MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 28 June 2005.....
.....[redacted].....
Sec. Research Graduate School Committee

Addendum

p 187 line 1 **3-Bromo-*N*-(*tert*-butyloxycarbonyl)-propylamine (137)**

Compound **137** should be referenced with:

Lee, B. H.; Miller, J. *J. Org. Chem.* **1983**, *48*, 24-31.

p 191 line 10 **4-*N*-(*tert*-Butyloxycarbonyl)aminobutanol (140)**

Compound **140** should be referenced with:

Mattingly, P. G. *Synthesis* **1990**, 366-368.

p 192 line 5 **4-Bromo-*N*-(*tert*-butyloxycarbonyl)-butylamine (142)**

Compound **142** should be referenced with:

Berrée, F.; Bazureau, J-P.; Michelot, G.; Le Corre, M. *Synth. Commun.* **1999**, *29*, 2685-2693.

p 196 line 14 **(147)** should be **(147)**[280]

p 197 line 10 **(145)** should be **(145)**[280]

The Preparation and Evaluation of *N*-Acetylneuraminic Acid Derivatives as Probes of Sialic Acid-Recognizing Proteins

A thesis submitted for a Degree of

DOCTOR OF PHILOSOPHY

at Monash University (Parkville Campus)

by

Silvana Ciccotosto, B.App.Sc., M.Pharm.

Department of Medicinal Chemistry

Monash University (Parkville Campus)

December 2004

In memory of my father, Michele Ciccotosto.

Table of Contents

CHAPTER 2

The design and synthesis of influenza virus hemagglutinin inhibitors

CHAPTER 3

The design and synthesis of an N*-acetyl neuraminic acid analogue to probe α(2,8)-sialyltransferase activity.*

CHAPTER 4

Purification of sialic acid-recognizing proteins (SARPs)

CHAPTER 5

Experimental

Abstract

Sialic acids are a family of 9-carbon naturally occurring acidic carbohydrates; the most abundant being 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (*N*-acetylneuraminic acid, Neu5Ac). Their significant role in several important biological processes has received considerable attention in recent years, primarily due to their interactions with specific enzymes and proteins. In addition to many essential biological functions within humans, the presence of cell-surface sialic acids is utilised to great advantage by certain bacteria, viruses and protozoa, leading to various human diseases states. This thesis reports the design and synthesis of novel biological probes for a number of sialic acid-recognizing proteins, with the emphasis of utilising Neu5Ac as the main design template. This thesis begins with an overview on the biology of sialic acids, and describes how they are intimately involved in a number of disease states.

The inhibition of influenza virus sialidase is now well recognised to cause a significant decrease in the spread of the influenza virus *in vivo*. Chapter 2 describes an alternative approach to the inhibition of the influenza virus by targeting influenza virus hemagglutinin, a sialic acid binding protein required by the virus for the initial adhesion to host cells. The design and synthesis of both monovalent and multivalent inhibitors have been investigated. Preliminary biological evaluation and molecular modelling of the monovalent inhibitor suggests that a multivalent approach may show higher inhibition due to the trimeric receptor binding site of influenza virus hemagglutinin.

Polymeric α(2,8)-linked sialosides are recognised as essential components of a number of cells, notably NCAM, and play important roles in development, neuronal plasticity, and certain metastatic tumours. Chapter 3 illustrates several approaches towards the design and synthesis of Neu5Ac analogues either as potential inhibitors of α(2,8)-sialyltransferases, or for use in detecting α(2,8)-sialyltransferase activity. The ability to selectively chemically modify the C-8 position of Neu5Ac was investigated, using a strategy that ultimately required Neu5Ac to be in an unnatural, but rigid, $^{5}C_{2}$ conformation, instead of the usual $^{2}C_{5}$ conformation. This work resulted in the successful synthesis of an 8-*O*-alkylated Neu5Ac derivative.

The design and synthesis of thiosialosides for the purification of sialic acid-recognizing proteins is outlined in Chapter 4. These thiosialosides were coupled to adsorbent supports (epoxy-activated Sepharose 6B and CNBr-activated Sepharose 4B) and then successfully utilized to purify the sialic acid-recognizing proteins *Vibrio cholerae* sialidase, a sialyltransferase from rat liver, sialidase-L from leech and *Trypanosoma cruzi trans*-sialidase.

Chapter 5 brings together all of the chemical and biological data which supports the information provided in the preceding chapters in this thesis.

Statement of Originality

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university, and to the best of my knowledge and belief does not contain any material previously published or written by another person except where due reference is made in the text. This thesis is less than 100,000 words in length, excluding tables, graphs, appendices and references.

[redacted]

Silvana Ciccotosto

December 2004

Acknowledgements

I am thankful to my supervisor, Prof. Mark von Itzstein for offering me the opportunity to undertake this degree. His guidance, support and encouragement are truly appreciated.

Special thanks to my two associated supervisors, Mr Jeff Dyason who left me early into my candidature, but then returned to help me in the final stage of my thesis, and Dr Milton Kiefel who had no problem in allowing me to share his dishwashing bucket in the lab. I would like to thank both supervisors for their invaluable support, guidance and encouragement.

I would like to thank the biochemists, Dr Samia Abo, Annette Alafaci, Wendy Stewart and Kaylene Quelch for allowing me to use their facilities in the lab and their excellent contribution to the biochemistry results in this thesis. I would also like to thank Dr Jenny Wilson for teaching me the ropes in NMR spectroscopy and allowing me invaluable experience on the amazing instruments.

Special thanks to Dr Robin Thomson for all the support and inspirational late night discussions in the lab. Also thanks to the technical staff, Milica Radanovic and Brendan Mackey.

I would like to thank all my colleagues at the VCP especially Ben, Fi, Ag (thanks so much for proof reading), Joe, Juliette, Justin and Faith Josephine Rose. Their friendship, support and interest they shared in my work made research so enjoyable.

I am also thankful to Stuart Thomson for providing mass spectrometry over the years.

I would like to acknowledge the Department of Medicinal Chemistry, Monash University for the receipt of a scholarship which was greatly appreciated.

Special thanks to my friends Nicole, Sara, Maryanne and Marie for their encouragement and friendship over the years we have known each other. To my new friends, the mothers from the Redlands, Helen, Jo and Bea for helping me in babysitting and encouraging me to finish

this thesis. I would like to especially thank Gloria for looking after the children and giving me the time to finish off the thesis.

Thanks to my dearest mother who has done so much for me over the years, her patience, love and support has been outstanding. I would like to thank my brother, Joe, for being there when I needed him. To my darling little boys, Michael and Daniel, thanks for trying not to wake me up at 5.30am every morning after staying up late at the computer. Hopefully one day you both will understand what a thesis really is.

Finally, I would like to thank my husband David, for the late night chemistry discussions and for your invaluable time in proofreading. Also thank you for your patience, support, encouragement and loving friendship, without which, the completion of this thesis would not have been possible.

Communications and Publications

Communications

Ciccotosto, S., von Itzstein, M., and Dyason, J.. Potential inhibitors of Haemagglutinin: an alternative approach to the design and Synthesis of anti-influenza drugs. Monash-Deakin annual Minisymposium on Medicinal Chemistry at the Victorian College of Pharmacy, Monash University, Parkville Campus, Victoria, **1995.** (Oral presentation)

Abo S., Stewart, W., Ciccotosto, S., Kiefel, M.J., and von Itzstein, M.. Purification of *N*-acetylneuraminic acid-recognising enzymes by affinity chromatography. XVIII International Carbohydrate Symposium, Milan, Italy, **1996.** (Poster presentation)

Ciccotosto, S., and von Itzstein, M., Design and Synthesis of novel *s*-linked *N*-acetylneuraminic acid analogues. XVIII International Carbohydrate Symposium, Milan, Italy, **1996.** (Poster presentation)

Publications

Ciccotosto, S. and von Itzstein, M.. Synthesis of Methyl 5-acetamido-3,4,5-trideoxy-4-guanidinyl-D-*glycero*-D-*galacto*-2-nonulopyranosidonic acid (4-deoxy-4-guanidino Neu5Acα2Me). *Tetrahedron Letters,* 36:5405-5408, **1995.**

Ciccotosto, S., Kiefel, M. J., Abo, S., Stewart, W., Quelch, K., and von Itzstein, M.. Synthesis and evaluation of *N*-acetylneuraminic acid-based affinity matrices for the purification of sialic acid-recognizing proteins. *Glycoconjugate Journal,* 15:663-669, **1998.**

Abo, S., Ciccotosto, S., Alafaci, A., and von Itzstein, M.. The synthesis and evaluation of novel sialic acid analogues bound to matrices for the purification of sialic acid-recognising proteins. *Carbohydrate Research,* 322:201-208, **1999.**

Abbreviations

Ac_2O	acetic anhydride
AIBN	α,α-azodiisobutylronitrile
Arg	arginine
Asn	asparagine
ATP	adenosine triphosphate
$BF_3{\cdot}Et_2O$	borontrifluoride diethyl ether
BHA	protease bromelain release soluble fragment of hemagglutinin
BOC	di-*tert*-butyl dicarbonate
br	broad resonance (NMR spectroscopy)
Brosyl chloride	4-bromobenzene sulfonyl chloride
BSA	bovine serum albumin
Bu_3SnH	tri-*n*-butyltin hydride
$[(Bu_3Sn)_2O]$	tributyltin oxide
°C	degrees Celsius
$CaCl_2$	calcium chloride
Calcd	calculated
$CF_3SO_3Si(CH_3)_3$	trimethylsilyl trifluoromethanesulfonate
CH_3CN	acetonitrile
CH_3COOH	acetic acid
CMP	cytidine monophosphate
COSY	correlation spectroscopy
CTP	cytidine triphosphate
Cys	cysteine
δ	chemical shift in parts per million (NMR spectroscopy)
d	doublet (NMR spectroscopy)
Dansyl chloride	5-(dimethylamino)-1-naphthalenesulfonyl chloride
DCM	dichloromethane
dd	doublet of doublets (NMR spectroscopy)
ddd	doublet of doublet of doublets (NMR spectroscopy)
DMF	dimethylformamide
DMP	dimethoxypropane
DNBS-Cl	2,4-dinitrobenzenesulfonyl chloride
EDTA	ethylenediamine tetraacetate
EI	electron impact (mass spectrometry)
eq	equivalent
Et_2NH	diethylamine
FAB	fast atom bombardment (mass spectrometry)
GalNAc	*N*-acetyl-D-galactosamine
GlcNAc	*N*-acetyl-D-glucosamine or 2-acetamido-2-deoxy- D-glucosamine
Gln	glutamine
Glu	glutamic acid
h(s)	hour(s)
HA	hemagglutinin
$HClO_4$	perchloric acid
HEF	hemagglutinin esterase fusion
His	histidine
HMQC	heteronuclear multi quantum coherence (NMR spectroscopy)
HPLC	high performance liquid chromatography
HRFABMS	high resolution fast atom bombardment mass spectrometry
HRMS	high resolution mass spectrometry
IR	infrared
J	coupling constant in Hz (NMR spectroscopy)
K_i	dissociation constant of inhibitor

K_m	Michaelis-Menten constant	PSA	polysialic acid
LDA	lithium diisopropylamide	q	quartet (NMR spectroscopy)
Leu	leucine	RBS	receptor binding site
LHMDS	lithium hexamethyldisilazide	*Rf*	retention factor (in chromatography)
LR	low Resolution (mass spectrometry)	RNA	ribonucleic acid
m/z	mass to charge ratio (mass spectrometry)	RNP	ribonucleoprotein
ManNAc	*N*-acetylmannosamine	rt	room temperature
ManNAc6P	*N*-acetylmannosamine 6-phosphate	s	singlet (NMR spectroscopy) and second (unit of time)
MeOTf	methyl triflate	SARPs	sialic acid recognizing proteins
MHz	megahertz	Ser	serine
MOMCl	chloromethylmethylether	SiaT	sialyltransferase
mp	melting point	t	triplet (NMR spectroscopy)
MS	mass Spectrometry	*T.cruzi*	*Trypanosoma cruzi*
N,N-DMF	*N,N*-dimethyl formamide	TBMS-Cl	*tert*-butyldimethysilyl chloride
NA	sialidase	TFA	trifluoroacetic acid
NaCl	sodium chloride	Thr	threonine
NaN_3	sodium azide	tlc	thin layer chromatography
NaOAc	sodium acetate	TMS	trimethylsilyl, tetramethylsilane
NBS	*N*-bromosuccinimide	tosyl group	*p*-toluenesulfonate
NCAM	neural cell adhesion molecule	Trp	tryptophan
NMR	nuclear magnetic resonance	TS	*trans*-sialidase
PAGE	polyacrylamide gel	Tyr	tyrosine
PCH	1H-pyrazole-1-carboxamidine hydrochloride	UDP	uridine diphosphate
PDC	pyridinium dichromate	UDP-GlcNAc	UDP-*N*-acetylglucosamine
PEP	phosphenolpyruvate	*V.c* sialidase	*Vibrio cholerae* sialidase
$(Ph)_3P$	triphenylphosphine		
P_i	inorganic phosphate		
Piv	trimethylacetyl chloride, pivaloyl chloride		
PP_i	inorganic diphosphate		
ppm	parts per million (NMR spectroscopy)		
Pro	proline		

Sialic Acid Abbreviations

CMP-Neu5Ac	Cytidine-5'-monophospho-5-*N*-acetyl-D-neuraminic acid
Colominic acid	$(NeuAc\alpha(2,8))_n$
G_{M1}	Galβ(1,3)GalNAcβ(1,4)[Neu5Acα(2,3)]Galβ1→Cer
KDN	3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid or 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid
KI-8110	5-fluoro-2',3'-*O*-isopropylidene-5'-*O*-(4-*N*-acetyl-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-methoxycarbonyl-D-*glycero*-α-D-*galacto*-octapyranosyl)uridine
MU	4-Methylumbelliferyl
MUN	4-Methylumbelliferyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid
Neu	5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid or neuraminic acid
$Neu5,9Ac_2$	*N*-acetyl-9-*O*-acetylneuraminic acid, 5-*N*-acetyl-9-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid
Neu5Ac	*N*-acetylneuraminc acid , 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid
Neu5Ac2en	5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid
$Neu4,5Ac_2\alpha2Me$	4-*O*-acetyl-5-*N*-acetyl-α-D-neuraminide
$Neu4,5,7,8,9Ac_5\alpha1Me$	methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate
Neu5Ac9P	*N*-acetylneuraminic acid-9-phosphate, 5-*N*-acetyl-9-phosphate-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid
Neu5Gc	*N*-glycolyl-neuraminic acid, 5-(2-hydroxyacetamido)-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid
Neu4,5,7,8,9Ac2βCl	methyl (5-*N*-acetyl-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosyl)-onate chloride
Sialyl Lewis a (sLe^a)	Neu5Acα(2,3)Galβ(1,3)[Fucα(1,4)]GlcNac
Sialyl Lewis x (sLe^x)	Neu5Acα(2,3)Galβ(1,4)[Fucα(1,3)]GlcNac
Sialyllactose	Neu5Acα(2,6)Galβ(1,4)Glc
Zanamivir	2,3-didehydro-2,4-dideoxy-4-guanidinyl-*N*-acetylneuraminic acid

CHAPTER 1

Introduction

1.1 Carbohydrates

Carbohydrates have long been known as a source of energy, however, as components of cell membranes their biological role has not been well understood. This undoubtedly renders them as one of the most undervalued and least medicinally explored group of biomolecules. Ubiquitous in nature, carbohydrates are found in all classes of living organisms in an exceptionally diverse range of biological processes.

The extraordinary complexity and structural diversity of carbohydrates in cells allows them to be effective carriers of information. In fact, carbohydrates can carry more information per unit weight compared to either nucleic acids or proteins.[1] The majority of carbohydrates are usually conjugated to other molecules found in cells, such as proteins and lipids, to give glycoproteins and glycolipids, respectively. These glycoconjugates are a major component of the outer surface of mammalian cells and form the glycocalyx.[1,2]

It is well known that carbohydrates participate in a range of biological roles such as fertilization, immune defence, viral replication, parasitic infection, cell growth, cell-cell adhesion, degradation of blood clots, malignant transformation and inflammation.[2] Figure 1 illustrates some of the important biological phenomena that involve cell surface carbohydrates.

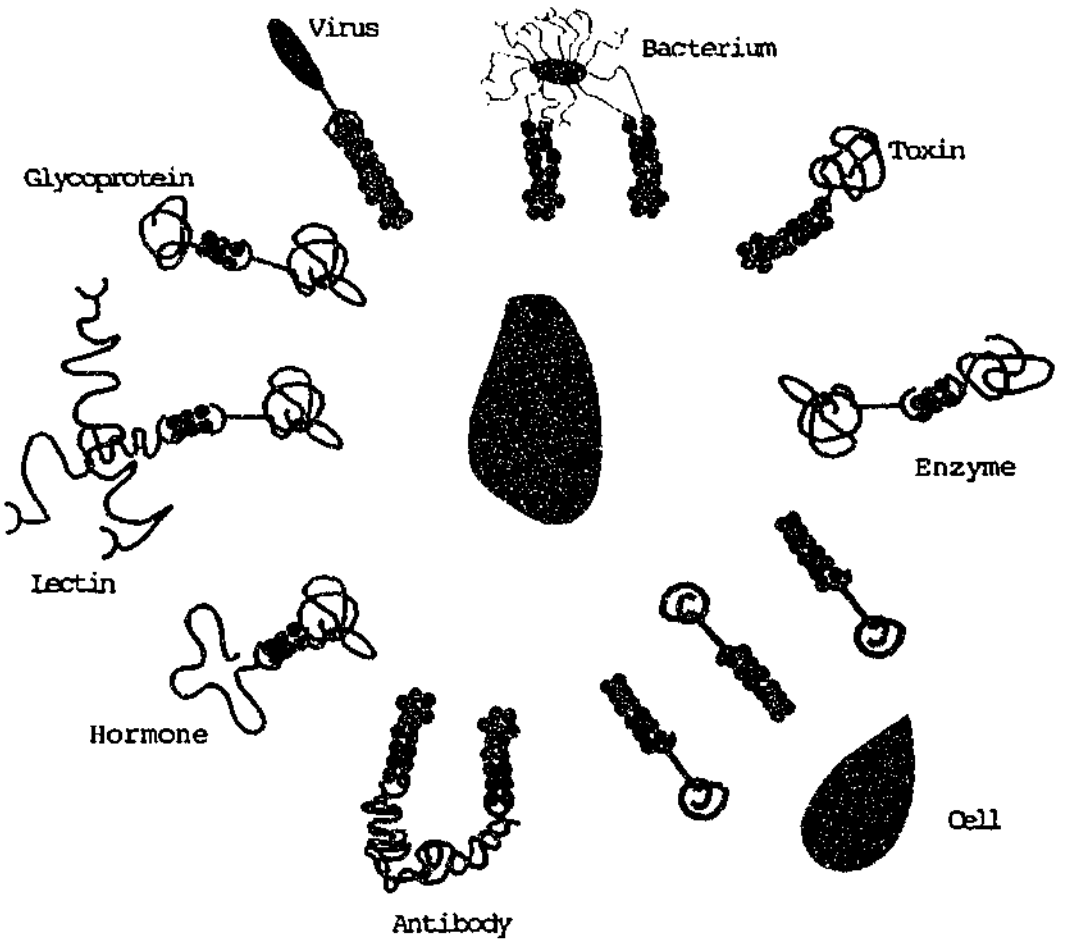


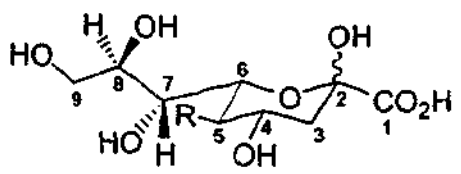
Figure 1. Cell surface carbohydrates involvement as biological receptors for other cells, toxins, viruses, hormones and bacteria. (Prepared by J.C.Dyason, adapted from Borman[3])

One important class of carbohydrates are the sialic acids. This group of biomolecules which have been found to be involved in a plethora of biological processes (*vide infra*) is the focal point of this thesis.

1.2 Sialic Acids

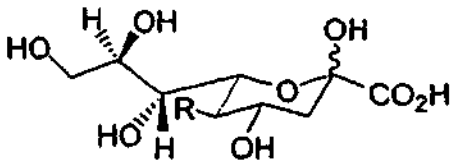
During the mid-1930s to late 1940s, various acidic amino sugars were independently discovered in the laboratories of Klenk (1935-1939)[4,5], Blix (1936)[4,5] and Gottschalk (1949).[4,5] These unique sugars were naturally named differently by each group. However, following structural elucidation studies in the early 1950s, it soon became apparent that

although three novel carbohydrates had initially been discovered, they were, in fact, all structurally similar.[4,5] To avoid any further confusion that may have arisen in this fledging area of carbohydrate chemistry, the above three researchers agreed to call the unsubstituted acidic amino sugar neuraminic acid (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid, Neu (1)).[5] In one case, neuraminic acid was discovered in the salivary gland mucin by Blix, from which the term sialic acid (from the Greek word *sialos* meaning saliva)[6,7] was derived and Klenk isolated neuraminic acid from brain glycolipids (neuro + amine + acid).[7] The name sialic acid was coined to encompass all derivatives of 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (**1**).[5]



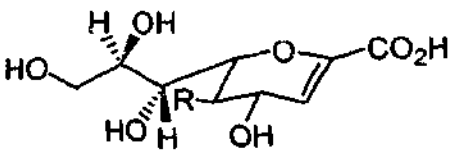
1 R = NH_2 (Neu)

Since the initial discovery of **1**, another 42 sialic acids have been isolated[8] and a total of 50 sialic acids have been identified.[7,9] Of the 43 sialic acids isolated, the one predominantly found in nature is 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (*N*-acetylneuraminc acid, Neu5Ac (**2**)).[8] Two other key sialic acids are *N*-glycolyl-neuraminic acid (Neu5Gc, (**3**)) which bears a hydroxylated *N*-acetyl group at C-5 and 3-deoxy-D-*glycero*-D-*galacto*-2-nonulsonic acid (KDN, (**4**)) which contains a 5-hydroxyl group instead of 5-amino group again at C-5. The diversity of sialic acids that exist in nature are merely derivatives of Neu5Ac (**2**), Neu5Gc (**3**) or KDN (**4**).[8-10]

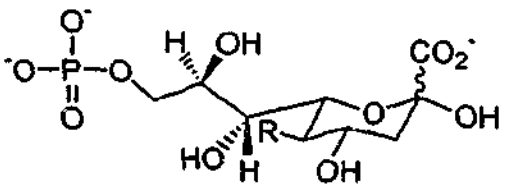


2 R = $NHC(O)CH_3$ (Neu5Ac)
3 R = $NHC(O)CH_2OH$ (Neu5Gc)
4 R = OH (KDN)

Sialic acids (except for unsaturated sialic acids (e.g. 2-deoxy-2,3-didehydro derivatives like Neu5Ac2en (**5**)) and *N*-acetylneuraminic acid-9-phosphate Neu5Ac9P (**6**)) mainly occur at the terminal position as components of glycoconjugates, oligosaccharides and polysaccharides (polysialic acids).[8,11,12] Being located in this prominent position allows sialic acids to be readily accessible to a wide range of biological processes such as recognition, masking, and protection (*vide infra*).



5 R = NHAc
Neu5Ac2en



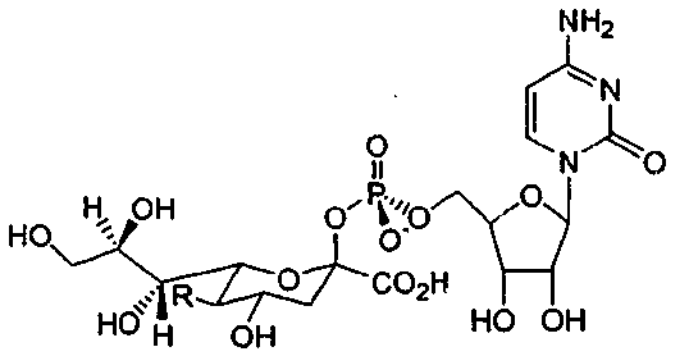
6 R = NHAc
Neu5Ac9P

1.2.1 Structural features of Sialic Acids

Sialic acids are acidic 9-carbon sugars. The salient features of this class of carbohydrates are the amino group at position 5 (except in the case of KDN (**4**) which contains a hydroxyl moiety) and the carboxyl group at position 1 which imparts a negative charge on the molecule at high pH and endows it with properties similar to other organic acids.[10] Usually the amino group is *N*-acetylated to give 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid, Neu5Ac (**2**).

In general, sialic acids form a pyranose (six-membered) ring in solution *via* intramolecular hemiketal condensation. Sialic acids predominantly exist in the $^2C_5$ chair-like conformation[7,13] and due to the hydrogen bonding between HO-7 and HO-8, the glycerol side chain is remarkably rigid.[8] In the case of free Neu5Ac (**2**), the anomeric hydroxyl group at C-2 is mainly axial and the carboxylic group mainly equatorial.[14]

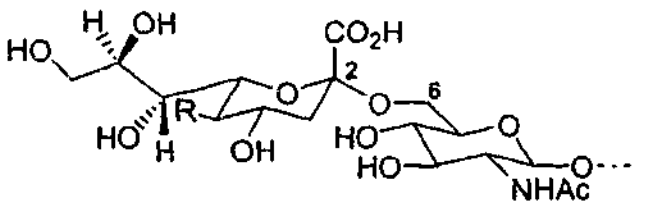
When bound to glycoconjugates, sialic acids are typically attached through the C-2 anomeric hydroxyl group and they tend to exist in the α anomeric form, whilst free acids in solution tend to adopt the β anomeric form (> 93%).[15] For nucleotide-bound sialic acids, the β anomeric form exists at the glycosidic bond.[16] This is exemplified by the activated form of Neu5Ac (**2**) which is is linked to CMP in the β anomeric form which gives Neu5Acβ2CMP or CMP-Neu5Ac (**7**) and is required for the biosynthesis of sialosyl glycosides.[7,8,14,17]



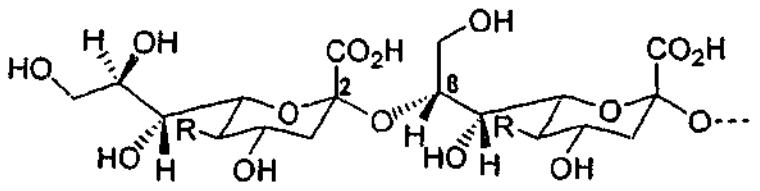
7 R = NHAc
CMP-Neu5Ac

As components of glycoconjugates, the linkage patterns for sialic acids are generally α(2,3)- or α(2,6)- to either D-galactose (Gal) or *N*-acetyl-D-galactosamine (GalNAc).[8,11,14] For *N*-acetyl-D-glucosamine (GlcNAc), α(2,6)- glycosidic bonds to sialic acids have also been observed (e.g. Neu5Acα(2,6)GlcNAc (**8**)).[8,11] In some cases polymeric forms of

sialic acids (also known as polysialic acids) have been found.[8] Generally, α(2,9)- and α(2,8)-linked homopolymers or alternating α(2,8)- and α(2,9)-linked polymers for Neu5Ac (**2**) and Neu5Gc (**3**) are found in glycoproteins and gangliosides.[8] Some unusual Neu5Gc-containing sialyl polysaccharides such as NeuGcα(2,5-$O_{glycoiyl}$)Neu5Gc have been isolated from the jelly coat of sea urchin eggs.[18,19] α(2,8)- Glycosidic bonds are typically observed in carbohydrates coupled to other sialic acids like Neu5Ac (**2**) as illustrated by structure **9**. Indeed, this linkage has been found in the cell surface of resident polysialic acids.[12,20]



8 R = NHAc
Neu5Acα(2,6)GlcNAc



9 R = NHAc
Neu5Acα(2,8)Neu5Ac

1.2.2 Distribution of Sialic Acids in nature

The literature is replete with the distribution of sialic acids in nature.[7,9,14,21] Generally speaking, sialic acids are found in the higher invertebrates or vertebrates. Thus, varying amounts and structural forms of sialic acids have been found in microorganisms, insects, on cell membranes and in all body fluids of mammals (see Table 1).[3,21] Sialic acids are also known to occur in some protozoa (*i.e. Trypanosoma cruzi*)[14], viruses (*i.e.* rabies and influenza)[8], and certain bacteria (*i.e. Escherichia coli, Neisseria meningitis,* and *Salmonella* strains).[14] In humans, sialic acids such as Neu5Ac (**2**) together with *O*-

acetylated and *O*-lactylated analogues have been identified[9] (see Table 1 for examples). Furthermore, sialic acids such as Neu5Gc (**3**) have been detected in colon, gastric and liver cancer and mammary carcinoma in humans.[21]

Table 1. Some naturally occurring sialic acids.[8,22]

Full Name	Abbreviation	Comments
Neuraminic acid	Neu	does not exist in free form
N-acetyl-neuraminic acid	Neu5Ac	found in higher animals (echinoderms to man), some bacteria and parasites
N-glycolyl-neuraminic acid	Neu5Gc	most higher animals
2-Keto-3-deoxy-nonulosonic acid	KDN	sperm and fish eggs
7-*O*-acetyl-*N*-acetyl-neuraminic acid	$Neu5,7Ac_2$	found in higher animals and some bacteria
7-*O*-acetyl-*N*-glycolyl-neuraminic acid	Neu5Gc7Ac	widespread in higher animals except in humans and birds
9-*O*-acetyl-*N*-acetyl-neuraminic acid	$Neu5,9Ac_2$	widespread in higher animals and some bacteria
9-*O*-acetyl-*N*-glycolyl-neuraminic acid	Neu5Gc9Ac	widespread throughout higher animals except in humans and birds
2,3-didehydro-2,6-anhydro-*N*-acetyl-neuraminic acid	Neu5Ac2en	found in biological fluids and tissues

1.2.3 Biological characteristics of sialic acids

Due to their existence on the cell surfaces, sialic acids act as strong, protective agents in living cells and organisms. The diverse functions of sialic acids are not completely known[7], however, it is clear that this class of carbohydrates plays significant roles in a wide range of biological processes.[7-10] It is not the intention of this introduction to provide the

reader with a detailed analysis of all the biological functions of sialic acids. Instead, an overview on various aspects of their biological roles will be discussed. Contributing factors to their biological significance include the negative charge of the carboxylate; bulkiness; hydrophilicity and location in exposed terminal positions, both in glycoconjugates and cell membranes.[23,24] These characteristic features facilitate the involvement of sialic acids in three broad biological roles that include:

i) *Physico-chemical effects on glycoconjugates and cell membranes.*

The surface of most cells is heavily sialylated.[10,14,25] For example, approximately 10^7 sialic acid residues are situated at the surface of a single human-erythrocyte.[10,14,25] The carboxylate of sialic acids is fully ionised under physiological conditions which renders them to be negatively charged (pK_a ca. pH 2.6)[8], and allows the cell to form a charged sheath which can both facilitate aggregation, through calcium bridging, or prevent aggregation, by electrostatic repulsion. The negative charge contributes significantly to the rigidity of the cell surface which is caused by the repulsive interactions. This results in an increase in the viscosity of mucins, which, in turn, can influence, or stabilize the conformations, physical properties and biological functions of proteins and glycoproteins.[8,10,23,25] Furthermore, the negative charge of sialic acids aids in the binding and transport of positively charged molecules, including pharmaceuticals, as well as the attraction and repulsion of cells and molecules.[9]

The prevention of erythrocyte aggregation is an excellent example of the repulsive effect induced by surface sialic acids. This was shown in an experiment whereby desialylation of the surface sialic acids caused erythrocytes to readily aggregate.[8] Similarly erythrocytes,

which are undersialylated have been implicated in the development of vascular disease resulting from diabetes.[8]

The negative charge of sialic acids not only aids binding of cationic compounds with physiological and pharmacological significance to macromolecules and cells but is also responsible for the Ca^{2+}-binding sites in muscle cells.[10,23,26] It has been reported[8,21] that both the glycerol side chain and the negatively charged carboxylate are responsible for binding of Ca^{2+} to sialic acid containing gangliosides. This complex has been suspected of playing an important role in the functioning of nerve tissues.

Nerve tissue glycocongugates are located as clusters on neuronal and synaptic membranes in the vicinity of a membrane-bound calcium pump, effectively enabling the supply of these ions for neuronal cells and the formation of Ca^{2+}-ganglioside complexes.[8,21] The negative charge of such complexes has an important effect on the activity of the nerve cells. Thus, formation of the complex may produce contraction of the presynaptic membrane and inhibit the release of transmitter substances, whereas dissociation may cause conformational changes in the presynaptic membrane.[14,21]

In neurological disorders such as schizophrenia, senile dementia or Alzheimer's disease, a decrease of gangliosides has been observed in the grey matter of the brain. After successful treatment of the disease, the sialic acid content restores to normal levels due to induction and up regulation of sialic acids in synaptosomal gangliosides.[21,27]

ii) Masking of recognition sites.

The critical positioning of sialic acids on glycoconjugate molecules and cell surfaces facilitates the protective role played by these compounds. Sialic acids mask penultimate carbohydrates, usually galactose and *N*-acetylgalactosamine, located in the glycan chains of glycoproteins and gangliosides.[8,21,23] For example, erythrocytes are red blood cells covered by a layer of sialic acid residues.[9,10] With sialic acids themselves being attached to galactose, the galactose residues can be exposed by removing the protective sialic acids by the action of serum sialidase. The unmasked blood cells are then bound to galactose-specific macrophages and phagocytosed (see Figure 2).[9,10] Sialic acids thus protect erythrocytes from degradation through masking of the subterminal galactose residues.[8,9,23] This type of mechanism is observed for other blood cells such as thrombocytes, leucocytes[10], and lymphocytes.[9]

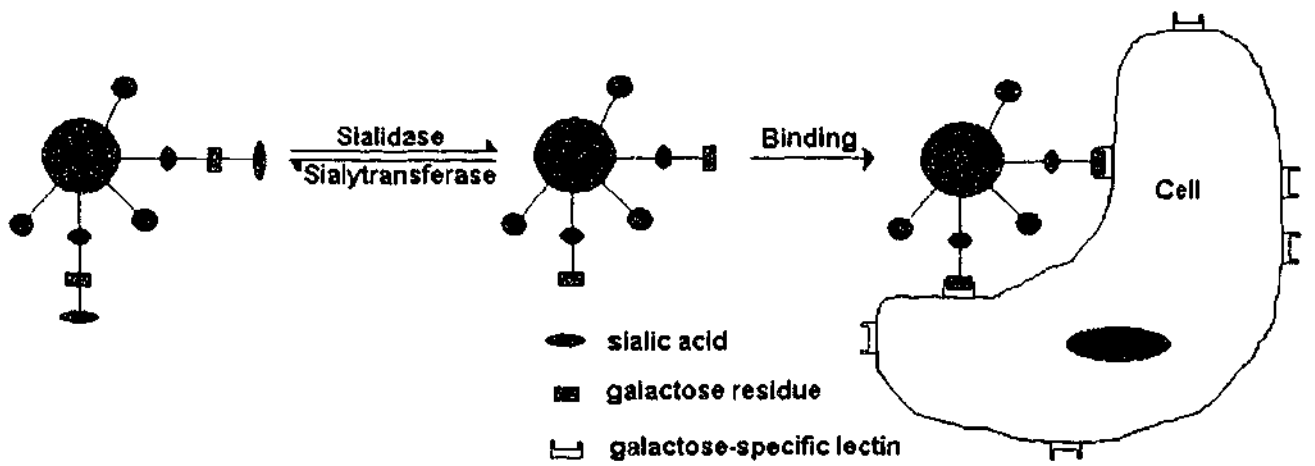


Figure 2. The unmasking of erythrocytes, allowing the initial step to phagocytosis (Adapted from Schauer[23]).

The reversible binding to cells *via* sialic acids and galactose is regulated by sialidase and sialyltransferases.[8,23] This reversible binding operates in cell communication during growth, differentiation, aging, malignant cell transformation and metastasis.[8,23] Sialic acids

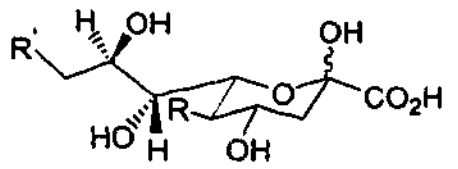
have the ability to protect host cells and inhibit possible infection by bacteria *via* the masking of the corresponding recognition sites.[8]

The above mechanism could also be applied for the elimination of malignant cells. In the case of tumour cells, terminal galactose residues that would otherwise inhibit further cell growth and spreading are masked by sialic acids. An increase in malignancy results as the galactose moieties are effectively hidden from the immune defence system.[8-10] The higher degree of sialylation observed for these tumour cells is accompanied by an immunosuppressive effect that corresponds to higher sialyltransferase activity.[10]

In the case of antigens, sialic acids have been found to also mask associated antigenic sites and thus weaken immuno-reactivity.[23] This causes the masked antigen to be unrecognized by the immune system. However, once the antigen is desialylated due to aging or the action of microbial or viral sialidases, the cells become 'non-self' and are vulnerable to destruction by macrophages, or are prone to form autoantibodies which may result in autoimmune diseases such as Gonococci (*Neisseria gonorrhoeae*).[10,23]

iii) Acting as receptors for binding and adhesion.

The recognition of sialic acids by various non-pathogenic and pathogenic toxins (e.g. from *Vibrio cholerae*), viruses (e.g. influenza, rotavirus), bacteria (e.g. *Helicobacter pylori*) and protozoa (e.g. *Trypansoma cruzi*) can result in adhesion and lead to infection of the cell.[10,21] Pathogens have different specificities when binding to sialic acids on host cells, for example influenza C virus recognizes Neu5,9Ac_2 (**10**) and Sendai virus recognizes Neu5Ac (**2**).[7,8]



10 R = $NHC(O)CH_3$, R' = $OC(O)CH_3$
Neu5,9Ac_2

Glycosphingolipids which bear sialic acids are classified as gangliosides.[28] Gangliosides have been recognized in the regulation of cell function[28], and cell growth[24,28] and are also known to bind to toxins.[24] The ganglioside G_{M1} is a receptor for cholera toxin and found to modulate ion transport and affect cell growth.[29,30]

The level of interest in sialic acid-interactions has significantly increased over the past ten years. This has no doubt been due, in part, to their critical involvement in propagation of infectious diseases such as influenza. Many of these interactions occur *via* adhesion of the pathogen, a process mediated by sialic acid-binding proteins called sialo lectins.[7]

Lectins are pervasive proteins which bind to mono- and oligosaccharides reversibly and with high specificity by hydrophobic and hydrogen bonds.[31,32] Mammalian lectins have been classified into different groups on the basis of structural features of the lectins themselves, and the types of carbohydrate ligands that are recognized. In the following sub-sections an overview of some of the well-known lectins is provided.

1.2.3.1 Vertebrate pathogen lectins

As previously mentioned (*vide supra*) sialic acids play a significant role in the initiation and propagation of bacterial and viral infections. This process is initiated following their recognition by bacterial and viral lectins. A range of pathogens (viruses, bacteria and

protozoa) express lectins that recognize various sialic acid ligands which can lead to entry into the host cells.[7] In some instances, the pathogens not only use sialic acid ligands but also other cell surface molecules for attachment.[7] Binding between these pathogenic lectins and their ligands may depend on the presence of a few functional groups of the oligosaccharide.[10] Generally, the lectins may bind to low-affinity ligands that are present in high density on a cell surface as well to a low number of high-affinity ligands.[10] This important quality enables fine tuning of cell-cell interactions as required for very specific recognition processes.[10] Examples of some lectins will be discussed below.

1.2.3.1.1 Adhesins

Lectins which are attached to the surface of bacterial pathogens are typically called adhesins.[7] Some examples of bacteria that have resident adhesins are *Helicobacter pylori* (an etiological agent for peptic ulcers) and *E. coli* strain K99.[7] Although many of the bacterial pathogens have high specificity towards sialic acids, some are not so specific (e.g. recognition of GlcNAc by *Pasteurella haemolytica*).[7] Adhesins may also exist as soluble sialic acid-binding lectins which are typically toxins such as the cholera toxin (secreted by *Vibrio cholerae*).[7]

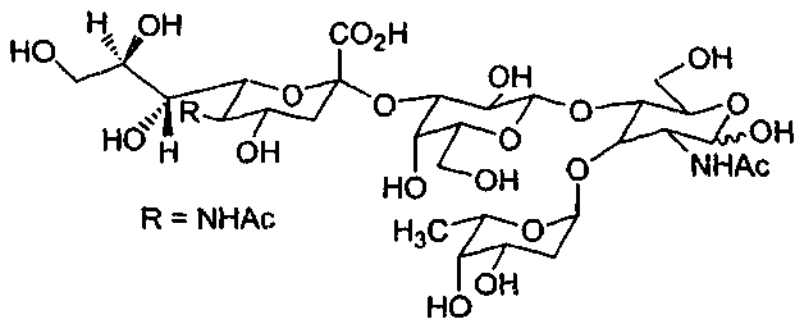
1.2.3.1.2 Hemagglutinin

Viral sialic acid-recognizing lectins (commonly known as hemagglutinins) are usually capable of agglutinating red blood cells.[7] A number of viruses utilize sialic acids for attachment to host cells.[7,8] The hemagglutinins (HAs) of influenza virus (A, B, and C), Newcastle disease virus, human coronavirus, Sendai virus and others have been isolated and shown to bind to sialic acids.[7] The most well-known and well studied HA is from the influenza A virus which will be discussed in more detail in Chapter 2.

1.2.3.1.3 Selectins

Selectins (selective carbohydrate-binding proteins) are a family of C-type lectins which generally contains a shared structural motif and requires calcium for carbohydrate recognition.[7] This family comprises three proteins and are named after the cell type from which they are identified *viz*, endothelium (E), platelets (P) and lymphocytes (L).[14] All three selectins contain a NH_2-terminal domain enabling them to recognize carbohydrate ligands.[33,34] Moreover, they all play a role in the adhesion process of white blood cells to specific endothelia called the "rolling stage". Essentially, white blood cells flow into the bloodstream and then slow down by rolling along the endothelial lining of the vessel and eventually penetrate into the tissue, which may be damaged by a lack of oxygen (e.g. transplants or inflammation).[9] The effect is caused by interactions with sialic acid-containing ligands and recognition of the appropriate ligand for adhesion.[21,35]

Selectins interact with varying degrees of affinity, *via* their binding pockets, with key functional sugar ligands assumed to be fucose, galactose and sialic acid parts of sialylated Lewis blood group epitopes, sialyl-Lewis x (sLe^x **11**) and E- and P-selectins for sialyl-Lewis a (sLe^a). The interactions of selectins with sialyloligosaccharides such as sLe^x have been reported to be associated with early stages of the inflammatory-response mechanism and tumour metastasis.[21,27,36] Knowing the natural substrates for selectins has expedited development of inhibitors to ameliorate inflammatory diseases[37,38] and prevent metastasis of tumours.[9]



11 Sialyl Lewis x (sLe^X)
(Neu5Acα(2,3)Galβ(1,4)[Fucα(1,3)]GlcNAc)

1.2.3.1.4 Siglecs

Recently, a subset of the immunoglobulin (Ig) superfamily has been discovered to behave as sialic acid binding lectins and have been defined as a new class of adhesion receptors known as Siglecs.[7,39,40] Siglecs recognize sialic acid linkages expressed on the cell surface of a wide variety of cell types.[41] At least eleven different Siglecs are present in humans which may be divided into two subgroups; i) sialoadhesin (Siglec-1), CD22 (Siglec-2), myelin-associated glycoprotein (MAG, Siglec-4a), Schwann cell myelin protein (SMP, Siglec-4b) and ii) CD33 (Siglec-3) related Siglecs.[42]

Sialoadhesins are found on specific macrophage sub-populations of murine bone marrow, spleen, and lymph nodes.[10,21] The function of sialoadhesin is thought to be in the trafficking of leucocytes in lymphatic organs and the development of myeloid cells in the bone marrow. On cell surfaces of glycoproteins and glycolipids, sialoadhesin recognizes the sequence Neu5Acα(2,3)Galβ(1,3)GalNAc.[10,21]

Other Siglecs are involved in the regulation of recognition of white blood cell types and macrophages and thus are regulators of the immune system.[9] They also recognize different sialic acids with varying glycosidic linkages. For example MAG preferentially binds to *N*-acetyl neuraminic acid in α(2,3)- linkages, whereas CD22 recognizes α(2,6)- linkages.[9,42]

1.3 Biosynthesis of sialic acids

As mentioned earlier, all sialic acids (excluding certain bacteria-specific sialic acids) are essentially derivatives of either Neu5Ac (**2**) or KDN (**4**). Although the biosynthetic pathway for KDN appears to be very similar to Neu5Ac, the metabolic enzymes are not well characterized[7] hence the discussions below will focus on the biosynthesis of Neu5Ac (**2**). As such, the biosynthesis of Neu5Ac will be used as a starting point in the ensuing discussions.

The biosynthesis of sialic acids involves a long pathway whereby the principal metabolic precursor is glucose (Glu, (**12**)).[8,13,24] In the case of Neu5Ac (**2**) specifically, the first key step involves epimerisation of *N*-acetylglucosamine (GlcNAc, (**13**)) to *N*-acetylmannosamine (ManNAc, (**14**)) by UDP-GlcNAc 2-epimerase [EC 5.1.3.14]. This is followed by phosphorylation at C-6 of the amino sugar by ManAc 6-kinase to form *N*-acetylmannosamine 6-phosphate (ManNAc6P, (**15**)) in the cytosol.[43] Condensation of ManNAc6P (**15**) with phosphoenol pyruvate to Neu5Ac9P (**6**) is accomplished with the aid of sialic acid 9-P synthase.[44-46] Subsequent dephosphorylation by *N*-acetylneuraminate-9-phosphate phosphatase [EC 3.1.3.29] affords the final product Neu5Ac (**2**).[13,24]

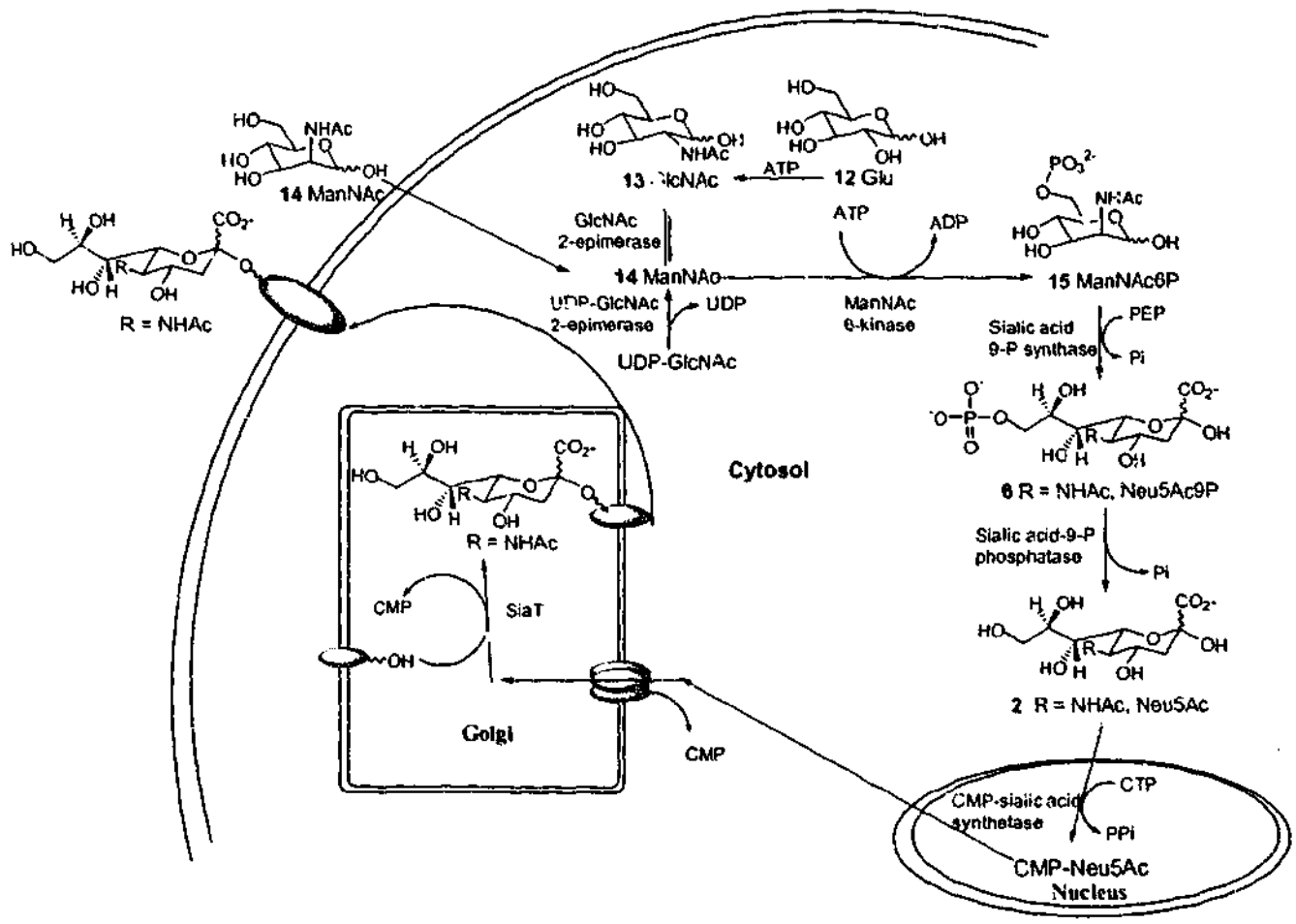


Figure 3. Biosynthesis of sialic acids. (Adapted from C.L. Jacobs *et al*[44])

(Abbreviations: UDP, uridine diphosphate; UDP-GlcNAc, UDP-*N*-acetylglucosamine; CMP, cytidine monophosphate; CTP, cytidine triphosphate; SiaT, sialyltransferase; PEP, phosphenolpyruvate; Pi, inorganic phosphate; PPi, inorganic diphosphate; ATP, adenosine triphosphate.)

