sialic acid moiety, decoupling of the H-9'a doublet of doublets at δ 4.29 ppm collapsed the double doublet of doublets at δ 5.22 (H-8') along with the doublet of doublets at δ 4.01 (H-9'b) and irradiation of the H-6' doublet of doublets at δ 4.08 ppm perturbed the doublet of doublets at δ 5.34 (H-7') as well as the quartet at δ 4.17 (H-5'). Irradiating the H-9b doublet of doublets at δ 4.01 ppm, which was assigned by the previous perturbation of H-9'a, simultaneously collapsed the double doublet of doublets at δ 5.22 along with the doublet of doublets at δ 4.29. In order to distinguish the H-3e/H-3a pair from the H-3'e/H-3'a pair, two decoupling experiments were carried out, thus, irradiation of the H-3e doublet of doublets at δ 2.52 ppm (H-3e) revealed that this proton was coupled to the triplet of doublets at δ 5.30 (H-4) as well as the doublet of doublets at δ 1.97 (H-3a) while irradiation of the H-3'a doublet of doublets at δ 1.80 ppm collapsed the multiplet at δ 5.34 (H-4') and the doublet of doublets centered at δ 2.43 (H-3'e). The assignment of the H-3a/H-3e/H-4 signals to the sialic acid moiety bearing the methyl aglycon was based on the general observation that conversion to an anomeric halo-sialoside results in a corresponding downfield shift for both H-3e and H-3a. Upon conversion of the acetate **14b** to the chloride **15**, the resonances at δ 2.52 and 1.97 ppm were shifted to δ 2.82 and 2.30 ppm respectively.



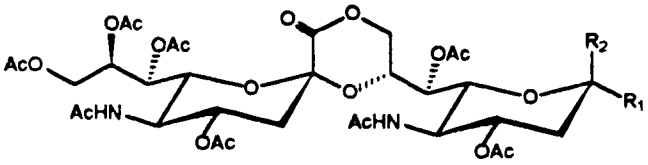
12

Ac_2O/Py



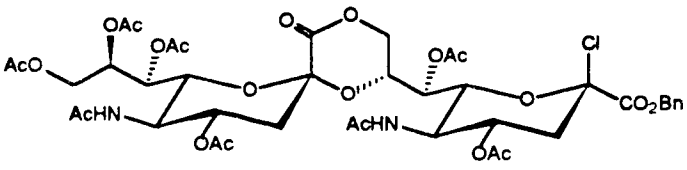
13a R_1 = OAc, R_2 = CO_2H
13b R_1 = CO_2H, R_2 = OAc

BnBr/KF



14a R_1 = OAc, R_2 = CO_2Bn
14b R_1 = CO_2Bn, R_2 = OAc

AcCl/HCl



15

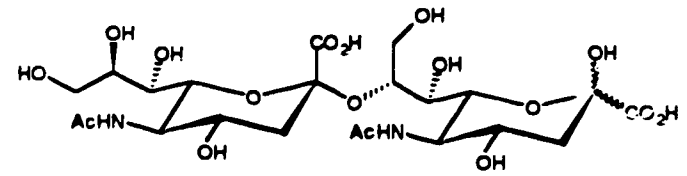
Conversion of the major β-acetate **14b** into the chloride **15** was carried out by direct application of the AcCl-$CHCl_3$-HCl conditions described earlier for the monosialyl derivative **11**. The ^1H NMR spectrum of **15** showed a downfield shift for H-3a (δ = 2.30 ppm) which is consistent with that displayed by both of the corresponding monosialyl 2β-chloro derivatives **4** and **11**. It should also be pointed out that separation of the α/β acetate mixture is not a necessary requirement since both products can serve as direct precusors to the desired chloro derivative. The crude chloride was used directly in the subsequent sialylation reactions without further manipulation.

4.2.2 Synthesis of a Di-sialyl Chloride Donor as the Di-benzyl Ester 18

The dibenzyl esters **17a** and **17b** along with some lactone **14b** were obtained in 3%, 41% and 12% yield respectively by initial *p*-toluenesulfonic acid catalyzed acetylation of **12** to provide the α/β mixture of di-acids **16a** and **16b**, followed by esterification (BnBr-KF/anhydrous DMF) and careful chromatography over silica (for a comparison of chemical shift values for **10a**, **10b**, and **17b**, see Table 8). Treatment of the β-acetate **17b** with AcCl-$CHCl_3$-HCl as before, afforded the crude chloride **18** (>85%, ^1H NMR) which again displayed the charcteristic downfield shift in the ^1H NMR spectrum for H-3a (δ = 2.22 ppm). The dibenzyl ester chloride, namely **18**, appeared to be more stable than the monobenzyl ester lactone chloride **15**, thus it was anticipated that slightly higher yields may be realized by employing the di-benzyl ester di-sialyl donor. Furthermore, the earlier reaction of **13b** with HCl was problematic in that the mixture turned into a gel and the subsequent isolated yield of **15** was relatively low (84%) considering the fact that only an aqueous wash was employed in the purification step.

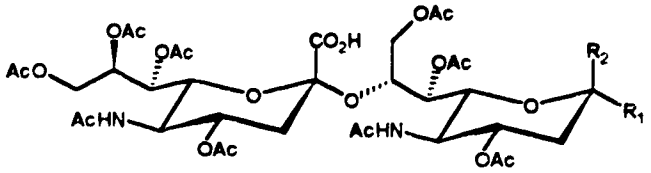
4.2.3 Reaction of 18 With a Primary Alcohol

With the desired di-Neu5Ac donor now available, reaction of the chloride **18** with excess methanol using $AgClO_4$-TMU in anhydrous THF[166] provided the α-methyl glycoside **19a** as the major anomeric component together with some β-methyl glycoside **19b** (α/β = 2.75:1) in 75% combined yield after separation of the anomeric mixture by column chromatography over silica gel. The α-methyl glycoside **19a** was sequentially deblocked in two steps; (1) H_2, 5% Pd-C; (2) NaOMe/MeOH to give **20a**, most probably as the di-ammonium salt upon



12

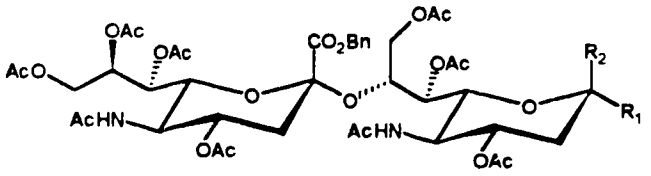
Ac_2O/H^+



16a R_1 = OAc, R_2 = CO_2H
16b R_1 = CO_2H, R_2 = OAc

+ **13a,b**

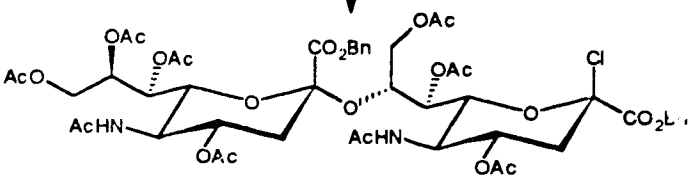
$BnBr/KF$



17a R_1 = OAc, R_2 = CO_2Bn
17b R_1 = CO_2Bn, R_2 = OAc

+ **14a,**

AcCl/HCl

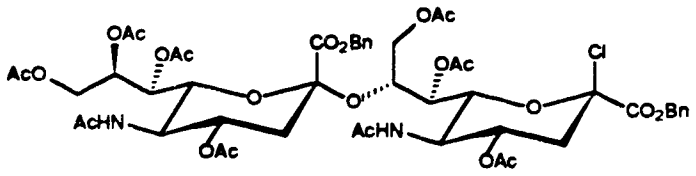


18

chromatography over silica gel using 8:1:1 isopropanol-ammonia-water as the elution solvent. Likewise, subjection of the β-anomer **19b** to the same reaction sequence provided **20b**. The structural assignments for **20a** and **20b** are based

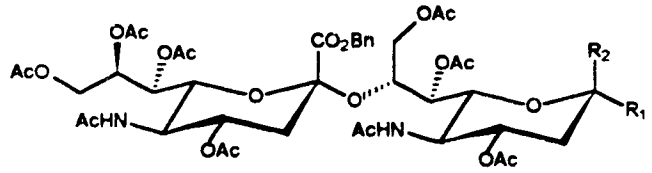
Table 8: Comparison of Proton Shifts For 10a, 10b, and 17b (ppm from TMS).

Neu5Ac			Di-Neu5Ac		
β-10b	Shift (δ)	J (Hz)	β-17b	Shift (δ)	J (Hz)
H-3e	2.56	13.25, 5.0	H-3e	2.53	13.0, 4.5
H-3a	2.1	13.25, 11.25	H-3a	1.98	13.0 (t)
H-4	5.25	m	H-4	5.34	m
H-5	4.11	m	H-5	3.95	10.5 (q)
H-6	4.11	m	H-6	4.18	10.5, 2.0
H-7	5.38	5.5, 2.0	H-7	5.28	2.5 (t)
H-8	5.09	6.5, 5.5, 2.5	H-8	4.69	9.5, 2.5 (dt)
H-9	4.45	12.5, 2.5	H-9	4.58	12.5, 2.5
H-9	4.15	12.5, 6.5	H-9	4.12	12.5, 9.5
NH	5.25	9.5	NH	6.08	9.5
Bn	5.24	12.0	Bn	5.24	12.0
Bn	5.17	12.0	Bn	5.19	12.0
α-10a			α		
H-3e	2.55	13.0, 4.5	H-3e	2.67	13.0, 4.5
H-3a	2.08	13.0, 12.5	H-3a	1.79	13.0 (t)
H-4	4.97	12.5, 10.5, 4.5	H-4	4.85	13.0, 10.5, 4.5
H-5	4.16	10.5 (q)	H-5	4.02	10.5 (q)
H-6	4.75	10.5, 2.5	H-6	3.66	10.5, 2.5
H-7	5.38	7.0, 2.5	H-7	5.27	9.5, 2.5
H-8	5.18	7.0, 5.5, 2.5	H-8	5.42	9.5, 5.5, 2.5
H-9	4.36	12.5, 2.5	H-9	4.23	12.5, 2.5
H-9	4.06	12.5, 5.5	H-9	4.08	12.5, 5.5
NH	5.26	10.5	NH	5.01	10.0
Bn	5.21	12.0	Bn	5.33	12.0
Bn	5.12	12.0	Bn	5.11	12.0



18

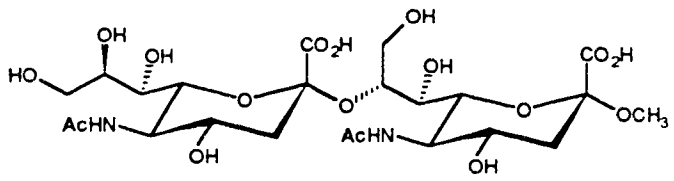
AgOTf | CH_3OH



19a $R_1 = OCH_3$, $R_2 = CO_2Bn$
19b $R_1 = CO_2Bn$, $R_2 = OCH_3$

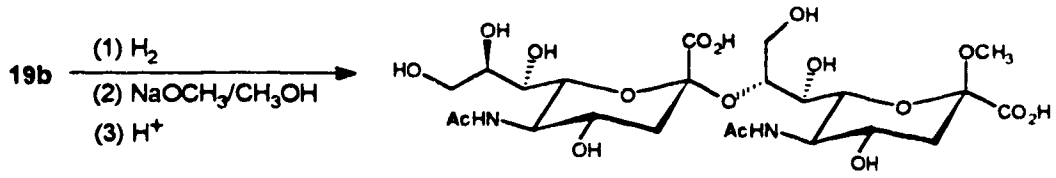
on the chemical shifts of their corresponding equatorial H-3e protons and are in good agreement with those predicted from empirical rules.[167] Our reasoning for this assignment was as follows; (1) in aqueous solutions, sialic acid exists almost exclusively (92-95%) in the β-configuration and the shift of H-3e(α) at δ 2.75 ppm is downfield from that of H-3e(β) generally located at 2.2 δ ppm;[112] (2) likewise, Neu5Acα(2→8)Neu5Ac **12** displayed signals at δ 2.75 ppm, for H-3'e and δ 2.2 ppm for H-3e and; (3) the shift of H-3e for the corresponding α– and β-methyl glycosides of Neu5Ac were shown previously to resonate at δ 2.69 and 2.36 ppm respectively[111] and this fact further supported our observed downfield shift of H-3e to δ 2.74 ppm for the α–sialoside **6a** when compared to δ 2.35 ppm for the β–sialoside **6b** described earlier. Compound **20a** showed downfield shifts

19a (1) H_2 (2) $NaOCH_3/CH_3OH$ (3) H^+



20a

for both of the sialic acid equatorial protons H-3e and H-3e' at δ 2.78 and δ 2.61 ppm indicative of an α-methyl glycoside while compound **20b**, which showed shifts at δ 2.76 ppm, for H-3e' and δ 2.30 ppm for H-3e, was assigned the β-methyl glycoside structure. These assignments are in good agreement with recent high field proton NMR studies performed on α-(2→8)-linked homo-sialooligosaccharides by Michon and coworkers.[113]



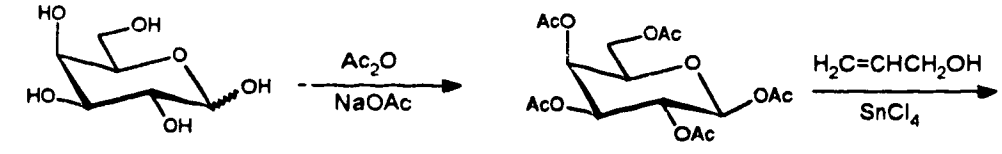
20b

Thus, preparation of the di-Neu5Ac donor **18** followed by its subsequent transformation into methyl glycosides has successfully demonstrated its reactivity with simple primary alcohols as described in this section. Hopefully, the utility of this donor can be expanded to include glycosidic reactions with secondary alcohols, in which case this donor may prove to be useful for the preparation of gangliosides such as GD_2 and GD_3. The utility of both **15** and **18** to this end will be explored in an upcoming section.

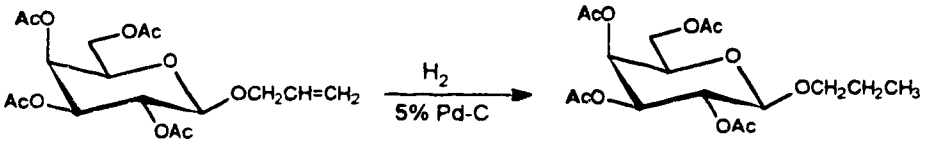
4.2.4 Reaction of 18 With a Secondary Alcohol

In order to complete our model studies, synthesis of the terminal trisaccharide sequence of the relevant melanoma associated ganglioside GD_3, α-**D**-Neu5Ac-(2→8)-α-**D**-Neu5Ac-(2→3)-β-**D**-Gal-(1→4)-β-**D**-Glc-(1→1)-Cer by employing the novel α-(2→8) disialyl glycosyl donor **18** described in the previous section is demonstrated in this section, herein providing an example of the reaction of this disialyl donor with a secondary alcohol. A key step in our approach towards the synthesis of di-sialyl gangliosides involves the introduction of the di-Neu5Ac unit with α-stereoselectivity onto the appropriate glycosyl acceptor. In the previous section, we demonstrated the utility of di-Neu5Ac synthon **18** with methanol. Now, in this section, we describe the reaction of this donor with a galactose derivative, thereby providing the terminal trisaccharide portion of the important GD_3 ganglioside.

"Disconnection" of the terminal trisaccharide sequence revealed the possibility of a synthetic route employing di-sialyl donor **18** along with the monosaccharide glycosyl acceptor **26**. Acetylation of β-D-galactose using the traditional sodium acetate and acetic anhydride[168] conditions yielded compound **22** and preparation of the required galactose acceptor was then carried out in a straightforward manner in four overall steps as follows. Reaction of allyl alcohol and β-D-galactose pentaacetate in the presence of tin (IV) chloride, according to the procedure of Banoub and Bundle[169] provided the allyl glycoside **23**[170] which upon subsequent 5% Pd-on-carbon catalyzed hydrogenation yielded the propyl derivative **24**.[171]

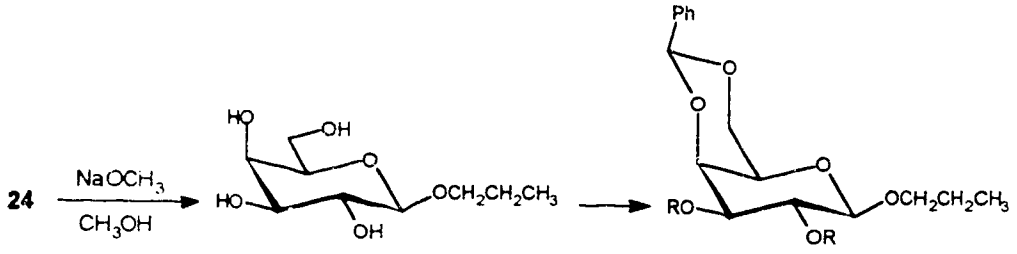


21 **22**



23 **24**

De-*O*-acetylation of **24** (sodium methoxide in dry methanol) led to the tetrol **25**,[172] which was then selectively blocked at the 4,6-*O*-positions by treatment with α,α'-dimethoxytoluene and catalytic *p*-toluenesulfonic acid in 2:1 acetonitrile-*N*,*N*-dimethylformamide to give the acceptor **26**. For future comparison purposes, a small amount of **26** was acetylated (2:1 pyridine-acetic

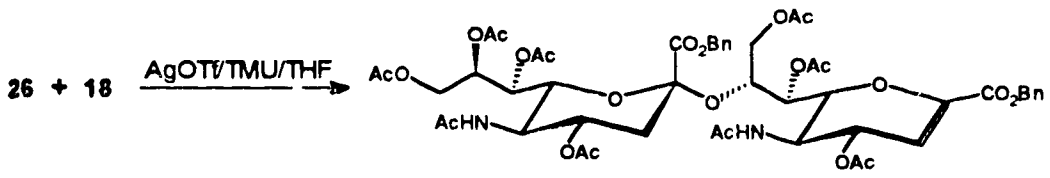


25

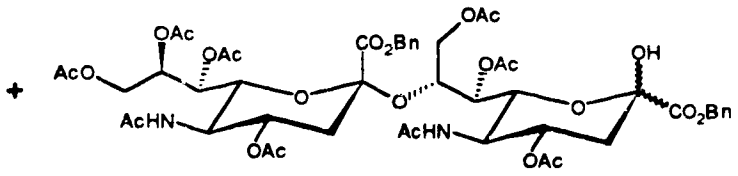
26 R = H
27 R = Ac

anhydride) to give **27**, which upon ^{1}H NMR analysis, displayed characteristic deshielded doublet of doublets at δ 5.39 ppm ($J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 8.0 Hz) and 4.96 ppm ($J_{3,2}$ = 10.5 Hz, $J_{3,4}$ = 3.5 Hz) for H-2 and H-3 respectively.

The glycosidation step was next carried out with the chloride donor **18** (1 equiv) and excess diol acceptor **26** (2 equiv) in the presence of silver trifluoromethanesulfonate and 1,1,3,3-tetramethylurea in anhydrous tetrahydrofuran at -30 °C under inert atmosphere.[173] As expected, the major component of the reaction mixture was the 2,3-dehydro compound **28**, formed by elimination of HCl from **18**, along with some hydrolysis product **29**. However, separation of the complex mixture by column chromatography over silica gel gave impure **30** and



28



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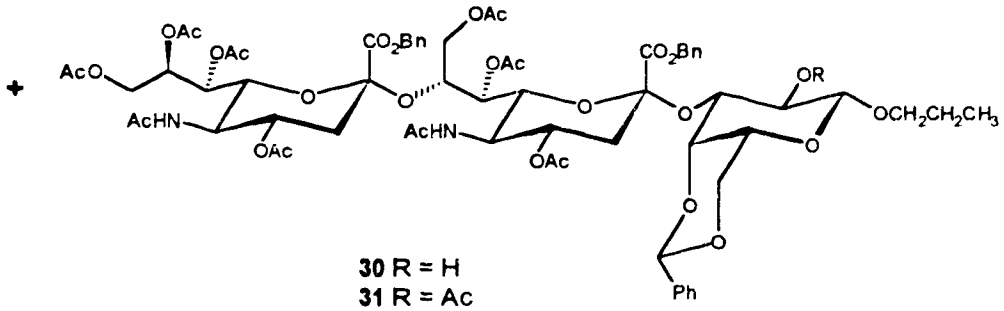
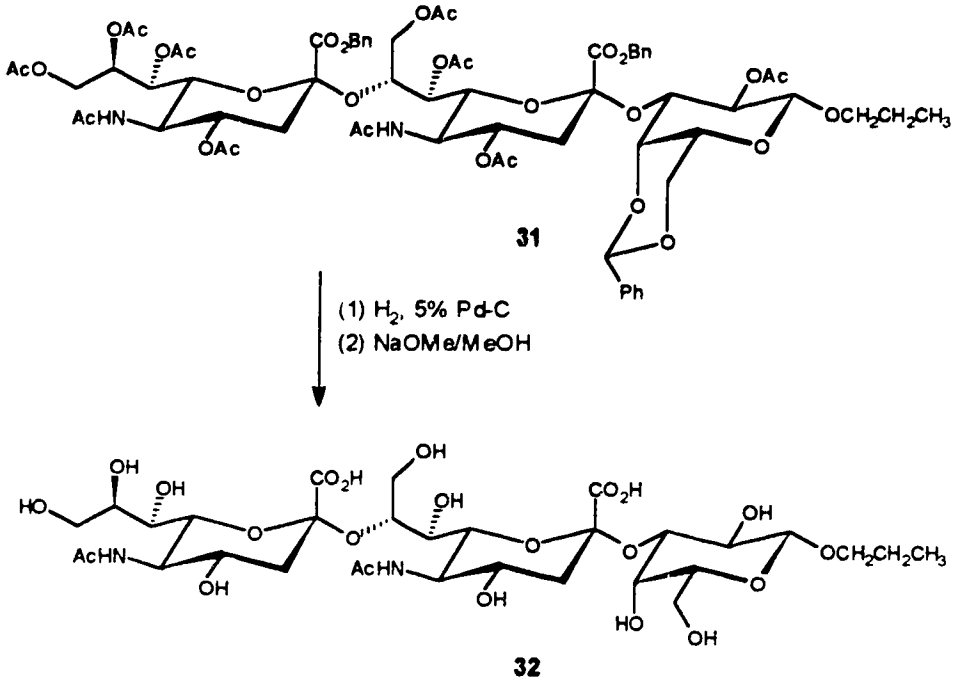


30 R = H
31 R = Ac

subsequent acetylation (2:1 pyridine-acetic anhydride) followed by rechromatography over silica were prerequisites in order to obtain a pure sample of trisaccharide **31** (14% overall yield based on starting chloride **18**). The ^{1}H NMR spectrum of **31** displayed doublet of doublet signals centered at δ 2.76 and 2.81 ppm respectively for the equatorial H-3e's of the corresponding sialic acid

moieties as well as the anticipated ten acetate singlets. Although the downfield signal for the galactose H-2 proton at δ 5.22 ppm was partially obscured by the benzyl proton resonances, the regiochemistry of the (2→3) glycosidic linkage was confirmed by decoupling the clearly visible galactose H-3 and H-1 signals at δ 4.55 and 4.41 ppm respectively.

Routine deblocking of **31**; (1) H_2, 5% Pd-C, MeOH, trace HOAc; (2) NaOMe/MeOH) proceeded in 68% overall yield to give 13 mg of **32** after chromatography over silica gel using 8:1:1 isopropanol-ammonia-water as eluant.



The ^1H NMR spectrum of **32** displayed two doublet of doublets at δ 2.81 and 2.65 ppm for the equatorial H-3e protons of the sialic acid residues. As was noted earlier for the spectra of the methyl glycosides of di-Neu5Ac obtained in D_2O, the corresponding H-3e resonances for the α-anomer were at δ 2.78 and 2.61 ppm, while resonances for these protons for the β-anomer were at δ 2.76 and 2.30 ppm. Thus, it appears that the trisaccharide **32** possesses the correct α -stereochemistry. The same pattern has been observed in the spectra of other sialic acid derivatives in D_2O and the chemical shift of H-3e is generally found to be greater than δ 2.5 ppm in the case of α-monosialosides[108] but less than δ 2.5

ppm for the corresponding β-analogues as mentioned earlier. Similar observations have also been reported for α-(2→8)-linked sialic acid oligosaccharides.[174]

We have now expanded our approach in this section to include a secondary alcohol (the 3 position of galactose) yielding the terminal trisaccharide portion of GD_3, namely derivative **32**. Recently, it has been suggested that this epitope represents an important binding region for certain anti-GD_3 monoclonal antibodies which recognize melanoma cells.[175]

These results suggested to us that it may be possible to expand this approach to lactose which would ultimately lead to the complete GD_3 carbohydrate sequence. Since virtually no β-(2→3) glycoside was detected in our reaction mixture, it can be postulated that participation of the solvent (THF instead of CH_3CN in Figure 5)[176] may in part be responsible for the α-stereoselectivity observed. Moreover, due to the low glycosylation yield obtained using the silver triflate-tetramethylurea procedure, it may be anticipated that reactions employing either thioglycosides[147] or *S*-xanthates[177] would provide better glycosidation yields. The 2,3-dehydro derivative **28** may also be a useful starting material for strategies employing C-3 stereocontrolling functionalities such as phenylseleno and phenylthio auxiliaries,[142] while the hydroxy derivative **29** can be recycled back to the fully acetylated derivative **17b**.

4.3 Synthesis and Conjugation of Lactose-*p*-NTFA Derivatives

4.3.1 Synthesis of the 3',4' Diol 37 as Sialyl Acceptor

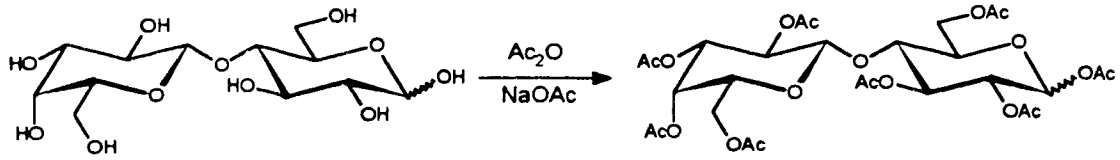
In order to synthesize GM_3 (and hopefully GD_3) with a linking arm suitable for subsequent conjugation to carrier proteins in order to provide antigens and/or solid supports for utility in affinity chromatography, the 3',4' diol **37** was chosen as the target sialyl acceptor. This disaccharide displays a simple protecting group pattern with the equatorial 3'-OH group of galactose free for sialylation while the competing axial 4'-OH group is anticipated to be dormant. Removal of the blocking groups from **37**, would provide the target GM_3 while elaboration to the ganglioside GM_2 would require no further blocking group manipulations. Finally, linking this carbohydrate derivative to other molecules could be achieved by utilizing the para-nitro group of the *p*-nitrophenethyl aglycon as a latent amino moiety[178] which can be subsequently activated in one

of several ways. It may even be possible to radioiodinate the aromatic ring of the aglycon of **59** for metabolic studies.

This identical sequence of reactions has previously been applied by Sugimoto and Ogawa[138] to the 3'4' diol per-O-benzylated lactose derivative (see previous chapter benzyl glycoside **1**). These investigators performed the key glycosidation reaction of this ideally protected lactose derivative with chloride **4** in the presence of $HgBr_2$-$Hg(CN)_2$ in 1,2-dichloroethane as solvent and obtained the β-linked trisaccharide along with the desired α-linked compound in a ratio of β/α = 2.5/1.

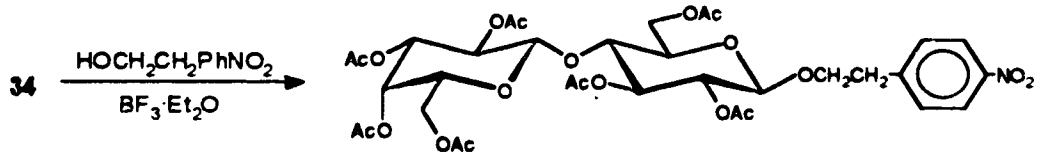
We anticipated that by employing our conditions using anhydrous THF as the solvent and silver as the initiator, solvent participation would direct the stereochemistry primarily to the desired α-stereoisomer. Furthermore, it was our intent to use the corresponding benzyl ester chloride **11**, since in our experience, this chloride was more stable than the corresponding methyl ester chloride **4**, a property that would greatly be appreciated for future application of this strategy to the disialyl donor.

Lactose **33** was first acetylated, by employing the sodium acetate and acetic anhydride conditions[168] mentioned earlier, to give the fully protected lactose derivative **34** in 88% yield, presumably as an α/β mixture. This mixture was used directly in the subsequent Lewis acid catalyzed glycosidation reaction.



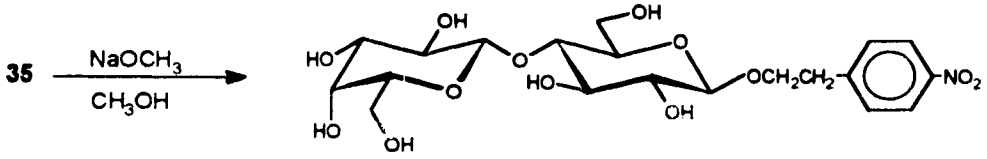
33 **34**

Thus, reaction of **34** with 4-nitrophenethyl alcohol (PNPE-OH) by activation with $BF_3{\cdot}Et_2O$ provided the desired β-glycoside **35** in 64% yield after purification by column chromatography over silica gel. Next, de-O-esterification by treatment with methanolic sodium methoxide gave the lactose-PNPE derivative **36** in



35

quantitative yield. Application of the conditions reported by Sugimoto and Ogawa to this lactose derivative sequentially proceeded as follows; (1) 2,2 dimethoxypropane, catalytic *p*-toluenesulfonic acid, dry DMF;[179,180] (2) NaH, benzyl bromide; and (3) 4:1 acetic acid-water, 80 °C, upon which the 3'4' diol **37** along with the 4'6' diol were obtained in 23% and 12% yield respectively.



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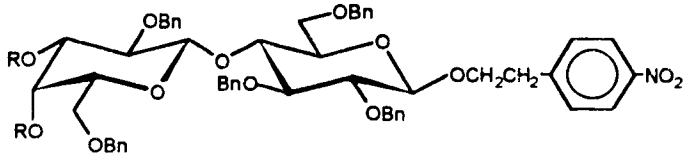
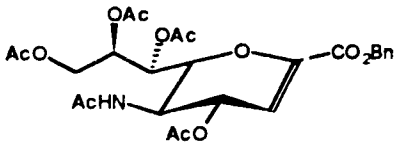
That this regiochemical assignment was indeed correct was confirmed by treatment of a small amount ot **37** with 2:1 pyridine-acetic anhydride to give the diacetate **38**. The ^{1}H NMR spectrum of **38** displayed a downfield shifted doublet



37 R = H
38 R = Ac

at δ 5.38 ppm ($J_{4',3'}$ = 3.5 Hz) for H-4' along with a double doublet signal at δ 4.86 for H-3' ($J_{3',2'}$ = 10.0 and 3.5 Hz for coupling to H-2' and H-4' respectively). These assignments were further confirmed by decoupling experiments. Thus, decoupling of the doublet of doublets centered at δ 4.86 ppm caused a collapse of the doublet at δ 5.38 along with a perturbation in the 5 proton multiplet located at δ 3.58-3.25. Closer inspection of this latter region then allowed for the assignment of the H-2' doublet of doublets centered at δ 3.51 ppm.

Now that both the sialyl donor and acceptor were in hand, introduction of diol **37** to the benzyl ester chloride **11** was initiated in the presence of silver trifluoromethanesulfonate by adapting previously reported glycosylation conditions.[181] Unfortunately, unlike the reported reaction employing methyl ester chloride **4**, the reaction at hand yielded only the unsaturated compound **39** along with hydrolysis product **40** and recovered diol **37**. It thus appears that unfavorable steric interactions between the bulky benzyl ester on the donor and the C-2' benzyl ether protecting group of the acceptor play a governing role in the outcome of this reaction leading exclusively to the α,β-elimination product of **39**. This competing reaction, as can be expected, is always the major by-product of such sialylation reactions. Thus, in order to employ the di-benzyl ester feature which, as was mentioned earlier, possesses favorable stability characteristics, the acceptor molecule would have to now be redesigned.



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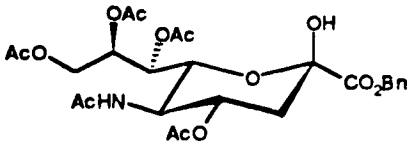


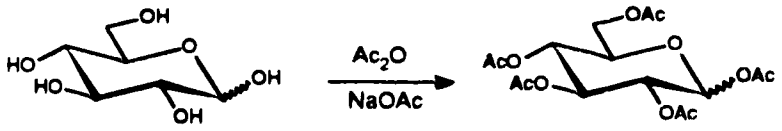
40

Since the observed steric hindrance appeared to involve the bulky nature of the ester moiety along with the formidable size of the protecting group residing at the C-2 position of the galactose unit of lactose diol **37**, it could be inferred that approach of the two reactants was from this side of the molecule and therefore, any reduction in the size of either group should lead to the desired glycosylation product. In our case, since we wanted to maintain the benzyl ester protection strategy for the sialyl donor, we decided to build a 2',3'-diol derivative in which the 4'- and 6'-positions were blocked and the 2'- and 3'-positions were now free. Hopefully, reaction would proceed with the desired regiochemistry as in the case of model monosaccharide **26**, (*i.e.*mainly at the desired 3' position).

4.3.2 Synthesis of 2',3' Diol 52 as a Sialyl Acceptor

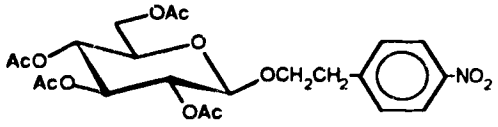
Our first approach to the 2',3' diol lactose sialyl acceptor involved a stepwise construction of the diol unit from monosaccharide building blocks. Thus

acetylation of α,β-D-glucose **41** using acetic anhydride and sodium acetate[168] provided glucose pentaacetate **42** as an α/β mixture in approximately 65% yield



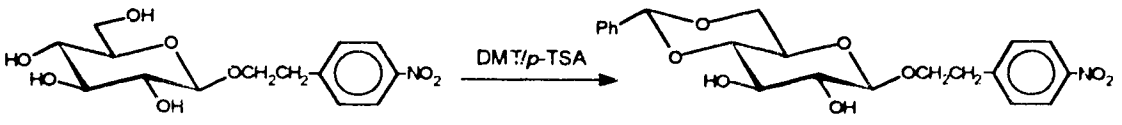
41 **42**

after recrystallization and this mixture was used directly in the subsequent glycosylation step. Using boron trifluoride etherate as the Lewis acid, glycosylation of the mixture **42** in the presence of *p*-nitrophenethyl alcohol led to the glucose derivative **43**. Since this product was indistinguishable from starting material in many of the TLC solvent systems investigated, the crude **43** was subjected to de-*O*-acetylation ($NaOCH_3/CH_3OH$) to give **44**, and treatment of

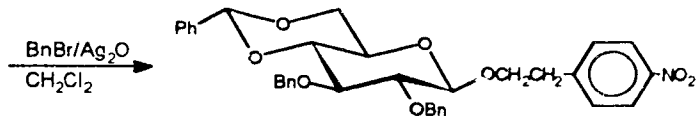


43

this tetrol with α,α'-dimethoxytoluene and catalytic *p*-toluenesulfonic acid yielded the 4,6-*O*-protected glucose-PNPE derivative **45** in 89% isolated yield. Etherification of this diol under mildly basic conditions employing silver oxide as the base and benzyl bromide as the *O*-alkylation reagent provided **46** in 90% yield from **45**.

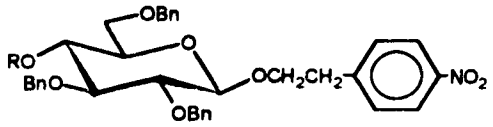


44 **45**



46

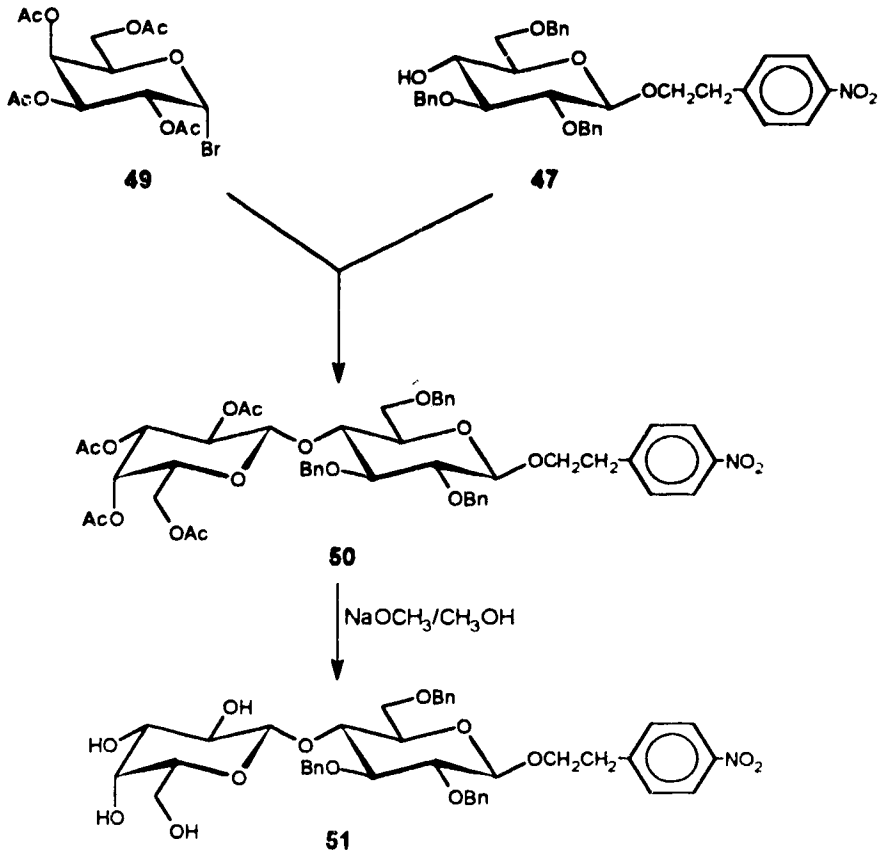
Compound **46** was now suitably set up for reduction with sodium cyanoborohydride under the conditions described by Garegg and Hultberg.[182] As anticipated, this reduction, when carried out in the presence of HCl, proceeded with a high degree of regioselectivity to give compound **47** (70%) wherein the 4-OH group was free for further elaboration to the corresponding lactose derivative. The regiochemistry of the reduction process was confirmed by acetylation of a small amount of this material to fully blocked glucose-PNPE derivative **48**.



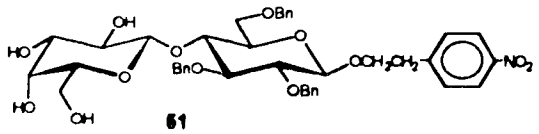
47 R = H
48 R = Ac

The ^{1}H NMR spectrum of this derivative displayed the anticipated downfield shift for H-4 to δ 4.96 ppm. Also, irradiation of the complex triplet centered at δ 4.96 ppm caused the collapse ot the triplet at δ 3.58 to a doublet. Likewise, irradiation of the triplet at δ 3.58 ppm caused the collapse of the triplet at δ 4.96, thus confirming the signals for H-4 and H-3. Decoupling of the doublet of doublets centered at δ 3.44 ppm simultaneously collapsed the triplet at δ 3.58 to a doublet as well as the doublet at δ 4.42 to a singlet confirming the signals for H-2, H-3, and H-1, thus the regiochemical assignment of **47** was indeed correct.

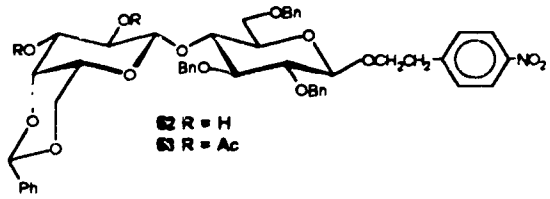
Glycosidation of **47** with acetobromogalactose **49** (which was readily obtained in crystalline form from the precursor β-acetate **22** by treatment with HBr in acetic acid) in the presence of silver salts yielded the fully blocked lactose-PNPE derivative **50** in good yield (75%). This derivative possessed all benzyl ether blocking groups on the glucose moiety and all ester protecting groups on its galactose unit, thus, the desired 2',3'-diol could now be obtained in a relatively straightforward manner. Compound **50** underwent de-*O*-esterification to give tetrol **51** (83%). Simultaneous protection of the 4'- and 6'-hydroxy groups was subsequently realized by reaction with α,α'-dimethoxytoluene and catalytic



p-toluenesulfonic acid as before to yield the desired sialyl acceptor as the 2',3'-diol **52** (90% yield). This compound was further characterized as its di-acetate derivative **53** whose ^{1}H NMR spectrum displayed two downfield shifted doublet of doublets at δ 5.30 and 4.74 ppm for H-2' and H-3' respectively.

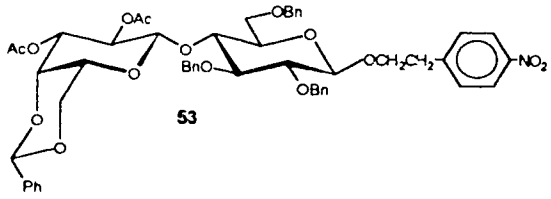


51

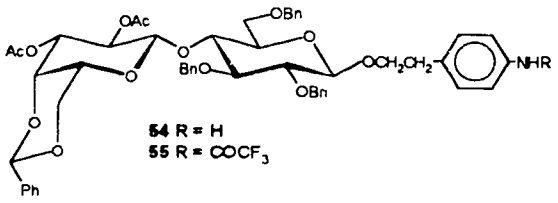


52 R = H
53 R = Ac

Due to our earlier observation that reduction of the *p*-nitro functionality during hydrogenation of the deblocked lactose derivative (*e.g.* **36**) was somewhat problematic, it was decided to work out the deblocking protocol on fully blocked lactose-PNPE derivative **53** at this stage before proceeding with the sialylation reaction sequence. The nitro moiety of di-acetate derivative **53** was converted to the free amine **54** by treatment with $Zn/CuSO_4$ in tetrahydrofuran-acetic acid-water[183] and this reaction was easily monitored by TLC using ninhydrin reagent to detect the product. *N*-trifluoroacylation of this amine by treatment with pyridine-trifluoracetic anhydride then gave the *p*-*N*-trifluoroacetamido (*p*-NTFA) lactose derivative **55** (79% from **53**) in which the

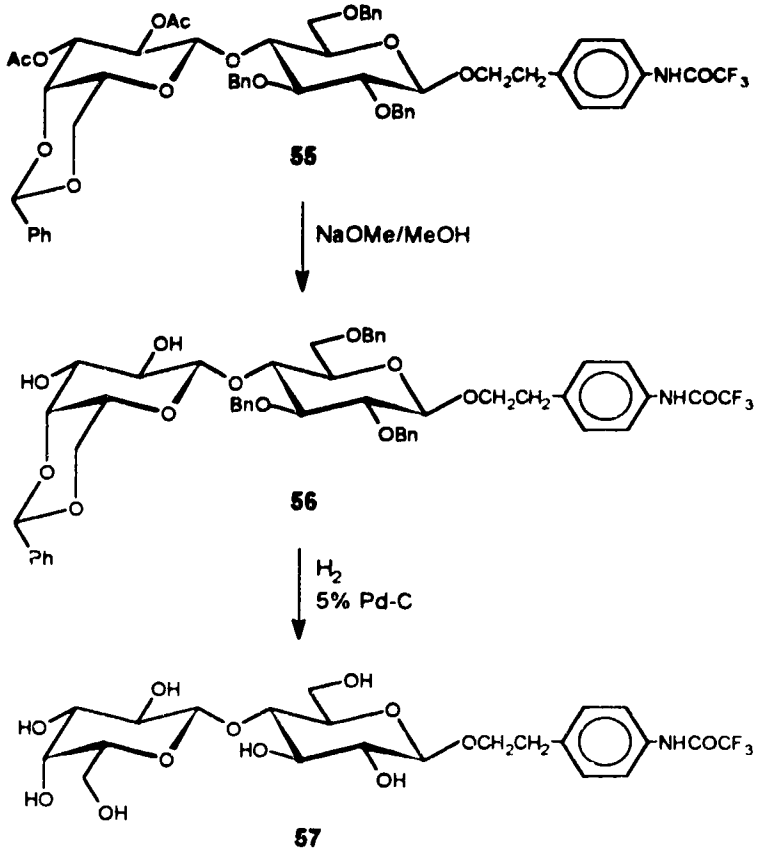


53



54 R = H
55 R = $COCF_3$

masked amino group could be liberated at a later stage for conjugation to carrier molecules. De-*O*-acetylation of **55** proceded quantitatively to provide diol **56**, which could also serve as a sialic acid acceptor in the event that the reduction of

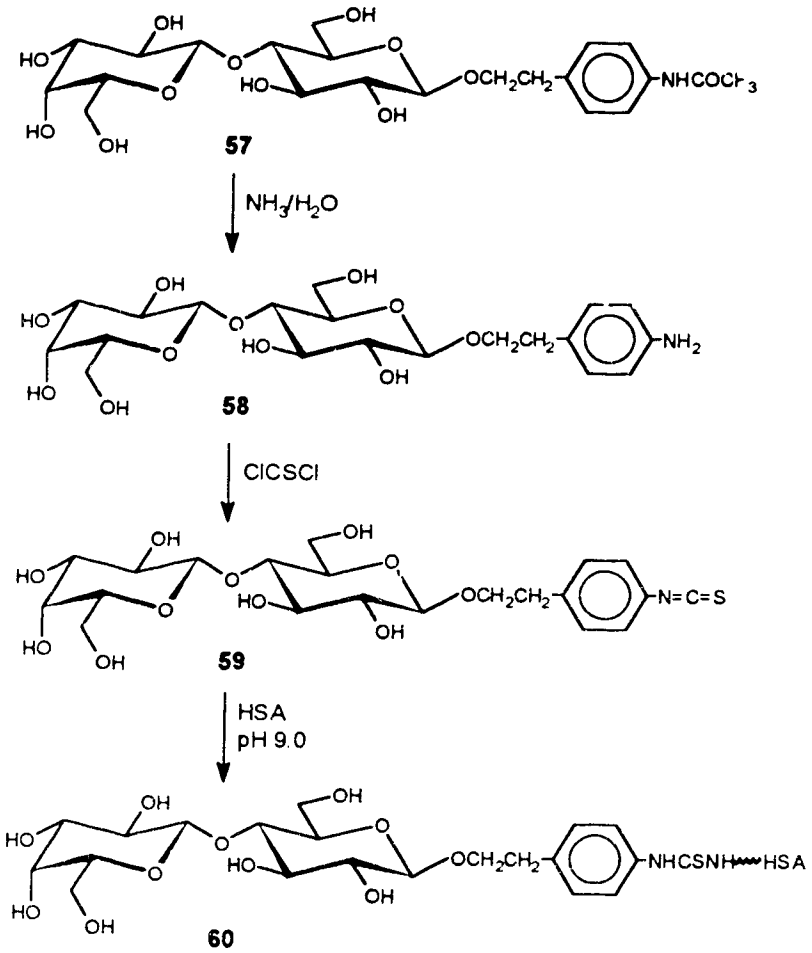


the *p*-nitro group in the presence of a sialic acid moiety proved problematic. Hydrogenolysis of compound **56** using 5% palladium-on-carbon as the catalyst proceeded cleanly when a small amount of acetic acid was added to the reaction mixture and led to the deprotected lactose derivative **57** in 90% yield as its phenethyl *p*-NTFA glycoside.

4.3.3 Conjugation of Lactose-*p*-NTFA

In order to conjugate the *p*-NTFA phenethyl lactose **57** to carrier proteins, the latent amino functionality was first revealed by removal of the *N*-trifluoroacetyl protection with 3:1 water-ammonia to give **58**, and secondly, activated by treatment with thiophosgene[178] under carbonate buffered conditions to provide the isothiocyanate derivative **59**. This compound was now suitably activated for coupling to the amino groups of proteins (*e.g.* the ε-amino

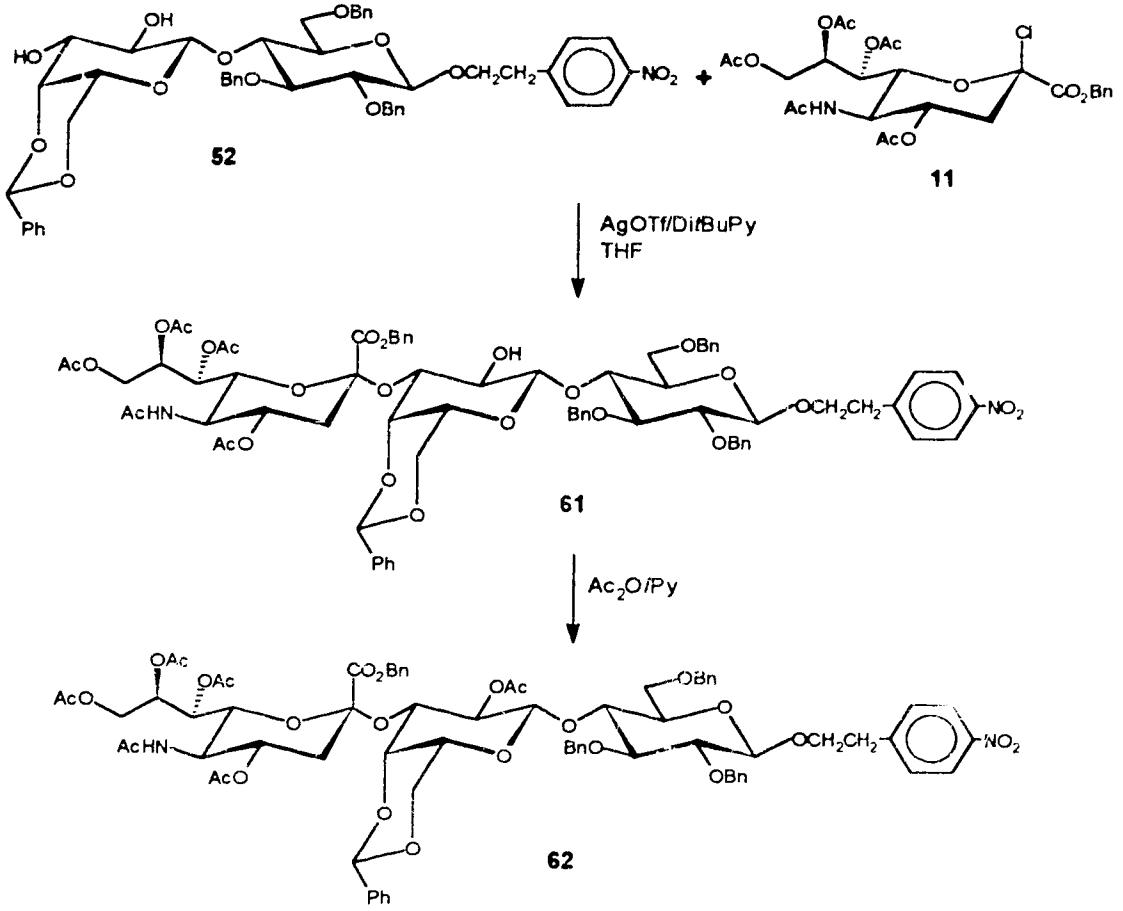
functionality of lysine). This whole sequence of events could be readily monitored by TLC using ninhydrin to develop the spots. Finally, conjugation was effected by stirring a solution of **59** and HSA in sodium bicarbonate-sodium carbonate buffer (pH 9). The resulting glycoconjugate **60** was separated from the low molecular weight by-products by exhaustive dialysis (Amicon, YM-10 membrane) against water and isolated as a powder by subsequent lyophilization. The conjugation ratio was estimated by the phenol-sulfuric acid assay,[184] and the resulting absorbance A_T = 0.276 corresponded to a substitution ratio of N = 12 (Graph 3, Appendix 1). Since deblocking and conjugation to a carrier protein proceeded well, our next goal was to apply this sequence to the synthesis of GM_3.



4.4 Synthesis and Conjugation of GM_3-*p*-NTFA Derivative 67

4.4.1 Sialylation of 2',3' Diol 52

In direct contrast to the sialylation reaction involving the 3',4' diol **37**, introduction of the 2',3'-diol **52** to sialic acid donor **11** (1.68 equiv) in the presence of silver salts led to a crude mixture containing starting diol **52** along with sialic acid by-products **39** and **40** and the desired sialoside **61**. Unfortunately, trisaccharide **61** could not be cleanly separated from this complex mixture, and column chromatography over silica gel at this stage only allowed for



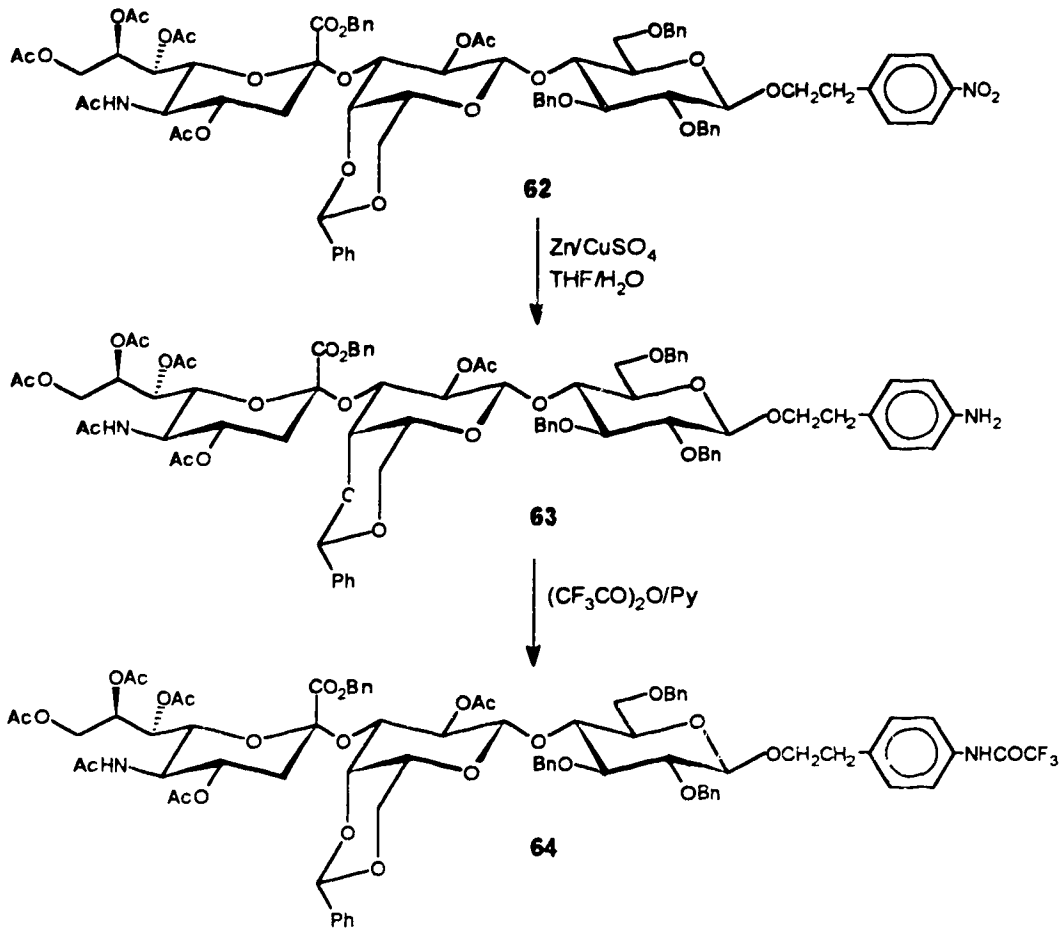
the separation of the 2-OH Neu5Ac derivative **40** from the remainder of the mixture. This hydroxy derivative can be recycled by re-acetylation to its precursor, namely pentaacetate **10b**, or directly transformed to chloride donor **11** with acetyl chloride.

Partial separation of diol **52** from the remaining mixture was accomplished by crystallization from chloroform leaving a mixture consisting mainly of the unsaturated sialic acid derivative **39** and the desired compound **61**. This mixture was then acetylated using 2:1 pyridine-acetic anhydride and the resulting product was readily separated into its components **39** and **62** by column chromatography over silica gel. The desired sialoside **62** was thus obtained in 29% overall yield from **52** and was now suitably derivatized for confirmation of the regioselectivity of the sialylation reaction by ^{1}H NMR spectroscopy. Thus, the acetylation reaction had served a twofold purpose.

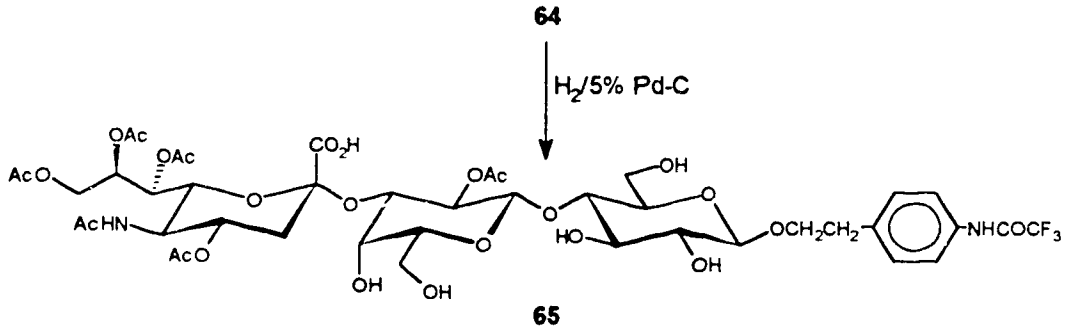
Extensive homonuclear decoupling experiments provided confirmation of several of the proton NMR resonances in the spectrum of trisaccharide **62** and in particular, the downfield shift of H-2' to δ 5.15 ppm was firmly established thus confirming the regioselectivity of this glycosidation reaction. The blocked GM_3 was now subjected to the same sequence of events previously worked out for the deprotection of disaccharide **53**. First, the nitro moiety was converted into the *p*-NTFA derivative by the two step sequence involving initial treatment with $Zn/CuSO_4$ in tetrahydrofuran-acetic acid-water to give the free amine **63** followed by protection of this functionality as the *p*-NTFA derivative by reaction with pyridine-trifluoracetic anhydride in order to provide the desired trisaccharide **64** (90% yield from the fully protected nitro derivative **62**).