To retain bioavailabilty, however, Neu5Ac must be activated by CMP-sialic acid synthase [EC 2.7.7.43] to the corresponding CMP-sialic acid for incorporation into complex carbohydrates[8,24,44,45] inside the nucleus (Figure 3). The final step aims at priming sialic acids for transferral to the terminal position of a specific carbohydrate, ie the oligosaccharide chain of a nascent glycoconjugate.[10,47,48] This reaction which takes place in the Golgi apparatus of vertebrates, and plasma membrane in the case of bacteria, is facilitated by sialyltransferases.[47,48]

There are over 15 sialyltransferases that have been isolated and characterized.[7,10,47] Each sialyltransferase transfers sialic acids to a particular acceptor to give specific linkages: α(2,3)-, α(2,6)-, α(2,8)- and α(2,9)- type linkages.[6,8,47] The different linkages which may be formed, results in a plethora of different oligosaccharides (mostly in terminal positions).[8] Other sialic acids besides Neu5Ac (**2**) can be transferred by sialyltransferases. These include Neu5Gc (**3**), *O*-acetylated species and synthetic Neu5Ac derivatives.[7,8] The role of sialyltransferases is discussed in more detail in section 3.2.

The bound sialic acid may be further modified by *O*-acetylation or *O*-methylation before the mature glycoconjugate is transported to the cell surface.[10] Furthermore, the bound sialic acid can be removed by the key enzyme sialidase (NA).[8,10] At this stage, metabolism and catabolism of sialic acids is observed (with NA being one of the important enzymes in the catabolism of sialic acids). Both the metabolism and catabolism of sialic acids is rather complex, however, attention on some of the important enzymes involved in both processes will be discussed below.

1.4 Metabolism and catabolism of sialic acids

A finely balanced array of enzymes is essential for the metabolism of sialoglycoconjugates in order that cells and tissues may function properly. Any imbalance in such systems can lead to severe diseases or genetic disorders.[8,10] There is an array of enzymes which are involved with the synthesis and degradation of sialic acids and are distributed among different compartments of the cell.[10] The three main enzymes involved in the catabolism of sialic acids are: *O*-acetylesterases, NAs and lyases and are further discussed below.[8]

1.4.1 Sialylate-*O*-acetylesterases [EC 3.1.1.53]

Sialic acids with *O*-acetyl groups cause an inhibitory affect on NAs and sialic acid-specific lyases. Esterases occur in viruses, bacteria, humans and vertebrates.[10] They have been isolated and characterized from influenza C virus, bovine brain, and horse and rat livers.[8,10,21] To commence turnover of *O*-acetylated sialic acids, esterases act on such carbohydrates prior to the action of NAs.[8] They have been found to hydrolyze the ester linkage between the acetyl and the sialic acid hydroxyl groups.[10] Generally, these enzymes hydrolyse 9-*O*-acetyl groups from free and glycosidically bound sialic acids.[8,10,21] Due to the ability for 7-*O*-acetyl groups to migrate non-enzymatically to position 9, they could also be cleaved by esterases.[10] In the case of the 7,8,9-tri-*O*-acetylated derivatives of sialic acids, de-*O*-acetylation by the influenza C virus esterases occurs only after migration of the other *O*-acetyl groups to the primary hydroxyl group at C-9.[8]

1.4.2 Sialidases [EC 3.2.1.18]

Sialidases [EC 3.2.1.18] (also known as neuraminidases, NAs (*vide supra*)) play an important role in both the metabolism and catabolism of sialic acids. Like their sialyl substrates, NAs are widely distributed in mammalian species, viruses, bacteria, fungi, mycoplasma, and protozoa.[10,49] Their primary function is to hydrolyze the *O*-glycosidic linkages between the terminal sialic acids of complex carbohydrates or sub-terminal glycoconjugates.[8,21,49] Typically NAs hydrolyse either the α(2,6), α(2,3), or α(2,8) linkages found in glycoconjugates, but at different rates.[8] Several studies have shown that NAs from *V. cholerae, C. perfringens, Bifidobacterium*[50,51], influenza virus[52], and *Salmonella typhimurium*[53] release sialic acids in the α-anomeric form after which they slowly mutarotate to the more stable β-form. The free sialic acid can then be either further

degraded by acylneuraminate lyases (*vide infra*) or re-transferred onto glycoconjugates by sialyltransferases (see Chapter 3) after activation to CMP-Neu5Ac (7) (see Figure 3).[10]

NAs are also known for their pathogenic roles in the life cycles of bacteria and viruses. Interestingly, their role in bacteria has also been found to be nutritional.[49,54] Bacterial NAs have been identified to break down mucin barriers, hence allowing entry for the bacterium into the body as in the development of pneumonia by *Streptococcus pneumoniae*.[54] Viral NAs mediate the proliferation and invasion of host cells. Unequivocally the most extensively studied[49,55] examples are the influenza virus A and B NAs which have been implicated in the release of aggregrated viral progeny (*vide infra*, Chapter 2).[56]

1.4.3 Sialate-pyruvate lyase [Neu5Ac aldolase EC 4.1.3.3]

Sialic acids liberated by NA are further degraded by lyases to form acylmannosamines and pyruvate.[10,57] Sialate-pyruvate lyases are located in the cytosol of mammalian cells.[10] The main function of these enzymes is the degradation of sialic acids, thus regulating the recycling of this carbohydrate.[10,57]

1.5 Synthesis of sialic acids

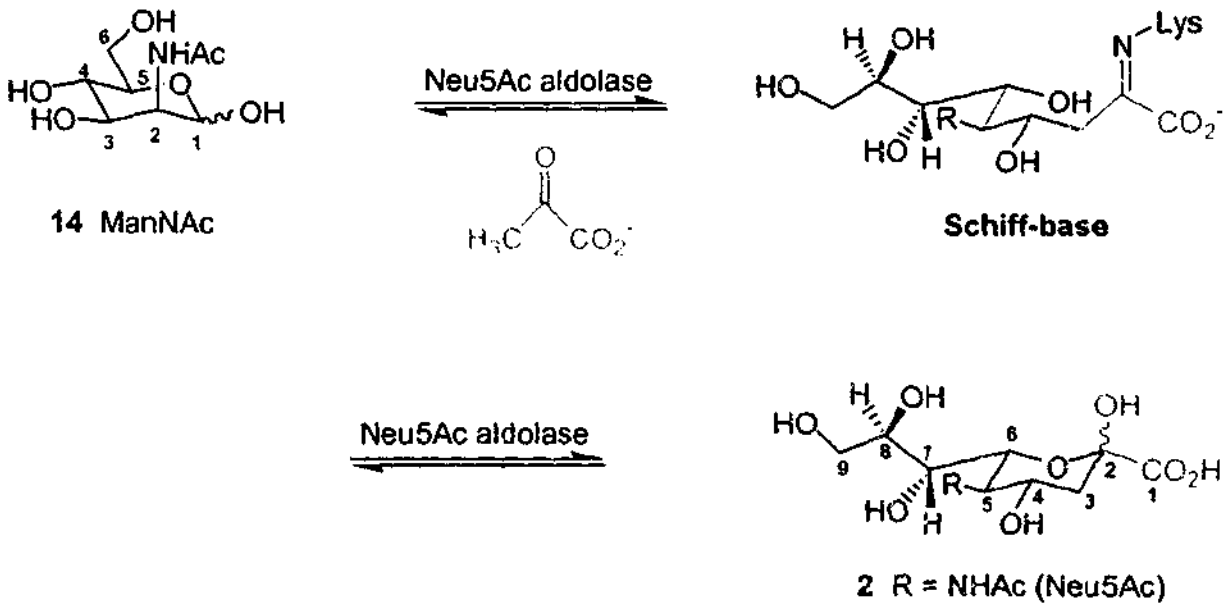
Sialic acids have been isolated from a number of natural sources including edible bird's nest (~2% by weight)[14,58], submaxillary gland mucins (9-36% by weight)[14] and egg yolk.[59] The small amounts found in nature and an increase in interest of sialic acids has resulted in the need to find a more practical source e.g. enzymatic and chemical synthesis. The use of both these methods has achieved the modification of every carbon of the Neu5Ac (**2**)

moiety.[60] The next section will discuss both chemical and enzymic methods for the preparation of sialic acids.

Research on sialic acids has grown enormously over the past few years and has resulted in the synthesis of a number of derivatives. Several comprehensive reviews concerned with sialic acid chemistry have been published.[43,60-62]

1.5.1 Enzymatic synthesis of sialic acids

Several protocols have been established for the enzymic synthesis of sialic acids. The most common treats *N*-acetyl-D-mannosamine (ManNAc, (**14**)) with Neu5Ac aldolase (*N*-acylneuraminate pyruvate lyase; [EC 4.1.3.3]) in the presence of pyruvate (Scheme 1). Although Neu5Ac aldolase is responsible for the catabolism of sialic acids *in vivo*, the equilibrium can be pushed to favour the formation of the aldol product by using excess pyruvate.[57,63] The aldolase forms a Schiff's base with a lysine residue and pyruvate then reacts with ManNAc (**14**). It is assumed that the imidazole group of a histamine residue protonates the aldehyde group of the acceptor substrate.[64,65]

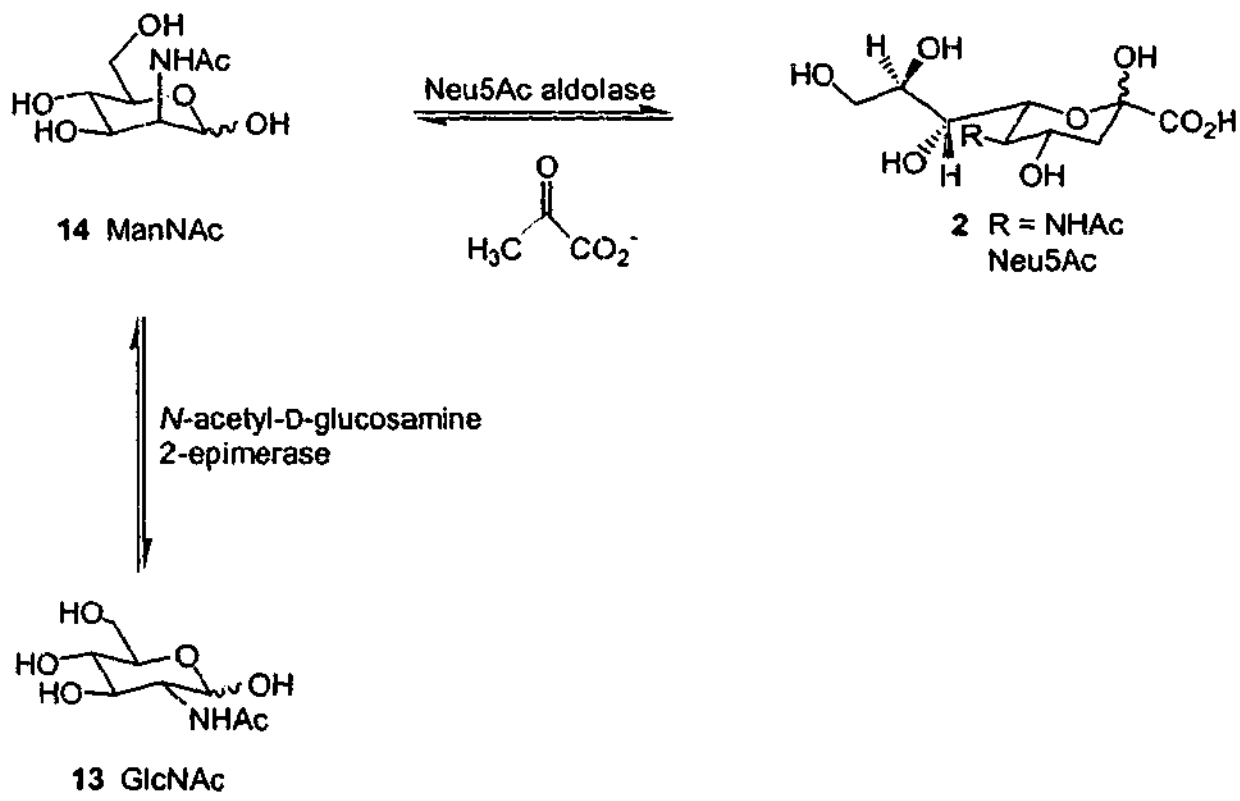


14 ManNAc

2 R = NHAc (Neu5Ac)

Scheme 1

Since ManNAc (**14**) is expensive and difficult to obtain in large quantities, Simon *et al*[66] generated Neu5Ac (**2**) from the less expensive GlcNAc (**13**) which was subjected to a base-catalysed epimerisation.[66,67] Kragl and coworkers[68] reported a continuous synthesis of Neu5Ac (**2**). Their method entailed dissolving the two enzymes, *N*-acetyl-D-glucosamine 2-epimerase [EC 5.1.3.8] and Neu5Ac aldolase with a substrate solution containing GlcNAc (**13**) and pyruvate in an enclosed "enzyme membrane reactor"[68] (Scheme 2).

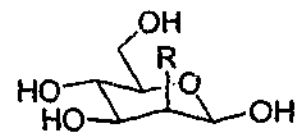


Scheme 2

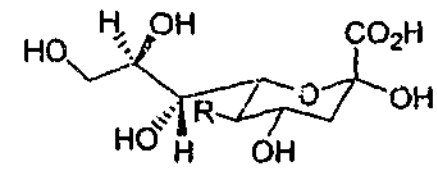
Maru *et al* [69] described a large scale production of *N*-acetylneuraminic acid using technology similar to that of Kragl.[63] Thus, *N*-acetyl-D-glucosamine 2-epimerase was expressed in quantities to support the relatively inexpensive production of Neu5Ac (**2**) with an overall yield of 77% from GlcNAc (**13**).[69]

The aldolase has been found to be specific for pyruvate as donor, however, a variety of acceptor substrates is plausible.[64,65] Particular modifications around the periphery of

ManNAc are tolerated by Neu5Ac aldolase, most notably at C-2 (including OH), C-4 and C-6 to give rise to Neu5Ac modified at C-5, C-7 and C-9 respectively.[65,70] For example, the modification of ManNAc (**14**) to give ManCBz derivative **16** has been found to be a substrate of Neu5Ac aldolase. This gives the Neu5CBz derivative **17** which may be utilized as a key intermediate for the synthesis of *N*-acyl derivatives (in which Sparks *et al*[71] used to develop inhibitors of influenza virus adhesion).



16 R = NHCBz
ManCBz



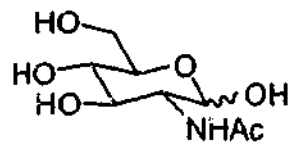
17 R = NHCBz
Neu5CBz

Not only are different analogues of ManNAc (**14**) good substrates for Neu5Ac aldolase, but also D-mannose and its' derivatives which afford 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (KDN, (**4**)) and related compounds.[65,72,73] Reviews by von Itzstein *et al*[43,61] discuss the range of different substituents at C-2, C-4 and C-6 accepted by the aldolase enzyme as substrates of ManNAc (**14**) or mannose[74] to give C-5, C-7 and C-9 substituted Neu5Ac and KDN derivatives, respectively. Modifications at C-3 are not accepted by Neu5Ac aldolase[64] except for 3-deoxymannose which produced a mixture of four acids upon exposure to Neu5Ac aldolase.[75]

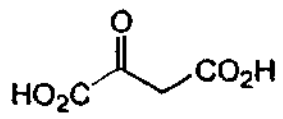
Recently, the Bertozzi group was able to synthesize sialic acid analogues with ketone side chains of various lengths at the *N*-acyl position, employing the Neu5Ac aldolase-catalyzed condensation of pyruvate and the corresponding mannosamine derivative.[76] Furthermore, these researchers were able to incorporate these sialic acid derivatives into cell-surface glycans.[76]

1.5.2 Chemical synthesis of sialic acids

Chemical synthesis of Neu5Ac (**2**) has a major advantage over enzyme synthesis in that it is possible to produce compounds which may not be accepted by the aldolase. The first reported synthesis of Neu5Ac (**2**) was by Cornforth and coworkers in 1958.[77] Cornforth's original synthesis involved condensation of *N*-acetylglucosamine (GlcNAc (**13**)) with oxaloacetic acid (**18**) (oxobutanedioic acid) in the presence of a base, to give, after decarboxylation, Neu5Ac (**2**) in 1-2% yield.[77]

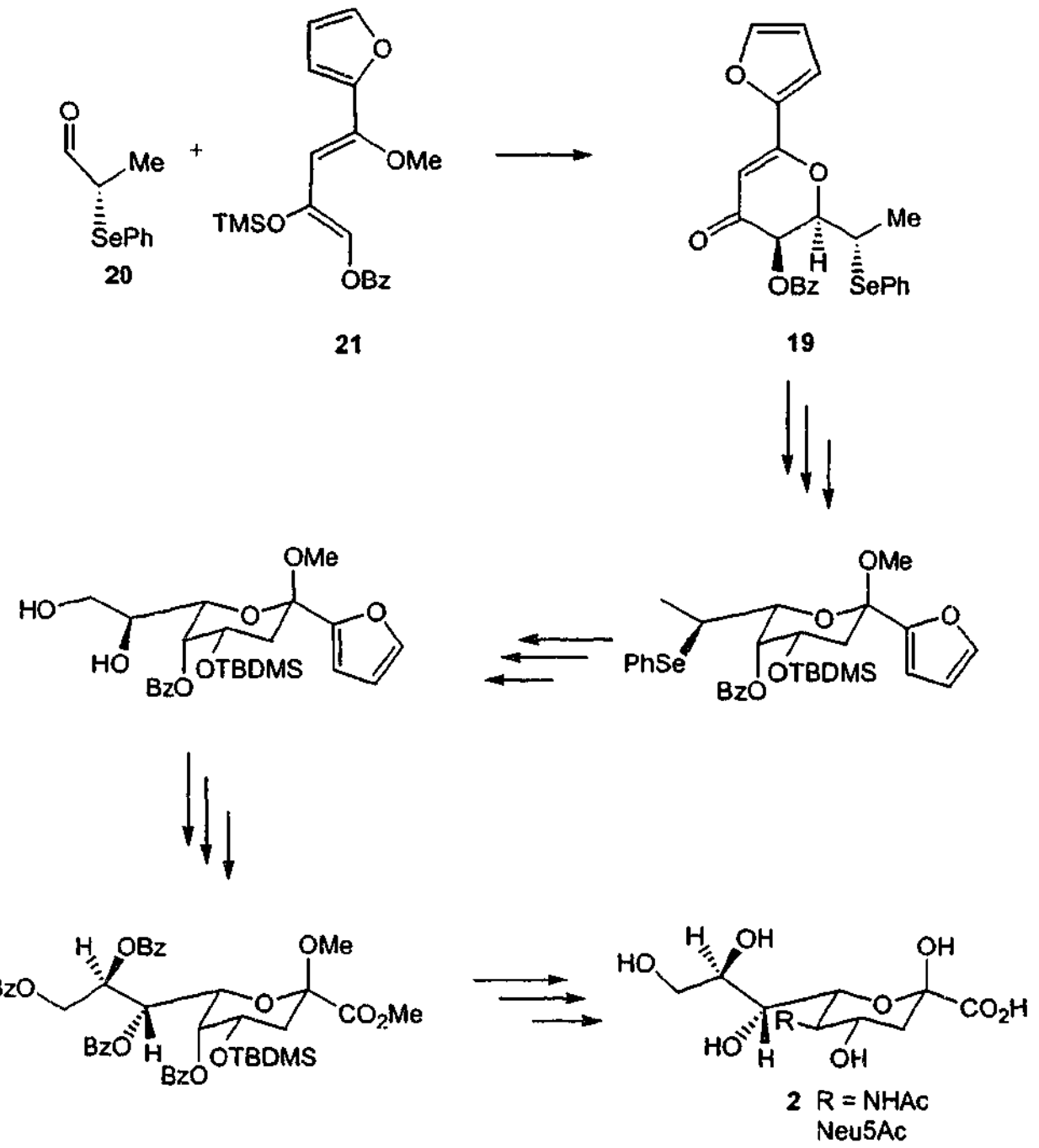


13 GlcNAc



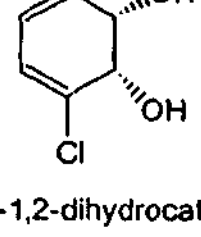
18

Improvements in procedures and yields have been accomplished[25,78,79] with one example being the first total synthesis of *N*-acetylneuraminic acid from non-carbohydrate precursors reported in 1988 by Danishefsky *et al.*[80] The multi-step sequence starts with the isolation of the key intermediate **19** obtained from the cyclocondensation of the (*S*)-seleno aldehyde (**20**), and the furyl diene (**21**) (Scheme 3).



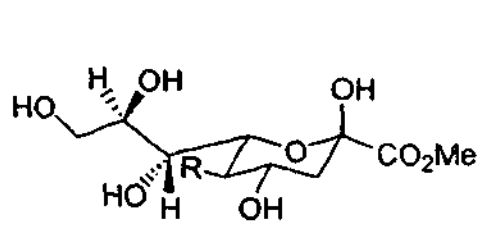
Scheme 3. Danishefsky's synthesis of Neu5Ac from a non-carbohydrate precursor.[80]

A recent report by Banwell *et al*[81] also described the synthesis of Neu5Ac (**2**) and KDN (**4**) from non-carbohydrate sources. In this case the starting material is an enantiopure *cis*-1,2-dihydrocatechol (**22**) and also involves a multi-step synthesis to produce Neu5Ac (**2**). Although this method is impracticable for large scale work, it provides an opportunity to prepare a wide range of ^{17}O-, ^{13}C- and/or ^{2}H- labelled Neu5Ac (**2**) derivatives.[81]

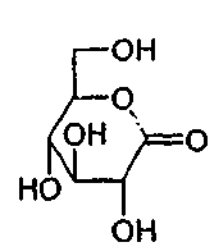


22 *Cis*-1,2-dihydrocatechol

Several groups have reported the use of indium-mediated allylation of ManNAc (**14**) with either ethyl α-(bromomethyl) acrylate[82] or α-(bromomethyl) acrylic acid[83] to obtain Neu5Ac (**2**). This short and efficient procedure has been employed to synthesise analogues of Neu5Ac (**2**).[84] Other reports on the synthesis of Neu5Ac (**2**) and its derivatives has included Diels-Alder reactions[85], alkylation[86] and Wittig chemistry.[87] The recent synthesis of Neu5Ac methyl ester (**23**) by Liu *et al*[88] involves a low cost and readily available starting material, D-glucono-δ-lactone (**24**). This particular synthesis also enables gram scale production of Neu5Ac methyl ester (**23**).[88]



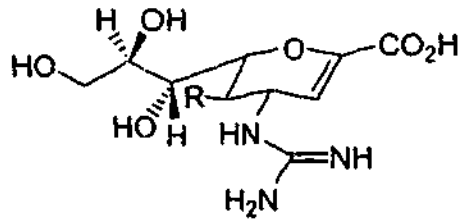
23 R = NHAc



24 D-Glucono-δ-lactone

N-acetylneuraminic acid has been modified at every position of Neu5Ac moiety. It is important to note the significance of modified sialic acids to target specific sialic acid-dependent processes. An excellent review by Zbiral[60] provides a detailed overview of the modifications to sialic acids. In more recent times, the design and development of sialic acid derivatives have been explored as probes for specific sialic acid-recognizing proteins (SARPs). For example, the development of the anti-influenza drug 2,3-didehydro-2,4-dideoxy-4-guanidinyl-*N*-acetylneuraminic acid (Zanamivir®, trade name of Relenza™ (**25**))

prompted numerous reports relating to the structural modifications of 2-deoxy-2,3-didehydro-sialic acids.[6,43,89] Indeed, the literature is replete with different ways of developing sialic acid-based therapeutics for selected disease states. Excellent reviews on carbohydrate-based therapeutics have been prepared by von Itzstein *et al*[6,43,61], Kiefel *et al*[62], Musser *et al*[27], Roy[37,90], Simanek *et al*[38] and Zbiral.[60]



25 R = NHAc
Relenza™

1.6 Probes for sialic acid-recognizing proteins (SARPs)

As mentioned earlier (*via supra*), the significant roles sialic acid plays in biological systems has prompted the widespread study of SARPs. These proteins recognize sialic acids for either metabolism or adhesion. Such recognition sites are of fundamental importance in an organism's development, cell-cell communication, as well as cell and protein targeting. They are also involved in the progression of a variety of disease states such as viruses and metastasis of tumours.

Some of these SARPs e.g. HAs (Chapter 2), sialyltransferases (Chapter 3), NAs, and cellular adehesion proteins have been extensively researched. However, there are several SARPs that have not been identified and characterised with much success due to technical difficulties faced by glycobiologists and glycochemists. These difficulties include the availability of systems for amplification of minute amounts of carbohydrates to facilitate

structural analysis and synthesis. Hence, studies centred about isolation of these SARPs for subsequent characterisation is explored in Chapter 4.

Several of the SARPs known to date are considered potential targets for the development of new drugs. It has been possible to map certain substrate specificities by utilising modified sialic acids and sialoglycocongugates. Useful relationships are thus gathered for estimating the minimum structural requirements for protein interactions and ultimately, optimisation and development of a pseudo-sialic acid that not only mimics the natural substrate, but also inhibits its harmful and sometimes life-threatening actions.

1.7 Scope of thesis

This thesis will focus primarily on the design and synthesis of some novel Neu5Ac analogues and their use in the purification of certain SARPs. These main objectives are detailed below:

- To explore the crystal structure of influenza virus HA in an effort to prepare potential monovalent and bivalent Neu5Ac analogues that may act as inhibitors of influenza virus. (Chapter 2)

- To design a fluorescent labelled Neu5Ac analogue that will potentially block and detect α2,8-sialyltransferases in order to study various sialic acid-recognition phenomena. (Chapter 3)

- To prepare a range of affinity matrices containing Neu5Ac analogues as the immobilised ligand for the purification of a number of SARPs in large quantities. (Chapter 4)

CHAPTER 2

The design and synthesis of influenza virus hemagglutinin inhibitors

2.0 Introduction to the Influenza Virus

A letter sent from Lord Randolph to Lord Cecil in November 1562[91]

" It ys a plague in their heades that have yt, and a soreness in their stomackes, with a great coughe, that remayneth with some longer, with others shorter tyme, as it findeth apte bodies for the nature of the disease. The queen kept her bed six days. There was no appearance of danger, nor manie that die of the disease, excepte some olde folkes."

Influenza, also commonly known as the flu, is a viral disease that has afflicted mankind through time, (*vide supra*). In the last century, three influenza pandemics caused millions of deaths worldwide, social disruption and profound economic losses. One of the most dramatic outbreaks was between 1918 and 1919. This particular pandemic swept across the world in three waves, claiming the lives of 20 million people and perhaps affecting the lives of one hundred times that number.[91,92] It has even been suggested that this outbreak was responsible, in part, for the end of World War 1;[92] there was simply not enough manpower left in either Europe or America and other continents to sustain the war effort. More recently, in excess of 17 000 deaths were recorded in England and Wales in 1976, and 26 000 deaths in the UK during the outbreak of 1989-1990.[93] This ever-present danger is still stalking humanity today. Even in a country with modern and accessible medical facilities such as the USA, the influenza virus was listed among the top 10 causes of death

in 1997.[94] More recently, in 2004 the World Health Organization (WHO) reported a total of 31 fatalities from Thailand (10) and Viet Nam (21).[95]

Since mid-December 2003 to early March 2004, eight Asian countries have confirmed outbreaks of a highly pathogenic avian influenza virus.[96] During January and February 2004, more than 100 million birds have either died of the disease or been culled. This figure is greater than the total number of combined poultry killed in the world's previous five largest outbreaks.[97] Currently there are concerns the H5N1 avian virus may combine with a human virus and unleash another deadly pandemic upon today's society.[98] Vaccination can be used as the first line of defence. However, due to the antigenic properties of the virus (*vide infra*), there is no guarantee that any vaccine can protect from viral infection.[99]

2.1 Pathogenicity of the Influenza Virus

The influenza virus is particularly harmful to pregnant women in the third trimester, the very young or aged, and those with chronic pulmonary and heart disease.[61,100] This disease is generally unpleasant with the most common symptoms being headaches, cough, chills, myalgia (muscle aches), catarrh (profuse discharge from nose and eyes), fever and aching limbs.[93,99] Complications such as viral pneumonia and meningitis can occur as a result. .

Influenza is still a major killer in the developed world.[93] Not only does this virus have a high mortality rate but also a significantly high morbidity that correlates to losses in economic terms due to a reduced workforce.[99] These considerations, coupled with the likelihood of another pandemic, have made the design of a therapeutic drug against influenza a priority for today's society.

All influenza strains are negative stranded RNA chains and belong to the family of orthomyxoviruses which are further classified, on serological differences, into three distinct types: A, B, and C.[55,99,101] Type A is the most prevalent and is responsible for the irregular major epidemics and pandemics. Type B is generally associated with minor localised epidemics while type C causes milder, less serious, cases of the disease to erupt.[102] Annual outbreaks of influenza are due to minor changes in the surface proteins of the virus progeny that enables the virus to evade the immunity humans have developed after previous infections with the virus or in response to vaccination.[103]

Two types of antigenic properties have been recognized in the virus: antigenic 'shift' and antigenic 'drift'. Antigenic shift is a radical change of the antigenic properties of the surface proteins leading to the emergence of new pandemic strains. Drift may result from a number of local minor alterations in the viral surface proteins which may cause epidemics.[104,105] Type A viruses are further classified into subtypes according to their antigenic shift characteristics based on HA and NA subtypes. In viruses that infect avian species, 15 subtypes of HA and 9 of NA have been identified.[106] Of the 15 HA subtypes (H1-H15), H3 and H7 also infect equines; H1, H2 and H3 infect humans; H1, H3, and H9 infect swine.[107] It has been discovered that some strains of avian viruses occasionally originate from humans, for example the H5 influenza outbreak in Hong Kong in 1997.[106]

Influenza type B viruses also contain both HA and NA surface glycoproteins. Type B viruses have been isolated from humans and seals and are not divided into subtypes.[107] Type C viruses have been isolated from humans and swine. However, unlike type A and type B viruses, they possess only one type of glycoprotein, HA-esterase-fusion (HEF), which contains all three functions of: receptor binding, H; membrane fusion, F; and receptor destroying-esterase activity, E.[107]

During this century three subtypes of influenza A have caused pandemics in man *viz.* the H1N1 subtype responsible for the 1918 pandemic; the H2N2 (Asia flu) which appeared in 1957 and H3N2 (Hong Kong flu) subtype which occurred in 1968.[101] Vaccination programs against prevalent influenza strains have been used to protect humans at risk of the virus.[99] However, mutations in the antigenic HA and NA proteins can occur at any time thereby affording new viral strains and effectively nullifying any protection derived from vaccines. Because of the propensity of the influenza virus to mutate, vaccines raised against certain strains cannot be guaranteed to protect against new ones.[94,99] Influenza viruses that infect humans initially derive from avian viruses either directly by cross-species infection or by gene reassortment during mixed infections.[107] In the light of this, the design of an antiviral agent is best met by inhibiting the physiology of that portion of the virus that does not alter.

2.2 The influenza virion

An electron microscopic study of the influenza virion has revealed a sphere approximately $1x10^3$ Å in diameter.[91] Beneath the lipid envelope of the virus particle is the membrane protein, which surrounds the core of the ribonucleoprotein (RNP).[108] The RNP consists of eight separate single-stranded ribonucleic acid (RNA) of negative polarity that make-up the genome of the virus, which is coated with a nucleoprotein (NP), forming helical structures that contain, as minor components, the three viral polymerase proteins PB2, PB1 and PA.[61,100,108,109] The influenza virus RNA polymerase, in conjunction with the single strand genomic structure, is subject to a high error rate which manifests as a poor-editing function. The resultant formation of minor mutations, which escape antibody recognition, cause antigenic drift, and allows influenza virus infection to prevail in individuals with previous exposure to different influenza strains.[110,111] The occurrence of antigenic shift is

believed to be due to co-infection of a human strain and an avian strain resulting in genetic resorting of the viral RNA.[101,104]

The two glycoproteins HA and NA which exist on the surface of the influenza virus (*vide supra*) are present on the virus as spike-like projections anchored in the lipid bilayer membrane by hydrophobic bases (Figure 4).[55,61,91,100] On each virion there are, on average, 500 HA and 100 NA spikes.[91,100] HA exists as a symmetric trimer and extends ~ 13.5 nm (135 Å) from the lipid membrane of the virion.[112,113] NA exists as a tetramer which extends ~ 6.0 nm (60 Å) from the virion surface.[112] Both glycoproteins appear to play essential roles in the infection cycle.

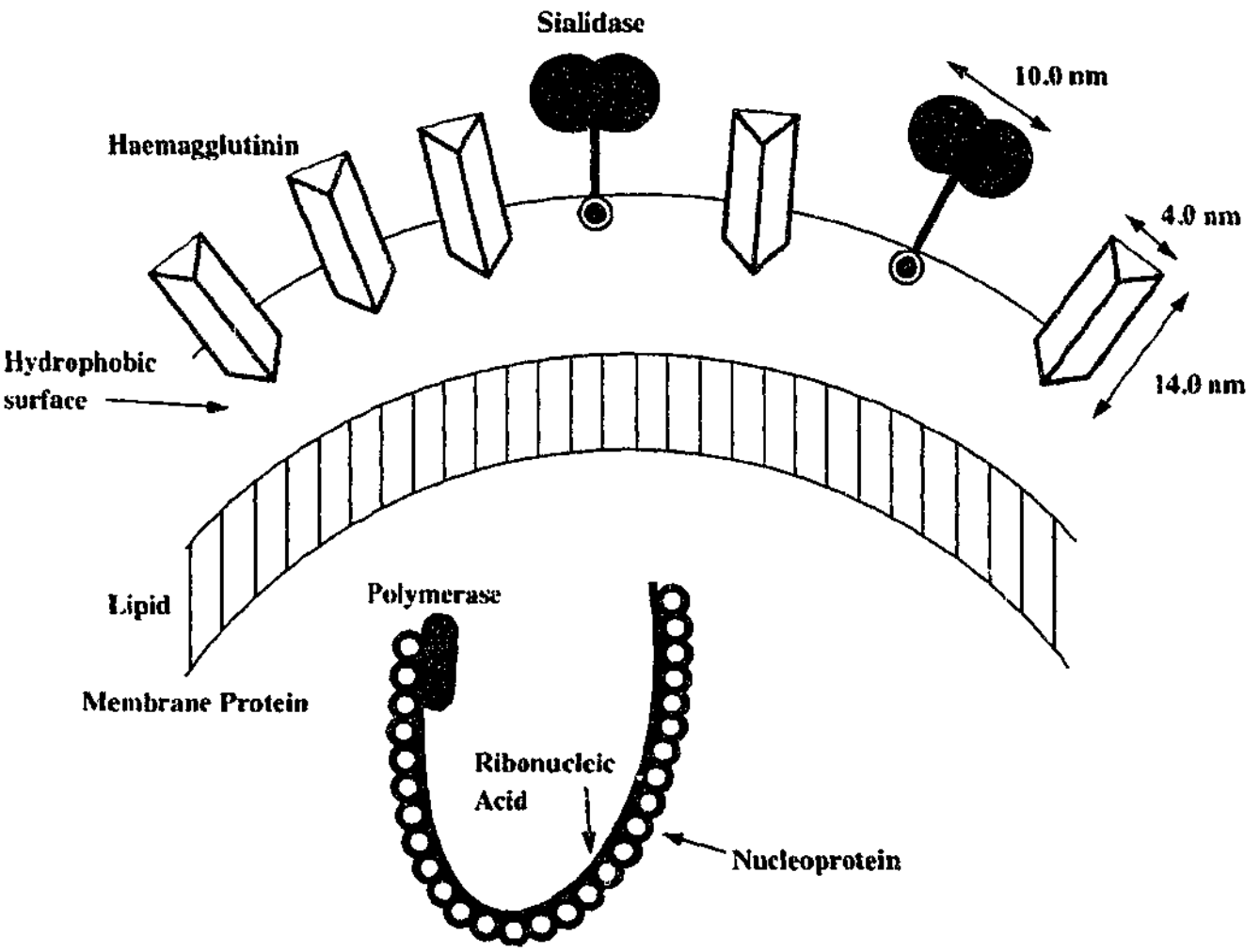


Figure 4. Cross-section of an influenza virus particle. (Prepared by J. C. Dyason)

2.3 Influenza virus infection cycle

The first step in the infection cycle involves adhesion of the virus to the *N*-acetylneuraminic acid receptors on the surface of a healthy cell. The adhesion process involves the recognition of Neu5Ac (**2**) located at the terminal position of oligosaccharides.[106,114] Adhesion to the target cell and subsequent penetration is mediated by the hemagglutinin glycoprotein. The key carbohydrate responsible for this process is the Neu5Ac (**2**) sialic acid.[55,106,115]

Adhesion and penetration is followed by receptor-mediated endocytosis of the virus particle upon which fusion of the viral membrane with the endosomal membrane is mediated to allow release of the RNP core into the cytoplasm and transported to the nucleus, the site of viral transcription and replication. After the assembly of the virion progeny at the surface of the cell, NA assists in their release from the infected cell by catalysing the cleavage of α-*O*-glycosidic linked Neu5Ac (**2**) (Figure 5).[109,114,116] Thus, α-glycosides of Neu5Ac (**2**) are both substrates for NA and ligands for HA. The freed viral progeny binds to other non-infected cells and the influenza virus cycle is repeated.

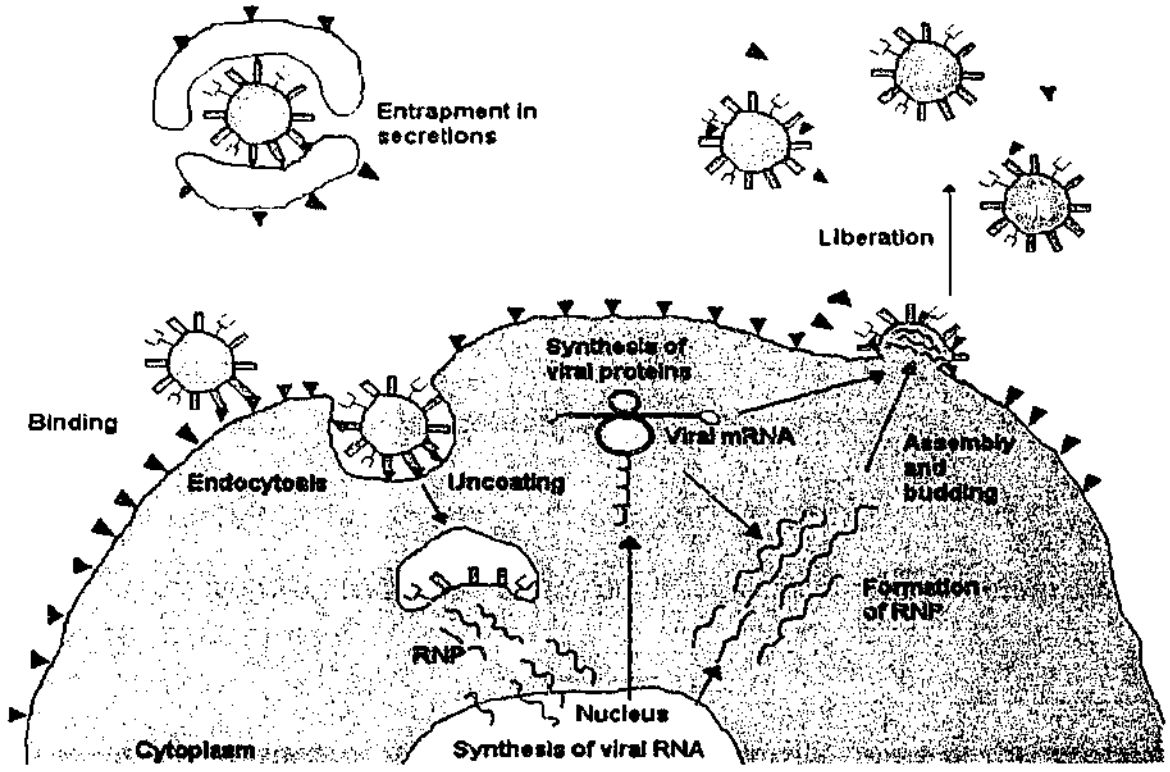


Figure 5. Influenza virus cycle.

▼ *N*-acetylneuraminic acid, ▯ Hemagglutinin, ⍊ Sialidase, RNP-ribonucleoprotein segments (Adapted from Gubareva *et al*[116])

2.4 Influenza virus sialidase (neuraminidase; *N*-acetylneuraminate glycohydrolase, EC 3.2.1.18)

As discussed in Chapter 1 (*vide supra*), NAs in general, play important roles in both the metabolism and catabolism of sialic acids. Influenza virus NA in particular, is well known for its pathogenic properties in the influenza virus cycle (*vide supra*). This is evident in the literature by the excellent articles which describe influenza virus NA's important features.[117-121]

Detergent-released NA preparations indicate that the NA head is a mushroom shaped tetramer and has a molecular weight of 24 kDa.[55,118] At 2.9 Å resolution, four identical circular subunits are arranged in a square planar configuration (100x100x60 Å). These

units are anchored into the viral membrane *via* a long thin stalk.[118] The heads are found to possess full enzymatic and antigenic properties.[100] Each subunit possesses a β-sheet propeller structure and a surface depression surrounded by conserved residues which has been identified to be the active site.[118]

2.4.1 The functions of influenza virus sialidase

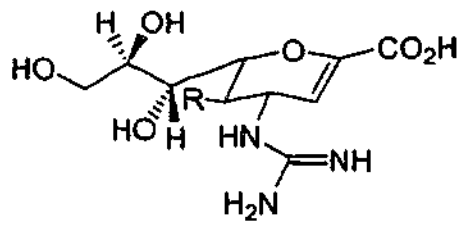
It is believed that there are two major roles for influenza virus NA in the infection cycle:[55]

1. It may assist in the transport of the virus through the mucosal lining of the respiratory tract, thus expediting the virus to the target epithelial cell. The ability of NA cleaving the terminal sialic acid residues on the mucosal sialoglycoproteins, can lead to impairment of the viscosity of the protective mucous layer of the upper respiratory apparatus.[10]

2. The more important and most discussed role for influenza virus NA is to facilitate cleavage of α-*O*-glycosidic linked sialic acid residues situated on the glycoconjugates of the host-cell surface thus, liberating the virion particle from the infected cell.[56,120-122] Newly formed virions may also contain sialic acids on the oligosaccharides of HA and NA. Removal of these terminal sugars prevents the HA of one virion binding to the sialic acids on an adjacent virion, thus reducing the propensity of the virus particles to self-aggregate.[56,120-122]

These significant roles have made the influenza virus NA a target for inhibition of the influenza virus disease. Before the crystal structure of NA was elucidaed in 1983 by Varghese *et al*[118], random screening of potential inhibitors did not produce an active drug

against the virus.[94] In 1993, von Itzstein *et al* were able to probe the active site of influenza NA based on the crystal structure utilising computer modelling. From this followed the conception, design and synthesis of an inhibitor for both influenza A and B virus NAs: 2,3-didehydro-2,4-dideoxy-4-guanidinyl-*N*-acetylneuraminic acid (GG167 or Zanamivir®, (**25**)).[117,118] Zanamivir® (**25**), which has the trade name of Relenza™, has been approved for general use in the USA, Europe, Australasia and Japan.[121]



25 R = NHAc
Relenza™

2.5 Influenza virus hemagglutinin

The HA glycoprotein, which has a molecular weight of 220 kDa, is present on the virus as a triangular rod-shaped glycoprotein, varying in radius from 15 to 40 Å.[113] HA is a trimer of identical subunits, each comprising two glycopolypeptides linked by a single disulfide bond, HA1 (328 residues) and HA2 (221 residues).[113,123,124] Following receptor binding at the cell surface, the influenza virus is internalized into cells by endocytosis, and the low pH of the endosome triggers conformational changes in HA that culminate in fusion.[125] During viral infection, the fusion process which is activated in the endosomes at a low pH, between pH 5 and pH 6, involves cleavage of a biosynthetic precursor, HA0.[113] Cleavage of HA0 generates the carboxy-terminus of HA1 and the amino-terminus of HA2 and is necessary for viral infection.[113] Incubation of HA with the protease bromelain releases a soluble fragment of hemagglutinin called BHA, with which the crystal structure was

elucidated in 1981.[126] The pH of a BHA solution is adjusted to 5 to allow BHA to undergo a conformational change associated with membrane fusion.[127]

There are three HA1 and three HA2 polypeptide chains in the membrane-bound HA homotrimer.[128] In essence, the structure of HA comprises 2 main sections; (i) an elongated stem region which contains residues of both HA1 and HA2 extending from the membrane surface; (ii) a globular region which contains entirely of HA1 is located on top of the elongated stem region.[114] The globular domain region contains an eight-strand anti-parallel sheet and houses the receptor binding site (RBS) and binds influenza to the cells (see Figure 6).[114,125] The HA1 chain harbours the RBS and binds influenza to the cells while HA2 is the subunit that induces membrane fusion.[125]

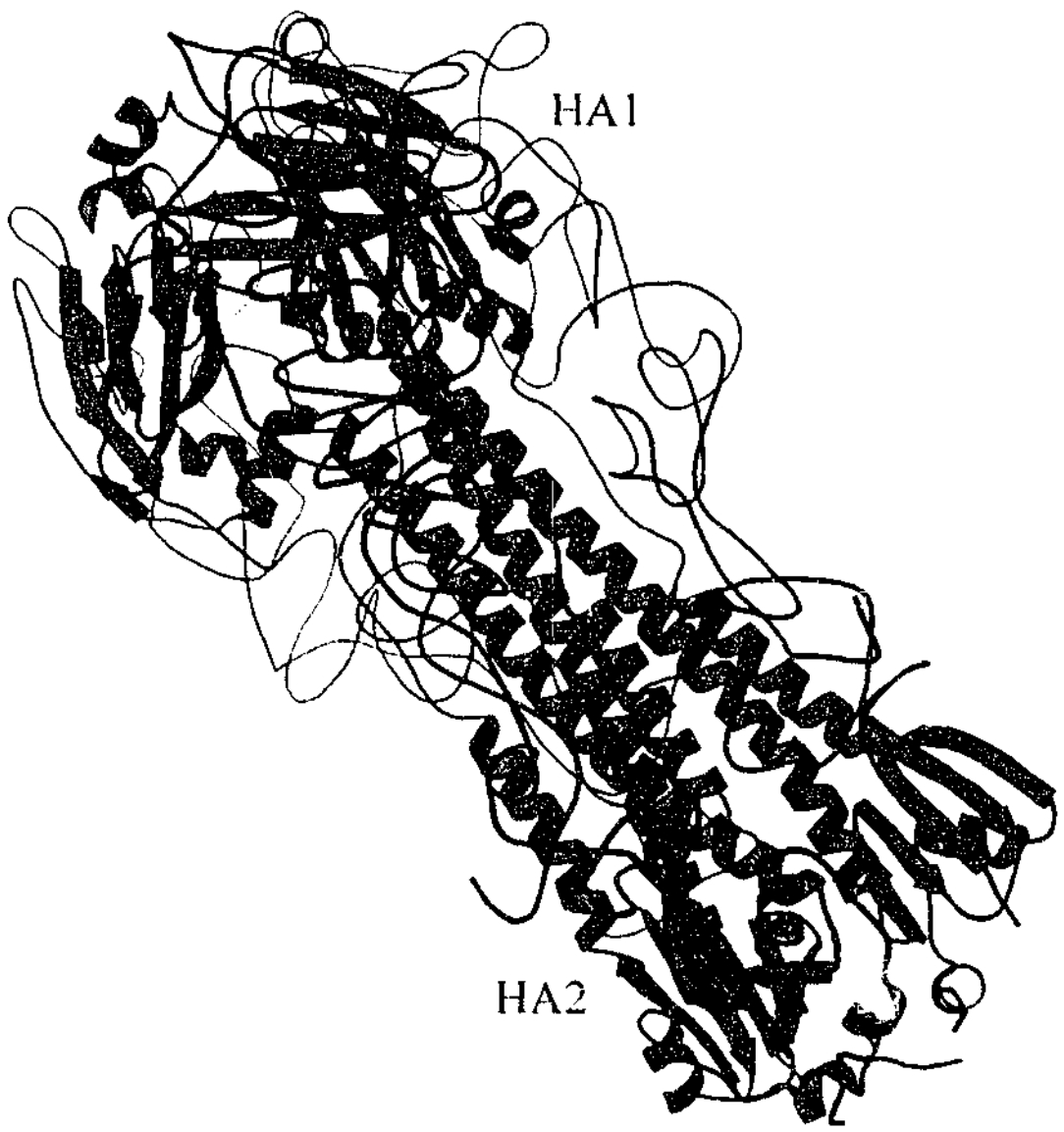


Figure 6. Three-dimensional structure of influenza virus hemagglutinin-detailing both HA1 and HA2 domains. (Prepared by J. C. Dyason, adapted from Kraulis[129])

2.5.1 The functions of influenza virus hemagglutinin

There are effectively three major functions of the influenza virus HA in the infection cycle:

1. Primarily to recognize and bind terminal sialic acid residues comprising carbohydrate side chains to initiate the virus-cell interaction.[114,123,128]

2. Following receptor-mediated endocytosis of the virus-receptor complexes, the HA then mediates the entry of the cell into the cytoplasm by a membrane-fusion

event. The membrane fusion is thought to be mediated by a conformational change in the HA, triggered at the low pH (between 5 and 6) of the endosome.[125,128]

3. The HA molecule is the major surface antigen of the virus against which neutralising antibodies are produced and as a consequence undergoes antigenic variation leading to recurrent epidemics and respiratory disease.[114,123]

2.5.2 The receptor-binding specificity of influenza virus hemagglutinin

Although Neu5Ac (**2**) is recognized by different strains of influenza virus, the recognition of a naturally occurring sialyloligosaccharide sequence by the viral HA depends on its subtype.[130] The two major linkages between *N*-acetylneuraminic acid and the penultimate galactose residues of carbohydrate side chains found in nature are Neu5Acα(2,3)Gal and Neu5Acα(2,6)Gal.[106] Different HAs have different recognition specificities to these linkages from viruses infecting different species; viruses from humans recognize the α(2,6)- linkage to galactose, those from avians and equines recognize α(2,3)- linkages, while swine appears to recognize both.[131] Recent examples are the 1997 Hong Kong avian virus containing a H5 subtype HA which binds to *N*-acetylneuraminic acid preferentially with α(2,3)- linkage, and the 1999 Hong Kong avian virus containing the HA of the H9 subtype which binds preferentially to sialic acids in the α(2,6)- linkage.[131]

Receptor specificity for H3 subtypes has been extensively studied. From various investigations, it has been established that human H3 isolates preferentially bind to erythrocytes containing the Neu5Acα(2,6)Gal,[130,132,133] whereas avian and equine influenza isolates bind cells containing the Neu5Acα(2,3)Gal.[130,133] This was attributed to the

substitution of amino acid 226, being Leu in the human isolates and Gln in avian isolates.[130]

2.5.3 The receptor-binding site of influenza virus hemagglutinin

The receptor-binding site (RBS) of influenza virus HA is a shallow pocket located on the membrane-distal end of each subunit and is composed of amino acids (Tyr 98, His 183, Glu 190, Trp 153, Leu 194) that are largely conserved in the numerous strains of the virus.[114,115] The phenolic hydroxyl group of Tyr 98 and the aromatic ring of Trp 153 form the bottom of the pocket (Figure 7). Glu 190 and Leu 194 project down in a short α-helix to define the rear of the site with His 183 and Thr 155. Residues 134 to 138 form the 'right' side of the site, while residues 224 to 228 form the 'left' side of the site.[115] Other conserved residues (Cys 97, Pro 99, Cys 139, Pro 147, Tyr 195, Arg 229) behind the pocket seemingly afford architectural integrity, not being in a position to interact with the receptor.[114] The conserved pocket of amino acids is surrounded by antigenically variable antibody binding sites.[115]

The natural substrate for the RBS of HA is Neu5Ac (**2**) indicating that this monosaccharide is the dominant component of the influenza virus receptor for which a binding site is conserved during antigenic evolution of the HA.[115] The X-ray crystal structure of HA perfused with sialyllactose was resolved by Weis *et al* in 1988.[115]

Binding affinity studies between HA and Neu5Ac, based on a molecular model of Neu5Ac (**2**) bound to complexes of α(2,6) sialyllactose (Neu5Acα(2,6)Galβ(1,4)Glc) (a trisaccharide with the same composition and linkages as the termini of many cell oligosaccharides) and α(2,3) sialyllactose, were performed to 3-Å-resolution.[115] In both

cases, Neu5Ac (**2**) was found to bind in the HA RBS in essentially the same way.[115] The position of Neu5Ac (**2**) in the HA RBS was determined, and possible atomic interactions of Neu5Ac (**2**) with the protein predicted from the corresponding inter-atomic distances.[115] A number of predictions were substantiated by direct binding experiments in solution.[115] Interestingly, the side-chain residues in the HA RBS that interact with Neu5Ac (**2**) (with the exception of residues 226 and 228), are either invariant or chemically similar for 86 influenza A virus strains from nine HA subtypes.[134]

A number of sialylated oligosaccharides were used as receptor analogues for crystal studies of the HA RBS.[106] Despite the sialic acid analogue in these complexes, the structure and orientation of the natural substrate Neu5Ac (**2**) in the HA RBS are all essentially identical; one side of the pyranose ring faces the base of the RBS, and the axial carboxylate, the acetamido nitrogen, and the 8- and 9-hydroxyl groups of the glycerol side chain, face into the site and form hydrogen bonds with the polar atoms of the conserved amino acid residues.[106,115] Each substituent of the Neu5Ac (**2**) moiety interacts with the HA RBS except for the ring atoms and the 4-hydroxyl group. The 4-hydroxyl group projects out of the active site and appears not to participate in binding.[106,115] A conserved Ser 136 forms a hydrogen bond with the carboxylate, which in turn is hydrogen bonded to the amide of peptide 137; His 183 and Glu 190 form hydrogen bonds with the 9-hydroxyl group, and Tyr 98 forms a hydrogen bond with the 8-hydroxyl group.[106] The acetamido carbonyl and the 7-hydroxyl group bond to each other and form van der Waals contacts with Leu 194.[106] The acetamido methyl group is in van der Waals contact distance with the six-membered ring of conserved Trp 153, and the 5-acetmido nitrogen forms a hydrogen bond with the carbonyl of the peptide 135.[106] The conserved amino acids Trp 153, Leu 194 and His 183 form a non-polar surface complimentary to, and in contact with, a non-polar surface on Neu5Ac (**2**) afforded by C-9, C-7 and the acetamido methyl group (Figure 7).[115]

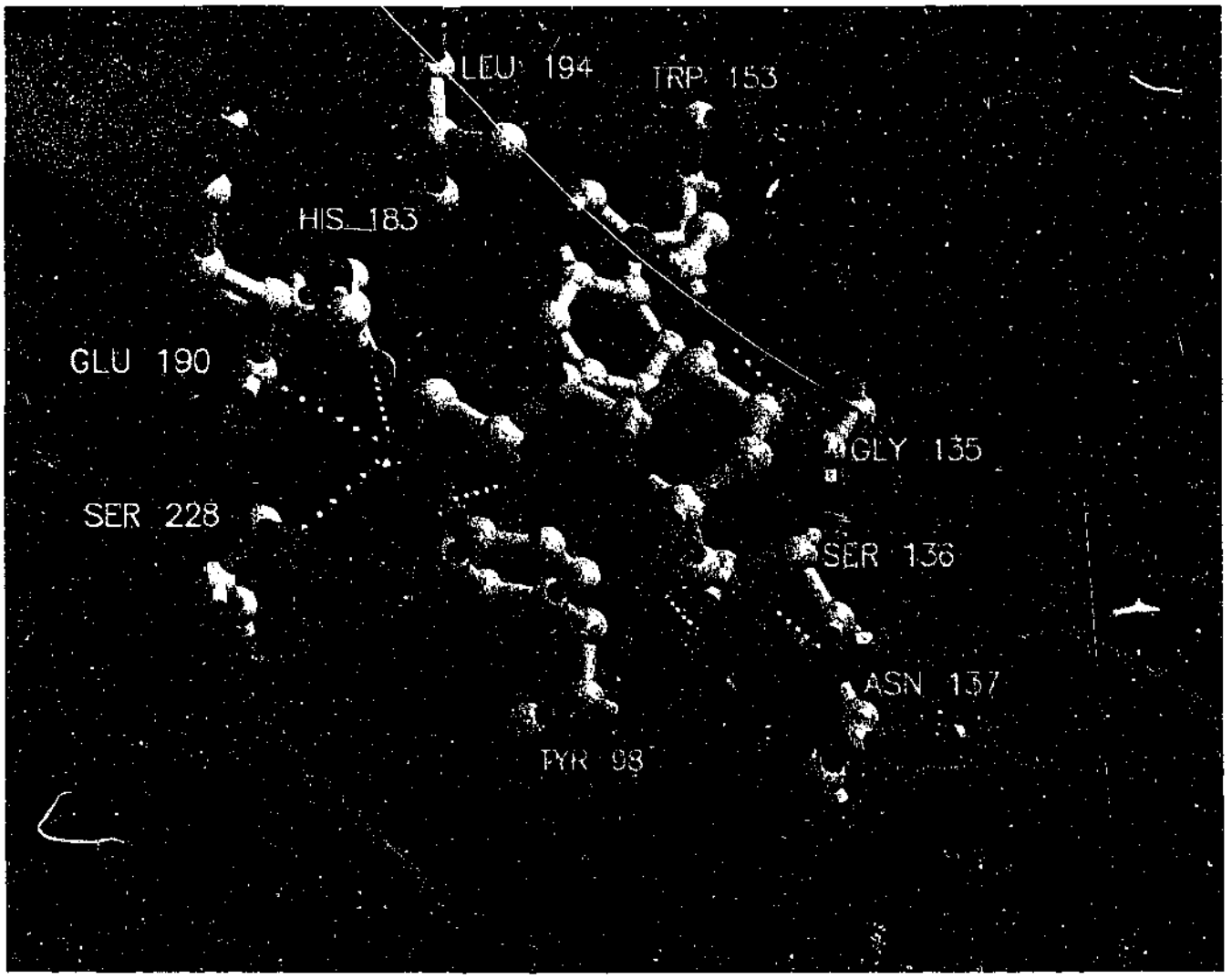


Figure 7. Crystal structure of hemagglutinin-Neu5Ac complex. (Prepared by J. C. Dyason, adapted from Merritt *et al*[135] and Taylor *et al*[136])

Rather weak binding affinities for the HA glycoprotein with the natural substrate Neu5Ac (**2**) (NMR titration dissociation constant (K_d) 2.1 mM) have been found.[128] The addition, removal and modification of all the substituents on Neu5Ac (**2**) was assessed by binding assays and the importance of each functional group deduced from the results. It was found that the axial carboxylate,[130,132,137] the 5-acetamido group[71,132,137] and the glycerol side-chain[132,137] constituted a substantial amount of the interaction energy with the RBS.

2.6 Inhibition of influenza virus HA

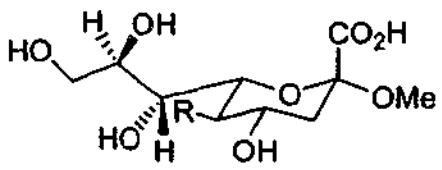
There are many approaches described in the literature that report the inhibition of sialic acid binding to influenza virus HA. Of particular interest are monovalent sialosides,[60,71,130,132,137-139] polymeric sialosides,[112,140-142] neoglycoconjugates,[143] multivalent inhibitors,[37,144,145] dendrimers,[146-150] bivalent sialosides[151] and clustering inhibitors.[152] Because the natural substrate, Neu5Ac (**2**), has very weak binding affinity for the HA glycoprotein, one possible approach is to develop a structurally modified Neu5Ac derivative with a greater binding affinity. Despite the low affinity of Neu5Ac (**2**) towards HA, part of the virulence factor of the influenza virus is the ability for HA to bind in simultaneous events. Therefore, another approach would be to inhibit HA by using bivalent or multivalent sialosides and obviate the multi-binding process.

2.6.1 Monovalent inhibition of hemagglutinin

Given that the dominant component occupying HA's RBS is *N*-acetylneuraminic acid and this natural substrate has a relatively weak binding affinity with HA, it is conceivable that a sialic acid analogue possessing a higher binding affinity than Neu5Ac (**2**) could be synthesised. Potential inhibitors with higher affinity for the sialoside-binding site of HA would theoretically disrupt the initial attachment process and therefore prevent or limit influenza virus infection.

An inhibitor commonly used as a standard for HA-receptor binding affinity is Neu5Acα2Me (**26**). Its HA inhibition assay shows it has a K_i of 2.0 mM[130] or K_d 2.8 mM.[128] This inhibitor has been employed as the benchmark inhibitor in most of the reports describing inhibition studies on HA (for example[134]). There are many potential inhibitors

that have been reported from various groups such as Zbiral,[60] Kelm *et al*,[137] Matrosovich *et al*,[132] Sparks *et al*,[71] Pritchett *et al*,[130] Toogood *et al*,[138] Weinhold *et al*[139] and Watowich *et al*.[134]

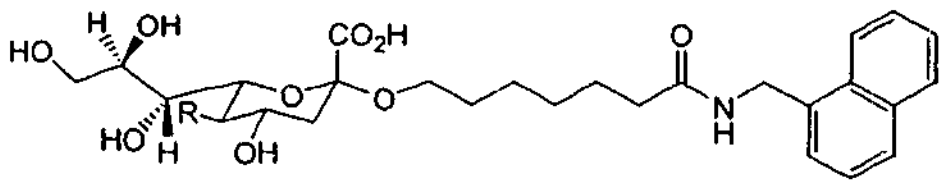


26 R = NHAc, K_i = 2.0 mM
Neu5Acα2Me

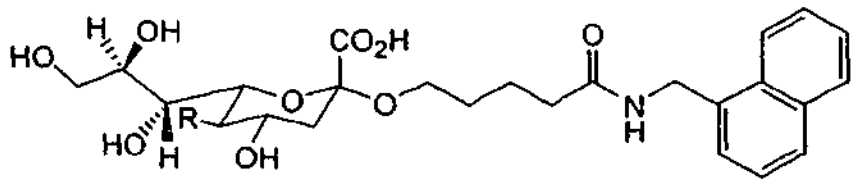
Some of the more potent HA inhibitors reported are shown below in Figure 8. Crystal structures of each, as a complex with BHA, have been elucidated by Watowich *et al*.[134] This has allowed a better insight into the interactions governing the observed binding affinities and also postulates for potential inhibitors possessing superior binding affinities. By way of example, compounds **27** and **28** developed by Toogood *et al*,[138] displayed strong binding with 50- and 25-fold-higher affinity (K_i = 44 μM and 88 μM, respectively) than Neu5Acα2Me (**26**) to HA (see Figure 8).[106] When complexed with BHA, their crystal structures indicated that tighter binding was related to the length of the aglycon moiety.[134] Compound **29** (developed by Weinhold & Knowles[139]), showed that the dansyl group in the C-4 position contributed to its high affinity (K_i = 300 μM).[134] This was attributed to the carbonyl oxygen of the 4-*O*-glycyl group hydrogen bonding to the side chain of the Ser 145.[134] However, addition of the 4-*O*-dansyl group to **27** (to obtain **30**), resulted in an increase in affinity by ~10-fold compared with **27** itself.[134] To date, **30** has one of the highest reported affinities for a monosialoside binding to HA.

Interestingly, viral NA did not cleave the glycosidic bond of the 4-substituted α-sialosides **29** and **30**.[139] However, incorporation of a dansyl group at the anomeric position and a free

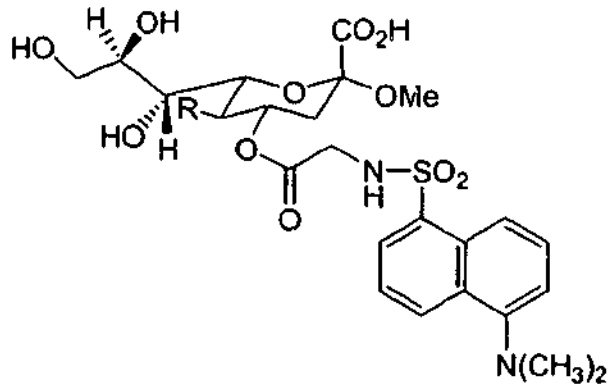
hydroxyl group at the 4 position, promotes hydrolysis by viral NA which shows NA sensitivity.[139] NMR experiments have also shown that the 4-acetyl group in Neu4,5Ac_2α2Me (**31**) does not interact with the HA protein.[128]



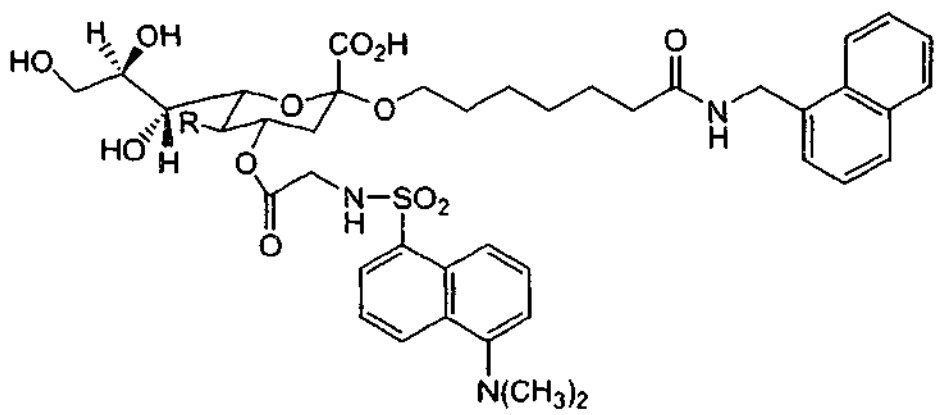
27 R = NHAc, K_i = 44 μM



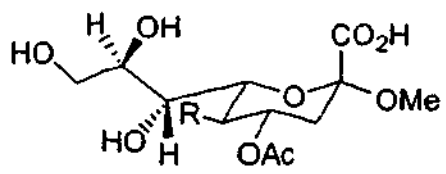
28 R = NHAc, K_i = 88 μM



29 R = NHAc, K_i = 300 μM



30 R = NHAc



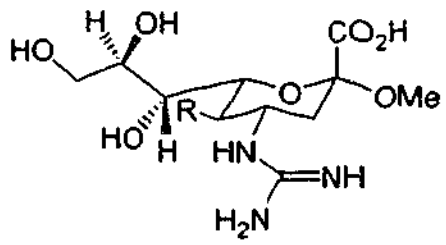
31 R = NHAc
Neu4,5Ac$_2$α2Me

Figure 8. Some of the more potent inhibitors of HA reported to date.

2.7 Molecular modelling of 4-deoxy-4-guanidino-Neu5Acα2Me (32)

From the results described above with monovalent HA inhibitors, Watowich *et al*[134] have proposed 4-deoxy-4-guanidino-Neu5Acα2Me (**32**) as a potential inhibitor for influenza virus HA. They described from preliminary investigations that **32** should bind in the active site resulting in a higher binding compared to the natural substrate Neu5Ac (**2**).[134] The orientation of the 4-guanidino group was designed to form hydrogen bonds to the carbonyl oxygen of the Gly 135 and Ser 145 in an effort to stabilise the complex and increase the binding affinity relative to Neu5Acα2Me (**26**).[134] The increase in affinity supposedly arises following formation of a salt bridge between the guanidinyl group and the highly conserved Asp 145 of equine H3 strains.[134]

We believed there was value in evaluating any possible interactions between 4-deoxy-4-guanidino-Neu5Acα2Me (**32**) and the RBS of influenza virus HA specifically by computational methods. Molecular modelling is extensively used in order to gain a better understanding of the molecular structure and the possible biological activity of carbohydrates.[136] The rational design of several carbohydrate-based inhibitors of influenza virus NA has been conceived with the aid of molecular modelling. The most notable of these being the design of Relenza™ (**25**), a potent inhibitor of influenza virus NA.[47]



32 R = NHAc
4-Deoxy-4-guanidino-Neu5Acα2Me

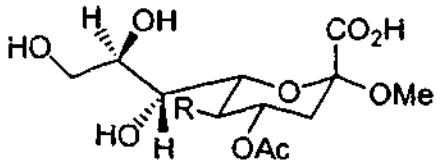
Exploration of the conformational properties of 4-deoxy-4-guanidino-Neu5Acα2Me (**32**) in the RBS of influenza virus HA and determination of the distances for each potential interaction in the RBS *via* molecular modelling was undertaken. Initially, the 4-deoxy-4-guanidino-Neu5Acα2Me (**32**) molecule was constructed and then minimized in the influenza virus HA RBS. The minimisation of the ligand **32** in the RBS was performed using the program Discover (see Appendix A). This program uses the Consistent Valence Forcefield to describe the interaction between the ligand and the protein. The minimisation process is required to remove any bad contacts introduced during the building of the ligand and to produce a complex that is suitable for molecular dynamics.

Molecular dynamics was then used to search for possible conformations showing the best binding modes (see Appendix B for program utilized). In the case of the influenza virus NA and its inhibitors, it has been shown that this approach can give a good indication of the inhibitory potential of the ligands.[136] Both molecular mechanics and molecular dynamics programs used in this transcript are described in the experimental section 5.2.

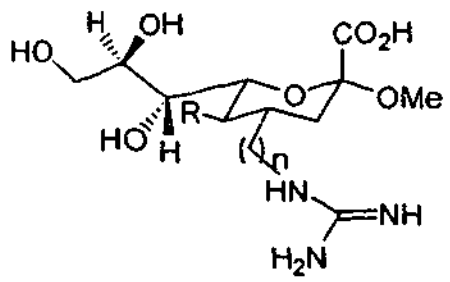
2.7.1 Discussion and results

The molecular dynamics protocol resulted in fifteen different frames containing different conformations of the 4-deoxy-4-guanidino-Neu5Acα2Me ligand (**32**) in the HA RBS. The most negative interaction energy is indicative of the best conformation for ligand **32** in the HA RBS. The conformation having the best interaction energy of -104 kcal/mol has been used as a starting point in the ensuing discussion. The combined molecular mechanics and molecular dynamics results for **32** have been depicted in Figure 9.

Computer modelling results indicate that 4-deoxy-4-guanidino-Neu5Acα2Me (**32**) does not interact with the Ser 145 as predicted by Watowich *et al.*[134] Rather they show that when 4-deoxy-4-guanidino-Neu5Acα2Me (**32**) is in the HA binding site, the 4-guanidino group is actually quite rigid and not flexible enough to have any interactions with Ser 145 (see Figure 9). However, there are some interactions between the 4-guanidino and the backbone oxygen of Gly 135. As a cross reference, the same molecular dynamics protocol was performed for Neu4,5Ac$_2$α2Me (**31**) in the binding site. Molecular modelling results for Neu4,5Ac$_2$α2Me (**31**) indicated that the 4-OAc has some degree of flexibility which enables interaction with the Ser 145 (see Figure 10). This flexibility could also be possible for 4-deoxy-4-guanidino-Neu5Acα2Me (**32**) if there was an extra carbon (or more carbons) in the molecule between the guanidino group and the pyranose ring as illustrated for compound **33.** The hypothetical ligand, **33,** may allow the guanidino group to interact with the Ser 145 in the HA RBS and possibly have greater affinity towards influenza virus HA.



31 R = NHAc
Neu4,5Ac$_2$α2Me



33 R = NHAc

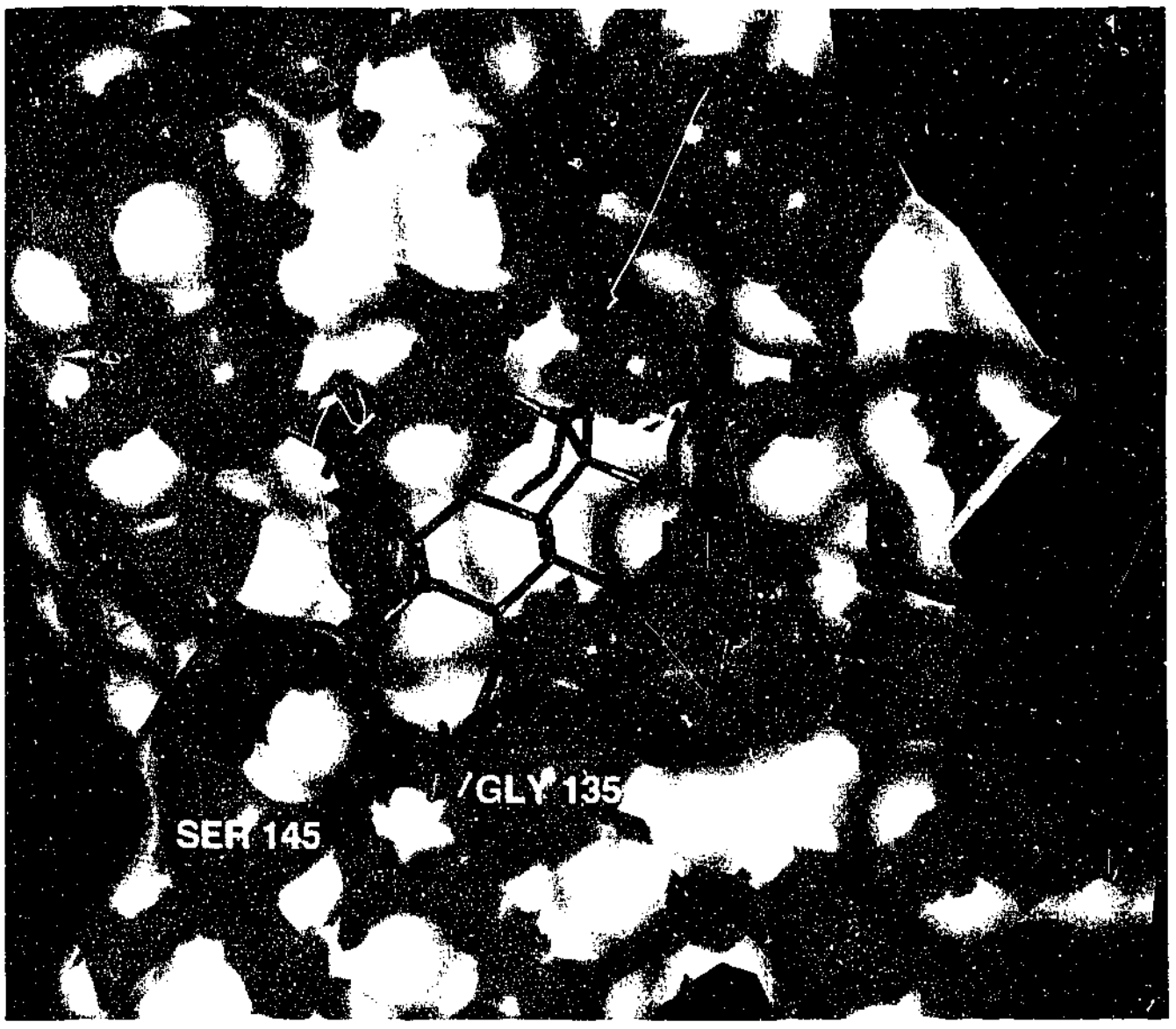


Figure 9. Molecular modelling structure of BHA receptor-binding site complexed with 4-deoxy-4-guanidino-Neu5Acα2Me (**32**).

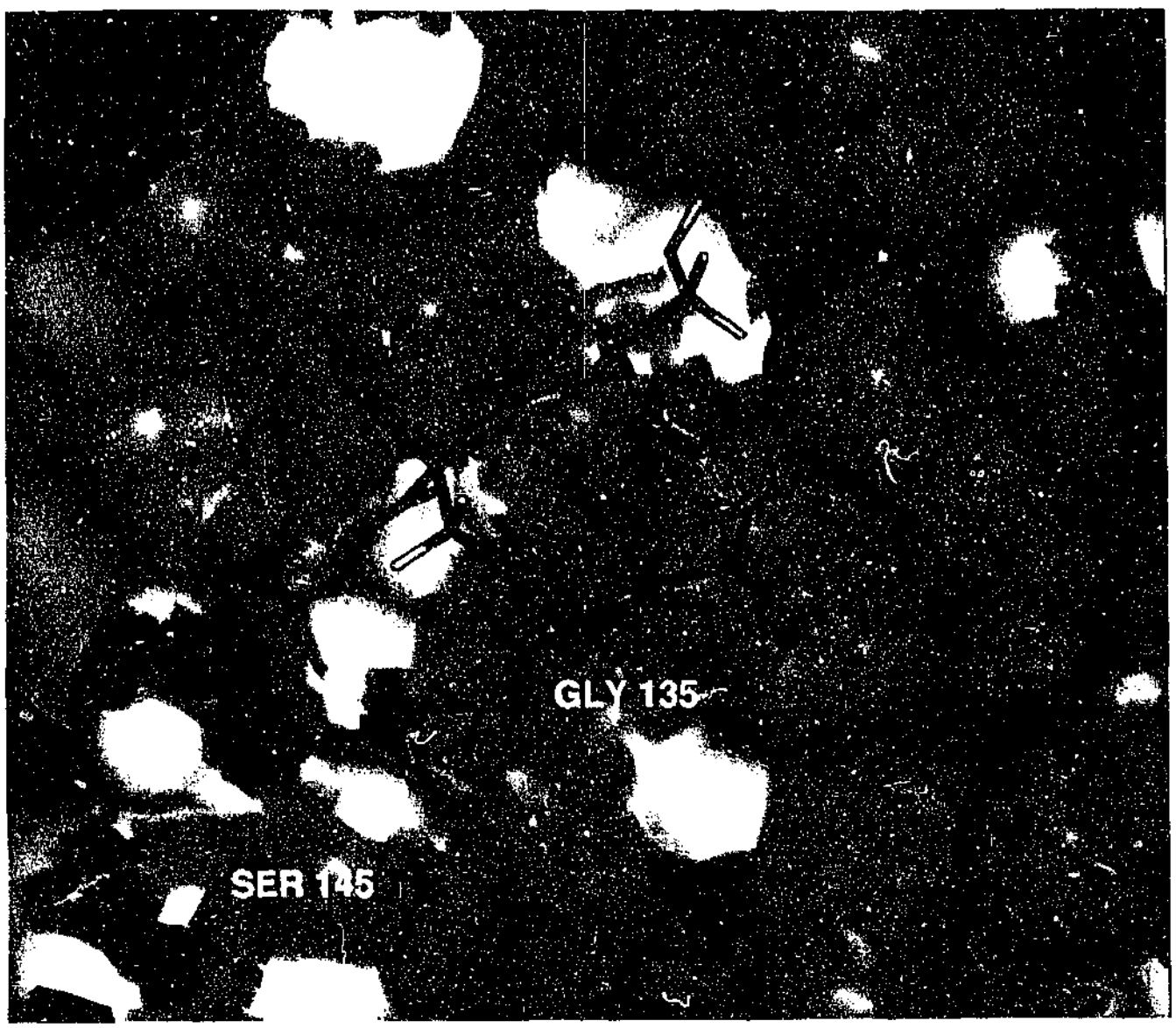
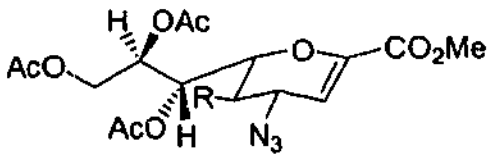


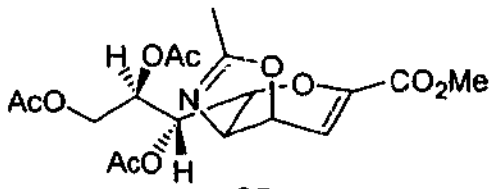
Figure 10. Molecular modelling structure of BHA receptor-binding site complexed with Neu4,5Ac$_2$α2Me (**31**).

2.8 Retrosynthesis of 4-deoxy-4-guanidino-Neu5Acα2Me (32)

Compound **34** was envisaged as the starting material to synthesize the target compound **32**. It can be produced in large quantities and, most importantly for a precursor to **32**, is a C-4 substituted sialic acid. There are a few reports on the synthesis of the key intermediate **34** which will be discussed below.

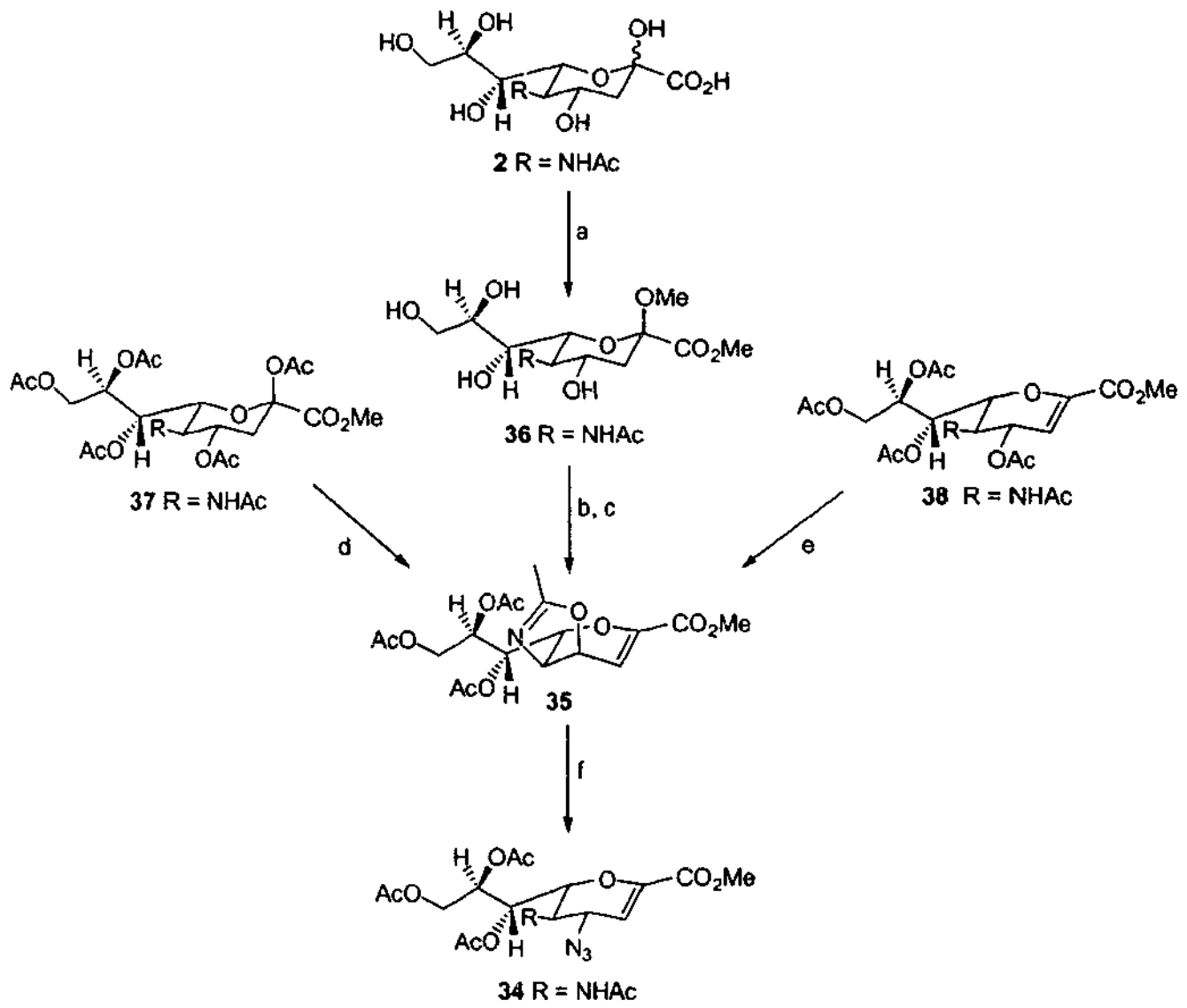


34 R = NHAc



35

The most common precursor for **34** is the oxazoline sialic acid **35.** One method for the development of **35** involves the initial treatment of Neu5Ac (**2**) with MeOH and acidic resin under reflux to obtain the β-methyl glycoside methyl ester of Neu5Ac (**36**) (see section 3.4.1 for synthesis).[153,154] Facile acetolysis of the β-methyl ketoside methyl ester of Neu5Ac (**36**) in the presence of acid followed by work-up with $NaHCO_3$[155] afforded high yields (82%) of the oxazoline derivative **35** (Scheme 4). In 1991, Schreiner *et al*[156] developed a method for the formation of oxazoline sialic acid (**35**) by treating the peracetylated methyl ester of Neu5Ac (**37**) with trimethylsilyl trifluoromethanesulfonate ($CF_3SO_3Si(CH_3)_3$) in acetonitrile (CH_3CN) (Scheme 4). Another method by von Itzstein *et al*[157] involves the treatment of Neu4,5,7,8,9$Ac_5$2en1Me (**38**) with boron trifluoride diethyl ether and methanol to form **35** in 96% yield. Since then, the oxazoline derivative **35,** has been utilized to develop C-4 substituted sialic acid derivatives.[155-158] Thus, treatment of the oxazoline intermediate with azidotrimethylsilane (Me_3Si-azide), formed the 4-azido sialic acid derivative **34** in excellent yield (82%).[157] The above strategy provides a direct entry into C-4 nitrogen-substituted α- and β-methyl sialosides. Alternative route from sialic acid require a substantial amount of protection and deprotection and are therefore less attractive. Moreover, introduction of nitrogen would have to be achieved by activation of C-4 (see for example[159]).

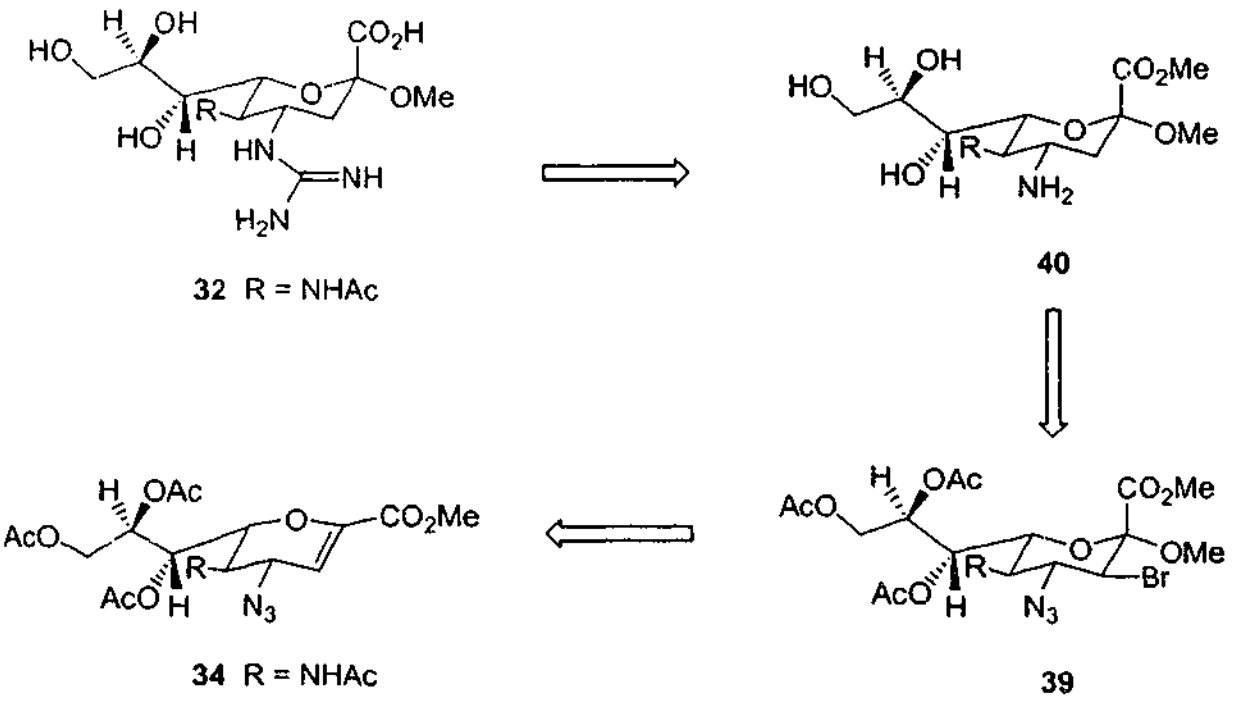


Reagents and conditions: (a) H^+, MeOH, reflux;[153,154] (b) Ac_2O, conc. H_2SO_4; (c) saturated $NaHCO_3$, (82%);[155] (d) $CF_3SO_3Si(CH_3)_3$, CH_3CN, 50°C, 2 hs, 82%;[156] (e) $BF_3.Et_2O$, CH_2Cl_2, MeOH, 16 hs, 25-30°C, 96%;[157] (f) $Me_3Si\text{-}N_3$, *t*-BuOH, 80°C, 4 h, 82%.[157]

Scheme 4

Since it was possible to obtain large quantities of **34**, the retrosynthesis of **32** was undertaken using this intermediate as outlined in Scheme 5. Initial addition to the double bond at the C-2 and C-3 carbon to obtain **39**, was accomplished *via* bromomethoxylation across the C-2, C-3 double bond as described in the literature by Okamoto *et al.*[160] The final two steps involve the simple reduction of the C-4 azide to form the respective amine **40**, which then can be transformed to the 4-guanidino target compound **32.** The final guanidination step utilizing the respective amino group as precursor has been discussed in the literature by von Itzstein,[158,161] Bamford[162] and Chandler.[163] Each have employed

different conditions to obtain yields ranging from 13%[158] to 57%.[161] Bernatowicz *et al*[164] were able to use 1H-pyrazole-1-carboxamidine hydrochloride for guanidination reactions and obtained most of their products in good yields (~72%). 1H-pyrazole-1-carboxamidine hydrochloride (PCH) in the presence of imidazole was investigated for the guanidination reaction in an effort to improve the work-up of literature[158,161-163] procedures and improve yields.

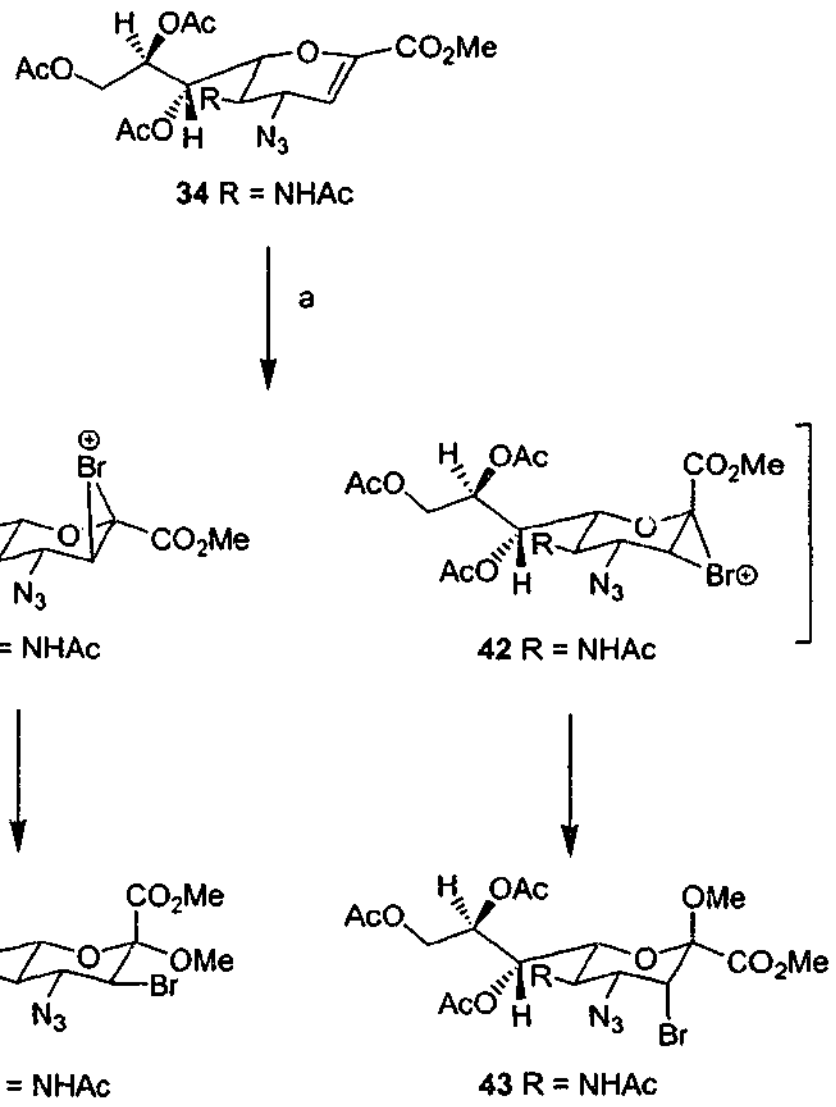


Scheme 5. Retrosynthesis of **32**.

2.8.1 Synthesis of Methyl 5-acetamido-3,4,5-trideoxy-4-guanidinyl-D-*glycero*-D-*galacto*-2-nonulopyranosidonic acid (4-deoxy-4-guanidino-Neu5Acα2Me) (32).[165]

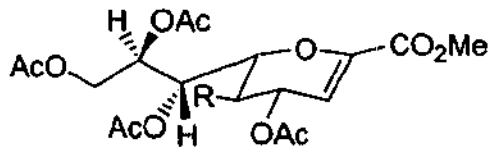
The strategy for the synthesis of **32** is outlined in Schemes 6 and 7.[165] Thus, treatment of **34** with *N*-bromosuccinimide (NBS) in methanol at room temperature resulted in the formation, *via* the bromonium ion intermediates **41** and **42** (across the C2-C3 double bond), of a diastereomeric mixture of **39** and **43**.

^{1}H NMR spectroscopy data showed that the crude product was composed of a 1:1 ratio of α and β isomers **39** and **43** respectively. Therefore, no stereocontrol was evident. Interestingly, bromomethoxylation of the peracetate derivative Neu5Ac2en1Me **(44)** has been reported to give a 4:6 diastereomeric mixture of the expected products, **45** and **46**[160] (see Figure 11). Slight variations in temperature had a significant effect on the reaction rate and formation of by-products. At temperatures below ambient a decrease in reaction rate was observed while partially de-*O*-acetylated by-products were formed at higher temperatures. Isomers **39** and **43** were readily separated by flash chromatography and isolated in an excellent overall yield of 87 %.

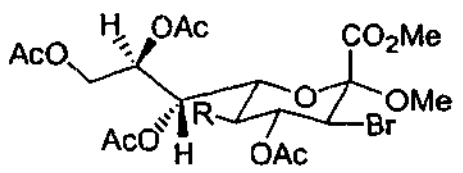


Reagents and conditions:(a) NBS, MeOH, rt, 16 hs, 87%.