Although the ^{1}H NMR spectrum of trisaccharide **64** was nearly identical to that of the nitro derivative **62**, the two proton doublet at $\approx \delta$ 8.0 ppm normally associated with the 2-(*p*-nitrophenyl)ethyl derivatives had now shifted upfield. As well, the ^{19}F NMR spectrum of **64** displayed a fluorine singlet at δ -75.84 ppm.

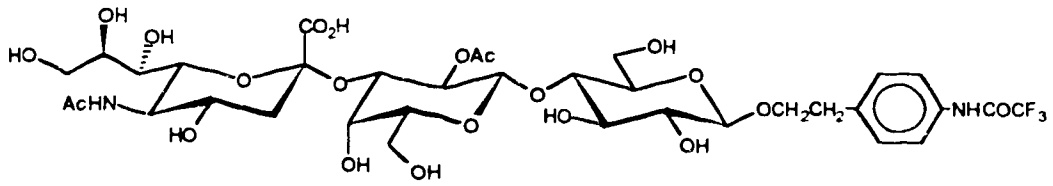
Blocked GM_3 **64** now basically possesses two types of protecting groups; (1) those removable by hydrogenolysis; and (2) those removable by de-*O*-esterification. Sequential deprotection of a methanolic solution of **64** by hydrogenation over wet 5% palladium-on-carbon containing a small amount of



acetic acid accomplished the removal of the first type of blocking groups to give the intermediate partially deprotected compound **65**.

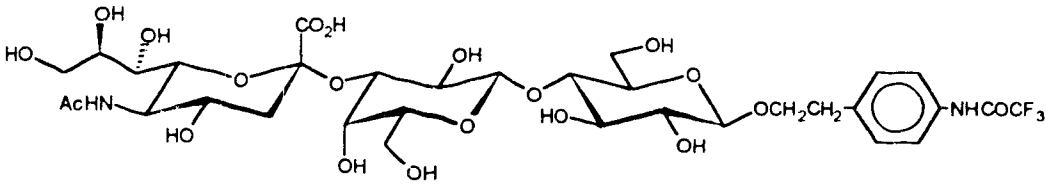


Subsequent de-*O*-acetylation with dilute sodium methoxide in methanol provided a product that was homogeneous by TLC (65:35:4 chloroform-methanol-water), however, the proton NMR spectrum of this product revealed that it was actually a mixture of two components in a ratio of about 2:1. The major component **66** possessed a downfield doublet at δ 4.93 ppm (dd, 1H, J_d = 10.5 Hz, J_d = 8.0 Hz) along with a 3 proton singlet at δ 2.25 ppm strongly suggesting that the acetate group at H-2' had remained intact. After a suitable solvent system for monitoring the de-*O*-acetylation reaction was found, treatment of **66** with a more concentrated solution of sodium methoxide in methanol led to



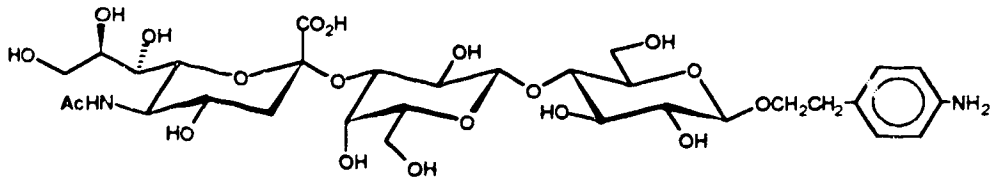
66

the disappearance of this compound along with the concommitant appearance of two lower R_f compounds, one of which was already present as the second component of the crude **66** mixture. Neutralization of this reaction mixture followed by subsequent separation by column chromatography over silica gel gave the totally deblocked trisaccharide **67** (62%) followed by the ninhydrin



67

positive amino compound **68** (12%) in 74% combined yield from the fully blocked *p*-NTFA derivative **64**. From the observed sluggish de-*O*-esterification of the acetylated hydroxyl group situated at C-2 of the galactose moiety, it appears that the topology of **66** (and therefore presumably **67**) is such that the sialic acid moiety has reduced the steric accessibility to this region of the trisaccharide.



68

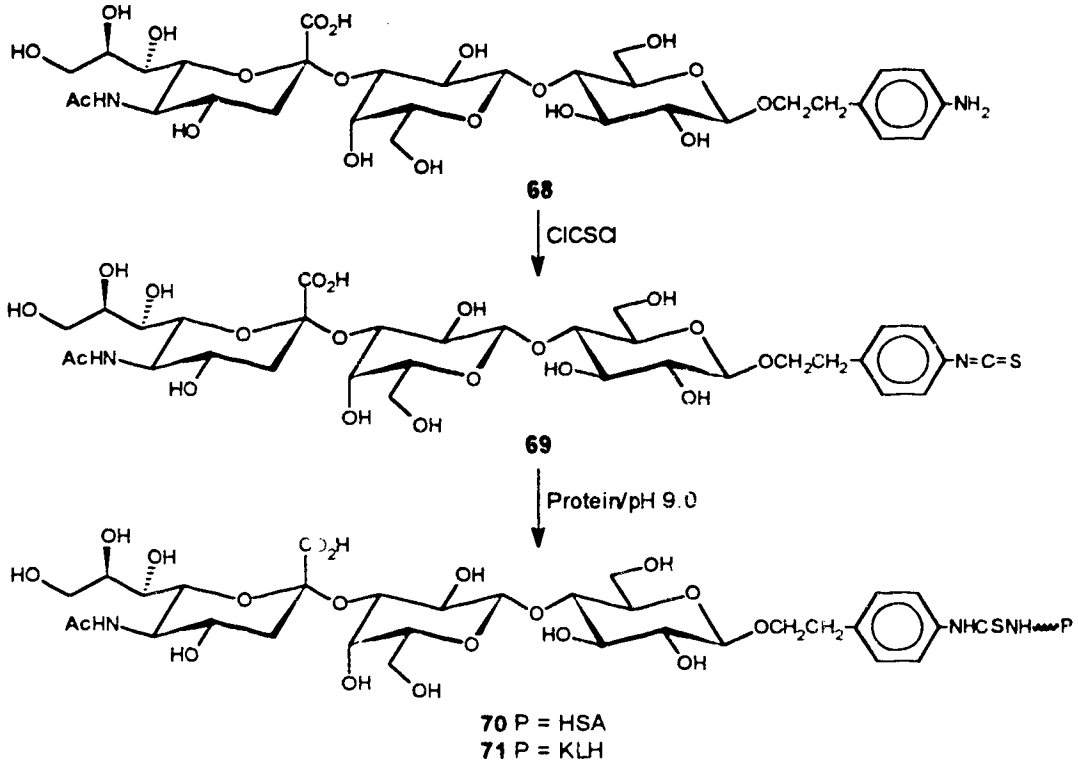
In any case, that the regiochemistry of the sialylation reaction was indeed correct was further evidenced by the downfield shift of the H-3' resonance of **67** to δ 4.09 ppm. Furthermore, this compound turned out to be the second component in the crude **66** mixture. The α-stereochemistry of the glycosylation reaction was firmly established by the fact that the signal for H-3"e of the sialic acid residue was found to be located at δ 2.74 ppm as discussed earlier and the remaining spectral data was consistent with the proposed structure. The amino derivative **68** could be either reprotected to give **67** or used directly in the conjugation reactions discussed in the next section.

4.4.2 Conjugation of GM_3-*p*-NTFA 67

In order to conjugate the GM_3-*p*-NTFA derivative to carrier proteins to provide antigens for immunization studies, compound **67** was treated with 1:1 ammonia-water for 20 h at room temperature to yield the ninhydrin positive amino derivative **68**. This derivative was activated for conjugation by treatment with thiophosgene to give the active isothiocyanate **69** which was used immediately in the conjugation reaction without further purification or characterization. Introduction of isothiocyanate **69** to HSA in pH 9 aqueous carbonate buffer for 96 h at room temperature gave the desired GM_3-HSA conjugate **70** after exhaustive dialysis and lyophilization.

Analysis for the sialic acid content of this glycoconjugate using the resorcinol-HCl assay is shown in Graph 4 (Appendix 1) from which an observed absorbance of A_T = 1.11 corresponds to a substitution ratio of N = 13. Likewise, conjugation of **69** to KLH following the same protocol as described for HSA yielded the GM_3-KLH conjugate **71**, and from the resorcinol-hydrochloric acid

assay shown in Graph 5 (Appendix 1), the absorbance A_T = 0.367 corresponds to a substitution ratio of N = 1044.



In this way, neoglycoconjugates were obtained which could be used as antigens for the generation of mAbs. Furthermore, isothiocyanate **69** could in theory be conjugated to solid supports possessing amino groups for the purpose of affinity purification of the resulting monoclonal antibodies. As well, the aromatic moiety of the spacer arm in the parent lactose derivative may lend itself to labeling with radioactive iodine.

4.5 Synthesis and Conjugation of Lactose-4M3P Derivatives

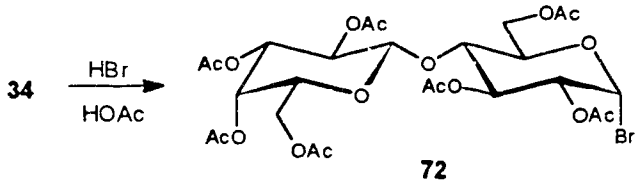
4.5.1 Synthesis of Lactose-4M3P 74

Although the conjugation of GM_3-*p*-NTFA proceeded reasonably well, combined with the fact that deprotection required several steps including the transformation of the nitro functionality into a blocked amino functionality, was the additional observation that de-*O*-protection was complicated due to the sensitivity of *p*-NTFA group to basic conditions. As well, the TLC of the

isothiocyanate revealed several components and it should be pointed out, that such a complex TLC profile was not observed for the formation of the lactose-isothiocyanate derivative **59**. This complex behavior was therefore attributed to the presence of the sialic acid moiety. Thus, before committing to the synthesis of the more valuable GD_3 ganglioside using this aglycon, it was decided to explore the synthesis of GM_3 with an alkenic linking arm such as employed in the earlier sialic acid work. The synthesis of carbohydrate derivatives as alkenyl glycosides has been reported by Bernstein and Hall[155] as mentioned earlier, and this linking arm can be activated under mild conditions for subsequent conjugation to carrier proteins for the preparation of semi-synthetic glycoconjugates.

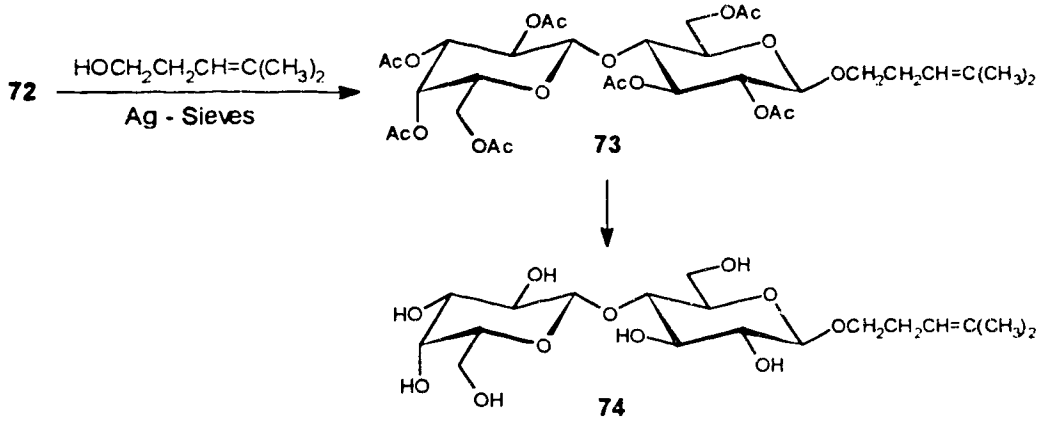
With these considerations in mind, we chose as a spacer, the 4-methyl-3-pentenyl arm. The alkenic moiety of this linking arm serves as a latent aldehyde group which can be unmasked under the mild ozonolysis conditions as was described earlier for the sialic acid derivatives, and subsequent covalent attachment to the ε-amino groups of lysine groups of the carrier protein can then be formalized by reduction of the resulting Schiff base with sodium cyanoborohydride.[185] Although 4-methyl-3-penten-1-ol (4M3P) is relatively expensive, use of this particular alkene was justified based on the fact that this aglycon would provide minimum interference with key signals in the proton NMR spectrum of the various derivatives, a property that would be particularly valuable in the interpretation of the spectra encountered in the more complex GD_3 series. Unreacted alcohol could be recovered and the total synthesis described herein should in theory, be compatible with other alkenic spacers.

Treatment of per-*O*-acetylated lactose **34** with HBr following the procedure described by Hudson and Kunz[186] led to the corresponding acetobromolactose **72**. Glycosylation was accomplished by first treating powdered activated molecular sieves (4 Å) with silver trifluoromethanesulfonate in toluene to form silver-sieves.[187] The supernatant from this reaction mixture



72

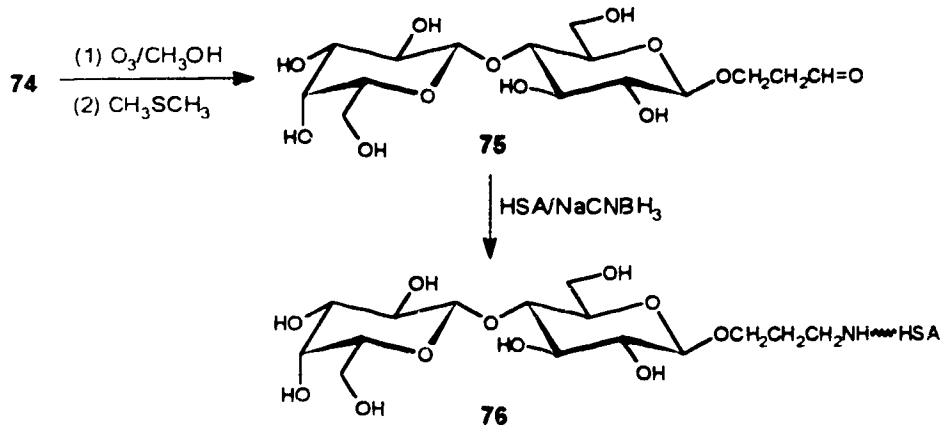
was tested for reactivity with benzyl bromide prior to decanting under argon and was found to be negative after a reaction period of a few hours. Glycosylation of **72** with 4-methyl-3-penten-1-ol (1.12 equiv) using the freshly prepared silver catalyst as promotor proceeded smoothly to give the lactose-4M3P derivative **73** in 45% yield post purification by column chromatography. The yield for this reaction step was not optimized and based on the observation that the reaction proceeded well according to its TLC profile, the lower isolated yield was attributed to losses during chromatography. De-*O*-acetylation with methanolic sodium methoxide led to the lactose-4M3P derivative **74** in quantitative yield.



4.5.2 Conjugation of Lactose-4M3P 74

Before proceeding further, the conjugation efficiency of the lactose-4M3P derivative was tested. Thus, ozonolysis of a methanolic solution of **74** at -15 °C followed by reduction of the resulting ozonide with methyl sulfide[161] gave the corresponding aldehyde **75** which was not isolated, but was employed directly in the subsequent conjugation step. Reductive amination of **75** with human serum albumin in phosphate buffered saline at pH 7.5 with $NaCNBH_3$ led to the lactose-HSA conjugate **76**.

The extent of conjugation was then estimated by the phenol-sulfuric acid assay and from Graph 6 (Appendix 1), an absorbance A_T = 0.09, corresponded to a substitution ratio of N = 4. When the ozonolysis was performed at -70 °C for 2-3 min, the reaction to aldehyde proceeded cleanly as estimated by TLC. Conjugation to HSA in phosphate buffered saline, pH 5.9, led to **76**.

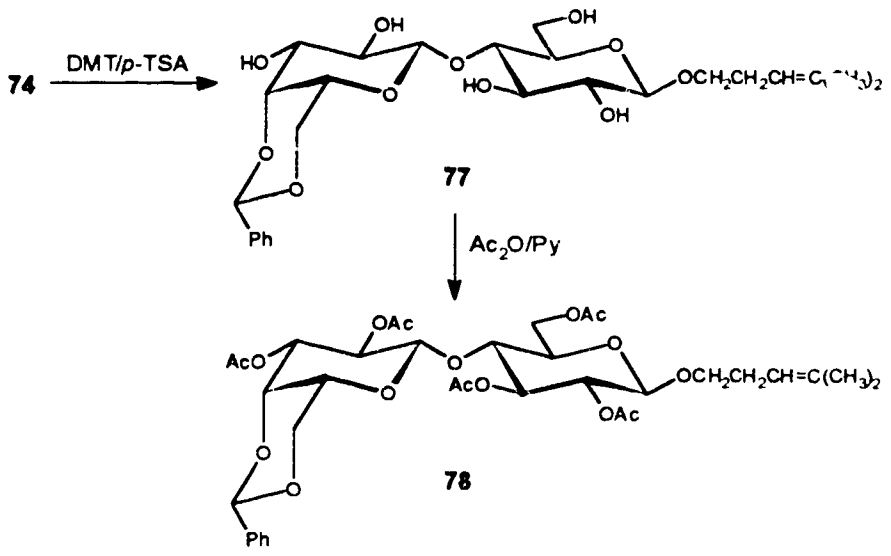


In this case, the carbohydrate content implicated by an absorbance $A_{\uparrow}$ = 0.181 from Graph 6 resulted in a conjugation ratio of N = 10. This improved reductive amination reaction under the latter conditions was attributed to a reduction in the rate of the competing α,β-elimination at pH 5.9 along with a possible concomitant increase in concentration of the free aldehyde.

4.5.3 Synthesis of 2,3,2',3' Tetrol 82

In order to prepare a sialyl acceptor possessing minimum steric interference from blocking groups, particularly at C-2, and in theory, allowing for the bulky size of the sialic acid donor in conjunction with the relative nucleophilicity of the secondary alcohol to influence the regioselectivity in the corresponding glycosidation reaction, we chose tetrol **82** as the target acceptor.

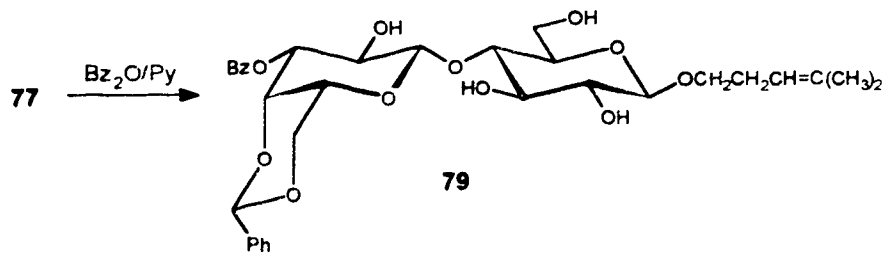
Simultaneous protection of the 4- and 6-hydroxyl groups of the galactose moiety of **74** by reaction with α,α'-dimethoxytoluene and catalytic *p*-toluenesulfonic acid in dry acetonitrile at 60 °C proceeded as anticipated to give the lactose-4M3P derivative **77** which was characterized as its per-*O*-acetylated derivative **78** by subsequent treatment with 2:1 pyridine-acetic anhydride. The ^1H NMR spectrum of the fully blocked lactose-4M3P derivative **78** displayed the expected 5 acetate singlets and the remaining signals were consistent with the proposed structure. It was hoped that treatment of **77** with a mild acylating agent such as benzoic anhydride would allow for discrimination between the primary



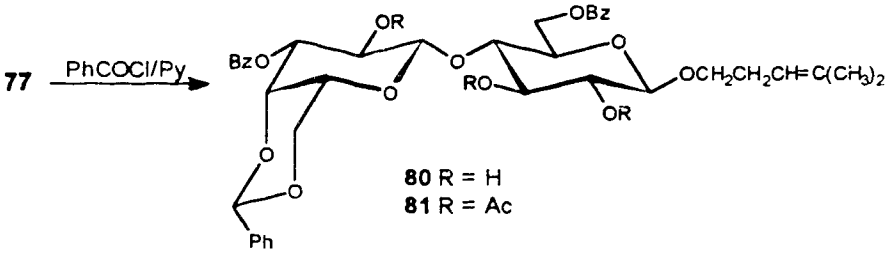
hydroxyl at C-6 and the secondary C-3' hydroxyl group of **77** leading directly to the desired tetrol **82**. However, when **77** was reacted with benzoic anhydride-pyridine under an inert atmosphere, the reaction was extremely sluggish at room temperature and we observed that even at 60 °C, greater than stoichiometric amounts (≈ 3 equiv) of the acylating reagent were required.

It may be argued that moisture had inadvertently been present, nevertheless, isolation of the major component of this reaction by chromatography over silica gel gave a product whose ^{1}H NMR spectrum was not consistent with the target tetrol. Although only one hydroxy group had been acylated during the course of this reaction, the downfield shift of H-3' to δ 5.16 ppm indicated that this secondary hydroxyl was the more reactive center and that the product of the reaction was tetrol **79** and not the desired tetrol **82**.

Treatment of pentol **77** under the more reactive benzoyl chloride-pyridine conditions did not show selectivity between the primary and secondary hydroxyl groups even at -70 °C, thus, **77** was converted to its di-benzoyl triol derivative by treatment of this compound with benzoyl chloride (2 equiv) in 2:1 chloroform-



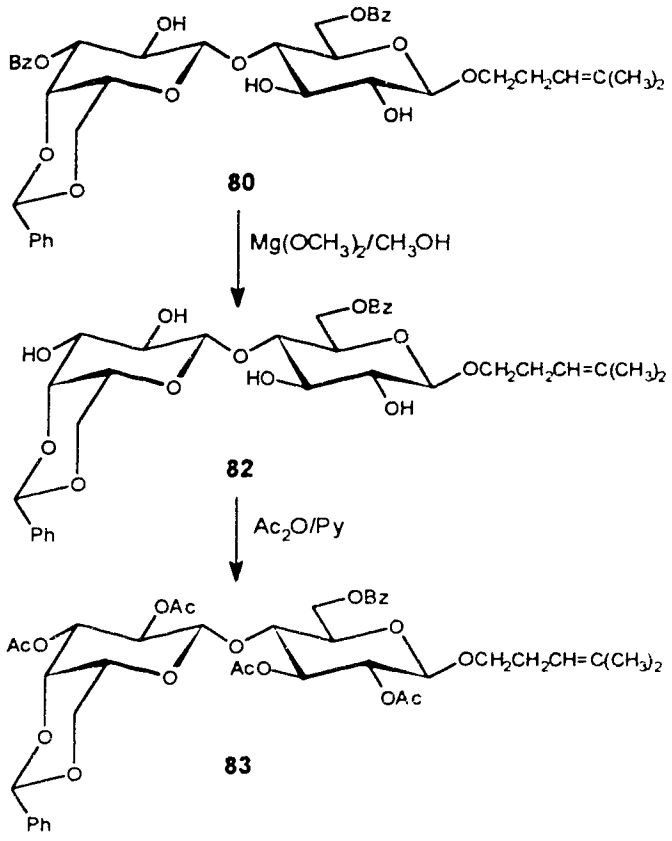
pyridine at -70 °C to provide the triol **80** in 49% yield along with a mixture of products at various stages of *O*-acylation. This mixture could be recycled to starting material **77** by de-*O*-acylation with methanolic sodium methoxide. The ^{1}H NMR spectral assignments were assisted by homonuclear decoupling experiments, thus, decoupling of the doublet of doublets centered at δ 5.12 ppm (H-3') collapsed the doublet at δ 4.49 (H-4') and the doublet of doublets at δ 4.28 (H-2') while decoupling of the the doublet of doublets centered at δ 4.96 ppm (H-6a) collapsed the doublet of doublets at δ 4.40 (H-6b) and multiplet at δ 3.69 (H-5) indicating that the hydroxyl functions at H-3' and H-6 were acylated. Since there are 5 potential sites of acylation, triol **80** was further characterized as its fully *O*-acetylated derivative **81** by treatment with 2:1 pyridine-acetic anhydride.



The ^{1}H NMR spectrum revealed three anticipated acetate singlets at δ 2.06, 2.04, and 1.99 ppm, and further structural assignments were aided by decoupling experiments performed on this derivative. Decoupling of the doublet of doublets centered at δ 5.46 ppm (H-2') caused a collapse in the doublet of doublets at δ 4.96 (H-3') along with the doublet at δ 4.57 (H-1') while simultaneous decoupling of the overlapping signals for H-2 and H-3' centered at δ 4.96 ppm collapsed signals at δ 5.28 (H-3), 4.53 (H-1), 5.46, and 4.45 (H-4'). Finally, decoupling of the triplet at δ 5.28 ppm produced a collapse in the doublet of doublets at δ 4.96 and triplet at δ 3.96 (H-4). These results indicated that

acetylation had occured at the 2,3 and 2' hydroxyl moieties and aided in further confirmation of the benzoylation pattern of **81**.

Now with triol **80** in hand, it was further hoped that de-*O*-acylation would mimic to some extent the rate of acylation, and that by tailoring the reaction conditions, some selectivity in ester hydrolysis could be achieved. With this hope in mind, the mild magnesium methoxide reagent was prepared and treatment of **80** with this reagent at 5 °C resulted in a major component that migrated (TLC) between de-*O*-acylated **77** and triol **80**. Purification to homogeneity of the reaction mixture by column chromatography over silica gel refunded starting material **80** (trace), compound **82** (85%), and **77** (trace). The ^{1}H NMR spectrum of tetrol **82** was clearly different from that of the mono-benzoyl

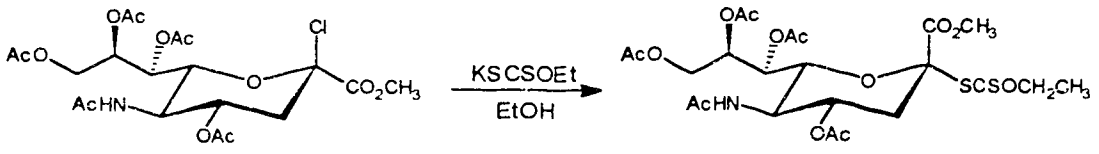


derivative **79** and irradiation of the doublet of doublets centered at δ 4.91 ppm (H-6a) of this tetrol caused the perturbation of signals located at δ 4.44 (H-6b) and δ 3.69 (H-5). Furthermore, assignment of the matching pairs H-6a-H-6b and H-6'a-H-6'b was aided by the examination of the cross peaks displayed in the 2D-COSY spectrum of the target tetrol (Appendix 3). Again, acetylation of a small amount of **82** with 2:1 pyridine-acetic anhydride yielded fully blocked lactose-4M3P derivative **83**. Along with the four acetate singlets at δ 2.06, 2.05, 2.04, and 2.02 ppm observed in the ^{1}H NMR spectrum of this derivative, decoupling of the downfield shifted H-3' doublet of doublets centered at δ 4.77 ppm produced a collapse in the doublet of doublets at δ 5.26 (H-2') as well as in the doublet at δ 4.28 (H-4'). Likewise, simultaneous decoupling of the H-2' doublet of doublets and H-3 triplet at δ 5.26 ppm caused a perturbation in signals at δ 4.77, 4.48 (H-1'), 4.96 (H-2), and 3.92 (H-4).

4.6 Synthesis and Conjugation of GM_3-4M3P Derivatives

4.6.1 Attempt at Sialylation With a Xanthate Donor

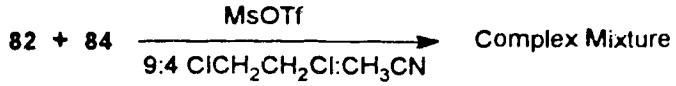
With sialyl acceptor **82** now accessible through a short synthetic sequence, several attempts were made to try to improve the yield of the subsequent sialylation step. First, attempts to activate the pentenyl sialoside **5a** for glycosylation with a model acceptor using N-iodosuccinimide/*t*-butyldimethylsilyl trifluoromethanesulfonate to generate an active iodinium species under the reaction conditions originally described by Fraser-Ried and coworkers[159] were unsuccessful. This approach however, was not extensively explored. Secondly, in view of a recent communication by Lonn and Stenvall[188] regarding the application of a new high yielding sialylation reaction, the stable crystalline xanthate derivative **84** was synthesized directly from sialyl chloride **4**



4 **84**

following the procedure published by Marra and Sinay.[189]

The newly reported glycosylation procedure involves activation of xanthate **84** by *S*-thioalkylation with methylsulfenyl trifluoromethanesulfonate, which can in turn be generated *in situ* by the reaction of methylsulfenyl bromide with silver trifluoromethanesulfonate present in the reaction mixture. Due to the anticipated sensitivity of 4M3P derivatives towards halogens, we decided to prepare the activating species directly rather than *in situ*. This was accomplished by reaction of methyl disulfide with bromine followed by introduction of the freshly generated methylsulfenyl bromide to excess silver trifluoromethanesulfonate as described by Dasgupta and Garegg.[190] Dropwise addition of freshly prepared solution of methylsulfenyl trifluoromethanesulfonate in 1,2 dichloroethane to a mixture of tetrol **82** and xanthate **84** in 9:4 acetonitrile-dichloroethane at -50 °C led to a complex mixture as evidenced by the complex TLC profile of the reaction mixture. This outcome was likely due to the sensitive nature of the protecting groups and/or linking arm of acceptor molecule **82**, and further work using this approach was abandoned.



4.6.2 Synthesis of GM_3-4M3P 88

Reaction of tetrol **82** with 2.0 equivalents of sialic acid donor **11** under the conditions described earlier for lactose-PNPE derivative **52**, as described earlier led to the formation of a new product, presumably trisaccharide **85**, in 36% yield. As before, this product was fully acetylated to compound **86** in order to assist in establishing the regiochemistry of the reaction.

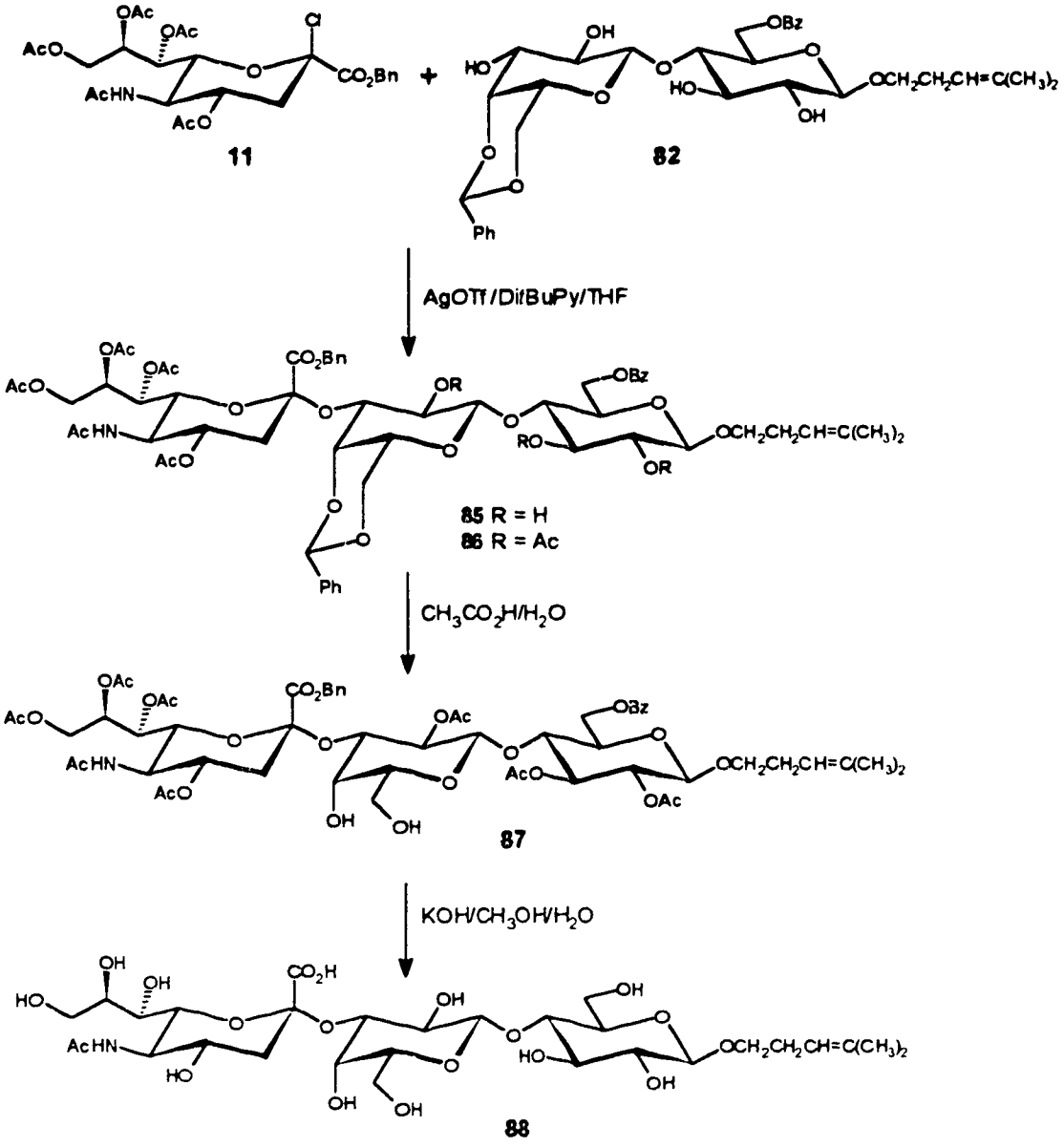
Due to the four possible sites of glycosidation, extensive homonuclear decoupling experiments were carried out on the fully blocked GM_3 derivative **86**, thus, decoupling of the H-8" double double doublets centered at δ 5.54 ppm collapsed the doublet of doublets at δ 5.37 (H-7"), 4.42 (H-9"a), and 4.06 (H-

9"b). The fact that this signal was located downfield from H-4" at δ 4.83 ppm served as preliminary evidence that the newly formed glycosidic linkage was in the α configuration.

Decoupling of the downfield shifted H-3 triplet located at δ 5.24 ppm collapsed the doublet of doublets at δ 4.93 (H-2) and the triplet at δ 3.90 (H-4). Irradiation of the benzyl methylene doublet at δ 5.22 ppm revealed its coupled geminal benzyl methylene doublet at δ 5.06 and also aided in the selection of the decoupling frequency for irradiation of the H-2' doublet of doublets centered at δ 5.09. As expected, irradiation of this position subsequently perturbed the doublet at δ 4.70 ppm (H-1') and the doublet of doublets at δ 4.33 (H-3'). Irradiation of the doublet of doublets located at δ 4.93 ppm (H-2) caused the anticipated collapse of the triplet at δ 5.24 (H-3) as well as the doublet at δ 4.51 (H-1) while confirmation of the H-2' signal at δ 5.09 ppm was obtained by decoupling the H-1' doublet centered at δ 4.70. Likewise, decoupling of the H-1 doublet centered at δ 4.51 ppm confirmed the H-2 doublet of doublets at δ 4.93. Finally, irradiation of the multiplet for H-5 centered at δ 3.75 ppm caused the simultaneous collapse of doublet of doublets at δ 4.83 (H-6a) and 4.32 (H-6b) along with the triplet at δ 3.90.

Three additional acetate singlets were observed in the ^{1}H NMR spectrum of **86**, and when viewed in conjunction with the previously summarized decoupling experiments, provided additional support that acetylation had occurred at the 2-,3- and 2'-OH sites. Thus, the regiochemical assignment that the sialylation reaction had occurred at the 3'-site was indeed correct. The downfield location of the sialic acid H-8" signal relative to the H-4" resonance served as preliminary evidence that the desired α-stereoselectivity had most likely been attained (see previous chapter). Lack of identification of the corresponding β-sialoside may be indicative of either low yield of this product or decomposition of this product under the reaction conditions back to starting tetrol **82** along with unsaturated Neu5Ac derivative **39**.

Removal of the benzylidene protection of blocked trisaccharide **86** using 4:1 acetic acid-water at 60 °C gave after purification by chromatography over silica gel, pure 4',6'-diol **87** (66%), recovered **86** and several more polar eluting by-products. The recovered starting material could readily be resubjected to the


11
82
AgOTf/DitBuPy/THF
85 R = H
86 R = Ac
CH_3CO_2H/H_2O
87
KOH/CH_3OH/H_2O
88

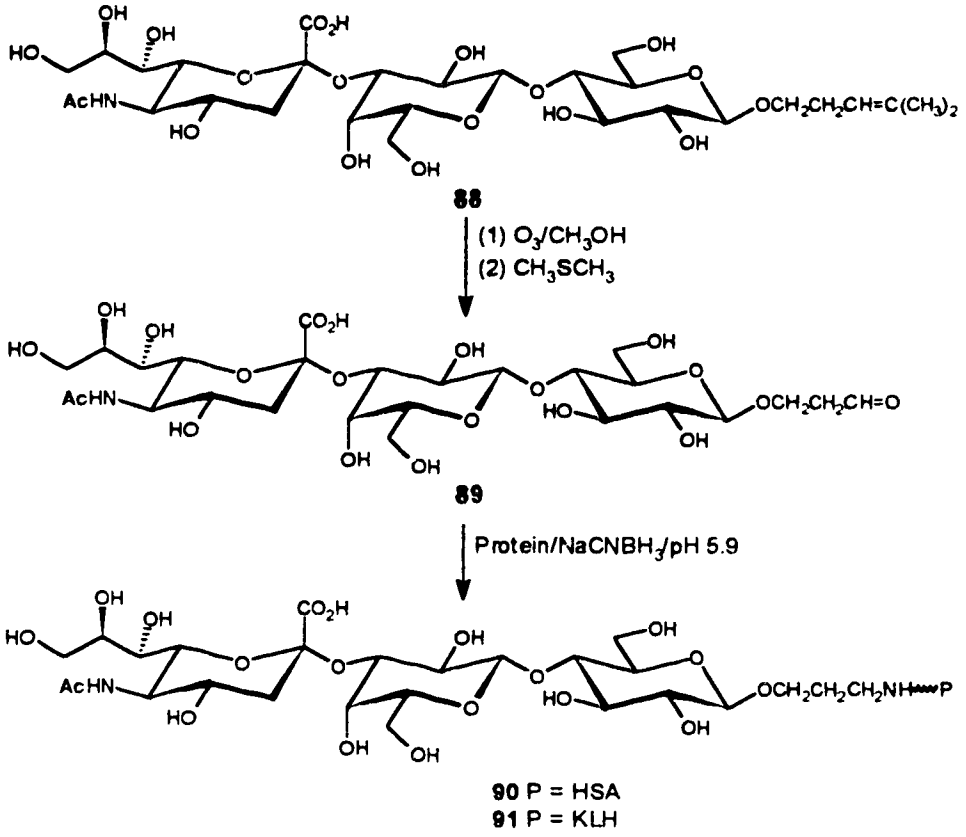
acetal deprotection conditions.

Interestingly enough, removal of the ester protection from the more polar mixture led to **87**, implying that along with removal of the acetal protection, some concomitant ester hydrolysis had occurred. It should also be pointed out that protection of the 6'-hydroxyl group of **87**, which is expected to be quite facile, provides an intermediate ideally suited for the synthesis of the GM_2 ganglioside. Hydrolysis of the remaining ester moieties from diol **87** using 0.5 M KOH in 4:1 methanol-water proved to be uneventful and provided the target GM_3-4M3P derivative **88** in quantitative yield.

4.6.3 Conjugation of GM_3-4M3P 88

As described earlier for the lactose-4M3P derivative **74**, ozonolysis of **88** at -70 °C followed by subsequent reduction of the resulting ozonide with methyl sulfide gave the aldehyde **89**, possibly in the hydrated form (see earlier discussion on **7** page 58), which was not purified or further characterized due to its unstable nature, but was used directly in the following conjugation step. Conjugation of **89** to human serum albumin by reductive amination in phosphate buffered saline at pH 7.5 using sodium cyanoborohydride as the reducing agent failed to provide the glycoconjugate **90**. This fact was verified by the absence of sialic acid in the final conjugate as indicated by a negative resorcinol-hydrochloric acid assay result. It appears that the α,β-elimination reaction was extant even at neutral pH as evidenced by the accumulation of a lower R_f material (thought to be the reducing sugar) during the course of the conjugation reaction.

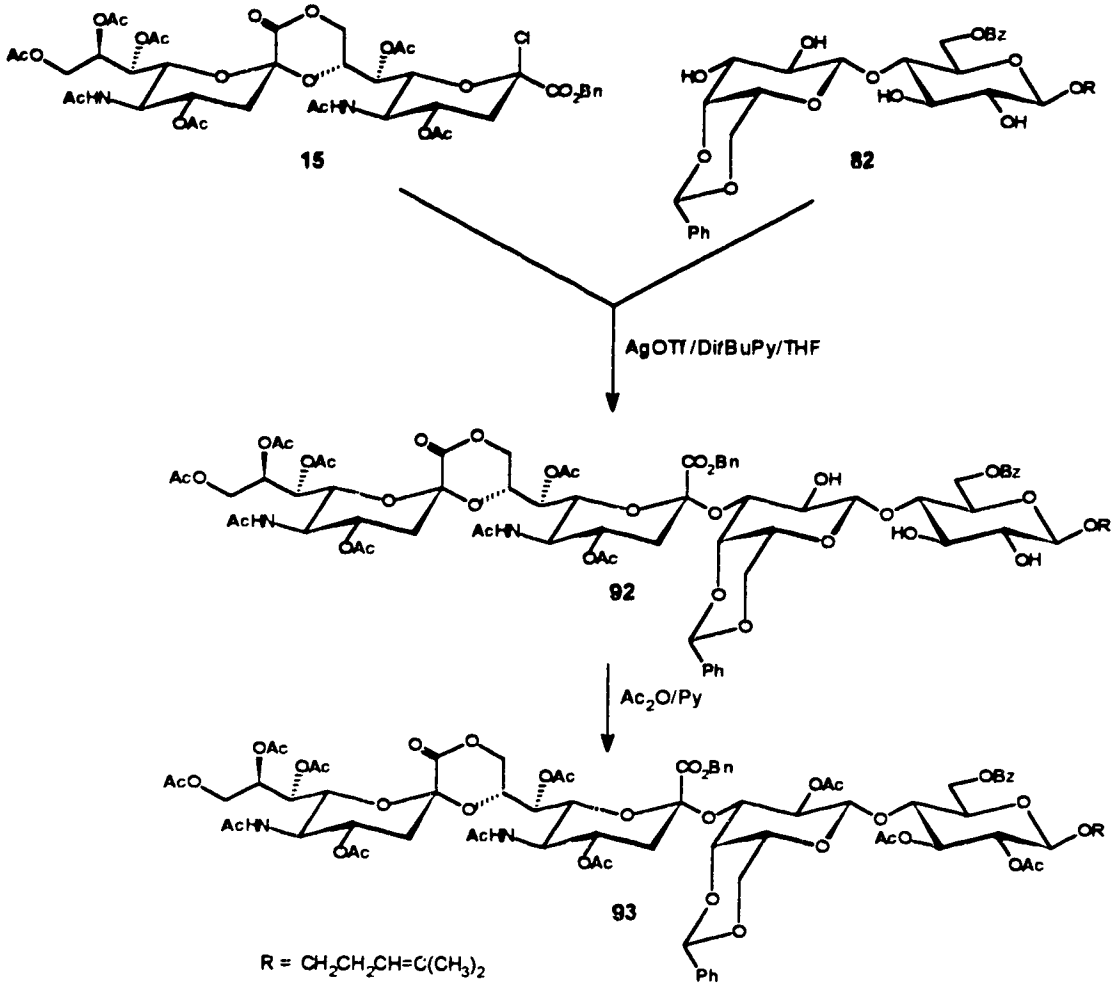
Repetition of the reaction in PBS buffer at pH 5.9 yielded the desired sialoglycoconjugate **90**, and from the analysis of the sialic acid content shown in Graph 7 (Appendix 1), an absorbance A_T = 0.556 yielded a conjugation ratio of N = 5. Similarily, conjugation of **89** to keyhole limpet hemocyanin under identical conditions led to conjugate **91**. From the resorcinol-hydrochloric acid assay results shown in Graph 8 (Appendix 1), the absorbance A_T = 0.556 implied a conjugation ratio of N = 200. Thus, suitable GM_3 glycoconjugates were obtained for immunization work in mice and this strategy also allowed for future expansion into the GM_2 series.



4.7 Synthesis and Conjugation of GD_3-4M3P Derivatives

4.7.1 Synthesis of GD_3-4M3P 95 From Di-Neu5Ac Donor 15

Since lactone formation upon acetylation of di-Neu5Ac **12** is quite facile and synthesis of the **14a/14b** mixture is straightforward, we decided to carry out the sialylation procedure in a two (lactose unit) plus two (di-Neu5Ac unit) fashion using di-Neu5Ac lactone donor **15**. Thus, reaction of this chloride, which unfortunately displayed poor solubility characteristics, with tetrol **82** (1.2 equiv) using silver trifluoromethanesulfonate as halophile under the same conditions as described for the synthesis of GM_3-4M3P, gave a crude mixture containing tetrasaccharide **92**. This mixture could not be separated and was therefore purified to homogeneity after subsequent acetylation to fully blocked GD_3-4M3P **93**.



Although the yield of **93** was extremely low (≈ 6%), the ^{1}H NMR data for this derivative was consistent with the proposed structure. Along with the presence of the expected 11 acetate singlets, decoupling experiments strongly suggested that the regiochemistry of the glycosidation reaction was indeed correct. Thus, decoupling of the doublet centered at δ 4.51 ppm (H-1) collapsed the doublet of doublets at δ 4.94 (H-2) while irradiation of the doublet at δ 4.36 ppm (H-1') collapsed the doublet of doublets at δ 5.06 (H-2'). Irradiation of the doublet of doublets centered at δ 5.06 ppm collapsed the doublet of doublets centered at δ 4.10 (H-3') along with the doublet at δ 4.36. Likewise, decoupling of the doublet of doublets centered at δ 4.94 ppm collapsed the triplet centered

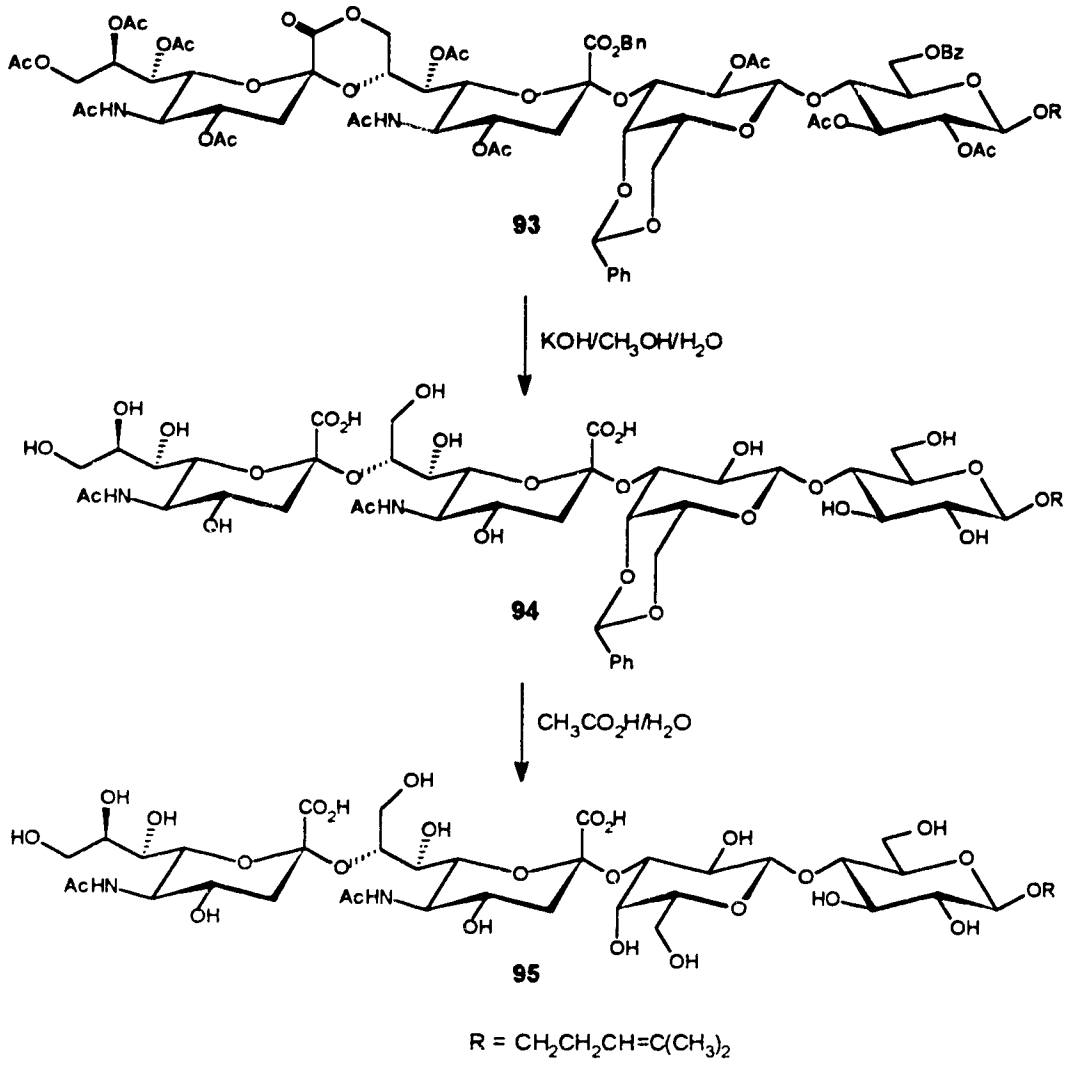
at δ 5.22 (H-3) along with the doublet at δ 4.51. These decouplings revealed that acetylation subsequent to sialylation had occurred at the 2-,3-, and 2'-hydroxyl moieties. Several assignments for spectral resonances of this complex NMR spectrum were aided by a single 2D-COSY experiment. This technique was especially valuable in establishing the H-3e/H-3a/H-4 connectivities. In summary, from the 2D-COSY spectrum (Appendix 3), the H-3"e doublet of doublets at δ 2.49 ppm located the H-4" triplet of doublets at δ 5.37 as well as the obscured H-3"a signal at ≈ δ 2.01 while the H-3"'a triplet at δ 1.82 ppm was useful in locating the H-4"' triplet of doublets at δ 4.99 and the H-3"'e doublet of doublets at δ 2.69 ppm. It should be pointed out that although the yield of sialylation is poor with the preferred route leading to the corresponding unsaturated di-Neu5Ac derivative via trans elimination of HCl, the yield may further be compromised by gradual lactone hydrolysis on the silica gel column.

Deprotection of **93** was carried out in a stepwise manner and initial de-*O*-esterification with 0.5 M potassium hydroxide in 4:1 methanol-water proceeded cleanly to give the 4',6'-*O*-benzylidene compound **94** in 79% yield after purification by chromatography over silica gel. Next, hydrolysis of the acetal functionality was performed by treatment with 4:1 acetic acid-water at 60 °C. The resulting crude mixture containing the fully deprotected GD_3-4M3P **95** derivative was again subjected to de-*O*-esterification due to the slow formation of a faster migrating component as detected by TLC (possibly lactone formation). Tetrasaccharide **95** was subsequently purified to homogeneity by chromatography over silica gel.

The FAB mass and NMR spectral data for compound **95** were uniformly consistent with the proposed structure. The downfield shift of the H-3 of galactose to δ 4.10 ppm lent further credence to the assigned regiochemistry while the shifts for the H-3 equatorial protons at δ 2.79 and 2.67 ppm corroborated the α-stereochemical assignments for both of the sialic acids.

Retrosynthesis of the GD_3 tetrasaccharide revealing two disaccharide fragments, a lactose unit and a di-Neu5Ac unit, resulted in a strategy leading to the complete synthesis of the carbohydrate component of this important melanoma-associated ganglioside marker. Although both synthons possessed simple protecting group profiles (thus simplifying deprotection), each accessible from minimal manipulation of the starting disaccharides, future studies directed

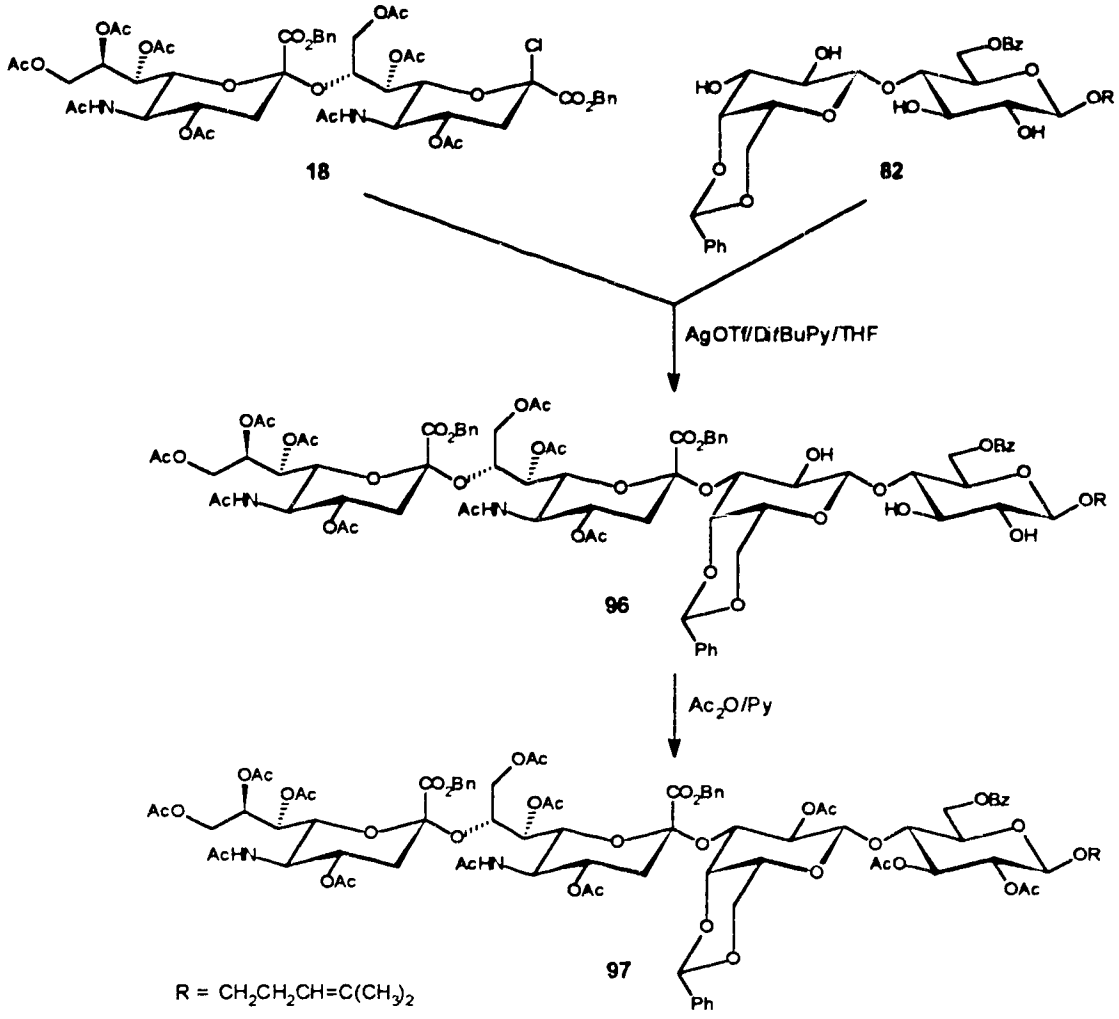
towards optimization of the sialylation reaction are much needed in order to make this approach more attractive for larger scale preparations.