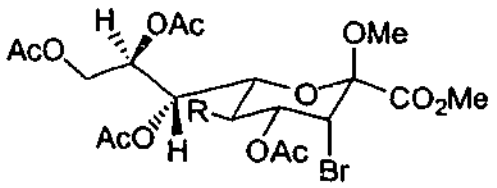
Scheme 6[165]



44 R = NHAc



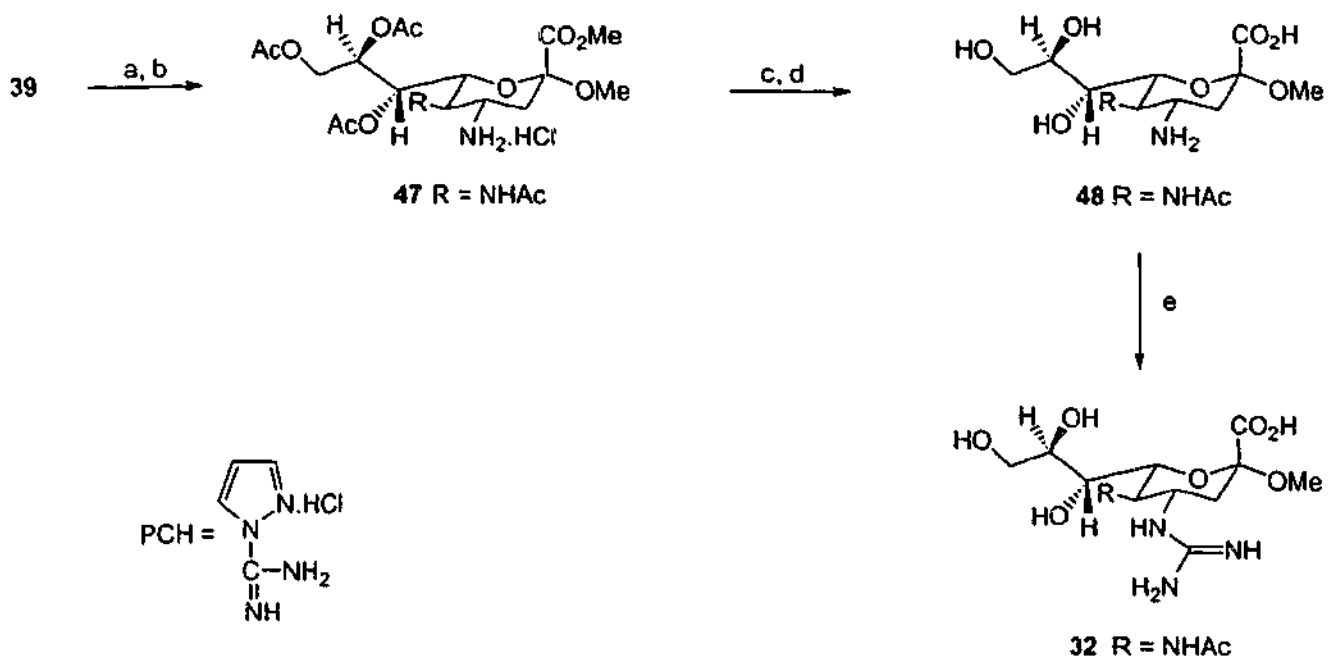
45



46

Figure 11

Comparison of chemical shifts with similar compounds and calculation of the apparent coupling constant between H-3 and H-4 protons aided the characterisation for each isomer, **39** and **43**. The H-3 proton of the α-OMe isomer **39** is in the axial position while that for the β-OMe isomer **43** is equatorial (Scheme 6). The expected coupling constant between H-3ax and H-4 for **39** is within the order of 10-12Hz, whereas, the coupling between H-3eq and H-4 in **43** is expected to be in the range of 3-5Hz.[160] The ^{1}H NMR signal for the methoxy group on the anomeric carbon for the α isomer (3.51 ppm) is downfield compared to the β isomer (3.27 ppm). This is consistent with analogous literature compounds.[160] The structure of compound **39** was also confirmed by IR spectroscopy which displayed a halogen band at 748cm^{-1} and another band at 2108 cm^{-1} typical of an azide functional group.[166] Mass spectroscopic analysis provided further evidence for the structure with the presence of the isotopic [^{79}Br (M + 1)$^{+}$] and [^{81}Br (M + 1)$^{+}$] peaks arising from the bromine atoms at *m/z* 567 and 569 respectively.



Reagents and conditions:(a) Bu_3SnH, AIBN(cat.), Dioxane; (b) Dil. HCl, 79%; (c) NaOMe, MeOH, 91%; (d) NaOH, 91%; (e) Imidazole, PCH, H_2O, 18%.

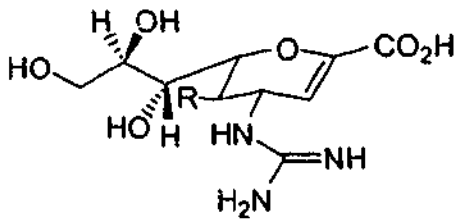
Scheme 7. Synthesis of HA inhibitor **32**.[165]

Clean debromination with concomitant azide reduction of the α-methylketoside (**39**) was achieved following treatment with tri-*n*-butyltin hydride and a catalytic amount of α,α-azodiisobutylronitrile (AIBN) in anhydrous dioxane at 80 °C. After acidification with dilute HCl, the corresponding 4-amino hydrochloride salt, **47** was afforded in 79 % isolated yield (Scheme 7). Formation of the hydrochloride salt of the respective amine allowed removal of any residual tri-*n*-butyltin hydride by-products. The structure of **47** was supported by ^{1}H NMR spectroscopy where the appearance of the H-3 axial proton resonance at 1.64 ppm was observed. Other resonances were observed at 2.52 ppm corresponding to the H-3 equitorial proton and 2.82 ppm for **47** due to an upfield shift of H-4 (from 4.38 ppm for **39**). This intermediate was also found to be ninhydrin positive and IR spectroscopy clearly indicated a loss of the azide and halogen bands. The amino substituent was still intact however as indicated by a band at 3448 cm^{-1}.

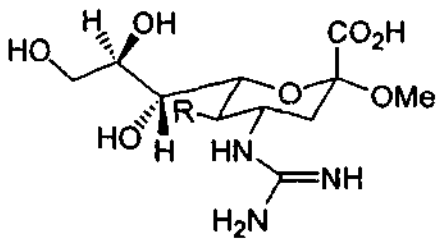
Deacetylation followed by subsequent saponification of **47** using standard conditions[167] afforded **48** in both high purity and yield (91%). Formation of **48** was confirmed by ^{1}H NMR spectroscopy with the downfield shift of H-4 (from 2.82 ppm for **47**) to 3.07 ppm for **48**. IR spectroscopy also supported formation of **48** with the appearance of the free carboxylic acid group at 1618 cm^{-1}. The free amine, 4-amino-4-deoxy-Neu5Acα2Me (**48**), was then treated with 1*H*-pyrazole-1-carboxamidine hydrochloride (PCH) in the presence of imidazole to afford the desired target, **32**, in a moderate yield of 18 %. The presence of the guanidino group in **32** was confirmed by ^{13}C NMR spectroscopy with the appearance of a new signal at 158.7 ppm for the C-guanidino (which is in agreement with the literature[158,161-163]). Mass spectroscopy also confirmed the structure with a $(M + 1)^{+}$ ion peak at *m/z* 365.

2.9 Binding studies of 4-deoxy-4-guanidino-Neu5Acα2Me (32)

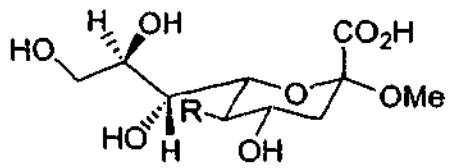
Preliminary binding studies were performed on 4-deoxy-4-guanidino-Neu5Acα2Me (**32**) by Yasuo Suzuki's group in Japan,[168] to determine if this compound had potential as an inhibitor of the influenza virus. An unsubstituted methyl glycoside compound **26** (prepared by Dr M. J. Kiefel) and the well known influenza inhibitor Zanamivir (**25**) (Relenza™, see Figure 12) were also tested in the binding studies and used to compare results.



25 R = NHAc
Relenza™



32 R = NHAc
4-Deoxy-4-guanidino-Neu5Acα2Me

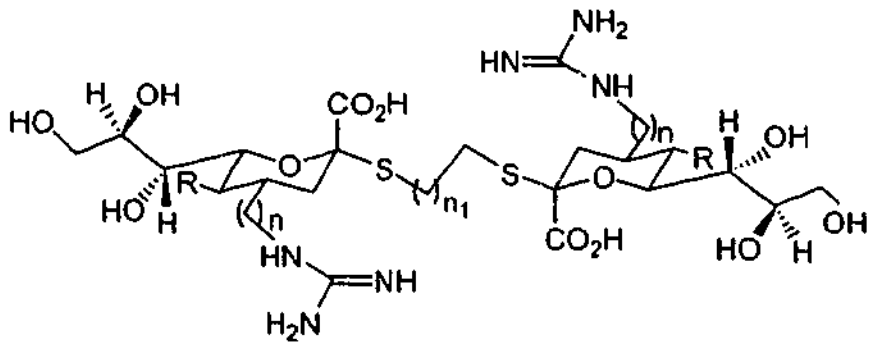


26 R = NHAc
Neu5Acα2Me

Figure 12

2.9.1 Results and Discussion

The experimental data showed that compound **32** and Relenza™ **(25)** prevented the cytopathic effect of the virus, but not compound **26**.[168] The binding studies indicate that compound **32** inhibits only in the late stage of the growth of influenza virus but not the onset stage where virus infection prevails by attachment of HA with its ligands.[168] This indicates that compound **32** contains more activity against NA inhibition than against HA. These results confirm that monovalent compounds do not significantly inhibit HA as expected. Additionally, due to compound **32** showing activity against NA may indicate that this sialoside is refractory towards NA activity. For future studies, the development of multivalent derivatives of compound **32,** such as compound **49** (where n and n_1 would need to be determined for optimal activity) may exhibit better results.



49 R = NHAc

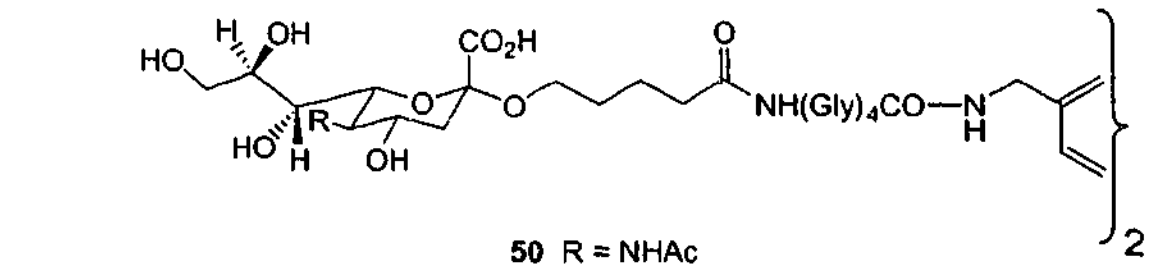
2.10 Multivalent inhibition of HA

It has been established from previous work that the position of the sialic acid group and the interactions between the protein and sialic acid are the same for 2- or 4- substituted sialic acid derivatives,[134] sialyllactose and other sialyloligosaccharides bound in the HA RBS.[169] Binding of soluble α-sialoside monomeric derivatives to HA is very weak (K_D ~ 2 mM)[147], compared to that of the influenza virus to target cells. This is a consequence of the virus's interaction with cell-surface sialic acid groups which develop strong polyvalent binding with multiple copies of HA on the virus.[147] Non-physiological and potentially toxic concentrations of soluble monomeric sialic acids are required to competitively disrupt the polyvalent interactions and prevent viral endocytosis into the cells.[147] To obviate these issues, polyvalent inhibitors having the potential to interact with multiple HA moieties on a single virus, which leads to increased binding affinity, have been developed.[147] There have been reports on inhibiting the HA glycoprotein utilising bivalent α-sialoside ligands.[151,152,170] The success of this method presumably takes advantage of the trimeric nature of HA. This approach considers the potential of multiple binding of Neu5Ac (**2**) to HA on the surface of a cell. Therefore, multivalent compounds may potentially act as inhibitors with increased affinity towards HA *via* co-operational effects. Such polyvalent interactions are supported by the observation that glycoproteins (e.g fetuin, α_2-

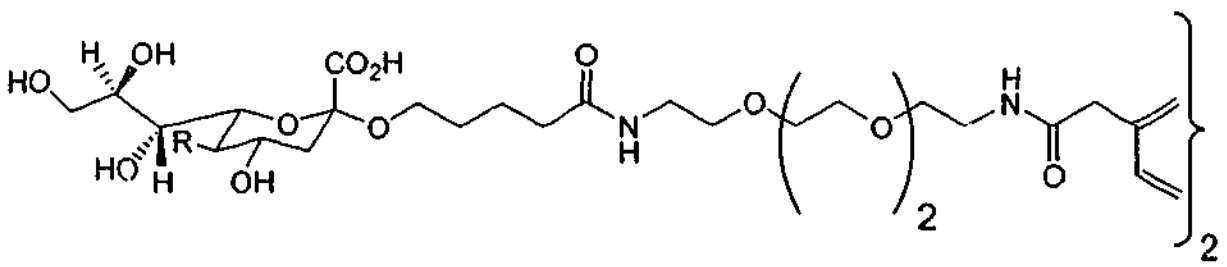
macroglobulins) and synthetic polymers containing high contents of sialic acid are strong inhibitors of HA.[140,171,172]

Some of the more potent bivalent inhibitors are compounds **50** and **51** which display exceptional affinity towards HA (see Figure 13).[151,170] Both compounds showed moderate-to-good enhanced affinity towards influenza virus HA when compared to Neu5Acα2Me (**26**) (~100 fold more for **50** and ~20 fold more for **51**).[170] Inhibition in this case was observed for whole influenza virus and not just for BHA.[170] This binding potency was not observed for the monovalent analogues of **50** and **51**.[170] As such, inhibition may be due to **50** or **51**, bridging with sialic acid loci on different HAs at the viral surface.[170] Other derivatives with different chain lengths were tested for inhibition however, compounds **50** and **51** were the most active within their respective series.[170] Notably, both have the same span length of 57 Å.[170]

There are many reports of other types of polyvalent systems, for example, neoglycoconjugates,[37,143,173] heptasaccharides,[152] glycopeptides,[143] glycopolymers,[37] liposomes[37] and dendrimers.[37,143,174] However, neoglycoconjugates and glycopolymers therapeutic utilization may be limited due to their high immunogenicity.[143] Recent articles by Roy[37,90] describe multivalent compounds related to *N*-acetylneuraminic acid.



50 R = NHAc



51 R = NHAc

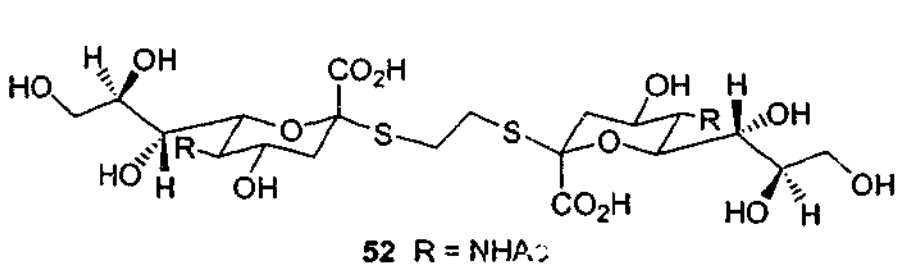
Figure 13

Despite the possible inhibitory properties of multivalent carbohydrates, they have the potential to generate unwanted antibody formation and, in the case of liposomes, can be incorporated unspecifically into cell membranes.[143] Therefore, when planning a synthesis, the design elements involving spatial and conformational optimization for all specific carbohydrate-protein interactions must be taken into account. Some of these polyvalent systems have been found to be either too long to show any inhibition or too bulky which would cause other side effects under *in vivo* conditions.[143]

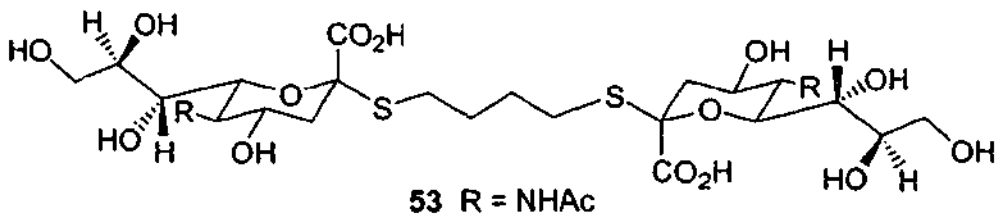
Another factor when considering HA inhibitors is that activity can be reduced due to the cleavage of sialic acid by the action of viral NA.[175] Obviously, the potential to act as an inhibitor increases with increasing resistance to NA.[175]

2.10.1 Design of multivalent inhibitors

The development of inhibitors presenting multiple copies of Neu5Ac (**2**) to the virus was investigated based on the premise that multivalent interaction is necessary for tighter binding of the substrate to the virus. Potential inhibitors were also simultaneously designed to be resistant to the action of viral NA (*vide supra*). Since thiosialosides are believed to be resistant to NA-catalysed hydrolysis,[176,177] α-thioketosides **52** and **53** were synthesized in an effort to combine HA and NA inhibition (see Figure 14). To avoid any issues associated with steric bulk, ethyl and butyl spacer arms were incorporated into **52** and **53** respectively. Both **52** and **53** were designed with different spacer arms to determine whether the span of these compounds had any effect on activity.



52 R = NHAc



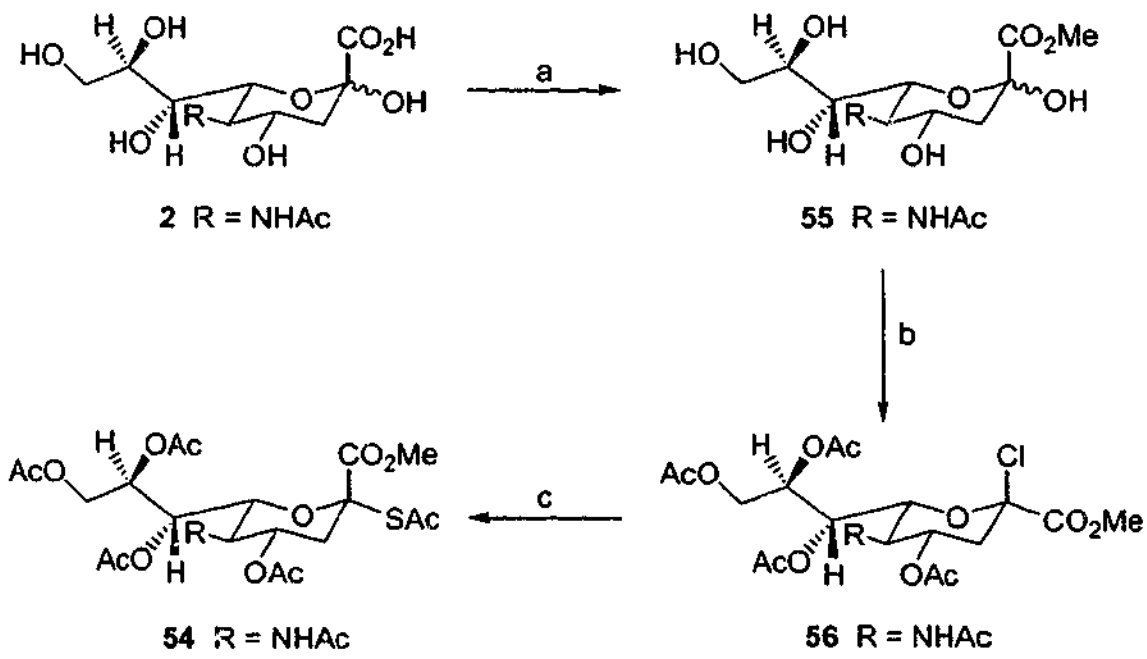
53 R = NHAc

Figure 14

2.10.1.1 Retrosynthesis of dithiosialosides

The general strategy for the synthesis of thioglycosides of Neu5Ac relies on the key precursor 2-thioacetyl Neu5Ac derivative **54**. Commercially available Neu5Ac (**2**) was initially esterified by treatment with an acidic resin in MeOH to give the Neu5Ac methyl

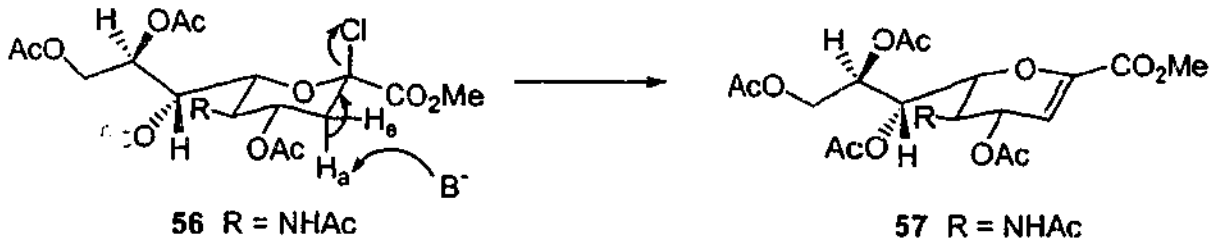
ester (**55**) in near quantitative yield (Scheme 8). Subsequent treatment of **55** with neat AcCl[153,178] for 60 h afforded Neu5Ac2βCl1Me (**56**). The use of saturated solutions of AcCl with HCl has also been used to prepare **56**.[179,180] In most cases crude **56** is used in subsequent reactions without further purification. Reaction of **56** with potassium thioacetate as reported by Hasegawa[167] leads to inversion of configuration about the anomeric carbon (C-2) to yield the thioacetate derivative **54** in moderate overall yield (~ 65%). Notable ^{1}H NMR signals include the SC(O)Me three proton singlet at 2.23 ppm and the one proton doublet at 2.59 ppm ($J_{3eq,3ax}$ 12.9, $J_{3eq,4}$ 4.5 Hz, H-3eq). These are characteristic signals for **54** in its α-anomer form (the H-3eq resonates further upfield in the β-anomeric form).



Reagents and conditions:(a) MeOH, Amberlite IR-120(H^+), 48 hs; (b) AcCl, 60 hs; (c) KSAc,CH_2Cl_2, 0°- rt; N_2, 24 hs.

Scheme 8

The 2,3-didehydro derivative **57** can be formed as a by-product by β-elimination of the H-3ax proton *trans*-diaxial to the chloro group at C-2 of **56**, during formation and storage (Scheme 9).

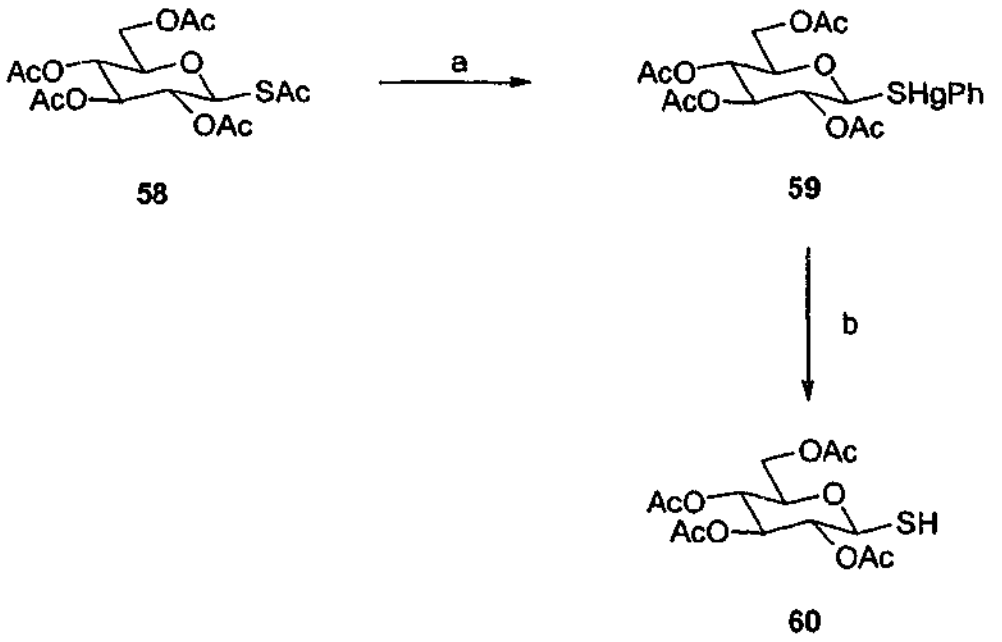


56 R = NHAc **57** R = NHAc

Scheme 9

Purification of **54** is accomplished by chromatography followed by recrystallization. Storage under an inert atmosphere at -20°C ensures that decomposition does not occur for a number of weeks. The thioacetate **54** can then be de-thioacetylated and coupled to the appropriate activated acceptors.

Methods exist in the literature which describes successful thiodeacetylation.[151,181-184] Demercuration, as shown in Scheme 10 has been used to selectively deacetylate anomeric thioacetates such as **58.**[183] The thiol generated **60** can then be coupled to the appropriate acceptor.[183] Although this procedure is reported to be high yielding,[183] it requires the use of extremely hazardous materials.

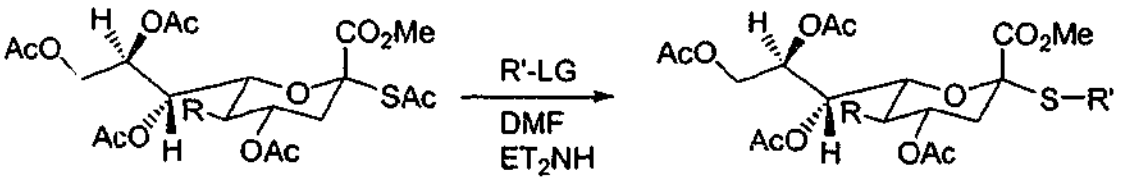


58 **59** **60**

Reagents and conditions: (a) PhHgOAc, EtOH; (b) H_2S, EtOH.

Scheme 10[183]

von Itzstein and coworkers[185] have described a mild and efficient procedure for the synthesis of thioglycosides of *N*-acetylneuraminic acid. In the simplest form, selective *in situ* thiodeacetylation of the 2-thioacetyl Neu5Ac derivative **54** using diethylamine (Et_2NH) in DMF at room temperature initially occurs followed by coupling of the resultant thiolate with activated acceptors such as alkyl halides, activated carbohydrates and nucleosides.[185-188]

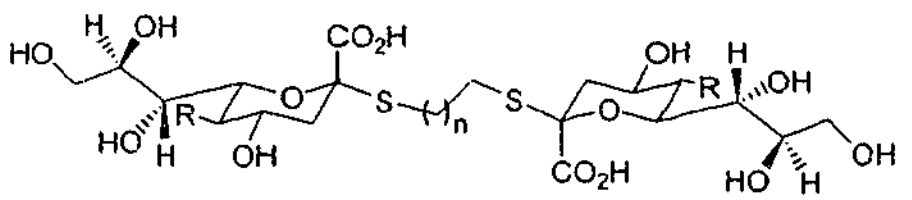


54 R = NHAc **61** R = NHAc

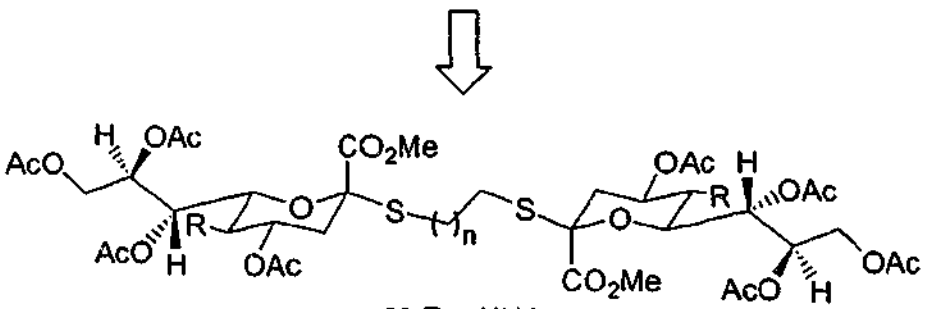
LG = OTf, halide

Scheme 11

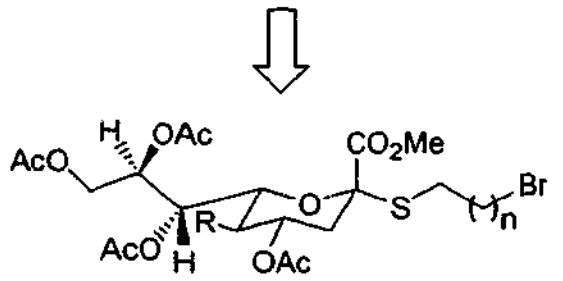
This methodology was utilized to prepare the dithiosialosides as outlined in retrosynthetic terms in Scheme 12. The reactions for the synthesis of the dithiosialosides involves the initial coupling of the key precursor 2-thioacetyl Neu5Ac derivative **54** with an alkyl halide to obtain the monothiosialoside **62** followed by a second addition of **54** to obtain the dithiosialoside **63**. Deacetylation and subsequent saponification of the coupled products ultimately affords the target compounds, **52** and **53.**



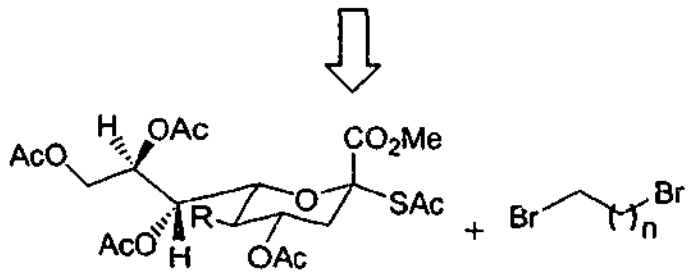
52 R = NHAc, n = 1
53 R = NHAc, n = 3



63 R = NHAc



62 R = NHAc

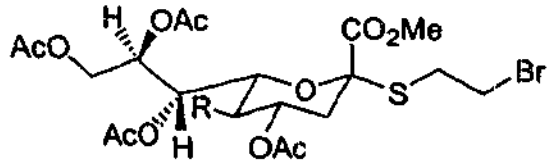


54 R = NHAc

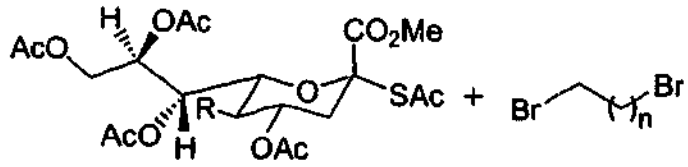
Scheme 12

2.11 Synthesis of multivalent inhibitors

Preparation of the bivalent sialoside **64** was initially attempted using an Et_2NH promoted coupling between two equivalents of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulo-pyranosonate **54** (prepared according to a published literature procedure[189]) and one equivalent of commercially available 1,2-dibromoethane (Scheme 13).[185] However, it was found that upon workup, the yield of the target compound **64** was only 20% after purification. To improve on the low yield, another approach was undertaken. This involved the addition of one equivalent of **54** to 1,2-dibromomethane followed by the isolation of the intermediate **65**. Again, a low yielding reaction of the desired product resulted. This prompted a third attempt that did not involve isolating the intermediate **65**, from the reaction mixture but rather adding a second equivalent of thioacetylated Neu5Ac (**54**). Smooth formation of the novel α-thiosialosides **64** and **66** in 45% and 39% yield respectively was observed using these conditions (Scheme 13). The ^1H NMR spectra of **64** and **66** are consistent with the structures shown. In particular, the presence of a four proton multiplet centred at 2.84 ppm for the alkyl protons (H-1a, 2a) and (H-1b, 2b) for **64** and 2.52-2.56 ppm for the alkyl H-1 and H-4 protons in the case of **66** is indicative of a CH_2 attached to a sulfur in a thioether moiety[166] (see experimental section 5.3.1 for ^1H NMR assignments). Deacetylation and subsequent saponification of the α-thiosialoside (**64**) furnished **52** in near quantitative yield (99%). Again the ^1H NMR spectrum of **52** is consistent with the structure outlined, having the correct number of hydrogens for the Neu5Ac moiety and alkyl protons. Deprotection of **66** was not undertaken due to time constraints.

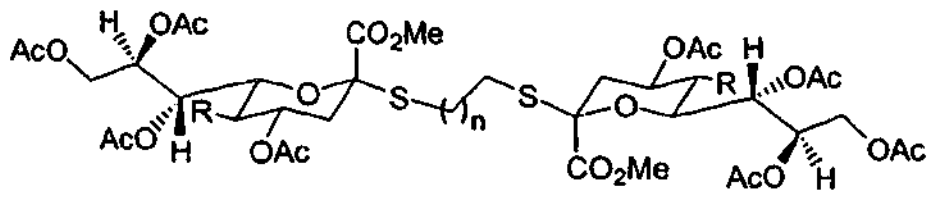


65 R = NHAc



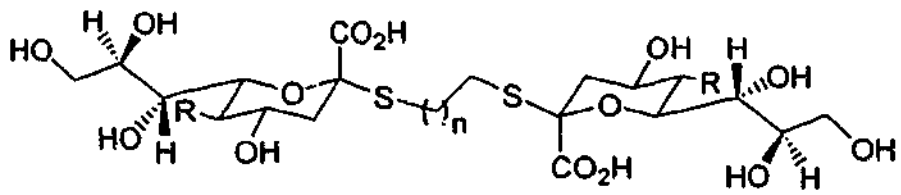
54 R = NHAc

a



64 R = NHAc, n = 1, 45%
66 R = NHAc, n = 3, 39%

b, c



52 R = NHAc, n = 1, 99%

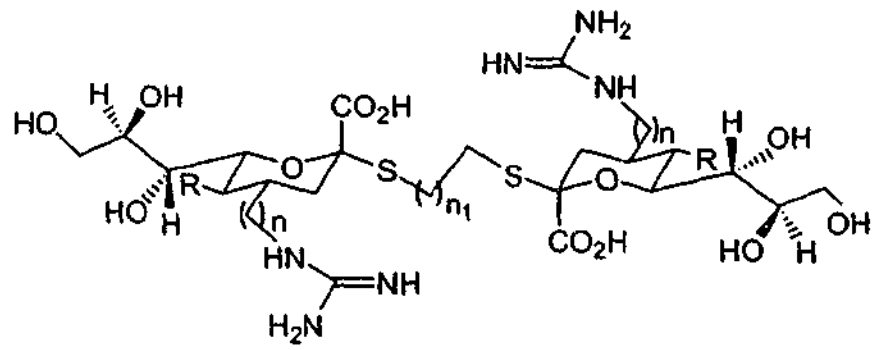
Reagents and conditions: (a) DMF, Et_2NH; (b) NaOMe, MeOH; (c) H_2O, NaOH.

Scheme 13

2.12 Conclusion

In summary, molecular modelling studies on a potential monovalent inhibitor, 4-deoxy-4-guanidino-Neu5Acα2Me (**32**), indicates that the 4-guanidino group on the molecule is not flexible enough to interact with the RBS of HA. Preliminary biological studies showed no significant inhibition of HA although, limited inhibition of NA was observed. The biological investigation also revealed greater inhibition of NA by **32**, compared with the 4-

hydroxy analogue (**26**). This increased inhibition is presumably due to the 4-guanidino substituent. The affinity of **32** may be increased by developing a bivalent derivative such as **49** which would have greater flexibility in the RBS and perhaps better binding properties with HA. These results suggest that the 4-guanidino substituent is a viable platform from which future analogues may be designed.



49 R = NHAc

It is hoped that inhibition studies yet to be undertaken on the bivalent thiosialoside compound **52**, will show that higher density or multivalent attack of HA would result in greater inhibition without being destroyed by NA. Alternatively, these compounds may act as bifunctional inhibitors, which simultaneously inhibit the binding of HA and compete with NA in stopping the progression of the influenza virus.

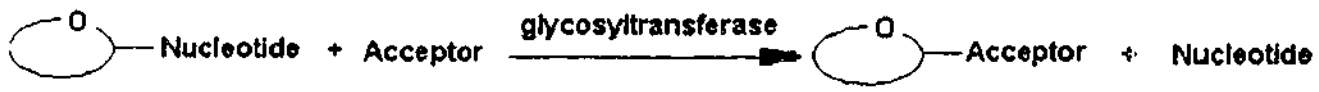
All the above compounds may be used as model ligands for probing sialic acid-recognizing proteins. Potentially, more potent inhibitors may be developed using the results obtained from this study. This could be achieved by synthesizing derivatives in the bivalent form of compound **32** such as **49**. Alternatively, bivalent analogues of **52** and **53** having larger spans may be investigated. Ultimately, the goal of either approach could lead to concomitant inhibition of HA and the influenza virus.

CHAPTER 3

The design and synthesis of an N*-acetyl neuraminic acid analogue to probe α(2,8)-sialyltransferase activity.*

3.1 Introduction to Glycosyltransferases

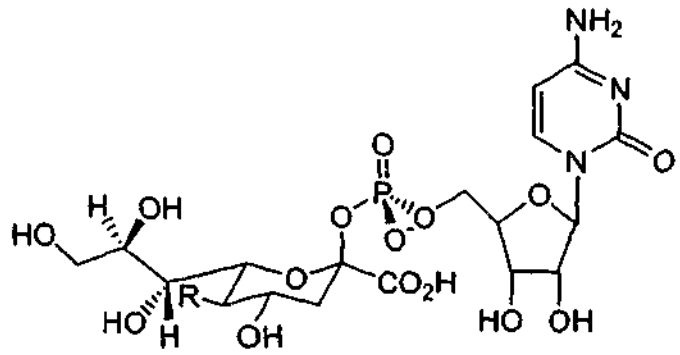
As previously discussed (section 1.2.1), sialic acids are widespread in eukaryotic cells and are frequently found as α-ketosidically linked terminal sugar residues for both *O*- and *N*-linked glycoproteins and glycolipids.[190,191] Sialic acid residues may also be linked internally to form oligopolysialic acid chains in both prokaryotic and eukaryotic organisms[191] in the endoplasmic reticulum and Golgi apparatus.[192] Ultimately, the "make up" of cells is typically composed of diverse linkages at the outermost positions of oligosaccharide chains. The oligosaccharide chains themselves are formed by the action of a series of glycosyltransferases.[8] Glycosyltransferases are a group of enzymes that catalyse the synthesis of specific glycosides by transferring a monosaccharide from an activated nucleotide-sugar donor substrate to a growing carbohydrate chain acceptor substrate, as shown below in Scheme 14.[193,194]



Scheme 14. Diagrammatic representation of the action of glycosyltransferases leading to the synthesis of oligosaccharide chains.[193,194]

The acceptor substrate may be another sugar residue, a polypeptide, or a lipid depending on the specificity of the transferase.[193] The specificity of the transferases is dependent upon

the donor and acceptor substrates in the formation of the carbohydrate chains on the cell surface.[194] These glycosyltransferases are typically grouped into families based on the type of carbohydrate they transfer (for example galactosyltransferase, sialyltransferase, fucosyltransferase and *N*-acetylgalactosaminoyltransferase).[194] The nucleotide moiety may be a diphosphonucleotide (DPN) (for example urdine diphosphate (UDP) serves as a donor substrate for glucose, galactose and other sugars), or it could be a monophosphate (for example cytidine monophosphate (CMP) serves as a donor substrate for Neu5Ac (**2**)).[193] This chapter examines those sialyltranferases known to transfer CMP-Neu5Ac (**7**).

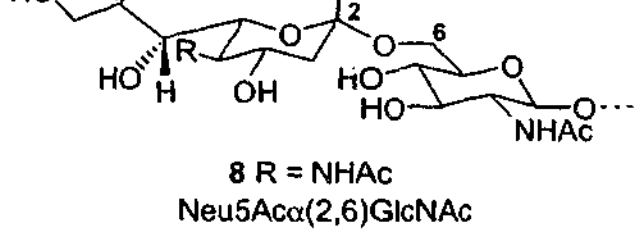


7 R = NHAc
CMP-Neu5Ac

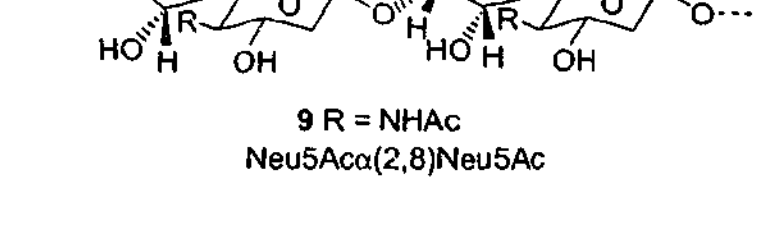
3.2 Sialyltransferases

Sialyltransferases represent a family of glycosyltransferases that catalyses the transfer of sialic acids from the corresponding substrate, cytidine monophosphate-Neu5Ac (CMP-Neu5Ac (**7**)), to carbohydrates of glycoproteins and glycolipids in an α-glycosidic linkage (α(2,3)-, α(2,6)-, α(2,8)-, or α(2,9)-[6,8,47] (*vide supra*) (e.g. compound **8** and **9**).[193,195] This is illustrated below (Scheme 15) where the *HO*-acceptor is the carbohydrate to which the sialic acid Neu5Ac (**2**) is being transferred. The incorporation of the sialic acid onto terminal oligosaccharides is not only regulated by sialyltransferases but also by the availability of the substrate CMP-Neu5Ac (**7**).[196] Sialyltransferases are highly specific when transferring a sialic acid to an oligosaccharide acceptor to form a single anomeric and

positional linkage.[13,197,198] In the case of sialyltransferases, only sialic acids which are β-glycosidically linked to CMP are recognized as donor substrates.[47]



8 R = NHAc
Neu5Acα(2,6)GlcNAc



9 R = NHAc
Neu5Acα(2,8)Neu5Ac

CMP-β-Neu5Ac + *HO*-acceptor → Neu5Ac-α-*O*-acceptor + CMP-*H*

Scheme 15

The transfer of the sialic acid Neu5Ac (**2**) onto glycoconjugates is a chain termination event and can be affected by inhibition of sialyltransferase enzymes or the transport of the substrate CMP-Neu5Ac (**7**) in the Golgi vesicles.[192] Sialyltransferases are located in the membranes of both the endoplasmic reticulum and Golgi apparatus.[10,195] The amino acid sequence for 18 distinct members of the sialyltransferase family is available.[20] This topic has been reviewed recently by Harduin-Lepers *et al.*[199] Although there are common features with the topology of the sialyltransferases, the amino acid sequences exhibit very little in homology.[194] The general structure of sialyltransferases consists of an *N*-terminal cytoplasmic domain, a hydrophobic signal membrane anchor domain, a luminal stem domain and a large *C*-terminal catalytic domain.[6,194,200] The degree of homology that does occur in sialyltransferase involves 50 conserved amino acids called the 'sialyl motif', located in the central region of the protein. The 'sialyl motif' is thought to be involved in

the binding of the common donor substrate, CMP-Neu5Ac (**7**).[6] Table 2 summarizes the mammalian sialyltransferases cloned to date, along with the acceptor specificity.[20] and references therein

Table 2. Some mammalian sialyltransferases.[20] and references therein

Sialyltransferase	Other name(s)	Acceptor specificity
ST3Gal I	ST3O, ST3GalA.1, SiaT4a	Galβ1,3GalNAc
ST3Gal II	ST3GalA.2, SAT4, SiaT4b	Galβ1,3GalNAc
ST3Gal II	ST3(N)	Galβ1,3(4)GalNAc
ST3Gal IV	STZ, SAT3, SiaT4c	Galβ1,3GalNAc and Galβ1,4GalNAc
ST3Gal V	GM3 synthase	Galβ1,4Glc-Cer
ST3Gal VI		Galβ1,4GlcNAc
ST6Gal I	SiaT1, α2,6ST, ST6(N)	Galβ1,4GlcNAc
ST6GalNAc I		GalNAc, Galβ1,3GalNAc and Siaα2,3Galβ1,3GalNAc
ST6GalNAc II		GalNAc, Galβ1,3GalNAc and Siaα2,3Galβ1,3GalNAc
ST6GalNAc III		Siaα2,3Galβ1,3GalNAc
ST6GalNAc IV		Siaα2,3Galβ1,3GalNAc
ST6GalNAc V	GD1α synthase	GM1b
ST6GalNAc VI	GD1α, GT1aα, GQ1bα synthase	GM1b, GT1b
ST8Sia I	GD3 synthase, SAT II	GM3
ST8Sia II	STX	Sia Galβ1,4GlcNAc
ST8Sia III		Siaα2,3Galβ1,4
ST8Sia IV	PST-1	Sia Galβ1,4GlcNAc
ST8Sia V	SAT V, SAT III	GM1b, GD1a, GT1b, GD3

Various sialyltransferases have been found in diverse locations and are involved in a number of important biological events.[193] All of the roles sialyltransferases play in biological systems cannot be covered within the scope of this thesis, but those mentioned below provide an explanation for the high level of interest in this topic over recent years.

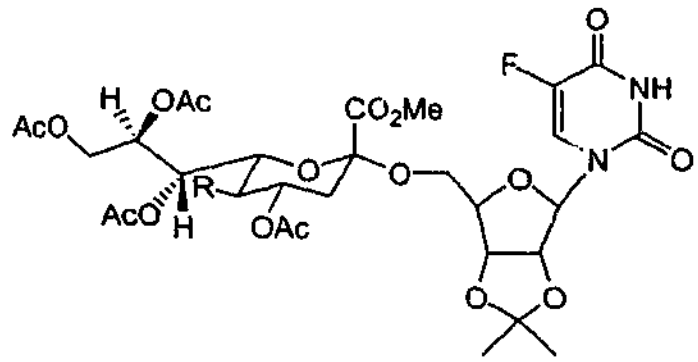
Expression, activity and specificity of sialyltransferases, which catalyze terminal sialylation in the biosynthesis of glycoconjugate glycan structures, are of growing interest in biochemistry.[201] For example sialyltransferases play a critical role in the pharmacological relevance in the sialylation of glycoproteins and glycolipids on normal and cancerous cell surfaces.[197] Furthermore, some sialyltransferases preside over sialylation of cell surface sialoglycoproteins and sialoglycosphingolipids of lymphocytes and macrophages which makes them primary targets for regulation of inflammation and tumour metastasis.[197]

A correlation between increased cell surface sialylation and cancer has been reported.[47,202] Specifically, the amount of sialic acid present on the cell surface through sialyltransferase enzymes or by the transport of the substrate CMP-Neu5Ac (7) is increased on malignant transformation.[192,202] As discussed in Chapter 1, this could be due to the ability of sialic acids to mask galactose residues that would otherwise inhibit further cell growth.[23] The masking of galactose moieties not only effectively hides them from the immune defence system but also results in a hypersialylated cell surface. This increase in sialic acids present on cell surfaces is thought to play a key role in influencing the tumourigenic and metastatic potential of tumour cells.[192] As mentioned earlier, the incorporation of sialic acid is regulated by the sialyltransferase enzymes and by the availability of the substrate CMP-Neu5Ac (7).[196] From these observations, the development of an inhibitor to stop metastasis through intervention in the function of the sialyltransferase may be a useful therapeutic tool.

3.2.1 Inhibition of sialyltransferases

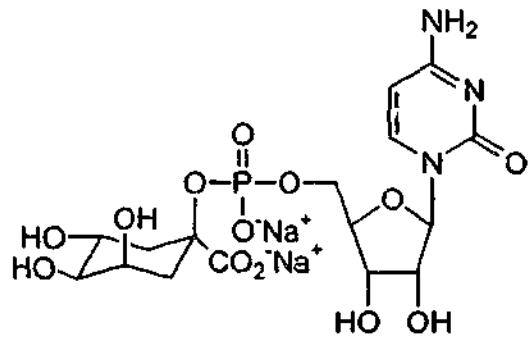
Little is known about inhibitors of sialyltransferases, although such compounds are interesting because of their potential biological implications with respect to sialylation, e.g. to study tumour biology and cancer therapy.[8] At present, a few sialyltransferases inhibitors have been developed,[203,204] although, none have yet shown any significant activity in halting the sialylation process.