4.7.2 Synthesis of GD_3-4M3P from Di-Neu5Ac Donor 18

In an effort to try to improve the yield of sialylation with a di-Neu5Ac donor, we explored the reaction of tetrol **82** (1 equiv) with the di-benzyl ester donor **18** (1 equiv), which displayed improved solubility characteristics when compared to lactone donor **15**. Under silver catalyzed initiation as before, reaction led to a mixture of **28**, **29**, **82**,and **96**, along with a minor unidentified

component. Only the unsaturated di-Neu5Ac derivative **28** could be separated from this complex mixture by chromatography over silica gel at this stage, thus, the mixture containing the remainder of the components was necessarily acetylated. Upon column chromatography over silical gel, disaccharide **83** (resulting from acetylation of **82**) eluted first, sequentially followed by a small amount of an unknown compound, desired tetrasaccharide **97**, and finally, acetate **17b** (from acetylation of **29**), which could readily be recycled to **18**. The unknown component was identified by ^{1}H NMR spectroscopy, and its spectrum



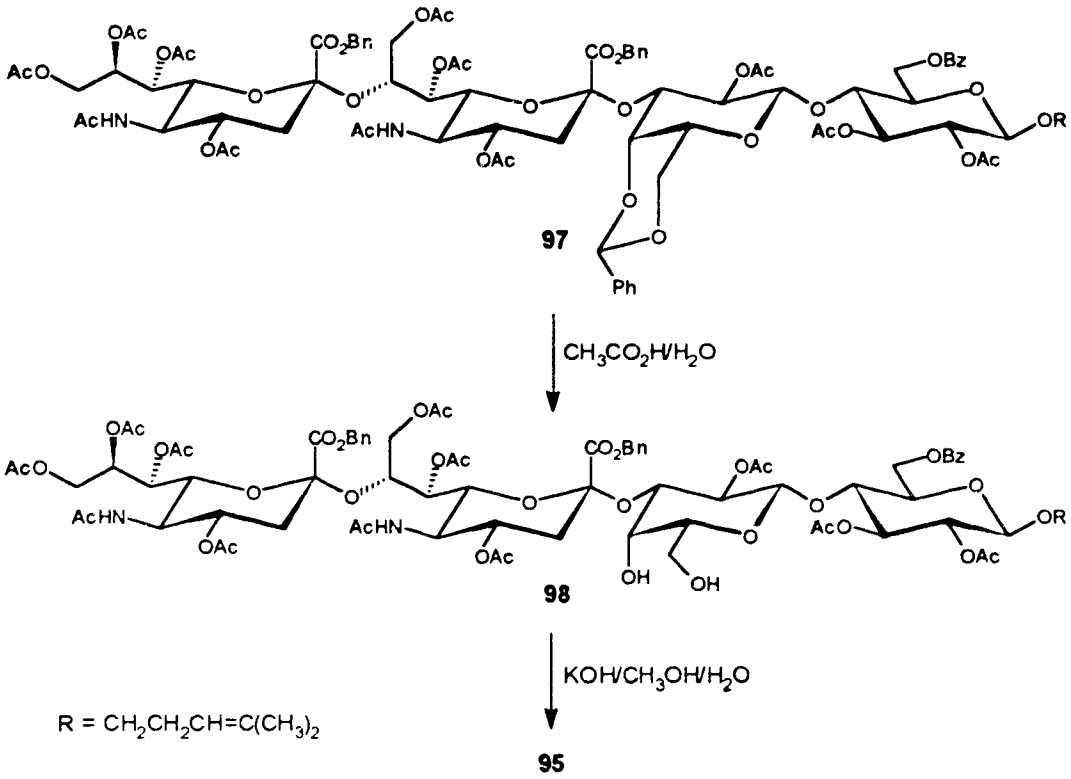
was found to be identical to the fully blocked GM_3-4M3P derivative **86**. Thus, it appears that a small amount of sialic acid donor **11** may have been formed by cleavage of **17b** under the acidic reaction conditions employed for chloride formation.

In any case, the desired fully blocked GD_3-4M3P derivative **97** was obtained in relatively poor yield (12%), however, it should be noted that the yield had roughly doubled by using the di-ester donor **18** as compared to ester-lactone donor **15**. There is no doubt that other means of activation of the anomeric center of di-Neu5Ac such as conversion to thioglycoside[147] or possibly phosphate[191] need to be explored in order to optimize reaction yields. Our primary objective however, was to provide biologically active glycoconjugates, thus, further optimization was not pursued.

The regiochemical portrait of the sialylation reaction was again established by ^{1}H NMR spectroscopy as before. Along with the notably high degree of similarity of the spectrum of **97** when compared with the model trisaccharide **31**, the anomeric centers could be defined by decoupling of the H-1 doublet centered at δ 4.50 ppm which collapsed the doublet of doublets at δ 4.95 (H-2) while irradiation of the H-1' doublet at δ 4.62 ppm collapsed the H-2' doublet of doublets at δ 5.10. With both H-2 signals now identified, decoupling of the H-2' doublet of doublets centered at δ 5.10 ppm collapsed the doublet of doublets centered at δ 4.57 (H-3') along with the doublet at δ 4.62. Likewise, decoupling of the H-2 doublet of doublets centered at δ 4.95 ppm collapsed the triplet centered at δ 5.22 (H-3) along with the doublet at δ 4.50. The acetylation pattern deduced from the proton spectrum of **97** strongly suggested that hydroxyl moieties at positions 2,3 and 2' had been acetylated implying that sialylation had occurred at the desired H-3' position. Further structural assignments were obvious from the cross peaks in the 2D-COSY spectrum (Appendix 3), from which it is apparent that the H-2' doublet of doublets at δ 5.10 ppm is coupled to the H-1' doublet at δ 4.62 while the H-2 doublet of doublets at δ 4.95 ppm is coupled to the H-1 doublet at δ 4.50, consistent with the results of the prior decoupling experiments. As well, the sialic acid acetamido NH'' at δ 6.25 ppm is coupled to its sialic acid H-5'' multiplet at δ 3.95 while the sialic acid acetamido NH''' located at δ 5.07 ppm is coupled to its corresponding sialic acid H-5''' quartet at δ 4.05. Other apparent connectivities include the coupling of the

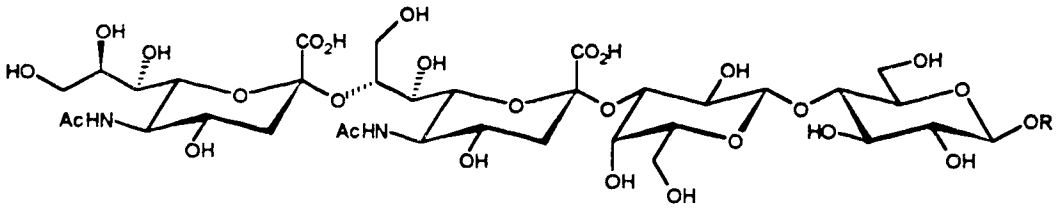
glucose H-6a doublet of doublets at δ 4.72 ppm to the glucose H-6b doublet of doublets at δ 4.43 and the H-5 double doublet of doublets at δ 3.71, and the geminal coupling of galactose H-6a doublet of doublets at δ 4.12 ppm to the corresponding H-6b doublet of doublets at δ 3.67.

Removal of the benzylidene acetal from the blocked tetrasaccharide **97** using 4:1 acetic acid-water at 60 °C gave recovered **97** (15%) followed by the desired 4',6'-diol **98** in 62% yield after purification by chromatography over silica gel. It is apparent that simple protection of the primary hydroxyl group of the galactose unit of this derivative would lead to an intermediate suitable for the synthesis of the important neuroblastoma-associated ganglioside GD_2. De-*O*-esterification of **98** with 0.5 M KOH in 4:1 methanol-water proceeded smoothly to provide the target GD_3-4M3P derivative **95** in 93% yield. Also, the order of deprotection could be reversed thus, de-*O*-esterification of **97** as the first deprotection step led to **94** in quantitative yield, and this derivative was then treated with CH_3CO_2H/H_2O as before to yield the title tetrasaccharide **95**.

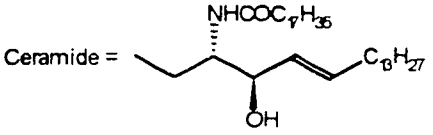


4.7.3 Synthesis of GD_3-Reducing Sugar 99

In order to unequivocally establish that both the stereochemical assignment and regiochemical portrait of the novel synthetic GD_3-4M3P derivative **95** are indeed correct, it would be ideal to compare this structure to natural GD_3. Due to the ceramide aglycon present on the natural ganglioside, liposome formation occurs spontaneously in aqueous solvents, thus, it is not possible to obtain a well resolved ^1H NMR spectrum of this material in D_2O. For this reason, the spectra of gangliosides are generally taken in 98:2 DMSO-d_6:D_2O at 45 °C. Under these conditions however, the trend that the H-3e proton resonates at δ > 2.5 ppm for the α-sialoside and δ < 2.5 ppm for the β-sialoside established in aqueous media no longer applies. Thus, obtaining the ^1H NMR spectrum of **95** in DMSO as solvent may not directly assist in confirmation of the stereochemistry of the newly formed α-(2→3) linkage. With these considerations in mind, a methanolic solution of GD_3-ceramide **103** obtained from natural

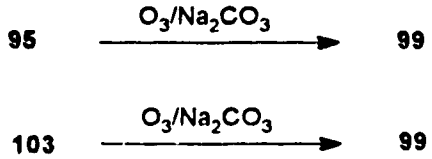


95 R = $CH_2CH_2CH{=}C(CH_3)_2$
99 R = H
103 R = Ceramide



sources was first treated with ozone at -15 °C and then stirred in aqueous 0.01 M sodium carbonate at room temperature overnight as described by Schwarzmann and Sandhoff.[192] This procedure served to effect in a sequential manner, ozonide reduction, rearrangement, and subsequent α,β-elimination to

provide the reducing sugar **99** after purification by column chromatography over silica gel. Compound **99** was analyzed by mass spectroscopy (negative FAB) and by ^1H NMR spectroscopy in D_2O after allowing for mutarotation in this solvent overnight. The molecular weight for compound **99** was calculated to be 924.8 (FAB, negative ion, TEA matrix, *m/z* 923.87 M-1$^-$) and the proton spectrum of the equilibrated solution of the reducing sugar displayed the anticipated 2:1

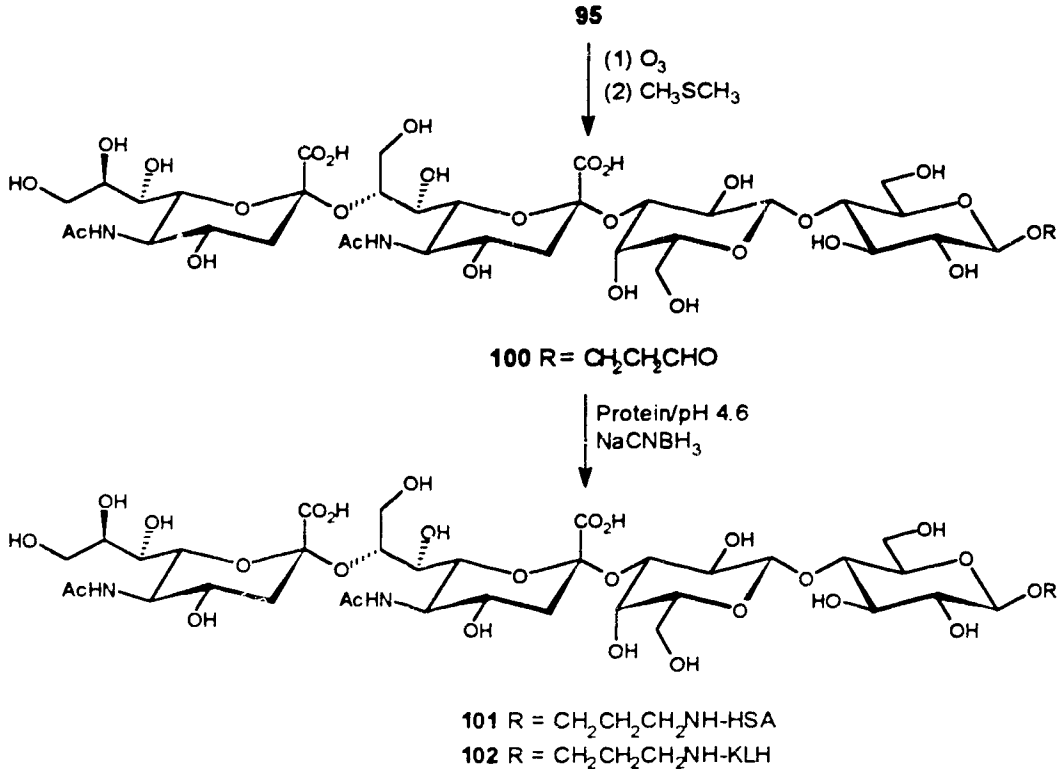


β/α anomeric mixture. Similarily, synthetic derivative **95** was subjected to ozonolysis followed by reduction to the corresponding aldehyde and α,β-elimination in aqueous 0.01 M sodium carbonate. Column chromatography over silica yielded a product with identical mass and proton spectra to **99** obtained from the natural source material lending unequivocal support to the correct structural assignment for GD_3-4M3P derivative **95**.

4.7.4 Conjugation of GD_3-4M3P

The steps required for conjugation of GD_3-4M3P to protein carriers were similar to those employed earlier for conjugation of GM_3-4M3P. Thus ozonolysis of **95** at -70 °C followed by subsequent reduction of the resulting ozonide to the aldehyde with methyl sulfide gave aldehyde **100**, again presumably as a dimethyl acetal. This product was not purified or further characterized due to its unstable nature, but was used directly in the following conjugation step. Conjugation of **100** to human serum albumin by reductive amination in phosphate buffered saline at pH 5.9 or in acetate buffer at pH 4.65 using $NaCNBH_3$ as the reducing agent yielded the desired sialoglycoconjugate **101**. Analysis for sialic acid content using the resorcinol-HCl method is shown in Graph 9 (Appendix 1), from which an absorbance A_T = 1.12 corresponds to a conjugation ratio of N = 9.2 for the reaction at pH 5.9 while an absorbance A_T =

1.31 corresponds to a conjugation ratio of N = 11.2 for the reaction at pH 4.65. Similarily, conjugation of **100** to keyhole limpet hemocyanin by reductive amination at pH 4.65 provided conjugate **102**, and from the resorcinol-hydrochloric acid assay results shown in Graph 10 (Appendix 1), an absorbance A_T = 0.809 implied a conjugation ratio of N = 654.



4.7.5 Attempts To Prepare 9-OAc-GD_3-4M3P

As was mentioned in chapter 2 (see Figure 3), 9-OAc-GD_3 is also considered to be a melanoma associated antigen. We have attempted to prepare 9-OAc-GD_3-4M3P from **95** by two routes; (1) $CH_3C(OCH_3)_3$/*p*-toluenesulfonic acid/DMSO or DMF then H^+;[193] and (2) *N,N*-dimethyacetamide dimethylacetal/*p*-toluenesulfonic acid/DMSO then silica gel.[194] Neither route has yielded the desired compound, however, both procedures have led to a similar mixture of two components. Upon preliminary examination of this mixture by ^1H NMR, one of the components displayed a downfield shifted sialic acid H-4 proton while the corresponding downfield shifted signal for the other component remains to be identified. It should be pointed out, that neither component displayed downfield shifted patterns characteristic of sialic acid H-9 protons typically observed in model compounds.[195] Further work on this step is essential, and it is possible that enzymatic acylation (acyl CoA) may be the preferred route for the preparation of 9-OAc-GD_3-4M3P from **95**.

4.7.6 Summary of Synthetic Work

The synthesis of lactose and GM_3 trisaccharide as a *p*-NTFA glycoside has been reported herein and subsequent conjugation of these compounds has provided the lactose-HSA conjugate for screening and the GM_3-HSA and -KLH glycoconjugates for immunization work. Since the thionyl chloride conditions are quite harsh for sensitive molecules such as GM_3, an effort to carry out conjugations under milder conditions was explored and for this reason, the 4-methyl-3-pentenyl linking arm was chosen and incorporated into our synthetic strategy. This linking arm was shown to be useful in conjugation to protein carriers as shown by the preparation of the lactose-HSA conjugate and provided little or no interference with signals in the proton NMR spectra of the various synthetic intermediates. Furthermore, this aglycon functioned as an anomeric masking agent which could be deprotected to give the corresponding reducing sugar in excellent yield thereby allowing for comparison with naturally derived material.

The lactose-4M3P derivative was prepared in a unique manner by employing a "silver sieves catalyst" generated *in situ*, and this compound was then transformed into a sialyl acceptor suitable for the synthesis of gangliosides

in three simple steps. Application of this lactose-4M3P acceptor resulted in the successful synthesis of a novel GM_3 derivative and subsequent conjugation provided the GM_3-HSA and -KLH sialoglycoconjugates for immunization work. Also, the utility of a previously unreported di-Neu5Ac donor for sialylation of primary and secondary alcohols has been demonstrated. Furthermore, this material may serve as a convenient synthon readily available from colominic acid and may be of potential commercial value in the scale up of ganglioside synthesis since the gene for colominic acid producing *E. Coli* strain has recently been cloned. [211] Application of two synthons, the lactose-4M3P acceptor **82** and either of the two di-NeuAc donors (lactone ester **15** or di-ester **18**) described herein, successfully resulted in the total synthesis of the tetrasaccharide portion of the melanoma associated ganglioside GD_3 after which the corresponding HSA and KLH glycoconjugates for immunization were prepared.

In view of the fact that only one step is required for the protection of the primary hydroxyl moieties of intermediates **87** and **98** for application towards the synthesis of the imporant melanoma associated structures GM_2 and GD_2 respectively, our approach seems to be a versatile one capable of leading to a series of melanoma associated antigens. In fact, the carbohydrate sequences of all four of these aforementioned gangliosides can be built up from just four basic building blocks, three of which we have described herein, namely lactose acceptor **82**, sialic acid donors **11** and **18** (or the equivalent donor **15**). The fourth building block is the known 2-azido galactose (or equivalent) synthon and is required for the synthesis of GM_2 and GD_2. Thus, these four synthons should allow for successful entry into this entire family of complex carbohydrate antigens.

As was mentioned in the previous chapter, the ganglioside GD_3 can be found as the *O*-acetyl derivative at the 9-position of the terminal sialic acid residue. Also, several lactones are possible from this tetrasaccharide, thereby multiplying the number of potential antigenic epitopes generated from this one molecule. The full potential of the synthetic approach may be realized by the synthesis of unnatural analogues possessing substitutions at key positions on the target gangliosides leading to more stable compounds not available via biological means. For example, replacement of the 9-OH group of the terminal Neu5Ac unit with an azido group would lead to a synthon which could be

reduced to the amino group in the final synthetic stages and subsequently *N*-acetylated. The resultant 9-NHAc should in theory, mimic the naturally occurring 9-OAc derivative and should display much improved stability in mild alkali not displayed by the *O*-acetylated compound. Extension of this idea to include synthons possessing an azido group at the 9-position of the inner sialic acid would lead to the lactam equivalent of the corresponding lactone by cyclic amide formation between the resulting unmasked 9-NH_2 functionality and the carbonyl moiety of the terminal sialic acid. Increased stability towards hydrolysis could be realized in this way since the lactone derivatives are readily hydrolyzed in aqueous solutions. Further extension of this methodology would include substitution of the galactose unit of GD_3 with 2-azido galactose to ultimately provide the lactam between the 2-position of galactose and the carbonyl group of the inner Neu5Ac unit. Also, several permutations of these various synthons can be envisioned whereby even the potentially unstable 9'''-OAc di-lactone could be prepared as its more stable unnatural 9'''-NHAc di-lactam mimic, a feat which may be difficult to achieve by biological means.

Although the yield of the key glycosidation step for the synthesis of GD_3 reported herein is admittedly low, improved yields may be realized by the enzymatic $\alpha(2\rightarrow8)$ addition of the terminal sialic acid to precursor GM_3. As a second approach, if large amounts of these materials are required for clinical applications such as immunotherapy, it may be possible to prepare anti-idiotypes against mAbs recognizing the GM_3- and/or GD_3-ganglioprotein conjugates. The resulting anti-idiotypes, which bear the image of the ganglioside, would necessarily need to be screened and evaluated by inhibition studies using the original carbohydrate antigens such as **88** and **95**.

Hopefully, the antigens **90**, **91**, **101**, and **102** will lead to the generation of clinically relevant mAb(s) for RAID and/or RAIT and subsequent detailed studies on the nature of the binding of the corresponding mAb to **88** or **95** on a molecular level by X-ray crystallography could then be possible. Finally, due to the fact that these derivatives have ideal aqueous behavior along with the fact that NMR studies are now possible on antibody Fab fragments, it may be possible to probe the nature of antigen/antibody binding in the natural aqueous environment using these synthetic derivatives. In any case, the work reported in

this thesis represents only a small, but first, step towards achieving these ultimate goals.

4.8 Preliminary Biological Results

With antigens in a form suitable for both the generation of monoclonal antibodies and subsequent screening now in hand, attention was next focussed on immunization of mice. The ultimate hope was to obtain prospective murine mAbs which recognized the corresponding immunogens and cross-reacted with melanoma. That such mAbs may inherently possess additional traits above and beyond that of functioning as transport vehicles for radiochemicals is dramatically highlighted by a recent report employing the anti-GD_3 mAb R24 in combination with a pharmacologic agent behaving as an endotoxin.[196] In fact, the observation of cancer regression in association with severe infection dates back at least a century.[197] Since that time, such an approach towards cancer management has gradually given way to chemotherapy and radiation therapy regimens. However, with the recent finding that endotoxin-provoked tumor regression can be induced by recombinant tumor necrosis factor-α (rTNF-α) through induction of inflammatory destruction of the tumor,[198] zeal for application of mAbs as therapeutics has intensified. The combination of rTNF-α, which has the ability to induce the production of H_2O_2 via stimulation of neutrophils which become attached to the extracellular matrix or endothelium,[199] with mAb R24, which has been shown to trigger inflammation by deposition of complement and neutrophil infiltration at tumor sites, has led to interesting results. Thus, it may now be possible to invoke the cytotoxic properties of neutrophils that have localized at the tumor site. The highlight of this eight patient clinical trial was the unexpected event that one patient with advanced disease treated in this way, developed an acute massive tumor lysis syndrome within just hours post treatment and subsequently displayed extensive necrosis of bulky tumors in multiple visceral sites. (Typically, induction of hemorrhagic tumor necrosis is associated with severe bacterial infections and is a bodily mechanism for selective tissue destruction mediated by inflammatory events). This extremely rare observation in patients with metastatic solid tumors is the first reported case[196] of such action in patients, and may well be the first documented case of hemorrhagic tumor necrosis in patients undergoing rTNF-α or mAb treatment.

4.8.1 Generation of Murine MAbs

In view of the forgoing observations, it was fortunate that our immunization work was underway. Our preliminary immunization results are disclosed herein (see materials and methods), and a more detailed account should appear elsewhere.[200] Thus, six week old male BALB/c were immunized with GM_3-KLH **91** or GD_3-KLH **102**, and positive clones were identified by ELISA using the corresponding HSA clones **90** and **101** respectively. Screening against KLH was also carried out in order to rule out clones directed towards carrier protein epitopes. Four clones with positive reactivity for GM_3 were obtained and one, B213.1 was selected for recloning. Likewise, three GD_3-reactive clones were generated and B212.1 was subsequently recloned. Determination of the antibody subclass of these newly generated immunoglobulins revealed that both were of the IgM class. Both mAbs were screened against several irrelevant carbohydrate antigens as shown in Table 9.

As well, both monoclonal antibodies were screened for reactivity towards ganglioside antigens. Gangliosides GM_2 and GD_2 were obtained via enzymatic

Table 9: Reactivity of B212.1 and B213.1 Towards Carbohydrate Antigens

Antigen	B212.1 (anti-GD_3)	B213.1 (anti-GM_3)
Neu5Acα2→3Galβ1→3(Fucα1→4)GlcNAcβ (SLe^a)	negative	negative
GalNAcα-HSA (Tn)	negative	negative
Galβ1→3GalNAcα-KLH (T)	negative	negative
Neu5Acα2→6GalNAcα-KLH (STn)	negative	negative
Galβ1→3(Neu5Acα2→6)GalNAcα-KLH (ST)	negative	negative
Neu5Ac-HSA	negative	negative

hydrolysis of GM_1 and GD_{1b} respectively, by treatment with β-galactosidase from bovine testes. The gangliosides thus obtained were purified to homogeneity by column chromatography over silica gel, and the alkenic moiety of the ceramide aglycon was exploited for conjugation. Treatment of methanolic solutions of GM_2 and GD_2 with ozone at -15 °C and subsequent reduction of the ozonide with methyl sulfide was carried out according to established protocol.[201] The resulting aldehyde (or acetal equivalent) was introduced to the carrier protein and covalent attachment was formalized by sodium cyanoborohydride reduction. The resulting conjugates were first dialyzed against TRIS/EDTA/2% aqueous DOC, then exhaustively dialyzed against water and lyophilization provided the desired GM_2- and GD_2-KLH antigens. Reactivity of mAbs B212.1 and B213.1 towards various ganglioside antigens is shown in Table 10.

As can be seen from this Table, monoclonal antibody B213.1 reacts primarily with GM_3-antigens (ELISA, Abs ≈ 0.70), however, some cross-reactivity with GD_3-antigens (ELISA, Abs ≈ 0.30) is also observed. On the other hand, anti-GD_3 mAb B212.1 reacts with GD_3-antigens (ELISA, Abs ≈ 0.50), and shows cross-reactivity with GM_3-antigens (ELISA, Abs ≈ 0.10). Neither of these two antibodies recognize the unsubstituted KLH carrier alone.

Table 10: Reactivity of B212.1 and B213.1 Towards Ganglioside Antigens

Antigen	B212.1 (anti-GD_3)	B213.1 (anti-GM_3)
KLH	negative	negative
GM_2-KLH	negative	negative
GM_3-KLH	weak	positive
GM_3-HSA	negative	positive
GD_2-KLH	negative	negative
GD_3-KLH	positive	positive
GD_3-HSA	positive	positive

As these results are preliminary, a critical decision will have to be made as to whether re-immunization should be attempted in order to try to obtain the more favorable IgG clones. In any event, the reactivity of these two mAbs towards various melanoma cell lines needs to be investigated.

4.3.2 Potential Utility of GM_3- and GD_3-Antigens and MAbs

Upon completion of this work, it has been reported[202] that gangliosides conjugated to keyhole limpet hemocyanin display superior immunogenic properties in animal models and these conjugates are potentially useful for application as cancer vaccines for immunization of melanoma patients. Thus, synthetic GD_3-KLH conjugates may provide valuable information on optimizing immunogenic properties for active specific immunotherapy of cancer.

A novel approach towards inducing immunity against tumors involves the application of anti-idiotypic monoclonal antibody vaccines and an excellent review article on this subject is available.[203] In this regard, the anti-idiotypic antibody, in theory, represents a peptide mimic of the original ganglioside antigen. In fact, an anti-idiotypic mAb designated 4C10 raised against an anti-GM_3 mAb has been described by Yamamoto and colleagues[204] who further showed that mice immunized with 4C10 developed an anti-GM_3 response. Subsequently, an anti-idiotypic anti-GD_3 mAb, BEC2, has also been generated,[205] and immunization of rabbits with this material induced the more long-lived IgG response, indicating possible T-cell involvement.

Exploitation of melanoma-associated gangliosides is moving ahead on several fronts (radioimaging, mAb therapy, radiolabeled mAb therapy, active specific immunotherapy, and anti-idiotype immunotherapy). With several potential applications for the GM_3- and GD_3-antigens and corresponding mAbs, an understanding on the molecular level of the interactions of immunodominant epitopes of the ligand with its specific receptor is essential. Determination of preferred binding conformations by NMR and molecular modelling may reveal amino acid sequences which mimic these antigens, which may prove to be useful in immunization work against metastatic melanoma. Before even considering extension into these applications, we still need to firmly establish the reactivity of the B212.1 and B213.1 mAbs towards the corresponding

immunogens as well as melanoma cells and demonstrate their utility in diagnosis. Although our efforts represent only a minute step in the direction towards developing new diagnostic (and possibly therapeutic) pharmaceuticals for melanoma, hopefully, the antigens and monoclonal antibodies described herein may be of some value in providing insights on the molecular level, as to the nature of important binding events involved in such processes.

5. MATERIALS AND METHODS

5.1 General Procedures

Specific rotations were determined with an Optical Activity LTD AA-100 polarimeter at 23 °C using a path length of 1 dcm. ^1H NMR spectra were recorded with a Bruker AM 300 or AM 400 spectrometer, or in some cases, a Varian 500 MHz spectrometer, in solutions of $CDCl_3$ unless otherwise noted. The shift values are expressed in ppm downfield from the internal signal for Me_4Si or in the case of D_2O, HOD δ 4.8 at ambient temperature. The notation used for the designation of protons is adapted from Figure 4 wherein nonsuperscripted designators represent sugar I, while ('), (''), and (''') represent sugars II, A, and C respectively. ^{13}C NMR were recorded at 75.47 MHz, and ^{19}F NMR at 376.5 MHz as either D_2O or $CDCl_3$ solutions unless otherwise specified. Assignments were aided by the J-MOD technique.[206,207] ^{19}F NMR chemical shifts are expressed in ppm from $CFCl_3$. Fast atom bombardment (FAB)-mass spectra were obtained with a Kratos AE1 MS9 mass spectrometer in the positive ion mode using either HCOOH/glycerol or Cleland matrix or in the negative ion mode using TEA as the matrix. Visible and UV absorptions were measured with a Hewlett Packard 8452A diode array spectrophotometer. Melting points were determined with a Gallenkamp MFB-585 melting point apparatus and are uncorrected. Analytical TLC was performed on Silica Gel F_{254} plates (Merck, Darmstadt) with detection by UV light or by charring with sulfuric acid and column chromatography was carried out using Kieselgel 60H (Merck, Darmstadt) packed in Michel-Miller HPLPLC columns (Ace Glass, NJ). Ozone was produced with a Welsbach T-408 ozinator. Toluene and tetrahydrofuran were distilled from sodium benzophenone ketyl under argon. Dichloromethane, chloroform, acetonitrile and *N,N*-dimethylformamide were distilled from P_2O_5 and stored over molecular sieves 3Å. Methanol was distilled from Mg under argon and likewise stored over molecular sieves 3Å. Concentrations were performed *in vacuo*.

5.2 Experimental

Neu5Ac (1) and *O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid (12). Colominic acid (2 g) was dissolved in distilled water (40 mL) and the pH of the solution was adjusted to ~ 2.0 with 0.1 M aqueous hydrochloric acid (~ 4 mL). The solution was heated to 75 °C (oil bath) for 75 min and then allowed to cool to room temperature. The pH of the solution was then adjusted to ~ 7.5 with 0.1 M aqueous sodium hydroxide and water was removed by evaporation under high vaccum. The resulting solid was dissolved in a minimum amount of 0.01 M Tris-HCl buffer and applied to a column of Sephadex A-25 (~ 40 g, DEAE-Sephadex ion exchange resin) previously equilibrated with this buffer. The column was then eluted with 0.01 M Tris-HCl buffer (500 mL) followed by gradient elution with aqueous NaCl (0.1 to 0.6 M). Fractions with similar R_f by TLC in 7:1:2 isopropanol-ammonia-water were pooled. After five identical runs on this 2 g scale, the combined amount of the first eluant, namely **1** was 4.38 g (43%). The combined second eluant was chromatographed over silica gel (90 g) using 8:1:1 isopropanol-ammonia-water gave 1.2 g (12%) of **12**, presumably as the di-ammonium salt. The resulting solid was lyophilized from water to give **12** as an amorphous white solid: R_f 0.29 (7:1:2 isopropanol-ammonia-water, R_f of compound **1**: 0.42), ^1H NMR (D_2O, HOD 4.8, HOD suppression) δ 4.0-3.5 (m, 14 H), 2.75 (dd, 1H, $J_{3e',3a'}$ = 12.5 Hz, $J_{3e',4'}$ = 4.5 Hz, H-3e'), 2.2 (dd, 1H, $J_{3e,3a}$ = 13.0 Hz, $J_{3e,4}$ = 5.0 Hz, H-3e), 2.04 (s, 3H, Ac), 2.0 (s, 3H, Ac), 1.72 (dd, 1H, $J_{3a,3e}$ = 13.0 Hz, $J_{3a,4}$ = 12.0 Hz, H-3a), (t, 1H, J_t = 12.5 Hz, H-3a').

5-Acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-nonulopyranosylonic acid (2b) and α-anomer (2a). A solution of sialic acid **1** (2.5 g, 8.08 mmol) in pyridine (20 mL) and acetic anhydride (11 mL) was stirred at room temperature for 18 h. The reaction mixture was diluted with toluene and evaporated. After coevaporation with toluene followed by deionization, filtration, and removal of solvents, 4.52 g of crude **2** was obtained as a tan colored powder which was used directly in the next step without further purification: R_f 0.34 (65:35:8 chloroform-methanol-water).

Methyl 5-acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-nonulopyranosylonate (3b) and α-anomer (3a). The crude acid

mixture **2a,b** (4.5 g, 8.66 mmol) was dissolved in *N,N*-dimethylformamide (30 mL) containing potassium fluoride (1 g, 17.33 mmol) and methyl iodide (2.7 g, 19 mmol) and the resulting solution was stirred at room temperature for 20 h. After this time, acetic acid (4 mL) was added to consume the excess methyl iodide and the reaction mixture was stirred for an additional 20 min after which time, the solvents were removed and the residue was coevaporated with toluene. The resulting oil was dissolved in chloroform (500 mL) and washed with saturated aqueous sodium bicarbonate (2×), dried (sodium sulfate), filtered, and the solvents were removed to give a yellowish foam. Column chromatography over silica gel (300 g) using 20:10:1 hexane-ethyl acetate-ethanol as eluant then switching to 15:10:1 hexane-ethyl acetate-ethanol gave 289 mg (7%) of **3a** followed by 3 g (66%) of **3b**. Compound **3a** was obtained as a white amorphous powder upon lyophilization from benzene; R_f 0.16 (10:10:1 hexane-ethyl acetate-ethanol): ^{1}H NMR δ 5.38 (dd, 1H, $J_{7,8}$ = 7.5 Hz, $J_{7,6}$ = 2.5 Hz, H-7), 5.32 (d, 1H, $J_{NH,5}$ = 10.5 Hz, NH), 5.20 (ddd, 1H, $J_{8,7}$ = 7.5 Hz, $J_{8,9b}$ = 5.5 Hz, $J_{8,9a}$ = 2.5 Hz, H-8), 5.02 (ddd, 1H, $J_{4,3a}$ = 12.5 Hz, $J_{4,5}$ = 10.0 Hz, $J_{4,3e}$ = 4.5 Hz, H-4), 4.7 (dd, 1H, $J_{6,5}$ = 10.5 Hz, $J_{6,7}$ = 2.5 Hz, H-6), 4.35 (dd, 1H, $J_{9a,9b}$ = 12.5 Hz, $J_{9a,8}$ = 2.5 Hz, H-9a), 4.16 (q, 1H, J_q = 10.5 Hz, H-5), 4.06 (dd, 1H, $J_{9b,9a}$ = 12.5 Hz, $J_{9b,8}$ = 5.5 Hz, H-9b), 3.77 (s, 3H, CH_3O), 2.56 (dd, 1H, $J_{3e,3a}$ = 13.0 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e), 2.12 (s, 3H, Ac), 2.11(s, 3H, Ac), 2.11 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.10 (s, 3H, Ac), and 1.90 (s, 3H, Ac). The signal for H-3a was obscured by the acetate signals. Likewise, lyophilization of **3b** from benzene gave a white amorphous solid; R_f 0.15 (10:10:1 hexane-ethyl acetate-ethanol): ^{1}H NMR δ 5.38 (dd, 1H, $J_{7,8}$ = 5.0 Hz, $J_{7,6}$ = 1.5 Hz, H-7), 5.32 (d, 1H, $J_{NH,5}$ = 9.5 Hz, NH), 5.25 (ddd, 1H, $J_{4,3a}$ = 12.0 Hz, $J_{4,5}$ = 10.0 Hz, $J_{4,3e}$ = 5.0 Hz, H-4), 5.08 (ddd, 1H, $J_{8,9b}$ = 6.5 Hz, $J_{8,7}$ = 5.0 Hz, $J_{8,9a}$ = 2.5 Hz, H-8), 4.5 (dd, 1H $J_{9a,9b}$ = 12.5 Hz, $J_{9a,8}$ = 2.5 Hz, H-9a), 4.12-4.11 (m, 5H, OMe, H-5, H-6), 4.11 (dd, 1H, $J_{9b,9a}$ = 12.5 Hz, $J_{9b,8}$ = 6.5 Hz, H-9b), 2.55 (dd, 1H, $J_{3e,3a}$ = 13.5 Hz, $J_{3e,4}$ = 5.0 Hz), 2.15, 2.15, 2.05, 2.04, 2.04 (6s, 6×3H, 6 Ac).

Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-chloro-2,3,5-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonate (4).[139] To a solution of **3a,b** (3.2 g, 6 mmol) in chloroform (40 mL) cooled to 0-5 °C (ice-water bath) was added acetyl chloride (15 mL) and water (250 μL). The mixture was allowed to slowly warm to room temperature, then stirred overnight. The reaction mixture was again cooled to 0-5 °C and ice water was added. The two phase mixture

was then transferred to a separatory funnel with the aid of chloroform and the layers were separated. The organic solution was washed with water, saturated sodium bicarbonate (2×), dried (sodium sulfate), filtered, and the solvents were removed. The resulting foam was lyophilized from benzene to give 2.7 g (89%) of **4** as an amorphous tan colored solid: R_f 0.18 (10:10:1 hexane-ethyl acetate-ethanol): ^{1}H NMR δ 5.49 (dd, 1H, $J_{7,8}$ = 7.0 Hz, $J_{7,6}$ = 2.0 Hz, H-7), 5.48 (m, NH, obscured by H-7 and H-4), 5.41 (ddd, 1H, $J_{4,3a}$ = 11.5 Hz, $J_{4,5}$ = 10.5 Hz, $J_{4,3e}$ = 5.0 Hz, H-4), 5.18 (ddd, 1H, $J_{8,7}$ = 7.0 Hz, $J_{8,9b}$ = 6.0 Hz, $J_{8,9a}$ = 2.5 Hz, H-8), 4.45 (dd, 1H, $J_{9a,9b}$ = 12.5 Hz, $J_{9a,8}$ = 2.5 Hz, H-9a), 4.38 (dd, 1H, $J_{6,5}$ = 10.5 Hz, $J_{6,7}$ = 2.0 Hz, H-6), 4.23 (q, 1H, J_q = 10.5 Hz, H-5), 4.09 (dd, 1H, $J_{9b,9a}$ = 12.5 Hz, $J_{9b,8}$ = 6.0 Hz, H-9b), 3.9 (s, 3H, OCH_3), 2.8 (dd, 1H, $J_{3e,3a}$ = 13.5 Hz, $J_{3e,4}$ = 5.0 Hz, H-3e), 2.3 (dd, 1H, $J_{3a,3e}$ = 13.5 Hz, $J_{3a,4}$ = 11.5 Hz, H-3a), 2.15 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.07 (s, 3H, Ac), and 1.93 (s, 3H, Ac).

Methyl (4-pentenyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)onate (5a) and β-anomer (5b). To a stirred solution of 4-penten-1-ol (1.03 mL, 10 mmol), silver trifluoromethanesulfonate (3.9 g, 15 mmol), and powdered molecular sieves 4Å (6 g) in anhydrous dichloromethane (10 mL) cooled to -25 °C, was added dropwise, a solution of chloride **4** (2.55 g, 5 mmol) in dry dichloromethane (5 mL). The mixture was then slowly allowed to warm to 0 °C and stirred at this temperature for 72 h. The reaction mixture was filtered through Celite®, diluted with dichloromethane, washed successively with water, saturated aqueous sodium bicarbonate, dried (sodium sulfate), filtered, and the solvents were removed. Column chromatography over silica gel (300 g) using 20:10:1 hexane-ethyl acetate-ethanol then 15:10:1 hexane-ethyl acetate-ethanol as eluant followed by rechromatography over silica gel (300 g) employing 70:1 chloroform-methanol as eluant gave first, 270 mg (10%) of **5b** followed by 1.54 g (55%) of **5a**. Compound **5a** was obtained as an amorphous white solid after lyophilization from benzene: R_f 0.31 (20:1 chloroform-methanol, two developments); $[\alpha]_D$ -20.8° (*c* 1, chloroform); ^{1}H NMR δ 5.8 (ddt, 1H, J_d = 17.0 Hz, J_d = 10.0 Hz, J_t = 6.0 Hz, $\underline{H}C{=}CH_2$), 5.39, (ddd, 1H, $J_{8,7}$ = 8.5 Hz, $J_{8,9b}$ = 5.5 Hz, $J_{8,9a}$ = 2.5 Hz, H-8), 5.32 (dd, 1H, $J_{7,8}$ = 8.5 Hz, $J_{7,6}$ = 1.75 Hz, H-7), 5.15 (d, 1H, $J_{NH,5}$ = 9.5 Hz, NH), 5.01 (dq, 1H, J_d = 17.0 Hz, J_q = 1.5 Hz, $HC{=}C\underline{H}_2$), 4.98 (dm, 1H, J_d = 10.0 Hz, J_m < 1.0 Hz, $HC{=}C\underline{H}_2$), 4.85 (ddd, 1H, $J_{4,3a}$ = 13.0 Hz, $J_{4,5}$ = 9.5 Hz,

$J_{4,3e}$ = 4.5 Hz, H-4), 4.31, (dd, 1H, $J_{9a,9b}$ = 12.5 Hz, $J_{9a,8}$ = 2.5 Hz, H-9a), 4.11 (dd, 1H, $J_{9b,9a}$ = 12.5 Hz, $J_{9b,8}$ = 5.5 Hz, H-9b), 4.1 (m, 1H, H-6, obscured by H-9b and H-5 protons), 4.08 (q, 1H, J_q = 10.0 Hz, H-5), 3.79 (s, 3H, CH_3O), 3.78 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 3.24 (dt, 1H, J_d = 9.5 Hz, J_t = 6.5 Hz, OCH_2), 2.59 (dd, 1H, $J_{3e,3a}$ = 12.5 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e), 2.13 (s, 3H, Ac), 2.12 (s, 3H, Ac), 2.11 (m, 2H, CH_2), 2.05 (s, 3H, Ac), 2.04 (s, 3H, Ac), 1.95 (t, 1H, $J_{3a,3e,4}$ = 12.5 Hz, H-3a), 1.89 (s, 3H, Ac), 1.64 (m, 1H, CH_2); ^{13}C NMR δ 171.00, 170.63, 170.18, 170.13, 170.00, and 168.51 (6 C=O), 138.02 ($H\underline{C}=CH_2$), 114.82 ($HC=\underline{C}H_2$), 98.68 (C-2), 72.42, 69.15, 68.66, and 67.36 (C-4,6,7,8), 64.21 and 62.33 (OCH_2 and C-9), 52.61 (CH_3O), 49.47 (C-5), 38.07 (C-3), 29.93 and 28.73 (2 CH_2), 23.17 and 21.07 (2 $\underline{C}H_3C=O$), 20.81 (2 $\underline{C}H_3C=O$), 20.76 ($CH_3C=O$). Some of the spectral assignments are tentative.

Anal. Calcd for $C_{25}H_{37}O_{13}N$ (559.56): C, 53.66; H, 6.67; N, 2.50. Found: C, 53.48; H, 6.79; N, 2.50.

Likewise, compound **5b** was obtained as an amorphous white solid after lyophilization from benzene: R_f 0.32 (20:1 chloroform-methanol, two developments); $[\alpha]_D$ -19.1° (c 1, chloroform); ^1H NMR δ 5.81 (ddt, 1H, J_d = 17.0 Hz, J_d = 10.0 Hz, J_t = 6.5 Hz, $\underline{H}C=CH_2$), 5.40 (dd, 1H, $J_{7,8}$ = 3.5 Hz, $J_{7,6}$ = 2.5 Hz, H-7), 5.32 (d, 1H, $J_{NH,5}$ = 10.5 Hz, NH), 5.27 (dd, 1H, $J_{4,3a,5}$ = 10.5 Hz, $J_{4,3e}$ = 5.0 Hz, H-4), 5.20 (ddd, 1H, $J_{8,9b}$ = 7.5 Hz, $J_{8,7}$ = 3.5 Hz, $J_{8,9a}$ = 2.5 Hz, H-8), 5.05 (dq, 1H, J_d = 17.0 Hz, J_q = 1.5 Hz, $HC=C\underline{H}_2$), 5.0 (dm, 1H, J_d = 10.0 Hz, J_m < 1.0 Hz, $HC=C\underline{H}_2$), 4.80 (dd, 1H, $J_{9a,9b}$ = 12.5 Hz, $J_{9a,8}$ = 2.5 Hz, H-9a), 4.12 (dd, 1H, $J_{9b,9a}$ = 12.5 Hz, $J_{9b,8}$ = 7.5 Hz, H-9b), 4.11 (q, 1H, J_q = 10.5 Hz, H-5), 3.92 (dd, 1H, $J_{6,5}$ = 10.5 Hz, $J_{6,7}$ = 2.5 Hz, H-6), 3.8 (s, 3H, CH_3O), 3.48 (dt, 1H, J_d = 9.5 Hz, J_t = 6.5 Hz, OCH_2), 3.35 (dt, 1H, J_d = 9.5 Hz, J_t = 6.5 Hz, OCH_2), 2.47 (dd, 1H, $J_{3e,3a}$ = 12.5 Hz, $J_{3e,4}$ = 5.0 Hz, H-3e), 2.15 (s, 3H, Ac), 2.12 (m, 2H, CH_2), 2.08 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.89 (s, 3H, Ac), 1.87 (dd, 1H, $J_{3a,3e}$ = 12.5 Hz, $J_{3a,4}$ = 10.5 Hz, H-3a), 1.68 (m, 2H, CH_2); ^{13}C NMR δ 171.03, 170.69, 170.48, 170.18, 170.18, and 167.52 (6 C=O), 137.77 ($H\underline{C}=CH_2$), 115.13 ($HC=\underline{C}H_2$), 98.47 (C-2), 72.21, 71.79, 68.94, and 68.52 (C-4,6,7,8), 63.42 and 62.41 (OCH_2 and C-9), 52.58 (CH_3O), 49.42 (C-5), 37.34 (C-3), 29.64 and 28.63 (2 CH_2), 23.12, 20.99, and 20.84 (3 $\underline{C}H_3C=O$), 20.73 (2 $\underline{C}H_3C=O$). Some of the spectral assignments are tentative.

Anal. Calcd for $C_{25}H_{37}O_{13}N$ (559.56): C, 53.66; H, 6.67; N, 2.50. Found: C, 53.48; H, 6.80; N, 2.50.

4-Pentenyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid (6a). A solution of **5a** (1.34 g, 2.36 mmol) in 20 mL of 4:1 methanol-water, 0.5 M in potassium hydroxide, was stirred overnight at room temperature. The reaction mixture was deionized with Dowex® (H^+) resin (3 g), filtered and the solvents were removed. Column chromatography over silica gel (125 g) using 65:35:2 chloroform-methanol-water as eluant gave 889 mg (97%) of **6a** as a powder; R_f 0.25 (65:35:5 chloroform-methanol-water); $[\alpha]_D$ -0.4° (c 1, methanol): ^1H NMR (D_2O, HOD 4.8), δ 5.89 (ddt, 1H, J_d = 17.5 Hz, J_d = 10.0 Hz, J_t = 6.5 Hz, $\underline{H}C=CH_2$), 5.08 (dq, 1H, J_d = 17.5 Hz, J_q = 2.0 Hz, $HC=C\underline{H}_2$), 5.01 (dq, 1H, J_d = 10.0 Hz, J_q < 1.0 Hz, $HC=C\underline{H}_2$), 3.90-3.78 (m, 2H), 3.76 (dt, 1H, J_d = 9.5 Hz, J_t = 6.5 Hz, OCH_2), 3.72-3.62 (m, 4H), 3.59 (dd, 1H, J_d = 9.0 Hz, J_d = 2.0 Hz), 3.46 (dt, 1H, J_d = 9.5 Hz, J_t = 6.5 Hz, OCH_2), 2.74 (dd, 1H, $J_{3e,3a}$ = 12.5 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e), 2.15 (m, 2H, CH_2), 2.02 (s, 3H, Ac), 1.65 (m, 3H, CH_2, and H-3a); ^{13}C (D_2O, HOD lock), δ 175.33 and 174.36 (2 C=O), 139.61 ($H\underline{C}=CH_2$), 115.48($HC=\underline{C}H_2$), 101.41 (C-2), 73.30, 72.49, 68.99, and 68.99 (C-4,6,7,8), 65.10 and 63.32 (OCH_2 and C-9), 52.66 (C-5), 41.16 (C-3), 30.13 and 28.97 (2 CH_2), 22.75 ($\underline{C}H_3C=O$). Some of the spectral assignments are tentative.

Molecular weight for $C_{16}H_{26}O_9NNa$: calcd 399.3, found (FAB, positive ion, Cleland matrix): *m/z* 400 $(M+1)^+$, Na salt.

4-Pentenyl 5-acetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonic acid (6b). A solution of **5b** (94 mg, 0.17 mmol) in 3 mL of 4:1 methanol-water, 0.5 M in potassium hydroxide, was stirred overnight at room temperature. The reaction mixture was neutralized with excess acetic acid and the solvents were removed. Column chromatography over silica gel (5 g) using 65:35:2 chloroform-methanol-water as eluant gave 63 mg (98%) of **6b** as a powder: R_f 0.13 (65:35:5 chloroform-methanol-water); $[\alpha]_D$ -32° (c 1, methanol); ^1H NMR (D_2O, HOD 4.8), δ 5.9 (ddt, 1H, J_d = 17.5 Hz, J_d = 10.0 Hz, J_t = 6.5 Hz, $\underline{H}C=CH_2$), 5.07 (dq, 1H, J_d = 17.5 Hz, J_q = 2.0 Hz, $HC=C\underline{H}_2$), 4.98 (dq, 1H, J_d = 10.0 Hz, J_q = 1.0 Hz, $HC=C\underline{H}_2$), 4.05 (ddd, 1H, $J_{4,3a}$ = 12.0 Hz, $J_{4,5}$ = 9.5 Hz, $J_{4,3e}$ = 5.0 Hz), 3.91 - 3.76 (m, 4H), 3.64 (dd, 1H, J_d = 12.0 Hz, J_d = 5.5 Hz), 3.59-3.49 (m, 2H), 3.29 (dt, 1H, J_d = 9.0 Hz, J_t = 6.0 Hz, OCH_2), 2.35 (dd, 1H,

$J_{3e,3a}$ = 12.5 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e), 2.11 (m, 2H, CH_2), 2.02 (s, 3H, Ac), 1.65 (m, 3H, CH_2, and H-3a); ^{13}C (D_2O, HOD lock), δ 176.3 and 175.66 (2 C=O), 140.06 ($H\underline{C}=CH_2$), 115.33 ($HC=\underline{C}H_2$), 100.93 (C-2), 71.22, 71.08, 69.03, and 67.78 (C-4,6,7,8), 64.24 and 63.59 (OCH_2 and C-9), 52.97 (C-5), 40.79 (C-3), 30.61 and 29.04 (2 CH_2), 22.96 ($\underline{C}H_3C=O$). Some of the spectral assignments are tentative.

Molecular weight for $C_{16}H_{26}O_9NNa$: calcd 399.3, found (FAB, positive ion, Glycerol/HCl): *m/z* 400 $(M+1)^+$, Na salt and *m/z* 416 $(M+1)^+$, K salt.

4-Oxobutyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid (7). A solution of sialyl derivative **6a** (25 mg, 0.066 mmol) in methanol (5 mL) was cooled to -70 °C (dry ice-acetone bath), and ozone was bubbled through the solution for 2-3 min until the solution displayed a slight but distinct blue color. The excess ozone was then removed by bubbling argon through the mixture for approximately 10-15 min. To the reaction mixture was added methyl sulfide (10 μL, 0.132 mmol) after which the reaction was allowed to warm to room temperature and the solvents were then removed. The residue was triturated with ethyl ether (3×5mL) and then dried *in vacuo* to provide 25 mg (quantitative) of **7** as a powder: R_f 0.22 (65:35:5 chloroform-methanol-water): ^1H NMR (D_2O, HOD 4.8, HOD suppression) δ 4.9 (t, 1H, J_t = 6.0 Hz), 3.9-3.4 (m, 9H), 2.72 (dd, 1H, $J_{3e,3a}$ = 12.5 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e), 2.01 (s, 3H, Ac), 1.75-1.60 (m, 5H); ^{13}C (D_2O, HOD lock), δ 175.95 and 174.49 (2 C=O), 108.83 (hydrated aldehyde ?), 101.41 (C-2), 73.44, 72.62, 69.13, and 69.08, (C-4,6,7,8), 65.08 and 63.44 (OCH_2 and C-9), 52.76 (C-5), 41.24 (C-3), 28.28 (CH_2), 25.14 (CH_2), 22.89 ($\underline{C}H_3C=O$). The extra peak in the spectrum is assumed to be due to solvent molecules and spectral assignments for **8** are tentative. Due to the fact that this compound was somewhat unstable, it was used directly in the next step without further purification.

Sialylated HSA (8). A solution of human serum albumin (75 mg, Sigma), and crude aldehyde **7** (23 mg) in phosphate buffered saline (PBS, pH 7.8) was stirred at room temperature for 40 min after which time sodium cyanoborohydride (34 mg, 0.54 mmol) was added and the reaction mixture stirred for 96 h. Diafiltration of the reaction mixture using an Amicon 50 mL stirred cell equipped with a YM 10 ultrafiltration membrane filter, against water was carried out for a total of 14 exchanges. Concentration followed by lyophilization gave 71 mg of

glycoconjugate **8** as a fluffy white powder. The sialic acid content of **9** was then estimated using the resorcinol-hydrochloric acid assay and from Graph 1, for A_T = 0.495, N = 20.

Preparation of KLH. Commercial keyhole limpet hemocyanin (*Megathura crenulata*, Calbiochem) was obtained as a mixture of protein (approximately 60%) in BES buffer and magnesium sulfate. KLH (500 mg) was dissolved in PBS buffer pH 7.8 and then transferred to an Amicon stirred cell equipped with a YM 10 ultrafiltration membrane filter. The solution was dialyzed successively against PBS then water to give 225 mg of KLH after concentration and lyophilization. The resulting protein was then used for conjugation reactions.

Sialylated KLH (9). A solution of KLH (60 mg) and aldehyde **7** (20 mg) in PBS pH 7.8 was stirred at room temperature for 45 min after which time sodium cyanoborohydride (34 mg, 0.54 mmol) was added and stirring was continued for an additional 72 h. Exhaustive dialysis (Amicon, YM 10 ultrafiltration membrane) against water gave 53 mg of the sialylated-KLH conjugate as a fluffy white solid. The sialic acid content of **9** was then estimated using the resorcinol-hydrochloric acid assay and from Graph 2, for A_T = 0.238, N = 740.

Resorcinol-Hydrochloric Acid Assay for Sialic Acid.[162,163]

(a) Preparation of the resorcinol reagent: An aqueous resorcinol stock solution was prepared by dissolving resorcinol (2 g, recrystallized from toluene and dried *in vacuo*) in distilled water (100 mL). Likewise, an aqueous 0.1 M copper sulfate solution was prepared by dissolving 1.6 g of anhydrous copper sulfate in 100 mL of water. The resorcinol reagent solution was then prepared by careful addition of the resorcinol stock solution (10 mL) to 80 mL of concentrated sulfuric acid containig 250 μL of the copper sulfate stock solution and the resulting solution volume was adjusted to 100 mL with distilled water. The mixture was allowed to stand at room temperature for 4 h with occasional agitation and then stored at 0-4 °C until used.

(b) Preparation of standards and sample as solutions: The purified protein (HSA or KLH), sialic acid compound (*e.g.*, **6a**) standard, and the corresponding sialoglycoconjugate (*e.g.*, **7** or **8**) were each prepared as 1 mg/mL aqueous solutions.

(c) Assay procedure: Eight Pyrex® culture tubes (13×100 mm, screw cap 13-415) were labelled and charged with the following; 500 μL of protein blank standard solution, 40, 60, 80, and 100 μL of sialic acid standard solution and sialoconjugate 500 μL (3×). The solution volume of each tube was brought to 1.5 mL with distilled water and resorcinol reagent (2 mL) was added to each tube. The tubes were sealed, vortexed for 1 min, and then incubated at 100-105 °C for 15 min. After this time, the tubes were rapidly cooled under a stream of cold tap water, then 85:15 butyl acetate-butanol (2 mL) was added to each tube, the tubes sealed and vortexed for 1 min and the tubes cooled in an ice-water bath for 15 min to further enhance colorimetric intensity. The spectrophotometer wavelength was set to 580 nm and the instrument zero was set with 85:15 butyl acetate-butanol. The absorbance of the blue organic (upper) layer for each of the standards and samples was measured and the absorbance versus μg of sialic acid for the sialic acid standards was plotted (see Appendix 1 for graphs). From the corrected absorbance of the samples, the amount of sialic acid in the sialoconjugate could be estimated and the N value (# of moles of carbohydrate/# moles of carrier) was approximated as follows:

$\mathbf{A_T}$ = True Abs. (sample) = Abs. (sample) - Abs. (protein std.)

From the standard plot and the $\mathbf{A_T}$ value, the amount of sialic acid $\mathbf{W_s}$ (μg) can be found. Then,

$\mathbf{N_s}$ (# μM of sialic acid) = $\mathbf{W_s}$ / MW sialic acid derivative

Amount of protein in sample is then approximated as;

$\mathbf{W_p}$ (μg) = 500 - $\mathbf{W_s}$ (μg)

From which;

$\mathbf{N_p}$ (# μM of protein) = $\mathbf{W_p}$ / MW protein

MW = 6.7×10^4 for HSA and 6.7×10^6 for KLH.

It follows that;

N value (# of moles of carbohydrate / # moles of carrier) = $\mathbf{N_s}$ / $\mathbf{N_p}$

Benzyl 5-acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-nonulopyranosylonate (10b) and α-anomer (10a). The crude acid mixture **2a,b** (4.2 g, 8.09 mmol) was dissolved in *N,N*-dimethylformamide (30 mL) containing potassium fluoride (930 mg, 16.16 mmol) and benzyl bromide (2.77 g, 16.2 mmol) and the resulting solution was stirred at room temperature for 48 h. After this time, acetic acid (4 mL) was added to consume the excess benzyl bromide and the reaction mixture was stirred for an additional 3 h. After this time, the solvents were evaporated and the residue was coevaporated with toluene. The resulting oil was dissolved in chloroform (500 mL) and washed with saturated aqueous sodium bicarbonate solution (2×), dried (sodium sulfate), filtered, and the solvents were removed to give a yellowish foam. Column chromatography over silica gel using 15:10:1 hexane-ethyl acetate-ethanol as eluant then switching to 10:10:1 hexane-ethyl acetate-ethanol gave 159 mg of impure **10a** followed by 4.11 g (83%) of **10b**. Compound **10a** was obtained in a pure state after rechromatography over silica gel using 60:1 chloroform-methanol as eluant: R_f 0.23 (19:1 chloroform-methanol) and lyophilization from benzene; $[\alpha]_D$ +30° (*c* 0.3, chloroform);^{1}H NMR δ 7.33 (m, 5H, aromatic), 5.38 (dd, 1H, $J_{7,8}$ = 7.5 Hz, $J_{7,6}$ = 2.5 Hz, H-7), 5.26 (d, 1H, $J_{NH,5}$ = 10.5 Hz, NH), 5.21 (d, 1H, J_d = 12.0 Hz, benzyl), 5.18 (ddd, 1H, $J_{8,7}$ = 7.0 Hz, $J_{8,9b}$ = 5.5 Hz, $J_{8,9a}$ = 2.5 Hz, H-8), 5.12 (d, 1H, J_d = 12.0 Hz, benzyl), 4.97 (ddd, 1H, $J_{4,3a}$ = 12.5 Hz, $J_{4,5}$ = 10.5 Hz, $J_{4,3e}$ = 4.5 Hz, H-4), 4.75 (dd, 1H, $J_{6,5}$ = 10.5 Hz, $J_{6,7}$ = 2.5 Hz, H-6), 4.36 (dd, 1H, $J_{9a,9b}$ = 12.5 Hz, $J_{9a,8}$ = 2.5 Hz, H-9a), 4.16 (q, 1H, J_q = 10.5 Hz, H-5), 4.06 (dd, 1H, $J_{9b,9a}$ = 12.5 Hz, $J_{9b,8}$ = 5.5 Hz, H-9b), 2.55 (dd, 1H, $J_{3e,3a}$ = 13.0 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e), 2.08 (dd, 1H, $J_{3a,3e}$ = 13.0 Hz, $J_{3a,4}$ = 12.5 Hz, H-3a), 2.13 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.07 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.02 (s, 3H, Ac),1.89 (s, 3H, Ac).

Anal. Calcd for $C_{28}H_{35}O_{14}N$: C, 55.17; H, 5.79; N, 2.30. Found: C, 55.43; H, 5.73; N, 2.22.

Likewise, lyophilization of **10b** from benzene gave a white amorphous solid: R_f 0.12 (15:10:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ -41.3° (*c* 0.3, chloroform); ^{1}H NMR δ 7.35 (m, 5H, aromatic), 5.38 (dd, 1H, $J_{7,8}$ = 5.5 Hz, $J_{7,6}$ = 2.0 Hz, H-7), 5.28 (d, 1H, $J_{NH,5}$ = 9.5 Hz, NH), 5.25 (m, 1H, H-4), 5.24 (d, 1H, J_d = 12.0 Hz, benzyl), 5.17 (d, 1H, J_d = 12.0 Hz, benzyl), 5.09 (ddd, 1H, $J_{8,9b}$ = 6.5 Hz, $J_{8,7}$ = 5.5 Hz, $J_{8,9a}$ = 2.5 Hz, H-8), 4.45 (dd, 1H $J_{9a,9b}$ = 12.5 Hz, $J_{9a,8}$ = 2.5

Hz, H-9a), 4.11 (m, 2H, H-5 and H-6), 2.56 (dd, 1H, $J_{3e,3a}$ = 13.25 Hz, $J_{3e,4}$ = 5.0 Hz), 2.1 (dd, 1H, $J_{3a,3e}$ = 13.25 Hz, $J_{3a,4}$ = 11.25 Hz, H-3a), 2.13 (s, 3H, Ac), 2.11 (s, 3H, Ac), 2.11 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.9 (s, 3H, Ac).

Anal. Calcd for $C_{28}H_{35}O_{14}N$: C, 55.17; H, 5.79; N, 2.30. Found: C, 54.81; H, 5.73; N, 2.22.

Benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-chloro-2,3,5-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonate (11). To a solution of **10b** (3.05 g, 5 mmol) in chloroform (30 mL) cooled to 0-5 °C (ice-water bath) was added acetyl chloride (15 mL) and water (250 μL). The mixture was allowed to slowly warm to room temperature, then stirred overnight. The reaction mixture was again cooled to 0-5 °C and ice water was added. The two phase mixture was then transferred to a separatory funnel with the aid of chloroform and the layers were separated. The organic solution was washed with water, saturated sodium bicarbonate (2×), dried (sodium sulfate), filtered, and the solvents were removed. The resulting foam was lyophilized from benzene to give 2.87 g (98%) of **11** as an amorphous solid: R_f 0.15 (15:10:1 hexane-ethyl acetate-ethanol) and 0.59 (3:2 chloroform-acetone); $[\alpha]_D$ -63° (*c* 1, chloroform); ^{1}H NMR δ 7.4 (m, 5H, aromatic), 5.48 (dd, 1H, $J_{7,8}$ = 7.0 Hz, $J_{7,6}$ = 2.5 Hz, H-7), 5.45 (d, 1H, J_d = 10.5 Hz, NH), 5.40 (ddd, 1H, $J_{4,3a}$ = 11.0 Hz, $J_{4,5}$ = 10.5 Hz, $J_{4,3e}$ = 5.0 Hz, H-4), 5.37 (d, 1H, J_d = 12.0 Hz, benzyl), 5.22 (d, 1H, J_d = 12.0 Hz, benzyl), 5.18 (ddd, 1H, $J_{8,7}$ = 7.0 Hz, $J_{8,9b}$ = 5.5 Hz, $J_{8,9a}$ = 2.5 Hz, H-8), 4.41 (dd, 1H, $J_{9a,9b}$ = 12.5 Hz, $J_{9a,8}$ = 2.5 Hz, H-9a), 4.38 (dd, 1H, $J_{6,5}$ = 10.5 Hz, $J_{6,7}$ = 2.5 Hz, H-6), 4.21 (q, 1H, J_q = 10.5 Hz, H-5), 4.1 (dd, 1H, $J_{9b,9a}$ = 12.5 Hz, $J_{9b,8}$ = 5.5 Hz, H-9b), 2.8 (dd, 1H, $J_{3e,3a}$ = 13.5 Hz, $J_{3e,4}$ = 5.0 Hz, H-3e), 2.3 (dd, 1H, $J_{3a,3e}$ = 13.5 Hz, $J_{3a,4}$ = 11.5 Hz, H-3a), 2.12 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.01 (s, 3H, Ac),1.9 (s, 3H, Ac).

***O*-(Benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylono-1',9-lactone)-(2→8)-5-acetamido-2,4,7-tri-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonate (14b) and α-anomer (14a).** A solution of **12** (105 mg, 0.165 mmol) in 2:1 pyridine-acetic anhydride (3 mL) was stirred at room temperature for 18 h. The solvents were removed and the residue containing **13a** and **13b** was coevaporated with toluene, taken up in ethyl acetate, deionized with Amberlite® IR 120(H^+) resin

(500 mg), filtered and the solvents were removed and then the residue was coevaporated with toluene to give an amorphous powder. A solution of this material in dry *N,N*-dimethylformamide (4 mL) containing anhydrous potassium fluoride (38 mg, 0.66 mmol) and benzyl bromide (171 mg, 1.0 mmol) was stirred at room temperature for 72 h. The solvents were then removed under high vacuum and the residue taken up in chloroform, washed with water, saturated sodium bicarbonate solution, saturated sodium chloride solution, dried (sodium sulfate), filtered and the solvents were removed. Column chromatography over silica gel (5 g) using 10:10:1 hexane-ethyl acetate-ethanol and then changing to 6:6:1 gave first 20 mg (12%) of material (possibly **14a**) followed by 85 mg (53%) of **14b**. Lactone **14b** was an amorphous powder: R_f 0.11 (4:4:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ -36.6° (*c* 1, chloroform); ^{1}H NMR δ 7.40-7.30 (m, 5H, aromatic), 5.88 (m, 1H, NH),* 5.52 (d, 1H, $J_{NH',5'}$ = 10.0 Hz, NH'),* 5.34 (m, 1H, H-4'),* 5.34 (dd, 1H, $J_{7',8'}$ = 9.5 Hz, $J_{7',6'}$ = 2.5 Hz, H-7'),* 5.30 (td, 1H, J_t = 10.5 Hz, $J_{4,3e}$ = 5.0 Hz, H-4),* 5.22 (ddd, 1H, $J_{8',7'}$ = 9.5 Hz, $J_{8',9'b}$ = 5.5 Hz, $J_{8',9'a}$ = 2.5 Hz, H-8'),* 5.22 (s, 2H, $C\underline{H}_2C_6H_5$), 5.17 (dd, 1H, $J_{7,8}$ = 8.0 Hz, $J_{7,6}$ = 1.5 Hz, H-7),* 4.45 (dd, 1H, $J_{9a,9b}$ = 12.5 Hz, $J_{9a,8}$ = 8.5 Hz, H-9a),* 4.35 (m, 1H, H-8),* 4.35 (m, 1H, H-9b),* 4.30 (dd, 1H $J_{9'a,9'b}$ = 12.5 Hz, $J_{9'a,8'}$ = 2.5 Hz, H-9'a),* 4.18 (m, 1H, H-5),* 4.17 (m, 1H, H-6), 4.17 (q, 1H, J_q = 10.5 Hz, H-5'),* 4.08 (dd, 1H, $J_{6',5'}$ = 10.5 Hz, $J_{6',7'}$ = 2.5 Hz, H-6'),* 4.01 (dd, 1H, $J_{9'b,9'a}$ = 12.5 Hz, $J_{9'b,8'}$ = 5.5 Hz, H-9'b),* 2.52 (dd, 1H, $J_{3e,3a}$ = 13.0 Hz, $J_{3e,4}$ = 5.0 Hz, H-3e),* 2.43 (dd, 1H, $J_{3'e,3'a}$ = 13.0 Hz, $J_{3'e,4'}$ = 5.0 Hz, H-3'e),* 2.18 (s, 3H, Ac), 2.16 (s, 3H, Ac), 2.13 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.00 (s, 3H, Ac), 1.97 (dd, 1H, $J_{3a,3e}$ = 13.0 Hz, $J_{3a,4}$ = 11.5 Hz, H-3a),* 1.92 (s, 3H, Ac), 1.89 (s, 3H, Ac), 1.80 (dd, 1H, $J_{3'a,3'e}$ = 13.0 Hz, $J_{3'a,4'}$ = 11.5 Hz, H-3'a). Some of the spectral assignments are tentative. *These assignments were confirmed by decoupling experiments, thus, decoupling of the doublet centered at δ 5.88 (NH) caused the collapse of the multiplet at δ 4.20 (H-5) while irradiation of the doublet at δ 5.52 (NH') likewise affected the quartet at δ 4.17 (H-5'); decoupling of the doublet of doublets at δ 5.17 (H-7) resulted in the collapse of a multiplet at δ 4.35 (H-8); irradiation of the doublet of doublets at δ 4.45 (H-9a) disturbed resonances at δ 4.37-4.32 which were presumably due to the H-9b and H-8 signals; decoupling of the doublet of doublets at δ 4.29 (H-9'a) collapsed the double doublet of doublets at δ 5.22 (H-8') along with the doublet of doublets at δ 4.01 (H-9'b); irradiation of the doublet of doublets at δ 4.08 (H-6') perturbed the doublet of doublets at δ 5.34 (H-7') as well as the quartet at δ 4.17 (H-5');

decoupling of the doublet of doublets at δ 4.01 (H-9'b) simultaneously collapsed the double doublet of doublets at δ 5.22 along with the doublet of doublets at δ 4.29; decoupling of the doublet of doublets at δ 2.52 (H-3e) revealed that it was coupled to the triplet of doublets at δ 5.30 (H-4) as well as the doublet of doublets at δ 1.97 (H-3a); and irradiation of the doublet of doublets at δ 1.80 (H-3'a) collapsed the multiplet at δ 5.34 (H-4') and the doublet of doublets centered at δ 2.43 (H-3'e).

Anal. Calcd for $C_{43}H_{54}O_{23}N_2$: C, 53.42; H, 5.63; N, 2.90. Found; C, 53.52; H, 5.81; N, 3.01.

***O*-(Benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylono-1',9-lactone)-(2→8)-5-acetamido-2-chloro-4,7-di-*O*-acetyl-2,3,5-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosyl-onate (15).** To a solution of **14b** (248 mg, 0.256 mmol) in chloroform (6 mL) was added dropwise acetyl chloride (6 mL) and the stirred mixture was cooled to 5 ° C . Cold water (150 μL) was added and the mixture was stirred at room temperature for 18 h during which time a gel had formed. The reaction mixture was diluted with chloroform, treated with ice-water and the layers were separated. The organic layer was washed with water, saturated aqueous sodium bicarbonate solution, dried (sodium sulfate), filtered and the solvents were removed to give 204 mg of **15** (84%) as a white solid after lyophilization from benzene: R_f 0.12 (4:4:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ -45° (*c* 1, chloroform); ^{1}H NMR δ 7.40-7.30 (m, 5H, aromatic), 5.56 (d, 1H, J_d = 10.0 Hz, NH or NH'), 5.44 (td, 1H, $J_t \approx$ 11.5 Hz, $J_{4,3e}$ = 5.0 Hz, H-4), 5.41 (td, 1H, $J_t \approx$ 11.5 Hz, $J_{4',3'e}$ = 5.5 Hz, H-4'), 5.35 (d, 1H, J_d = 12.5 Hz, C$\underline{H}_2$C$_6$H$_5$), 5.34 (dd, 1H, $J_{7',8'}$ = 9.0 Hz, $J_{7',6'}$ = 2.0 Hz, H-7'), 5.30 (d, 1H, J_d = 10.0 Hz, NH or NH'), 5.27 (d, 1H, J_d = 12.5 Hz, C$\underline{H}_2$C$_6$H$_5$), 5.26 (dd, 1H, $J_{7,8}$ = 8.0 Hz, $J_{7,6}$ = 2.0 Hz, H-7), 5.14 (ddd, 1H, $J_{8',7'}$ = 9.0 Hz, $J_{8',9'b}$ = 5.0 Hz, $J_{8',9'a}$ = 2.5 Hz, H-8'), 4.54 (dd, 1H, $J_{9a,9b}$ = 12.0 Hz, $J_{9a,8}$ = 10.5 Hz, H-9a), 4.47 (dd, 1H, J_d = 10.5 Hz, J_d = 2.0 Hz, H-6 or H-6'), 4.36 (dd, 1H, $J_{9b,9a}$ = 12.0 Hz, $J_{9b,8}$ = 3.0 Hz, H-9b), 4.29 (dd, 1H, $J_{9'a,9'b}$ = 12.5 Hz, $J_{9'a,8'}$ = 2.5 Hz, H-9'a), 4.24 (m, 1H), 4.23 (q, 1H, J_q = 10.0 Hz, H-5'), 4.18 (m, 1H), 4.02 (dd, 1H, $J_{9'b,9'a}$ = 12.5 Hz, $J_{9'b,8'}$ = 5.0 Hz, H-9'b), 3.78 (dd, 1H, J_d =10.5 Hz, J_d = 2.0 Hz, H-6 or H-6'), 2.82 (dd, 1H, $J_{3e,3a}$ =13.5 Hz, $J_{3e,4}$ = 5.0 Hz, H-3e), 2.35 (dd, 1H,$J_{3'e,3'a}$ =13.5 Hz, $J_{3'e,4'}$ = 5.5 Hz, H-3'e), 2.30 (dd,1H, $J_{3a,3e}$ =13.5 Hz, $J_{3a,4}$ =11.5 Hz, H-3a), 2.16 (s, 3H, Ac), 2.14 (s, 3H, Ac),

2.06 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.95 (s, 3H, Ac), 1.89 (s, 3H, Ac), 1.86 (dd,1H, $J_{3'a,3'e}$ =13.5 Hz, $J_{3'a,4'}$ =11.5 Hz, H-3'a).

***O*-(5-Acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-5-acetamido-2,4,7,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonic acid (16b) and α-anomer (16a).** A suspension of **12** (750 mg, 1.18 mmol) and acetic anhydride (20 mL) was cooled to -5 °C and a solution of *p*-toluenesulfonic acid monohydrate (580 mg, 3.05 mmol) in acetic anhydride (5 mL) was added using a syringe. The reaction was stirred at this temperature for 150 h after which time pyridine (25 mL) was added and stirring was continued at -5 °C for an additional 18 h. The solvents were removed and the residue was coevaporated with toluene. Flash chromatography over silica gel (30 g) using 95:15:5 ethyl acetate-methanol-water gave a crude mixture of **16a** and **16b** which was used directly in the next step without further purification or characterization.

***O*-(Benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→8)-benzyl 5-acetamido-2,4,7,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonate (17b) and α-anomer (17a).** The crude mixture containg **16b** and **16a** was dissolved in *N*,*N*-dimethylformamide (24 mL) containing anhydrous potassium fluoride (1.16 g, 20 mmol) and benzyl bromide (3.42 g, 20 mmol) and the mixture was stirred at room temperature for 48 h. To the reaction mixture was added excess acetic acid to destroy the remaining benzyl bromide. The solvents were removed and the residue was then taken up in chloroform, washed with saturated aqueous sodium bicarbonate solution (2×), dried (sodium sulfate), filtered and the solvents were removed. Column chromatography over silica gel (35 g) using first hexane-ethyl acetate-ethanol 10:10:1 then gradiently switching to 4:4:1 gave first some material (possibly **17a**), followed by 550 mg (42%) of β-acetate **17b** and finally 157 mg of lactone **14b** (14%). The β-acetate **17b** was obtained as a white solid after lyophilization from benzene: R_f 0.19 (5:5:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ +4.8° (*c* 1, chloroform); ^{1}H NMR δ 7.38-7.30 (m, 10H, aromatic), 6.08 (d, 1H, $J_{NH,5}$ = 9.5 Hz, NH),* 5.42 (ddd, 1H, $J_{8',7'}$ = 9.5 Hz, $J_{8',9'b}$ = 5.5 Hz, $J_{8',9'a}$ = 2.5 Hz, H-8'),* 5.35 (m, 1H, H-4),* 5.33 (d, 1H, J_d = 12.0 Hz, C$\underline{H}_2$C$_6$H$_5$), 5.28 (t, 1H, J_t = 2.5 Hz, H-7),* 5.27 (dd,1H, $J_{7',8'}$ = 9.5 Hz, $J_{7',6'}$ = 2.0 Hz, H-7'),* 5.22 (d, 1H, J_d = 12.0 Hz, C$\underline{H}_2$C$_6$H$_5$), 5.19 (d, 1H, J_d = 12.0 Hz, C$\underline{H}_2$C$_6$H$_5$), 5.11

(d, 1H, J_d = 12.0 Hz, $C\underline{H}_2C_6H_5$), 5.01 (d, 1H, $J_{NH',5}$ = 10.0 Hz, NH'),* 4.85 (ddd, 1H, $J_{4',3'a}$ = 13.0 Hz, $J_{4',5'}$ = 10.5 Hz, $J_{4',3'e}$ = 4.5 Hz, H-4'),* 4.69 (dt, 1H, $J_{8,9}$ = 9.5 Hz, J_t = 2.5 Hz, H-8),* 4.58 (dd, 1H, $J_{9a,9b}$ = 12.5 Hz, $J_{9a,8}$ = 2.5 Hz, H-9a),* 4.23 (dd, 1H, $J_{9'a,9'b}$ = 12.5 Hz, $J_{9'a,8'}$ = 2.5 Hz, H-9'a),* 4.18 (dd, 1H, $J_{6,5}$ = 10.5 Hz, $J_{6,7}$ = 2.0 Hz, H-6),* 4.12 (dd, 1 H, $J_{9b,9a}$ = 12.5 Hz, $J_{9b,8}$ = 9.0 Hz, H-9b),* 4.08 (dd, 1H, $J_{9'b,9'a}$ = 12.5 Hz, $J_{9'b,8'}$ = 5.5 Hz, H-9'b),* 4.02 (q, 1H, J_q = 10.5 Hz, H-5'),* 3.95 (q, 1H, J_q = 10.5 Hz, H-5),* 3.66 (dd, 1H, $J_{6',5'}$ = 10.5 Hz, $J_{6',7'}$ = 2.5 Hz, H-6'),* 2.67 (dd, 1H, $J_{3'e,3'a}$ = 13.0 Hz, $J_{3'e,4'}$ = 4.5 Hz, H-3'e),* 2.53 (dd, 1H, $J_{3e,3a}$ = 13.0 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e),* 2.19 (s, 3H, Ac), 2.18 (s, 3H, Ac), 2.13 (s, 3H, Ac), 2.07 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.0 (s, 3H, Ac), 1.98 (t, 1H, J_t = 13.0 Hz, H-3a),* 1.97 (s, 3H, Ac), 1.87 (s, 3H, Ac), 1.79 (t, 1H, J_t = 13.0 Hz, H-3'a).* *These assignments were confirmed by decoupling experiments, and in summary, decoupling of the doublet centered at δ 6.08 (NH) caused the collapse of the quartet at δ 3.95 (H-5) while irradiation of the doublet at δ 5.01 (NH') likewise affected the quartet at δ 4.02 (H-5'); simultaneous decoupling of both of the doublet of doublets at δ 5.28 (H-7), and δ 5.27 (H-7') collapsed signal pairs at δ 4.69 (H-8), 4.18 (H-6) and δ 5.42 (H-8'), 3.66 (H-6') respectively; irradiation of the double doublet of doublets at δ 5.42 (H-8') collapsed signals at δ 5.27 (H-7'), 4.23 (H-9'a), and 4.08 (H-9'b); decoupling of the multiplet located at δ 5.35 (H-4) perturbed the quartet at δ 3.95 (H-5), the doublet of doublets at δ 2.53(H-3e), and the triplet at δ 1.97 (H-3a); decoupling of the double doublet of doublets at δ 4.85 (H-4') simultaneuosly affected signals at δ 4.02 (H-5'), 2.67 (H-3'e), and 1.79 (H-3'a); irradiation of the double doublet of doublets at δ 4.69 (H-8) indicated that this proton was coupled to protons resonating at δ 5.28 (H-7), 4.58 (H-9a), and 4.12 (H-9b); decoupling of the doublet of doublets at δ 4.58 (H-9a) confirmed couplings to resonances at δ 4.69 (H-8) and the corresponding geminal proton δ 4.12 (H-9b) while decoupling of the doublet of doublets at δ 4.23 (H-9'a) likewise confirmed couplings to resonances at δ 5.42 (H-8') and the corresponding geminal proton δ 4.08 (H-9'b); irradiation of the quartet at δ 3.95 (H-5) simutaneously perturbed signals at δ 6.08 (NH), 5.35 (H-4), and 4.18 (H-6); irradiation of the doublet of doublets at δ 3.66 (H-6') collapsed signals at δ 5.27 (H-7') and 4.02 (H-5'); decoupling of the doublet of doublets situated at δ 2.67 collapsed signals at δ 4.85 (H-4') and 1.79 (H-3'a); irradiation of the doublet of doublets at δ 2.53 (H-3e) revealed that this proton was coupled to protons at δ 5.35 (H-4) and 1.98 (H-

3a); and, decoupling of the triplet at δ 1.79 (H-3'a) resulted in simultaneous collapse of signals at δ 4.85 (H-4') and 2.67 (H-3'e).

O-(Benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→8)-benzyl 5-acetamido-4,7,9-tri-*O*-acetyl-2-chloro-2,3,5-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonate (18). Compound **17b** (224 mg, 0.2 mmol) was dissolved in chloroform (5 mL), cooled to 5 °C, and acetyl chloride (5 mL) was added. After 10 min, water (160 μL) was added and the mixture was then stirred at room temperature for 18 h. After this time, the reaction mixture was diluted with chloroform (15 mL), cooled to 5 °C and ice added portionwise until the bubbling subsided. The layers were separated and the organic layer was washed with water, saturated aqueous sodium bicarbonate solution, water, dried (sodium sulfate), filtered and the solvents were removed to give 216 mg (quantitative) of **18** after freeze-drying from benzene: R_f 0.20 (5:5:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ -14° (*c* 1, chloroform); ^{1}H NMR δ 7.40-7.30 (m, 10H, aromatic), 6.60 (d, 1H, $J_{NH,H5}$ = 10.5 Hz, NH), 5.41 (ddd, 1H, $J_{4,3a}$ = 11.0 Hz, $J_{4,5}$ = 10.5 Hz, $J_{4,3e}$ = 4.5 Hz, H-4), 5.38 (t, 1H, J_t = 2.0 Hz, H-7), 5.33 (m, 1H, H-8'), 5.33 (d, 1H, J_d = 12.0 Hz, $C\underline{H}_2C_6H_5$), 5.30 (d,1H, J_d = 12.0 Hz, $C\underline{H}_2C_6H_5$), 5.28 (dd,1H, $J_{7',6'}$ = ?, $J_{7',8'}$ = 2.0 Hz, H-7'), 5.27 (d, 1H, J_d = 12.0 Hz, $C\underline{H}_2C_6H_5$), 5.18 (d, 1H, J_d = 12.0 Hz, $C\underline{H}_2C_6H_5$), 5.02 (d, 1H, $J_{NH',5'}$ = 10.0 Hz, NH'), 4.87 (ddd, 1H, $J_{4',3a}$ = 12.5 Hz, $J_{4',5'}$ = 10.5 Hz, $J_{4',3e'}$ = 4.5 Hz, H-4'), 4.65 (dt, 1H, $J_{8,9}$ = 8.0 Hz, J_t = 2.0 Hz, H-8), 4.50 (dd, 1H, $J_{6,5}$ = 10.5 Hz, $J_{6,7}$ = 2.0 Hz, H-6), 4.45 (dd, 1H, J_d = 12.5 Hz, J_d = 2.5 Hz, H-9 or H-9'), 4.36 (dd, 1H, J_d =12.5 Hz, J_d = 2.5 Hz, H-9 or H-9'), 4.13 (q, 1H, J_q = 10.5 Hz, H-5 or H-5'), 4.10 (m, 2 H, H-9 and/or H-9'), 4.05 (q, 1H, J_q = 10.5 Hz, H-5 or H-5'), 3.80 (dd, 1H, $J_{6',5'}$ = 10.5 Hz, $J_{6',7'}$ = 2.0 Hz, H-6'), 2.85 (dd, 1H, $J_{3e,3a}$ = 13.5 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e), 2.68 (dd, 1H, $J_{3'e,3'a}$ = 12.5 Hz, $J_{3'e,4'}$ = 4.5 Hz, H-3'e), 2.22 (dd, 1H, $J_{3a,3e}$ = 13.5 Hz, $J_{3a,4}$ = 11.0 Hz, H-3a), 2.15 (s,3H, Ac), 2.10 (s, 3H, Ac), 2.08 (s, 3H, Ac), (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.97 (s, 6H, 2 × Ac), 1.95 (s, 3H, Ac), 1.87 (s, 3H, Ac). The position of H-3'a was obscured by acetate signals.

Anal. Calcd for $C_{52}H_{64}O_{25}N_2$: C, 55.91; H, 5.77; N, 2.51. Found; C, 56.15; H, 5.95; N, 2.60.

Methyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→8)-benzyl 5-acetamido-4,7,9-tri-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate (19a) and β-OCH_3 isomer (19b). A solution of methanol (13 mg, 0.41 mmol), Drierite ® (100 mg), 1,1,3,3-tetramethylurea (19 mg, 0.16 mmol), and anhydrous tetrahydrofuran (400 μL) was stirred at room temperature for 2 h. To the mixture was added chloride **18** (40 mg, 0.037 mmol) and the mixture was cooled to -30 °C. A solution of silver perchlorate (15 mg, 0.073 mmol) in 2:1 tetrahydrofuran-toluene (300 μL) was added dropwise and the reaction was stirred at this temperature for 15 min, then slowly warmed to 0 °C and stirred overnight. The reaction mixture was diluted with chloroform, filtered through Celite®, then washed with saturated aqueous sodium bicarbonate, dried (sodium sulfate), filtered and the solvents were removed. Column chromatography over silica gel (10 g) using first chloroform, then 100:1 chloroform-methanol gave 22 mg (55%) of **19a** followed by 8 mg (20%) of **19b.** Compound **19a** was obtained as a white solid after lyophilization from benzene: R_f 0.39 (19:1 chloroform-methanol); ^1H NMR δ 7.4-7.30 (m, 10H, aromatic), 6.42 (d, 1H, $J_{NH,5}$ = 9.5 Hz, NH), 5.48 (dd, 1H, J_d = 9.5, J_d = 5.0, J_d = 3.0 Hz), 5.33 (d, 1H, J_d = 12.5 Hz, $C\underline{H}_2C_6H_5$), 5.3 (m, 2H), 5.28 (s, 2H, $C\underline{H}_2C_6H_5$), 5.18 (d, 1H, J_d = 12.5 Hz, $C\underline{H}_2C_6H_5$), 5.12 (d, 1H, $J_{NH',5'}$ = 10.0 Hz, NH'), 4.97 (ddd, 1H, J_d = 13.0 Hz, J_d = 10.0 Hz, J_d = 4.5 Hz), 4.93 (m, 1H), 4.84 (ddd, 1 H, J_d = 13.0 Hz, J_d = 10.5 Hz, J_d = 4.5 Hz, H-4 or H-4'), 4.61 (dd, 1H, J_d = 12.0 Hz, J_d = 2.5 Hz), 4.24 (m, 3H), 4.15-4.00 (m, 3H), 3.84 (dd, 1H, J_d = 10.5 Hz, J_d = 2.0 Hz), 3.4 (s, 3H, OCH_3), 2.71 (dd, 1H, J_d = 13.0 Hz, J_d = 4.5 Hz, H-3e or H-3'e), 2.70 (dd, 1H, J_d = 13.0 Hz, J_d = 4.5 Hz, H-3e or H-3'e), 2.18 (s, 3H, Ac), 2.15 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.07 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.01 (s, 3H, Ac), 1.93 (s, 3H, Ac), 1.92 (t, 1H, J_t = 13.0 Hz H-3a or H-3'a), 1.90 (s, 3H, Ac), 1.89 (t, 1 H, J_t = 13.0 Hz, H-3a or H-3'a). Similarily, lyophilization of **19b** from benzene gave a white solid: R_f = 0.38 (19:1 chloroform-methanol); ^1H NMR δ 7.40-7.30 (m, 10H, aromatic), 6.18 (bd, 1 H, J_d = 9.5 Hz, NH or NH'), 5.37 (ddd, 1H, J_d = 9.5 Hz, J_d = 6.0 Hz, J_d = 2.5 Hz), 5.35-5.25 (m, 5H), 5.22 (d, 1H, J_d = 12.5 Hz, $C\underline{H}_2C_6H_5$), 5.15 (d, 1H, J_d = 12.5 Hz, $C\underline{H}_2C_6H_5$), 5.1 (d, 1H, J_d = 10.0 Hz, NH or NH'), 4.9 (dd, 1H, J_d = 12.0 Hz, J_d = 2.5 Hz), 4.82 (m, 2H), 4.26 (dd, 1H, J_d = 12.5 Hz, J_d = 2.5 Hz), 4.05 (m, 4H), 3.72 (dd, 1H, J_d = 10.5, J_d = 1.5 Hz), 3.48 (s, 3H, OCH_3), 2.7 (dd, 1H, J_d = 13.0 Hz, J_d = 4.5 Hz, H-3e or H-3'e), 2.47 (dd, 1H, J_d = 13.0 Hz, J_d = 5.0 Hz, H-3e or H-3'e), 2.19 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.09 (s,

3H, Ac), 2.07 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.01 (s, 3H, Ac), 1.90 (s, 3 H, Ac), 1.87 (s, 3H, Ac), 1.84 (t, 1H, J_t = 13.0 Hz, H-3a or H-3'a), 1.79 (t, 1H, J_t = 13.0 Hz, H-3a or H-3'a).

Methyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid (20a). A mixture of **19a** (18 mg) in 3:1 toluene-methanol (4 mL) and 5% palladium-on-carbon (50 mg, 55% moisture content) was hydrogenated at 1 atm, room temperature for 2 h. The mixture was filtered through Celite® and the solvents were removed. The resulting material was dissolved in methanol (3 mL) containing sodium metal (2.5 mg) and the mixture was stirred at room temperature overnight. The reaction mixture was neutralized with acetic acid and the solvents were removed. Column chromatography over silica gel (5 g) using 8:1:1 isopropanol-ammonia-water gave 11 mg of **20a**, presumably as the di-ammonium salt: R_f 0.29 (7:1:2 isopropanol-ammonia-water); ^{1}H NMR (D_2O, HOD) δ 4.2 (m, 1H), 4.18 (dd, 1H, J_d = 12.0 Hz, J_d = 3.5 Hz), 3.98-3.88 (m, 6H), 3.75-3.50 (m, 6H), 3.25 (s, 3H, OCH_3), 2.78 (dd, 1H, J_d =12.0 Hz, J_d = 4.5 Hz, H-3e or H-3'e), 2.61 (dd, H, J_d = 12.5 Hz, J_d = 4.5 Hz, H-3e or H-3'e), 2.17 (s, 3H, Ac), 2.12 (s, 3H, Ac), 1.73 (t, 1H, J_t = 12.0 Hz, H-3a or H-3'a), 1.58 (t, 1H, J_t = 12.5 Hz, H-3a or H-3'a).

Methyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-5-acetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosylonic acid (20b). A mixture of **19b** (6 mg) in 1:1 ethyl acetate-methanol (1mL), and 5% palladium-on-carbon (30 mg, 55% moisture content), was hydrogenated at 1 atm, and room temperature for 1 h. The reaction mixture was then filtered through Celite® and the solvents were removed. The residue was dissolved in methanol (1mL) containing sodium metal (1 mg) and the reaction was stirred at room temperature for 2 h. The reaction mixture was then neutralized with acetic acid and the solvents were removed. Column chromatography over silica gel (1.5 g) using 8:1:1 isopropanol-ammonia-water as eluant gave 2 mg of **20b,** presumably as the di-ammonium salt: R_f 0.21 (7:1:2 isopropanol-ammonia-water): ^{1}H NMR (D_2O, HOD) δ 4.17 (m, 1H), 4.07 (dd, 1H, J_d = 12.0 Hz, J_d = 2.5 Hz), 3.95 (ddd, 1H, J_d =13.0 Hz, J_d = 10.5 Hz, J_d = 5.0 Hz), 3.86-3.80 (m, 5H), 3.75- .55 (m, 6H), 3.20 (s, 3H, OCH_3),

2.76 (dd, 1H, J_d = 12.5 Hz, J_d = 4.5 Hz, H-3e or H-3'e), 2.30 (dd, 1H, J_d = 13.0 Hz, J_d = 4.75 Hz, H-3e or H-3'e), 2.05 (s, 3H, Ac), 2.0 (s, 3H, Ac).

β-D-Galactose Pentaacetate (22). A suspension of galactose **21** (45 g, 0.25 mol), sodium acetate (30.73g, 0.375 mol) and acetic anhydride (350 mL, 3.75 mol) was stirred at reflux for 1 h. After cooling to room temperature, the excess acetic anhydride was quenched by the dropwise addition of water. The mixture was then transferred to separatory funnel with the aid of dichloromethane and washed successively with water (3×) and saturated aqueous sodium bicarbonate solution, then dried (sodium sulfate), filtered and the solvents were removed. The resulting solid was recrystallized from ethyl acetate-hexane to provide **22**: $[\alpha]_D$ +24° (*c* 1, chloroform, lit.[208] 23°); ^{1}H NMR δ 5.7 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 5.43 (dd, 1H, $J_{4,3}$ = 3.5 Hz, $J_{4,5}$ = 1.0 Hz, H-4), 5.35 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2), 5.08 (dd, 1H, $J_{3,2}$ = 10.5 Hz, $J_{3,4}$ = 3.5 Hz, H-3), 4.16 (m, 2H, H-6a and H-6b), 4.08 (m, 1H, H-5), 2.18 (s, 3H, Ac), 2.16 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.00 (s, 3H, Ac).

Allyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (23).[170] A solution of β-D-galactose pentaacetate **22**, (3.9 g, 10 mmol) in anhydrous dichloromethane (80 mL) was cooled to -10 °C, then a solution of tin (IV) chloride (1.3 mL, 11 mmol) in dichloromethane (10 mL) was added, and the mixture was stirred at this temperature for 10 min. To the reaction mixture was added a solution of allyl alcohol (890 µL, 13 mmol) in dichloromethane (10 mL), then the mixture was stirred at -10 °C for 3 h and finally at room temperature overnight. The reaction mixture was then diluted with dichloromethane, washed with water, saturated sodium bicarbonate, dried (sodium sulfate), filtered and the solvents were removed. Column chromatography over silica gel, using 4:1 hexane-ethyl acetate as eluant gave 1.74 g (45%) of **23** as an oil: R_f 0.64 (2:1 hexane-ethyl acetate); $[\alpha]_D$ -17.7° (*c* 1, chloroform); ^{1}H NMR δ 5.90 (m, 1H, allyl CH), 5.41 (dd, 1H, $J_{4,3}$ = 3.5 Hz, $J_{4,5}$ = 1.5 Hz, H-4), 5.30 (dq, 1H, J_d = 17.5 Hz, J_q = 1.5 Hz, allyl CH), 5.24 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2), 5.22 (dq, 1H, J_d = 10.5 Hz, J_q = 1.5 Hz, allyl CH), 5.03 (dd, 1H, $J_{3,2}$ = 10.5 Hz, $J_{3,4}$ = 3.5 Hz, H-3), 4.53 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 4.37 (m, 1H, allyl CH), 4.16 (dd, 1H, $J_{6,6}$ = 12.0 Hz, $J_{6,5}$ = 7.0 Hz, H-6), 4.13 (dd, 1H, $J_{6,6}$ = 12.0 Hz, $J_{6,5}$ = 7.0 Hz, H-6), 4.10 (m, 1H, allyl CH), 3.92 (dt, 1H, $J_{5,6}$ = 7.0 Hz, $J_{5,4}$ = 1.5 Hz, H-5), 2.16 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.05 (s, 3H, Ac), 1.99 (s, 3H, Ac).

Anal. Calcd for $C_{17}H_{24}O_{10}$: C, 52.57; H, 6.23. Found: C, 52.58; H, 6.08.

Propyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (24). A mixture of allyl glycoside **24** (1.14 g, 2.94 mmol) and 5% palladium-on-carbon (2 g, 55% moisture content) in methanol (30 mL) was hydrogenated at 1 atm, room temperature for 2 h. The mixture was filtered through Celite® and the solvents were removed to give 1.03 g (90%) of **24** as an oil: R_f 0.66 (2:1 hexane-ethyl acetate); $[\alpha]_D$ -14.6° (*c* 1, chloroform, lit.[171] -13.5°).

Anal. Calcd for $C_{17}H_{26}O_{10}$: C, 52.30; H, 6.71. Found: C, 52.09; H, 6.61.

Propyl β-D-galactopyranoside (25). Crude **24** (1.03 g, 2.6 mmol) was dissolved in methanol (20 mL) containing sodium (90 mg, 3.9 mmol) and the mixture was stirred at room temperature overnight. The mixture was then deionized with Amberlite® IR 120 (H^+) resin (2 g), filtered, and the solvents were removed to give 586 mg (quantitative) of **25**: R_f 0.2 (6:1 chloroform-methanol); mp 113-114 °C; $[\alpha]_D$ -21° (*c* 1, methanol); lit.[172]: mp 110-112 °C; $[\alpha]_D$ -20.3° (*c* 1, methanol).

Anal. Calcd for $C_9H_{18}O_6$: C, 48.64; H, 8.16. Found: C, 47.83; H, 8.27.

Propyl 4,6-*O*-benzylidene-β-D-galactopyranoside (26). The crude tetrol **26** (311 mg, 1.4 mmol) was dissolved in 2:1 acetonitrile-*N,N*-dimethylformamide (12 mL) containing α,α'-dimethoxytoluene (462 mg, 2.8 mmol) and *p*-toluenesulfonic acid monohydrate (18 mg, 0.09 mmol). After stirring at room temperature for 16 h, the reaction mixture was neutralized with excess triethylamine and the solvents were removed. The residue was dissolved in chloroform, washed with saturated aqueous sodium bicarbonate solution, dried (sodium sulfate), filtered and concentrated. Column chromatography over silica gel, using chloroform then 100:1 chloroform-methanol as eluant, gave 263 mg (61%) of **26** as an amorphous solid: R_f 0.77 (6:1 chloroform-methanol); mp 162-164 °C; $[\alpha]_D$ -41.1° (*c* 1, chloroform); ^1H NMR δ 7.55-7.30 (m, 5H, aromatic), 5.55 (s, 1H, benzylidene), 4.34 (dd, 1H, $J_{6,6}$ = 12.5 Hz, $J_{6,5}$ = 1.5 Hz, H-6), 4.21 (dd, 1H, $J_{4,3}$ = 3.5 Hz, $J_{4,5}$ = 1.0 Hz, H-4), 4.08 (dd, 1H, $J_{6,6}$ = 12.5 Hz, $J_{6,5}$ = 1.75 Hz, H-6), 3.94 (dt, 1H, J_d = 9.5 Hz, J_t = 6.5 Hz, OCH_2), 3.80-3.70 (m, 2H), 3.48 (m, 1H), 3.47 (dt, 1H, J_d = 9.5 Hz, J_t = 6.5 Hz, OCH_2), 2.60 (bm, OH), 1.67 (m, 2H, CH_2), 0.95 (t, 3H, J_t = 7.5 Hz, CH_3).

Molecular weight for $C_{16}H_{22}O_6$: calcd 310.34, found (CI): *m/z* 310.9.

Anal. Calcd for $C_{16}H_{22}O_6$: C, 61.92; H, 7.15. Found: C, 61.40; H, 7.10.

Propyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranoside (27). A small portion of **26** (28 mg) was acetylated in 2:1 pyridine-acetic anhydride and purified by column chromatography over silica gel using 3:1 hexane-ethyl acetate as eluant to provide **27** as an amorphous solid: mp 92-93 °C; $[\alpha]_D$ +61.1° (*c* 1, chloroform); ^{1}H NMR δ 7.55-7.35 (m, 5H, aromatic), 5.50 (s, 1H, benzylidene), 5.39 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2), 4.96 (dd, 1H, $J_{3,2}$ = 10.5 Hz, $J_{3,4}$ = 3.5 Hz, H-3), 4.49 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 4.38 (dd, 1H, $J_{4,3}$ = 3.5 Hz, $J_{4,5}$ = 0.5 Hz, H-4), 4.33 (dd, 1H, $J_{6,6}$ = 12.5 Hz, $J_{6,5}$ = 1.5 Hz, H-6), 4.06 (dd, 1H, $J_{6,6}$ = 12.5 Hz, $J_{6,5}$ = 1.75 Hz, H-6), 3.90 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 3.51 (q, 1H, J_q = 1.5 Hz, H-5), 3.44 (dt, 1H, J_d = 9.5 Hz, J_t = 6.5 Hz, OCH_2), 2.08 (s, 3H, Ac), 2.06 (s, 3H, Ac), 1.60 (m, 2H, CH_2), 0.91 (t, 3H, J_t = 7.5 Hz, CH_3).

Anal. Calcd for $C_{20}H_{26}O_8$: C, 60.90; H, 6.65. Found: C, 60.62; H, 6.65.

Propyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→8)-*O*-(benzyl 5-acetamido-4,7,9-tri-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-2-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranoside (31). To a mixture of diol **26** (50 mg, 0.16 mmol), silver trifluoromethanesulfonate (50 mg, 0.19 mmol), 1,1,3,3-tetramethylurea (24 mg, 0.2 mmol), Drierite® (250 mg) and dry tetrahydrofuran (1.5 mL) at -30 °C, was added a solution of chloride **18** (85 mg, 0.079 mmol) in toluene (200 μL). The solution was stirred at this temperature for 30 min, then at -5 °C overnight. The reaction mixture was then diluted with chloroform, filtered through Celite® and the combined organic filtrate washed with saturated aqueous sodium bicarbonate, dried (sodium sulfate), filtered and the solvents were removed. Column chromatography over silica gel, using 15:10:1 hexane-ethyl acetate-ethanol followed by rechromatography with 115:1 chloroform-methanol, gave 2,3-dehydro compound **28**, then hydrolysis product **29**, and finally compound **30** as crude mixture which was directly acetylated (2:1 pyridine-acetic anhydride, 3 mL). The product was chromatographed over silica gel using 115:1 chloroform-methanol to give **31** (14 mg, 14% based on chloride >85% as estimated by ^{1}H NMR) as a powder after

lyophilization from benzene: R_f 0.33 (19:1 chloroform-methanol); ^1H NMR δ 7.45-7.25 (m, 15H, aromatic), 6.25 (d, 1H, $J_{NH',5'}$ = 10.0 Hz, NH'),* 5.47 (ddd, 1H, $J_{8'',7''}$ = 10.0 Hz, $J_{8'',9''b}$ = 5.0 Hz, $J_{8'',9''a}$ = 3.0 Hz, H-8''),* 5.37 (bs, 1H, H-7'), 5.33 (d, 1H, J_d = 12.0 Hz, $C\underline{H}_2C_6H_5$), 5.31 (dd, 1H, $J_{7'',8''}$ = 10.0 Hz, $J_{7'',6''}$ = 1.5 Hz, H-7''),* 5.24 (s, 2H, $C\underline{H}_2C_6H_5$), 5.23 (dd, 1H, $J_{2,3}$ = 10.0 Hz, $J_{2,1}$ = 8.0 Hz, H-2),* 5.19 (d, 1H, J_d = 12.0 Hz, $C\underline{H}_2C_6H_5$), 5.10 (d, 1H, $J_{NH'',5''}$ = 10.5 Hz, NH''), * 5.10 (dd, 1H, $J_{9'a,9'b}$ = 12.0 Hz, $J_{9'a,8'}$ = 2.0 Hz, H-9'a),* 4.99 (bm, 1H, H-4'),* 4.95 (dd, 1H, J_d = 9.5 Hz, $J_{8',9'a}$ = 2.0 Hz, H-8'),* 4.93 (s, 1H, benzylidene), 4.87 (ddd, 1H, $J_{4'',3''a}$ = 13.5 Hz, $J_{4'',5''}$ = 10.5 Hz, $J_{4'',3''e}$ = 4.5 Hz, H-4''),* 4.55 (dd, 1H, $J_{3,2}$ = 10.0 Hz, $J_{3,4}$ = 3.5 Hz, H-3),* 4.41 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1),* 4.23 (dd, 1H,$J_{9''a,9''b}$ = 12.5 Hz, $J_{9''a,8''}$ = 5.0 Hz, H-9''a),* 4.19 (dd, 1H, $J_{9''b,9''a}$ = 12.5 Hz, $J_{9''b,8''}$ = 3.0 Hz, H-9''b),* 4.18 (dd, 1H, $J_{6a,6b}$ = 12.5, $J_{6a,5}$ < 1.0 Hz, H-6a), 4.09 (q, 1H, J_q = 10.5 Hz, H-5''),* 4.04 (dd, 1H, $J_{9'b,9'a}$ = 12.0 Hz, $J_{9'b,8'}$ = 9.5 Hz, H-9'b),* 4.01 (m, 2H, H-5' and H-6'), 3.83 (dd, 1H, $J_{6'',5''}$ = 10.5 Hz, $J_{6'',7''}$ = 1.5 Hz, H-6''),* 3.81 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2),* 3.78 (dd, 1H, $J_{6b,6a}$ = 12.5 Hz, $J_{6b,5}$ < 1.0 Hz, H-6b), 3.60 (d, 1H, $J_{4,3}$ = 3.5 Hz, H-4),* 3.41 (dt, 1H, J_d = 9.5 Hz, J_t = 6.5 Hz, OCH_2),* 3.31 (bs, 1H, H-5), 2.81 (dd, 1H, $J_{3'e,3'a}$ = 13.0 Hz, $J_{3'e,4'}$ = 5.0 Hz, H-3'e),* 2.76 (dd, 1H, $J_{3''e,3''a}$ = 13.0 Hz, $J_{3''e,4''}$ = 4.5 Hz, H-3''e),* 2.18 (s, 3H, Ac), 2.17 (s, 3H, Ac), 2.16 (s, 3H, Ac), 2.13 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.049 (s, 3H, Ac), 2.00 (s, 3H, Ac), 1.93 (s, 3H, Ac), 1.91 (s, 3H, Ac), 1.88 (t, 1H, J_t = 13.0 Hz, H-3''a),* 1.86 (t, 1H, J_t = 13.0 Hz, H-3'a),* 1.55 (m, 2H, propyl CH_2), 0.88 (t, 3H, J_t = 7.5 Hz, CH_3). The assignments for some signals are tentative. *These assignments were confirmed by decoupling experiments, and in summary, decoupling of the doublet centered at δ 6.25 (NH) caused the collapse of the signal at δ 4.01 (H-5'); irradiation of the doublet doublet of doublets at δ 5.47 (H-8'') simultaneously perturbed signals at δ 5.31 (H-7''), 4.23 (H-9''a), and 4.19 (H-9''b); decoupling of the doublet of doublets at δ 5.23 (H-2) caused the collapse of the resonances at δ 4.55 (H-3) and δ 4.41 (H-1); simultaneous irradiation of the NH'' and H-9'a signals at δ 5.01 caused the collapse of sets of signals at δ 4.09 (H-5''), δ 4.95 (H-8') and 4.04 (H-9'b) respectively; simultaneous decoupling of the H-4' and H-8' resonances centered at δ 4.97 collapsed related sets of signals at δ 4.01 (H-5'), 2.81 (H-3'e), and 1.86 (H-3'a), and the δ 5.01 (H-9'a) and 4.04 (H-9'b) pair respectively; irradiation of the doublet doublet of doublets at δ 4.87 (H-4'') caused perturbation in signals at δ 4.09 (H-5''), 2.76 (H-3''e), and 1.88 (H-3''a); meanwhile, decoupling of the doublet of doublets located at δ 4.55 (H-3) caused the collapse

of the doublet of doublets at δ 5.23 (H-2) as well as the doublet located at δ 3.60 (H-4); decoupling of the doublet located at δ 4.41 (H-1) collapsed the doublet of doublets at δ 5.23 (H-2); simultaneous decoupling of the signals for the geminal methylene protons H-9"a and H-9"b at δ 4.20 collapsed the signal at δ 5.47 (H-8"); simultaneous irradiation of the signals for H-5' and H-5" at δ 4.05 collapsed acetamido NH doublets at δ 6.25 and 5.10; decoupling of the resonance centered at δ (H-6") confirmed its coupling to signals at δ 5.31 (H-7") and 4.09 (H-5"); decoupling of the doublet of doublets at δ 2.81 (H-3'e) collapsed signals at δ 4.99 (H-4') and 1.86 (H-3'a) while decoupling of the doublet of doublets at δ 2.76 (H-3"e) collapsed signals at δ 4.87 (H-4") and 1.88 (H-3"a); and finally, decoupling of the propyl CH_2 multiplet at δ collapsed the geminal methylene protons at δ 3.81 and 3.41 along with the methyl triplet at δ 0.88.

Data for 2,3-dehydro di-Neu5Ac **28**: ^{1}H NMR δ 7.40-7.30 (m, 10H, aromatic), 6.61 (d, 1H, J_d = 10.0 Hz, NH or NH'), 5.97 (d, 1H, J_d = 2.5 Hz, H-3), 5.61 (dd, 1H, J_d = 9.5 Hz, J_d = 2.5 Hz), 5.30 (s, 2H, $C\underline{H}_2C_6H_5$), 5.28-5.25 (m, 4H), 5.18 (d, 1H, J_d = 12.5 Hz, $C\underline{H}_2C_6H_5$), 5.11 (d, 1H, J_d = 10.5 Hz, NH or NH'), 4.85 (ddd, 1H, $J_{4',3a'}$ = 13.0 Hz, $J_{4',5'}$ = 9.5 Hz, $J_{4',3a'}$ = 4.5 Hz, H-4'), 4.79 (ddd, 1H, J_d = 8.0 Hz, J_d = 5.5 Hz, J_d = 3.0 Hz), 4.54 (dd, 1H, J_d = 12.0 Hz, J_d = 2.5 Hz), 4.44-4.36 (m, 3H), 4.18 (dd, 1H, J_d = 12.5 Hz, J_d = 8.5 Hz), 4.00 (q, 1H, J_q = 10.5 Hz, H-5 or H-5'), 3.95 (m, 1H), 3.81 (dd, 1H, J_d = 10.5, J_d = 2.5 Hz), 2.68 (dd, 1H, $J_{3e',3a'}$ = 13.0 Hz, $J_{3e',4'}$ = 4.5 Hz, H-3e'), 2.15 (s, 3H, Ac), 2.09 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.01 (s, 3H, Ac), 1.99 (s, 3H, Ac), 1.95 (s, 3H, Ac), 1.85 (s, 3H, Ac). The position of H-3'a was obscured by acetate signals.

Data for 2-OH di-Neu5Ac **29**: ^{1}H NMR δ 7.50-7.30 (m, 10H, aromatic), 5.71 (d, 1H, J_d = 2.5 Hz), 5.54 (d, 1H, J_d = 10.0 Hz, NH or NH'), 5.45 (ddd, 1H, $J_{8',7'}$ = 9.5 Hz, $J_{8',9'}$ = 5.5 Hz, $J_{8',9'}$ = 2.5 Hz, H-8'), 5.34 (d, 1H, J_d = 12.5 Hz, $C\underline{H}_2C_6H_5$), 5.31 (dd, 1H, J_d = 9.5 Hz, J_d = 2.5 Hz), 5.30 (m, 1H), 5.26 (s, 2H, $C\underline{H}_2C_6H_5$), 5.13 (m, 2H), 5.05 (d, 1H, J_d = 10..5 Hz, NH or NH'), 4.86 (ddd, 1H, J_d = 13.0 Hz, J_d = 10.5 Hz, J_d = 4.5 Hz), 4.55 (dt, 1H, J_d = 8.0 Hz, J_t = 2.5 Hz, H-8), 4.38-4.32 (m, 2H), 4.14-4.08 (m, 2H), 4.04 (q, 1H, J_q = 10.5 Hz, H-5 or H-5'), 3.98 (dd, 1H, J_d = 12.5 Hz, J_d = 5.5 Hz), 3.65 (dd, 1H, J_d = 10.5 Hz, J_d = 2.5 Hz), 2.71 (dd, 1H, J_d = 13.5 Hz, J_d = 5.0 Hz, H-3 or H-3'), 2.28 (dd, 1H, J_d = 13.5 Hz, J_d = 5.0 Hz, h-3 or H-3'), 2.21 (s, 3H, Ac), 2.105 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.03 (s, 3H,

Ac), 2.02 (s, 3H, Ac), 2.01 (s, 3H, Ac), 1.99 (s, 3H, Ac), 1.85 (s, 3H, Ac), 1.77 (s, 3H, Ac). The position of H-3a and H-3'a was obscured by acetate signals.

Propyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranoside (32). A solution of **31** (30 mg) and 5% palladium-on-carbon (60 mg, 55% moisture content) in methanol containing a few drops of acetic acid was hydrogenated at 1 atm, room temperature for 18 h. The reaction yielded one major product by TLC (R_f 0.085, 80:15:5 ethyl acetate-methanol-water) along with a minor product (R_f 0.15). The mixture was filtered through Celite® and the solvents were removed. Column chromatography over silica gel (4.5 g), using 100:15:5 ethyl acetate-methanol-water as eluant, gave 18 mg of material which was de-*O*-acetylated using sodium methoxide in methanol, neutralized and finally purified by column chromatography over silica gel (1.5 g) using 8:1:1 isopropanol-ammonia-water as eluant to give 13 mg of **32** (68% overall yield from **30**, presumably as the di-ammonium salt) as a white solid after lyophilization from water: R_f = 0.14 (65:35:8 chloroform-methanol-water) and R_f 0.45 (7:1:2 isopropanol-ammonia-water); ^{1}H NMR (D_2O, HOD = 4.80, 297°K) δ 4.47 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 4.11 (dd, 1H obscured by a second proton, J_d = 10.0 Hz, J_d = 3.0 Hz, possibly H-3), 4.01 (d, 1H, J_d = 3.0 Hz, possibly H-4), 3.95-3.50 (m), 2.81 (dd, 1H, $J_{3e,3a}$ = 13.0 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e' or H-3e"), 2.65 (dd, 1H, $J_{3e,3a}$ = 12.5 Hz, $J_{3e,4}$ = 4.0 Hz, H-3e' or H-3e"), 2.05 (s, 3H, Ac), 2.04 (s, 3H, Ac), 1.85 (t, 1H, J_t = 12.5 Hz, H-3a' or H-3a"), 1.75 (t, 1H, J_t = 13.0 Hz, H-3a' or H-3a"), 1.65 (m, 2H,C$\underline{H}_2$CH$_3$), 0.91 (t, 3H, J_t = 7.5 Hz, CH_3).

Molecular weight for $C_{31}H_{52}O_{22}N_2$: calcd 804.6, found (FAB, positive ion, thioglycerol matrix): *m/z* 805.2 $(M+1)^+$.

β-D-Lactose octaacetate (34). A suspension of lactose **33** (34.2 g, 35 mmol), sodium acetate (16.4 g, 70 mmol) and acetic anhydride (225 mL, 2.36 mol) was heated to 140 °C (oil bath) for 2 h, then allowed to slowly cool to room temperature and finally to 5 °C. The excess acetic anhydride was quenched by the dropwise addition of water (500 mL) and stirring was continued at room temperature overnight. The mixture was then transferred to separatory funnel with the aid of dichloromethane and washed successively with water (3×) and saturated aqueous sodium bicarbonate (2×), dried (sodium sulfate), filtered and

the solvents were removed to give 60.7 g (90%) of **34** which was used directly in the next step without further purification. A small amount of this material was crystallized from ethanol to give a 5:1 β:α mixture: mp 87-89 °C (lit[209] 90 °C); R_f 0.45 (10:10:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ +5.2° (*c* 1, chloroform, lit.[209] $[\alpha]_D$ +4.3°); ^{1}H NMR δ 5.67 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1), 5.35 (dd, 1H, $J_{4',3'}$ = 3.5 Hz, $J_{4',5'}$ = 1.0 Hz, H-4'), 5.25 (dd, 1H, $J_{3,2}$ = 9.5 Hz, $J_{3,4}$ = 9.0 Hz, H-3), 5.12 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'), 5.05 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 8.5 Hz, H-2), 4.95 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.48 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.45 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 9.0 Hz, H-6a), 4.14 (dd, 1H, $J_{6'a,6'b}$ = 11.0 Hz, $J_{6'a,5'}$ = 7.0 Hz, H-6'a), 4.13 (dd, 1H, $J_{6b,6a}$ = 12.0 Hz, $J_{6b,5}$ = 4.5 Hz, H-6b), 4.08 (dd, 1H, $J_{6'b,6'a}$ = 11.0 Hz, $J_{6'b,5'}$ = 7.0 Hz, H-6'b), 3.87 (dt, 1H, J_t = 6.0 Hz, $J_{5',4'}$ = 1.0 Hz, H-5'), 3.82 (t, 1H, J_t = 9.0 Hz, H-4), 3.76 (ddd, 1H, $J_{5,4}$ = 9.0 Hz, $J_{5,6b}$ = 4.5 Hz, $J_{5,6a}$ = 2.0 Hz, H-5), 2.16 (s, 3H, Ac), 2.12 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.07 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.03 (s, 3H, Ac), 1.97 (s, 3H, Ac).