Inhibitors of sialyltransferases or the transport of CMP-Neu5Ac (7) could potentially impede the biological pernicious characteristics of hypersialylation. Inhibitors of sialyltransferases have generally been developed as analogues of CMP-Neu5Ac which is the natural substrate for sialyltransferase. The classical example is the fully protected CMP-Neu5Ac derivative, 5-fluoro-2′,3′-*O*-isopropylidene-5`-*O*-(methyl 4-*N*-acetyl-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-D-*glycero*-α-D-*galacto*-nonulopyranosyl)uridine, KI-8110 (67). It has been shown that KI-8110 (67) does not reduce the incidence of metastases by hindering sialyltransferase activity, but rather by inhibiting the transport of CMP-sialic acid into the Golgi vesicles thereby depleting the donor substrate pool of sialyltransferases.[47] This was also observed with Golgi vesicles from human liver and colorectal cancer cells.[205] Treatment of cancerous cell lines with KI-8110 (67) appeared to reduce the formation of hepatic metastases by colorectal carcinoma cell lines in the nude mouse model.[196] From these results, it has been suggested that reduced sialylation of adhesion molecules such as carcinoembryonic antigen, may change the biology of the tumour cell and prevent the implantation of the cells into distant sites thus resulting in a reduced incidence of metastases.[196]

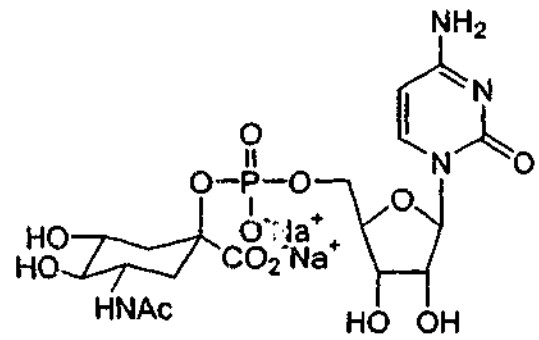


67 R = NHAc
KI-8110

Other inhibitors of sialyltransferase that have been reported are tunicamycin and swainsonine.[206] Both have been shown to reduce metastases in tumour cell lines.[205,207-209] Schaub *et al*[203] have designed the quinic acid derivatives **68** and **69**, which displayed potent α(2,6)-sialyltransferase inhibitory action. Recently this group designed new quinic acid derivatives to inhibit sialyltransferase by mimicking the potential transition state of CMP-Neu5Ac‡ (**70**) *via* the introduction of a double bond in the quinic acid moiety (see Figure 15).[204] These new derivatives exhibit an increased level of binding to sialyltransferase compared to CMP-Neu5Ac.[204] An example of the new quinic acid-based inhibitors is illustrated below as compound **71** (see Figure 15).



68



69

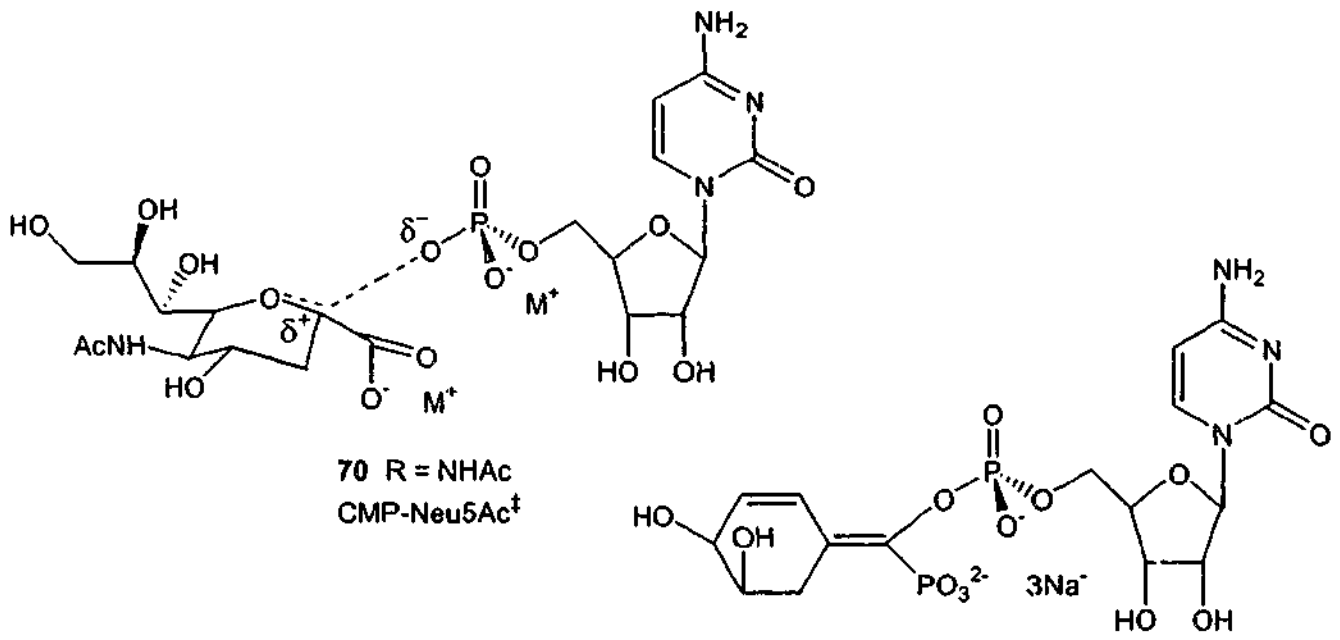


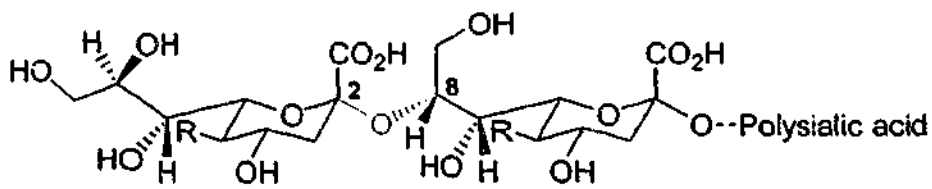
Figure 15

As shown in table 2, there are close to twenty sialyltransferases which have been cloned to date. This thesis is predominantly focused on the α(2,8)-sialyltransferases. Little is known about the biological functions of α(2,8)-sialyltransferase, however, what follows below is a brief overview of the presumed role of α(2,8)-sialyltransferases in biological systems.

3.2.2 α(2,8)-Sialyltransferase biological functions

It is known that α(2,8)-linked sialic acids exist on the embryonic form of vertebrate neural cell adhesion molecules (NCAM) in the form of polysialic acid (PSA (**72**)).[191,197] Polysialic acid is a homopolymer in which tens of sialic acid residues are connected *via* an α(2,8)-linkage in a linear array[12,20,210-212] with predominant *N*-linked units in mammals being Neu5Ac (**2**) and Neu5Gc (**3**).[12,211] A main function of PSA is the promotion of plasticity in cell-cell interactions.[12] In mammalian cells, PSA is generally found on glycoproteins such as NCAM and the neurotoxin responsive Na^+ channel.[213] NCAM is abundant in the embryonic brain and modulates embryonic development by mediating cell-

cell adhesive interactions in processes like neurite fasciculation, neuromuscular interaction and cell migration.[12,191,210-212] The embryonic form of NCAM is particularly rich in PSA whilst the adult form of NCAM has significantly reduced amounts of sialic acid, mainly in the brain regions.[20,211]



72 R = NHAc

Although the amount of polysialylated NCAM is reduced in adult tissue, re-expression of the polysialylated form has been reported in the case of several tumours such as medulloblastomas and Wilms' tumour.[12,211] In addition, high expression of polysialylated NCAM has been discovered in human neuroblastoma cells and in small lung carcinoma cells.[12] Therefore, polysialylated NCAM is potentially an onco-developmental antigen that may enhance the metastatic potential of malignant cells.[191,211]

Polysialylation is mediated by two distinct sialyltransferases, namely ST8Sia II and ST8 IV.[12,20] The production of PSA by these two sialyltransferases is dependent upon the histological origin of the tumour.[20] For example, neuroblastoma cells preferentially express ST8Sia II, while acute myeloid leukaemia cell lines preferentially express ST8Sia IV.[20]

Although it is has been suggested that the expression of polysialyltransferases is regulated at the transcriptional level,[12] there is little known concerning the catalytic mechanism of polysialyltransferases.[12,191] Methods to assay the activity of sialyltransferase(s) involved in the synthesis of this polymer have been developed in order to study the control of the

expression of PSA.[211] It is known that α(2,8)-sialyltransferases are detected in both normal cell and cancerous cell surfaces.[176]

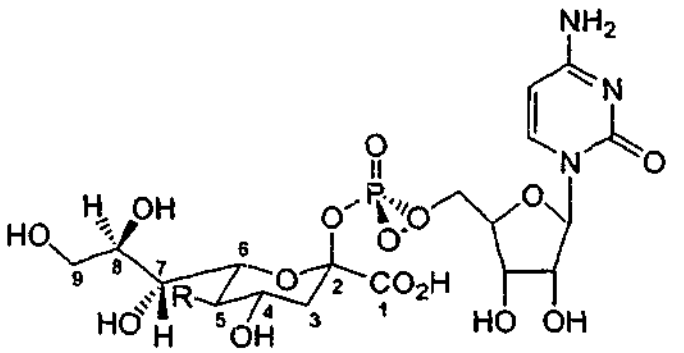
3.3 The design of a potential donor substrate and inhibitor of α(2,8)-sialyltransferases

From the previous discussion, it would be plausible to use α(2,8)-sialyltransferases as a reliable marker for diagnosis and prognosis in some cancer patients. Additionally, the development of a drug which could block the incorporation of sialic acids into cell surface oligosaccharides which contain metastatic potential would be a useful therapeutic tool.

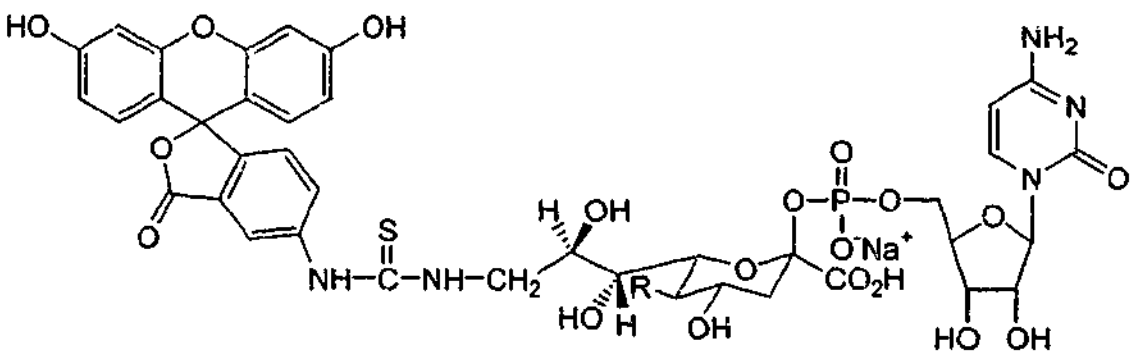
In the past, detection of sialyltransferase activity required radiometric assays, employing radioactive atoms such as ^{3}H or ^{14}C on the nucleotide of CMP-Neu5Ac (7).[195,211,214] Usually, the activity of the enzymes is very low in crude tissue preparations, cellular extracts, or body fluids which could make detection difficult.[195] Hence, the donor substrate CMP-Neu5Ac (7) requires radiolabeling with high specific activity at saturated concentrations. Current radiolabeling assays available for sialyltransferase activity require either precipitation, ion-exchange chromatography, or adsorption of the glycoprotein product formed with subsequent liquid scintillation photometry.[214] These tedious procedures are laborious due to the handling of radioactive compounds.[214] Thus, the development of a donor substrate for easy detection and high sensitivity of sialyltransferases is highly desirable.

Binding of donor substrates to sialyltransferases requires the CMP moiety.[203,204] Any structural modifications in the phosphate group and the C-4, C-5[215] and C-9[216] positions of

the Neu5Ac (**2**) moiety are tolerated by the sialyltansferases.[203,204] There are some reports that CMP-9-fluoresceinyl-Neu5Ac analogues (e.g. compound **73**) have been used successfully in sialyltransferase assays.[201,214,217] Gross *et al*[214] showed that the CMP-9-fluoresceinyl-Neu5Ac donor has a detection limit 200-1000 fold lower compared to the ^{3}H-labeled CMP-Neu5Ac donor radiometric assay. Hence, the utilization of CMP-9-fluoresceinyl-Neu5Ac is appropriate for the detection of very low concentrations of sialyltransferases. The successful incorporation of the fluoresceinyl-Neu5Ac (**73**) derivative with α(2,6)-sialyltransferase on the galactose acceptor sites was found to be identical to the natural Neu5Ac donor.[217]



7 R = NHAc
CMP-Neu5Ac



73 R = NHAc

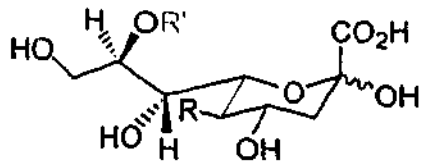
An assay used to detect α(2,8)-sialyltransferases activity has been developed which incorporated the ^{3}H labelled CMP-Neu5Ac as the donor substrate.[211] The successful assay

was performed with the utilisation of (NeuAcα(2,8))$_n$ (colominic acid) as the acceptor substrate.[211] The activity of α(2,8)-sialyltransferase was determined in rat brain and different tumour cell lines. Interestingly, results showed the existence of α(2,8)-sialyltransferase that acted only once on an acceptor carrying a terminal sialic acid residue to afford a single Siaα(2,8)Sia dissaccharide unit.[211]

Other reports which successfully utilised CMP-9-fluorescent-Neu5Ac donors have only dealt with α(2,3)-[195,217] and α(2,6)-sialyltransferases.[195,201,214,217] These results together with the knowledge that α(2,8)-sialyltransferases are active in the progression of metastasis, prompted us to develop a molecule which could mimic the natural substrate of α(2,8)-sialyltransferases and halt cell proliferation of abnormal cancerous cells.

The design of the target compound must ideally encompass two functional features. Primarily, the target molecule would have detectable properties once it has been successfully linked onto polysialylated NCAM. The other feature of the target compound would entail the inhibition of α(2,8)-sialyltransferase in transferring any further Neu5Ac (2) molecules onto polysialylated NCAM.

Given that α(2,8)-sialyltransferases are involved in the linkage of sialic acids in an α(2,8) manner to oligosaccharides, a potential inhibitor would be a compound that had an inert and detectable substituent at the 8-OH position of the sialic acid moiety, such as compound 74. This type of analogue would stop α(2,8)-sialyltransferase in over expressing polysialylated NCAM, as well as function as a detectable substrate. The literature is replete with different types of plausible detectable groups that can be used.[195,214,218]



74 R = NHAc
R' = **Fluorescent group**

To overcome the methodological difficulties inherent in common sialyltransferase assays and in chemical labelling of sialoglycans together with potential blocking of the Neu5Ac transfer by α(2,8)-sialyltransferase, an *O*-fluorescent group was initially designed to be placed at the C-8 position of Neu5Ac. Having the fluorescent "TAG" group at the C-8 position on the Neu5Ac moiety of CMP-Neu5Ac could create a donor for α(2,8)-sialyltransferase which could incorporate the 8-TAG-Neu5Ac onto a developing polysialic acid to facilitate detection for diagnostic and prognostic purposes of cancerous cell lines (see Figure 16 for possible incorporation of the 8-fluorescent-Neu5Ac by α(2,8)-sialyltransferase). Thus, a C-8 TAG derivative that could be a substrate with concomitant potential to block the addition of Neu5Ac onto polysialic acid by α(2,8)-sialyltransferase is compound **75**.

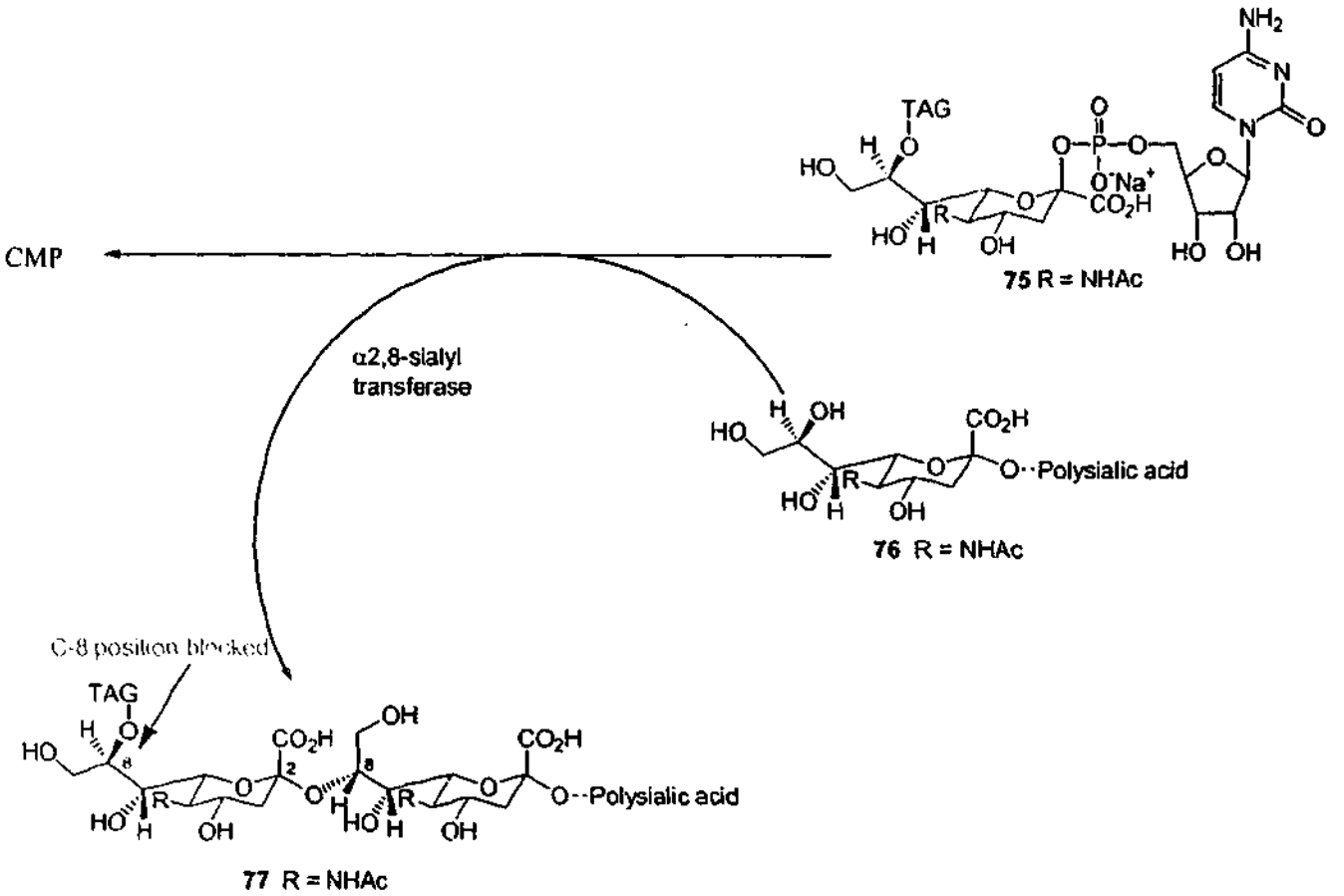
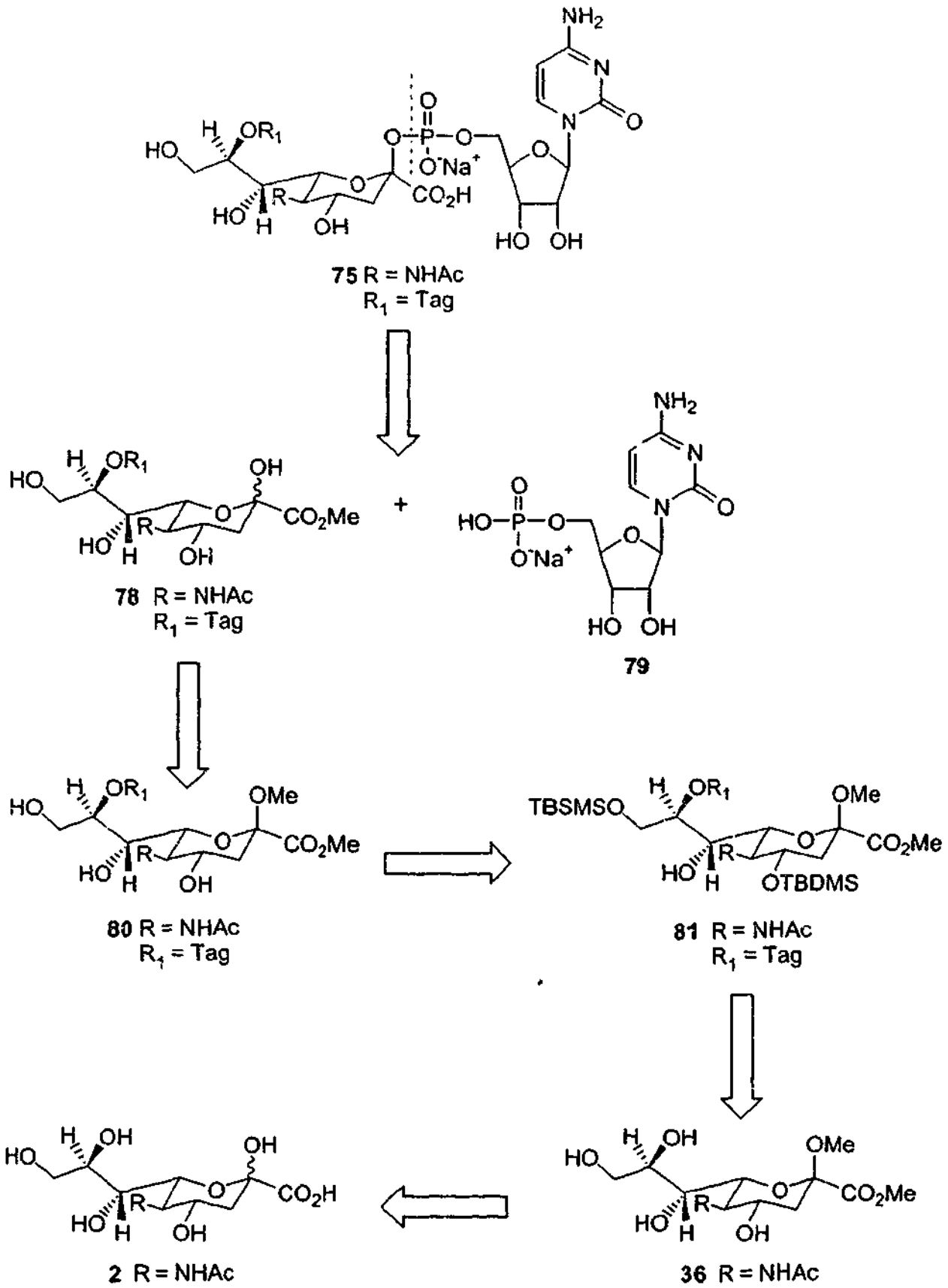


Figure 16. The proposed incorporation of the 8-TAG-Neu5Ac (**75**) onto polysialic acid by α(2,8)-sialyltransferase.

3.4 Retrosynthesis of 8-TAG compound

The proposed synthesis of **75** is shown in retrosynthetic terms in Scheme 16 and the obvious disconnection involves the coupling between the carbohydrate **78** and the nucleoside **79**. The carbohydrate needs to contain a free hydroxyl at the C-2 anomeric position prior to activation for coupling with the nucleoside (*vide infra*). Due to the low reactivity at the C-8 position (*vide infra*), most of the Neu5Ac (**2**) substituents need to be protected before the TAG molecule is placed at the C-8 position. Firstly, it is important to protect the C-1 carboxyl group and C-2 anomeric position on the carbohydrate moiety, to give **36**, before any further manipulations can take place on Neu5Ac. Furthermore, protection at the C-9 and C-4 positions is required as they are known to be more reactive

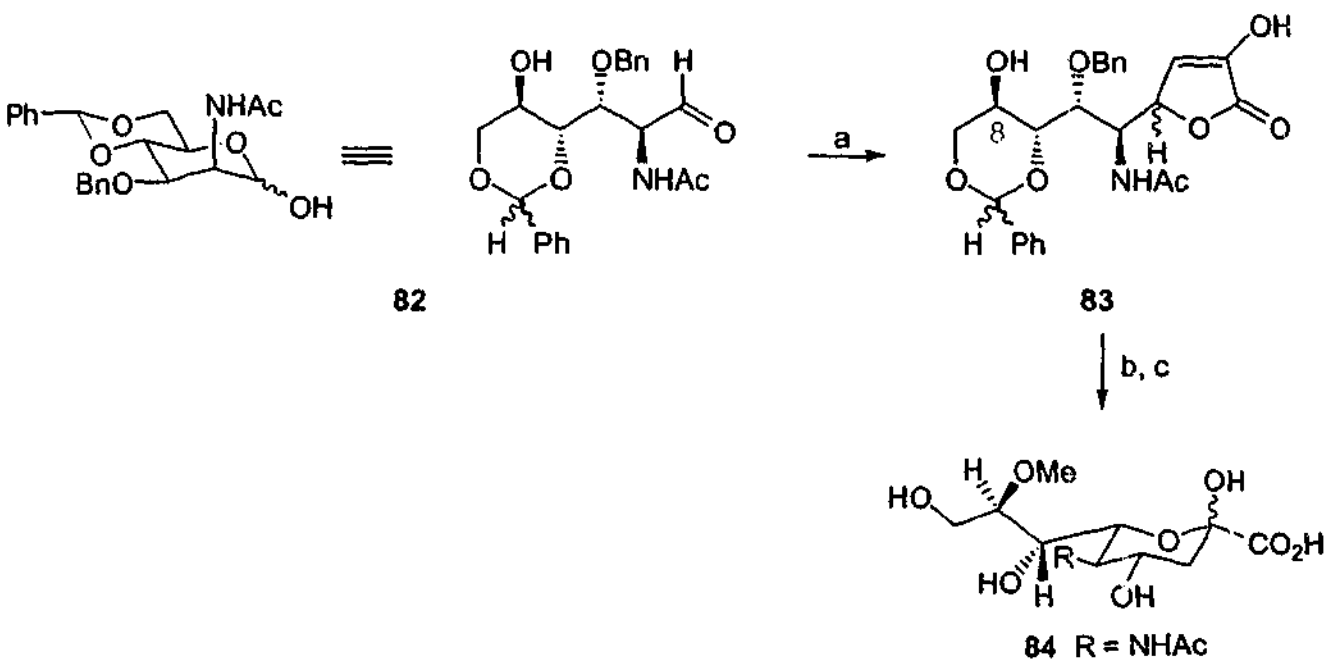
than C-8.[43] The most common protecting groups for carbohydrate hydroxyls are acetate, benzoate, silyl and benzyl.[43] The silyl group was chosen in this case as the methodology has been well documented by Brandstetter *et al.*[219]



Scheme 16. Retrosynthesis of 8-TAG compound **75**.

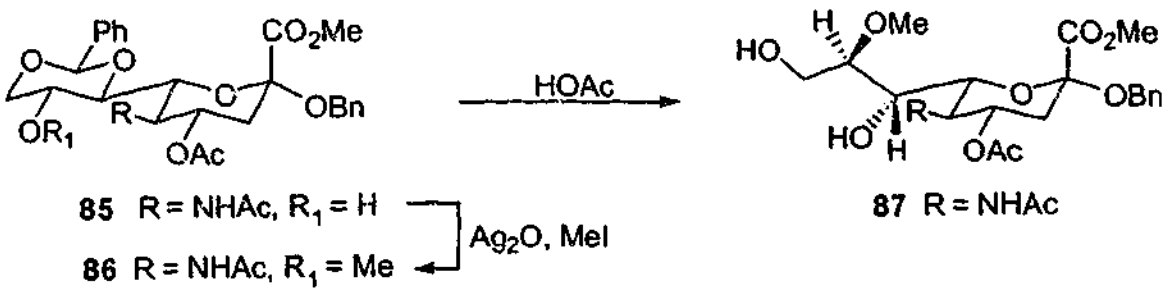
O-Sialylation at the 8-OH position of Neu5Ac (**2**) has been reported[220-222] as has the 8-*O*-methylation of Neu5Ac (**2**).[223,224] In the latter example treatment of the precursor 3-*O*-benzyl-4,6-*O*-benzylidene-*N*-acetyl-D-mannosamine **82** with potassium di-*tert*-butyl oxobutanedioate afforded the protected intermediate, 6-*O*-benzyl-7,9-*O*-benzylidene-*N*-acetyl-D-neuraminic acid γ-lactone **83.** This was then methylated at C-8 using MeI with Ag_2O in a methanolic solution and subsequently deprotected to give the 8-*O*-methyl-Neu5Ac derivative **84** (Scheme 17). A different approach for the 8-*O*-methylation was accomplished by Dufner *et al.*[224] This involved the formation of a benzylidene Neu5Ac intermediate **85** which was methylated using MeI with Ag_2O to give **86** and then debenzylidenated to obtain the Neu5Ac 8-OMe product **87** (Scheme 18).

David *et al*[75] and Augé *et al*[70] found that treating 2-acetamido-2-deoxy-5-*O*-methyl-D-mannose (**88**) with Neu5Ac aldolase to enzymatically synthesize the 8-*O*-methyl-Neu5Ac did not proceed very well. This was apparently due to the 2-acetamido-2-deoxy-5-*O*-methyl-D-mannose (**88**) being inert to Neu5Ac aldolase, possibly due to steric hindrance incurred by the many bond rotations on the enzyme involved in the transformation process to form the respective 8-*O*-methyl-Neu5Ac.[70,75] Thus, an enzymatic approach for 8-*O*-alkylation would not be viable in this synthesis. Perhaps, 8-*O*-alkylation could be accomplished *via* a more direct chemical approach onto the sialic acid, making this a challenging synthesis!

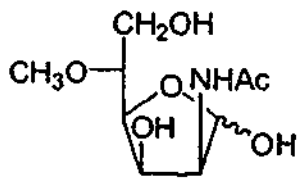


Reagents and conditions: (a) potassium di-*tert*-butyl oxobutanedioate (b) MeI, Ag_2O, MeOH (c) H_2/Pd, NaOH, H^+ resin

Scheme 17. Synthesis of 8-*O*-Methyl Neu5Ac (**84**). [223]



Scheme 18[224]



88

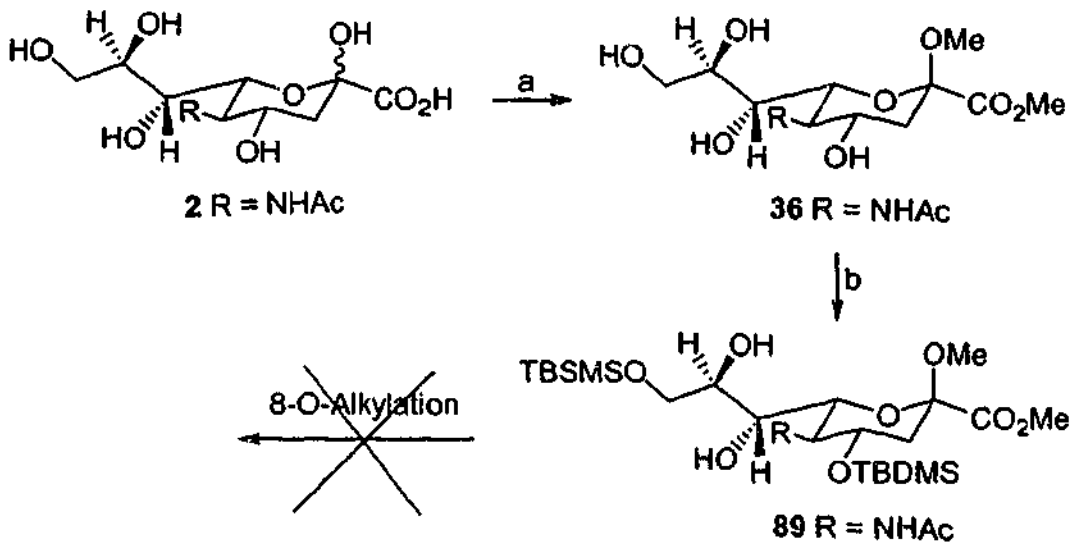
3.4.1 Results and Discussion

Based on the retrosynthesis presented in Scheme 16, we felt that the most direct route for the synthesis of **75** was to firstly prepare the 8-TAG-Neu5Ac **78** intermediate. Initially, the

sugar molecule needs to be protected before the incorporation of the TAG moiety at the 8 position of Neu5Ac. As described in section 2.8 (*vide supra,* Scheme 4), the preparation of the Neu5Ac1,β2Me$_2$ **36** was performed under standard conditions (Scheme 19).[153,154] A methanolic solution of Neu5Ac (**2**) was heated to reflux in the presence of an acidic catalyst, to afford β-methyl glycoside methyl ester derivative **36** in high yield. ^{1}H NMR examination of the product confirmed formation of **36** by the presence of the methyl ester and methyl glycoside groups resonanting at 3.77 and 3.23 ppm, respectively. The melting point (138-140°C) and yield (83%) was slightly higher than the previous literature report (which was 115-130°C for melting point and 58% yield).[153] Compound **36** was treated with *tert*-butyldimethysilyl chloride (TBDMS-Cl) and imidazole in DMF to afford the disilyl ether **89** in an isolated yield of 36%. ^{1}H NMR spectroscopic analysis confirmed the incorporation of two silyl ether groups with resonances observed at 0.08 (6H) and 0.09 (6H) ppm. ^{13}C NMR showed slight upfield shift for the C-9 (65.3 for **36** to for 64.7 ppm for **89**). Although the isolated yield was slightly lower compared to the literature (48%),[219] it was quantitative with respect to recovered starting material.

The next step, involving *O*-alkylation at the 8-OH position, is quite challenging due to the unreactive nature of the hydroxyl group in the 8 position.[220,221,225] Initially, the substituent that was devised to be utilised in the CMP-8-TAG-Neu5Ac (**75**) analogue was an alkyl-napthyl group, due to its high fluorescent characteristics. Since compound **89** contains both acid (TBDMS) and base (COOMe) labile groups, it was important to employ neutral reaction conditions which would not displace the existing protecting groups when considering 8-*O*-alkylation. Classical examples are silver (I) oxide (Ag_2O) and silver (II) oxide (AgO) which are known as weak bases[226] and have been shown to provide electrophilic catalysis for *O*-alkylations.[226] Preliminary investigations into the alkylation utilising Ag_2O in DMF, showed that a bulky group, such as 2-(bromomethyl)napthalene,

did not attach at the 8 position (see Table 3 for conditions). Possible explanations may include steric hindrance of the alkylating reagent or low reactivity at the C-8 position.



Reagents and conditions: (a) H^+, MeOH, reflux, 83%;
(b) TBDMS-Cl, Imidazole, DMF, 30%.

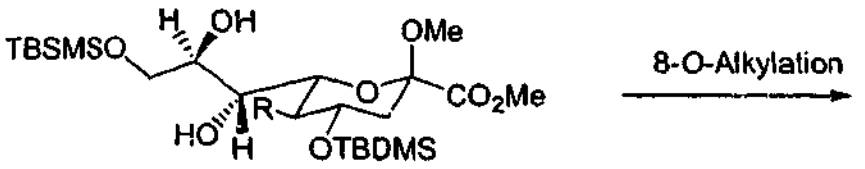
Scheme 19. Attempted synthesis of 8-TAG Neu5Ac.

To address the question of steric encumbrance, benzyl bromide (which is a less sterically hindered alkylating reagent) was used. However, none of the desired product was obtained (following literature methodology[227]) in the presence of Ag_2O in DMF (Table 3). The same reaction was attempted with Ag_2O in acetonitrile, but also gave no desired product.

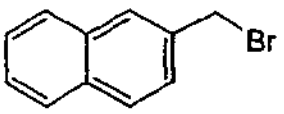
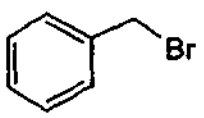
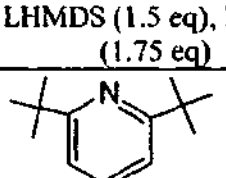
The reactivity of the 8-OH group in **89** was investigated by the attempted introduction of the less bulky methyl group *via* alkylation with methyliodide in the presence of Ag_2O in acetonitrile (CH_3CN). However, once again none of the desired product was obtained. *O*-alkylation of the substrate **89** was also attempted with methyliodide in the presence of a non-nucleophilic base, lithium hexamethyldisilazide (LHMDS). Again, no desired compound was formed (Table 3).

In an attempt to ascertain why no 8-*O*-alkylation was observed, the powerful alkylating agent methyl triflate was reacted with the substrate **89** utilising standard conditions (2,6-di-*tert*-butyl-4-methylpyridine in dichloromethane).[228] However, no desired product was obtained even after stirring the reaction mixture for 30 hs at 30°C. The results and conditions are summarised in Table 3.

Table 3. Attempted 8-*O*-alkylation of the Neu5Ac derivative **89.**



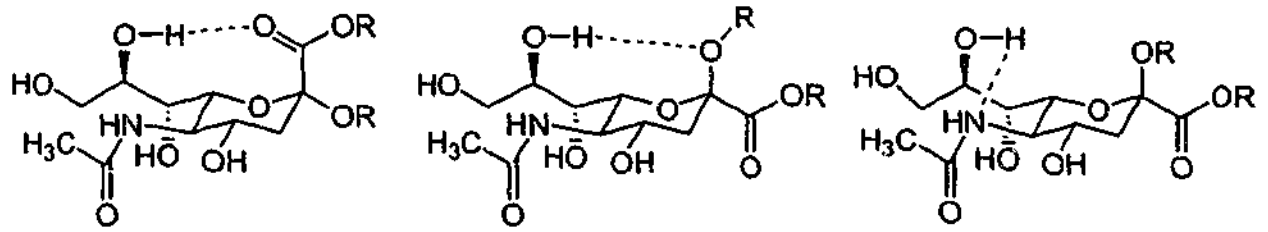
89 R = NHAc

Alkylating agent	Reagent(s)	Solvent	Temperature	Result*
 (1.2 eq - 2 eq)	Ag_2O (2 eq)	DMF	RT	NR
	Ag_2O (2 eq)	DMF	60°C	NR
	Ag_2O (2 eq)	DMF	100°C	Decomposition
	K_2CO_3(1.5 eq), KI (cat.)	CH_3CN	RT	NR
	NaH (4.15 eq)	DMF	0°C – RT	Decomposition
	Ag_2O (2 eq)	CH_2Cl_2	RT	NR
 (1.1 eq - 2 eq)	Ag_2O (2 eq)	DMF	RT	NR
	Ag_2O (2 eq)	DMF	RT - 60°C	Decomposition
	Ag_2O (2 eq)	DMF	100°C	Decomposition
	Ag_2O (2 eq)	CH_3CN	RT - 60°C	NR
	LDA (2.2 eq)	THF	0 - 5°C	Decomposition
CH_3I (3 eq – 5 eq)	Ag_2O (5 eq)	CH_3CN	Reflux	NR
	LHMDS (1.5 eq), HMPA (1.75 eq)	THF	-78°C - RT	Decomposition
MeOTf (5 eq)	 (5 eq)	CH_2Cl_2	RT - 30°C	NR

*** NR means that >90% unreacted starting material was recovered. The reagent amounts used in the alkylating reactions are indicated in brackets.**

These results are in accordance with Schmidt's[220,221,225] observations which indicate that the 8-OH group of Neu5Ac derivatives possessing a $^2C_5$ conformation, such as compounds 90(A-C) shown in Figure 17, have low reactivity due to obvious steric effects and the interaction of the 8-OH with the C-1 carboxyl or 2-OR (R = Me, Ac, H, etc) moieties.[221,229]

Another possible interaction that could lower the 8-OH group reactivity is with the 5-acetamido group *via* the formation of hydrogen bonds (**90(C)**) (Figure 17).[220,221,229]



90(A) **90(B)** **90(C)**

Figure 17

3.5 The 1,7-lactone 8-TAG intermediate approach

Having failed to obtain any 8-*O*-alkylation on the bi-silylated derivative **89**, we decided to explore an alternative approach that may address the low reactivity of the 8-OH. It has previously been reported that sialylglycoconjugates are susceptible to intramolecular esterification due to the proximity of the carboxylic acid group to neighbouring hydroxyl substituents.[229,230] For example, if the carboxylic acid group is in the equatorial position, cyclization occurs either at the C-4 or C-7 hydroxyl forming the 1,4-lactone **91(D)** and the 1,7-lactone **91(E)**, respectively (Figure 18).[230]

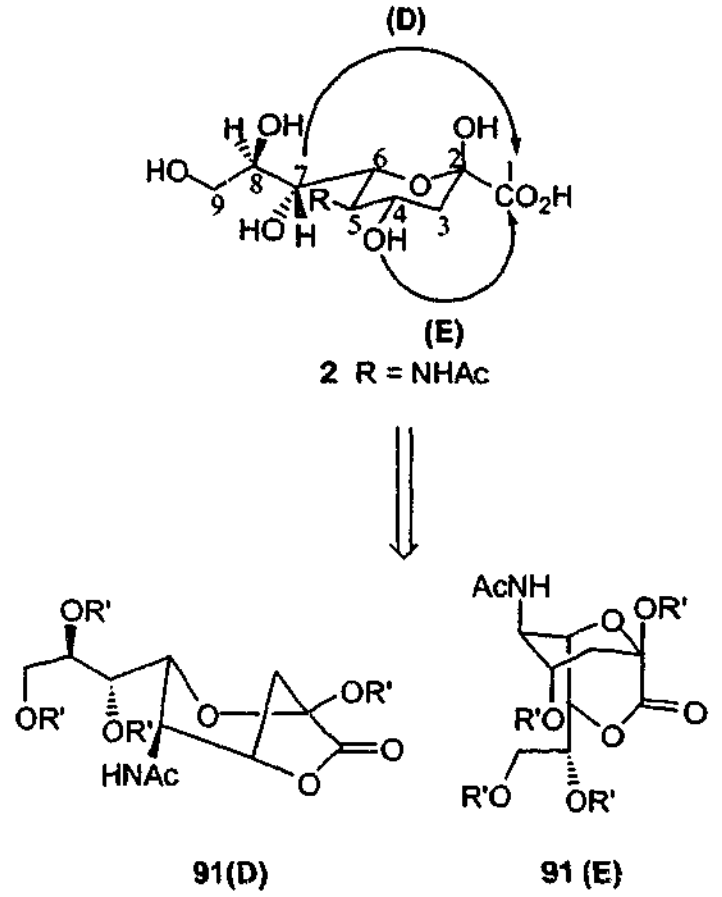
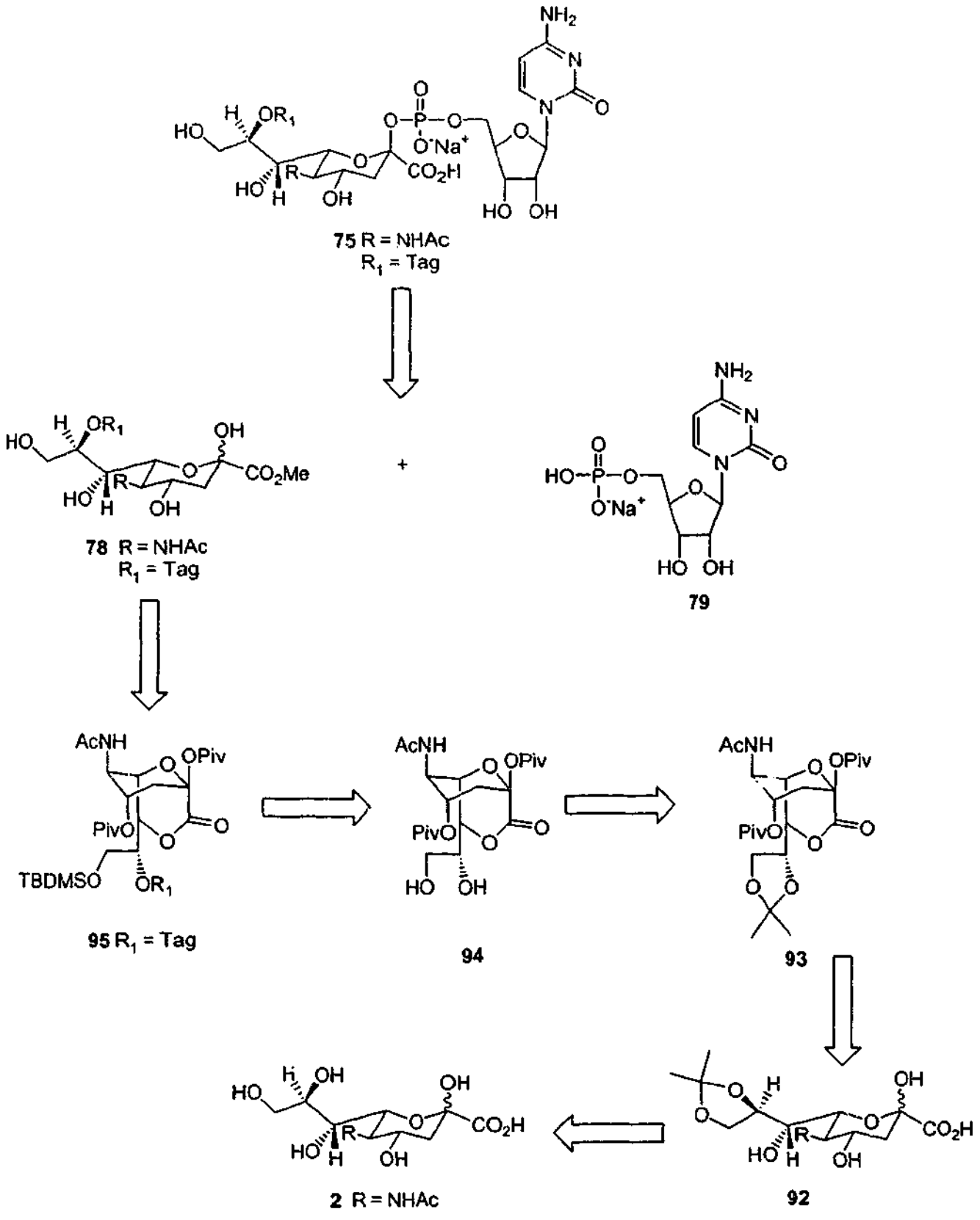


Figure 18

3.5.1 Retrosynthesis of 1,7-lactone 8-TAG

Based upon the observation of others, and our own preliminary attempts at 8-*O*-alkylation on the sialoside **89**, it was felt that a 1,7-lactone derivative of Neu5Ac may be better suited to enable 8-*O*-alkylation to occur. Therefore, our revised synthetic strategy is shown in retrosynthetic terms in Scheme 20.



Scheme 20. Alternative retrosynthesis of compound **75**.

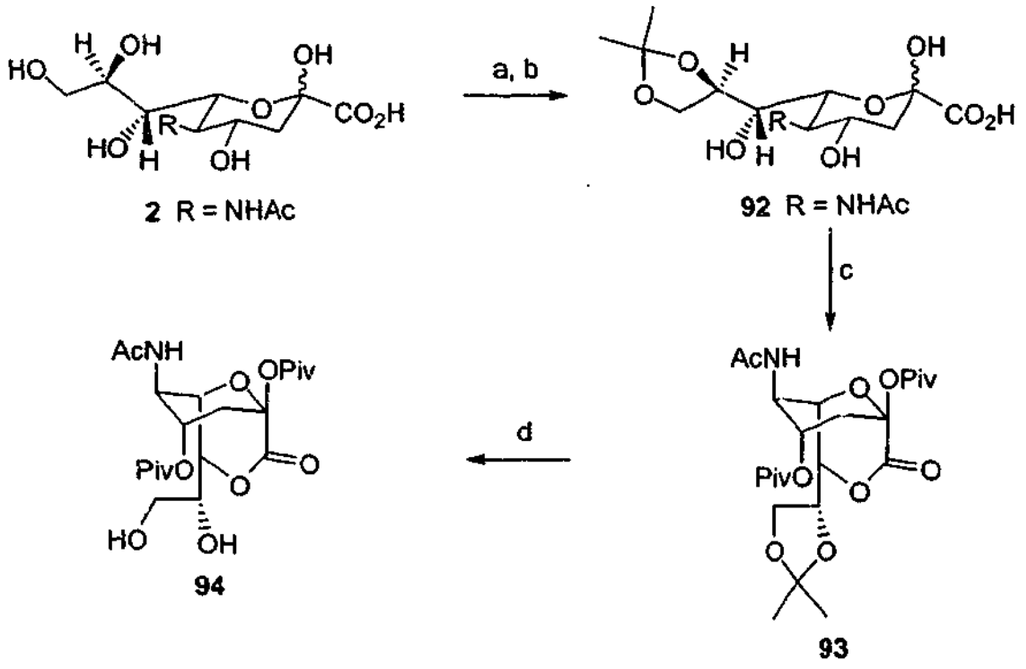
Treatment of 8,9-*O*-isopropylidene Neu5Ac derivative **92**, with trimethylacetyl chloride (pivaloyl chloride) in pyridine should afford the lactonised product **93** having the $^{5}C_{2}$ conformation.[220] This conformation provides enough rigidity about the pyranose ring to

prevent the C-8 hydroxyl from participating in any intramolecular hydrogen bonding, thereby making it potentially more reactive.[225] Therefore, after deprotection of the 8,9-*O*-isopropylidene group on the lactone **93**, easier access to the 8-OH group for alkylation reactions should result for **94**.