2-(*p*-Nitrophenyl)ethyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (35). A solution of the lactose octaacetate **34** (21 g, 31 mmol) in anhydrous dichloromethane (100 mL) was cooled to O °C under argon and a solution of boron trifluoride etherate (23 mL, 186 mmol) in anhydrous dichloromethane (23 mL) was added dropwise. The resulting reaction mixture was stirred at this temperature for 20 h, after which time a solution of saturated aqueous sodium bicarbonate (200 mL) was added dropwise and stirring continued for 1 h. The layers were separated and the organic layer was washed with saturated aqueous sodium bicarbonate solution, dried (sodium sulfate), filtered, and the solvents were removed. Column chromatography over silica gel (900 g) using 30:10:1 hexane-ethyl acetate-ethanol gave 24.3 g (64%) of **35**: R_f 0.11 (20:10:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ -7.7° (*c* 1.0, chloroform); ^{1}H NMR δ 8.12 (m, 2H, aromatic), 7.35 (m, 2H, aromatic), 5.35 (dd, 1H, $J_{4',3'}$ = 3.5 Hz, $J_{4',5'}$ = 1.0 Hz, H-4'), 5.15 (t, 1H, J_t = 9.0 Hz, H-3), 5.10 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'), 4.95 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.88 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2), 4.51 (dd, 1H, J_d = 12.0 Hz, J_d = 2.0 Hz, H-6 or H-6'), 4.49 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.45 (d, 1H, J_d = 8.0 Hz, H-1' or H-1), 4.18-4.03 (m, 4H, H-6b, H-6'a, H-6'b, OCH_2), 3.87 (dt, 1H, J_t = 7.0 Hz, $J_{5',4'}$ = 1.0 Hz, H-5'), 3.79 (t, 1H, J_t = 9.5 Hz, H-4), 3.68 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.59 (ddd, 1H, $J_{5,4}$ = 9.5

Hz, $J_{5,6b}$ = 4.5 Hz, $J_{5,6a}$ = 2.0 Hz, H-5), 2.99 (m, 2H, CH_2), 2.15 (s, 3H, Ac), 2.11 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.03 (s, 3H, Ac), 1.97 (s, 3H, Ac), 1.89 (s, 3H, Ac).

Anal. Calcd for $C_{34}H_{43}O_{20}N$: C, 51.98; H, 5.52; N, 1.78. Found: C, 51.98; H, 5.48; N, 1.66.

2-(*p*-Nitrophenyl)ethyl *O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (36). A solution of **35** (3.1 g, 3.9 mmol) in methanol containing sodium metal (230 mg, 10 mmol) was stirred at room temperature for 20 h. The reaction mixture was deionized with Amberlite® IR-120 H^+ resin, filtered and the solvents were removed. Column chromatography over silica gel using 65:35 chloroform-methanol gave 1.89 g of **36** as a slightly yellowish solid: R_f 0.36 (65:35:2 chloroform-methanol-water); ^1H NMR (D_2O, HOD = 4.80, 297 °K) δ 8.2 (d, 2H, J_d = 8.5 Hz, aromatic), 7.52 (d, 2H, J_d = 8.5 Hz, aromatic), 4.48 (d, 1H, J_d = 8.0 Hz, H-1'), 4.42 (d, 1H, J_d = 8.0 Hz, H-1), 4.20 (dt, 1H, J_t = 10.5 Hz, J_d = 6.5 Hz, OCH_2), 4.0 (dt, 1H, J_t = 10.5 Hz, J_d = 6.5 Hz, OCH_2), 3.95 (dd, 1H, J_d = 11.0 Hz, J_d = 1.0 Hz, H-6 or H-6'), 3.91 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'), 3.81 - 3.55 (m, 10 H), 3.52 (dd, 1H, J_d = 10.0 Hz, J_d = 8.0 Hz, H-2'), 3.27 (m, 1H, H-2), 3.1 (t, 2H, J_t = 6.5 Hz, CH_2).

Molecular weight for $C_{20}H_{29}O_{13}N$: calcd 491.44, found (FAB, positive ion, Cleland matrix): *m/z* 492 $(M+1)^+$.

2-(*p*-Nitrophenyl)ethyl *O*-(2,6-di-*O*-benzyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (37). A suspension of *p*-nitrophenethyl β-lactose **36** (1.8 g, 3.68 mmol), 2,2-dimethoxypropane (1.04 g, 10 mmol) and *p*-toluenesulphonic acid monohydrate (100 mg) in dry *N,N*-dimethylformamide (20 mL) was stirred at room temperature for 72 hours. Excess triethylamine was added and the solvents were removed under high vacuum. The crude mixture was dissolved in dry *N,N*-dimethylformamide and sodium metal (1g, 42 mmol) was added. The mixture was stirred at room temperature for 45 min and then cooled to 0 °C. Benzyl bromide (4 mL, 33.6 mmol) was added dropwise and the solution was allowed to warm to room temperature and finally stirred overnight. The rection mixture was quenched with excess methanol and the solvents were removed. The resulting foam was dissolved in 85:15 acetic acid-water and heated to 80 °C for 3 h. The solvents

were removed and the resulting dark foam was purified by column chromatography to give 800 mg (23%) of 3',4'-diol followed by 400 mg (11.5%) of presumably the 4',6'-diol. The 3',4'-diol **37** was lyophilized from benzene; R_f 0.05 (3:1 hexane-ethyl acetate); $[\alpha]_D$ +6.9° (*c* 1, chloroform): ^{1}H NMR δ 7.45-7.15 (m, 29 H, aromatic), 4.98 (d, 1H, J_d = 11.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.95 (d, 1H, J_d = 12.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.91(d, 1H, J_d = 11.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.80 (d, 1H, J_d = 12.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.77 (d, 1H, J_d = 11.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.71 (d, 1H, J_d = 11.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.66 (dd, 1H, J_d = 12.0 Hz, J_d = 2.5 Hz), 4.61 (d, 1H, J_d = 12.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.49 (d, 1H, J_d = 7.5 Hz, H-1 or H-1'), 4.47 (d, 1H, J_d = 12.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.45 (d, 1H, J_d = 12.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.44 (d, 1H, J_d = 7.5 Hz, H-1 or H-1'), 4.39 (d, 1H, J_d = 12.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.02 (t, 1H, J_t = 9.0 Hz), 3.94 (bt, 1H, H-4'), 3.83 (dd, 1H, J_d = 11.0 Hz, J_d = 4.0 Hz, H-6 or H-6'), 3.77 (dd, 1H, J_d = 11.0 Hz, J_d = 2.0 Hz, H-6 or H-6'), 3.61 (dd, 1H, J_d = 10.0 Hz, J_d = 6.5 Hz), 3.59 (t, 1H, J_t = 9.0 Hz), 3.52 - 3.32 (m, 9H), 2.50 (d, 1H, J_d = 3.5 Hz, OH-4'), 2.41 (d, 1H, J_d = 4.5 Hz, OH-3').

2-(*p*-Nitrophenyl)ethyl *O*-(3,4-di-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galacto-pyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (38). A solution of **37** (20 mg, 0.02 mmol) in 2:1 pyridine-acetic anhydride (3 mL) was stirred at room temperature overnight. The solvents were removed and the residue was coevaporated with toluene. Column chromatography over silica gel (1.5 g) using 4:1 hexane-ethyl acetate as eluant gave 18 mg (83%) of **38** as a white powder after lyophilization from benzene; R_f 0.25 (3:1 hexane-ethyl acetate); $[\alpha]_D$ -16.6° (*c* 1, chloroform): ^{1}H NMR δ 7.40-7.20 (m, 29 H, aromatic), 5.38 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'),* 4.95 (d, 2H, J_d = 12.0 Hz, 2 × OC$\underline{H}_2$C$_6$H$_5$), 4.91 (d, 1H, J_d = 11.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.86 (dd, 1H, $J_{3',2'}$ = 10.0 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'),* 4.73 (m, 1H), 4.73 (d, 1H, J_d = 11.5 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.70 (d, 1H, J_d = 11.5 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.61 (d, 1H, J_d = 12.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.59 (d, 1H, J_d = 11.5 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.51 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.49 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.46 (d, 1H, J_d = 12.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.42 (d, 1H, J_d = 12.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.20 (d, 1H, J_d = 12.0 Hz, OC$\underline{H}_2$C$_6$H$_5$), 4.02 (t, 1H, J_t = 9.0 Hz), 3.79 (dd, 1H, $J_{6a,6b}$ = 11.0 Hz, $J_{6a,5}$ = 4.0 Hz, H-6a), 3.71 (dd, 1H, $J_{6b,6a}$ = 11.0 Hz, $J_{6b,5}$ = 1.0 Hz, H-6b), 3.58-3.46 (m, 4H) 3.51 (dd, 1H, $J_{2',3'}$ = 10.0 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'),* 3.34 (ddd, 1H, $J_{5,4}$ = 9.5 Hz, $J_{5,6a}$ = 4.0 Hz, $J_{5,6a}$ = 1.0 Hz, H-5), 3.32-3.25 (m, 3H), 1.98 (s, 3H, Ac), 1.92 (s, 3H, Ac). *These assignments were further confirmed by decoupling experiments, thus, decoupling of the doublet of

doublets centered at δ 4.86 (H-3') caused a collapse of the doublet at δ 5.38 (H-4') along with a perturbation of the 5 proton multiplet at δ 3.58-3.46.

Attempt at Sialylation of the 3',4'-Diol 37. A mixture **37** (94 mg, 0.1mmol), silver trifluoromethanesulfonate (57 mg, 0.22 mmol), 1,1,3,3-tetramethylurea (70 mg, 0.6 mmol) and Drierite® (500 mg) in anhydrous tetrahydrofuran (4 mL) was stirred at room temperature for 4 h under argon to ensure dryness. After cooling to -30 °C, a solution of **11** chloride (118 mg, 0.2 mmol) in anhydrous toluene (500 μL) was added dropwise and the mixture was allowed to slowly warm to 0 °C, then stirred overnight at this temperature. The reaction mixture was then filtered through Celite® and the organic layer was washed with saturated aqueous sodium bicarbonate solution, dried (sodium sulfate), filtered, and concentrated. Column chromatography over silica gel (5 g) using 3:2 hexane-ethyl acetate gave recovered diol **37** followed by the unsaturated (major) and 2-OH derivatives of sialic acid **39** and **40** from elimination and hydrolysis respectively.

β-D-Glucose Pentaacetate (42). A suspension of α,β-D-glucose **41** (27g, 0.15 mol), sodium acetate (18.5 g, 0.23 mol) and acetic anhydride (210 mL, 2.25 mol) was stirred at reflux (oil bath 150 °C) for 1 hour, then cooled to room temperature and finally to 5 °C. The excess acetic anhydride was then quenched by the dropwise addition of water. The mixture was transferred to a separatory funnel with the aid of dichloromethane and washed successively with water (3×) and saturated aqueous sodium bicarbonate, then dried (sodium sulfate), filtered and the solvents were removed. The resulting solid was recrystallized from ethanol-ethyl ether to provide 38 g (65%) of **42** as an α/β mixture.

2-(*p*-Nitrophenyl)ethyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (43). A solution of **42** (19.5 g, 50 mmol), recrystallized *p*-nitrophenethyl alcohol (13.4 g, 80 mmol) and anhydrous dichloromethane (25 mL) was cooled to -10 °C under argon and then boron trifluoride etherate (25.2 mL, 200 mmol) was added slowly via an addition funnel. Upon completion of the addition, the reaction mixture was allowed to stir at this temperature for 5 h and then at room temperature overnight. The solution was again cooled to -10 °C then neutralized by the slow addition of saturated aqueous sodium bicarbonate solution. The layers were separated and the organic phase washed with water and concentrated. Since the product **43** was indistinguishable by TLC (R_f 0.54, 20:1

chloroform-methanol) from starting material **42**, this mixture was used directly in the next step without purification or further characterization.

2-(*p*-Nitrophenyl)ethyl β-glucopyranoside (44). De-*O*-acetylation of the crude mixture containing **43** was performed under standard Zemplen conditions using 1 M methanolic sodium methoxide (100 mL). After 18 h, the reaction was deionized with IR 120® (H^+) resin, filtered, and the solvents were removed. Column chromatography over silica gel using 15:1 chloroform-methanol, to gave 6 g of **44** (37% overall yield from **42**): R_f 0.1 (9:1 chloroform-methanol).

2-(*p*-Nitrophenyl)ethyl 4,6-*O*-benzylidene-β-D-glucopyranoside (45). A solution of tetrol **44** (5.6g, 17 mmol) and benzaldehyde dimethyl acetal (7.1 g, 46.8 mmol) in 1:1 acetonitrile-*N,N*-dimethylformamide (100 mL) containing a catalytic amount of *p*-toluenesulphonic acid monohydrate (162 mg, 0.85 mmol) was stirred at room temperature overnight then neutralized with excess triethylamine and evaporated to dryness. Column chromatography over silica gel using 10:5:1 hexane-ethyl acetate-ethanol gave 6.32 g (89%) of **45** as a white solid: R_f 0.34 (10:5:1 hexane-ethyl acetate-ethanol).

2-(*p*-Nitrophenyl)ethyl 2,3-di-*O*-benzyl-4,6-*O*-benzylidene-β-D-glucopyranoside (46). A mixture of diol **45** (4.17 g, 10 mmol), silver oxide (14 g, 60 mmol), and powdered Drierite® (5 g) in dry dichloromethane (80 mL) was cooled to 0 °C and a solution of benzyl bromide (5.13 g, 30 mmol) in dry dichloromethane (16 mL) was then added dropwise. Upon completion of the addition, the reaction mixture was stirred at room temperature in the dark for 72 h. After this time, methanol (1 mL) was added and stirring was continued for an additional 3 h in order to consume the excess benzyl bromide. The reaction mixture was then filtered through Celite®, the pad washed several times with dichloromethane, and the solvents were removed. Column chromatography over silica gel (150 g) using chloroform as eluant gave 5.35 g (90%) of **46**: R_f 0.21 (4:1 hexane-ethyl acetate); $[\alpha]_D$ -19.6° (*c* 0.5, chloroform); ^1H NMR δ 8.16 (d, 2H, J_d = 9.0 Hz, aromatic), 7.5-7.1 (m, 17H, aromatic), 5.57 (s, 1H, benzylidene), 4.90 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 4.77 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 4.68 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.60 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.51 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.34 (dd, 1H, $J_{6a,6b}$ = 10.5 Hz, $J_{6a,5}$ = 5.0 Hz, H-6a), 4.22 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 3.81 (dt, 1H, J_d = 9.5 Hz, J_t =

6.5 Hz, OCH_2), 3.78 (t, 1H, J_t = 10.0 Hz), 3.75 (m, 1H), 3.68 (t, 1H, J_t = 9.5 Hz), 3.77-3.45 (m, 2H), 3.03 (bt, 2H, J_t = 6.5 Hz, $OCH_2C\underline{H}_2$).

Anal. Calcd for $C_{35}H_{35}O_8N$ (597.64): C, 70.34; H,5.90; N,2.34. Found: C, 70.38; H, 5.94; N, 2.37.

2-(*p*-Nitrophenyl)ethyl 2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (47). A rapidly stirred mixture of **46** (5.25 g, 8.8 mmol), sodium cyanoborohydride (5.5 g, 88 mmol) and Drierite® (12 g) in dry tetrahydrofuran (90 mL) containing a crystal of methyl orange was cooled to 0 °C under an inert atmosphere. To the mixture was added dropwise, a solution of ethereal hydrochloric acid until bubbling subsided and the solution remained a red color. The reaction mixture was then diluted with chloroform, filtered through Celite® and the organic solution washed with aqueous saturated sodium bicarbonate solution (2×), brine, dried (sodium sulfate), filtered, and evaporated. Column chromatography over silica gel (300 g) using chloroform then 80:1 chloroform-methanol as eluent gave 3.67 g (70%) of pure **47**: R_f 0.37 (20:10:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ -9.5° (*c* 0.5, chloroform); ^{1}H NMR δ 8.05 (d, 2H, J_d = 9.0 Hz, aromatic), 7.4-7.1 (m, 17H, aromatic), 4.90 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 4.70 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 4.66 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.60 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 4.59 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.55 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 4.41 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.23 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 3.79 (dt, 1H, J_d = 9.5 Hz, J_t = 6.5 Hz, OCH_2), 3.77 (dd, 1H, $J_{6a,6b}$ = 10.5 Hz, $J_{6a,5}$ = 3.5 Hz, H-6a), 3.65 (dd, 1H, $J_{6b,6a}$ = 10.5 Hz, $J_{6b,5}$ = 5.0 Hz, H-6b), 3.58 (dt, 1H, J_t = 9.0 Hz, $J_{4,OH}$ = 2.0 Hz, H-4), 3.46 (ddd, 1H, $J_{5,4}$ = 9.0 Hz, $J_{5,6b}$ = 5.0 Hz, $J_{5,6a}$ = 3.5 Hz, H-5), 3.40 (dd, 1H, $J_{2,3}$ = 9.0 Hz, $J_{2,1}$ = 7.5 Hz, H-2), 3.38 (t, 1H, J_t = 9.0 Hz, H-3), 3.04 (bt, 2H, J_t = 6.5 Hz, $OCH_2C\underline{H}_2$).

Anal. Calcd for $C_{35}H_{37}O_8N$ (599.66): C, 70.10; H, 6.22; N, 2.34. Found: C, 69.79; H, 6.11; N, 2.33.

2-(*p*-Nitrophenyl)ethyl 4-*O*-acetyl-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (48). A small amount of **47** (16 mg) was treated with 2:1 pyridine-acetic anhydride (3 mL) for 18 h. The solvents were removed and the residue was coevaporated with toluene. The resulting oil was then taken up in chloroform, washed with saturated aqueous sodium bicarbonate, water, dried (sodium sulfate), filtered, and the solvents were removed. The resulting material was

purified by column chromatography over silica gel (1.4 g) using 4:1 hexane-ethyl acetate as eluant and the pooled fractions were combined to give 18 mg (95%) of **48**: R_f 0.14 (4:1 hexane-ethyl acetate); $[\alpha]_D$ -4.5° (*c* 0.25, chloroform); ^1H NMR δ 8.05 (d, 2H, J_d = 9.0 Hz, aromatic), 7.4-7.1 (m, 17H, aromatic), 4.96 (bt, 1H, J_t = 9.0 Hz, H-4),* 4.78 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.65 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 4.59 (bd, 2H, J_d ~ 12.0 Hz, 2 × $OC\underline{H}_2C_6H_5$), 4.51 (s, 2H, $OC\underline{H}_2C_6H_5$), 4.42 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.24 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 3.80 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.58 (t, 1H, J_t = 9.0 Hz, H-3),* 3.56-3.52 (m, 3H, H-5, H-6a, H-6b), 3.44 (dd, 1H, $J_{2,3}$ = 9.0 Hz, $J_{2,1}$ = 7.5 Hz, H-2),* 3.04 (bt, 2H, J_t ~ 6.5 Hz, $OCH_2C\underline{H}_2$), 1.83 (s, 3H, OAc). *These assignments were further confirmed by decoupling experiments, thus, irradiation of the complex triplet centered at δ 4.96 caused the collapse ot the triplet at δ 3.58 to a doublet, and likewise, irradiation of the triplet at δ 3.58 caused the collapse of the triplet at δ 4.96, thus confirming the signals for H-4 and H-3. Decoupling of the doublet of doublets centered at δ 3.44 caused the collapse of the triplet at δ 3.58 to a doublet as well as the doublet at δ 4.42 to a singlet confirming the signals for H-2, H-3, and H-1.

Acetobromogalactose (49). β-D-Galactose pentaacetate **22** (25 g, 64 mmol) was dissolved in glacial acetic acid (150 mL) and HBr gas was bubbled through the solution until saturation was acheived and the mixture was stirred at room temperature overnight. The reaction mixture was then diluted with dichloromethane (600 mL) and successively washed with water (several times), saturated aqueous sodium bicarbonate solution (2×), water, dried (sodium sulfate), filtered, and concentrated. The resulting yellowish foam was recrystallized from ethyl ether-hexane to give 12.9 g (49%) of **49** as a white solid: R_f 0.5 (1:1 hexane-ethyl acetate); ^1H NMR δ 6.72 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 5.55 (dd, 1H, $J_{4,3}$ = 3.5 Hz, $J_{4,5}$ = 1.0 Hz, H-4), 5.43 (dd, 1H, J_d = 11.0 Hz, J_d = 3.5 Hz, H-2 or H-3), 5.08 (dd, 1H, J_d = 11.0 Hz, J_d = 3.5 Hz, H-3 or H-2), 4.50 (t, 1H, J_t = 6.5 Hz, H-5), 4.22 (dd, 1H, $J_{6a,6b}$ = 11.0 Hz, $J_{6a,5}$ = 6.5 Hz, H-6a), 4.13 (dd, 1H, $J_{6b,6a}$ = 11.0 Hz, $J_{6b,5}$ = 6.5 Hz, H-6b), 2.18 (s, 3H, OAc), 2.16 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.03 (s, 3H, OAc).

2-(*p*-Nitrophenyl)ethyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (50). A mixture of **47** (3.6 g, 6 mmol), silver carbonate (8.27 g, 30 mmol), catalytic silver trifluoromethane-

sulfonate (77 mg, 0.3 mmol), and powdered Drierite® (15 g) in dry dichloromethane (40 mL) was cooled to -70 °C under argon. To the reaction mixture was added dropwise via syringe, a solution of **49** (4.94 g, 12 mmol) in dry toluene (15 mL) and the mixture was allowed to slowly warm to -10 °C and then stirred overnight. The reaction mixture was filtered through Celite® and the solvents were removed. Column chromatography over silica gel (300 g), using 2:1 hexane-ethyl acetate yielded 4.18 g (75%) of **50**: R_f 0.11 (2:1 hexane-ethyl acetate); $[\alpha]_D$ -1.3° (*c* 0.25, chloroform); ^{1}H NMR δ 8.05 (d, 2H, J_d = 9.0 Hz, aromatic), 7.40-7.10 (m, 15H, aromatic), 7.10 (m, 2H, aromatic), 5.25 (dd, 1H, $J_{4',3'}$ = 3.5 Hz, $J_{4',5'}$ = 1.0 Hz, H-4'), 5.10 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'), 4.91 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.81 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.78 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.74 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.61 (s, 2H, 2 × $OC\underline{H}_2C_6H_5$), 4.60 (d, 1H, J_d = 8.0 Hz, H-1' or H-1), 4.47 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.38 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.21 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 3.99 (dd, 1H, $J_{6'a,6'b}$= 11.0 Hz, $J_{6'a,5'}$ = 7.5 Hz, H-6'a), 3.93 (t, 1H, J_t = 9.5 Hz, H-4), 3.84 (dd, 1H, $J_{6'b,6'a}$ = 11.0 Hz, $J_{6'b,5'}$ = 6.0 Hz, H-6'b), 3.77 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 3.72 - 3.69 (m, 2H, H-6a and H-6b), 3.56 (t, 1H, J_t = 9.0 Hz, H-3), 3.52 (bdt, 1H, J_t ~ 7.0 Hz, $J_{5',4'}$ = 1.0 Hz, H-5'), 3.38 (dd, 1H, $J_{2,3}$ = 9.0 Hz, $J_{2,1}$ = 8.0 Hz, H-2), 3.37 (dt, 1H, J_d = 9.0 Hz, J_t = 2.5 Hz, H-5), 3.07-3.01 (m, 2H, $OCH_2C\underline{H}_2$), 2.09 (s, 3H, Ac), 1.98 (s, 3H, Ac), 1.96 (s, 3H, Ac), 1.95 (s, 3H, Ac).

Anal. Calcd for $C_{49}H_{55}O_{17}N$ (929.35): C, 63.27; H, 5.96; N, 1.51. Found: C, 62.88; H, 5.90; N, 1.48.

2-(*p*-Nitrophenyl)ethyl *O*-(β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (51). To a solution of **50** (4.0 g, 4.3 mmol) in dry methanol (30 mL) was added a solution of sodium metal (92 mg, 4 mmol) in dry methanol (10 mL) and the solution was stirred for 18 hours at room temperature. The mixture was neutralized with IR 120® (H^+) resin, filtered and the solvents were removed to give 3.2 g (quantitative) of **51** which was used directly in the next step without further purification or characterization: R_f 0.18 (5:5:1 hexane-ethyl acetate-ethanol).

2-(*p*-Nitrophenyl)ethyl *O*-(4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (52). A solution of tetrol **51** (3.2 g, 4.2 mmol), benzaldehyde dimethyl acetal (1.12 g, 7.35 mmol) and *p*-toluenesulphonic acid monohydrate (40 mg, 0.21 mmol) in dry acetonitrile (45 mL) was stirred at room temperature overnight. The reaction was quenched with

an excess of triethylamine and the solvents were removed to give a foam. Column chromatography over silica gel (250 g) using chloroform then 100:1 chloroform-methanol as eluant gave 2.96 g (83%) of **52**: R_f 0.22 (30:1 chloroform-methanol); $[\alpha]_D$ +1° (*c* 1, chloroform); ^{1}H NMR δ 8.05 (d, 2H, J_d = 9.0 Hz, aromatic), 7.50-7.30 (m, 20H, aromatic), 7.10 (m, 2H, aromatic), 5.45 (s, 1H, benzylidene), 4.99 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.90 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.71 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.65 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.61 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.58 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.55 (d, 1H, J_d = 7.5 Hz, H-1' or H-1), 4.40 (d, 1H, J_d = 7.5 Hz, H-1 or H-1'), 4.22 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 4.05 (dd, 1H, $J_{6'a,6'b}$ = 12.0 Hz, $J_{6'a,5'}$ = 1.0 Hz, H-6'a), 4.04-4.01 (m, 2H), 4.02 (t, 1H, J_t = 9.0 Hz, H-4), 3.79 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 3.78 (dd, 1H, $J_{6'b,6'a}$ = 12.0 Hz, $J_{6'b,5'}$ = 2.0 Hz, H-6'b), 3.76 (m, 1H), 3.68 (t, 1H, J_t = 9.0 Hz, H-3), 3.62 (bm, 1H), 3.50-3.44 (m, 3H), 3.42 (dd, 1H, $J_{2,3}$ = 9.0 Hz, $J_{2,1}$ = 7.5 Hz, H-2), 3.04 (m, 2H, $OCH_2C\underline{H}_2$), 2.88 (bs, 1H, OH), 2.41 (d, 1H, J_d = 9.0 Hz, OH).

Anal. Calcd for $C_{48}H_{51}O_{13}N$ (849.34): C, 67.82; H, 6.05; N, 1.65. Found: C, 67.58; H, 5.93; N, 1.68.

2-(*p*-Nitrophenyl)ethyl *O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (53). The diol **52** (170 mg, 0.2 mmol), was treated with 2:1 pyridine-acetic anhydride (6 mL) at room temperature for 18 hours. The reaction mixture was then evaporated to dryness and coevaporated several times with toluene. The residue was dissolved in dichloromethane, washed with saturated aqueous bicarbonate solution, water, dried (sodium sulfate), filtered, and the solvents were removed. The resulting material was purified by column chromatography over silica gel (10 g) using chloroform then 90:1 chloroform-methanol to give 168 mg (90%) of **53**: R_f 0.09 (2:1 hexane-ethyl acetate); $[\alpha]_D$ 13.1° (*c* 0.5, chloroform); ^{1}H NMR δ 8.05 (d, 2H, J_d = 9.0 Hz, aromatic), 7.50-7.10 (m, 22H, aromatic), 5.45 (s, 1H, benzylidene), 5.30 (dd, 1H, $J_{2',3'}$ = 10.5 Hz , $J_{2',1'}$ = 8.0 Hz, H-2'), 5.05 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.79 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.74 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.73 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.64 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.64 (s, 2H, 2 × $OC\underline{H}_2C_6H_5$), 4.48 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.38 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.23 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'), 4.20 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 4.15 (dd, 1H, $J_{6'a,6'b}$ =

12.5 Hz, $J_{6'a,5'}$ = 1.0 Hz, H-6'a), 3.91 (dd, 1H, $J_{4,5}$ = 9.5 Hz, $J_{9,0}$ = 9.0 Hz, H-4), 3.83 (dd, 1H, $J_{6'b,6'a}$ = 12.5 Hz, $J_{6'b,5'}$ = 1.5 Hz, H-6'b), 3.77 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.76-3.71 (m, 2H, H-6a and H-6b), 3.61 (t, 1H, J_t = 9.0 Hz, H-3), 3.38 (dd, 1H, $J_{2,3}$ = 9.0 Hz, $J_{2,1}$ = 7.5 Hz, H-2), 3.37 (m, 1H, H-5), 3.06-3.01 (m, 3H, H-5' and $OCH_2C\underline{H}_2$), 2.06 (s, 3H, Ac), 1.98 (s, 3H, Ac).

Anal. Calcd for $C_{52}H_{55}O_{15}N$ (934.01): C, 66.87; H, 5.94; N, 1.50. Found: C, 66.78; H, 5.88; N, 1.48.

2-(*p*-Aminophenyl)ethyl *O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (54). To a solution of **53** (159 mg, 0.170 mmol) and zinc (300 mg) in 6:2:0.3 tetrahydrofuran-acteic acid-water (1 mL) cooled to 0 °C under argon was added a solution of copper sulfate (0.6 mL of 1 g $CuSO_4.5H_2O$ in 100 mL H_2O) and stirring was continued for 30 min. The solution was filtered through Celite®, the pad was washed several times with dichloromethane, and the combined organic filtrate was washed successively with water, saturated aqueous sodium bicarbonate solution, dried (sodium sulfate), and filtered. The solvents were removed and the residue was coevaporated with toluene. The resulting crude **54** appeared as a ninhydrin positive spot by TLC (15:10:1 hexane-ethyl acetate-ethanol, R_f near baseline) and was used directly in the next step without further purification or characterization.

2-(*p*-Trifluoroacetamidophenyl)ethyl *O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (55). Crude **54** was dissolved in anhydrous dichloromethane (2 mL) and cooled to -20 °C under argon. A mixture of 2:1 pyridine-trifluoroacetic anhydride (160 µL) was then added via a syringe and the reaction continued for 30 min at this temperature. After removal of solvents, the residue was dissolved in dichloromethane, washed with saturated aqueous sodium bicarbonate, dried (sodium sulfate), filtered, and the solvents were removed. Column chromatography over silica gel (10 g) using chloroform then 90:1 chloroform-methanol yielded 134 mg of **55** (79% from **53**). R_f 0.44 (1:1 hexane-ethyl acetate); $[\alpha]_D$ +12.8° (*c* 0.5, chloroform); ^1H NMR δ 7.63 (bs, 1H, NH), 7.50-7.10 (m, 24H, aromatic), 5.45 (s, 1H, benzylidene), 5.30 (dd, 1H, $J_{2',3'}$ = 10.5 Hz , $J_{2',1'}$ = 8.0 Hz, H-2'), 5.02 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.78 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.74 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.72 (d,

1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.64 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.64 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.58 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.48 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.36 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.22 (dd, 1H, $J_{4',3'}$ = 3.5 Hz, $J_{4',5'}$ < 1.0 Hz, H-4'), 4.17 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 4.14 (dd, 1H, $J_{6'a,6'b}$ = 12.5 Hz, $J_{6'a,5'}$ = 1.0 Hz, H-6'a), 3.91 (t, 1H, J_t = 9.0 Hz, H-4), 3.82 (dd, 1H, $J_{6'b,6'a}$ = 12.5 Hz, $J_{6'b,5'}$ = 1.5 Hz, H-6'b), 3.79-3.72 (m, 2H, H-6a and H-6b), 3.73 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.60 (t, 1H, J_t = 9.0 Hz, H-3), 3.38 (dd, 1H, $J_{2,3}$ = 9.0 Hz, $J_{2,1}$ = 7.5 Hz, H-2), 3.37 (m, 1H, H-5), 3.03 (d, 1H, J_d = 1.5 Hz, H-5'), 2.94 (t, 1H, J_t = 7.0 Hz, $OCH_2C\underline{H}_2$), 2.05 (s, 3H, Ac), 1.97 (s, 3H, Ac). ^{19}F NMR δ -78.32 (d, J_d = 1.0 Hz), broadband proton decoupling caused the collapse of this doublet to a singlet.

Anal. Calcd for $C_{54}H_{56}O_{14}NF_3$ (1000.03): C, 64.84; H, 5.65; N, 1.40; F, 5.70. Found: C, 64.38; H, 5.60; N, 1.47.

2-(*p*-Trifluoroacetamidophenyl)ethyl *O*-(4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (56). To a solution of **55** (130 mg, 0.13 mmol) in dry methanol (3 mL) was added a solution of sodium metal (16 mg, 0.7 mmol) in dry methanol (1 mL) and the reaction mixture was stirred at room temperature for 3 h. The reaction mixture was then deionized with IR 120® (H^+) resin, filtered, and the solvents were removed. Column chromatography over silica gel (5 g) using chloroform, then 75:1 chloroform-methanol as eluant gave 113 mg (95%) of **56** as a white solid after lyophilization from benzene: R_f 0.17 (30:1 chloroform-methanol); $[\alpha]_D$ +2.6° (*c* 0.5, chloroform); ^1H NMR δ 7.67 (bs, 1H, NH), 7.50-7.10 (m, 24H, aromatic), 5.45 (s, 1H, benzylidene), 4.96 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.90 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.70 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.65 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.57 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.56 (d, 1H, J_d = 11.0 Hz, $OC\underline{H}_2C_6H_5$), 4.55 (d, 1H, J_d = 8.0 Hz, H-1' or H-1), 4.39 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.18 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 4.03 (dd, 1H, $J_{6'a,6'b}$ = 12.0 Hz, $J_{6'a,5'}$ = 1.0 Hz, H-6'a), 4.02-4.00 (m, 2H, H-6a and H-6b), 4.00 (t, 1H, J_t = 9.0 Hz, H-4), 3.80-3.71 (m, 3H), 3.66 (t, 1H, J_t = 9.0 Hz, H-3), 3.63-3.57 (m, 2H), 3.49 - 3.42 (m, 2H), 3.41 (dd, 1H, $J_{2,3}$ = 9.0 Hz, $J_{2,1}$ = 8.0 Hz, H-2), 2.97 (m, 2H, $OCH_2C\underline{H}_2$), 2.84 (bs, 1H, OH), 2.40 (d, 1H, J_d = 8.5 Hz, OH). ^{19}F NMR δ: -75.86 (d, J_d = 1.0 Hz).

Anal. Calcd for $C_{50}H_{52}O_{12}NF_3$ (915.96): C, 65.55; H, 5.73; N, 1.53; F, 6.23. Found: C, 64.69; H, 5.61; N, 1.59.

2-(*p*-Trifluoroacetamidophenyl)ethyl *O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (57). A mixture of diol **56** (110 mg, 0.12 mmol) and 5% palladium-on-carbon (200 mg) in 95:5 methanol-water containing acetic acid (few drops) was hydrogenated at 10 psi for 2 hours. The mixture was filtered through Celite® and the solvents were removed. Column chromatography over silica gel (5 g) using 65:35 chloroform-methanol then 65:35:2 chloroform-methanol-water as eluant gave 60 mg (90%) of **57**: R_f 0.42 (65:35:6 chloroform-methanol-water); $[\alpha]_D$ -8.1° (*c* 0.5, methanol); ^1H NMR (D_2O, HOD) δ 7.50-7.45 (d, 2H, aromatic), 7.40-7.35 (d, 2H, aromatic), 4.50 (d, 1H, J_d = 8.0 Hz, H-1'), 4.46 (d, 1H, J_d = 7.5 Hz, H-1), 4.17 (dt, 1H, J_d = 10.5 Hz, J_t = 6.5 Hz, OCH_2), 4.00-3.90 (m, 3H), 3.83-3.70 (m, 4H), 3.69-3.57 (m, 5H), 3.55 (dd, 1H, $J_{2,3}$ = 10.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2'), 3.30 (m, 1H, H-2), 2.97 (m, 2H, $OCH_2C\underline{H}_2$); ^{19}F NMR δ -75.7; ^{13}C NMR δ 158.09, 157.59, 138.31 (aromatic C), 133.82 (aromatic C), 130.62 (2 × aromatic C), 123.25 (2 × aromatic C), 103.77 (C-1), 102.91 (C-1'), 79.25 (C-4), 76.19 (C-5'), 75.59 (C-5), 75.23 (C-3), 73.62 (C-2), 73.38 (C-3'), 71.80 (C-2'), 71.44 ($O\underline{C}H_2$), 69.39 (C-4'), 61.85 (C-6'), 60.94 (C-C-6), 35.57 ($\underline{C}H_2C_6H_4$). Some of the assignments remain tentative and are based on data reported for the methyl glycoside.[200]

Molecular weight for $C_{22}H_{30}O_{12}NF_3$: calcd 557.47, found (FAB, positive ion, Cleland matrix): *m/z* 558.13 $(M+1)^+$.

2-(*p*-Aminophenyl)ethyl *O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (58). A solution of *N*-TFA lactose **57** (6 mg, 11 μmol) in 3:1 water-ammonia (2 mL) was stirred at room temperature overnight. After this time, analysis by TLC (65:35:6 chloroform-methanol-water) showed that the reaction was complete yielding one major ninhydrin positive spot (R_f 0.2). The solvents were evaporated and the residue was coevaporated with water. The resulting material was used directly in the next step without further purification or characterization.

2-(*p*-Isothiocyanophenyl)ethyl *O*-(β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (59). The amine **58** was dissolved in water (1 mL) containing sodium bicarbonate (6 mg, 0.07 mmol) and this solution was then added

dropwise to a solution of thiophosgene (4.1 μL, 55 μmol) in chloroform (1 mL). The resulting mixture was stirred vigorously at room temperature for one hour after which time the reaction was complete by TLC (R_f 0.41, 65:35:6 chloroform-methanol-water). The aqueous layer was then extracted with chloroform (3×1 mL) and the water was removed under high vacuum to give crude **59** which was used directly in the conjugation step without further purification or characterization.

Human Serum Albumin-Lactose Conjugate (60). A solution containing crude **59** and HSA (12 mg) in aqueous carbonate buffer (pH 9, 1:1 0.1 M $NaHCO_3$-0.01 M Na_2CO_3, 1.2 mL) was stirred at room temperature for 72 hours. Exhaustive dialysis (Amicon, YM 10 ultrafiltration membrane) against water followed by lyophilization provided 9 mg of conjugate **60**. The carbohydrate content of glycoconjugate **60** was estimated by the phenol-sulfuric acid assay and from Graph 3, for A_T = 0.276, N = 12.

Phenol-Sulfuric Acid Assay[184]

(a) Preparation of the phenol reagent: An aqueous 5% phenol stock solution was prepared by dissolving phenol (5 g, redistilled) in distilled water (100 mL) and this solution was stable for a long period of time.

(b) Preparation of standards and sample as solutions: The purified protein (HSA or KLH) as the protein standard, carbohydrate compound (*e.g.*, **59**) as the carbohydrate standard, and the corresponding glycoconjugate sample (*e.g.*, **60**) were each prepared as 1 mg/mL aqueous solutions.

(c) Assay procedure: Eight Pyrex® culture tubes (13×100 mm, screw cap 13-415) were labelled and charged with the following; 500 μL of protein blank standard solution, 30, 50, 70, and 90 μL of carbohydrate standard solution and glycoconjugate sample (500 μL, in triplicate). The solution volume of each tube was brought to 500 μL with distilled water and 5% phenol reagent (1 mL) was added to each tube. Concentrated sulfuric acid (4.5 mL) was added to the tube and the tube was sealed and vortexed for 1 min. Each tube was succesively treated in this manner and the samples were then allowed to sit at room temperature for 15 min. The spectrophotometer wavelength was set to 400 nm and the instrument zero was set with sulfuric acid. The absorbance of each of

the standards and samples was measured and the absorbance versus μg of glycoside for the carbohydrate standards was plotted (see Appendix 1 for graphs). From the corrected absorbance of the samples, the carbohydrate content of the glycoconjugate could be estimated and the N value (# of moles of carbohydrate/# moles of carrier) was approximated as follows:

A_T = True Abs. (sample) = Abs. (sample) - Abs. (protein std.)

From the standard plot and the A_T value, the amount of conjugated carbohydrate W_s (μg) can be found. Then,

N_s (# μM of carbohydrate) = W_s / MW carbohydrate derivative

Amount of protein in sample is then approximated as;

W_p (μg) = 500 - W_s (μg)

From which;

N_p (# μM of protein) = W_p / MW protein

MW = 6.7×10^4 for HSA and 6.7×10^6 for KLH.

It follows that;

N value (# of moles of carbohydrate / # moles of carrier) = N_s / N_p

2-(*p*-Nitrophenyl)ethyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonate)-(2→3)-*O*-(4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-gluco-pyranoside (61). A mixture of diol **52** (1.9 g, 2.24 mmol), silver trifluoromethanesulfonate (1.1 g 4.28 mmol), 2,6-di-*t*-butylpyridine (945 mg, 4.94 mmol), powdered Drierite® (3 g), and anhydrous tetrahydrofuran (12 mL) was strirred at room temperature under argon atmosphere for 8 h to ensure dryness. The reaction mixture was then cooled to -35 °C under argon and a solution of chloride **11** (2.2 g, 3.37 mmol) in dry toluene (4 mL) was then added dropwise. Upon completion of the addition, the reaction mixture was stirred for an additional 30 min at this temperature and then at 0 °C overnight. The reaction mixture was then diluted with chloroform and filtered through Celite®. The

organic filtrate was washed with saturated aqueous sodium bicarbonate, water, dried (sodium sulfate), filtered, and the solvents were removed to give a yellowish foam. This foam was then purified by column chromatography over silica gel (150 g) using 20:10:1 hexane-ethyl acetate-ethanol as eluant to give crude **61** contaminated with diol **52** and unsaturated compound **39**. A majority of the diol was separated out by crystallization from a chloroform solution of this mixture. The desired compound was purified to homogeneity after the subsequent acetylation step.

2-(*p*-Nitrophenyl)ethyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonate)-(2→3)-*O*-(2-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (62). The crude **61** (1.2 g) was treated with 2:1 pyridine-acetic anhydride (24 mL) at room temperature for 48 h and the mixture was then evaporated to dryness. After coevaporation with toluene, the resulting yellowish foam was purified by column chromatography over silica gel using 95:5 chloroform-methanol as eluant to provide 940 mg (29% overall yield from **52**) as a white solid after lyophilization from benzene: R_f 0.36 (20:1 chloroform-methanol); $[\alpha]_D$ +15.8° (*c* 0.5, chloroform); ^{1}H NMR δ 8.00 (d, 2H, J_d = 9.0 Hz, aromatic), 7.40-7.05 (m, 27H, aromatic), 5.58 (ddd, 1H, $J_{8'',7''}$ = 9.0 Hz, $J_{8'',9''b}$ = 6.0 Hz, $J_{8'',9''a}$ = 2.5 Hz, H-8''),* 5.36 (dd, 1H, $J_{7'',8''}$ = 9.0 Hz, $J_{7'',6''}$ = 2.5 Hz, H-7''),* 5.15 (dd, 1H, $J_{2',3'}$ = 10.0 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'),* 5.13 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.05 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.04 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.03 (d, 1H, J_d = 10.5 Hz, NH), 4.82 (s, 1H, benzylidene), 4.80 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'),* 4.80 (m, 1H, H-4''),* 4.65-4.55 (m, 4H, 4 × $OC\underline{H}_2C_6H_5$), 4.38 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1),* 4.35 (dd, 1H, $J_{9''a,9''b}$ = 12.5 Hz, $J_{9''a,8''}$ = 2.5 Hz, H-9''a),* 4.29 (dd, 1H, $J_{3',2'}$ = 10.0 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'),* 4.19 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 4.07 (q, 1H, J_q = 10.5 Hz, H-5''),* 3.97 (dd, 1H, $J_{9''b,9''a}$ = 12.5 Hz, $J_{9''b,8''}$ = 6.0 Hz, H-9''b),* 3.90 (dd, 1H, $J_{6'a,6'b}$ = 12.5 Hz, $J_{6'a,5'}$ < 1.0 Hz, H-6'a), 3.87 (dd, 1H, $J_{6a,6b}$ = 11.0 Hz, $J_{6a,5}$ = 1.5 Hz, H-6a), 3.84 (dd, 1H, $J_{6'',5''}$ = 10.5 Hz, $J_{6'',7''}$ = 2.5 Hz, H-6''),* 3.83 (t, 1H, J_t = 9.5 Hz, H-4), 3.78 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.72 (dd, 1H, $J_{6b,6a}$ = 11.0 Hz, $J_{6b,5}$ = 5.5 Hz, H-6b), 3.63 (t, 1H, J_t = 9.0 Hz, H-3),* 3.52 (dd, 1H, $J_{6'b,6'a}$ = 12.5 Hz, $J_{6'b,5'}$ = 1.0 Hz, H-6'b), 3.49 (ddd, 1H, $J_{5,4}$ = 9.5 Hz, $J_{5,6b}$ = 5.5 Hz, $J_{5,6a}$ = 1.5 Hz, H-5), 3.40 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'),* 3.36 (dd, 1H, $J_{2,3}$ = 9.0 Hz, $J_{2,1}$ = 8.0 Hz, H-2),* 3.05 (bs, 1H, H-5'), 3.04-3.00 (m, 2H, $OCH_2C\underline{H}_2$),

2.73 (dd, 1H, $J_{3"e,3"a}$ = 12.5 Hz, $J_{3"e,4"}$ = 4.5 Hz, H-3"e),* 2.20 (s, 3H, Ac), 2.12 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.00 (s, 3H, Ac), 1.88 (s, 3H, Ac), 1.80 (t, 1H, J_t = 12.5 Hz, H-3"a). *These assignments were further confirmed by decoupling experiments, thus, decoupling of the multiplet centered at δ 5.58 (H-8") collapsed doublet of doublets at δ 5.36 (H-7"), 4.35 (H-9"a), and 3.97 (H-9"b); irradiation of the doublet of doublets centered at δ 5.36 collapsed the multiplet at δ 5.58 and doublet of doublets at δ 3.84 (H-6"); irradiation of the doublet of doublets centered at δ 5.13 (H-2') collapsed the doublet at δ 4.80 (H-1') and the doublet of doublets at δ 4.29 (H-3'); decoupling of the multiplet centered at δ 4.80 (H-4") collapsed the quartet at δ 4.07 along with doublet of doublets at δ 2.73 (H-3"e) and 1.80 (H-3"a); irradiation of the doublet of doublets centered at δ 4.35 collapsed the multiplet at δ 5.58 along with the doublet of doublets at δ 3.97; decoupling of the doublet of doublets at δ 4.29 caused a collapse of the doublet of doublets at δ 5.13 along with the doublet at δ 3.40 (H-4'); and decoupling of the doublet of doublets at δ 3.36 (H-2) caused a collapse of the doublet at δ 4.38 (H-1) and the doublet of doublets at δ 3.63 (H-3).

Anal. Calcd for $C_{76}H_{84}O_{26}N_2$ (1440.53): C, 63.31; H, 5.88; N, 1.94. Found: C, 63.00; H, 5.77; N, 1.94.

2-(*p*-Aminophenyl)ethyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonate)-(2→3)-*O*-(2-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (63). A solution of **62** (930 mg, 0.65 mmol) in 15:2:0.6 tetrahydrofuran-acetic acid-water was cooled to 0 °C and zinc powder (2 g) was then added in one portion. To the rapidly stirred mixture was added 4 mL of aqueous copper sulfate solution (100 mg $CuSO_4 \cdot 5H_2O$ per 1 mL H_2O) and then the mixture was stirred at room temperature for 1 h. After dilution with chloroform, the reaction was filtered through Celite® and the organic filtrate was washed with saturated aqueous sodium bicarbonate, dried (sodium sulfate), filtered, and the solvents were removed. Finally, the residue was coevaporated with toluene to give crude **63**, R_f 0.26 (20:1 chloroform-methanol, visualization with ninhydrin spray) which was used directly in the next step without further purification or characterization.

2-(*p*-Trifluoroacetamidophenyl)ethyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonate)-(2→3)-*O*-(2-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (64). The crude amine **63** was dissolved in anhydrous dichloromethane (10 mL) containing anhydrous pyridine (525 μL, 6.5 mmol) and the reaction mixture was cooled to -20 °C under argon. To the reaction was added trifluoroacetic anhydride (305 μL, 2.17 mmol) and the reaction mixture was stirred at this temperature for an additional 10 min. The mixture was diluted with dichloromethane and then washed with saturated aqueous sodium bicarbonate solution, dried (sodium sulfate), filtered, and the solvents were removed. The resulting compound was then purified by column chromatography over silica gel (75 g) using 20:10:1 then 15:10:1 hexane-ethyl acetate-ethanol as eluant to give 880 mg (90%) of **64** as a white solid after lyophilization from benzene: R_f 0.1 15:10:1 (hexane-ethyl acetate-ethanol); $[\alpha]_D$ +1.7° (*c* 0.4, chloroform); ^{1}H NMR δ 7.70 (bs, 1H, NHTFA), 7.40-7.10 (m, 29H, aromatic), 5.57 (ddd, 1H, $J_{8'',7''}$ = 9.0 Hz, $J_{8'',9''b}$ = 6.0 Hz, $J_{8'',9''a}$ = 2.5 Hz, H-8''), 5.35 (dd, 1H, $J_{7'',8''}$ = 9.0 Hz, $J_{7'',6''}$ = 2.5 Hz, H-7''), 5.14 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'), 5.11 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 5.04 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 5.02 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 5.02 (d, 1H, J_d = 10.5 Hz, NH), 4.82 (s, 1H, benzylidene), 4.80 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 4.79 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.79 (m, 1H, H-4''), 4.62 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.61 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 4.59 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 4.53 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 4.37 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 4.36 (dd, 1H, $J_{9''a,9''b}$ = 12.5 Hz, $J_{9''a,8''}$ = 2.5 Hz, H-9''a), 4.29 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.16 (dt, 1H, J_d = 9.5 Hz, J_t = 6.0 Hz, OCH_2), 4.06 (q, 1H, J_q = 10.5 Hz, H-5''), 3.97 (dd, 1H, $J_{9''b,9''a}$ = 12.5 Hz, $J_{9''b,8''}$ = 6.0 Hz, H-9''b), 3.90 (dd, 1H, $J_{6'a,6'b}$ = 12.0 Hz, $J_{6'a,5'}$ < 1.0 Hz, H-6'a), 3.87 (dd, 1H, $J_{6a,6b}$ = 11.0 Hz, $J_{6a,5}$ = 1.5 Hz, H-6a), 3.83 (dd, 1H, $J_{6'',5''}$ = 10.5 Hz, $J_{6'',7''}$ = 2.5 Hz, H-6''), 3.82 (t, 1H, J_t = 9.5 Hz, H-4), 3.73 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.72 (dd, 1H, $J_{6b,6a}$ = 11.0 Hz, $J_{6b,5}$ = 5.5 Hz, H-6b), 3.62 (t, 1H, J_t = 9.0 Hz, H-3), 3.52 (dd, 1H, $J_{6'b,6'a}$ = 12.0 Hz, $J_{6'b,5'}$ = 1.0 Hz, H-6'b), 3.46 (ddd, 1H, $J_{5,4}$ = 9.5 Hz, $J_{5,6b}$ = 5.5 Hz, $J_{5,6a}$ = 1.5 Hz, H-5), 3.41 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'), 3.35 (dd, 1H, $J_{2,3}$ = 9.0 Hz, $J_{2,1}$ = 8.0 Hz, H-2), 3.05 (bs, 1H, H-5'), 2.92 (t, 2H, J_t = 7.0 Hz, $OCH_2C\underline{H}_2$), 2.73 (dd, 1H, $J_{3''e,3''a}$ = 12.5 Hz, $J_{3''e,4''}$ = 4.5 Hz, H-3''e), 2.20 (s, 3H, Ac), 2.11 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.00 (s, 6H, 2 × Ac), 1.87 (s, 3H, Ac), 1.79 (t, 1H, J_t = 12.5 Hz, H-3''a). ^{19}F NMR δ: -75.84 (s).

Anal. Calcd for $C_{78}H_{85}O_{25}N_2F_3$ (1506.54): C, 62.13; H, 5.69; N, 1.86. Found: C, 61.78; H, 5.53; N, 1.88.

2-(*p*-Trifluoroacetamidophenyl)ethyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonate)-(2→3)-*O*-(2-*O*-acetyl-4,6-β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (65). To a mixture of **64** (840 mg, 0.583 mmol), methanol (49 mL), and wet 5% palladium-on-carbon (1.5 g, wet with 1 mL of water) was added acetic acid (500 μL) and the mixture was hydrogenated at 5 psi, room temperature for 20 h. The reaction mixture was then filtered through Celite® and the solvents were removed to give 538 mg of **65** which was homogeneous by TLC, R_f 0.39 (65:35:3 chloroform-methanol-water). This material was directly subjected to de-*O*-acetylation without further purification or characterization.

2-(*p*-Trifluoroacetamidophenyl)ethyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonic acid)-(2→3)-*O*-(2-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (66). A small amount of **65** (17 mg) was treated with a solution of methanol (2 mL) containing sodium metal (5 mg) and the mixture was stirred at room temperature for 6 h. The reaction mixture was deionized with IR 120® (H^+) resin, filtered, and the solvents were removed. Column chromatography over silica gel (1.4 g) using 65:35 chloroform-methanol then 65:35:2 chloroform-methanol-water as eluant gave 12 mg of crude product: R_f 0.11 (65:35:4 chloroform-methanol-water). The ^1H NMR spectrum revealed that many of the peaks were doubled and this compound was a mixture of **66/67** ≈ 2/1. Compound **66** displayed the following characteristic peaks; ^1H NMR (D_2O, HOD suppression) δ 4.93 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'), 4.73 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.45 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 2.25 (s, 3H, OAc).

2-(*p*-Trifluoroacetamidophenyl)ethyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (67). A solution of **65** (538 mg) in dry methanol containing sodium metal (17 mg, 0.73 mmol) was stirred at room temperature overnight. After this time, a second portion of sodium (46 mg, 2 mM) in methanol (2 mL) was added and the reaction was monitored by TLC (R_f 0.15 and 0.12 (6:1.2:1 ethyl acetate-methanol-water) for **66** and **67** respectively). After 96 h, no **66** was detected by TLC, however a lower R_f ninhydrin positive

spot was now present. The reaction mixture was neutralized with acetic acid, filtered and the solvents were removed. Column chromatography over silica gel (40 g), using first 10:2:1 then 6:1.2:1 ethyl acetate-methanol-water as eluant gave pure **67** (305 mg, 62%) followed by the lower R_f product. Data for **67**: R_f 0.15 (6:1.2:1 ethyl acetate-methanol-water); $[\alpha]_D$ +0.9 (*c* 1, methanol) ; ^1H NMR (D_2O, HOD) δ 7.45-7.35 (m, 4H, aromatic), 4.50 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.46 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 4.12 (dt, 1H, J_d = 10.0 Hz, J_t = 6.5 Hz, OCH_2), 4.09 (dd, 1H, $J_{3',2'}$ = 10.0 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 3.97-3.51 (m), 3.53 (dd, 1H, J_d = 10.0 Hz, J_d = 8.0 Hz, H-2'), 3.27 (t, 1H, J_t = 9.0 Hz, H-2), 2.95 (t, 2H, J_t = 6.5 Hz, $OCH_2C\underline{H}_2$), 2.74 (dd, 1H, $J_{3''e,3''a}$ = 12.5 Hz, $J_{3''e,4''}$ = 4.5 Hz, H-3''e), 2.00 (s, 3H, Ac), 1.78 (t, 1H, J_t = 12.5 Hz, H-3''a); ^{19}F NMR δ: -75.70 (s); ^{13}C NMR δ 175.72 ($NH\underline{C}OCH_3$), 174.57 (C-1), 138.18 (aromatic C), 133.64 (aromatic C), 130.46 (2 × aromatic C), 123.12 (2 × aromatic C), 103.35 (C-1), 102.78 (C-1'), 100.51 (C-2''), 78.76 (C-4), 76.18 (C-3'), 75.84 (C-5'), 75.43 (C-5), 75.04 (C-3), 73.57 (C-6''), 73.46 (C-2), 72.45 (C-8''), 71.27 (OCH_2), 70.05 (C-2'), 69.02 (C-4''), 68.81 (C-7''), 68.17 (C-4'), 63.28 (C-9''), 61.69 (C-6'), 60.75 (C-6), 52.39 (C-5''), 40.33 (C-3''), 35.41 ($\underline{C}H_2C_6H_4$), 22.75 ($NHCO\underline{C}H_3$). Some of the ^{13}C assignments remain tentative and are based on data reported for the methyl glycoside,[210] as well as ^{13}C data obtained for compound **57**.

Molecular weight for $C_{33}H_{47}O_{20}N_2F_3$: calcd 848.73, found (FAB, negative ion, TEA matrix): *m/z* 847.4 $(M-1)^-$.

2-(*p*-Aminophenyl)ethyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (68). The lower R_f product obtained from the chromatography of **67** was purified by column chromatography over silica gel (9 g) using 65:35:4.5 chloroform-methanol-water as eluant to give 51 mg (12% from **65**) of **68** as a slightly yellowish powder. This compound was also synthesized by treatment of the corresponding *N*-TFA derivative **67** with 1:1 ammonia-water for 18 h at room temperature. Data for **68**: R_f 0.06 (65:35:4.5 chloroform-methanol-water, ninhydrin positive); ^1H NMR (D_2O, HOD) δ 7.35-6.85 (m, 4H, aromatic), 4.52 (d, 1H, J_d = 8.0 Hz, H-1' or H-1), 4.48 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.11 (dt, 1H, obscured by H-3', OCH_2), 4.11 (dd, 1H, $J_{3',2'}$ = 10.0 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 3.99-3.53 (m), 3.27 (t, 1H, J_t = 9.0 Hz), 2.85 (t, 2H, J_t = 7.0 Hz,

$OCH_2C\underline{H}_2$), 2.75 (dd, 1H, $J_{3"e,3"a}$ = 12.5 Hz, $J_{3"e,4"}$ = 4.5 Hz, H-3"e), 2.02 (s, 3H, Ac), 1.78 (t, 1H, J_t = 12.5 Hz, H-3"a).

2-(*p*-Isothiocyanophenyl)ethyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (69). A solution of **68** (15 mg, 20 μmol) in 0.1 M aqueous sodium bicarbonate solution (1 mL) was added dropwise to a rapidly stirred solution of thiophosgene (8 μL, 0.1 mM) in chloroform (1 mL) cooled to 0 °C and the reaction mixture was stirred at this temperature for an additional 15 min. The reaction mixture was diluted with chloroform (2 mL) and after stirring at 0 °C, the layers were separated and this process was repeated three more times. The aqueous solution containing crude **69** was used immediately in the subsequent conjugation step.

Human Serum Albumin-GM_3 Conjugate (70). To a solution of human serum albumin (20 mg) in aqueous carbonate buffer (pH 9, 1:1 0.1 M $NaHCO_3$-0.01 M Na_2CO_3, 2.0 mL) was added the aqueous solution containing **69** (1 mL) and the reaction was stirred at room temperature for 96 h. Exhaustive dialysis (Amicon, YM 10 ultrafiltration membrane) against water followed by lyophilization provided 22 mg of GM_3-HSA conjugate **70**. The sialic acid content of this conjugate was estimated using the resorcinol-HCl assay and from Graph 4, for A_T = 1.11, N = 13.

Keyhole Limpet Hemocyanin-GM_3 Conjugate (71). To a solution of keyhole limpet hemocyanin (23 mg) in aqueous carbonate buffer (pH 9, 1:1 0.1 M $NaHCO_3$-0.01 M Na_2CO_3, 2.3 mL) was added the aqueous solution containing **69** (1 mL) and the reaction was stirred at room temperature for 96 h. Exhaustive dialysis (Amicon, YM 10 ultrafiltration membrane) against water followed by filtration through a Millex®-PF 0.8 micron filter unit and lyophilization provided 15 mg of GM_3-KLH conjugate **71**. The sialic acid content of this conjugate was estimated using the resorcinol-HCl assay and from Graph 5, for A_T = 0.367, N = 1044.

Acetobromolactose (72). A solution of acetate **34** (47.5 g, 0.07 mol) in glacial acetic acid (140 mL) was saturated with HBr (gas), then stirred overnight at room temperature. After this time, the reaction mixture was transferred to a separatory funnel with the aid of dichloromethane (300 mL) and washed with

water until the pH of the aqueous solution was neutral. The organic solution was then washed with saturated aqueous sodium bicarbonate solution (2×), dried (sodium sulfate), filtered, and the solvents were removed. The resulting yellowish foam was crystallized from ethyl ether-hexane to give 36.7 g (75%) of **72**: mp 140-142 °C (lit[209] 141-142 °C); $[\alpha]_D$ +102° (*c* 1.0, chloroform, lit.[209] $[\alpha]_D$ +104°); ^{1}H NMR δ 6.52 (d, 1H, $J_{1,2}$ = 4.0 Hz, H-1), 5.56 (t, 1H, J_t = 9.5 Hz, H-3), 5.37 (dd, 1H, $J_{4',3'}$ = 3.5 Hz, $J_{4',5'}$ = 1.0 Hz, H-4'), 5.14 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'), 4.97 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.77 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 4.0 Hz, H-2), 4.51 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.50 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 1.5 Hz, H-6a), 4.21 (m, 1H, H-5), 4.18 (dd, 1H, $J_{6b,6a}$ = 12.0 Hz, $J_{6b,5}$ = 4.5 Hz, H-6b), 4.16 (dd, 1H, $J_{6'a,6'b}$ = 11.0 Hz, $J_{6'a,5'}$ = 6.5 Hz, H-6'a), 4.09 (dd, 1H, $J_{6'b,6'a}$ = 11.0 Hz, $J_{6'b,5'}$ = 7.0 Hz, H-6'b), 3.90 (dt, 1H, J_t = 7.0 Hz, $J_{5',4'}$ = 1.0 Hz, H-5'), 3.87 (t, 1H, J_t = 9.5 Hz, H-4), 2.17 (s, 3H, Ac), 2.14 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.07 (s, 3H, Ac), 2.06 (s, 3H, Ac), 1.97 (s, 3H, Ac).

4-Methyl-3-pentenyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (73). To a rapidly stirred slurry of powdered activated molecular sieves 4Å (25 g) and dry toluene (75 mL) was added dropwise, a solution of silver trifluoromethanesulfonate (20.6 g, 80 mmol) in dry toluene (100 mL) and the mixture was then stirred in the dark at room temperature for 5 h. The solution was tested at this point for silver trifluoromethanesulfonate by the addition of benzyl bromide to a 1 mL aliqot of filtered supernatant and was found to be negative. The solvent was decanted under a stream of argon and a solution of 4-methyl-3-penten-1-ol (2.25 g, 22.5 mmol) in dry dichloromethane (16 mL) was added. After stirring at room temperature for 1 h to ensure dryness, the reaction mixture was cooled to -70 °C under argon and a solution of **72** (14 g, 20 mmol) in dry dichloromethane (25 mL) was added with the aid of a syringe. The reaction mixture was allowed to slowly warm to -5 °C and stirred at this temperature overnight. After this time, the mixture was filtered through Celite® and the solvents were removed. The resulting oil was dissolved in dichloromethane (500 mL) and the organic solution was washed with saturated aqueous sodium bicarbonate solution, dried (sodium sulfate), filtered, and concentrated to give 14 g of foam. Column chromatography over silica gel using first 40:10:1, then 35:10:1 hexane-ethyl acetate-ethanol gave 6.5 g (45%) of **73** as a white foam: R_f 0.14 (30:10:1 hexane-ethyl acetate-

ethanol); $[\alpha]_D$ -15.2° (*c* 1, chloroform); ^{1}H NMR δ 5.28 (dd, 1H, $J_{4',3'}$ = 3.5 Hz, $J_{4',5'}$ < 1.0 Hz, H-4'), 5.20 (t, 1H, J_t = 9.5 Hz, H-3), 5.16 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'), 5.13 (tt, 1H, J_t = 7.0 Hz, J_t < 1.0 Hz, HC=C), 4.88 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.84 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2), 4.44 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.44 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 2.0 Hz, H-6a). ∅ 43 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.07 (dd, 1H, $J_{6'a,6'b}$ = 11.0 Hz, $J_{6'a,5'}$ = 6.5 Hz, H-6'a), 4.05 (dd, 1H, $J_{6b,6a}$ = 12.0 Hz, $J_{6b,5}$ = 5.0 Hz, H-6b), 4.04 (dd, 1H, $J_{6'b,6'a}$ = 11.0 Hz, $J_{6'b,5'}$ = 7.5 Hz, H-6'b), 3.84 (dt, 1H, J_t = 7.5 Hz, $J_{5',4'}$ < 1.0 Hz, H-5'), 3.80 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.79 (t, 1H, J_t = 9.5 Hz, H-4), 3.60 (ddd, 1H, $J_{5,4}$ = 9.5 Hz, $J_{5,6b}$ = 5.0 Hz, $J_{5,6a}$ = 2.0 Hz, H-5), 3.52 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 2.25 (q, 2H, J_q = 7.0 Hz, $OCH_2C\underline{H}_2$), 2.16 (s, 3H, Ac), 2.12 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.05 (s, 6H, 2 × Ac), 2.04 (s, 3H, Ac), 1.97 (s, 3H, Ac), 1.68 (bs, 3H, CH_3), 1.60 (bs, 3H, CH_3).

Anal. Calcd for $C_{32}H_{46}O_{18}$ (718.69): C, 53.48; H, 6.45. Found: C, 53.48; H, 6.54.

4-Methyl-3-pentenyl *O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (74). To a solution of **73** (5 g, 7 mmol) in dry methanol (75 mL) was added a solution of sodium metal (230 mg, 10 mM) in dry methanol (10 mL) and the resulting solution was stirred at room temperature overnight. The reaction mixture was then deionized with Amberlite® IR 120 (H^+) resin, the mixture filtered, and the solvents were evaporated to give 2.9 g (quantitative) of **74**. A small amount of this material (120 mg) was purified over silica gel (7 g) using 5:1 chloroform-methanol as eluant to give pure **74** (104 mg) as a white solid after lyophilization from water: R_f 0.08 (5:1 chloroform-methanol); $[\alpha]_D$ -16.1° (*c* 1, methanol); ^{1}H NMR (D_2O, HOD) δ 5.23 (tm, J_t = 6.5 Hz, J_m = 1.5 Hz, HC=C), 4.50 (d, 1H, J_d = 8.0 Hz, H-1'), 4.46 (d, 1H, J_d = 7.5 Hz, H-1), 3.98 (dd, 1H, J_d = 12.5 Hz, J_d = 2.0 Hz, H-6a or H-6'a), 3.93 (m, 1H), 3.90 (dt, 1H, J_d = 10.0 Hz, J_t = 7.0 Hz, OCH_2), 3.83-3.58 (m, 9H), 3.54 (dd, 1H, J_d = 10.0 Hz, J_d = 8.0 Hz, H-2'), 3.30 (m, 1H, H-2), 2.35 (q, 2H, J_q = 7.0 Hz, $OCH_2C\underline{H}_2$), 1.70 (bs, 3H, CH_3), 1.65 (bs, 3H, CH_3); ^{13}C NMR δ 136.68 [$(CH_3)_2\underline{C}$=C], 120.73 (H$\underline{C}$=C), 103.80 (C-1), 102.87 (C-1'), 79.30 (C-4), 76.20 (C-5'), 75.61 (C-5), 75.28 (C-3), 73.67 (C-2), 73.40 (C-3'), 71.82 (C-2'), 70.94 (OCH_2), 69.41 (C-4'), 61.87 (C-6'), 60.98 (C-6), 28.76 ($OCH_2\underline{C}H_2$), 25.69 (CH_3), 17.92 (CH_3). Some of the ^{13}C assignments remain tentative and are based on data reported for the methyl glycoside.[200]

Anal. Calcd for $C_{18}H_{32}O_{11}$ (424.44): C, 50.93; H, 7.60. Found: C, 50.45; H, 7.69.

Molecular weight for $C_{18}H_{32}O_{11}$: calcd 424.44, found (FAB, positive ion, Cleland matrix): *m/z* 425 $(M+1)^+$.

3-Oxopropyl *O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (75). A solution of **74** (10 mg, 24 μmol) in methanol (10 mL) was cooled to -15 °C and ozone gas was bubbled through the solution for 3 min. Argon was then passed throught the solution to remove the excess ozone. Excess methyl sulfide (6 μL, 82 μmol) was then added and stirring was continued for a few minutes at this temperature. The solvents were then removed to give **75**, R_f 0.17 (65:35:6 chloroform-methanol-water) which was used directly in the subsequent conjugation without further purification or characterization.

Lactose-HSA Conjugate (76). A solution of the aldehyde **75** (≈ 10 mg), and human serum albumin (30 mg) in phosphate buffered saline (3 mL), pH 7.5, was stirred at room temperature for 15 min. Sodium cyanoborohydride (10 mg, 160 μmol) was added and stirring was continued for an additional 72 h. Exhaustive dialysis (Amicon, YM 10 ultrafiltration membrane) against water followed by lyophilization provided 24 mg of lactose-HSA conjugate **76**. The carbohydrate content of glycoconjugate **76** was estimated by the phenol-sulfuric acid assay and from Graph 6, for A_T = 0.09, N = 4. When the ozonolysis reaction was performed at -70 °C for 2-3 min. and the conjugation was carried out in phosphate buffered saline at pH 5.9, the carbohydrate content for A_T = 0.181 from this same graph led to a conjugation ratio of N = 10.

4-Methyl-3-pentenyl *O*-(4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (77). A mixture of **74** (2.8 g, 6.6 mmol) and benzaldehyde dimethyl acetal (2.0 g, 13.2 mmol) in dry acetonitrile containing a catalytic portion of *p*-toluenesulfonic acid (63 mg, 0.33 mM) was heated to 60 °C for 30 min. After cooling to room temperature, the acid was quenched by the addition of excess triethylamine and the solvents were evaporated. The resulting solid was washed several times with ethyl ether and dried *in vacuo* to give crude **77** (3.3 g), R_f 0.50 (6:1 chloroform-methanol), which was characterized as its fully acetylated derivative **78**.

4-Methyl-3-pentenyl *O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galacto-pyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (78). A small amount of **77** (30 mg, 0.06 mmol) was dissolved in 2:1 pyridine-acetic anhydride (3 mL) and the reaction was stirred at room temperature overnight. After this time, the solvents were removed and the residue was coevaporated with toluene. The resulting compound was purified by column chromatography over silica gel (4 g) using 35:10:1 hexane-ethyl acetate-ethanol as eluant to give 36 mg of **78** (85%) after lyophilization from benzene: R_f 0.10 (35:10:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ +27.5° (*c* 1, chloroform); ^{1}H NMR δ 5.26 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'), 5.22 (t, 1H, J_t = 9.5 Hz, H-3), 5.06 (tm, 1H, J_t = 7.0 Hz, J_m = 1.0 Hz, HC=C), 4.92 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2), 4.88 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.51 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 2.0 Hz, H-6a), 4.48 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.47 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.33 (bd, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'), 4.30 (dd, 1H, $J_{6'a,6'b}$ = 12.5 Hz, $J_{6'a,5'}$ = 1.0 Hz, H-6'a), 4.12 (dd, 1H, $J_{6b,6a}$ = 12.0 Hz, $J_{6b,5}$ = 5.0 Hz, H-6b), 4.04 (dd, 1H, $J_{6'b,6'a}$ = 12.5 Hz, $J_{6'b,5'}$ = 1.0 Hz, H-6'b), 3.81 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.78 (t, 1H, J_t = 9.5 Hz, H-4), 3.60 (ddd, 1H, $J_{5,4}$ = 9.5 Hz, $J_{5,6b}$ = 5.0 Hz, $J_{5,6a}$ = 2.0 Hz, H-5), 3.46 (d, 1H, J_d = 1.0 Hz, H-5'), 3.44 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 2.26 (q, 2H, J_q = 7.0 Hz, $OCH_2C\underline{H}_2$), 2.12 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.68 (bs, 3H, CH_3), 1.60 (bs, 3H, CH_3).

Anal. Calcd for $C_{35}H_{46}O_{16}$ (722.72): C, 58.16; H, 6.42. Found: C, 58.07; H, 6.49.

4-Methyl-3-pentenyl *O*-(3-*O*-benzoyl-4,6-*O*-benzylidene-β-D-galacto-pyranosyl)-(1→4)-β-D-glucopyranoside (79). A mixture of disaccharide **77** (26 mg, 50 μmol) and benzoic anhydride (21 mg, 90 μmol) in 1:1 pyridine-chloroform (2 mL) was heated to 70 °C under argon overnight. After this time, a second portion of benzoic anhydride (14 mg, 60 μmol) in 1:1 pyridine-chloroform (1 mL) was added and heating was continued for 4 h. The reaction mixture was quenched with excess methanol, allowed to cool to room temperature and evaporated to dryness. The residue was taken up in chloroform, washed with saturated aqueous sodium bicarbonate solution, dried (sodium sulfate) and the solvents were removed. Column chromatography over silica gel (4 g) using 15:10:1 hexane-ethyl acetate-ethanol as eluant gave faster eluting components

followed by 12 mg of the major component **79** (39%): R_f 0.12 (10:10:1 hexane-ethyl acetate-ethanol); ^{1}H NMR δ 8.09 (dm, 2H, J_d = 8.0 Hz, J_m < 1.0 Hz, aromatic), 7.59 (m, 1H, aromatic), 7.50-7.42 (m, 3H, aromatic), 7.39-7.35 (m, 3H, aromatic), 5.51 (s, 1H, benzylidene), 5.16 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 5.10 (tm, 1H, J_t = 7.5 Hz, J_m < 1.0 Hz, HC=C), 4.77 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.48 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'), 4.32 (dd, 1H, $J_{6'a,6'b}$ = 12.0 Hz, $J_{6'a,5'}$ = 1.0 Hz, H-6'a), 4.30 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.26 (dd, 1H, J_d = 9.5 Hz, J_d = 8.0 Hz, H-2 or H-2'), 4.08 (dd, 1H, $J_{6'b,6'a}$ = 12.0 Hz, $J_{6'b,5}$ = 1.0 Hz, H-6'b), 4.02 - 3.90 (m, 1H), 3.89 - 3.82 (m, 2H), 3.80 (t, 1H, J_t = 9.0 Hz, H-4), 3.75 - 3.69 (m, 2H), 3.48 (dt, 1H, J_d = 9.5 Hz, J_t = 7.5 Hz, OCH_2), 3.44 - 3.37 (m, 2H), 2.30 (m, 2H, $OCH_2C\underline{H}_2$), 1.68 (bs, 3H, CH_3), 1.60 (bs, 3H, CH_3).

4-Methyl-3-pentenyl *O*-(3-*O*-benzoyl-4,6-*O*-benzylidene-β-D-galacto-pyranosyl)-(1→4)-6-*O*-benzoyl-β-D-glucopyranoside (80). A solution of the benzylidene compound **77** (3.38 g, 6.6 mmol) in 2:1 chloroform-pyridine was cooled to -70 °C under argon and a solution of benzoyl chloride (1.7 mL, 14.5 mmol) in chloroform (5 mL) was added dropwise over 5-10 min. Stirring was continued at this temperature for 1h. The solution was allowed to slowly warm to -50 °C, quenched with excess methanol (3 mL), and finally allowed to warm to room temperature. The solvents were evaporated and the residue was coevaporated with toluene (3×). The resulting foam was dissolved in chloroform (350 mL). The solution was washed with saturated aqueous sodium bicarbonate solution, dried (sodium sulfate), filtered, and concentrated. Column chromatography over silica gel (250 g) using 20:10:1 hexane-ethyl acetate-ethanol as eluant gave 1.54 g (28%) of faster eluting material (which can be recycled to **77** by de-*O*-acetylation) followed by 2.33 g (49%) of pure **80**: R_f 0.14 (30:1 chloroform-methanol); $[\alpha]_D$ +50.1° (*c* 1, chloroform); ^{1}H NMR δ 8.09 (dm, 2H, J_d = 8.0 Hz, J_m < 1.0 Hz, aromatic), 8.04 (dm, 2H, J_d = 8.0 Hz, J_m < 1.0 Hz, aromatic), 7.58 (m, 2H, aromatic), 7.48-7.42 (m, 6H, aromatic), 7.38 - 7.33 (m, 3H, aromatic), 5.51 (s, 1H, benzylidene), 5.12 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'),* 5.10 (tm, 1H, J_t = 7.0 Hz, J_m = 1.0 Hz, HC=C), 4.96 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 1.0 Hz, H-6a),* 4.54 (d, 1H, J_d = 7.5 Hz, H-1 or H-1'), 4.49 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'),* 4.40 (dd, 1H, $J_{6b,6a}$ = 12.0 Hz, $J_{6b,5}$ = 5.0 Hz, H-6b),* 4.37 (m, 1H), 4.35 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.32 (dd, 1H, $J_{6'a,6'b}$ = 12.5 Hz, $J_{6'a,5'}$ = 1.0 Hz, H-6'a), 4.28 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'),* 4.10 (dd, 1H, $J_{6'b,6'a}$ = 12.5 Hz, $J_{6'b,5}$ = 1.0 Hz, H-6'b), 3.88 (m, 1H), 3.73

(t, 1H, J_t = 9.0 Hz, H-4), 3.70 (m, 1H), 3.69 (m, 1H, H-5),* 3.58 (d, 1H, J_d = 3.5 Hz), 3.55 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.52 - 3.45 (m, 2H), 2.54 (d, 1H, J_d < 1.0 Hz), 2.36 (m, 2H, $OCH_2C\underline{H}_2$), 1.68 (bs, 3H, CH_3), 1.60 (bs, 3H, CH_3). *These assignments were confirmed by homonuclear decoupling experiments, thus, decoupling of the doublet of doublets centered at δ 5.12 (H-3') collapsed the doublet at δ 4.49 (H-4') and the doublet of doublets at δ 4.28 (H-2') while decoupling of the the doublet of doublets centered at δ 4.96 (H-6a) collapsed the doublet of doublets at δ 4.40 (H-6b) and multiplet at δ 3.69 (H-5).

Anal. Calcd for $C_{39}H_{44}O_{13}$ (720.77): C, 64.98; H, 6.15. Found: C, 64.44; H, 6.31.

4-Methyl-3-pentenyl *O*-(2-*O*-acetyl-3-*O*-benzoyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzoyl-β-D-glucopyranoside (81). A solution of triol **80** (25 mg, 35 μmol) in 2:1 pyridine-acetic anhydride (4.5 mL) was stirred at room temperature overnight. The solvents were evaporated and the residue was coevaporated with toluene. Column chromatography over silica gel (4 g) using 70:1 chloroform-methanol gave **81** in quantitative yield (30 mg) as a white amorphous solid after lyophilization from benzene: R_f 0.41 (30:1 chloroform-methanol); $[\alpha]_D$ +70.9° (*c* 1, chloroform); ^1H NMR δ 8.09 (dm, 2H, J_d = 8.0 Hz, J_m < 1.0 Hz, aromatic), 8.04 (dm, 2H, J_d = 8.0 Hz, J_m < 1.0 Hz, aromatic), 7.60-7.30 (m, 11H, aromatic), 5.46 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'),* 5.44 (s, 1H, benzylidene), 5.28 (t, 1H, J_t = 9.5 Hz, H-3),* 5.05 (tm, 1H, J_t = 7.0 Hz, J_m = 1.0 Hz, HC=C), 4.98 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2),* 4.96 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'),* 4.72 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 2.0 Hz, H-6a), 4.57 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), * 4.53 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1),* 4.45 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'),* 4.44 (dd, 1H, $J_{6b,6a}$ = 12.0 Hz, $J_{6b,5}$ = 5.0 Hz, H-6b), 4.32 (dd, 1H, $J_{6'a,6'b}$ = 12.5 Hz, $J_{6'a,5'}$ = 1.5 Hz, H-6'a), 4.02 (dd, 1H, $J_{6'b,6'a}$ = 12.5 Hz, $J_{6'b,5'}$ = 1.5 Hz, H-6'b), 3.96 (t, 1H, J_t = 9.5 Hz, H-4),* 3.81 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.76 (ddd, 1H, $J_{5,4}$ = 9.5 Hz, $J_{5,6b}$ = 5.0 Hz, $J_{5,6a}$ = 2.0 Hz, H-5), 3.45 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.40 (bs, 1H, H-5'), 2.25 (m, 2H, $OCH_2C\underline{H}_2$), 2.06 (s, 3H, Ac), 2.04 (s, 3H, Ac), 1.99 (s, 3H, Ac), 1.66 (bs, 3H, CH_3), 1.58 (bs, 3H, CH_3). *These assignments were further confirmed by decoupling experiments, thus, decoupling of the doublet of doublets centered at δ 5.46 (H-2') caused a collapse of the doublet of doublets at δ 4.96 (H-3') along with the doublet at δ 4.57 (H-1');

decoupling of doublet of doublets (H-2) and doublet of doublets (H-3') at δ 4.96 caused a collapse of signals at δ 5.28 (H-3), 4.53 (H-1), 5.46, and 4.45 (H-4'), and decoupling of triplet at δ 5.28 caused a collapse of the doublet of doublets at δ 4.96 and triplet at δ 3.96 (H-4).

Anal. Calcd for $C_{45}H_{50}O_{16}$ (846.88): C, 63.82; H, 5.95. Found: C, 63.58; H, 6.19.

Magnesium methoxide solution. A mixture of magnesium metal (1.22 g, 0.05 mmol, 70-80 mesh) and dry methanol (50 mL) was heated to 70 °C under argon and kept at reflux until all of the metal had been consumed. After an additional 1 h of heating, the mixture was allowed to cool to room temperature and quickly filtered through glass wool under a stream of argon. The solution was assumed to be 0.05 mM magnesium methoxide.

4-Methyl-3-pentenyl *O*-(4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-6-*O*-benzoyl-β-D-glucopyranoside (82). A solution of triol **80** (2.25 g, 3.12 mmol) in dry methanol (30 mL) was cooled to -15 °C under argon and magnesium methoxide solution (5 mL, 0.25 mmol) was added. The reaction mixture was stirred at this temperature for 30 min, then a second portion of $Mg(OCH_3)_2$ (5 mL) was added. The solution was stirred at 5 °C for an additional 30 min, then neutralized with excess acetic acid and concentrated. The residue was taken up in chloroform (300 mL), washed with saturated aqueous sodium bicarbonate solution, dried (sodium sulfate), filtered and the solvents were removed. Column chromatography over silica gel (125 g) using 30:1 chloroform-methanol as eluant gave 1.63 g (85%) of **82** as a white solid: R_f 0.50 (8.5:1 chloroform-methanol); $[\alpha]_D$ +25.5° (*c* 1, chloroform); ^{1}H NMR δ 8.05 (dm, 2H, J_d = 8.0 Hz, J_m < 1.0 Hz, aromatic), 7.58 (m, 1H, aromatic), 7.49-7.42 (m, 4H, aromatic), 7.38-7.35 (m, 3H, aromatic), 5.53 (s, 1H, benzylidene), 5.10 (tm, 1H, J_t = 7.0 Hz, J_m = 1.0 Hz, HC=C), 4.91 (dd, 1H, $J_{6a,6b}$ = 12.5 Hz, $J_{6a,5}$ = 1.5 Hz, H-6a),* 4.44 (dd, 1H, $J_{6b,6a}$ = 12.5 Hz, $J_{6b,5}$ = 6.0 Hz, H-6b),* 4.38 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.35 (d, 1H, J_d = 7.5 Hz, H-1 or H-1'), 4.29 (dd, 1H, $J_{6'a,6'b}$ = 13.0 Hz, $J_{6'a,5'}$ = 1.5 Hz, H-6'a),* 4.19 (dd, 1H, $J_{4',3'}$ = 3.5 Hz, $J_{4',5'}$ < 1.0 Hz, H-4'), 4.06 (dd, 1H, $J_{6'b,6'a}$ = 12.5 Hz, $J_{6'b,5'}$ = 2.0 Hz, H-6'b),* 3.89-3.81 (m, 2H), 3.72 (t, 1H, J_t = 9.5 Hz, H-4), 3.70-3.68 (m, 1H, H-5),* 3.65-3.61 (m, 1H), 3.56-3.45 (m, 4H), 2.35 (m, 2H, J_m = 7.0 Hz, $OCH_2C\underline{H}_2$), 1.69 (bs, 3H, CH_3), 1.61 (bs, 3H, CH_3). *Decoupling of doublet of doublets centered at δ 4.91 (H-6a) caused a

collapse of signals at δ 4.44 (H-6b) and 3.69 (H-5) and the matching pairs of H-6a-H-6b and H-6'a-H-6'b were further confirmed by 2D-COSY.

Anal. Calcd for $C_{32}H_{40}O_{12}$ (616.66): C, 62.32; H, 6.54. Found: C, 62.45; H, 6.37.

4-Methyl-3-pentenyl *O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-galacto-pyranosyl)-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzoyl-β-D-glucopyranoside (83). A solution of **82** (32 mg, 52 μmol) in 2:1 pyridine-acetic anhydride (3 mL) was stirred at room temperature overnight. The solvents were removed and the residue was coevaporated with toluene. Column chromatography over silica gel (4.5 g) using 75:1 chloroform-methanol gave 38 mg (93%) of **83** as a white amorphous solid after lyophilization from benzene: R_f 0.39 (15:10:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ +37.0° (*c* 1, chloroform); ^{1}H NMR δ 8.05 (dm, 2H, J_d = 8.0 Hz, J_m < 1.0 Hz, aromatic), 7.60 (m, 1H, aromatic), 7.50-7.42 (m, 4H, aromatic), 7.40-7.35 (m, 3H, aromatic), 5.44 (s, 1H, benzylidene), 5.26 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'),* 5.26 (t, 1H, J_t = 9.5 Hz, H-3), 5.04 (tm, 1H, J_t = 7.0 Hz, J_m = 1.0 Hz, HC=C), 4.96 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2), 4.77 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'),* 4.69 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 2.0 Hz, H-6a), 4.52 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 4.48 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'),* 4.41 (dd, 1H, $J_{6b,6a}$ = 12.0 Hz, $J_{6b,5}$ = 5.0 Hz, H-6b), 4.28 (dd, 1H, $J_{6'a,6'b}$ = 12.5 Hz, $J_{6'a,5}$ = 1.0 Hz, H-6'a), 4.28 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'),* 3.99 (dd, 1H, $J_{6'b,6'a}$ = 12.5 Hz, $J_{6'b,5'}$ = 1.5 Hz, H-6'b), 3.92 (t, 1H, J_t = 9.5 Hz, H-4), 3.80 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.73 (ddd, 1H, $J_{5,4}$ = 9.5 Hz, $J_{5,6b}$ = 5.0 Hz, $J_{5,6a}$ = 2.0 Hz, H-5), 3.44 (dt, 1H, J_d = 9.5 Hz, J_t = 7.5 Hz, OCH_2), 3.32 (d, 1H, J_d < 1.0 Hz, H-5'), 2.25 (m, 2H, $OCH_2C\underline{H}_2$), 2.06 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.66 (bs, 3H, CH_3), 1.57 (bs, 3H, CH_3).
*These assignments were further confirmed by decoupling experiments, thus, decoupling of the doublet of doublets centered at δ 4.77 (H-3') caused a collapse of the doublet of doublets at δ 5.26 (H-2') along with the doublet at δ 4.28 (H-4') while decoupling of doublet of doublets (H-2') and triplet (H-3) at δ 5.26 caused a collapse of signals at δ 4.77, 4.48 (H-1'), 4.96 (H-2), and 3.92 (H-4).

Anal. Calcd for $C_{40}H_{48}O_{16}$ (784.78): C, 61.16; H, 6.16. Found: C, 60.97; H, 6.25.

***O*-Ethyl *S*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate) dithiocarbonate (84).[189]** A solution of chloride **4** (740 mg, 1.45 mmol) and *O*-ethyl *S*-potassium dithiocarbonate (353 mg, 2.2 mmol) in anhydrous ethanol (15 mL) was stirred at room temperature in the dark for 30 h. The solvents were then removed and the residue was taken up in chloroform, washed with water, saturated aqueous sodium chloride solution, dried (sodium sulfate), filtered, and concentrated. Column chromatography over silica gel (35 g) using chloroform then 80:1 chloroform-methanol as eluant gave 590 mg (65%) of **84** as a solid. An analytical sample was recrystallized from anhydrous benzene and pure **84** was obtained as needles: R_f 0.17 (10:10:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ +77.3° (*c* 1, chloroform, lit[189] +79°); ^{1}H NMR δ 5.31 (m, 1H, H-8), 5.30 (m, 1H, H-7), 5.24 (d, 1H, $J_{NH,5}$ = 10.0 Hz, NH), 4.90 (ddd, 1H, $J_{4,3a}$ = 12.0 Hz, $J_{4,5}$ = 10.0 Hz, $J_{4,3e}$ = 4.5 Hz, H-4), 4.81 (dt, 1H, J_d = 10.5 Hz, J_t = 7.0 Hz, $OC\underline{H}_2CH_3$), 4.58 (dd, 1H, $J_{6,5}$ = 10.5 Hz, $J_{6,7}$ = 1.5 Hz, H-6), 4.53 (dt, 1H, J_d = 10.5 Hz, J_t = 7.0 Hz, $OC\underline{H}_2CH_3$), 4.33 (dd, 1H, $J_{9a,9b}$ = 12.5 Hz, $J_{9a,8}$ = 2.5 Hz, H-9a), 4.20 (dd, 1H, $J_{9b,9a}$ = 12.5 Hz, $J_{9b,8}$ = 5.0 Hz, H-9b), 4.03 (q, 1H, J_q = 10.5 Hz, H-5), 3.80 (s, 3H, MeO), 2.63 (dd, 1H, $J_{3e,3a}$ = 13.0 Hz, $J_{3e,4}$ = 4.5 Hz, H-3e), 2.15 (s, 3H, Ac), 2.14 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.01 (dd, 1H, $J_{3a,3e}$ = 13.0 Hz, $J_{3a,4}$ = 12.0 Hz, H-3a), 1.90 (s, 3H, Ac), 1.37 (t, 3H, J_t = 7.0 Hz, $OCH_2C\underline{H}_3$).

Molecular weight for $C_{23}H_{33}O_{13}NS_2$: calcd 595.62, found (FAB, positive ion, Cleland matrix): *m/z* 596 $(M+1)^+$.

Anal. Calcd for $C_{23}H_{33}O_{13}NS_2$ (595.62): C, 46.38; H, 5.58; N, 2.35; S, 10.77. Found: C, 46.23; H, 5.40; N, 2.35; S, 10.44.

Attempt at sialylation of the 2,3,2',3' tetrol 82 with 84. A mixture of tetrol **82** (205 mg, 0.33 mmol), xanthate **84** (356 mg, 0.6 mmol) and 400 mg of powdered molecular sieves 3Å in 2.5:1 anhydrous acetonitrile-dichloromethane (6 mL) was stirred for 2 h at room temperature to ensure dryness and then cooled to -45 °C under argon. To the reaction mixture was added dropwise via a syringe, a solution of methylsulfenyl trifluoromethanesulfonate (810 μL, 0.6 mmol, freshly prepared by reaction of methylsulfenyl bromide and silver trifluoromethanesulfonate[190]) in 1,2 dichloromethane. The reaction mixture was stirred for an additional 50 min at this temperature and quenched by the addition of an excess of diisopropylamine. The reaction was then filtered through Celite®

and subsequently analyzed by TLC (19:1 chloroform-methanol and 5:5:1 hexane-ethyl acetate-ethanol) which upon development, revealed a heterogeneous mixture thus further purification and characterization was not pursued.

4-Methyl-3-pentenyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonate)-(2→3)-*O*-(4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-6-*O*-benzoyl-β-D-glucopyranoside (85). A mixture of tetrol **82** (308 mg, 0.5 mmol), silver trifluoromethanesulfonate (308 mg, 1.2 mmol), 2,6-di-*t*-butylpyridine (276 mg, 1.4 mmol), powdered Drierite® (1 g), and dry tetrahydrofuran (4 mL) was strirred at room temperature under argon atmosphere for 6 h to ensure dryness. The reaction mixture was then cooled to -45 °C under argon and a solution of chloride **11** (586 mg, 1.0 mmol) in anhydrous toluene (1 mL) was then added dropwise. Upon completion of the addition, the reaction mixture was stirred for an additional 30 min at this temperature and then at 0 °C overnight. The reaction was then diluted with chloroform, filtered through Celite®, and the organic filtrate was then washed with saturated aqueous sodium bicarbonate solution, water, dried (sodium sulfate), filtered, and evaporated to give a yellowish foam. This foam was subsequently subjected to column chromatography over silica gel (34 g) using 10:10:1 hexane-ethyl acetate-ethanol as eluant to give 206 mg (36%) of **85** after lyophilization from benzene: R_f 0.1 (10:10:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ +8.1° (*c* 1, chloroform); ^{1}H NMR δ 8.05 (dm, 2H, J_d = 8.0 Hz, J_m < 1.0 Hz, aromatic), 7.55 (m, 1H, aromatic), 7.42-7.30 (m, 12H, aromatic), 5.42 (ddd, 1H, $J_{8'',7''}$ = 8.5 Hz, $J_{8'',9''b}$ = 6.0 Hz, $J_{8'',9''a}$ = 2.5 Hz, H-8''), 5.33 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.30 (dd, 1H, $J_{7'',8''}$ = 8.5 Hz, $J_{7'',6''}$ = 2.5 Hz, H-7''), 5.23 (bd, 1H, $J_{NH,5''}$ = 10.0 Hz, NH), 5.08 (tm, 1H, J_t = 7.0 Hz, J_m = 1.0 Hz, HC=C), 5.06 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.01 (m, 1H, H-4''), 5.00 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 2.0 Hz, H-6a), 4.93 (s, 1H, benzylidene), 4.56 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.48 (bs, 1H, OH), 4.41 (dd, 1H, $J_{6b,6a}$ = 12.0 Hz, $J_{6b,5}$ = 6.5 Hz, H-6b), 4.39 (dd, 1H, $J_{9''a,9''b}$ = 12.5 Hz, $J_{9''a,8''}$ = 2.5 Hz, H-9''a), 4.37 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.11 (dd, 1H, $J_{6'a,6'b}$ = 12.5 Hz, $J_{6'a,5'}$ = 1.5 Hz, H-6'a), 4.09 (dd, 1H, $J_{6'',5''}$ = 10.0 Hz, $J_{6'',7''}$ = 1.5 Hz, H-6''), 4.09 (m, 1H), 4.05 (dd, 1H, $J_{9''b,9''a}$ = 12.5 Hz, $J_{9''b,8''}$ = 6.0 Hz, H-9''b), 3.95 (q, 1H, J_q = 10.0 Hz, H-5), 3.88 - 3.78 (m, 3H), 3.72 (t, 1H, J_t = 9.5 Hz, H-4), 3.66 (dd, 1H, $J_{6'b,6'a}$ = 12.5 Hz, $J_{6'b,5'}$ = 1.0 Hz, H-6'b), 3.59 - 3.45 (m, 3H), 3.33 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'), 3.26 (bs,

1H, H-5'), 3.26 (bs, 1H, OH), 2.78 (dd, 1H, $J_{3"e,3"a}$ = 12.5 Hz, $J_{3"e,4"}$ = 4.5 Hz, H-3"e), 2.53 (bs, 1H, OH), 2.33 (m, 2H, $OCH_2C\underline{H}_2$), 2.15 (s, 3H, Ac), 2.14 (s, 3H, Ac), 2.02 (s, 3H, Ac), 1.97 (s, 3H, Ac), 1.97 (t, 1H, J_t = 12.5 Hz, H-3"a), 1.90 (s, 3H, Ac), 1.67 (bs, 3H, CH_3), 1.58 (bs, 3H, CH_3).

Anal. Calcd for $C_{58}H_{71}O_{24}N$ (1166.19): C, 59.74; H, 6.14; N, 1.20. Found: C, 59.15; H, 5.93; N, 1.20.

4-Methyl-3-pentenyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonate)-(2→3)-*O*-(2-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-2,3-*O*-acetyl-6-*O*-benzoyl-β-D-glucopyranoside (86). A solution of **85** (170 mg, 0.146 mmol) was dissolved in 2:1 pyridine-acetic anhydride (12 mL) and the mixture was stirred at room temperature overnight. The solvents were then removed and the residue was coevaporated with toluene. The resultant foam was subjected to column chromatography over silica gel (30 g) using chloroform, then 80:1 chloroform-methanol as eluant to give 182 mg (97%) of **86** as an amorphous white solid after lyophilization from benzene: R_f 0.13 (10:10:1 hexane-ethyl acetate-ethanol); $[\alpha]_D$ +19.6° (*c* 1, chloroform); ^{1}H NMR δ 8.05 (dm, 2H, J_d = 8.0 Hz, J_m < 1.0 Hz, aromatic), 7.58 (m, 1H, aromatic), 7.45 (m, 2H, aromatic), 7.40-7.30 (m, 10H, aromatic), 5.54 (ddd, 1H, $J_{8",7"}$ = 9.0 Hz, $J_{8",9"b}$ = 5.5 Hz, $J_{8",9"a}$ = 2.5 Hz, H-8"),* 5.37 (dd, 1H, $J_{7",8"}$ = 9.0 Hz, $J_{7",6"}$ = 2.5 Hz, H-7"),* 5.24 (t, 1H, J_t = 9.5 Hz, H-3),* 5.22 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$),* 5.09 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'),* 5.06 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$),* 5.04 (m, 1H, HC=C), 5.02 (d, 1H, $J_{NH,5"}$ = 10.5 Hz, NH),* 4.93 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2),* 4.83 (m, 1H, H-4"), 4.82 (dd, 1H, $J_{6a,6b}$ = 11.5 Hz, $J_{6a,5}$ = 2.0 Hz, H-6a),* 4.80 (s, 1H, benzylidene), 4.70 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'),* 4.51 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1),* 4.42 (dd, 1H, $J_{9"a,9"b}$ = 12.5 Hz, $J_{9"a,8"}$ = 2.5 Hz, H-9"a),* 4.33 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'),* 4.32 (dd, 1H, $J_{6b,6a}$ = 11.5 Hz, $J_{6b,5}$ = 6.0 Hz, H-6b),* 4.12 (dd, 1H, $J_{6'a,6'b}$ = 12.0 Hz, $J_{6'a,5'}$ = 1.0 Hz, H-6'a), 4.06 (dd, 1H, $J_{9"b,9"a}$ = 12.5 Hz, $J_{9"b,8"}$ = 5.5 Hz, H-9"b),* 4.00 (q, 1H, J_q = 10.5 Hz, H-5"),* 3.90 (t, 1H, J_t = 9.5 Hz, H-4),* 3.79 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.78 (dd, 1H, $J_{6",5"}$ = 10.5 Hz, $J_{6",7"}$ = 2.5 Hz, H-6"), 3.75 (m, 1H, H-5),* 3.70 dd, 1H, $J_{6'b,6'a}$ = 12.0 Hz, $J_{6'b,5'}$ = 1.0 Hz, H-6'b), 3.44 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.33 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'), 3.22 (bs, 1H, H-5'), 2.71 (dd, 1H, $J_{3"e,3"a}$ = 13.0 Hz, $J_{3"e,4"}$ = 4.5 Hz, H-3"e), 2.23 (m,

$OCH_2C\underline{H}_2$), 2.18 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.02 (s, 6H, 2 × Ac), 1.86 (s, 3H, Ac), 1.75 (t, 1H, J_t = 13.0 Hz, H-3"a), 1.66 (d, 3H, J_d < 1.0 Hz, CH_3), 1.57 (d, 3H, J_d < 1.0 Hz, CH_3). *These assignments were confirmed by decoupling experiments, thus, decoupling of the double double doublets centered at δ 5.54 (H-8") caused a collapse of doublet of doublets at δ 5.37 (H-7"), 4.42 (H-9"a), and 4.06 (H-9"b); decoupling of the triplet located at δ 5.24 (H-3) collapsed the doublet of doublets at δ 4.93 (H-2) and the triplet at δ 3.90 (H-4); irradiation of the benzyl methylene doublet at δ 5.22 caused the collapse of the corresponding geminal benzyl methylene doublet at δ 5.06; decoupling of the doublet of doublets centered at δ 5.09 (H-2') collapsed the doublet at δ 4.70 (H-1') and the doublet of doublets at δ 4.33 (H-3'); irradiation of the NH doublet at δ 5.02 collapsed the quartet at δ 4.00 (H-5"); decoupling of the doublet of doublets located at δ 4.93 (H-2) caused the collapse of the triplet at δ 5.24 and the doublet at δ 4.51 (H-1); decoupling of the doublet centered at δ 4.70 collapsed the doublet of doublets at δ 5.09; meanwhile, decoupling of the doublet centered at δ 4.51 (H-1) collapsed the doublet of doublets at δ 4.93; and finally, irradiation of the multiplet centered at δ 3.75 (H-5) caused the simultaneous collapse of doublet of doublets at δ 4.83 (H-6a) and 4.32 (H-6b) along with the triplet at δ 3.90.

Anal. Calcd for $C_{64}H_{77}O_{27}N$ (1292.31): C, 59.48; H, 6.01; N, 1.08. Found: C, 59.27; H, 6.03; N, 1.13.

4-Methyl-3-pentenyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonate)-(2→3)-*O*-(2-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3-*O*-acetyl-6-*O*-benzoyl-β-D-glucopyranoside (87). Blocked trisaccharide **86** (168 mg, 0.13 mmol) was dissolved in 4:1 acetic acid-water (10 mL) and stirred at 60 °C for 7 h and the reaction was monitored by TLC (5:5:1 hexane-ethyl acetate-ethanol). Upon completion of the reaction, the solvents were removed and the residue was coevaporated with toluene. Column chromatography over silica gel (30 g) using chloroform, then 85:1 chloroform-methanol gave 18 mg of recovered **86** (11%) followed by the desired diol **87** (105 mg, 66%): R_f 0.16 (19:1 chloroform-methanol); $[\alpha]_D$ -2.3° (*c* 1, chloroform); ^{1}H NMR δ 8.05 (dm, 2H, J_d = 8.0 Hz, J_m < 1.0 Hz, aromatic), 7.57 (m, 1H, aromatic), 7.45-7.30 (m, 12H, aromatic), 5.52 (ddd, 1H, $J_{8",7"}$ = 8.5 Hz, $J_{8",9"b}$ = 6.5 Hz, $J_{8",9"a}$ = 2.5 Hz, H-8"), 5.32 (dd, 1H, $J_{7",8"}$ = 8.5 Hz, $J_{7",6"}$ = 2.5

Hz, H-7"), 5.24 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.23 (t, 1H, J_t = 9.5 Hz, H-3), 5.14 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.04 (d, 1H, $J_{NH,5"}$ = 10.0 Hz, NH), 5.02 (m, 1H, HC=C), 4.99 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'), 4.91 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2), 4.81 (ddd, 1H, $J_{4",3"a}$ = 12.5 Hz, $J_{4",5"}$ = 10.5 Hz, $J_{4",3"e}$ = 4.5 Hz, H-4"), 4.76 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 2.0 Hz, H-6a), 4.69 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.50 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 4.40 (dd, 1H, $J_{9"a,9"b}$ = 12.5 Hz, $J_{9"a,8"}$ = 2.5 Hz, H-9"a), 4.32 (dd, 1H, $J_{6b,6a}$ = 12.0 Hz, $J_{6b,5}$ = 6.0 Hz, H-6b), 4.23 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.03 (q, 1H, J_q = 10.5 Hz, H-5"), 4.01 (t, 1H, J_t = 9.5 Hz, H-4), 3.95 (dd, 1H, $J_{9"b,9"a}$ = 12.5 Hz, $J_{9"b,8"}$ = 6.5 Hz, H-9"b), 3.84 (dd, 1H, $J_{6",5"}$ = 10.5 Hz, $J_{6",7"}$ = 2.5 Hz, H-6"), 3.77 (dt, 1H, J_d = 9.5 Hz, J_t = 6.5 Hz, OCH_2), 3.75 - 3.67 (m, 2H), 3.5 (bm, 1H), 3.43 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.33 (t, 1H, $J_t \approx$ 5.0 Hz), 3.07 (bd, 1H, $J_d \approx$ 2.0 Hz), 2.68 (dd, 1H, $J_{3"e,3"a}$ = 12.5 Hz, $J_{3"e,4"}$ = 4.5 Hz, H-3"e), 2.42 (bd, 1H, OH), 2.20 (m, 2H, $OCH_2C\underline{H}_2$), 2.17 (s, 3H, Ac), 2.09 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.01 (s, 3H, Ac), 2.00 (s, 3H, Ac), 1.86 (s, 3H, Ac), 1.80 (t, 1H, J_t = 12.5 Hz, H-3"a), 1.64 (bs, 3H, CH_3), 1.55 (bs, 3H, CH_3).

Anal. Calcd for $C_{57}H_{73}O_{27}N$ (1204.2): C, 56.85; H, 6.11; N, 1.16. Found: C, 56.45; H, 6.25; N, 1.18.

4-Methyl-3-pentenyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (88). A mixture of diol **87** (95 mg, 0.08 mmol) and 0.5 M potassium hydroxide in 4:1 methanol-water (6 mL) was stirred at room temperature overnight. The reaction mixture was then deionized with Dowex® (H^+) resin, filtered, and the solvents were removed. The resulting solid was purified by column chromatography over silica gel (5 g) using 65:35:2 then gradually changing to 65:35:6 chloroform-methanol-water as eluant yielding 55 mg (quantitative) of **88** as a white solid: R_f 0.21 (65:35:5 chloroform-methanol-water); $[\alpha]_D$ -1° (*c* 1, methanol); ^1H NMR (D_2O, HOD) δ 5.23 (tm, 1H, J_t = 7.0 Hz, J_m = 1.0 Hz, HC=C), 4.54 (d, 1H, J_d = 8.0 Hz, H-1'), 4.50 (d, 1H, J_d = 8.0 Hz, H-1), 4.12 (dd, 1H, $J_{3',2'}$ = 10.0 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.00 (dd, 1H, J_d = 12.0 Hz, J_d = 2.0 Hz, possibly H-6 or H-6'), 3.97 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'), 3.93 - 3.81 (m, 5H), 3.79 - 3.59 (m, 11H), 3.58 (dd, 1H, J_d = 10.0 Hz, J_d = 8.0 Hz, H-2'), 3.31 (t, 1H, J_t = 9.0 Hz, H-2), 2.77 (dd, 1H, $J_{3"e,3"a}$ = 12.5 Hz, $J_{3"e,4"}$ = 4.5 Hz, H-

3"e), 2.20 (q, 2H, J_q = 7.0 Hz, $OCH_2C\underline{H}_2$), 2.04 (s, 3H, Ac), 1.81 (t, 1H, J_t = 12.5 Hz, H-3"a), 1.72 (s, 3H, CH_3), 1.66 (s, 3H, CH_3); ^{13}C NMR δ 175.68 ($NH\underline{C}OCH_3$), 174.71 (C-1), 136.67 [$(CH_3)_2\underline{C}$=C], 120.73 ($H\underline{C}$=C), 103.52 (C-1), 102.88 (C-1'), 100.67 (C-2"), 79.17 (C-4), 76.35 (C-3'), 76.01 (C-5'), 75.61 (C-5), 75.24 (C-3), 73.73 (C-6"), 73.66 (C-2), 72.61 (C-8"), 70.92 (OCH_2), 70.22 (C-2'), 69.18 (C-4"), 68.97 (C-7"), 68.33 (C-4'), 63.45 (C-9"), 61.86 (C-6'), 60.95 (C-6), 52.55 (C-5"), 40.50 (C-3"), 28.75 ($OCH_2\underline{C}H_2$), 25.68 (CH_3), 22.89 ($NHCO\underline{C}H_3$), 17.90 (CH_3). Some of the ^{13}C assignments remain tentative and are based on data reported for the methyl glycoside,[210] and on the lactose derivative **74**.

Molecular weight for $C_{29}H_{49}O_{19}N$: calcd 715.29, found (FAB, negative ion, TEA matrix): *m/z* 714 $(M-1)^-$.

3-Oxopropyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (89). A solution of **88** (3.5 mg, 4.9 μmol) in methanol (4 mL) was cooled to -70 °C and ozone gas was bubbled through the solution for 2 min. Argon was then passed throught the solution to remove the excess ozone. The solvents were removed and the residue was dissolved in methanol (2 mL). After cooling again to -70 °C, excess methyl sulfide (6 μL, 82 μmol) was added. The cooling bath was removed and after stirring for 5 min, the solvents were removed to give **89**, R_f 0.33 (5:4:1 chloroform-methanol-aqueous 0.2% calcium chloride) which was used directly in the subsequent conjugation without further purification or characterization.

GM_3-HSA Conjugate (90). A solution of the aldehyde **89** (≈ 3.5 mg) and human serum albumin (14 mg) in phosphate buffered saline (1.4 mL), pH 5.93, was stirred at room temperature for 45 min. To the reaction mixture was added a freshly prepared solution of sodium cyanoborohydride (100 μL, 16 mg/mL, 25 μmol) in PBS (pH 5.93) and stirring was continued for an additional 72 h. Exhaustive dialysis (Amicon, YM 10 ultrafiltration membrane) against water followed by lyophilization provided 14 mg of GM_3-HSA conjugate **90**. The sialic acid content of glycoconjugate **90** was estimated by the resorcinol-hydrochloric acid assay and from Graph 7, for A_T = 0.556, N = 5.

GM_3-KLH Conjugate (91). A solution of the aldehyde **89** (6 mg, ≈ 9 μ mol), and keyhole limpet hemocyanin (16 mg) in phosphate buffered saline (1.6 mL), pH 5.93, was stirred at room temperature for 2 h. To the reaction mixture was added a freshly prepared solution of sodium cyanoborohydride (100 μL, 28 mg/mL, 45 μmol) in PBS (pH 5.93) and the reaction mixture was stirred at room temperature overnight. Exhaustive dialysis (Amicon, YM 10 ultrafiltration membrane) against water followed by lyophilization provided 13 mg of GM_3-HSA conjugate **91**. The sialic acid content of glycoconjugate **91** was estimated by the resorcinol-hydrochloric acid assay and from Graph 8, for A_T = 0.217, N = 200.

4-Methyl-3-pentenyl *O*-[benzyl 5-acetamido-8-*O*-(5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylono-1',9-lactone)-4,7-di-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate]-(2→3)-*O*-(4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-6-*O*-benzoyl-β-D-glucopyranoside (92). A mixture of tetrol **82** (152 mg, 0.25 mmol), silver trifluoromethanesulfonate (67 mg, 0.26 mmol), 2,6-di-*t*-butylpyridine (67 mg, 0.35 mmol), powdered Drierite® (1.9 g), and dry tetrahydrofuran (3 mL) was strirred at room temperature under argon atmosphere for 4.5 h to ensure dryness. The reaction mixture was then cooled to -45 °C under argon and a solution of chloride **15** (197 mg, 0.21 mmol) in 1:1 dry toluene-tetrahydrofuran (1 mL) was then added dropwise. Upon completion of the addition, the reaction mixture was stirred for an additional 30 min at this temperature and then at 0 °C overnight. The reaction was then diluted with chloroform, filtered through Celite® and the organic filtrate was then washed with saturated aqueous sodium bicarbonate solution, dried (sodium sulfate), filtered, and concentrated to give a yellowish solid. This foam was then subjected to flash column chromatography over silica gel (10 g) using 10:10:1 chloroform then 60:1 chloroform-methanol as eluant to give 95 mg of solid. The resulting solid contained tetrasaccharide **92** along with several other components, therefore, the mixture was acetylated prior to further purification or characterization: R_f 0.06 (4:4:1 hexane-ethyl acetate-ethanol).

4-Methyl-3-pentenyl *O*-[benzyl 5-acetamido-8-*O*-(5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylono-1',9-lactone)-4,7-di-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate]-(2→3)-*O*-(2-*O*-acetyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzoyl-β-D-glucopyranoside (93). A solution of crude triol **92** (93 mg) was acetylated in 2:1 pyridine-acetic anhydride (9 mL) overnight, after which time the solvents were evaporated and the residue was coevaporated with toluene. Column chromatography over silica gel (4 g) using 6:6:1 hexane-ethyl acetate-ethanol as eluant gave 22 mg of **93** as a white solid after lyophilization from benzene which was contaminated with a small amount of by-product: R_f 0.25 (4:4:1 hexane-ethyl acetate-ethanol, 2 developments); ^{1}H NMR δ 8.05 (dm, 2H, J_d = 8.0 Hz, J_m < 1.0 Hz, aromatic), 7.60 (m, 1H, aromatic), 7.50 (m, 2H, aromatic), 7.40-7.24 (m, 10H, aromatic), 5.59 (d, 1H, J_d = 10.0 Hz, NH or NH'),** 5.37 (td, 1H, $J_t \approx$ 11.0 Hz, $J_{4'',3''e}$ = 4.5 Hz, H-4''),** 5.34 (dd, 1H, $J_{7'',8''}$ = 9.5 Hz, $J_{7'',6''}$ = 2.0 Hz, H-7''), 5.28 (d, 1H, J_d = 10.0 Hz, NH or NH'),** 5.25 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 5.22 (t, 1H, J_t = 9.5 Hz, H-3),*,** 5.22 (dd, 1H, $J_{7''',8'''}$ = 8.0 Hz, $J_{7''',6'''}$ = 1.5 Hz, H-7'''), 5.20 (ddd, 1H, $J_{8''',7'''}$ = 8.0 Hz, $J_{8''',9'''b}$ = 5.0 Hz, $J_{8''',9'''a}$ = 3.0 Hz, H-8'''), 5.14 (d, 1H, J_d = 11.5 Hz, $OC\underline{H}_2C_6H_5$), 5.06 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'),*,** 5.05 (tm, 1H, J_t = 7.0 Hz, J_m = 1.0 Hz, HC=C), 4.99 (td, 1H, $J_t \approx$ 11.0 Hz, $J_{4''',3'''e}$ = 5.0 Hz, H-4'''),** 4.98 (s, 1H, benzylidene), 4.94 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2),*,** 4.65 (dd, 1H, $J_{6a,6b}$ = 11.5 Hz, $J_{6a,5}$ < 1.0 Hz, H-6a),** 4.56 - 4.55 (m, 2H), 4.51 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1),*,** 4.43 (dd, 1H, $J_{6b,6a}$ = 11.5 Hz, $J_{6b,5}$ < 1.0 Hz, H-6b),** 4.38 (m, 2H), 4.36 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'),*,** 4.28 (dd, 1H, J_d = 13.0 Hz, J_d = 3.0 Hz, H-9''a or H-9'''a), 4.22 (q, 1H, J_q = 10.5 Hz, H-5'' or H-5'''),** 4.10 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'),*,** 4.08 (dd, 1H, $J_{6'a,6'b}$ = 12.0 Hz, $J_{6'a,5'}$ < 1.0 Hz, H-6'a),** 4.02 (dd, 1H, J_d = 13.0 Hz, J_d = 5.0 Hz, H-9''b or H-9'''b), 4.00 (m, 1H), 3.96 (dd, 1H, J_d = 10.5 Hz, J_d = 1.5 Hz, H-6'' or H-6'''), 3.82 (m, 1H), 3.81 (t, 1H, J_t = 9.5 Hz, H-4),*,** 3.80 (m, 1H, OCH_2), ** 3.74 (m, 1H, H-5),** 3.65 (dd, 1H, $J_{6'b,6'a}$ = 12.0 Hz, $J_{6'b,5'}$ < 1.0 Hz, H-6'b),** 3.50 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'),** 3.46 (dt, 1H, J_d = 9.5 Hz, J_t = 6.5 Hz, OCH_2),** 2.95 (bs, 1H, H-5'), 2.69 (dd, 1H, $J_{3'''e,3'''a}$ = 13.0 Hz, $J_{3'''e,4'''}$ = 4.5 Hz, H-3'''e),** 2.49 (dd, 1H, $J_{3''e,3''a}$ = 13.5 Hz, $J_{3''e,4''}$ = 5.0 Hz, H-3''e),** 2.26 (q, 2H, J_q = 7.0 Hz, $OCH_2C\underline{H}_2$),** 2.14 (s, 3H, Ac), 2.11 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.05 (s, 6H, 2 × Ac), 2.03 (s, 3H, Ac), 2.02 (s, 3H, Ac), 2.01 (m, 1H, H-3''a),** 1.92 (s, 3H, Ac), 1.90 (s, 3H, Ac), 1.82 (t, 1H, J_t = 13.0 Hz, H-

3'''a),** 1.67 (s, 3H, CH_3), 1.57 (s, 3H, CH_3). Some of the spectral assignments are tentative. *These assignments were confirmed by decoupling experiments, thus, decoupling of the doublet centered at δ 4.51 (H-1) collapsed the doublet of doublets at δ 4.94 (H-2) while irradiation of the doublet at δ 4.36 (H-1') collapsed the doublet of doublets at δ 5.06 (H-2'); decoupling of the doublet of doublets centered at δ 5.06 collapsed the doublet of doublets centered at δ 4.10 (H-3') along with the doublet at δ 4.36, likewise, decoupling of the doublet of doublets centered at δ 4.94 collapsed the triplet centered at δ 5.22 (H-3) along with the doublet at δ 4.51. **The indicated couplings were established by cross peaks in the 2D-COSY spectrum (see Appendix 3) and are summarized as follows; the sialic acid acetamido NH at δ 5.59 was found to be coupled to the sialic acid H-5 multiplet at δ 4.00 while the sialic acid acetamido NH at δ 5.28 was coupled to the sialic acid H-5 quartet at δ 4.22; the H-2' doublet of doublets at δ 5.06 was found to be coupled to the H-1' doublet at δ 4.36 and the H-3' doublet of doublets at δ 4.10 while the H-2 doublet of doublets at δ 4.94 was found to be coupled to the H-3 triplet at δ 5.22 and the H-1 doublet at δ 4.51 consistent with the decoupling experiments; the glucose H-6a doublet of doublets at δ 4.65 was coupled to the glucose H-6b doublet of doublets at δ 4.43 along with the H-5 multiplet at δ 3.74; the H-4 triplet at δ 3.81 was found to be coupled to the H-3 triplet at δ 5.22; the H-6'b doublet of doublets at δ 3.65 was coupled to the H-6'a doublet of doublets at δ 4.08; the H-4' doublets at δ 3.46 was found to be coupled to the H-3' doublet of doublets at δ 4.10; the methylene doublet of triplets centered at δ 3.46 was used to locate the corresponding obscured geminal methylene proton at δ 3.80; and the H-3''e doublet of doublets at δ 2.49 located the H-4'' triplet of doublets at δ 5.37 as well as the obscured H-3''a signal at ≈ δ 2.01 while the H-3'''a triplet at δ 1.82 was useful in locating the H-4''' triplet of doublets at δ 4.99 and the H-3'''e doublet of doublets at δ 2.69.