3.5.2 Results and discussion

Before forming the 1,7-lactone derivative **93**, the 8- and 9 hydroxyls were protected as the isopropylidene derivative by treatment of Neu5Ac with dimethoxypropane (DMP) in dimethylformamide (DMF). During the isolation of the isopropylidene derivative **92**, it was important to neutralise the reaction mixture with base (dilute NaOH) or no product was observed. Compound **92** was isolated in quantitative yield (see Scheme 21) and was found to be pure by ^{1}H NMR. The isopropylidene methyl protons for **92** in the ^{1}H NMR spectrum appear at 1.46 and 1.51 ppm was also consistent with the ^{13}C NMR data showing the existence of the quaternary carbon for the same substituent at 100.5 ppm. Subsequent reaction of compound **92** with pivaloyl chloride in pyridine produced the Neu5Ac 1,7-lactone derivative, **93** in 75% yield after recrystallization from ethyl acetate/hexane. The characteristic H-7 proton was observed at 4.11-4.19 ppm which is consistent with similar derivatives reported by Tsvetkov *et al.*[220] The appearance of the pivaloyl groups in both the ^{1}H and ^{13}C NMR spectra (at 1.18 and 1.21 ppm for ^{1}H and 26.6 for $C(CH_3)_3$ and 38.6 ppm for $C(CH_3)_3$ in ^{13}C NMR) provided further evidence for the structure of **93**. Furthermore, correct analytical data was obtained for **93**. Deprotection of the 8,9-*O*-isopropylidene group in **93** was accomplished with 80% aqueous acetic acid at 60°C for 3 hs. Thin layer chromatography (TLC) indicated a lower R_f for the diol **94** compared to **93**, which would be expected due to the assumption that **94** would be the more polar compound in this case. Compound **94** was obtained in an excellent yield of 93% following

recrystallization. Assignments of the ^{1}H NMR spectrum for **94** showed a downfield shift for the H-7 proton (from 4.11- 4.19 ppm for **93**, to 4.45 ppm for **94**). Additionally, the H-9a, H-9b and H-8 protons all showed an upfield shift which could be due to the removal of the isopropylidene. The ^{13}C NMR spectrum of **94** showed the absence of the quaternary C from the isopropylidene substituent at 110.1 ppm. These results, together with the correct analytical data confirmed isolation of the diol **94.**

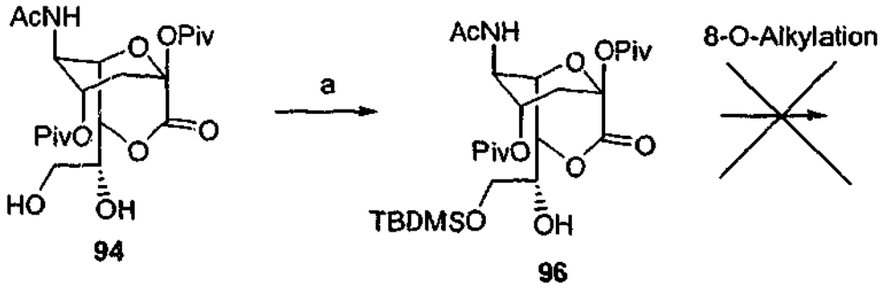


Reagents and conditions: (a) $Me_2C(OMe)_2$, DMF, H^+ resin; (b) 1M NaOH, pH =7; quant.; (c) PivCl, Pyridine, 75%; (d) 60% CH_3COOH, 60°C, 3 hs, 93%.

Scheme 21

Having prepared the diol derivative **94** in high yield, our attention turned to the 8-*O*-alkylation chemistry. In order to attempt this, the primary C-9 hydroxyl in **94** needed to be protected first. This was readily achieved by exposure of **94** to TBDMSCl and imidazole in DMF. After purification, the expected 9-*O*-silyl derivative **96** was obtained in 65% yield (Scheme 22). Both the ^{1}H and ^{13}C NMR spectra confirmed formation of the silyl ether with the appearance of the methyl singlets for $Si(CH_3)_2$ and $SiC(CH_3)_3$ seen in the ^{1}H NMR

at 0.11 and 0.91 ppm, respectively. The quaternary carbon from $SiC(CH_3)_3$ observed in the ^{13}C NMR spectrum was at 18.4 ppm which is consistent with the structure as shown. Mass spectrometry also demonstrated formation of the product with the detection of the correct molecular ion $[(M + 1)^+]$ at *m/z* 574.



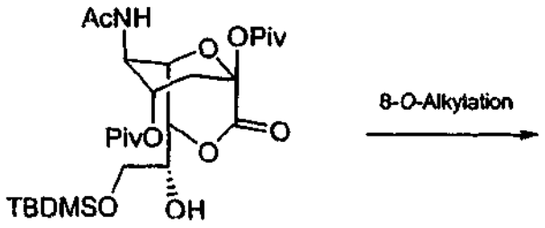
Reagents and conditions: (a) TBDMSCl, Imidazole, DMF, 65%.

Scheme 22

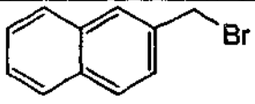
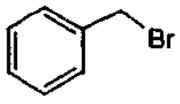
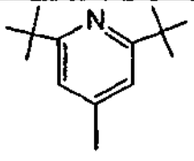
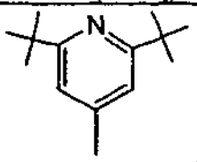
Compound **96** contains both acid and basic labile groups. The lactone may be opened and pivolyl groups removed under basic conditions whereas the TBDMS groups may be deprotected under acidic conditions. Hence, neutral reaction conditions must be found that do not disrupt the existing protecting groups when considering 8-*O*-alkylation. Indeed, the obvious way to deprotonate the 8-hydroxy of **96** would be to use a non-nucleophilic base such as lithium diisopropylamide (LDA) or lithium hexamethyldisilazide (LHMDS).

As can been seen from Table 4, there was no success in producing the desired alkylated product when **96** was treated with 2-bromomethylnapthalene and the non-nucleophilic base, lithium diisopropylamide (LDA). The reaction of **96** with benzyl bromide and either LDA or LHMDS, yielded no desired product. Unsuccessful *O*-alkylations were also observed when **96** was treated with benzyl bromide and either Ag_2O or AgO.

Table 4. Attempted 8-*O*-alkylation of **96**.



96

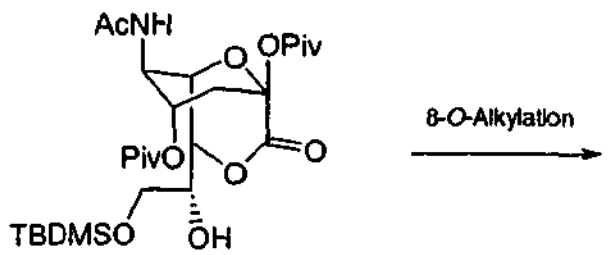
Alkylating Agent	Reagent(s)	Solvent	Temperature	Result*
 (1.1 eq)	LDA (1.2 eq)	THF	-78°C-RT	NR
	Ag_2CO_3 (1.1 eq)	Acetone	Reflux	NR
	NaH (2 eq)	DMF	0°C-RT	Decomposition
 (1.1 eq – 5 eq)	LDA (1.25 eq)	THF	-78°C-RT	NR
	LHMDS (1.25 eq), HMPA (1.5 eq)	THF	Reflux	Decomposition
	AgOTf (3 eq), LHMDS (1.5 eq), HMPA (1.75 eq)	THF	-70°C	Decomposition
	AgOTf (3 eq), LHMDS (1.5 eq), DMPU (1.75 eq)	THF	-70°C-RT	Decomposition
	AgOTf (5 eq), LHMDS (3 eq), HMPA (3.5 eq)	CH_3CN	-70°C-RT	Decomposition
	K_2CO_3 (2.4 eq), KI (cat.)	CH_3CN	RT	NR
	Ag_2O (2 eq)	DMF	RT	NR
	Ag_2O (2 eq)	THF	RT	NR
	Ag_2O (2 eq)	Acetone	Reflux	NR
	AgO (2 eq)	DMF	RT	NR
	Ag_2CO_3 (1.1 eq)	Acetone	Reflux	NR
	 AgOTf (2.6 eq)	Cyclohexane	-70°C-RT	NR
	 AgOTf (2.6 eq)	DMF	RT	NR
	$(Bu_3Sn)_2O$ (1 eq), $Bu_4N^+Br^-$ (1 eq)	Toluene	RT	Decomposition

*** NR means that >90% unreacted starting material was recovered. The reagent amounts used in the alkylating reactions are indicated in brackets.**

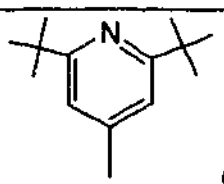
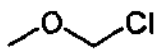
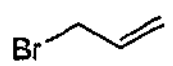
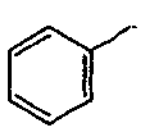
There are literature procedures which describe successful *O*-benzylation on carbohydrates with a triflate such as silver triflate and an organic base like, 2,6-di-*tert*-butyl-4-methylpyridine.[231,232] Similar conditions were applied for **96** in two different solvents (cyclohexane and DMF) however, only recovered starting material was obtained in both cases. Reports in the literature have also shown that tributyltin oxide [$(Bu_3Sn)_2O$] mediated alkylations on monosaccharide hydroxyl groups are successful and even have regioselectivity.[233,234] The application of the literature conditions[233,234] with benzyl bromide as the alkylating reagent on **96** unfortunately did not produce the desired product.

After unsuccessful *O*-alkylations with benzyl bromide, the powerful alkylating agent methyl triflate (MeOTf) was reacted with the substrate **96**, however, no *O*-alkylated product was obtained. Another strong alkylating agent, chloromethylmethylether (MOMCl), likewise yielded no *O*-alkylated product (Table 5).

Table 5. Attempted 8-*O*-alkylation of **96**.

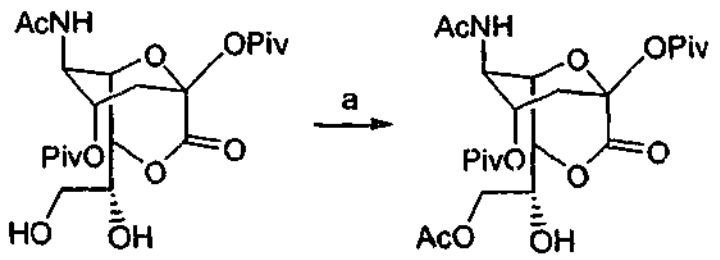


96

Alkylating Agent	Reagent(s)	Solvent	Temperature	Result*
MeOTf (5 eq)	 (5 eq)	CH_2Cl_2	RT	NR
 (3 eq)	LHMDS (1.5 eq), HMPA (1.75 eq)	THF	-40°C-RT	Decomposition
 (1.5 eq)	Ag_2O (2 eq)	THF	RT	NR
 NEt_3Cl (3 eq)	LHMDS (1.5 eq)	CH_3CN	RT - -60°C	NR

*** NR means that >90% unreacted starting material was recovered. The reagent amounts used in the alkylating reactions are indicated in brackets.**

As all these bases and other alkylating reagents yielded no desired product, a second approach was investigated. Here, a different intermediate containing a less bulky group in the 9- position of compound **94** was formed. The substituent employed in this case was an acetyl group, making **97** the precursor (Scheme 23).



94 **97**

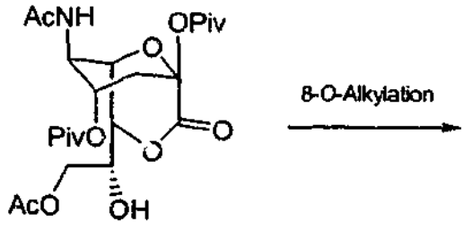
Reagents and conditions: (a) AcCl, Pyridine/CH_2Cl_2, 73%.

Scheme 23

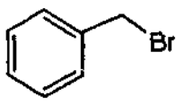
Acetylation on the 9-OH position was accomplished by treating **94** with acetyl chloride in a solution of pyridine and dichloromethane. Compound **97** was isolated in 73% yield after purification. Both ^{1}H and ^{13}C NMR supported formation of **97** with the appearance of the acetyl peak at 2.11 ppm (for ^{1}H NMR) and 20.6 ppm (for ^{13}C NMR). Other resonances in the ^{13}C NMR spectrum showed a downfield shift for the C-9 from 62.7 ppm for **94** to 65.1 ppm for **97**. Mass spectrometry also confirmed the isolation of the correct product with the detection of *m/z* 502 [$(M + 1)^{+}$] molecular ion.

Attempted 8-*O*-alkylation of the 9-*O*-acetyl derivative **97**, using both Ag_2O and [$(Bu_3Sn)_2O$] also failed to furnish any of the desired 8-*O*-alkylated product (Table 6). After a few attempts with different reagents and conditions (see Table 4 and Table 5) it was concluded that neither of these protecting groups on the 9-OH position would allow any 8-*O*-alkylation to occur. This prompted yet another approach towards compounds that may serve as useful probes for α(2,8)sialyltransferases to be developed.

Table 6. Attempted 8-*O*-alkylation of **97**.



97

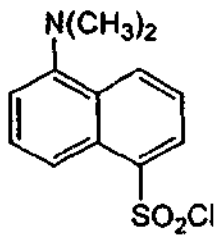
Alkylating Agent	Reagent(s)	Solvent	Temperature	Result*
 (1.1 eq)	Ag_2O (2 eq)	DMF	RT	NR
	$(Bu_3Sn)_2O$ (1 eq), $Bu_4N^{+}Br$ (1 eq)	Toluene	Reflux	NR

*** NR means that >90% unreacted starting material was recovered. The reagent amounts used in the alkylating reactions are indicated in brackets.**

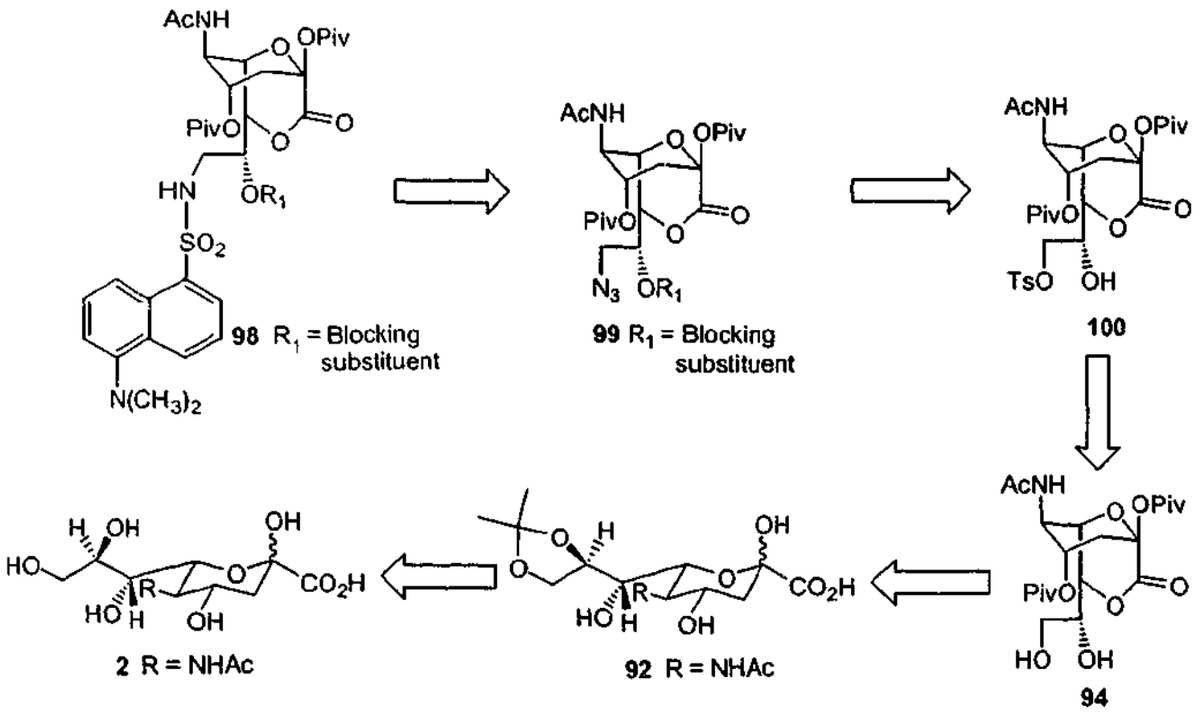
3.6 The 1,7-lactone 9-TAG, 8-substituted approach

The results from the preceding attempts indicated that it would be difficult to place a fluorescent group at the C-8 position on Neu5Ac. With the knowledge that α(2,8)sialyltransferases tolerate structural modifications at the C-9 position,[216] an alternative approach would then be to attach the fluorescent TAG substituent at the 9-OH and a blocking substituent (shown as R_1) at the 8-OH position as illustrated for compound **98** in Scheme 24.

For the 9 position, the fluorescent group, 5-(dimethylamino)-1-naphthalenesulfonyl chloride group (dansyl chloride) was selected due to its ability to be detected down to the picomolar range.[235] The dansyl group's fluorescence intensity depends on the polarity of the environment[236] which is important when considering fluorescence assays. As outlined in the retrosynthesis in Scheme 24, it was decided to place an azido group on the C-9 position of the 1,7-lactone intermediate **99** which could be transformed to the free amine. The azide would be an ideal substitutent because the introduction of the dansyl group no longer involves *O*-alkylation, but rather involves reaction of the C-9 amine with dansyl chloride to form the sulfonamide product **98**. A suitable precursor to the azido group would ideally contain a good leaving group, such as the *p*-toluene sulfonate anion (tosyl group) as shown in Scheme 24.



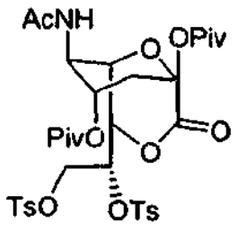
Dansyl chloride



Scheme 24. Retrosynthesis of the 9-TAG-8-substituted-1,7-lactone derivative **98**

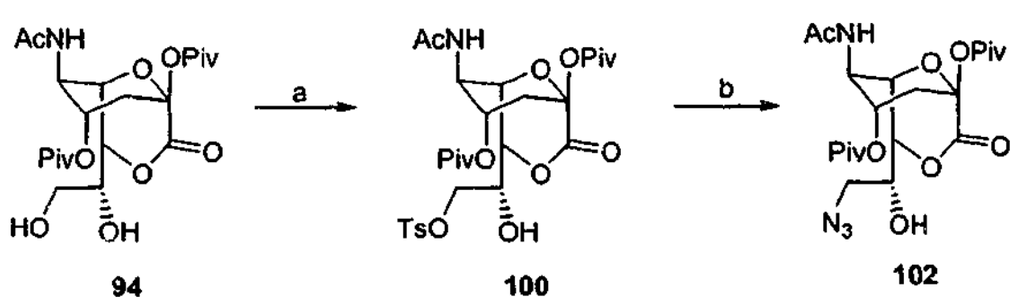
3.6.1 Results and discussion

Treatment of the diol **94** with *p*-toluene sulfonyl choride in pyridine at 0 °C for 3 hs afforded the desired product **100** in 55% yield after purification together with recovered starting material (40%). It was necessary to quench tosylation after 3 hs because of unwanted formation of the di-tosyl analogue **101.** Evidence for the mono-tosyl intermediate **100** was confirmed by ^{1}H, ^{13}C NMR, both showing the aromatic resonances attributed to the tosyl group at 7.34-7.39 ppm and 7.79-7.82 ppm for ^{1}H NMR and in the 128 to 146 ppm region in the ^{13}C NMR. The addition of the tosyl group on the C-9 carbon showed a downfield shift in the ^{13}C NMR spectrum for **100** (from 62.7 ppm for **94** to 70.6 ppm for **100**). Additional verification for compound **100** was obtained with mass spectroscopy showing the correct molecular ion.



101

With the selectively protected 9-OTs derivative **100** in hand, we decided to continue the sequence shown in retrosynthetic terms Scheme 24. Therefore, reaction of the 9-*O*-tosylate **100** with sodium azide in DMF at elevated temperatures afforded **102**. Optimal displacement occurred at temperatures between 80-100°C to produce **102** in a reasonable yield of 54% (Scheme 25). ^{1}H NMR spectroscopy showed that removal of the tosyl group in **100** caused an upfield shift for the both H-9 protons (from 4.35-4.45 ppm for **100** to 3.7 ppm for **102**). Similar results were observed in the ^{13}C NMR, with the C-9 resonance shifting from 70.6 ppm (for **100**) to 53.3 ppm (for **102**). IR spectroscopy supported the presence of an azide substituent on **102** with a band at 2105 cm^{-1}.



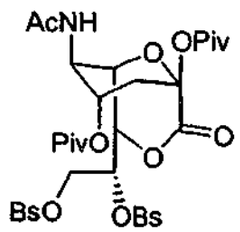
94 **100** **102**

Reagents and conditions: (a) TsCl, Pyridine, 0°C, 3 hs, 93%; (b) NaN_3, DMF, 80-100°C, 54%.

Scheme 25

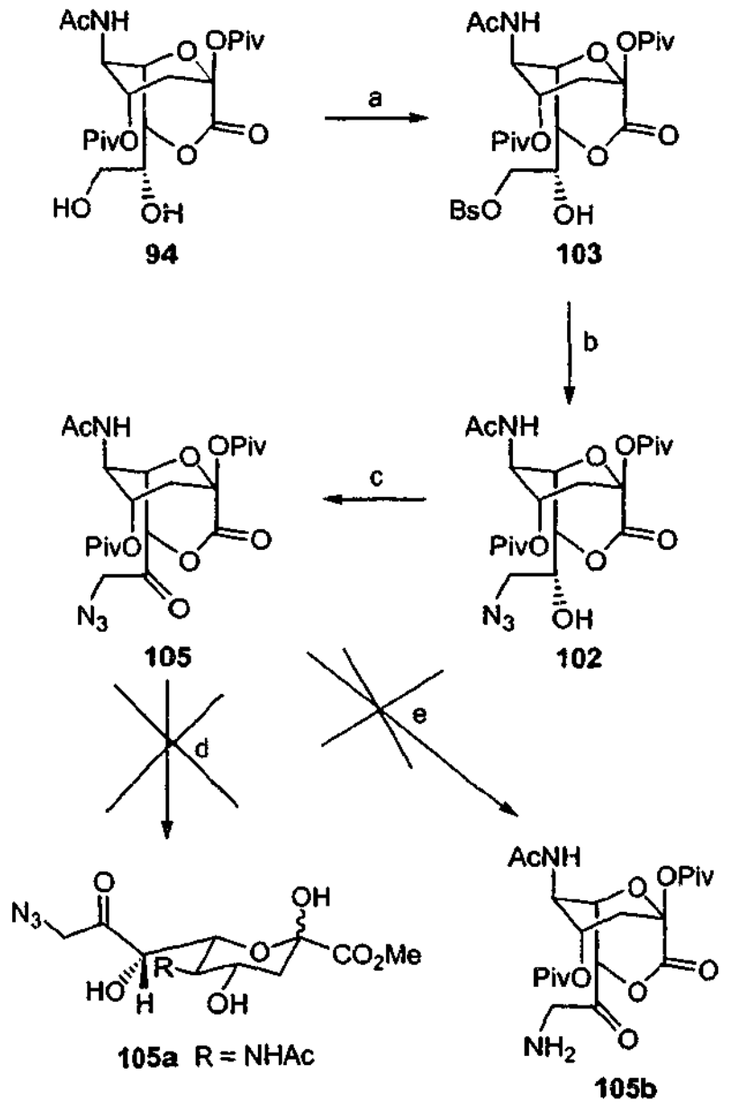
Given that the displacement reaction with the tosyl group did not go very cleanly, it was decided to try another nucleofuge similar to the tosyl group ie, 4-bromobenzene sulfonyl chloride (brosyl chloride). Following literature methodology[237], brosylation of **94**

proceeded smoothly in pyridine to afford **103** in 39% yield with recovered starting material (50%) after purification (Scheme 26). The brosylation reaction was quenched with water after 3 hs due to the unwanted formation of the di-brosyl analogue **104.** The mono-brosylated intermediate **103** was identified by diagnostic peaks of the aromatic region in the ^{1}H NMR at 7.73 and 7.81 ppm and ^{13}C NMR at 129 -134 ppm. A similar downfield shift at the C-9 postion (from 62.7 ppm for **94** to 71.5 ppm for **103**) was observed for the brosyl analog in the ^{13}C NMR spectrum. Correct analytical data provided further support for the **103** structure.



104

Displacement of the brosyl group in **103** with the azide was performed utilising similar conditions as for the tosyl derivative **100**. However, a lower temperature of 60°C was sufficient to obtain a quantitative yield of the azide **102**. Although brosylation is not as efficient as tosylation, the subsequent displacement with the azide is certainly more facile. Having successfully introduced the azide functionality at C-9, our attention turned back to attempted blocking of the C-8 hydroxyl. It was felt that instead of *O*-alkylation, which had proved quite difficult to accomplish, we would prepare the 8-oxo derivative **105** (see Scheme 26).



Reagents and conditions: (a) BsCl, pyridine, 0°C, 4 hs, 39%; (b) NaN_3, DMF, 60°C, quant.; (c) PDC/Ac_2O, CH_2Cl_2, RT, 6 hs, 73%; (d) NaOMe, MeOH; (e) H_2, Pd /C, MeOH.

Scheme 26

These type of 8-oxo compounds have been well documented by Zbiral as useful inhibitors for CMP-sialate synthase [EC 2.7.7.43].[238] Initially, oxidation at the 8-OH position was performed using the well known Swern Oxidation.[239,240] However, after a few attempts, only a moderate yield of 34% of the 8-oxo compound **105** was obtained. Another common method for oxidation utilises pyridinium dichromate (PDC) in dichloromethane[241] and this gave a yield of 73% of **105** (Scheme 26). The oxo compound **105,** was characterised with ^1H and ^{13}C NMR (with the quaternary C-8 peak at 201.7 ppm in the ^{13}C NMR spectrum) in

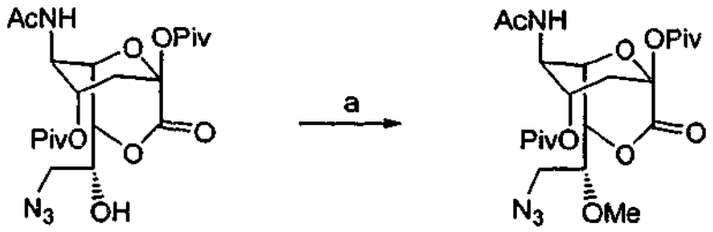
addition to a characteristic azide band at 2109 cm^{-1} by IR and the molecular ion (*m/z* 483 $[(M + 1)^{+}]$) by mass spectroscopy.

At this stage in the sequence, having successfully blocked the C-8 hydroxyl, we were ready to open the lactone ring in **105** to isolate the target compound **105a**. An initial attempt was adapted from Schmidt's[220] methodology, in that the lactone **105** was reacted with a solution of NaOMe in methanol. Unfortunately, extensive decomposition was observed due to the instability of the oxo functional group under alkaline conditions. Since it was necessary to perform the lactone opening under neutral conditions due to the presence of the ketone group in **105**, the scheme was altered and reduction of the azide was investigated.

Reduction of the azide under neutral conditions resulted in the formation of decomposition products, presumably due to the free amine being too alkaline for the pivaloyl-protected compound. To prevent decomposition of the pivaloyl protected Neu5Ac 1,7-lactone intermediate **105**, it is possible to hydrogenate the azide under similar conditions with some acetic acid to form the amine acetate salt. Unfortunately, utilising acetic acid in the reduction of **105** produced unidentifiable by-products.

Protecting the 8-oxo group would eliminate the restricted deprotection conditions. Thus, the 8-oxo group could conceivably be converted to the ketal. Ketal formation was attempted on **105** using literature methods[242] with methanol and acidic resin. However, starting material was obtained in all cases. Although this oxo derivative was not intended for the synthetic pathway, its production may be a gateway for other reactions that utilise a ketone adduct e.g. Grignard reaction.

At this stage of the project, it was decided once again to explore the formation of an 8-*O*-alkoxide group. A methoxy group, for example would be inert to both alkaline and acidic conditions and be much easier to handle. Thus, following a literature method,[243] compound **102** was treated with a solution of methyl iodide, $CaSO_4$ and Ag_2O under reflux. To our delight the 8-OMe derivative **106** was formed in excellent yields (84%) (Scheme 27). A three proton singlet peak at 3.48 ppm for ^1H NMR and a methoxy peak at 57.5 ppm in the ^{13}C NMR confirmed the formation of **106**. IR also indicated the azide substituent to be intact on compound **106** with a band at 2104 cm^{-1}. Further evidence for the formation of **106** was shown in the mass spectrometry data with the correct $[(M + 1)^+]$ molecular ion at *m/z* 499.



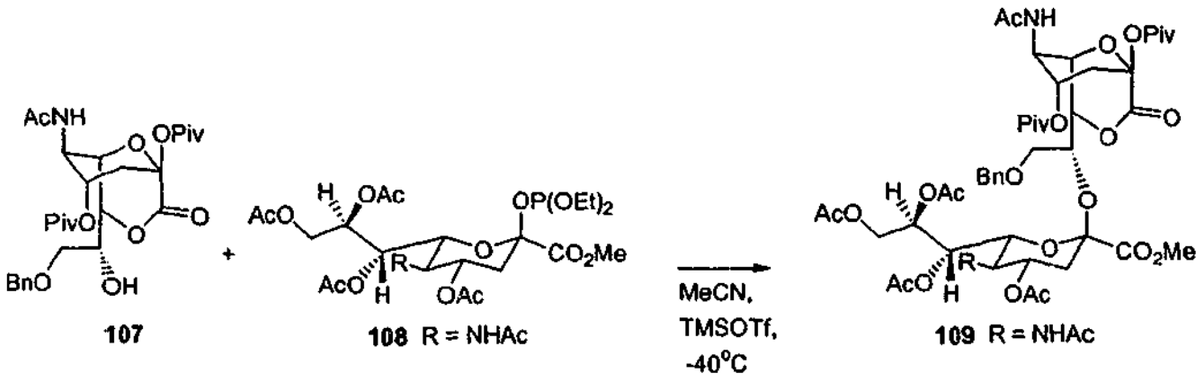
102 **106**

Reagents and conditions: (a) MeI, Ag_2O, $CaSO_4$, reflux, 48 hs, 84%.

Scheme 27

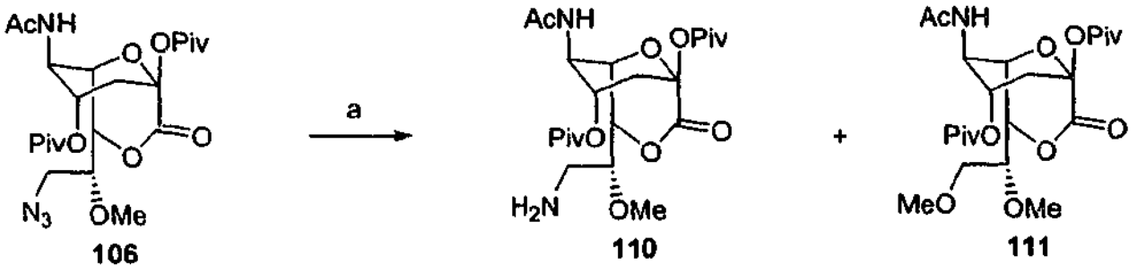
When compared to the same reaction using **96** as the substrate, it is tempting to speculate on the reasons for successful 8-*O*-alkylation in the case of **102.** The only difference between **96** and **102** is in the C-9 substituent. Thus it appears 8-*O*-alkylation is facilitated when there are no oxygen substitutents on the 9 position, presumably due to electronic effects. Interestingly, the reported 8-*O*-sialylation of **107** was successfully undertaken in the presence of a 9-*O*-benzyl group which gave a yield of 68%[220] (Scheme 28). These results may be indicating that steric encumbrance does not have an effect on these type of

reactions. Other *O*-sialylation reactions reported have had much lower yields due to low reactivity of the C-8.[229]



Scheme 28[220]

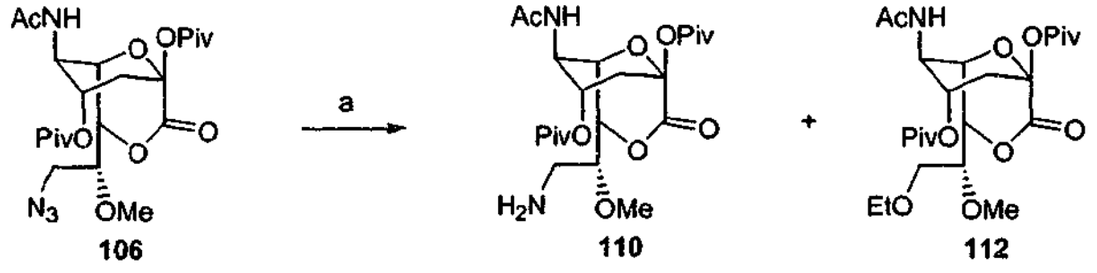
Having protected the 8-position as its methyl ether, the substrate **106** was now set up for successful reduction of the azide *via* hydrogenation under acidic conditions. The presence of the 8-OMe group avoids any potential chelation of the Pd catalyst following formation of the amine. Thus, Parr Hydrogenation (30Psi) of **106** with a small amount of glacial acetic acid in methanol yielded the free amine **110** in a reasonable yield (70%) (Scheme 29). The presence of the amino group in **110** was verified with positive ninhydrin test and mass spectroscopy demonstrating the correct molecular ion at *m/z* 473.5 [$(M + 1)^+$]. The reduction worked well on small scale (0.2 mmol), however, once the scale was increased to 0.8 mmol, the reaction did not work as well. Formation of a side-product, which seems to be **111** according to ^{1}H NMR (with the appearance of an additional three proton singlet at 3.45 ppm) became a major factor in the decreased yields of the desired material (Scheme 29).



Reagents and conditions: (a) MeOH, CH_3COOH, Pd/C, PARR/H_2, 30Psi, RT, 70%.

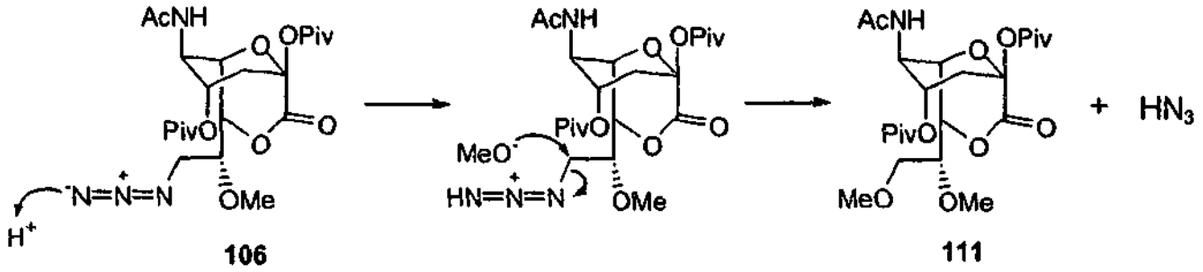
Scheme 29

The hydrogenation was repeated in ethanol in an attempt to reduce the formation of the side-product **111**. Again, formation of a similar product, the 9-ethoxy analogue **112** this time was obtained in 47% yield (Scheme 30). ^{1}H NMR and low-resolution mass spectrometry verified the formation of **112**. A possible mechanism for the formation of the side-products is the potential protonation of the azide (to form the hydrazoic acid HN_3 as leaving group) and then displacement by the alcohol (see Scheme 31). Using ethanol as the solvent instead of methanol, delivered the desired product **110** at a slightly lower yield of 47% than the methanol method (70%) for small scale reactions of ~ 0.2mmol.



Reagents and conditions: (a) EtOH, CH_3COOH, Pd/C, PARR/H_2, 30Psi, RT, 47%.

Scheme 30



Scheme 31

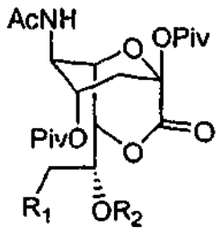
Another method was attempted for the reduction reaction to eliminate the by-products. Nagarajan and Ganem[189] claim a simple method for the reduction of azides which involves refluxing the azide and triphenyl phosphine (Ph_3P) in THF and water. Unfortunately, none of the desired amine was obtained when **106** was subjected to these conditions and ~90% of starting material was recovered.

An additional way of potentially optimizing the hydrogenation step is to avoid use of an alcoholic solvent, and carry out the reaction using neat acetic acid. These conditions remain to be investigated.

The ^{1}H NMR spectrum of **110** revealed distinguishable change in shift for most of the protons especially the H-3ax and H-3eq protons compared to the precursors **106, 102, 103** and **94**. Normally the H-3ax and H-3eq protons for the derivatives **106, 102, 103** and **94,** are directly adjacent to each other at 2.2-2.3 ppm, however, for **110,** the H-3eq has moved downfield to 3.03 ppm whilst the H-3ax proton (at 2.4 ppm), has not shifted significantly (see Table 7). Other protons which show significant changes in chemical shifts are the H-9a and H-9b protons. The H-9a proton has typically shifted > 0.4 ppm downfield and the H-9b proton has shifted > 0.5 ppm upfield compared to the derivatives listed in Table 7. Once **110** was transformed to the sulphonamide (ie derivative **113** or **114,** *vide infra*), the

unusual chemical shifts observed for **110** were alleviated and the familiar chemical shifts (which were seen for **106, 102, 103** and **94**) were observed for **113** and **114.**

Table 7. ^{1}H NMR Chemical shift data (in ppm) of 1,7-lactone analogues at 300MHz (*indicates at 500MHz) in $CDCl_3$.

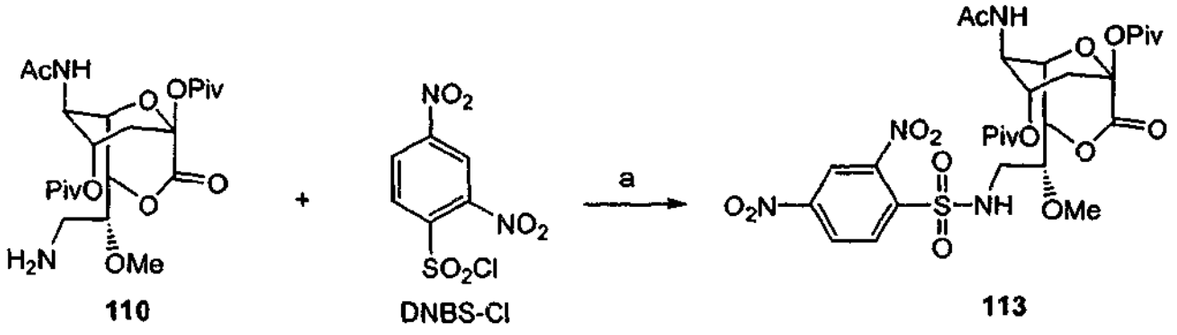


Compound	R_1	R_2	H-3ax	H-3eq	H-4	H-5	H-6	H-7	H-8	H-9a	H-9b
94*	OH	H	2.21	2.31	5.14	4.13-4.17	4.67	4.45	4.13-4.17	3.92	3.92
103	Bs	H	2.20	2.30	5.14	4.18	4.62	4.38-4.42	4.48-4.42	4.38-4.42	4.38-4.42
102*	N_3	H	2.21	2.26	5.13	4.10	4.62	4.37	4.26	3.70	3.70
106	N_3	Me	2.20	2.27	5.14	4.22	4.48-4.53	4.48-4.53	3.84-3.90	3.60	3.84-3.90
110*	NH_2	Me	2.34	3.03	5.08	4.39	4.17	4.65	3.33	3.19	4.87
113*	2,4-dinitro-phenyl	Me	2.23	2.28	5.11	4.15	4.30	4.38	3.70-3.78	3.70-3.78	3.70-3.78
114	Dansyl	Me	2.16	2.29	5.10	4.12	4.37	4.42	3.69	3.29-3.36	3.29-3.36

3.6.2 Attachment of the Fluorescent group at C-9

Although the synthesis of the free amine derivative **110** was not optimal, sufficient material was available to undertake the attachment of the fluorescent group. Initial investigations explored the possibility of carrying out a sulfonylation reaction using 2,4-dinitrobenzenesulfonyl chloride (DNBS-Cl) as the fluorescent group. Utilising similar

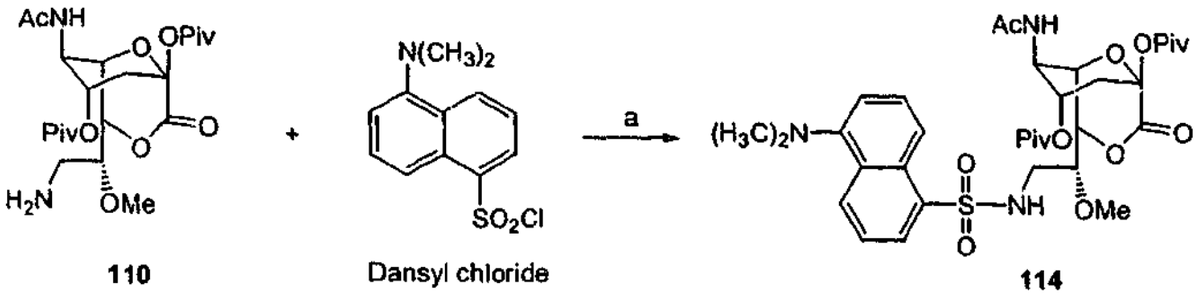
conditions to those reported by Reinhard *et al*,[235] the coupling reaction was performed at room temperature with pyridine as the solvent (Scheme 32). After stirring for 2 days, a further amount of 2,4-dinitrobenzene sulfonyl chloride (2 equivalents) was added to the reaction mixture. A total of 3 days was required before TLC showed no more starting material in the reaction mixture. After purification, the adduct **113** was isolated in 61% yield. ^{1}H and ^{13}C NMR confirmed the formation of **113** with the appearance of the aromatic peaks at 8.38, 8.54, and 8.69 ppm for the trisubstituted aromatic ring. Furthermore the 2,4-dinitrobenzene sulfonamide group in compound **113** caused a downfield shift for the C-9 peak from 46.0 ppm for **110** to 67.0 ppm for **113** in the ^{13}C NMR spectrum.



Reagents and conditions: (a) Pyridine, RT, 72 hs, 61%.

Scheme 32

The coupling reaction of **110** was repeated under similar conditions with dansyl chloride (Scheme 33). The reaction was stopped only after one day of stirring due to the disappearance of the starting material according to TLC. In this case the reaction did not proceed as well, with a 38% yield for the desired product **114**. The ^{13}C NMR spectrum for **114** showed an upfield shift for the C-9 peak from 46.0 ppm for **110** to 40.8 ppm for **114**. Both ^{1}H and ^{13}C NMR assignments were confirmed with 2-D experiments (ie COSY and HMQC respectively). Mass spectroscopy provided further evidence for the production of **114** with the correct molecular ion $[(M + 1)^{+}]$ at *m/z* 706.5.



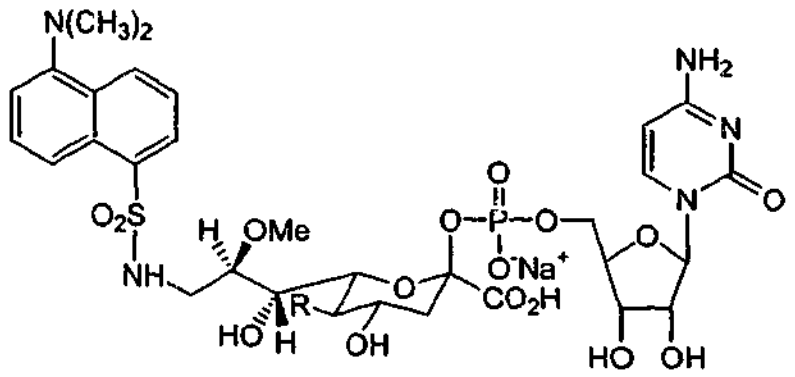
Reagents and conditions: (a) Pyridine, RT, 24 hs, 38%;

Scheme 33

3.7 Conclusion

Sialyltransferases are known to take part in many important biological systems however there are many unknown features about them. An important finding relating to most sialyltransferases is their ability to possibly hypersialylate cell surfaces which can lead to metastasis. This includes the α(2,8)sialyltransferases which take part in the progression of cancer and have been the main topic of study in this aspect of this PhD project.

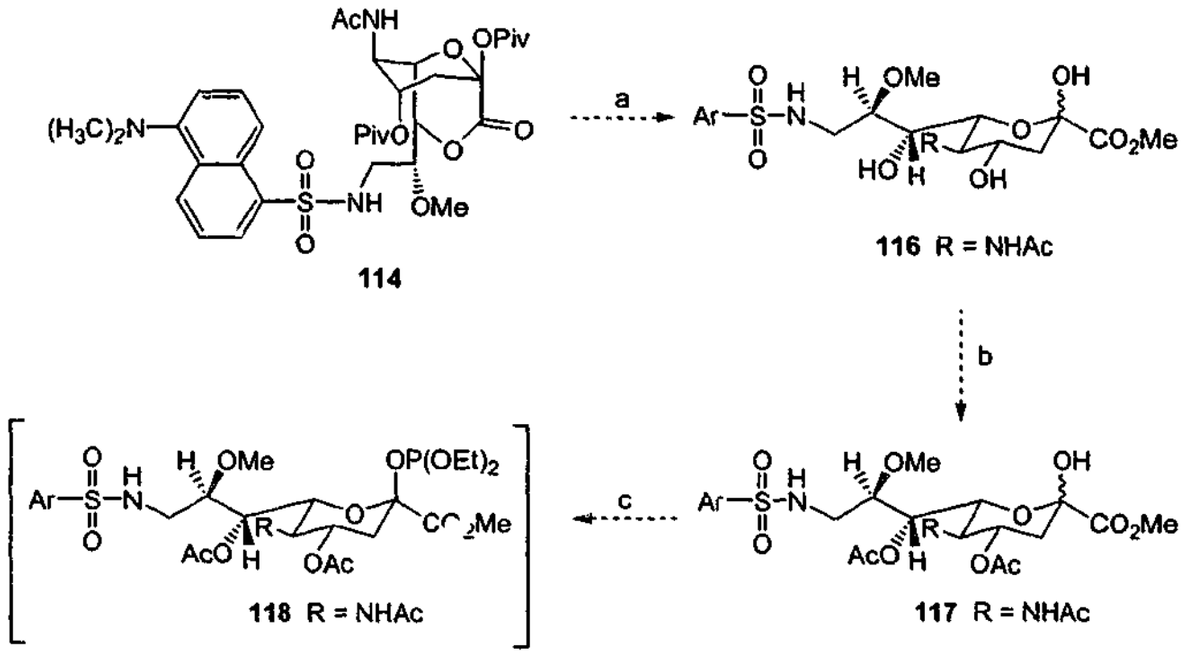
Synthesizing a compound that can not only mimic the natural substrate for α(2,8)sialyltransferases, but can also be used to detect transferase activity would be a significant step towards early diagnosis and combating diseases. Knowing that it is possible for sialyltransferases to recognize CMP-Neu5Ac analogues and have them transferred in biological systems, it is possible that the target compound **115** could be extremely beneficial in this kind of study.



115 R = NHAc

Due to time constraints, it was not possible to continue with this project; however, the subsequent steps are well known and will be discussed. A few investigations in the literature have discussed the formation of CMP-Neu5Ac analogues, which could be applied in the final steps of this project to obtain the new target compound **115**.