4-Methyl-3-pentenyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-(4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (94). To a solution of **93** (21 mg, 13 μmol) in methanol (2 mL) was added 500 μL of 0.5 M potassium hydroxide in 4:1 methanol-water and the reaction mixture was stirred at room temperature overnight. The reaction mixture was then neutralized with 500 μL of acetic acid and the solvents were removed. The resulting product was purified

by column chromatography over silica gel (1.1 g) using 65:35:10 then 60:40:10 chloroform-methanol-water as eluant to give 11 mg of **94**: R_f 0.2 (60:40:10 chloroform-methanol-water); ^1H NMR (D_2O, HOD) δ 7.58 (m, 2H, aromatic), 7.48 (m, 3H, aromatic), 5.73 (s, 1H, benzylidene), 5.23 (tm, 1H, J_t = 7.0 Hz, J_m < 1.0 Hz, HC=C), 4.65 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.51 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.41 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'), 4.28 (dd, 1H, $J_{3',2'}$ = 10.5 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.25 (bs, 1H, H-5'), 4.24 (dd, 1H, J_d = 12.5 Hz, J_d = 3.5 Hz), 4.16 (m. 1H), 4.05 (dd, 1H, $J_{6'a,6'b}$ = 12.5 Hz, $J_{6'a,5'}$ = 1.0 Hz, H-6'a), 3.94 - 3.59 (m, 20H), 3.58 (ddd, 1H, J_d = 12.5 Hz, J_d = 10.0 Hz, J_d = 4.5 Hz, H-4" or H-4'"), 3.32 (m, 1H, H-2), 2.79 (dd, 1H, J_d = 12.5 Hz, J_d = 4.5 Hz, H-3"e or H-3'"e), 2.66 (dd, 1H, J_d = 12.0 Hz, J_d = 4.5 Hz, H-3"e or H-3'"e), 2.36 (q, 2H, J_q = 7.0 Hz, $OCH_2C\underline{H}_2$), 2.07 (s, 3H, Ac), 2.05 (s, 3H, Ac), 1.76 (t, 1H, J_t = 12.5 Hz, H-3"a or H-3'"a), 1.73 (s, 3H, CH_3), 1.68 (t, 1H, J_t = 12.0 Hz, H-3"a or H-3'"a), 1.66 (s, 3H, CH_3).

4-Methyl-3-pentenyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (95). A solution of **94** (11 mg, 10 μmol) in 3:1 acetic acid-water (2.4 mL) was heated to 60 °C for 90 min and the solvents were then evaporated. The residue was dissolved in methanol (2 mL) containing 500 μL of 0.5 M potassium hydroxide in 4:1 methanol-water and the reaction mixture was stirred at room temperature overnight then neutralized with 500 μL of acetic acid and concentrated. The residue was purified by column chromatography over silica gel (1.2 g) using 65:35:2 then 6:4:1 chloroform-methanol-water as eluant to yield 5 mg of deblocked GD_3: R_f 0.14 (60:40:10 chloroform-methanol-water):$[\alpha]_D$ -5.7° (*c* 0.5, methanol); ^1H NMR (D_2O, HOD) δ 5.23 (tm, 1H, J_t = 7.0 Hz, J_m < 1.0 Hz, HC=C), 4.53 (d, 1H, J_d = 8.0 Hz, H-1'), 4.49 (d, 1H, J_d = 8.0 Hz, H-1), 4.17 (m, 1H), 4.14 (m, 1H), 4.10 (dd, 1H, $J_{3',2'}$ = 10.0 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.00 (dd, 1H, J_d = 12.5 Hz, J_d = 2.0 Hz), 3.98 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'), 3.93-3.59 (m, 20H), 3.58 (dd, 1H, $J_{2',3'}$ = 10.0 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'), 3.31 (t, 1H, $J_t \approx 8.5$ Hz, H-2), 2.79 (dd, 1H, J_d = 12.5 Hz, J_d = 4.5 Hz, H-3'"e), 2.67 (dd, 1H, J_d = 12.5 Hz, J_d = 4.5 Hz, H-3"e), 2.35 (q, 2H, J_q = 7.0 Hz, $OCH_2C\underline{H}_2$), 2.07 (s, 3H, NHAc'"), 2.03 (s, 3H, NHAc"), 1.76 (t, 1H, J_t = 12.5 Hz, H-3'"a), 1.74 (t, 1H, J_t = 12.0 Hz, H-3"a), 1.72 (s, 3H, CH_3), 1.65 (s, 3H, CH_3); ^{13}C NMR δ 175.86 (2 × $NH\underline{C}OCH_3$), 174.56 (C-1" or C-1'"), 174.12 (C-1"

or C-1'''), 136.67 [$(CH_3)_2\underline{C}$=C], 120.73 (H$\underline{C}$=C), 103.54 (C-1), 102.68 (C-1'), 101.43 (C-2'' or C-2'''), 101.21 (C-2'' or C-2'''), 79.01 (C-4), 78.85 (C-8''), 76.26 (C-3'), 75.98 (C-5'), 75.63 (C-5), 75.21 (C-3), 74.91 (C-6''), 73.70 (C-2'), 73.48 (C-6'''), 72.63 (C-8'''), 70.92 (OCH_2), 70.16 (2C, C-2' and C-7''), 69.31 (C-4''), 69.05 (C-4'''), 68.81 (C-7'''), 68.61 (C-4'), 63.46 (C-9''), 62.40 (C-9'''), 61.93 (C-6'), 60.92 (C-6), 53.13 (C-5''), 52.62 (C-5'''), 41.36 (C-3''), 40 29 (C-3'''), 28.75 ($OCH_2\underline{C}H_2$), 25.68 (CH_3), 23.15 ($NHCO\underline{C}H_3$''), 22.90 ($NHCOCH_3$'''), 17.91 (CH_3). Some of the ^{1}H assignments remain tentative and are based on data reported for the reducing sugar.[109] As wall, some of the ^{13}C assignments remain tentative and are based on data reported for the GM_3-methyl glycoside,[210] and on GM_3 derivative **88**.

Molecular weight for $C_{40}H_{66}O_{27}N_2$: calcd 1006.9, found (FAB, negative ion, TEA matrix): *m/z* 1005.2 $(M-1)^-$.

4-Methyl-3-pentenyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→8)-(benzyl 5-acetamido-2,4,7,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-*O*-(4,6-*O*-benzylidene-β-D-galactopyranosyl)-(1→4)-6-*O*-benzoyl-β-D-glucopyranoside (95). A mixture of tetrol **82** (37_ mg, 0.60 mmol), silver trifluoromethanesulfonate (193 mg, 0.75 mmol), 2,6-di-*t*-butylpyridine (172 mg, 0.9 mmol), powdered Drierite® (2 g), and dry tetrahydrofuran (4.5 mL) was stirred at room temperature under an argon atmosphere for 6 h to ensure dryness. The reaction mixture was then cooled to -45 °C under argon and a solution of chloride **18** (649 mg, 0.59 mmol) in dry toluene (1.5 mL) was added dropwise. Upon completion of the addition, the reaction mixture was stirred for an additional 30 min at this temperature and then at 0 °C overnight. After this time, the reaction mixture was diluted with chloroform and the solution was filtered through Celite®. The organic filtrate was then washed with saturated aqueous sodium bicarbonate, wa ed (sodium sulfate), filtered, and concentrated to give a yellowish foam. The foam was then subjected to flash column chromatography over silica gel (10 g) using 70:1 chloroform-methanol as eluant to give 460 mg of a mixture consisting mainly of starting tetrol **82**, hydrolysis product **29**, and tetrasaccharide **96**. This mixture was directly acetylated in the following step prior to any further purification or characterization.

4-Methyl-3-pentenyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*gaiacto*-2-nonulopyranosylonate)-(2→8)-(benzyl 5-acetamido-2,4,7,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-*O*-(2-*O*-acetyl-4,6-*O*-benzylidene-β-D-galacto-pyranosyl)-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzoyl-β-D-glucopyranoside (97). A solution of crude **96** (459 mg) was treated with 2:1 pyridine-acetic anhydride (24 mL) at room temperature overnight and then the mixture was coevaporated with toluene. Column chromatography over silica gel (10 g) using chloroform then 70:1 chloroform-methanol as eluant gave first, a small amount of **86**, followed by 128 mg of **97** (12% overall yield from chloride **18**) as a fluffy white solid upon lyophilization from benzene: R_f 0.25 (19:1 chloroform-methanol); $[\alpha]_D$ +40.4° (*c* 1, chloroform); ^{1}H NMR δ 8.03 (dd, 2H, J_d = 8.0 Hz, J_d = 1.0 Hz, aromatic), 7.58 (tm, 1H, J_d = 7.5 Hz, J_m < 1.0 Hz, aromatic), 7.45 (bt, 2H, J_t = 8.0 Hz, aromatic), 7.41-7.34 (m, 6H, aromatic), 7.32 - 7.28 (m, 9H, aromatic), 6.25 (d, 1H, $J_{NH'',5''}$ = 9.5 Hz, NH''),** 5.44 (ddd, 1H, $J_{8''',7'''}$ = 9.5 Hz, $J_{8''',9'''a}$ = 5.5 Hz, $J_{8''',9'''b}$ = 2.5 Hz, H-8'''), 5.36 (bs, 1H, H-7''), 5.33 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.29 (dd, 1H, $J_{7''',8'''}$ = 9.5 Hz, $J_{7''',6'''}$ = 1.5 Hz, H-7'''), 5.25 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.22 (t, 1H, J_t = 9.5 Hz, H-3),* 5.20 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.19 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.10 (dd, 1H, $J_{2',3'}$ = 10.0 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'),*,**5.07 (d, 1H, $J_{NH''',5'''}$ = 10.5 Hz, NH'''),** 5.03 (tm, 1H, J_t = 7.0 Hz, J_m < 1.0 Hz, HC=C), 4.96 (m, 1H, H-4'' or H-4'''),** 4.95 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2),*,**4.90 - 4.84 (m, 3H), 4.86 (s, 1H, benzylidene), 4.72 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 2.0 Hz, H-6a),** 4.62 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'),*,** 4.57 (dd, 1H, $J_{3',2'}$ = 10.0 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'),* 4.50 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1),*,** 4.43 (dd, 1H, $J_{6b,6a}$ = 12.0 Hz, $J_{6b,5}$ = 5.5 Hz, H-6b),** 4.23 (dd, 1H, $J_{9'''a,9'''b}$ = 12.5 Hz, $J_{9'''a,8'''}$ = 5.5 Hz, H-9'''a), 4.16 (dd, 1H, $J_{9'''b,9'''a}$ = 12.5 Hz, $J_{9'''b,8'''}$ = 2.5 Hz, H-9'''b), 4.12 (dd, 1H, $J_{6'a,6'b}$ = 12.0 Hz, $J_{6'a,5'}$ < 1.0 Hz, H-6'a), 4.05 (q, 1H, J_q = 10.5 Hz, H-5'''),** 3.95 (q, 1H, J_q = 10.0 Hz, H-5''),** 3.92 (dd, 1H, $J_{6'',5''}$ = 10.0 Hz, $J_{6'',7''}$ = 1.5 Hz, H-6''), 3.85 (dd, 1H, $J_{6''',5'''}$ = 10.5 Hz, $J_{6''',7'''}$ = 1.5 Hz, H-6'''), 3.82 (t, 1H, J_t = 9.5 Hz, H-4), 3.78 (m, 1H), 3.77 (dt, 1H, J_d = 9.5 Hz, $J_t \approx$ 7.0 Hz, OCH_2), 3.71 (ddd, 1H, $J_{5,4}$ = 9.5 Hz, $J_{5,6b}$ = 5.5 Hz, $J_{5,6a}$ = 2.5 Hz, H-5),** 3.67 (dd, 1H, $J_{6'b,6'a}$ = 12.0 Hz, $J_{6'b,5'}$ < 1.0 Hz, H-6'b), 3.46 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4'), 3.44 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.30 (bs, 1H, H-5'), 2.81 (dd, 1H, J_d = 12.5 Hz, J_d = 5.0 Hz, H-3''e or H-3'''e),** 2.80 (dd, 1H, J_d = 12.5 Hz, J_d = 5.0 Hz, H-3''e or H-3'''e),** 2.23 (m, 2H, $OCH_2C\underline{H}_2$), 2.19 (s, 3H, Ac), 2.17 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.05

(s, 3H, Ac), 2.02 (s, 3H, Ac), 2.01 (s, 6H, 2 × Ac), 2.00 (s, 3H, Ac), 1.99 (s, 3H, Ac), 1.94 (s, 3H, Ac), 1.90 (s, 3H, Ac), 1.89 (s, 3H, Ac), 1.87 (t, 1H, J_t = 12.5 Hz, H-3"a or H-3'"a), 1.77 (t, 1H, J_t = 12.0 Hz, H-3"a or H-3'"a), 1.65 (s, 3H, CH_3), 1.59 (s, 3H, CH_3). Some of the spectral assignments are tentative. *These assignments were confirmed by decoupling experiments, thus, decoupling of the doublet centered at δ 4.50 (H-1) collapsed the doublet of doublets at δ 4.95 (H-2) while irradiation of the doublet at δ 4.62 (H-1') collapsed the doublet of doublets at δ 5.10 (H-2'); decoupling of the doublet of doublets centered at δ 5.10 collapsed the doublet of doublets centered at δ 4.57 (H-3') along with the doublet at δ 4.62 likewise, decoupling of the doublet of doublets centered at δ 4.95 collapsed the triplet centered at δ 5.22 (H-3) along with the doublet at δ 4.50. **The indicated couplings were established by cross peaks in the 2D-COSY spectrum (see Appendix 3) and are summarized as follows; the sialic acid acetamido NH" at δ 6.25 was found to be coupled to the sialic acid H-5" multiplet at δ 3.95 while the sialic acid acetamido NH'" at δ 5.07 was coupled to the sialic acid H-5'" quartet at δ 4.05; the H-2' doublet of doublets at δ 5.10 was found to be coupled to the H-1' doublet at δ 4.62 while the H-2 doublet of doublets at δ 4.95 was found to be coupled to the H-1 doublet at δ 4.50, consistent with prior decoupling experiments; the glucose H-6a doublet of doublets at δ 4.72 was coupled to the glucose H-6b doublet of doublets at δ 4.43 along with the H-5 double doublet of doublets at δ 3.71; and the galactose H-6a doublet of doublets at δ 4.12 was coupled to the galactose H-6b doublet of doublets at δ 3.67.

Anal. Calcd for $C_{88}H_{106}O_{38}N_2$ (1799.8): C, 58.73; H, 5.94; N, 1.56. Found: C, 58.52; H, 6.23; N, 1.55.

4-Methyl-3-pentenyl *O*-(benzyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→8)-(benzyl 5-acetamido-2,4,7,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3)-*O*-(2-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzoyl-β-D-glucopyranoside (98). A solution of blocked tetrasaccharide **97** (85 mg, 47 μmol) in 3:1 acetic acid-water (6 mL) was heated to 60 °C and the reaction was monitored by TLC. After 5.5 h, the solvents were removed and the resulting mixture was purified by column chromatography over silica gel (4 g) using 60:1 then 50:1 chloroform-methanol as eluant to give first 13 mg (15%) of recovered **97** followed by 50 mg (62%) of the desired **98** as a

solid after lyophilization from benzene: R_f 0.16 (15:1 chloroform-methanol); $[\alpha]_D$ +16.7° (*c* 1, chloroform); ^{1}H NMR δ 8.03 (dd, 2H, J_d = 8.0 Hz, J_d = 1.0 Hz, aromatic), 7.60 (tm, 1H, J_d = 7.5 Hz, J_m < 1.0 Hz, aromatic), 7.45 (bt, 2H, J_t = 8.0 Hz, aromatic), 7.40-7.30 (m, 10H, aromatic), 6.30 (d, 1H, $J_{NH'',5''}$ = 10.0 Hz, NH''), 5.44 (ddd, 1H, $J_{8''',7'''}$ = 9.5 Hz, $J_{8''',9'''}$ = 5.5 Hz, $J_{8''',9'''}$ = 2.5 Hz, H-8'''), 5.37 - 5.31 (m, 3H), 5.27 (dd, 1H, $J_{7''',8'''}$ = 9.5 Hz, $J_{7''',6'''}$ = 2.0 Hz, H-7'''), 5.25 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.21 (t, 1H, J_t = 9.5 Hz, H-3), 5.19 (d, 1H, J_d = 12.0 Hz, $OC\underline{H}_2C_6H_5$), 5.09 (d, 1H, $J_{NH''',5'''}$ = 10.0 Hz, NH'''), 5.05 (dd, 1H, J_d = 12.0 Hz, J_d = 2.0 Hz), 5.01 (dd, 1H, $J_{2',3'}$ = 10.0 Hz, $J_{2',1'}$ = 8.0 Hz, H-2'), 5.01(m, 1H, HC=C), 4.96 (ddd, 1H, J_d = 12.5 Hz, J_d = 10.0 Hz, J_d = 4.5 Hz, H-4'' or H-4'''), 4.91 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{2,1}$ = 8.0 Hz, H-2), 4.86 (m, 1H, H-4'' or H-4'''), 4.85 (dd, 1H, J_d = 9.5 Hz, J_d = 2.0 Hz), 4.71 (dd, 1H, J_d = 11.5 Hz, J_d = 2.0 Hz), 4.61 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.49 (d, 1H, J_d = 8.0 Hz, H-1 or H-1'), 4.36 (dd, 1H, $J_{3',2'}$ = 10.0 Hz, $J_{3',4'}$ = 3.5 Hz, H-3'), 4.30 (dd, 1H, J_d = 12.0 Hz, J_d = 2.5 Hz), 4.30 (dd, 1H, J_d = 12.0 Hz, J_d = 5.5 Hz), 4.20 - 4.12 (m, 3H), 4.08 - 3.89 (m, 5H), 3.82 (dd, 1H, J_d = 10.5 Hz, J_d = 1.5 Hz), 3.80 (dd, 1H, J_d = 10.0 Hz, J_d = 2.0 Hz), 3.78 - 3.67 (m, 3H), 3.50 (bm, 1H), 3.42 (dt, 1H, J_d = 9.5 Hz, J_t = 7.0 Hz, OCH_2), 3.35 (bt, 1H, J_t = 7.0 Hz), 3.26 (bd, 1H, J_d = 2.5 Hz), 2.77 (dd, 1H, J_d = 12.5 Hz, J_d = 4.5 Hz, H-3''e or H-3'''e), 2.76 (dd, 1H, J_d = 12.5 Hz, J_d = 4.5 Hz, H-3''e or H-3'''e), 2.31 (m, 2H, $OCH_2C\underline{H}_2$), 2.17 (s, 3H, Ac), 2.13 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.09 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.02 (s, 6H, 2 × Ac), 1.96 (s, 3H, Ac), 1.95 (s, 3H, Ac), 1.92 (s, 3H, Ac), 1.90 (s, 3H, Ac), 1.88 (t, 1H, J_t = 12.5 Hz, H-3''a or H-3'''a), 1.83 (t, 1H, J_t = 12.5 Hz, H-3''a or H-3'''a), 1.66 (s, 3H, CH_3), 1.56 (s, 3H, CH_3). Some of the spectral assignments are tentative.

Synthesis of GD_3-4M3P (95) from (98). A solution of **98** (53 mg, 31 μ mol) was dissolved in methanol (2 mL) to which 0.5 M potassium hydroxide in 4:1 methanol-water (1 mL) was added and the mixture was stirred at room temperature overnight. The potassium hydroxide was then neutralized by the addition of excess acetic acid and the solvents were evaporated. Column chromatography over silica gel (4 g) using 65:35:6 then 6:4:1 chloroform-methanol-water as eluant provided 29 mg (93%) of the GD_3-4M3P derivative **95** (for spectral data, see prior synthesis of **95**).

***O*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside and α-anomer (99).**[109] A solution of GD_3-ceramide (8 mg, 5 μ mol) from bovine source (Genzyme) in methanol (5 mL) was cooled to ≈ -15 °C (dry ice/ethanol) and ozone was bubbled through the solution for 3 min. After this time, argon was passed through the solution for a few minutes, then the solvents were evaporated. The residue was taken up in 0.01 M aqueous sodium carbonate (2.5 mL) and the reaction was stirred at room temperature overnight. The reaction mixture was then neutralized with acetic acid and the solvents were evaporated. Column chromatography over silica gel (1 g) using 6:4:1 then 5:4:1 chloroform-methanol-water as eluant provided 2 mg (46%) of **99**: R_f 0.08 (5:4:1 chloroform-methanol-0.2% aqueous calcium chloride); ^1H NMR (D_2O, HOD, the sample was allowed to equlibrate overnight prior to spectral measurement and was found to be a 2:1 β/α mixture) δ 5.23 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1, α-anomer), 4.68 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1, β-anomer), 4.68 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.19 (dm, J_d = 13.0 Hz, J_m < 1.0 Hz), 4.16 (m, 1H), 4.11 (dt, J_d = 9.5 Hz, J_t = 3.0 Hz, H-3', α- and β-anomer), 4.01-3.56 (m, 22H), 3.30 (dd, $J_{2,3}$ = 9.0 Hz, $J_{2,1}$ = 8.0 Hz, H-2, β-anomer), 2.80 (dd, 1H, J_d = 12.5 Hz, J_d = 4.5 Hz, H-3'''e), 2.69 (dd, 1H, J_d = 12.5 Hz, J_d = 4.5 Hz, H-3"e), 2.09 (s, 3H, NHAc'''), 2.04 (s, 3H, NHAc"), 1.76 (t, 2H, J_t = 12.5 Hz, H-3'''a), 1.75 (t, 2H, J_t = 12.5 Hz, H-3"a). Some of the ^1H assignments remain tentative while others are based on data reported for this compound.[109]

Molecular weight for $C_{34}H_{56}O_{27}N_2$: calcd 924.8, found (FAB, negative ion, TEA matrix): *m/z* 923.87 $(M-1)^-$.

Synthesis of GD_3-reducing sugar (99) from (98). A solution of **98** (4 mg, 4 μmol) in methanol (4 mL) was colled to -70 °C (dry ice/acetone) and ozone was bubbled through the solution for 3 min. After this time, argon was passed through the solution for a few minutes and the solvents were evaporated. The residue was taken up in 0.01 M aqueous sodium carbonate (1.1 mL) and the reaction mixture was stirred at room temperature overnight. The reaction was neutralized with acetic acid and the solvents were evaporated. Column chromatography over silica gel (400 mg) using 6:4:1, then 5:4:1 chloroform-methanol-water as eluant provided 2.9 mg (79%) of **99**. The ^1H NMR and mass

spectra for **99** obtained in this manner were identical to that obtained from the reaction using GD_3 from natural source.

3-Oxopropyl *O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→8)-*O*-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (100). A solution of **95** (5 mg, 5 μmol) in methanol (5 mL) was cooled to -70 °C and ozone gas was bubbled through the solution for 2 min. Argon was then passed through the solution to remove the excess ozone. The solvents were evaporated and the residue was dissolved in methanol (2 mL), cooled to -70 °C, and excess methyl sulfide (6 μL, 82 μmol) was added. The cooling bath was removed and the reaction was stirred for 5 min then the solvents were evaporated. Trituration with ether (3×) followed by drying in vacuo gave approximately 5 mg of **100** which was used directly in the subsequent conjugation without further purification or characterization: R_f 0.2 (40:35:10 chloroform-methanol-water).

GD_3-HSA Conjugate (101). A solution of the aldehyde **100** (≈ 5 mg), and human serum albumin (14 mg) in phosphate buffered saline (1.4 mL) at pH 5.93 (or in acetate buffer at pH 4.65) was stirred at room temperature for 45 min, then a freshly prepared solution of sodium cyanoborohydride (100 μL, 16 mg/mL, 25 μmol) in PBS (pH 5.93 or 4.65) was added and stirring was continued for an additional 72 h. Exhaustive dialysis (Amicon, YM 10 ultrafiltration membrane) against water followed by lyophilization provided 13 mg of GD_3-HSA conjugate **101**. The sialic acid content of glycoconjugate **101** was estimated by the resorcinol-hydrochloric acid assay and from Graph 9, for A_T = 1.12, N = 9.2 (an absorbance A_T = 1.31 corresponds to a conjugation ratio of N = 11.2 for the reaction carried out in acetate buffer at pH 4.65).

GD_3-KLH Conjugate (102). A solution of the aldehyde **100** (6 mg, ≈ 6 μ mol) and keyhole limpet hemocyanin (16 mg) in acetate buffer (1.6 mL), pH 4.65, was stirred at room temperature for 15 min, then a freshly prepared solution of sodium cyanoborohydride (100 μL, 20 mg/mL, 32 μmol) in acetate buffer (pH 4.65) was added and the reaction mixture was stirred at room temperature for 60 h. Exhaustive dialysis (Amicon, YM 10 ultrafiltration membrane) against water followed by lyophilization provided 13 mg of GD_3-HSA conjugate **102**. The sialic

acid content of glycoconjugate **102** was estimated by the resorcinol-hydrochloric acid assay and from Graph 10, for A_T = 0.809, N = 654.

5.3 Immunization and Fusion.

Ganglioside antigens, as the corresponding KLH conjugates, were formulated into mg/mL solutions in PBS containing 22.5 μL/mL of Ribi adjuvant stock solution (Ribi Immunochemicals, Hamilton, MT). Male BALB/c mice (The Jackson Laboratory, Bar Harbor, ME), four to six weeks old, were given simultaneous s.c. injections (25 μL) on the inside of each hind leg. After receiving the priming dose, the mice were boosted with identical doses of the antigen solution at week four, then again at week eight. Finally, the mice received the last injection three days prior to sacrifice. The spleen cells were removed and dissociated into a single cell suspension.

The cell suspension containing the spleen cells was mixed with murine myeloma cell line Sp2/0 (American Type Culture Collection (ATCC), Rockville, MD) to a final 5:1 ratio of spleen:myeloma cells, then centrifuged and the supernatant was decanted. To the suspension was added 2 mL of PEG solution (10 g of PEG MW 4000, 1 mL DMSO, and 10 mL saline) and the mixture was incubated at 37 °C for a short time. After washing the cell suspension in culture medium, the cells were suspended in hypoxanthine/aminopterin/thymidine (HAT) supplemented culture medium.[200] Aliquots (50 μL) of this suspension were transferred to the wells of 96-well tissue culture plates and aliquots of the supernatant of spent medium from day six were evaluated by ELISA for reactivity towards the corresponding ganglioside-HSA conjugates.

Of the four potential anti-GM_3 clones thus obtained, one, namely B213.1, was selected for recloning. Similarily, of the three resulting anti-GD_3 clones, B212.2 was chosen for recloning. Recloning was carried out at a dilution of 1/3 cells per well and the precise details of this procedure appear elsewhere.[200] Preliminary isotype determination of B212.1 and B213.1 by ELISA using goat anti-mouse IgM and IgG (Southern Biotechnology Associates Inc., Birmingham, AL) revealed that both antibodies were of the IgM class.

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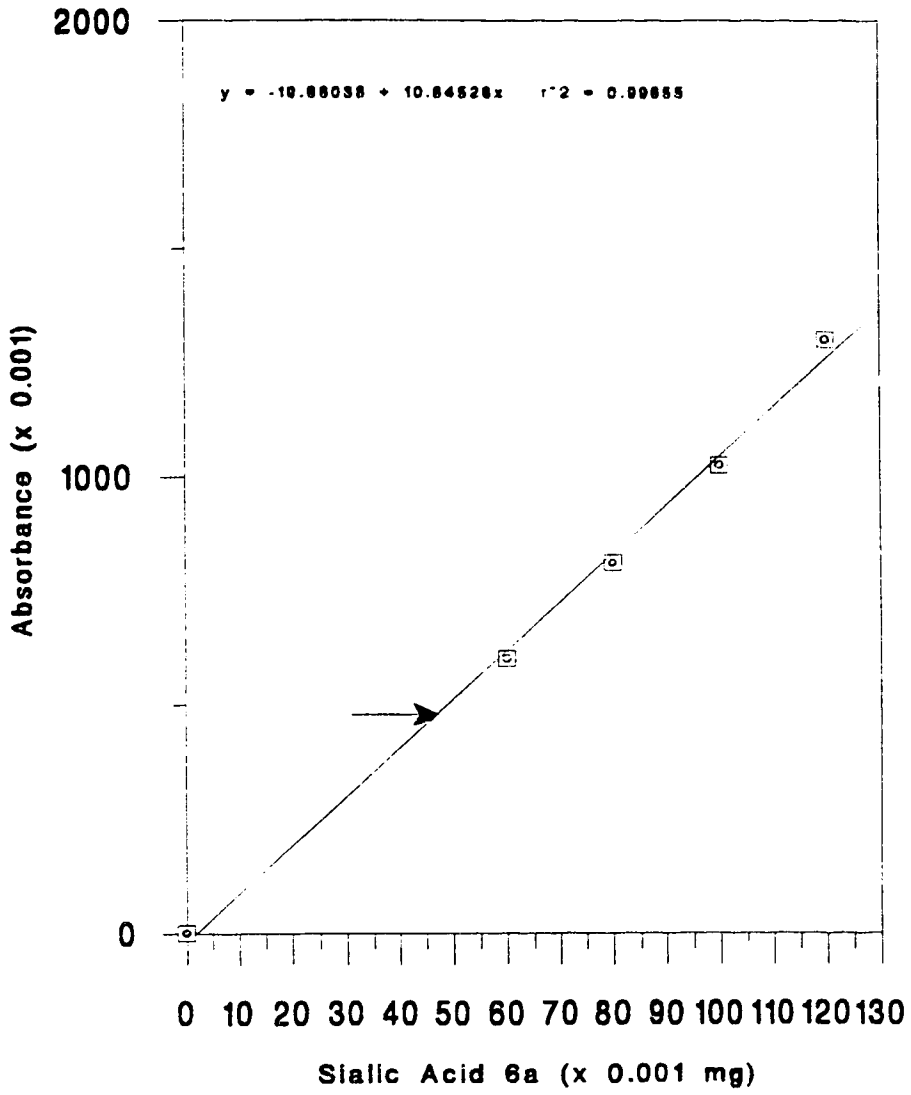
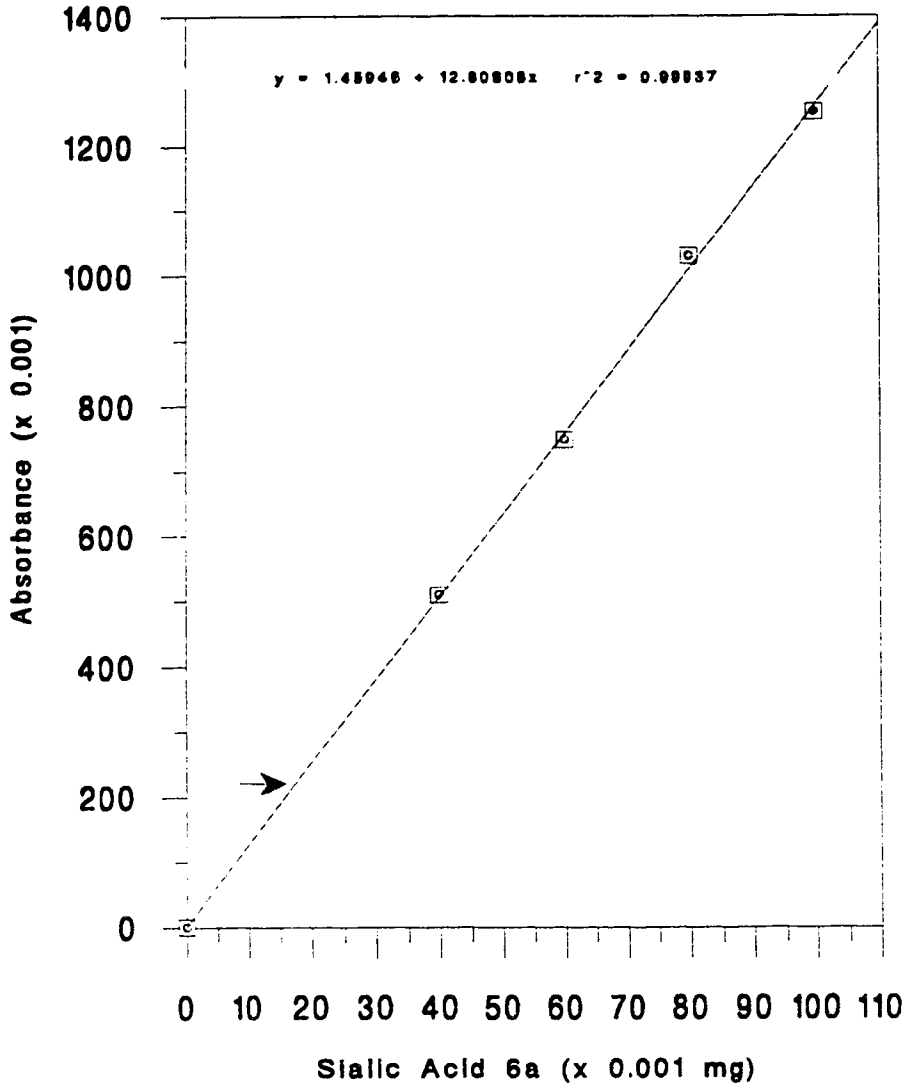
7. APPENDICES

Appendix 1: Carbohydrate Content

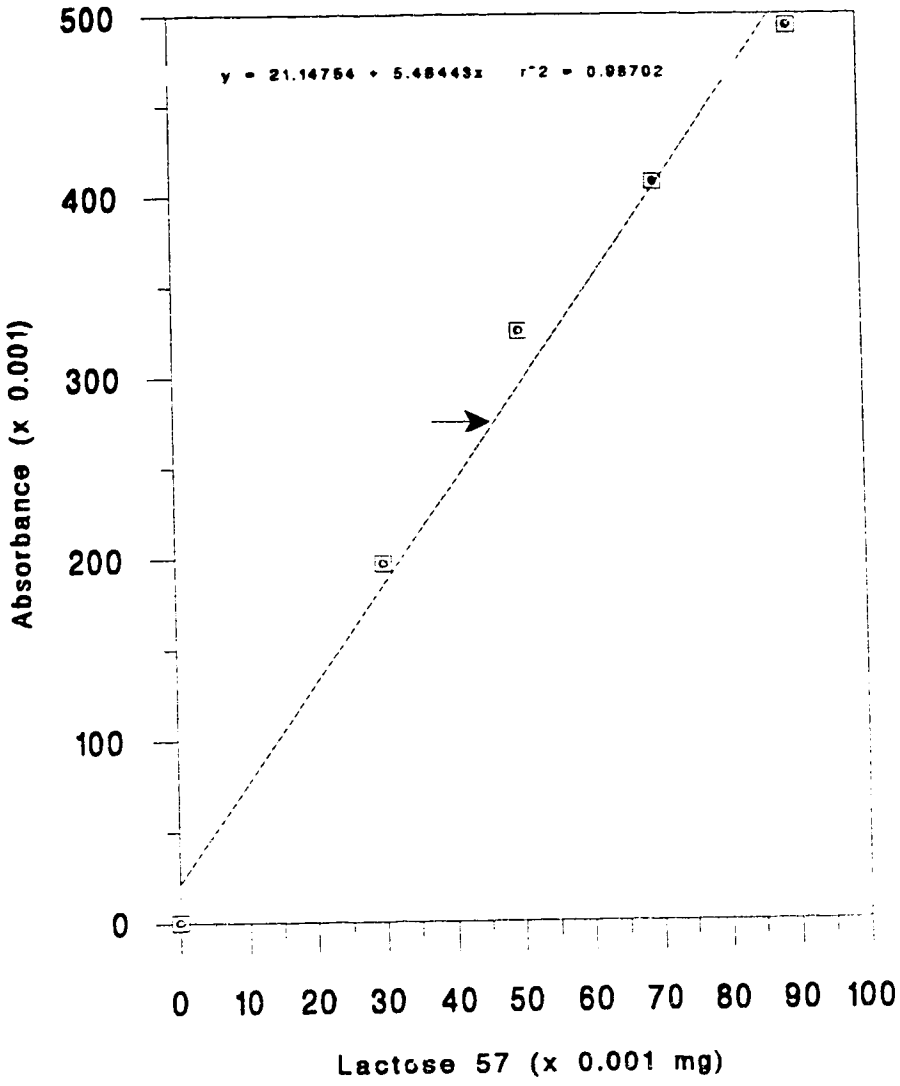
Graph 1: Sialic Acid - HSA 8



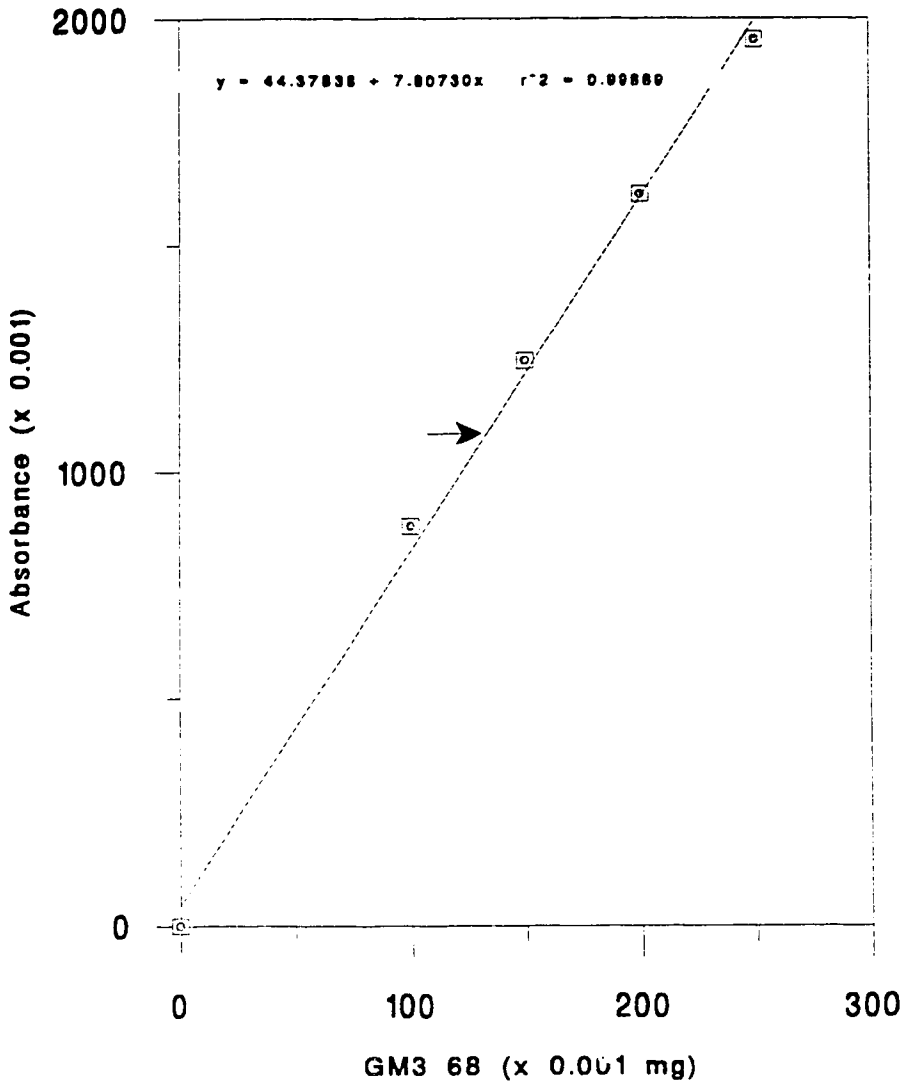
Graph 2: Sialic Acid - KLH 9



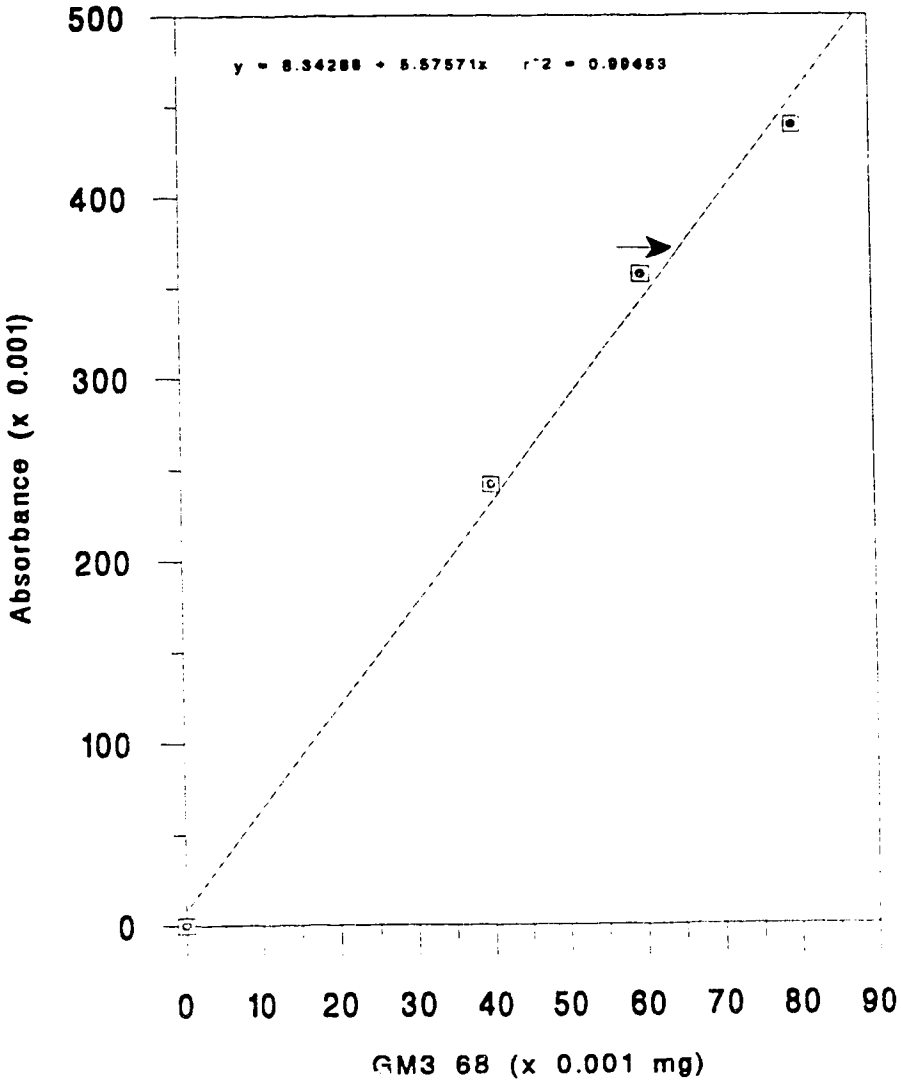
Graph 3: Lactose-HSA 60



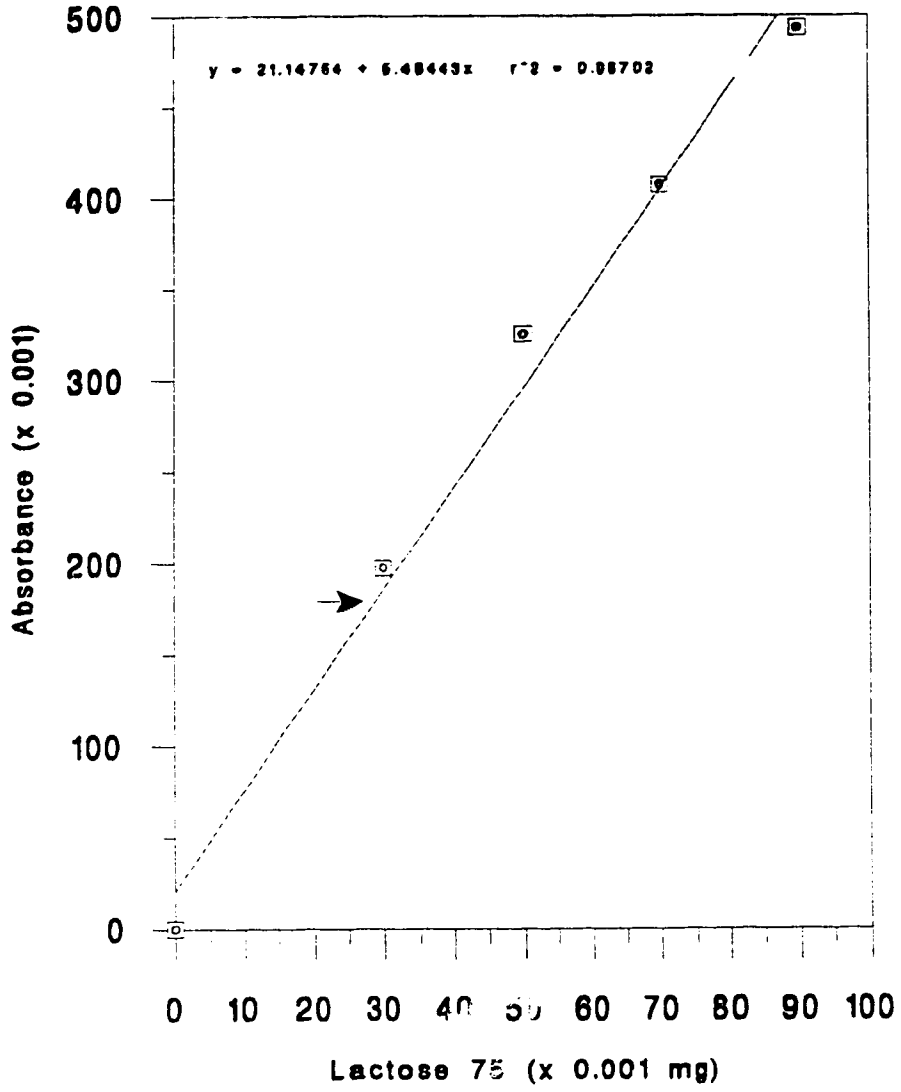
Graph 4: GM3-HSA 70



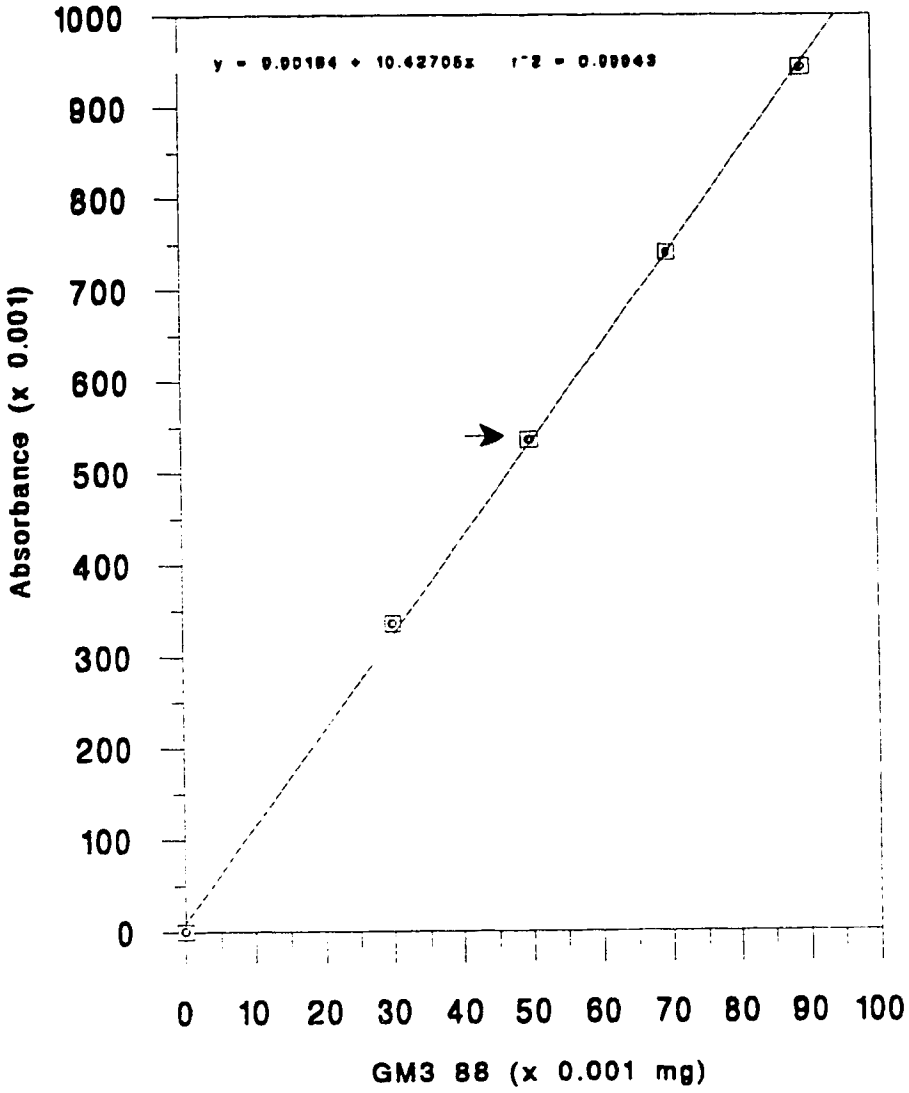
Graph 5: GM3-KLH 71



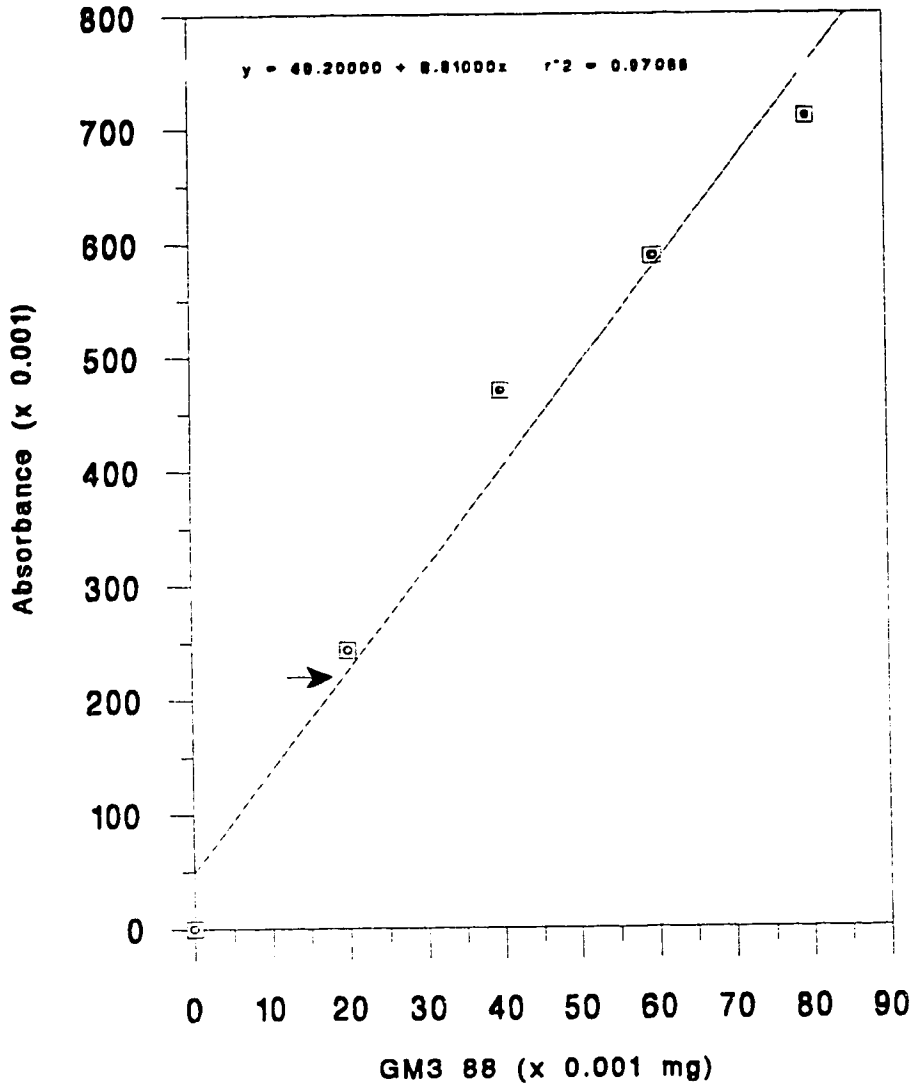
Graph 8: Lactose-HSA 76



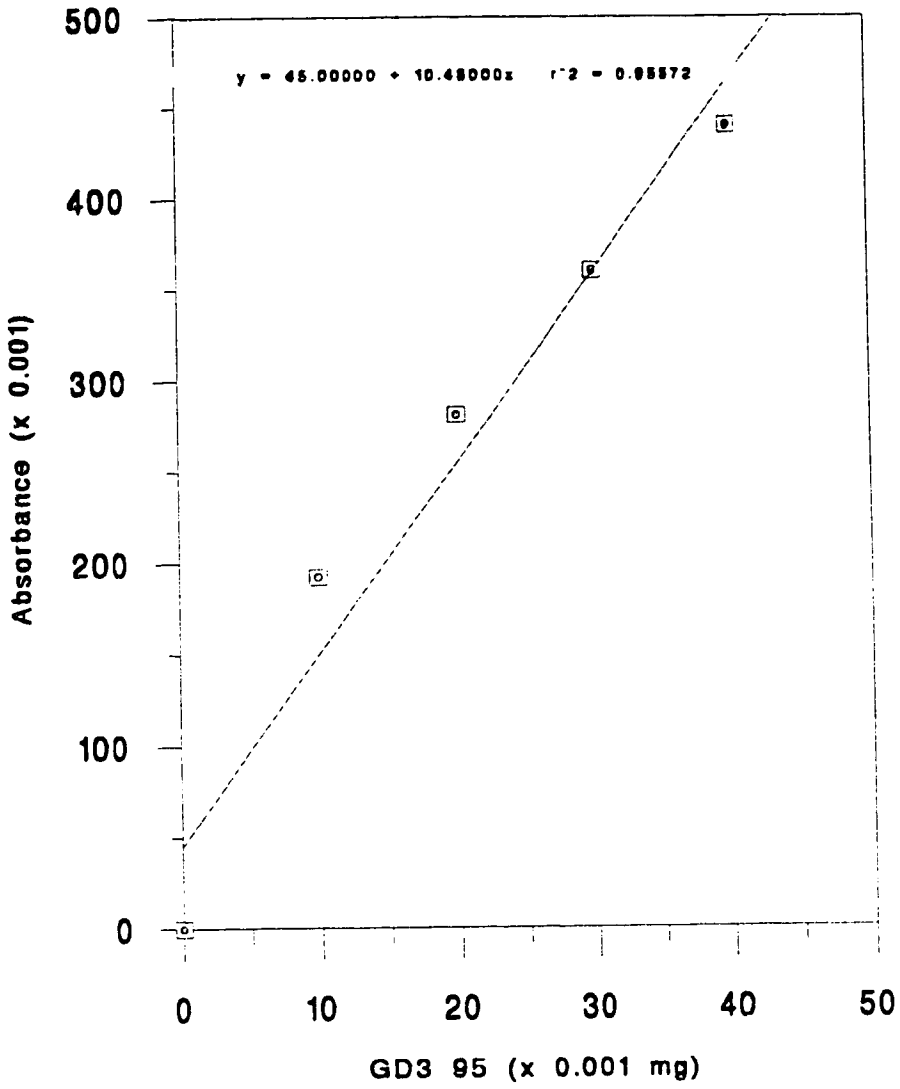
Graph 7: GM3-HSA 90



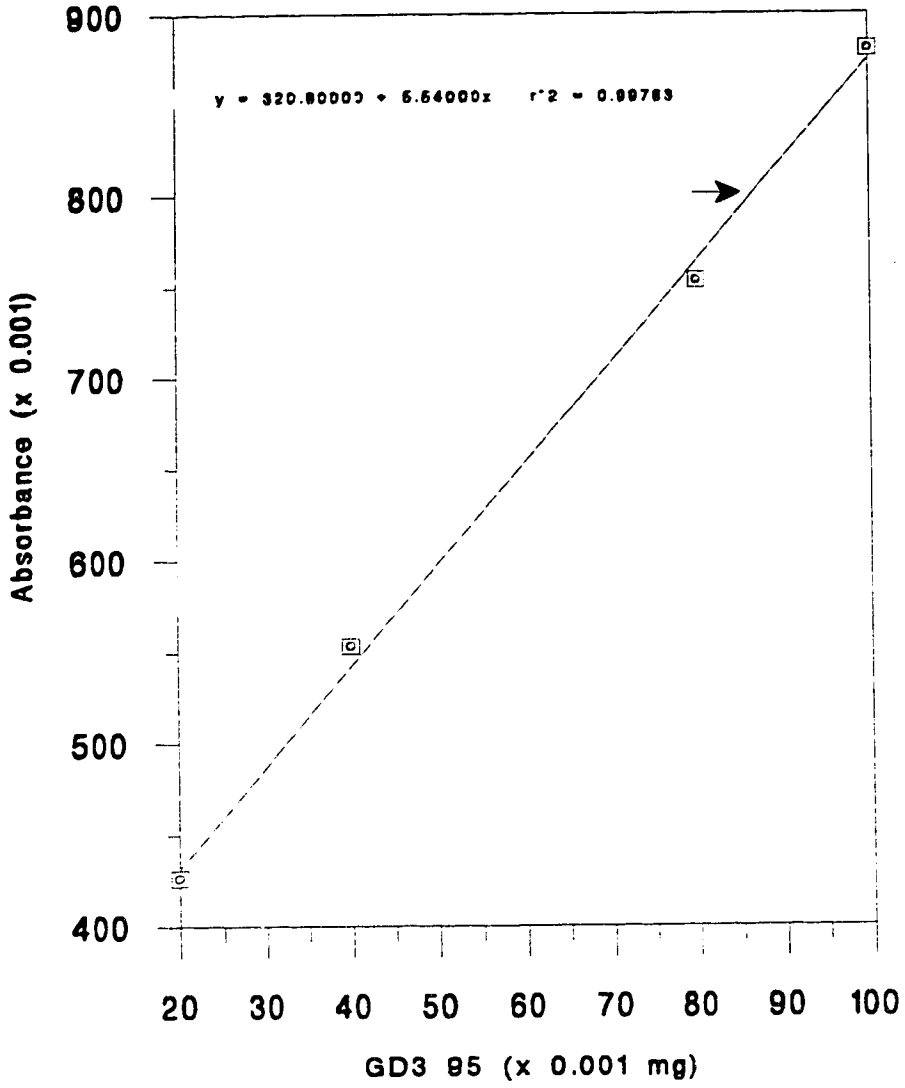
Graph 8: GM3-KLH 91



Graph 9: GD3-HSA 101

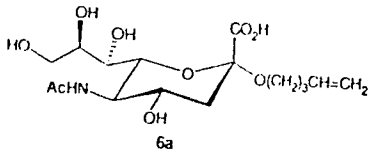


Graph 10: GD3-KLH 102



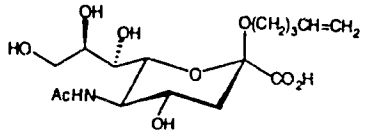
Appendix 2: ^{1}H NMR Spectra

300 MHz ^{1}H NMR Spectrum of **6a** (D_2O):



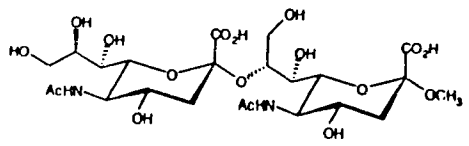
6a

300 MHz ^{1}H NMR Spectrum of **6b** (D_2O):



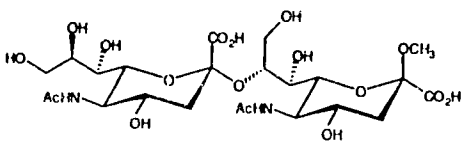
6b

400 MHz ^{1}H NMR Spectrum of **20a** (D_2O):



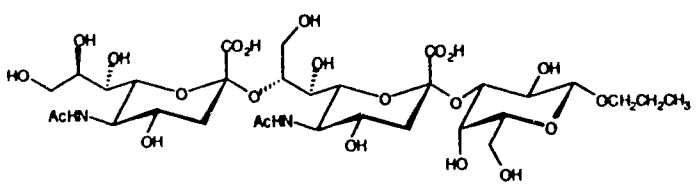
20a

400 MHz ^{1}H NMR Spectrum of **20b** (D_2O):



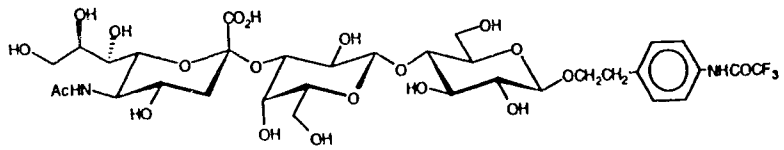
20b

400 MHz ^{1}H NMR Spectrum of **32** (D_2O):



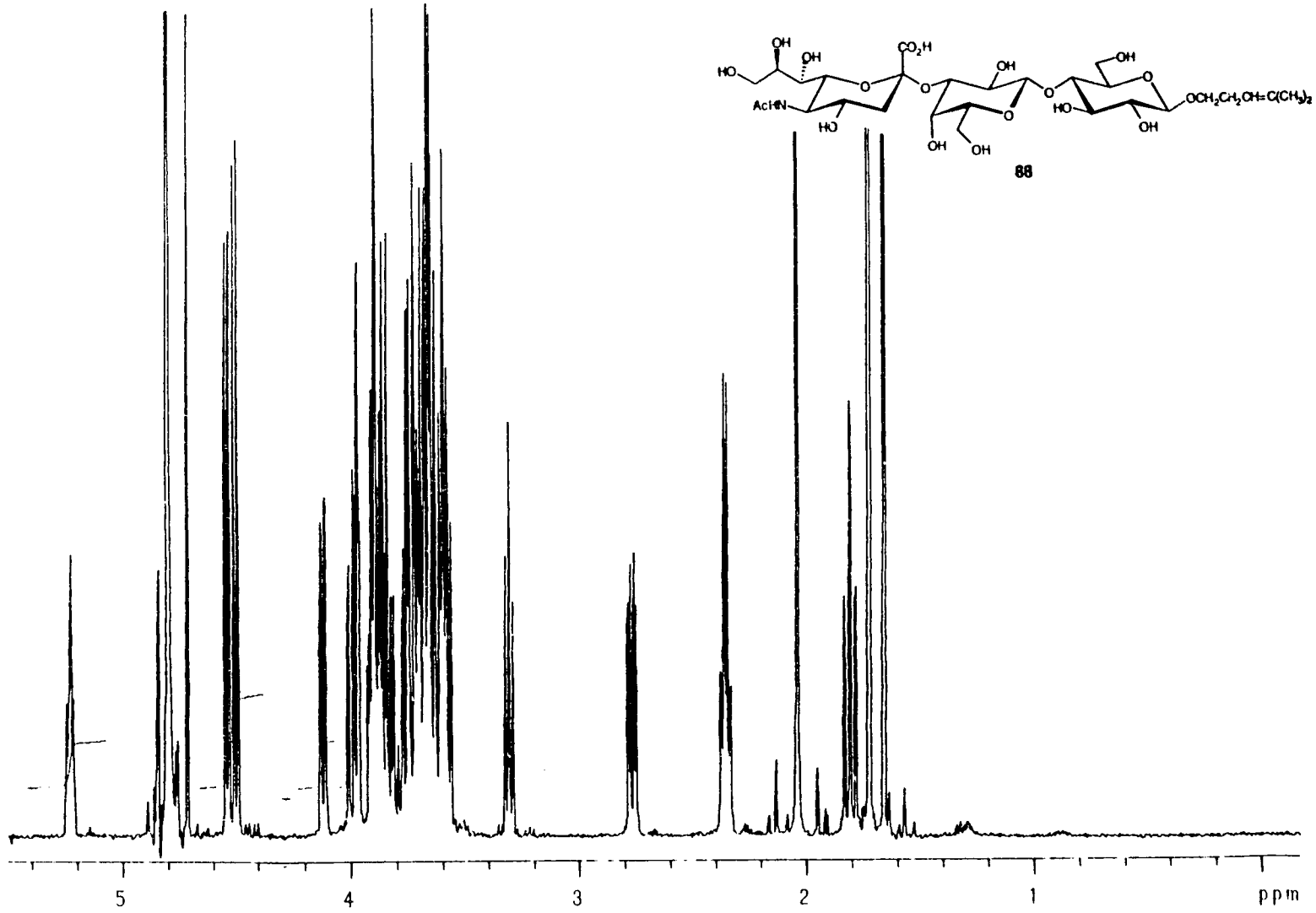
32

400 MHz ^{1}H NMR Spectrum of **67** (D_2O):



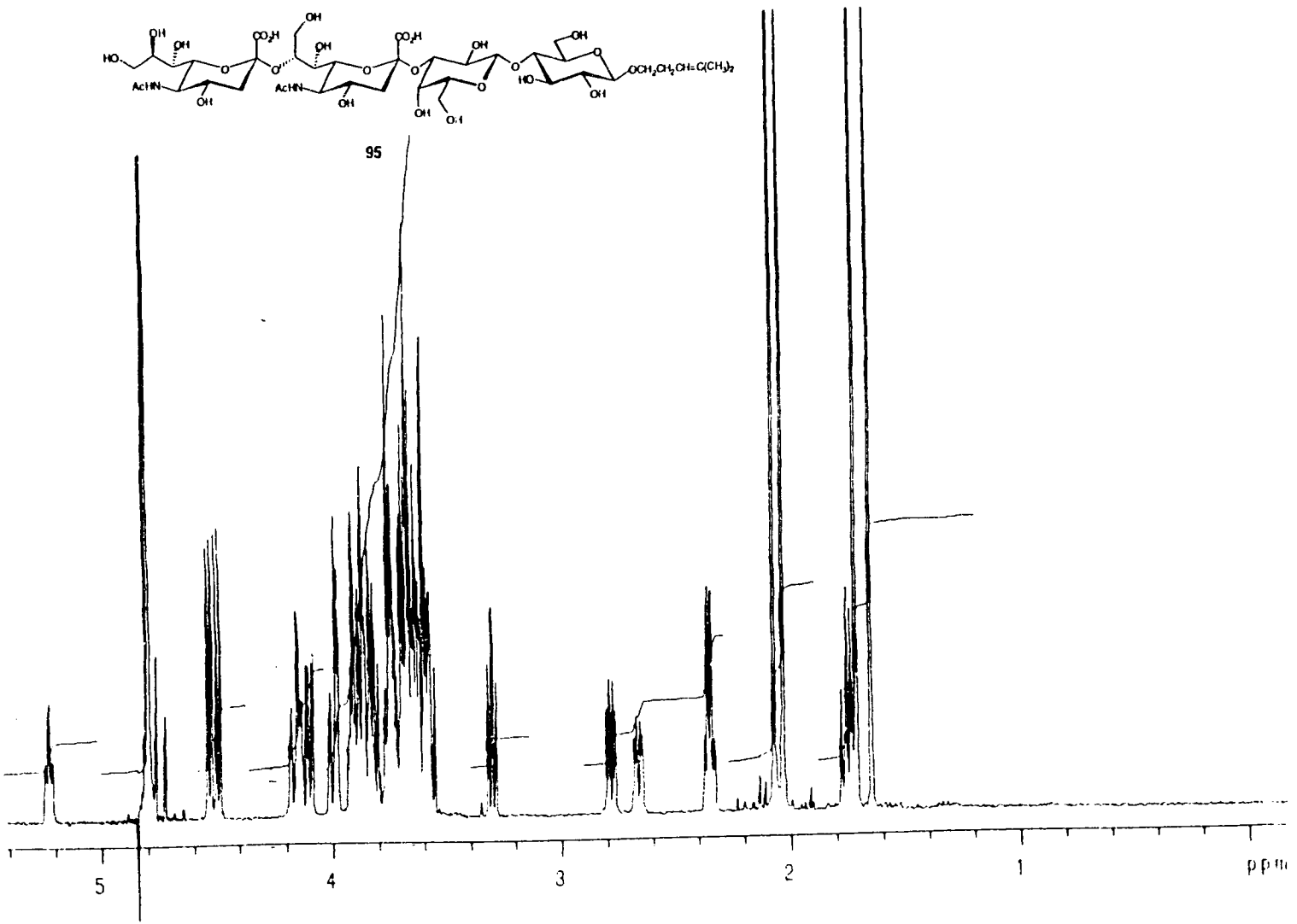
67

500 MHz ^{1}H NMR Spectrum of **88** (D_2O):

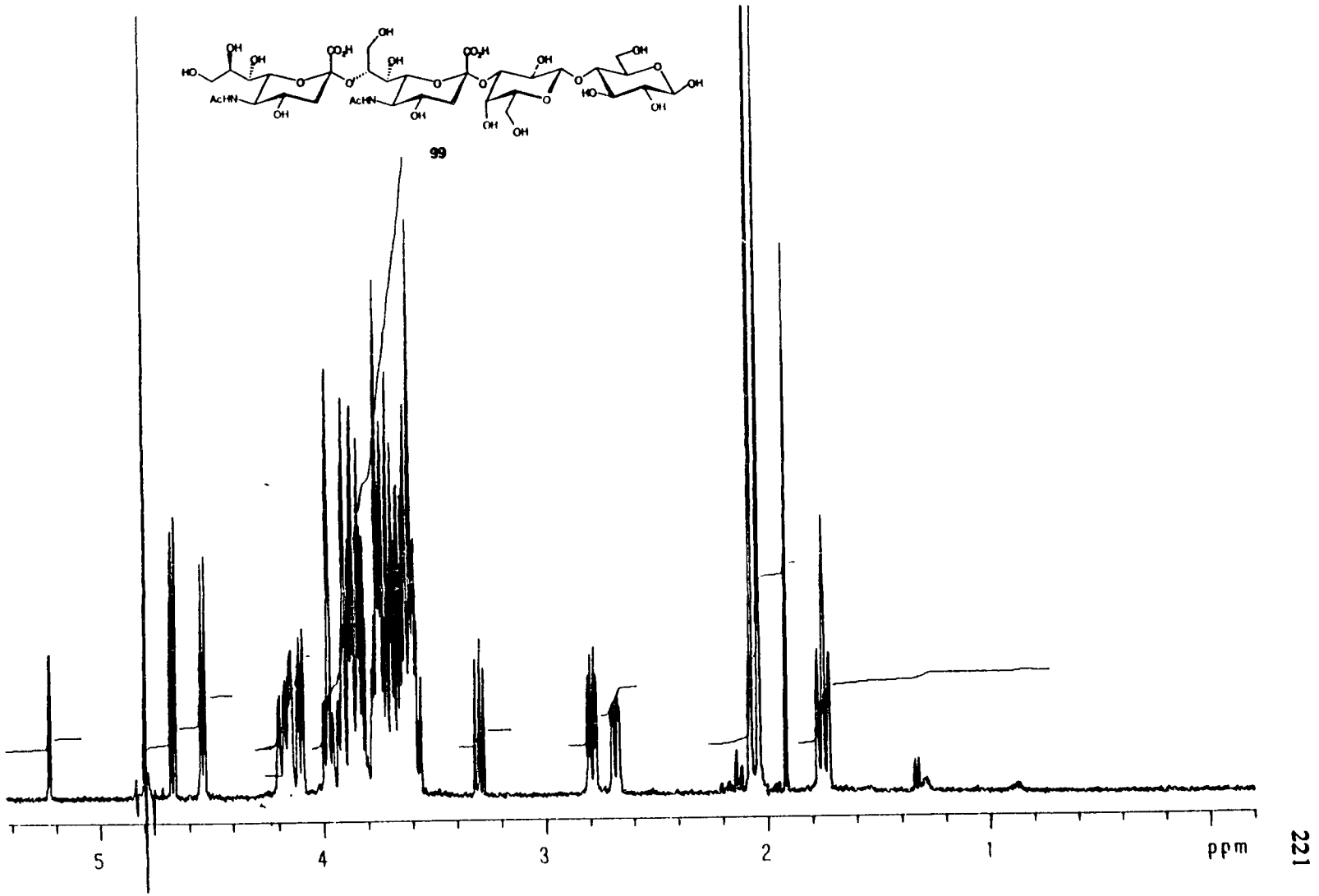


88

500 MHz ^{1}H NMR Spectrum of **95** (D_2O):

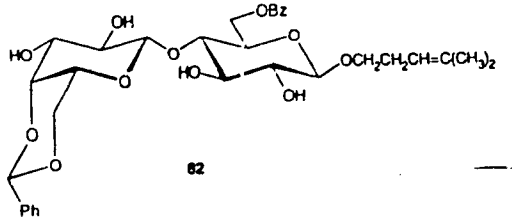


500 MHz ^{1}H NMR Spectrum of **99** (D_2O):

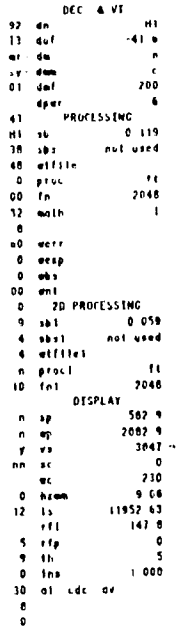


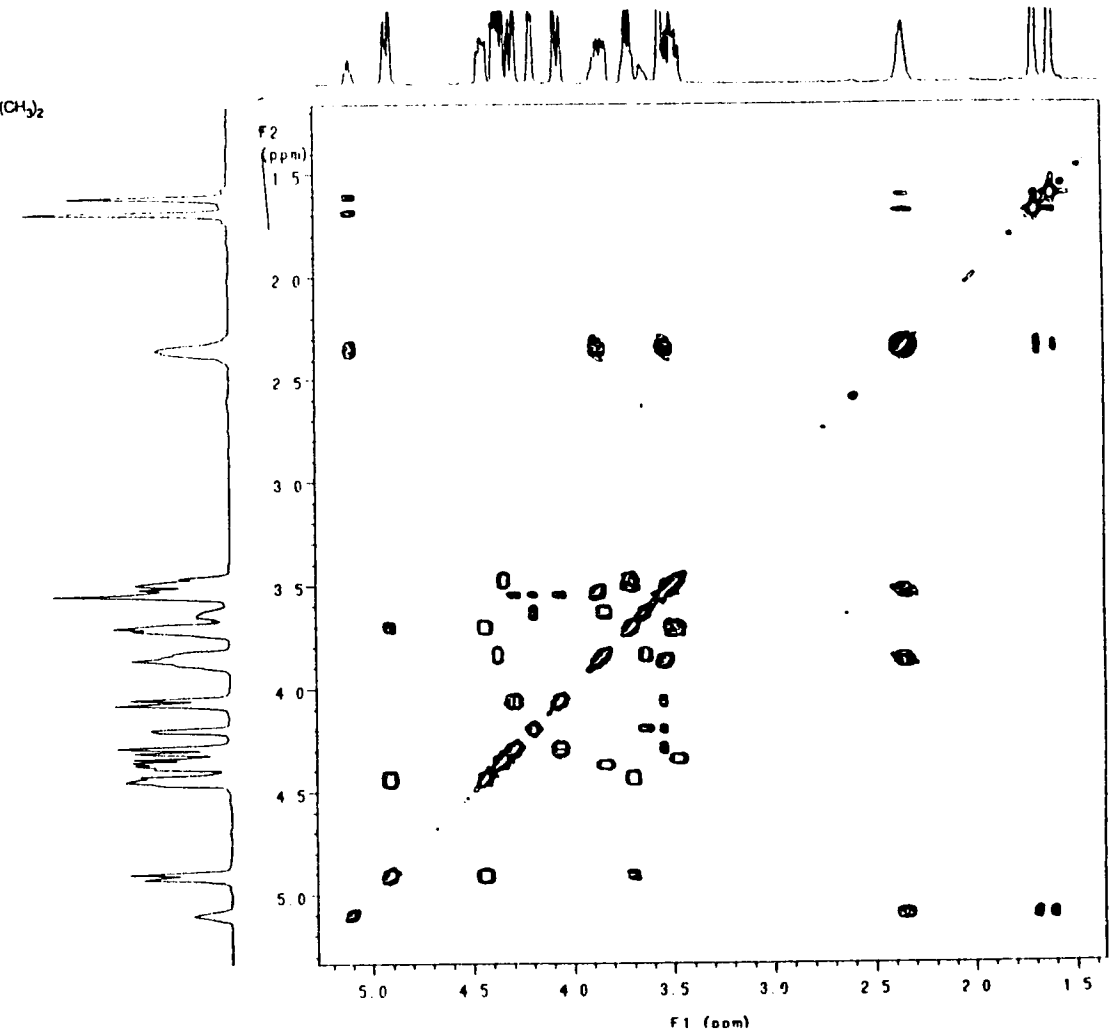
Appendix 3: 2D-COSY NMR Spectra

2D-COSY ^{1}H NMR Spectrum of **82**

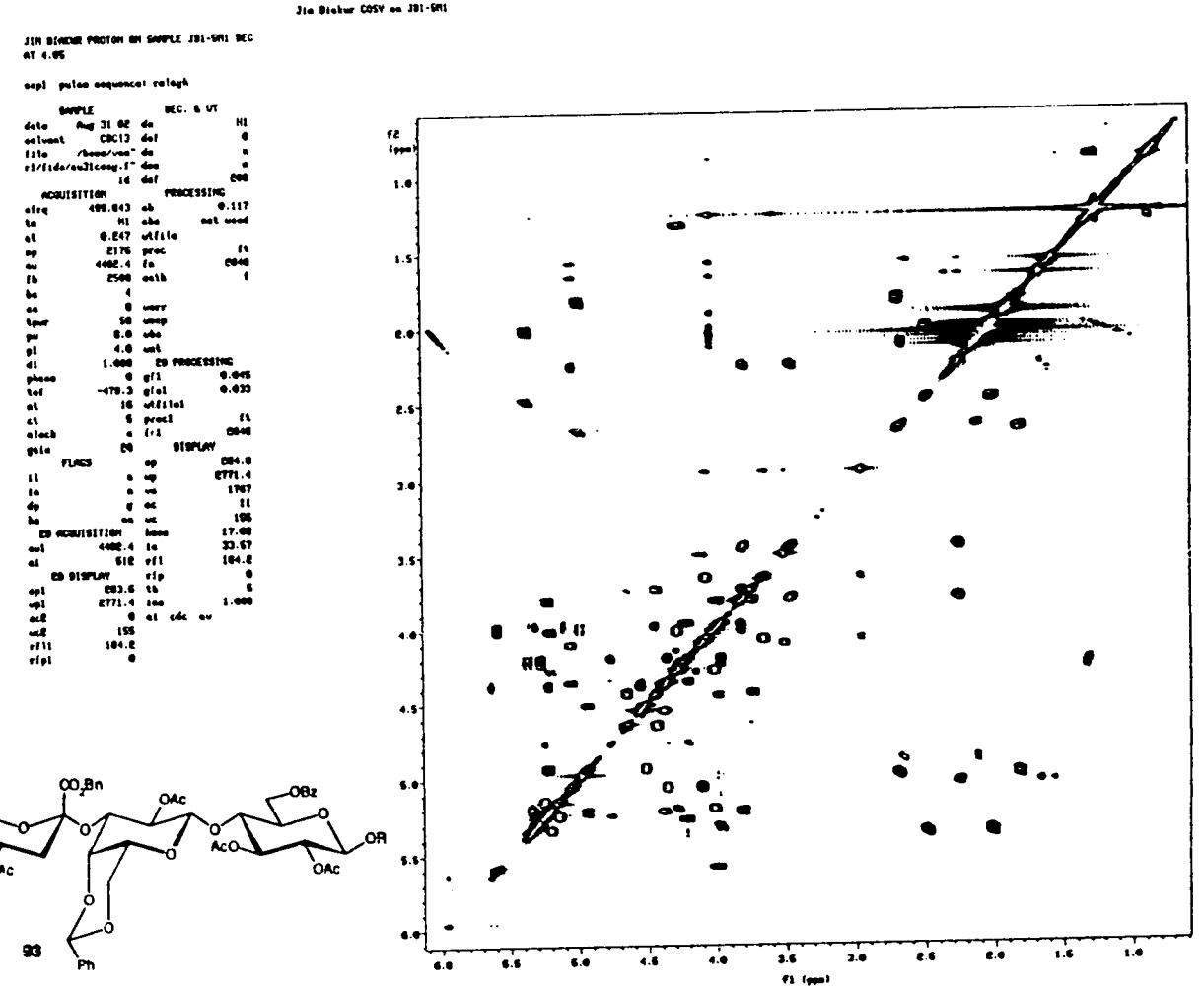


82



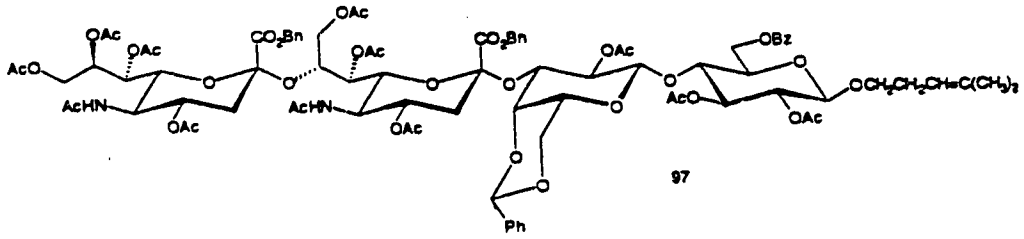


2D-COSY ^{1}H NMR Spectrum of **93**

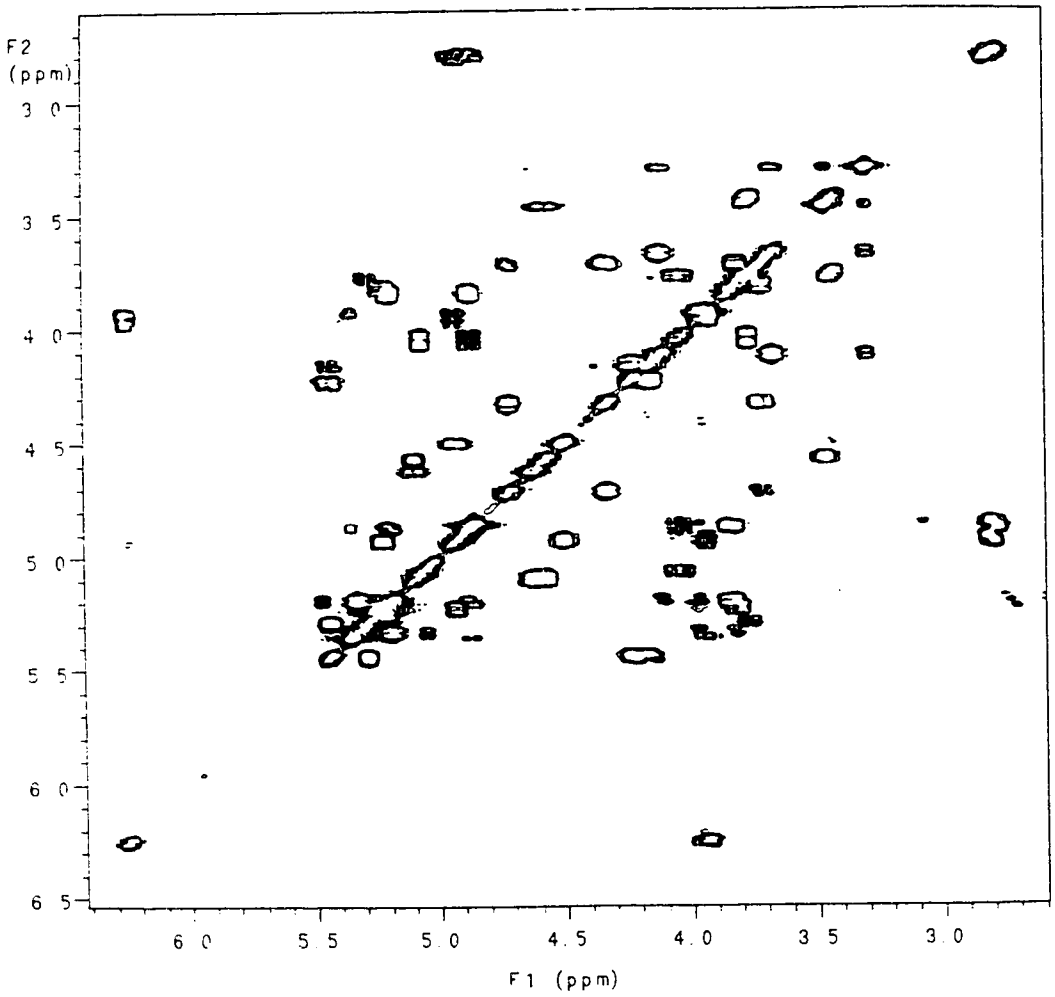


R = $CH_2CH_2CH{=}C(CH_3)_2$ **93**

2D-COSY ^{1}H NMR Spectrum of **97**

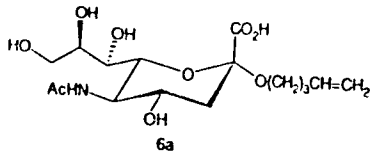


97

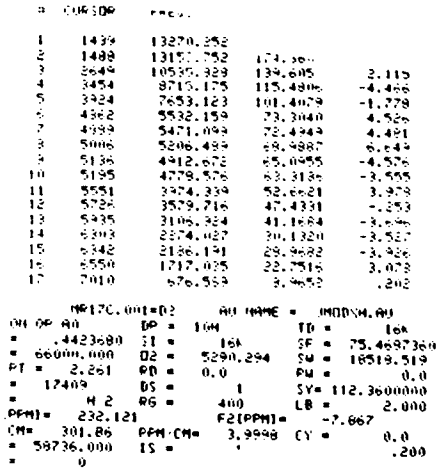


Appendix 4: ^{13}C NMR Spectra

75.47 MHz ^{13}C NMR Spectrum of **6a** (D_2O):



6a



1	1439	13270.252		
2	1408	13151.752	174.96	
3	3649	10535.329	134.605	2.115
4	3454	8715.175	115.480	-4.466
5	3924	7653.123	101.4079	-1.779
6	4362	5532.159	73.3040	4.526
7	4099	5471.099	72.4943	4.491
8	5006	5206.499	68.9887	6.644
9	5135	4912.673	65.0955	-4.526
10	5195	4779.576	63.3136	-3.555
11	5551	3574.339	52.6621	3.979
12	5726	3579.716	47.4331	-.253
13	5435	3106.324	41.1684	-3.696
14	6303	2374.027	30.1320	-3.527
15	6342	2186.191	28.9682	-3.926
16	6550	1717.035	22.7515	3.073
17	7010	676.559	8.9653	.202

```
      NR17C.001=D2          AU NAME =  IMODNM.AU
OH OP AQ          DP =  16H          TD =     16A
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=  6600m.000      D2 =   5290.294    SW =  16519.519
PT =   2.261      PD =    0.0        PW =       0.0
=   17409         DS =       1       SY= 112.3600000
=      M 2        RG =     400       LB =     2.000
PPM1=   232.121              F2[PPM]=    -7.867
CM=    301.86     PPM/CM=   3.9998   CY =       0.0
=  58736.000     IS =       '                  .200
=         0
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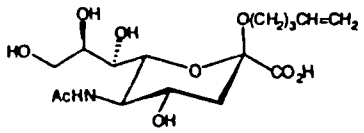
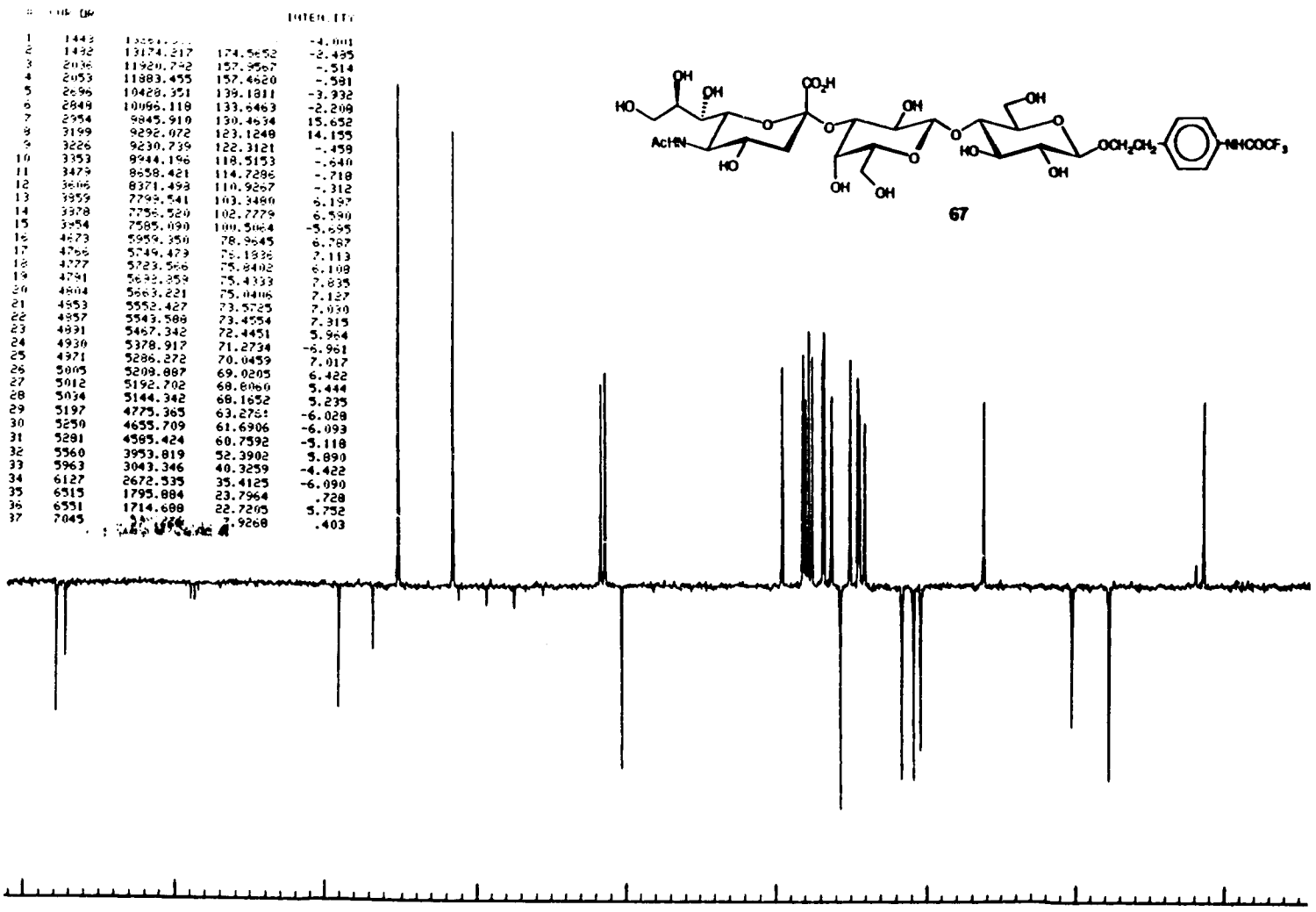
75.47 MHz ^{13}C NMR Spectrum of **6b** (D_2O):



6b

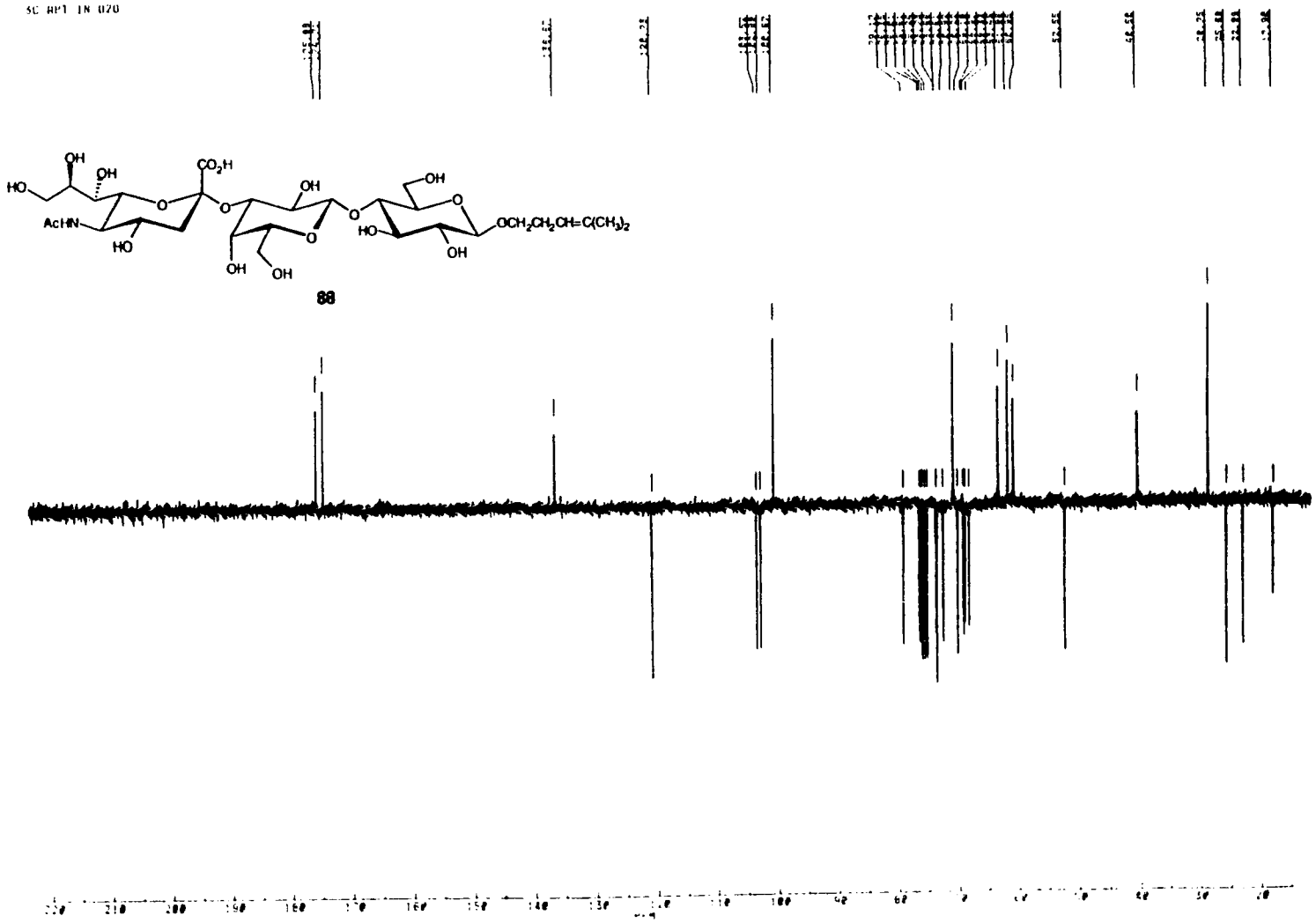
75.47 MHz ^{13}C NMR Spectrum of **67** (D_2O):



#				INTENSITY
1	1443	[illegible]		-4.001
2	1432	13174.217	174.5652	-2.485
3	2036	11920.742	157.9567	-.514
4	2053	11883.455	157.4620	-.581
5	2696	10428.351	138.1811	-3.932
6	2848	10066.118	133.6463	-2.209
7	2954	9845.910	130.4634	15.652
8	3199	9292.072	123.1248	14.155
9	3226	9230.739	122.3121	-.459
10	3353	8944.196	118.5153	-.640
11	3479	8658.421	114.7286	-.718
12	3606	8371.493	110.9267	-.312
13	3959	7799.541	103.3480	6.197
14	3978	7756.529	102.7779	6.590
15	3954	7585.090	100.5064	-5.695
16	4673	5959.350	78.9645	6.787
17	4766	5749.479	76.1836	7.113
18	4777	5723.566	75.8402	6.108
19	4791	5698.359	75.4333	7.835
20	4804	5663.221	75.0406	7.127
21	4853	5552.427	73.5725	7.030
22	4857	5543.508	73.4554	7.315
23	4891	5467.342	72.4451	5.964
24	4930	5378.917	71.2734	-6.961
25	4971	5286.272	70.0459	7.017
26	5005	5209.887	69.0205	6.422
27	5012	5192.702	68.8060	5.444
28	5034	5144.342	68.1652	5.235
29	5197	4775.365	63.2761	-6.028
30	5250	4655.709	61.6906	-6.093
31	5291	4565.424	60.7592	-5.118
32	5560	3953.819	52.3902	5.890
33	5963	3043.346	40.3259	-4.422
34	6127	2672.535	35.4125	-6.090
35	6515	1795.884	23.7964	.728
36	6551	1714.680	22.7205	5.752
37	7045	[illegible]	7.9268	.403

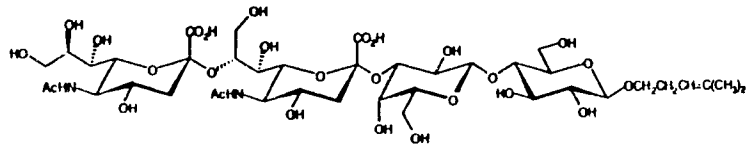
67

75.47 MHz ^{13}C NMR Spectrum of **88** (D_2O):

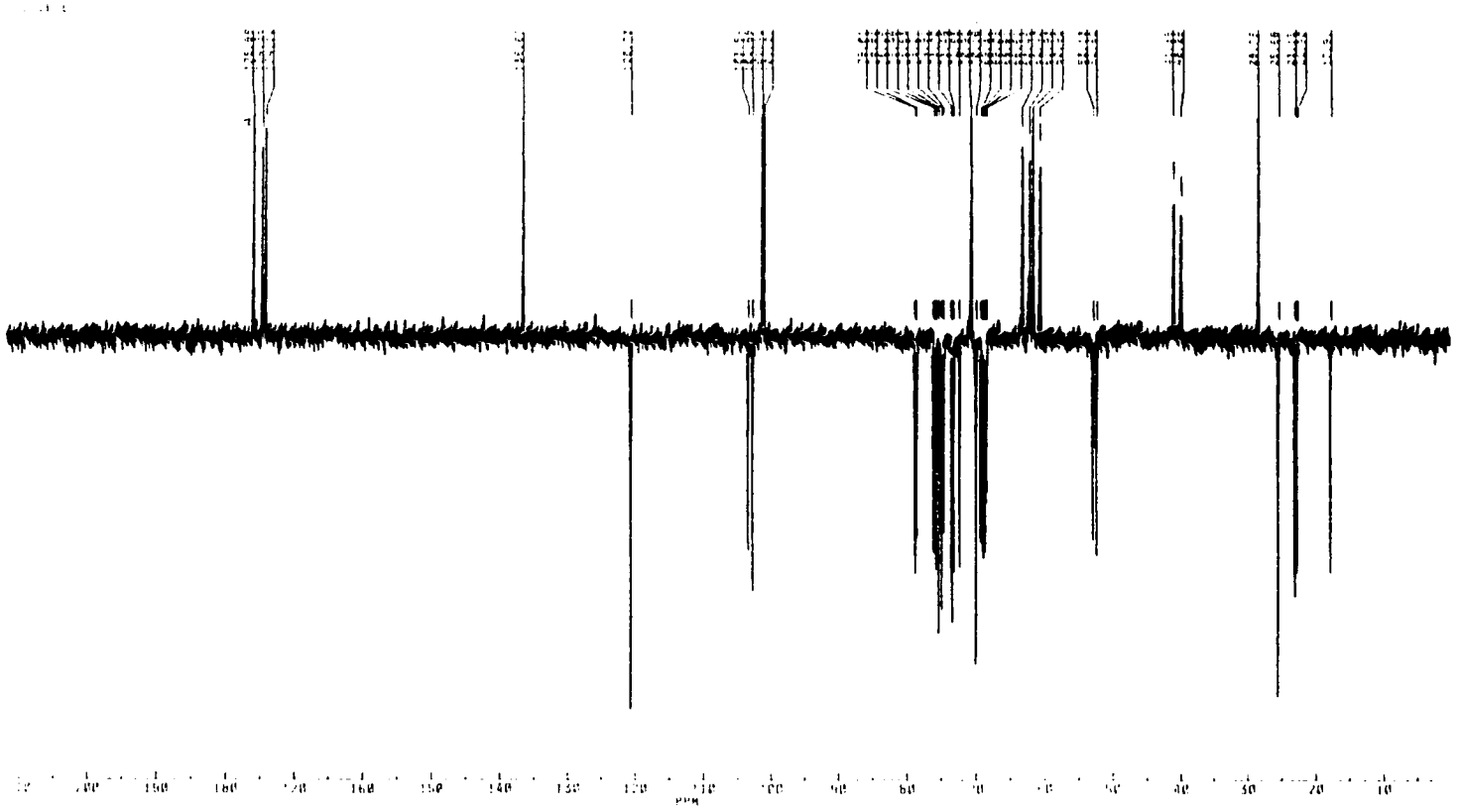


88

75.47 MHz ^{13}C NMR Spectrum of **95** (D_2O):

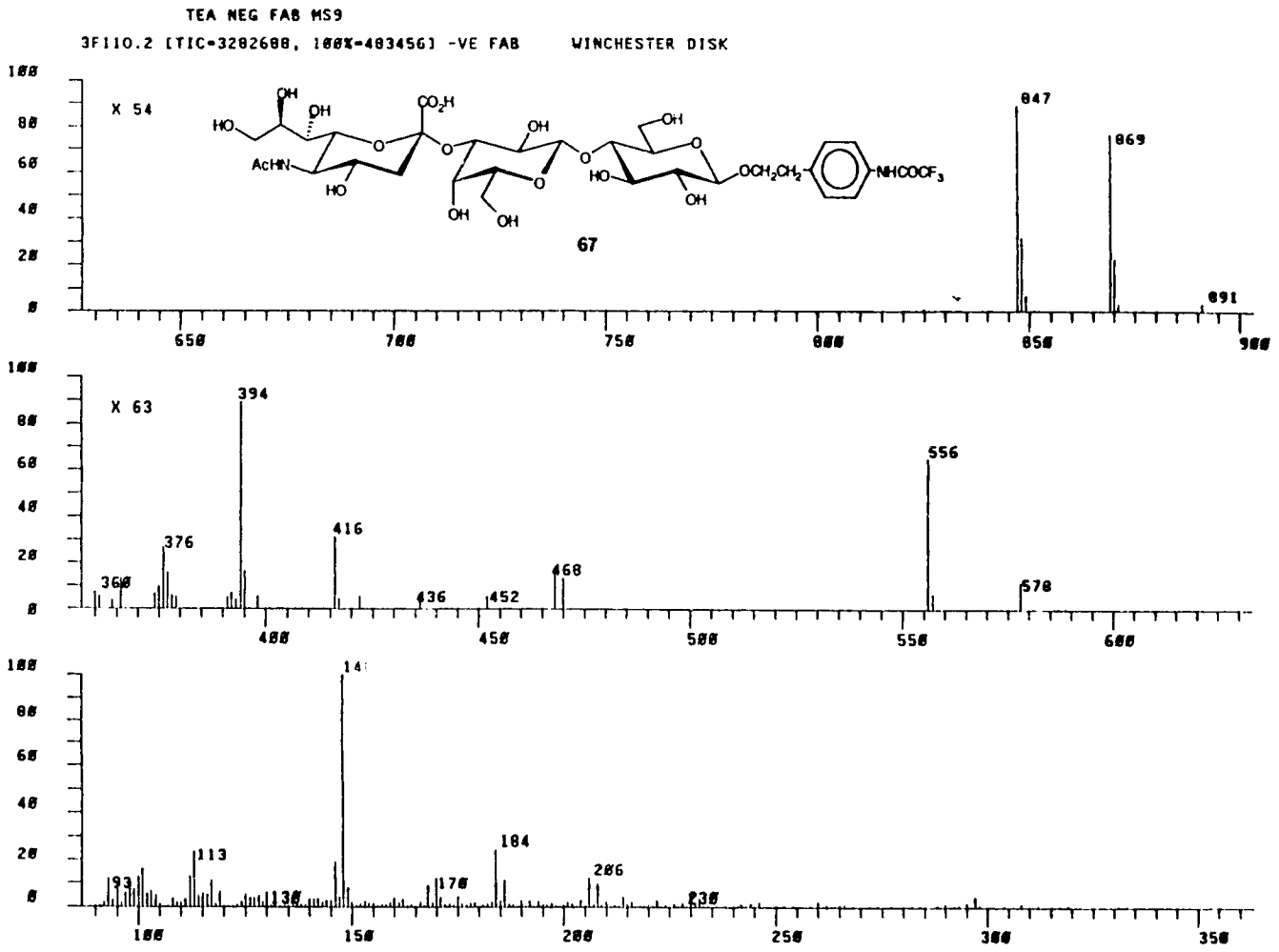


95

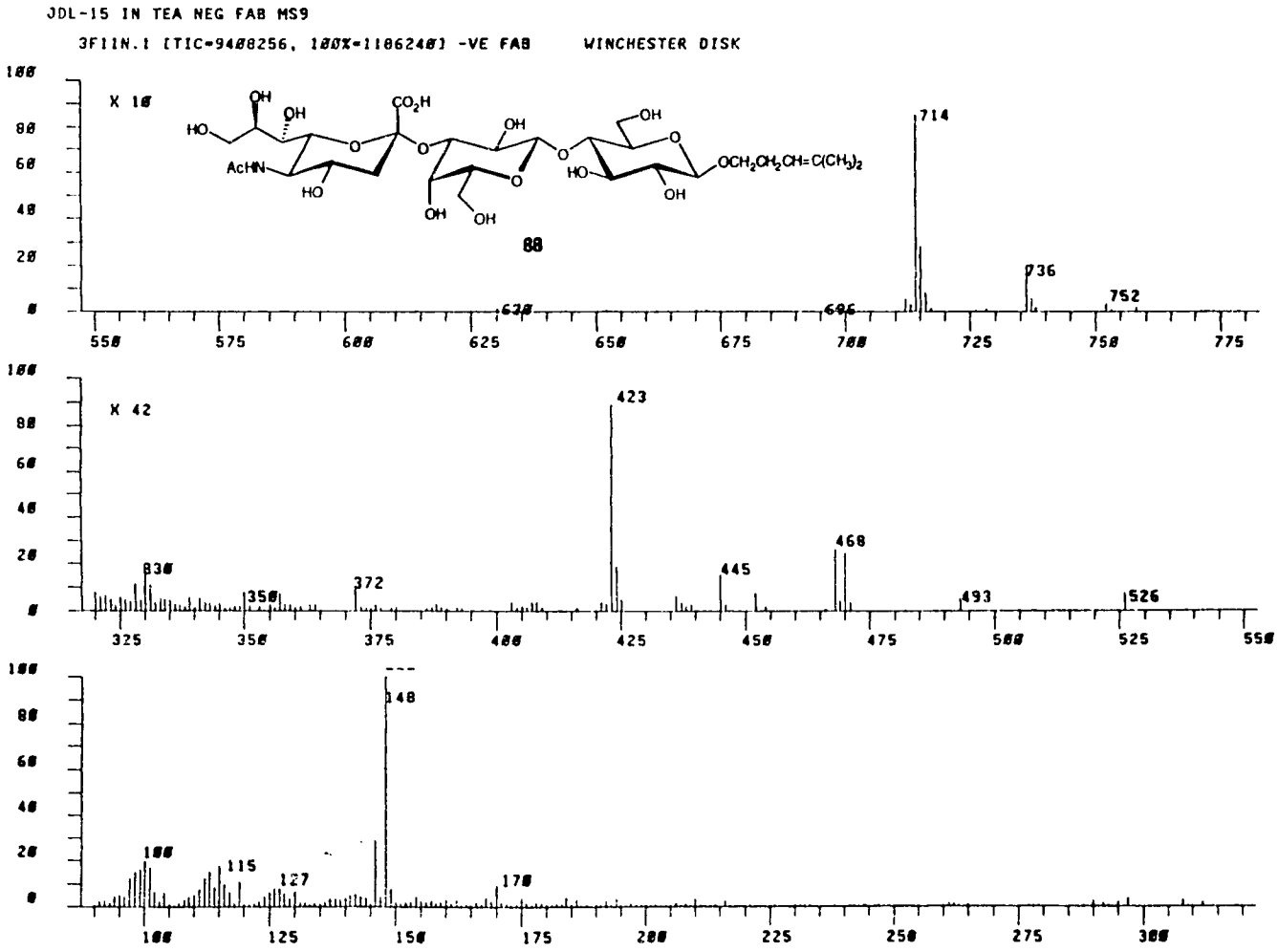


Appendix 5: FAB MS Spectra

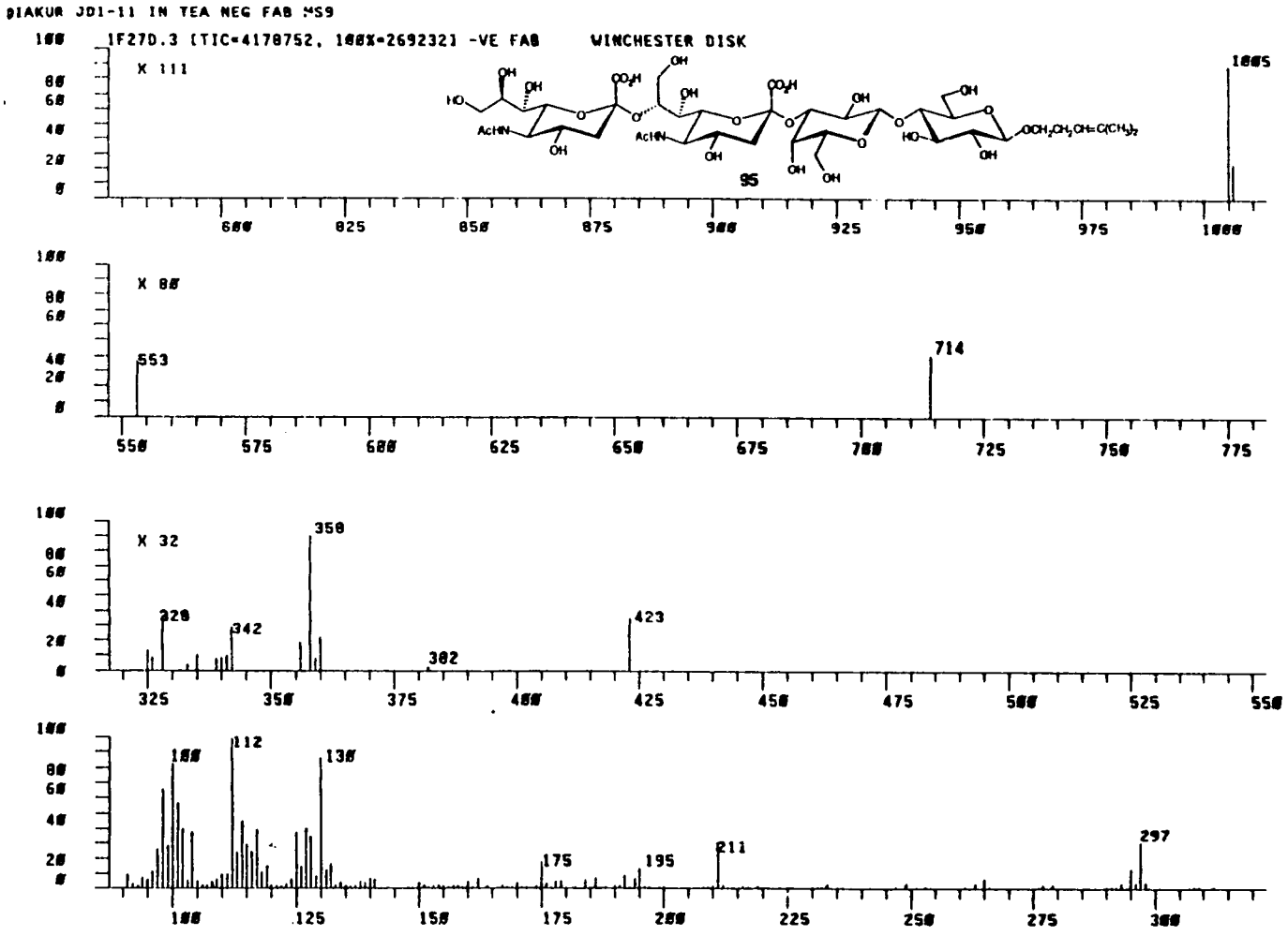
Negative Ion FAB-MS Spectrum of **67** (Triethanolamine Matrix):



Negative Ion FAB-MS Spectrum of **88** (Triethanolamine Matrix):



Negative Ion FAB-MS Spectrum of **95** (Triethanolamine Matrix):



Negative Ion FAB-MS Spectrum of **99** (Triethanolamine Matrix):