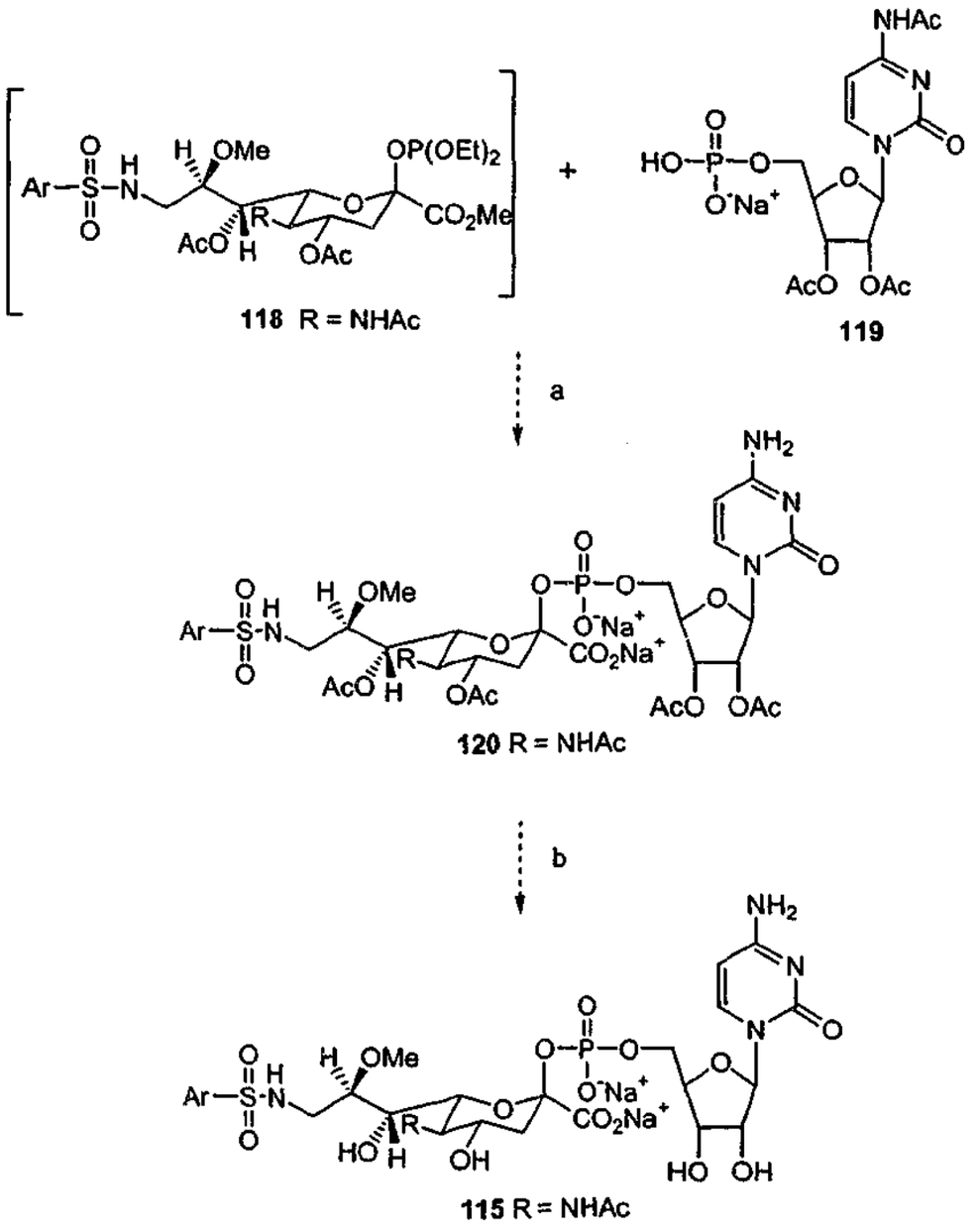
According to Schmidt,[220] the initial deprotection of the lactone ring may be accomplished by treatment with sodium methoxide in methanol. These conditions should convert the lactone **114** to the $^2C_5$ configured Neu5Ac derivative **116** as shown in Scheme 34. Subsequent reaction involving the selective protection of the Neu5Ac analogue **116** as described by Martin *et al*,[244] may be achieved by treatment with acetic anhydride (Ac_2O) and perchloric acid ($HClO_4$) in an acidic methanol solution to obtain **117** (Scheme 34). Compound **117** may also be achieved by fully acetylating compound **116** and then removing the anomeric acetate using hydrazine acetate (A. Liakatos, M. J. Kiefel and M. von Itzstein, unpublished results).



Reagents and conditions: (a) NaOMe, MeOH; (b) $HClO_4$, Ac_2O, MeOH, H^+; (c) $ClP(OEt)_2$, $EtN(i\text{-}Pr)_2$

Scheme 34

Activation of the sialic acid derivative **117** for coupling with CMP can be accomplished *via* the phosphite **118**[224,244-248] and then coupling with the tri-*O*-acetyl CMP derivative **119** will provide the desired 9-TAG, 8-blocked CMP-Neu5Ac derivative **120** (Scheme 35). The coupling reaction of the protected CMP and the sialyl phosphite has been well documented by Schmidt's group.[224,245,249] The final deprotection of the CMP-Neu5Ac derivative **120** may be accomplished by treating **120** with sodium methoxide/methanol and then water[245] to give the target compound **115** (Scheme 35).

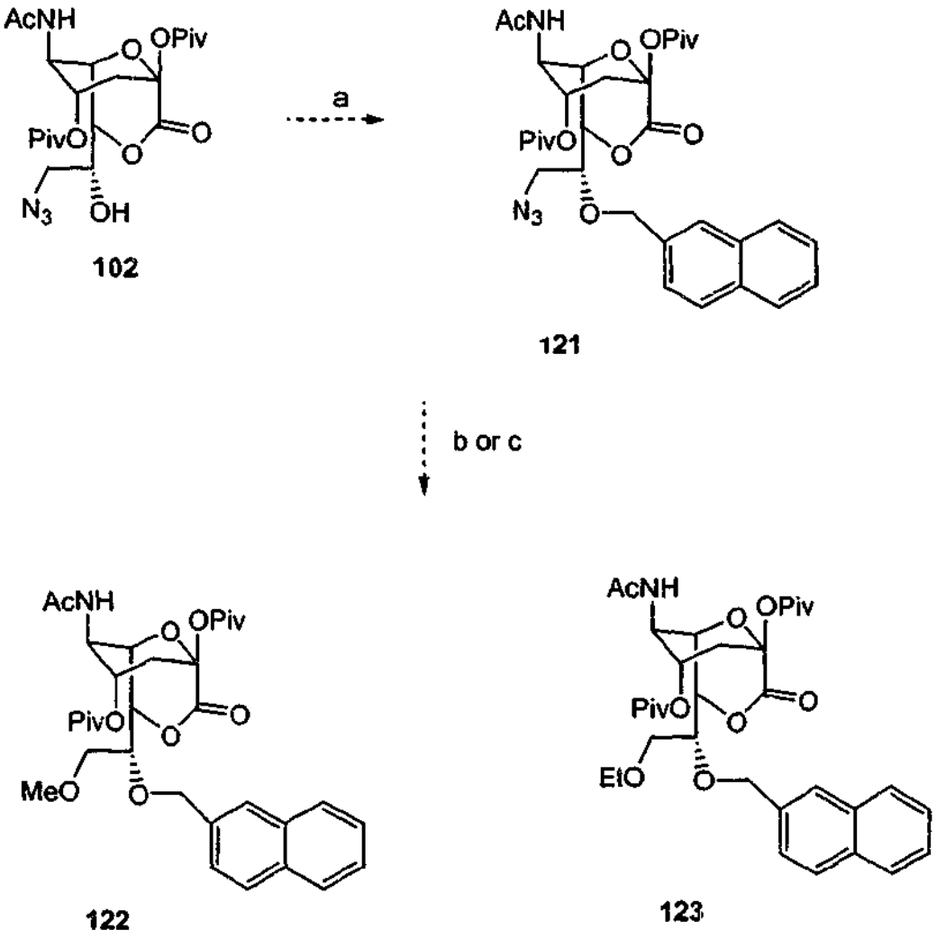


Reagents and conditions: (a) CH_3CN/THF/DMF (1:1:1), -15°C-RT; (b) NaOMe, MeOH.

Scheme 35

Further work could also be directed towards the synthesis of an analogue of the original target molecule, i.e. an 8-*O*-TAG derivative of **75** following the serendipitous observations that 8-*O*-alkylation is facilitated in the presence of a 9-azido group and that the same azide functionality can be converted to a 9-methoxy or -ethoxy moiety in good yield via hydrogenolysis.

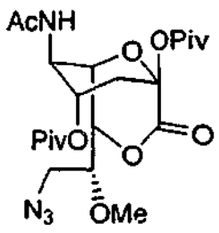
Thus, starting material, **102**, could be employed in the reaction with a suitable alkylating agent using similar conditions described for the *O*-methylated product, **106**, to afford the 8-*O*-TAG derivative, **121** (Scheme 36 below). Given the successful synthesis of **106**, 8-*O*-alkylation of **102** would be expected to proceed in the case of 2-bromomethyl naphthalene as the 9-position is no longer loaded with a potentially debilitating oxygenated moiety. Following alkylation at the 8-position with the fluorescent TAG, the original oxygenation pattern of sialic acid can be returned to the 9-position *via* replacement of the azide with an ether in a hydrogenolysis reaction to afford **122** or **123** (Scheme 36).



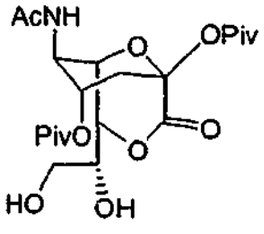
Reagents and conditions: (a) bromomethyl napthalene, Ag_2O, $CaSO_4$, reflux, 48 hs; (b) MeOH, CH_3COOH, Pd/C, PARR/H_2 , RT; (c) EtOH, CH_3COOH, Pd/C, PARR/H_2 , RT.

Scheme 36

Admittedly, this is a more circuitous route to *O*-alkylation at the 8-position. However, such an approach has merit if *O*-alkylation at this position is genuinely desired given the possible lack of reactivity in the presence of the neighbouring 9-hydroxy group. Both brosylation at the 9-position and subsequent displacement by azide proceed in good to high yield. Work-up of these reactions is also straightforward. Likewise, the alkylation and hydrogenolysis reactions *en route* to the target molecule following synthesis of **102** are high-yielding and experimentally uncomplicated. When considered in this light, the two additional steps from **94** in the overall synthesis are more than offset by obtaining the desired product, **121**, in good yield through facile reactions.



106



94

In conclusion, this investigation developed a key 8-oxo intermediate, **105**, which could prove useful should other (e.g. Grignard) reactions be considered for this type of substrate. Additionally, methylation conditions were found to afford the 8-OMe Neu5Ac derivative **106** in excellent yield (84%). Important issues in relation to the reactivity of the C-8 position of Neu5Ac which supports earlier reports by Schmidt's group[220,221,225] were also investigated and discussed.

CHAPTER 4

Purification of sialic acid-recognizing proteins (SARPs)

4.1 Introduction

As mentioned earlier (Chapter 1), the importance of sialic acids in biological systems has led to an increased interest in the proteins that recognize these acidic carbohydrates.[8,13,250] Information from the three dimensional structure of a sialic acid-recognizing protein obtained through X-ray crystallographic data can be utilized to obtain a greater understanding of protein-substrate/inhibitor interactions and aid in the design of potential inhibitors. The X-ray crystal structure of a number of sialic acid-recognizing proteins (SARPs), such as proteins from influenza virus hemagglutinin,[134] E-selectin[251] as well as several sialidases[49,118,120,252-254] and recently, *Trypanosoma rangeli* sialidase[255] and *Trypanosoma cruzi trans*-sialidase (*T. cruzi trans*-sialidase)[256] have been elucidated.

SARPs such as sialidases from pathogenic organisms are obvious targets for the design of potential therapeutic targets. The initial step towards designing the target compounds is the isolation of the specific SARP in a purified state and in sufficient quantity to facilitate obtaining the X-ray crystal structure. Once the crystal structure has been resolved, the enzyme/protein may be used for possible high throughput screening against potential inhibitors. Furthermore, other important biological information may be elucidated once an enzyme/protein is isolated in a purified state. The exploration of unknown biological roles or even the examination of known biological roles will typically require the enzyme/protein in a purified state.

As part of our interest in SARPs (Chapter 1) we required sufficient methods to purify these proteins. In particular as a result of our interest in the sialidases, *Vibrio cholerae* sialidase (*V.c.* sialidase), sialidase-L from leech, and *trans*-sialidase from *T. cruzi* and a sialyltransferase from rat liver. We have focused our attention on developing purification methods that would allow for the isolation of these proteins. A brief description on what is known about these SARPs will be outlined below. A description of sialyltransferase has been presented in Chapter 3 (section 3.2) and will not be discussed further.

4.2 *Vibrio cholerae* sialidase

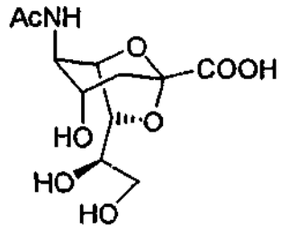
Cholera is a serious epidemic disease that can lead to death within a few hours. The disease is initiated by a toxin released after adhesion of the non-invasive *Vibrio cholerae* bacteria to the small intestine of the host.[254] One of the main roles of *V.c.* sialidase is cleavage of sialic acid from higher order gangliosides to give GM_1, the putative cholera toxin receptor.[254,257] This suggests that the sialidase may increase the virulence of *Vibrio cholerae* by increasing the number of receptor sites available.

In the case of *V.c.* sialidase, although the crystal structure is known,[253] its purification has been documented to involve exhaustive dialysis followed by chromatography.[258] Hence, an efficient method for purification of *V.c.* sialidase would be beneficial for further studies.

4.3 Sialidase-L

Sialidase-L from leech has been reported to produce 2,7-anhydro-Neu5Ac (**124**) instead of Neu5Ac from various sialoglycoconjugates.[259,260] This unusual sialidase also displayed specificity toward the hydrolysis of only the NeuAcα(2,3)Gal linkage without destroying

other sialosyl linkages such as NeuAcα(2,6)Gal.[259] Due to its characteristic linkage specificity, sialidase-L would be a useful tool to study the biological significance of the NeuAcα(2,3)Gal linkage and the structural elucidation of sialoglycoconjugates. This particular sialidase has been purified utilizing many centrifugation steps and columns.[259] A more efficient method for purification would be ideal.



124

4.4 *Trans*-sialidase from *T. cruzi*

Trans-sialidase (TS) is found in the parasite *Trypanosoma cruzi* (*T.cruzi*)[261] and is the etiological agent responsible for Chagas' disease, an incurable, debilitating disease that affects millions of people in Latin America.[261] Trypanosomes are unable to synthesize sialic acids and use their *trans*-sialidase to scavenge the acidic carbohydrate from host cell glycoconjugates and transfer it onto mucin-like acceptor molecules in the plasma membrane.[255,261,262] As such, *T. cruzi trans*-sialidase represents an ideal target for therapeutic intervention. Even though *T. cruzi trans*-sialidase behaves as a sialyltransferase in the presence of a suitable sugar acceptor substrate, the transfer reaction catalysed by *T. cruzi trans*-sialidase is different from that of typical sialyltransferase.[261] As discussed in section 3.1, sialyltransferases typically require sugar-nucleotides (e.g. CMP-Neu5Ac (7)) for the transfer reaction.[193,194,261] In the case for the *trans*-sialidase from *T.cruzi*, the enzyme does not require a nucleotide-bound sialic acid as the sialyl donor, but rather an α-linked glycoside of sialic acid (see Figure 19).[262] Although the

crystal structure of *T. cruzi trans*-sialidase has been solved, there are many unknown features about this sialidase which need to be explored. For example, why is this sialidase more efficient in transferring rather than hydrolysing terminal sialic acids?[261]

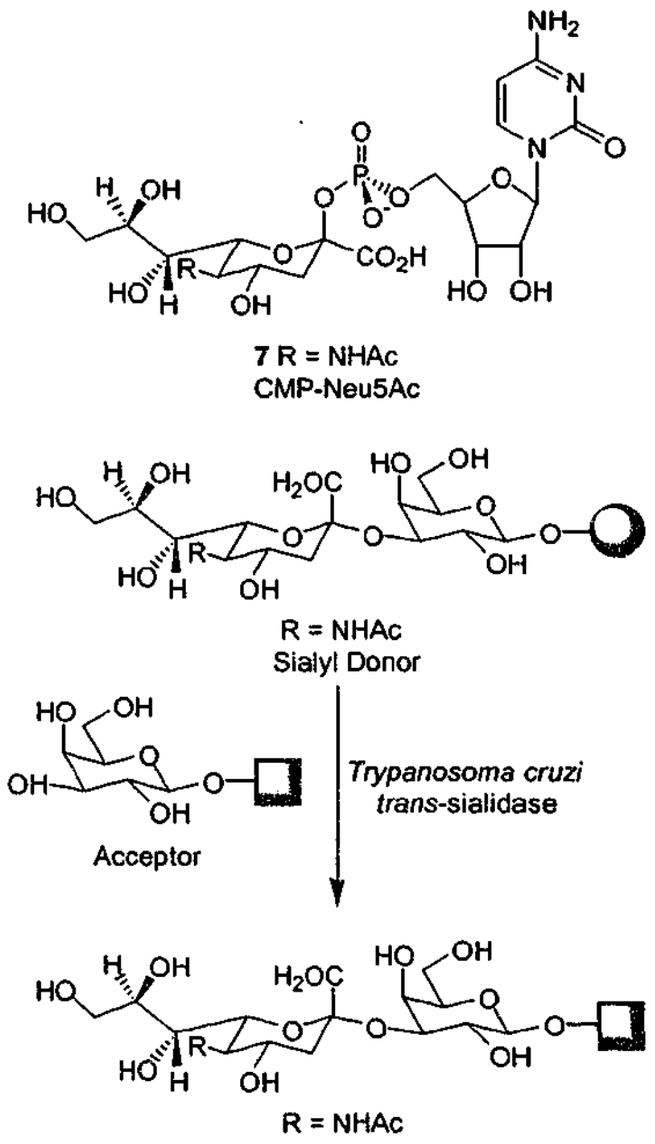


Figure 19. *Trypanosoma cruzi trans*-sialidase catalysed *N*-acetylneuraminic acid transfer.

(Adapted from Wilson *et al*[262])

4.5 Purification of Sialic acid-recognizing proteins (SARPs)

From the previous discussion it is reasonable to suggest that all of the sialidases discussed may be used as targets for therapeutic intervention. Although sialidases have been isolated

from viral, bacterial and eucaryotic sources, their structures and many of their physiological functions are still unknown, due primarily to the instability of these enzymes and the difficulty of their purification. In fact, most of the purification protocols employed are complicated, multi-step processes typically requiring initial precipitation of the enzyme followed by at least two individual chromatographic steps such as ion-exchange and gel filtration.[263-268]

The ongoing interest in sialic acid-recognizing proteins (SARPs), in particular sialidases, has prompted us to develop an efficient purification method for large amounts of these proteins. Ultimately, the purified proteins would facilitate structure determination and functional studies.

Affinity chromatography has long been considered an excellent method for protein purification due to the unique specificity inherent in a ligand-biomacromolecule interaction.[269] This technique depends on an affinity interaction *via* biological recognition rather than physico-chemical properties. Consequently it is suitable for preserving the biological activity of the isolated protein. Affinity chromatography also allows dilute solutions to be concentrated and is also capable of stabilizing the protein of interest when bound to a ligand for which it has a natural affinity.[269]

The technique applied in affinity chromatography involves the preparation of a column which contains an affinity support with desirable properties such as: inert, hydrophilic, easily derivatized, chemically and physically resistant and macroporous.[269] The derivatization of the support makes it specific to purify certain proteins. For example, a sialic acid ligand which is coupled to the support would have the potential to allow sialic acid-recognizing proteins to bind to the column. This is illustrated in Figure 20 which

shows an affinity support with a sialic acid ligand bound. When a crude mixture of the protein is placed onto the column, the sialic acid-recognizing protein attaches to the ligand on the support while the unwanted proteins are eluted from the column (see First Elution, Figure 20). When all of the unwanted proteins have eluted from the column, then the pure sialic acid-recognizing protein may be eluted from the column (see Second Elution, Figure 20).

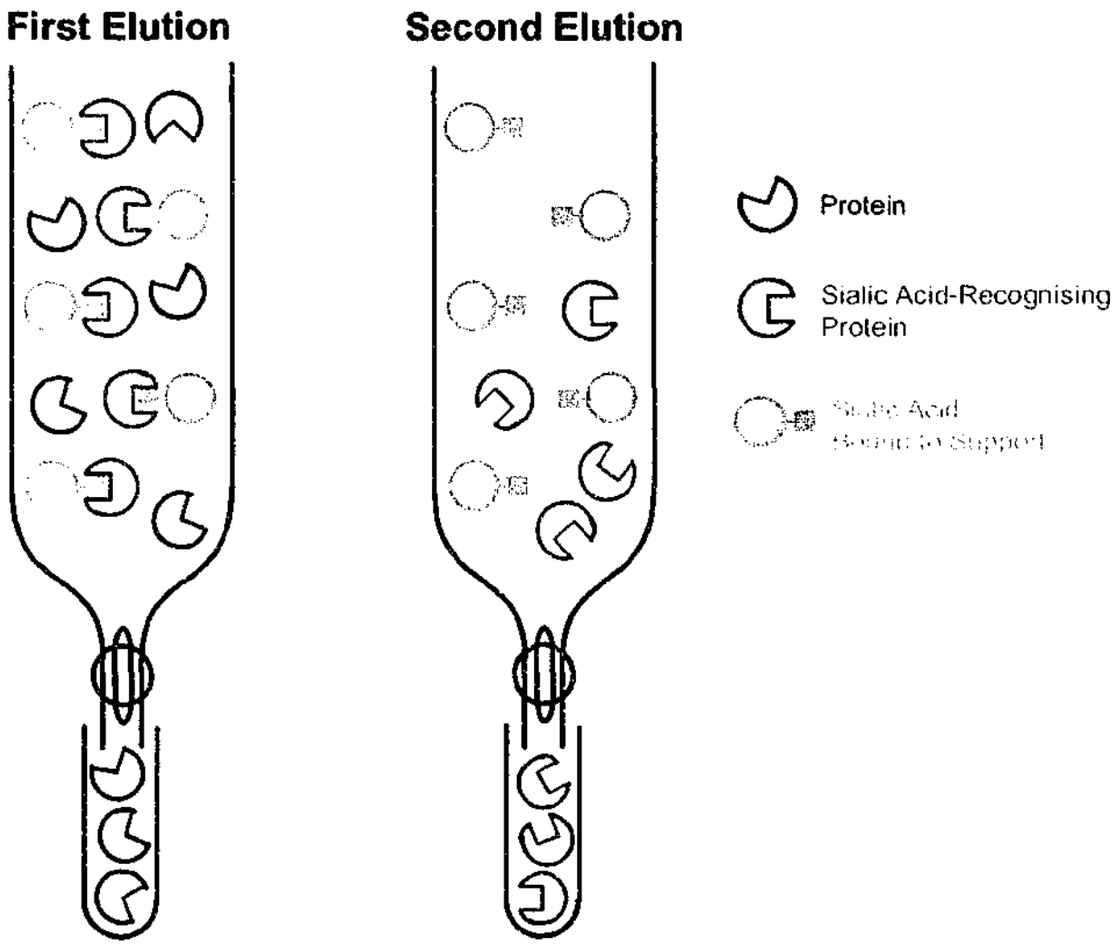
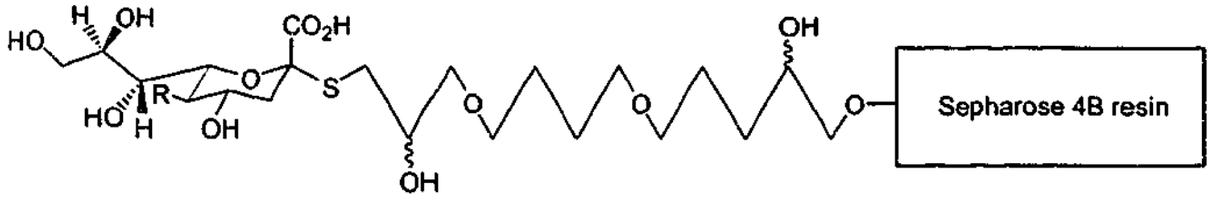


Figure 20. First Elution: Sialic acid-recognizing protein bound to support, whilst unwanted protein is eluted from column. **Second Elution:** Pure sialic acid-recognizing protein unbound from support and eluted from column.

Several affinity adsorbents have been described[263-268,270-277] and many are based on immobilizing some form of the natural substrate (i.e., synthetic *N*-acetyl-neuraminic acid derivatives[263-265,270-272] or glycoproteins which contain *N*-acetyl-neuraminic acid

residues[266,273-275]) onto a chromatographic support. However, a number of these affinity matrices suffer from either a lack of specificity or from hydrolysis of the immobilized ligand.[267,268,270,274,276,277] Suzuki *et al*[263] reported a method which overcame some of these limitations; an affinity adsorbent was prepared by attaching Neu5Ac to epoxy-activated Sepharose 4B *via* a thioglycoside linkage (see Figure 21).[263] Since thiosialosides are considered to be resistant to hydrolysis by sialidases,[177,278] affinity matrices based on such ligands, should be stable towards the enzymes being purified. The affinity matrix prepared by Suzuki and coworkers was able to absorb the overexpressed *Clostridium perfringens* sialidase, and gave a purification of 21% over three steps.[263]



R = NHAc

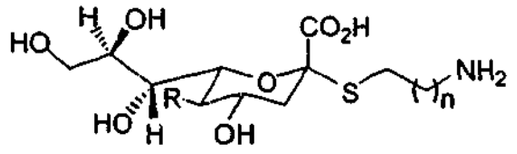
Figure 21. Affinity absorbent prepared by Suzuki *et al.*[263]

In the present investigation, sialic acid was used as the immobilized natural substrate on an affinity matrix in an effort to purify important SARPs for structure determination and functional studies. Previous work on the synthesis of novel α-thioketosides of *N*-acetylneuraminic acid[185-188] led to the preparation of an affinity matrix with an α-thiosialoside capable of attaching to an epoxy-activated Separose 6B.

4.6 Design of affinity matrices

A simple target molecule was the main objective in designing the ligand. Initially, it was decided to synthesise α-thiosialosides such as **125** because thiosialosides are thought to be

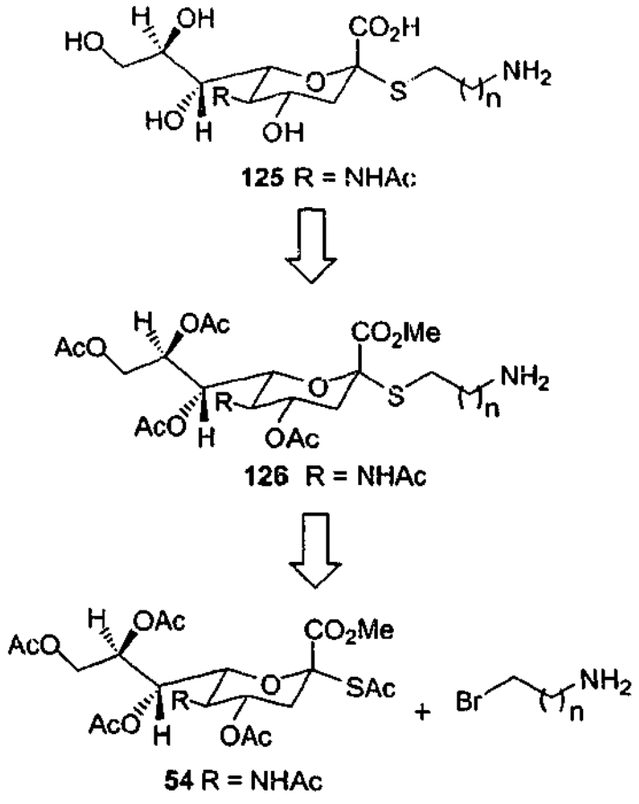
resistant to sialidases.[177,278] In order to develop a matrix that would ultimately purify SARPs with high specificity, we decided to investigate a series of ligands with different spacer (carbon chain) lengths. In order to efficiently attach the ligand to epoxy-activated Sepharose 6B, a thiosialoside was designed with spacer arms containing different carbon chain lengths and a terminal amino group (NH_2).



125 R = NHAc

4.6.1 Retrosynthesis of the ligands

Scheme 36 outlines the retrosynthesis for the target ligands **125**. The main step is the coupling of the well known 2-thioacetyl Neu5Ac derivative **54** with a simple halo alkylamine. For this series, readily available bromo alkylamines of different carbon chain lengths were used. Deacetylation and saponification of the coupled products should then afford the target compounds **125**.

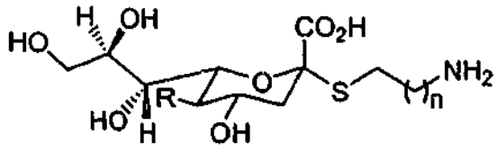


Scheme 37. Retrosynthesis of Ligands for Affinity Chromatography.

4.7 Synthesis of Ligands for Affinity Matrices[279,280]

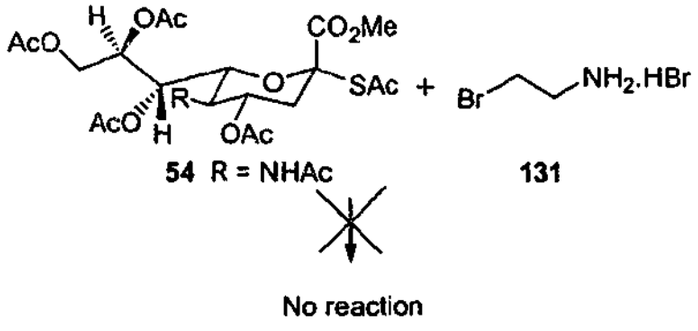
4.7.1 Synthesis of Thioketosides of *N*-Acetylneuraminic acid

The key intermediate for the synthesis of the target ligand **125** is the 2-thioacetyl Neu5Ac derivative **54** (methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate). The general strategy for the synthesis of **54** is outlined in section 2.10.1.1. The mild and efficient method[185] involving the utilisation of diethylamine (Et_2NH) in DMF at room temperature as described in section 2.10.1.1 was applied for the coupling reaction to form the thioketosides **127, 128, 129, 130.**



127 R = NHAc, n = 1,
128 R = NHAc, n = 2,
129 R = NHAc, n = 3,
130 R = NHAc, n = 4

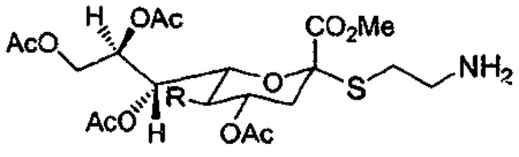
The Et_2NH promoted[185] coupling between methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulo-pyranosonate (**54**) (prepared according to a published procedure[27]) and commercially available 2-bromoethylamine hydrobromide (**131**) was attempted. However, this reaction failed to furnish any of the α-thiosialoside **132**, presumably due to the presence of the hydrobromide salt (Scheme 38).



54 R = NHAc **131**

No reaction

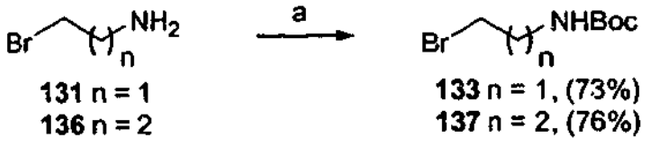
Scheme 38



132 R = NHAc

In an attempt to overcome this problem, the amino group was protected by reaction of an aqueous solution of 2-bromoethylamine hydrobromide (**131**) with di-*tert*-butyl dicarbonate in CH_3CN at pH 9 to give the Boc protected amine **133** in 73% yield after purification (Scheme 39). Exposure of an *N,N*-DMF solution of the 2-thioacetyl Neu5Ac derivative **54**

and the Boc protected amine **133** to Et_2NH resulted in smooth formation of the novel α-thiosialoside **134** in 77% yield (Scheme 40). The ^1H NMR spectrum of **134** is consistent with the structure shown. In particular, the presence of a two proton multiplet centred at 2.82 ppm (H-2) (see section 5.3.3 for proton assignments) is indicative of a CH_2 attached to sulphur.[166] Deacetylation and subsequent saponification of the α-thiosialoside **134** furnished **135** in near quantitative yield (99%). Deprotection of the aglycon alkylamine in **135** was achieved utilizing known methodology[281] with $BF_3{\cdot}Et_2O$ in CH_3COOH to afford the desired α-thiosialoside **127** (Scheme 40) in 64% yield after HPLC purification.[279] The thiosialoside **127**, which contains the appropriate functionality to facilitate attachment to a chromatographic support, is formed in an overall 49% yield from readily accessible starting materials.

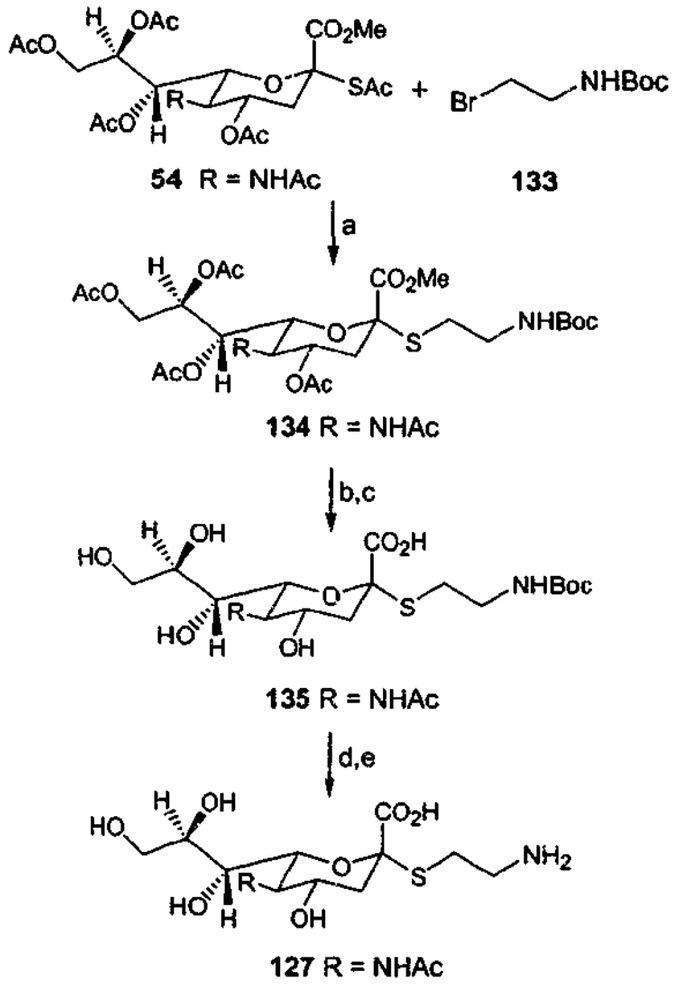


131 n = 1
136 n = 2

133 n = 1, (73%)
137 n = 2, (76%)

Reagents and conditions: (a) $(Boc)_2O$, CH_3CN, Et_3N, pH 9.

Scheme 39

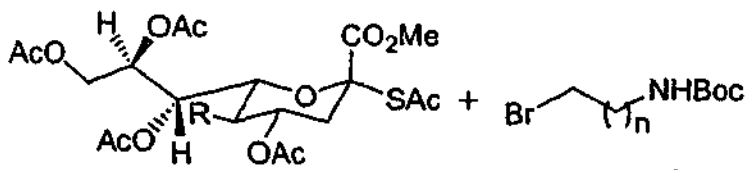


Reagents and conditions: (a) DMF, Et_2NH, 77%; (b) NaOMe, MeOH; (c) H_2O, NaOH,99%; (d) CH_3COOH, $BF_3{\cdot}Et_2O$; (e) Et_3N, pH = 7, 64%.

Scheme 40. Synthesis of the affinity ligand **127**.[279]

The synthesis of **128** is similar to that employed in the synthesis of **127**, and involves the coupling of the 2-thioacetyl Neu5Ac derivative (**54**) to the protected alkyl halide **137** (see Scheme 39 for formation of protected alkyl halide **137** and Scheme 41 for coupling reaction). Again, the ^1H NMR spectrum of 138 is consistent with the structure shown. Subsequent NaOMe mediated deacylation of the thiosialoside **138** followed by saponification gave **139** in 99% yield. If the deprotection of **139** was performed using $BF_3{\cdot}OEt_2$ in CH_3COOH as previously described[279], removal of the triethylammonium acetate salt became problematic and **128** was isolated in a modest 35% yield after

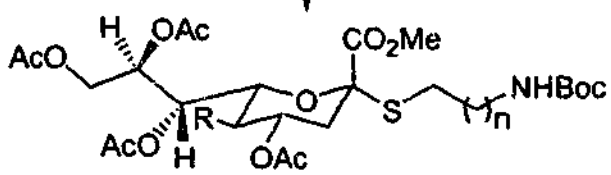
purification. Therefore, an alternative deprotection procedure was desired. Thus, deprotection of the aglycon alkylamine using neat TFA afforded the desired α-thiosialoside **128** in 71% yield. This increased the overall yield of **128** to 59%. ^{1}H NMR spectroscopy confirmed the formation of **128,** furthermore the ninhydrin test was positive for the free amine and IR showed the presence of the carboxylic group at 1626 cm^{-1}. Correct analytical analysis was obtained for both **138** and **128.**



54 R = NHAc

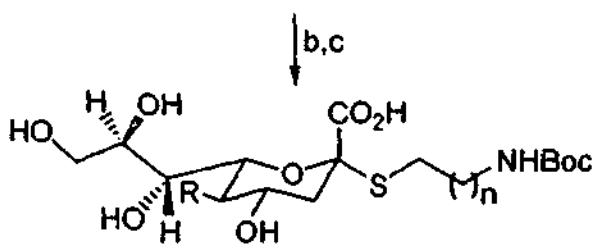
137 n = 2
142 n = 3
145 n = 4

a



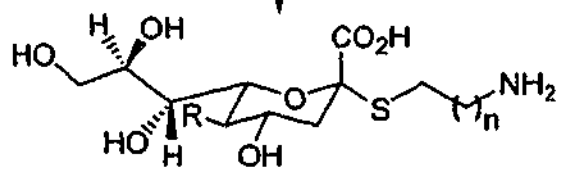
138 R = NHAc, n = 2 (87%)
143 R = NHAc, n = 3 (99%)
148 R = NHAc, n = 4 (80%)

b,c



139 R = NHAc, n = 2 (99%)
144 R = NHAc, n = 3 (87%)
149 R = NHAc, n = 4 (83%)

d

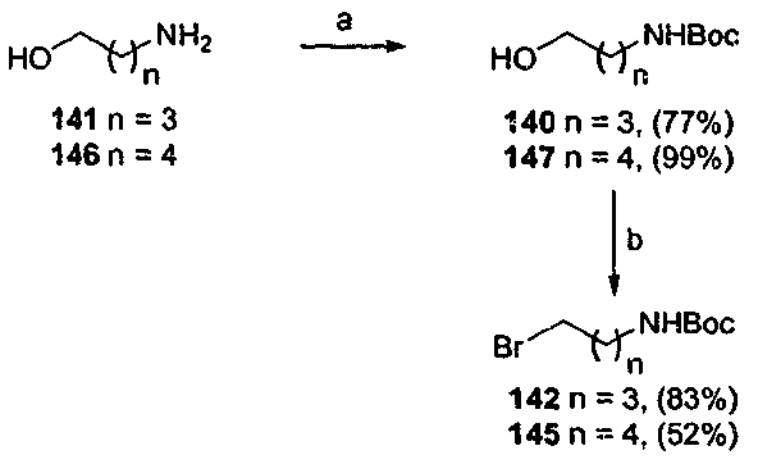


128 R = NHAc, n = 2 (71%)
129 R = NHAc, n = 3 (78%)
130 R = NHAc, n = 4 (85%)

Reagents and conditions: (a) DMF, Et_2NH; (b) NaOMe, MeOH; (c) H_2O, NaOH; (d) CF_3COOH.

Scheme 41. Synthesis of affinity ligands **128, 129** and **130.**[280]

For ligand **129**, bromo butylamine was not commercially available. However, it was possible to synthesize the Boc protected analogue **140** *via* 4-aminobutanol (**141**). The alcohol **140** was then converted to the bromo derivative **142** utilising non-acidic conditions with NBS/triphenylphosphine in dichloromethane[282] with a yield of 83% (Scheme 42).



Reagents and conditions: (a) $(Boc)_2O$, CH_3CN, Et_3N, pH 9; (b) NBS, $(Ph)_3P$, DCM, pyridine.

Scheme 42. Synthesis of Bromo intermediate **142** and **145**.

The synthesis of **129** was similar to that employed for **128,** and involved the coupling of the 2-thioacetyl Neu5Ac derivative **54** to the alkyl halide **142**[176,185-188,279,280] (Scheme 41). The coupling reaction produced **143** with an excellent yield of 99% after purification. Subsequent NaOMe mediated deacylation of the thiosialoside **143** followed by saponification gave **144**. Deprotection of the aglycon alkylamine using neat TFA afforded the desired α-thiosialoside **129** in 78% yield after HPLC purification. Correct analytical data was obtained for **129**. The overall yield for ligand **129** was found to be 43% when starting from **141** (Scheme 41).

The thiosialoside **130** was synthesized using similar conditions to that of **129**. Thus, the protected alkyl halide **145** was prepared utilising the same procedure as for **142** (Scheme

42). Formation of the Boc protected alkylamine **145** was accomplished with an overall yield of 51% (Scheme 42).

The subsequent reaction, which involves the coupling of the 2-thioacetyl Neu5Ac derivative **54** to the alkyl halide **145** to form the protected adduct **148**, had an excellent yield of 80%[185-188] (Scheme 41). Correct analytical analysis was obtained for the protected thiosialoside **148.** Deacylation of the α-thiosialoside **148** followed by saponification furnished **149** in 83% yield. The boc-protected α-thiosialoside **149** was treated with neat TFA to obtain the deprotected product **130**.[280] The crude mixture was successfully purified by HPLC to isolate the pure product **130** in 85% yield. ^{1}H NMR and ^{13}C NMR verified formation of product **130**.

4.8 Inhibition studies of compound 127 with *V.c.* sialidase

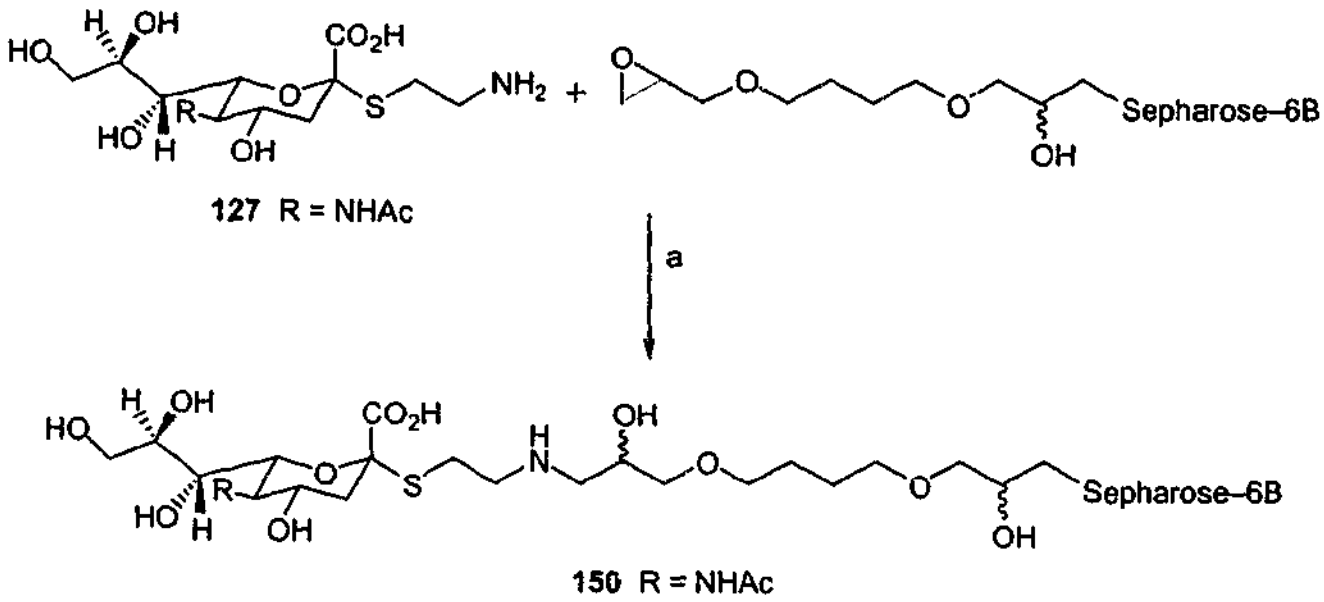
The biological studies were performed in conjunction with Dr Samia Abo, Wendy Stewart, Kaylene Quelch and Annette Alafaci.

Before the ligands were bound to the affinity matrix, compound **127** was analysed for inhibition of, or recognition by *V.c.* sialidase (i.e. determination of inhibition constant, K_i (*vide infra*)). These studies would provide some indication as to the suitability of the ligands for attachment to a chromatographic support and subsequent use as an affinity matrix for purifying SARPs. Thus, if selective recognition of the *V.c.* sialidase to the ligand bound to the affinity matrix occurred, then protein purification could potentially be facilitated.

The K_m of *V.c.* sialidase to 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (MUN) was calculated to be 80 μM from the double reciprocal Lineweaver-Burk plot (see experimental section 5.4.5). The K_i of the Neu5Ac α-thioketoside **127** to *V.c.* sialidase was in the order of 4×10^{-5} M. This good binding affinity suggests that compound **127** is a suitable candidate for an affinity adsorbent to purify *V.c.* sialidase.

4.9 Preparation of affinity matrices with epoxy-activated Sepharose 6B[279]

Initial studies entailed the coupling of Neu5Ac-α-thioketoside **127** to epoxy-activated Sepharose 6B which was achieved under alkaline conditions, using a procedure similar to that recommended by the manufacturer (see Chapter 5, Biochemistry section 5.4.3), to give the affinity matrix **150** (Scheme 43). The amount of **127** bound onto the Sepharose 6B was determined by measuring the absorbance at 220 nm against a standard curve of known amounts of **127** in 0.4 M Na_2CO_3 (pH 13.0), and was estimated to be 15%. The affinity matrix **150** is stable when stored at 4 °C in a buffer containing 0.02 % aq. NaN_3, and can be easily regenerated for repetitive use by three cycles of washing with 0.1 M Tris (pH 8.0) containing 0.5 M NaCl followed by 0.1 M NaOAc (pH 4.0) containing 0.5 M NaCl.



Reagents and conditions: (a) 0.4M Na_2CO_3, pH 13, RT, 16 hs

Scheme 43. Synthesis of affinity matrix **150**.[279]

4.9.1 Purification of *V.c.* sialidase using epoxy-activated Sepharose 6B matrix[279]

Previous reports[253] have indicated that *V.c.* sialidase requires Ca^{2+} for enzyme activity. The bivalent metal cation is possibly involved in the stabilisation of the enzyme-substrate complex. Not surprisingly, binding of *V.c.* sialidase onto affinity matrix **150** was found to be dependent upon the presence of Ca^{2+} in the buffer system. This was demonstrated in the absence of $CaCl_2$ where the sialidase was weakly bound. When subsequently eluted with 1 M NaCl/buffer, the sialidase was contaminated with the bulk of *E. coli* proteins (Figure 22A). Binding of the *V.c.* sialidase to the affinity column **150** was increased by the addition of 6 mM $CaCl_2$ to the equilibrated buffer, with only 7% of the enzyme activity lost in the initial wash step. Sequestering Ca^{2+} from the enzyme-substrate complex *via* addition of 1 mM EDTA to the elution buffer was necessary to elute *V.c.* sialidase from the column. Then, in an effort to reverse the inhibitory effects of the introduced EDTA on enzyme activity, those fractions containing 1 mM EDTA were assayed in the presence of

24 mM $CaCl_2$. The results obtained from affinity absorbent **150**, presented in Figure 22B, show that *V.c.* sialidase is obtained free of bulk protein, with a recovery rate of 60%.

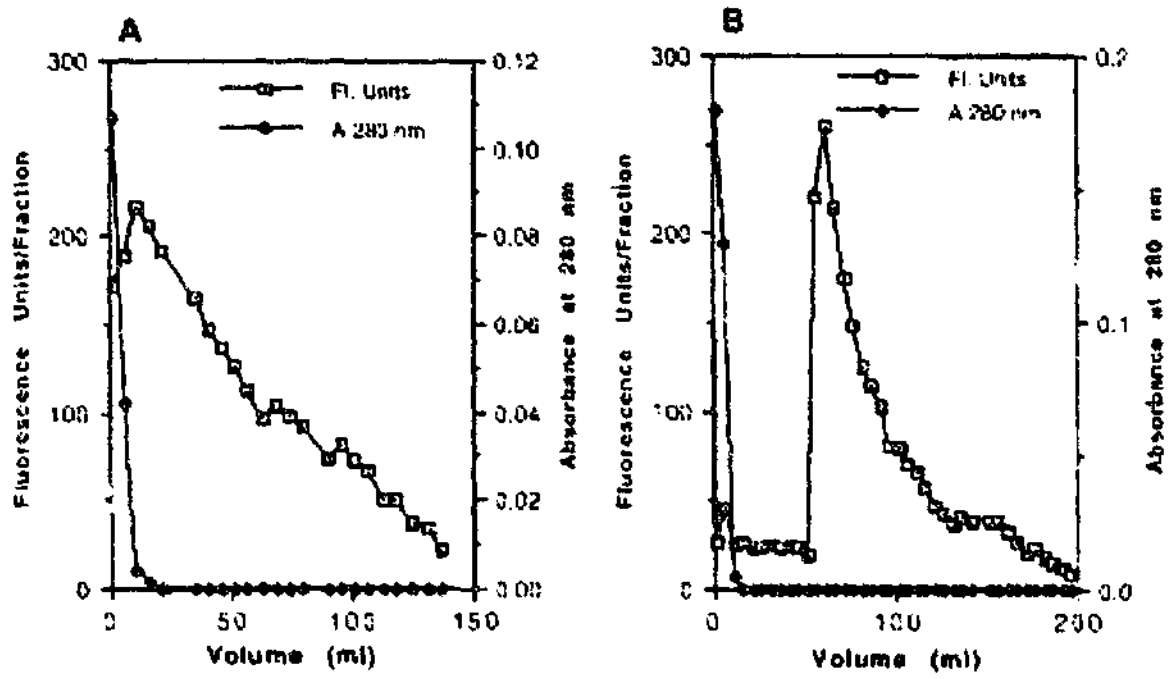


Figure 22. Chromatography of *V.c.* sialidase on affinity column **150** (A) without $CaCl_2$; (B) with 6mM $CaCl_2$ in loading buffer and 1mM EDTA in the elution buffer.

SDS-PAGE visualised with silver stain (Figure 23) showed one single band (illustrated in lane 6) with an apparent molecular weight at approximately 83 kDa which is in agreement with the literature for the weight of *V.c.* sialidase.[253] During this project it was found that the affinity matrix **150** can be used repeatedly without any overall loss of efficiency, suggesting the immobilised ligands are not cleaved by *V.c.* sialidase during the purification process.

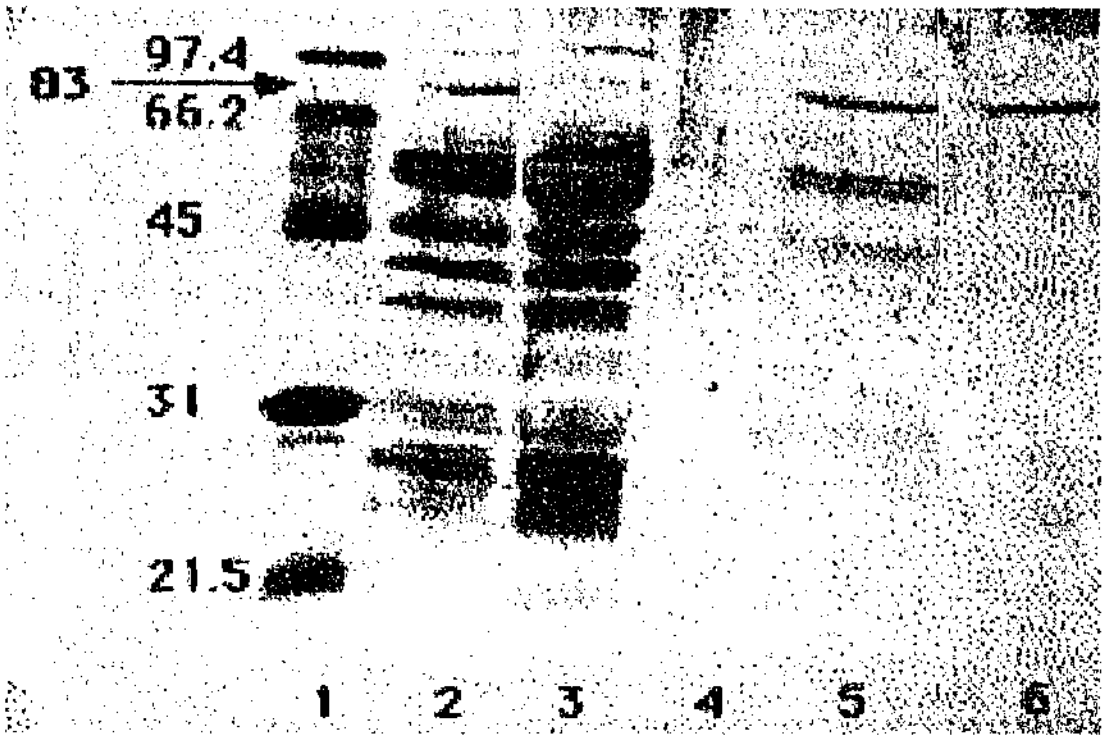
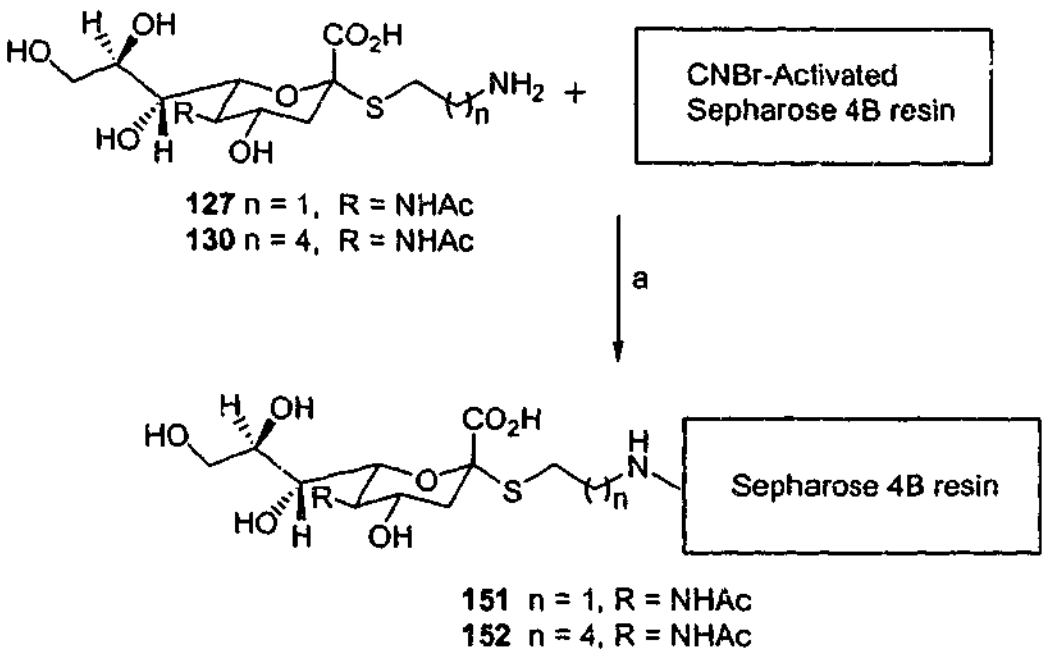


Figure 23. Affinity chromatography results obtained for **150** with 12.5% SDS-PAGE. Lane 1: MW standard (in kDa); lane 2: load on the column; lane 3: flow through; lane 4: wash step; lane 5: fractions 1 and 2 eluted with 1 M NaCl containing 1mM EDTA; lane 6: fractions 3 to 8 eluted with 1 M NaCl containing 1 mM EDTA showing 83 kDa is the MW of purified protein corresponding to *V.c.* sialidase recovered after elution.

Although preliminary results showed efficient purification of overexpressed *V.c.* sialidase in a one-step purification process with the Sepharose 6B affinity columns (*vide infra*), the overall yield of the coupling of the ligand to the resin (15%) was not particularly satisfactory. This led to the investigation of another adsorbent support, namely CNBr–activated Sepharose 4B. The original concept of using ligands with different span lengths to determine the efficacy of the matrix in the purification of a sialidase was again applied.

4.10 Preparation of affinity matrices with CNBr-activated Sepharose 4B[280]

The coupling of the thiosialosides **127** or **130** to CNBr-activated Sepharose 4B (see Experimental 5.4.6) was achieved efficiently with yields of over 75% (based on recovered starting material) (Scheme 44).



Reagents and conditions: (a) 0.4M $NaHCO_3$, pH 9, RT, 2-16 hs

Scheme 44. Synthesis of affinity columns **151** and **152**.[280]

The amount of **127** or **130** bound onto the Sepharose was determined by measuring the absorbance at 220 nm against a standard curve of known amounts of **127** or **130** in 0.1 M $NaHCO_3$ (pH 9), and was estimated to be over 75%. The affinity matrices **151** and **152** were observed to be stable when stored at 4 °C in a convenient buffer containing 0.02% aq. NaN_3. Both matrices were successfully reused several times without any significant changes in the efficiency of the purification process or the quality of the purified proteins. For an efficient reuse of the column, it is recommended, however, to regenerate the matrix

by three cycles of washing with 0.1 M Tris (pH 8.0) containing 0.5 M NaCl followed by 0.1 M NaOAc (pH 4.0) containing 0.5 M NaCl.

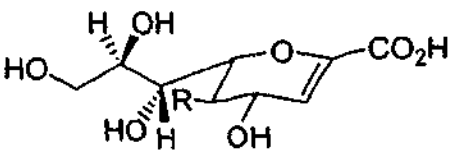
4.10.1 Purification of sialidases with CNBr Sepharose 4B matrices[280]

Initially, the efficiency of the affinity matrix **151** made by the coupling of **127** to CNBr-activated Sepharose 4B was compared to the efficiency of affinity matrix **150**, prepared by the coupling of **127** to epoxy-activated Sepharose 6B.[279] *V.c.* sialidase was used as a benchmark during this comparative study. Following affinity chromatography, it was found **151** not only had a higher capacity (more than 20-fold) to bind to *V.c.* sialidase than **150**, but also demonstrated higher efficiency in purifying *V.c.* sialidase in one step. Surprisingly, and contrary to our earlier observations (*vide infra*),[279] no Ca^{2+} was needed for the binding of the enzyme to matrix **151**. Similar results were obtained when matrix **152** was employed. The reasons behind the Ca^{2+} dependency by some resins, remains unknown. However, it is noteworthy that the epoxy-activated Sepharose 6B affinity matrix **150** has a span arm of 12 atoms, while the CNBr-activated Sepharose 4B affinity matrices **151** and **152** have no spacer arms. This may provide a possible explanation for this altered Ca^{2+} requirement, although it is equally likely that the higher number of available sites on the affinity adsorbents **151** and **152** (coupling efficiency > 75%, compared to epoxy-activated Sepharose 6B ~ 15%) and presumably improved overall binding capacity has also played a part in the Ca^{2+} dependency.

4.10.1.1 Purification of *V.c.* Sialidase with CNBr Sepharose 4B matrices[280]

V.c. sialidase was readily purified by both **151** and **152**, free of other contaminant proteins, with a recovery rate of 65%. Only 4% of the activity was lost in the wash step (Table 8, data for matrix **151** only shown). SDS-PAGE visualised with silver stain showed one

single band with an apparent molecular weight of approximately 83 kDa as previously reported (*vide supra*).[253,280] In an attempt to investigate the nature of the binding of *V. c.* sialidase to the affinity adsorbent, the known competitive sialidase inhibitor, Neu5Ac2en (**5**) was added at micromolar concentrations to the elution buffer. Under these conditions Neu5Ac2en (**5**) was able to elute the *V.c.* sialidase, indicating that the nature of the binding to the matrix was certainly of an affinity type. These results demonstrate that both affinity matrices **151** and **152** are highly efficient in purifying *V.c.* sialidase. The main difference between ligands **127** and **130** is that **130** has 3 extra carbons on the aglycon side chain, making the span length in matrix **152** slightly longer than in matrix **151**. This small difference in span length, on this occasion, does not appear to influence the quality or the yield of the purified sialidase enzyme.



5 R = NHAc
Neu5Ac2en

4.10.1.2 Purification of Rat Liver Sialyltransferase with CNBr-activated Sepharose 4B matrices[280]

Given these promising results with *V.c.* sialidase, the CNBr-activated matrices **151** and **152** were then used to purify other SARPs. Sialyltransferase from rat liver-derived Golgi-enriched fractions, which were bound to the affinity matrix **151** showed a recovery rate of over 47% of enzyme activity originally loaded onto the column being collected in the eluted fractions (Table 8). Elution of this activity was achieved at a NaCl concentration of ~ 0.6 M and was found to contain an enriched highly pure protein band by SDS-PAGE. The apparent molecular weight of this band was around 44-46 kDa which is consistent

with the size of an α(2,3)-sialyltransferase purified from rat liver.[283] Only 5% of enzyme activity was found in the preliminary washings.

Weinstein *et al*[283] describes a four to five step partial purification of sialyltransferases. Their results show isolation of α(2,6)-sialyltransferase and α(2,3)-sialyltransferase in 16 and 7 % yield respectively.[283] This illustrates that using affinity chromatography for the purification of sialyltransferase from rat liver as described above (*vide supra*) can isolate the enzyme with a higher recovery compared to other methods.

4.10.1.3 Purification of sialidase-L from Leech with CNBr-activated Sepharose 4B matrices[280]

Recombinant sialidase-L from Leech provided another example in demonstrating the efficiency of the affinity adsorbents for general SARP purification. Approximately 50% of the loaded enzyme activity was located in the eluted fractions for the affinity matrix **151** (Table 8). Another method for purification of sialidase-L from leech has been described by Chou *et al*[284] which had a recovery activity of 15 % for the enzyme. This indicates that the affinity chromatography method is far better than that described by Chou *et al.*[284] A single band of molecular weight 84 kDa (which is consistent for a sialidase-L enzyme from leech[284]) along with the BSA band at 67 kDa, added as a stabilizing agent for the enzyme, was detected by SDS-PAGE.

In this particular experiment, 42% of the enzyme activity was recovered in the initial wash fractions and from other experiments. It is possible that this was simply a direct result of column overloading. Further work is underway in optimizing the conditions for the purification of this enzyme. It should be noted that similar results were obtained for affinity column **152** (data not shown).

4.10.1.4 Purification of *trans*-sialidase from *T. cruzi* with CNBr-activated Sepharose 4B matrices[280]

In the case of *trans*-sialidase approximately 5% of the enzyme activity was detected in the initial washings for the affinity column **151**. The recovered activity in the eluted fractions reached 18% of the loaded enzyme activity (Table 8). This low recovery tends to suggest that a significant amount of enzyme activity has been lost during the purification procedure and is presumably due, in part, to the instability of the *trans*-sialidase. Adding BSA to the buffer was necessary to stabilise the enzyme activity during storage. As noted for sialidase-L, a similar outcome was observed when the affinity matrix **152** was employed (data not shown). Investigation into the effect of metal ions on *trans*-sialidase activity and stabilisation is underway. Preliminary findings suggest that metal ions such as Mn^{2+} may have a positive effect on the enzyme activity (S.Abo and M. von Itzstein, manuscript in preparation).

Table 8. Activity (%) recovered in the fractions after purification by affinity column **151**.

	% Activity	
	Eluted fractions	Wash fractions
V.c. sialidase	65	4
Sialyltransferase	47	< 5
Sialidase-L	49.6	42
Trans-sialidase	18	5

4.11 Conclusion

The synthesis of novel thiosialosides which contain a terminal amine group on the aglycon moiety have been developed. These novel compounds were successfully coupled through the amine group to different resins producing a variety of affinity matrices. These affinity media proved to be effective in purifying a number of sialic acid-recognizing proteins, including sialidases, in one step and in relatively high yield of purified protein. These affinity adsorbents are currently being used to purify other SARPs.

The ligand **127** has been successfully linked, through the amino group, to CNBr-activated Sepharose 4B with high coupling yield. This affinity matrix **151** purified *V.c.* sialidase in a comparable manner to the epoxy-activated Sepharose 6B matrix **150**.[279] It also proved to be effective in purifying the *trans*-sialidase from *T. cruzi*, sialyltransferase from rat liver and the recombinant Leech-sialidase, sialidase-L, all in relatively high yield.

In the case for *trans*-sialidase from *T. cruzi*, it was possible to utilize the affinity purified sialidase to perform ^{1}H NMR investigations.[262] This clearly demonstrates the benefit of using affinity matrices for the simple and efficient purification of SARPs. Such purification methods may be broadly applicable to other SARPs, thus facilitating not only NMR studies but other investigative techniques as well.

Recombinant rat sialidase and normal sialidase from Leech showed good binding affinity to matrix **152**. Nevertheless, binding and elution conditions need to be examined more thoroughly. In general, it would be anticipated that metabolically stable alkyl thiosialosides attach to affinity matrices would provide excellent purification media for enzymes such as sialidases. However, because multiple-binding domains are present in both

glycosyltransferases for example a sialic acid-recognising domain, a galactose/*N*-acetylgalactosamine-recognising domain and a nucleotide-recognising domain and in *trans*-sialidases such as sialic acid-recognising domain, a galactose/*N*-acetylgalactosamine-recognising domain, disaccharides such as glycosylsialosides may provide higher affinity when attached to matrices. Therefore, investigations aimed at producing affinity media with more specific ligands suitable for a variety of sialidases, sialyltransferases and *trans*-sialidases are being pursued.

CHAPTER 5

Experimental

5.1 General

Melting points were determined on a Mettler FP21 hot-stage melting point apparatus and are uncorrected. Infrared spectra were recorded on either a Hitachi 270-30 infrared spectrometer or a Bio-Rad Merlin using either sodium bromide or sodium chloride discs. Optical rotations were measured using a Jasco DIP-370 digital polarimeter. A concentration of 1 % refers to 10 mg/mL

^{1}H and ^{13}C (JMOD) NMR spectra were recorded on either a Brüker AM 300WB or 500WB mutinuclear magnetic resonance spectrometers. Chemical shifts (δ) were reported in parts per million (ppm) and are relative to the solvent as an internal reference as shown below:

Solvent	^{1}H Chemical Shift (number of peaks)	^{13}C Chemical Shift (number of peaks)
$CDCl_3$	7.27 (1)	77.23 (3)
D_2O	4.80 (1)	
d_4-MeOH	4.87 (1) 3.31 (5)	49.45 (7)

Where (*) is used the assignment is tentative, while (†) indicates overlapping signals. Multiplicities are denoted as s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), dd (doublet of doublets), ddd (doublet of doublet of doublets). In order to confirm assignments 2-D NMR experiments were performed using ^{1}H - ^{1}H COSY experiment for proton-proton interactions and the ^{1}H -^{13}C HMQC experiment for proton-carbon interactions.

Low resolution mass spectra were obtained using either a Jeol JMS-DX 300 mass spectrometer (LRFABMS; thioglycerol-glycerol matrix) or by electrospray ionisation (ESI) using a Micromass Platform II (LRESIMS). High resolution mass spectra were recorded using either a Jeol JMS-DX 300 mass spectrometer (HRFABMS; polyethylene glycol matrix) or on a Bruker Bio-Apex II spectrometer in ESI mode.

Microanalyses were performed by the Chemical and Microanalysis Services Pty. Ltd., Belmont, Australia or by the microanalysis service department of Chemistry at the University of Queensland.

Column chromatography was performed using E. Merck Silica Gel 60 (0.04-0.063 mm). Thin layer chromatography (tlc) was performed on aluminium plates coated with Silica Gel 60 F_{254} (E. Merck) and compounds visualised using 5 % H_2SO_4 in EtOH followed by heating to 200 °C. High-performance liquid chromatography (HPLC) purification was performed on a Waters 600E system controller, using reverse phase C_{18} Waters µBondapak™ columns (analytical: 8 x 100 mm, part no. WAT085721; preparative: 25 x 100 mm, part no. WAT085721), detecting at 230nm with Waters 484 tunable absorbance detector.

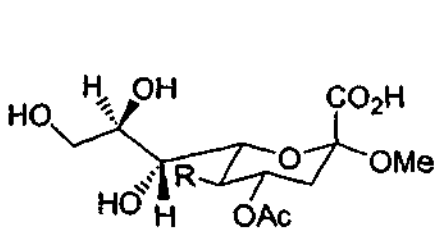
All solvents and reagents were distilled prior to use or were of analytical grade. Dried solvents were distilled under N_2 according to Perrin and Armarego.[285] Dowex-50W x 8 (H^+) was obtained from Aldrich Chemical Company, Inc. All commercially available starting materials were used without further purification. pH 4 buffer was purchased from Activon. Epoxy-activated Sepharose 6B and CNBr-activated Sepharose 4B were purchased from Pharmacia.

Methyl 5-acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-azido-3,4,5-trideoxy-D-*glycero*-D-*galacto*-non-2-enonate (4-azido-4-deoxy-Neu5,7,8,9Ac_4-2en1Me (**34**)) was prepared according to literature method.[157] 4-Methylumbelliferyl 5-acetamido-3,5-dideoxy-D-*glycero*- α-D-*galacto*-2-nonulopyranosidonic acid (MUN) and 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid (Neu5Ac2en (**5**)) were prepared according to literature methods.[286,287]

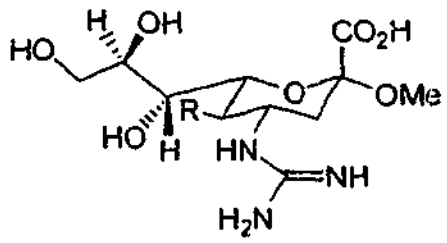
The *V.c.* sialidase clone, kindly provided by E. Vimr (University of Illinois, Urbana, Illinois) was expressed in *hemagglutinin* as described by Vimr *et al*[288] and Taylor *et al.*[258] The recombinant sialidase-L[284] was kindly supplied by Yu-Teh Li (Tulane University, School of Medicine, New Orleans, Louisiana). *Trans*-sialidase from *Trypanosoma cruzi*,[289] was kindly provided by M. E. A. Pereira (New England Medical Center Hospitals, Department of Medicine, Boston, Massachusetts).

5.2 Molecular modelling

Co-ordinates for bromelian-released hemagglutinin (BHA) were obtained from the Brookhaven Protein Data Bank access code 1HGI. This structure involves HA (bromelian digested) (obtained from the recombinant viral strain X-31 that carries the HA from the A/Aichi/2/1968 (H3N2) isolate) complexed with Methyl 5-acetamido-4-*O*-acetyl-3,5-dideoxy-D-*glycero*- α-D-*galacto*-2-nonulopyranosidonic acid (Neu4,5Ac$_2$α2Me (**31**)).[290] This complex was chosen because the Neu4,5Ac$_2$α2Me (**31**) ligand in the binding site, is very similar to the natural substrate Neu5Ac (**2**) and also similar to **32**. Initially, the 4-deoxy-4-guanidino-Neu5Acα2Me (**32**) ligand was modified in the binding site using Insight II with its position and ring conformation the same as observed for the starting ligand **31**. Since attention was focussed on one particular monomer, two out of the three monomers from the hemagglutinin trimer were deleted. Prior to minimisation, all of the carbohydrate molecules involved in glycosylation (except for the inhibitor, 4-deoxy-4-guanidino-Neu5Acα2Me (**32**)) and water molecules were deleted from the crystal structure. The Insight II program was used to add hydrogens to the crystal structure, assuming a pH 7. This resulted in arginines and lysines being positively charged while glutamic acid and aspartic acid residues were negatively charged and histidine were treated as neutral.



31 R = NHAc
Neu4,5Ac$_2$α2Me



32 R = NHAc
4-Deoxy-4-guanidino-Neu5Acα2Me

Calculations were performed on a Silicon Graphics Indigo R4000 XZ workstation with Insight II (version 97.0) and Discover (version 2.96) software. Within Discover, the forcefield incorporated into the minimisation program was the Consistent Valence Forcefield (CVFF) with a cut-off of 15 Å. CVFF was chosen because it is well characterised and is parameterised to reproduce protein properties. An initial energy minimisation was performed to relieve steric strain associated with the newly docked ligand, 4-deoxy-4-guanidino-Neu5Acα2Me (**32**). This minimisation protocol is a step-wise procedure involving the following:

i) fixing all the heavy atoms (*ie* C, N, O and S) to allow the movement of H atoms,

ii) fixing the backbone atoms C, N and O to allow the side chains to move,

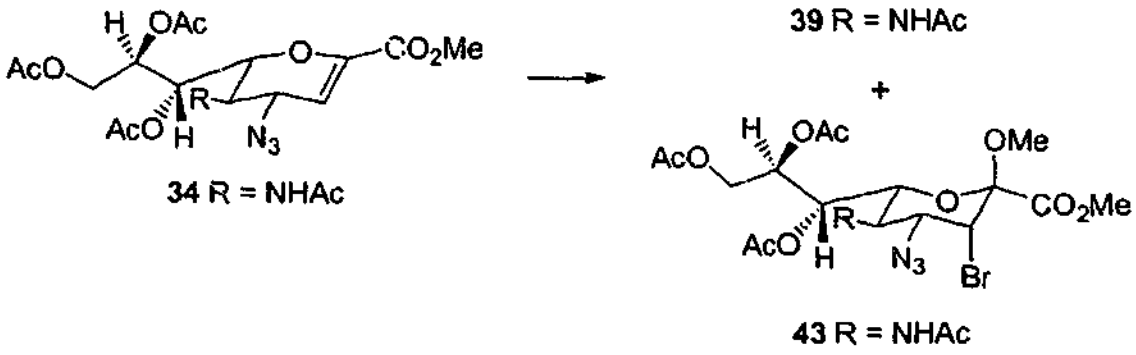
iii) periodically reducing the tether on the backbone which gradually reduces the applied force constant to allow the molecules to relax (see Appendix A).

A sphere of water was added to cap the binding site. Additionally, all residues more than 15 Å radius from the binding site were fixed into position. Calculations were performed using the algorithms steepest descents (in all constrained minimisations for up to 500 iterations) to relieve initial strain and then conjugate gradients were used until a final root mean square deviation (RMSD) of 0.01 was attained. Subsequent molecular dynamics calculations were then performed at 350 K using the leapfrog algorithm in Discover (see Appendix B). Fifteen frames were obtained from the molecular dynamics in which frame 7 contained the best interaction energy of -104 kcal/mol, between the 4-deoxy-4-guanidino-Neu5Acα2Me (**32**) and the receptor binding site (RBS).

5.3 Experimental

5.3.1 Synthesis of Hemagglutinin Compounds

Methyl (Methyl 5-Acetamido-7,8,9-tri-*O*-acetyl-4-azido-3-bromo-3,4,5-trideoxy-α-D-*erythro*-L-*gluco*-2-nonulopyranosid) onate (39)[165]



Compound **34**[157] (1.0 g, 2.2 mmol) and *N*-bromosuccinimide (0.47 g, 2.6 mmol) were dissolved in anhydrous MeOH (50 mL). The solution was then stirred at room temperature under an N_2 atmosphere. After 16 hs, the resulting mixture was concentrated in *vacuo*. The residue was taken up in EtOAc (150 mL) and successively washed with water (5 x 150 mL), saturated NaCl solution (150 mL), then dried (Na_2SO_4) and evaporated to dryness. ^{1}H NMR spectroscopy showed that the ratio of the isomers (**39** and **43**) in the crude mixture was 1:1. Separation of the isomers was achieved by column chromatography on silica gel (EtOAc:hexane, 1:1) and yielding the α isomer **39** as white crystals and the β isomer **43** as plateletes. The overall yield for both isomers was 87%.

Characterisation for α isomer **39**: Mp 189-190 °C; *Rf* 0.6 (EtOAc); $[\alpha]_D^{28}$ -78.4° (*c* = 1 MeOH); ν_{max} (NaCl) 2108 (azide), 1744 (COOMe), 748 (CBr) cm^{-1};

^{1}H NMR ($CDCl_3$): δ 2.03, 2.07 (9H, 2s, 3AcO), 2.11 (3H, s, AcNH), 3.51 (3H, s, OMe), 3.71 (2H, m, $J_{3ax,4}$ 10.8, $J_{5,4}$ 10.6, $J_{5,6}$ 10.8, $J_{5,NH}$ 9.3 Hz, H-3ax, H-5), 3.86 (3H, s, CO_2Me),

4.10 (1H, dd, $J_{9a,9b}$ 12.5, $J_{9a,8}$ 5.5 Hz, H-9a), 4.24 (1H, dd, $J_{9b,9a}$ 12.5, $J_{9b,8}$ 2.5 Hz, H-9b), 4.38 (1H, t, $J_{4,5}$ 10.6, $J_{4,3ax}$ 10.8 Hz, H-4), 4.79 (1H, dd, $J_{6,5}$ 10.8, $J_{6,7}$ 1.8 Hz, H-6), 5.26 (1H, dd, $J_{7,8}$ 8.8, $J_{7,6}$ 1.8 Hz, H-7), 5.35 (1H, ddd, $J_{8,7}$ 8.8, $J_{8,9a}$ 5.5, $J_{8,9b}$ 2.5 Hz, H-8), 5.62 (1H, d, $J_{NH,5}$ 9.3 Hz, NH);

^{1}H assignments were confirmed using ^{1}H -^{1}H COSY NMR spectroscopy.

^{13}C NMR: d 20.8 (3 OC(O)*Me*), 23.2 (NC(O)*Me*), 51.1 (C-5), 52.0 (C-3), 52.3 (CO_2*Me*), 52.6 (O*Me*), 62.3 (C-9), 65.3 (C-4), 67.3 (C-7), 68.6 (C-8), 72.0 (C-6), 98.8 (C-2), 168.0 (C-1), 169.5 (N*C*(O)Me), 170.3,170.5 (3 O*C*(O)Me);

LRFABMS: *m/z* 567 [^{79}Br $(M + 1)^+$] (33), 569 [^{81}Br $(M + 1)^+$] (35), 537(100), 509 (68), 463 (60), 414 (79); found: 567.09438 [$M + 1)^+$], $C_{19}H_{28}N_4O_{11}{}^{79}Br$ requires 567.09381.

Methyl (Methyl 5-Acetamido-7,8,9-tri-*O*-acetyl-4-amino-3,4,5-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid) onate hydrochloride

(47)[165]

A solution of **39** (170 mg, 0.3 mmol) in anhydrous dioxane (7.5 mL) was treated with tri-*n*-butyltin hydride (0.34 mL, 1.2 mmol) in the presence of a catalytic amount of AIBN under a nitrogen atmosphere. After stirring at 80°C for 12 h the reaction mixture was cooled to room temperature and concentrated under *vacuo*. The residue was partitioned between acetonitrile and hexane. The acetonitrile extract was washed with several portions of hexane and concentrated under reduced pressure. Column chromatography (EtOAc) on silica gel afforded the title compound as a yellow oil, which was taken up in water and

neutralised with 0.1 M HCl. All insoluble by-products were removed by centrifugation. The supernatant was lyophilised to afford **47** (118 mg, 79 %).

Mp 180-182°C (dec); R_f 0.53 (EtOAc:MeOH:H_2O; 7:2:1); $[\alpha]_D^{25}$ +3.6° (*c* 1, H_2O); ν_{max} (KBr) 3448 (amine), 1746 (COOMe) cm^{-1};

^1H NMR (D_2O): δ 1.64 (1H, t, $J_{3ax,3eq}$ 12.8, $J_{3ax,4}$ 2.8 Hz, H-3ax), 1.88 (3H, s, AcNH), 1.99, 2.06, 2.11 (9H, 3s, 3 AcO), 2.52 (1H, dd, $J_{3eq,3ax}$ 12.8, $J_{3eq,4}$ 4.1 Hz, H-3eq), 2.82 (1H, m, H-4), 3.24 (3H, s, OMe), 3.61 (1H, t, $J_{5,6}$ 10.4, $J_{5,4}$ 10.4 Hz, H-5), 3.77 (3H, s, CO_2Me), 4.11 (2H, m, H-9a, 6), 4.25 (1H, dd, $J_{9b,9a}$ 12.6, $J_{9b,8}$ 2.6 Hz, H-9b), 5.26 (1H, dd, $J_{7,8}$ 8.7, $J_{7,6}$ 1.8 Hz, H-7), 5.32 (1H, m, H-8);

^{13}C NMR (D_2O): δ 22.8, 23.2, 24.7 (3 OC(O)*Me*), 25.9 (NC(O)*Me*), 39.7 (C-3), 51.5 (C-4), 51.7 (C-5), 54.8 (O*Me*), 56.1 (CO_2*Me*), 64.6 (C-9), 70.0 (C-7), 71.0 (C-8), 74.9 (C-6), 101.6 (C-2), 71.2 (C-1), 175.1, 175.5, 176.4, 177.3 (3 O*C*(O)Me, N*C*(O)Me);

LRFABMS: *m/z* 463 [(M + 1)$^+$,100%], 414 (98).

Methyl 5-Acetamido-4-amino-3,4,5-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (48)[165]

47 R = NHAc **48** R = NHAc

Compound **47** (77 mg, 0.17 mmol) was treated with NaOMe (0.083 mmol) in anhydrous MeOH (4 mL) at room temperature under a nitrogen atmosphere. After stirring for 2 h, MeOH was removed under reduced pressure. Water (4 mL) was added to the residue and dilute NaOH solution was used to adjust the pH to 12. The reaction mixture was then stirred overnight at room temperature. The pH of the reaction mixture was adjusted to 7.0-7.5 with Dowex-50Wx8 (H^+) resin. After filtration, the filtrate was lyophilised to afford

the target compound **48** in a pure state according to NMR spectroscopy (49 mg, 91%); *Rf* 0.22 (EtOAc:*i*-PrOH:H_2O; 5:3:1); $[\alpha]_D^{25}$ -3.3° (*c* 1, H_2O);

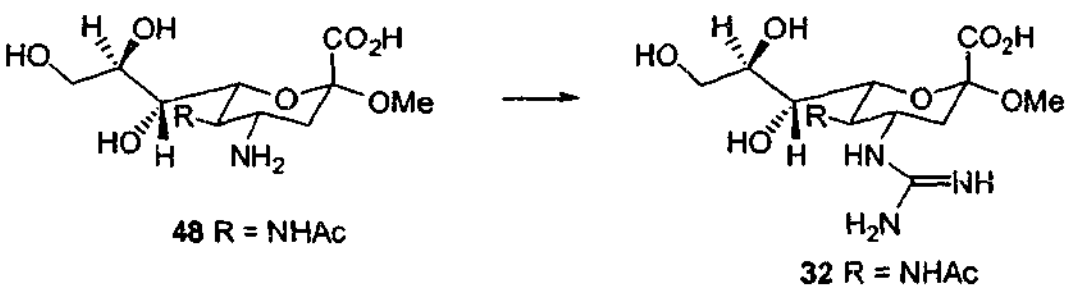
ν_{max} (KBr) 3456 (amine), 1618 (COO^-) cm^{-1};

^1H NMR (D_2O): δ 1.66 (1H, t, $J_{3ax,3eq}$ 12.7, $J_{3ax,4}$ 12.5 Hz, H-3ax), 1.9 (3H, s, AcNH), 2.68 (1H, dd, $J_{3eq,3ax}$ 12.7, $J_{3eq,4}$ 4.3 Hz, H-3eq), 3.07 (1H, ddd, $J_{4,3eq}$ 4.3, $J_{4,3ax}$ 12.5 Hz, H-4), 3.34 (3H, s, OMe), 3.63 (2H, m, $J_{9a,9b}$ 11.5, $J_{9a,8}$ 6.4 Hz, H-9a, 9b*), 3.85 (4H, m, H-5, 6*, 7, 8);

^{13}C NMR (D_2O): δ 24.8 (NC(O)*Me*), 40.5 (C-3), 52.5 (C-4), 52.8 (C-5), 54.3 (O*Me*), 65.3 (C-9), 70.9 (C-7*), 74.3 (C-8*), 75.8 (C-6*), 103.2 (C-2), 175.8 (C-1), 177.8 (N*C*(O)Me);

LRFABMS: *m/z* 323 [(M + 1)$^+$, 17%], 279 (64); HRFABMS: $C_{12}H_{23}N_2O_8$ requires 323.14545, found: 323.14732.

Methyl 5-Acetamido-3,4,5-trideoxy-4-guanidinyl-D-*glycero*-α-D-*galacto*-2-nonulo-pyranosidonic acid (32)[165]



To a solution of **48** (40 mg, 0.124 mmol) in water (7 mL) was added, imidazole (25 mg, 0.372 mmol) and 1*H*-pyrazole-carboxamidine hydrochloride (PCH) (36 mg, 0.248 mmol). After stirring for 43 h at 60°C, the reaction mixture was lyopholized. Flash chromatography on silica gel (EtOAc:*i*-PrOH:H_2O; 7:2:1) afforded the target compound **32** (25mg, 18%) as a white amorphous mass.

Mp 233-236 °C (dec); R_f 0.17 (EtOAc:*i*-PrOH:H_2O; 2:3:1); $[\alpha]_D^{28}$ -39.4° (*c* 1, MeOH);

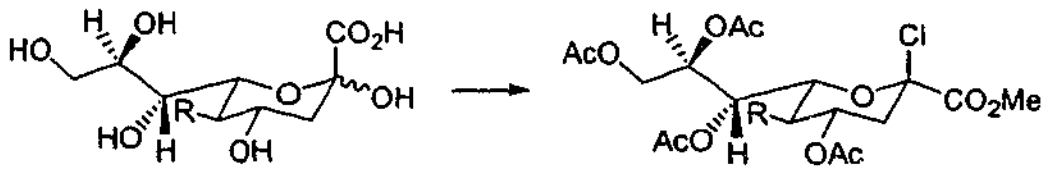
ν_{max} (NaCl) 3352 (amine), 1642 (COO^-) cm^{-1};

^1H NMR (CD_3OD): δ 1.61 (1H, t, $J_{3ax,3eq}$ 12.4, $J_{3ax,4}$ 11.9 Hz, H-3ax), 1.83 (3H, s, AcNH), 2.54 (1H, dd, $J_{3eq,3ax}$ 12.4, $J_{3eq,4}$ 2.8 Hz, H-3eq), 3.23 (3H, s, OMe), 3.54 (2H, m, H-6, 9a), 3.71 (3H, m, H-7, 8, 9b), 3.86 (2H, m, H-4, 5);

^{13}C NMR (CD_3OD): δ 22.9 (NC(O)*Me*), 39.4 (C-3), 52.1 (C-4/C-5), 52.4 (O*Me*), 53.0 (C-4/C-5), 64.7 (C-9), 70.1 (C-6), 73.8 (C-7/C-8), 74.5 (C-7/C-8), 101.8 (C-2), 158.7 (C-guan), 174.0 (C-1), 174.7 (N*C*(O)Me);

LRFABMS: *m/z* 365 [$(M + 1)^+$, 23%], 307 (43); HRFABMS: $C_{13}H_{25}N_4O_8$ requires 365.16724, found 365.16577.

Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosyl)onate chloride (56) [153]



2 R = NHAc **56** R = NHAc

To a suspension of Neu5Ac (**2**) (5.00 g 0.016 mol) in dry MeOH (450 mL) was added Dowex-50Wx8 (H^+) resin (125 mg) under N_2. The mixture was allowed to stir at room temperature for 48 h after which, the reaction was observed to be complete according to tlc (EtOAc:*i*-PrOH:H_2O 3:2:1, R_f 0.30). The reaction mixture was filtered and the resin washed thoroughly with MeOH (2x 70 mL). The filtrate was concentrated *in vacuo* and the residue dried under high vacuum to afford the methyl ester as a white solid (5.23 g, 100%). Acetyl chloride (200 mL, 2.8 mol) was then added and the reaction solution was stoppered securely and left to stir for 60 h at room temperature (CAUTION: HCl gas). Excess acetyl chloride was removed *in vacuo* the residual solvent was removed by azeotroping with toluene (3x 40 mL). The reaction mixture was dried under high vacuum

to isolate crude **56** as cream coloured solid (8.24 g, 100%) which was pure according to ^{1}H NMR spectroscopy and was used in subsequent reactions without further purification. [178,179]

^{1}H NMR ($CDCl_3$): δ 1.90 (3H, s, AcNH), 2.04, 2.05, 2.07, 2.11 (12H, 4s, 4AcO), 2.27 (1H, dd, $J_{3ax,3eq}$ 13.8, $J_{3ax,4}$ 11.1 Hz, H-3ax), 2.78 (1H, dd, $J_{3eq,3ax}$ 13.8, $J_{3eq,4}$ 4.8 Hz, H-3eq), 3.86 (3H, s, CO_2Me), 4.10 (1H, dd, $J_{9a,8}$ 6.0, $J_{9a,9b}$ 12.5, Hz, H-9a), 4.22 (1H, m, H-5), 4.34 (1H, dd, $J_{6,5}$ 10.8, $J_{6,7}$ 2.4 Hz, H-6), 4.45 (1H, dd, $J_{9b,8}$ 2.5, $J_{9b,9a}$ 12.5, Hz, H-9b), 5.14 (1H, ddd, $J_{8,7} = J_{8,9a}$ 6.0, $J_{8,9b}$ 2.5 Hz, H-8), 5.38 (1H, ddd, $J_{4,5} = J_{4,3ax}$ 11.1, $J_{4,3eq}$ 4.8 Hz, H-4), 5.46 (1H, dd, $J_{7,8}$ 6.0, $J_{7,6}$ 2.4 Hz, H-7), 5.62 (1H, d, $J_{NH,5}$ 10.0 Hz, NH);

Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (54) [167]

56 R = NHAc **54** R = NHAc

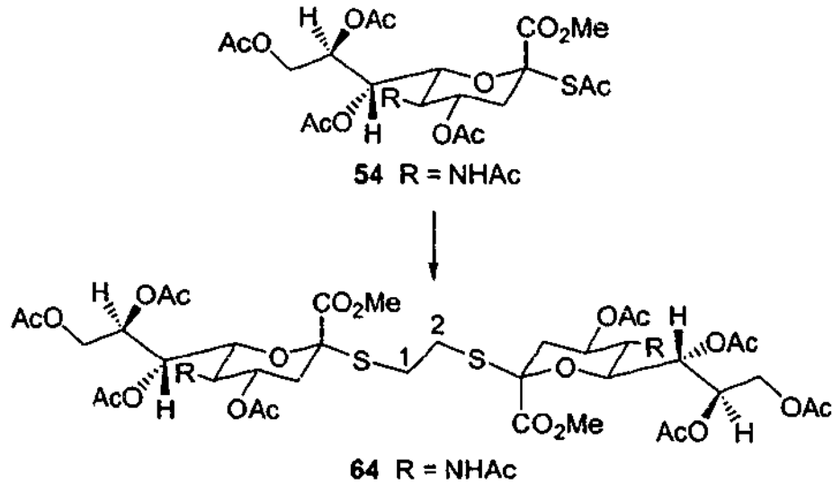
Potassium thioacetate (KSAc) (3.5 g, 0.013mol) was added to a solution of **56** in dry CH_2Cl_2 (40 mL) at 0°C under N_2. After stirring for 24 h at rt, the reaction mixture was concentrated in *vacuo* and the residue taken up in $CHCl_3$ (50 mL) washed with H_2O (2x 50 mL), dried ($MgSO_4$), filtered and concentrated. Column chromatography (EtOAc) followed by recrystallization (EtOAc/hexane) afforded compound **54** (2.9 g, 78%). ^{1}H NMR results of **54** were identical to those reported by Hasegawa *et al.* [167]

^{1}H NMR ($CDCl_3$): δ 1.83 (3H, s, AcNH), 2.04 (13H, m, 4AcO/H-3ax), 2.23 (3H, s, SAc), 2.59 (1H, dd, $J_{3eq,3ax}$ 12.9, $J_{3eq,4}$ 4.5 Hz, H-3eq), 3.75 (3H, s, CO_2Me), 4.03 (1H, dd, $J_{9a,9b}$ 12.3, $J_{9a,8}$ 6.0 Hz, H-9a), 4.12 (1H, m, H-5), 4.40 (1H, dd, $J_{9b,9a}$ 12.4, $J_{9b,8}$ 2.4 Hz, H-9b), 4.66 (1H, dd, $J_{6,5}$ 10.8, $J_{6,7}$ 2.3 Hz, H-6), 4.91 (1H, ddd, $J_{4,5} = J_{4,3ax}$ 11.1, $J_{4,3eq}$ 4.5 Hz, H-4),

5.22 (1H, ddd, $J_{8,7} = J_{8,9a}$ 6.2, $J_{8,9b}$ 2.5 Hz, H-8), 5.39 (1H, d, $J_{NH,5}$ 10.2 Hz, NH), 5.36 (1H, dd, $J_{7,8}$ 6.3, $J_{7,6}$ 2.3 Hz, H-7).

$C_{22}H_{31}NO_{13}S$ (549.55)

Methyl 2-*S*-ethyl (methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate) 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (64)



Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (**54**) (0.2 g, 0.36 mmol) and 1,2-dibromomethane (0.068 g, 0.36 mmol) were dissolved in dry *N,N*-DMF (5 mL) at ambient temperature under N_2. Dry Et_2NH (1.6 mL) was added to the reaction and after stirring for 8 h at room temperature, a further amount of **54** (0.2 g, 0.36 mmol) was added. After stirring for a further 16 h, the reaction mixture was concentrated under reduced pressure and the residue diluted with EtOAc (20 mL) and washed with pH 4 buffer (30 mL), H_2O (2 × 30 mL), dried (Na_2SO_4) and evaporated to dryness under reduced pressure. Column chromatography on silica gel (EtOAc:*i*-PrOH; 9:1) gave **64** (0.17 g, 45%) as a yellow coloured amorphous mass.

Mp 97-101°C; R_f 0.33 (EtOAc:*i*-PrOH; 9:1).

^{1}H NMR (500 MHz; $CDCl_3$): **Alkyl unit:** δ 2.75-2.79 (2H, m, H-1a, 2a), 2.89-2.93 (2H, m,

H-1b, 2a); **Neu5Ac unit**: δ 1.88 (6H, s, 2 × AcNH), 2.01 (2H, t, $J_{3ax,3eq}$ 12.8, $J_{3ax,4}$ 11.5 Hz, H-3ax), 2.04 (6H, s, 2 × AcO), 2.07 (6H, s, 2 × AcO), 2.15 (6H, s, 2 × AcO), 2.17 (6H, s, 2 × AcO), 2.71(2H, dd, $J_{3eq,3ax}$12.8, $J_{3eq,4}$ 4.6 Hz, H-3eq), 3.80 (8H, m, CO_2Me, H-6), 4.03 (2H, q, $J_{5,4}$ =$J_{5,6}$ =$J_{5,NH}$ =10.5 Hz, H-5), 4.12 (2H, d, $J_{9a,9b}$ 12.5 Hz, H-9a), 4.25 (2H, d, $J_{9b,9a}$ 12.5 Hz, H-9b), 4.85 (2H, ddd, $J_{4,3ax}$ 11.5, $J_{4,5}$ 10.6, $J_{4,3eq}$ 4.6 Hz, H-4), 5.17 (2H, d, , $J_{NH,5}$ 10.2 Hz, NH), 5.18-5.32 (4H, m, H-7, 8).

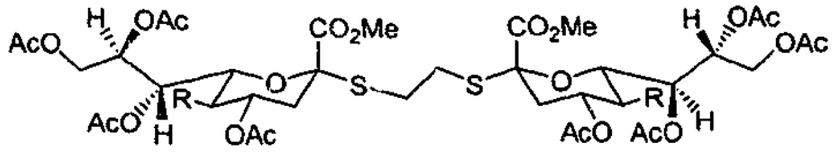
^{1}H assignments were confirmed using ^{1}H -^{1}H COSY NMR spectroscopy.

^{13}C NMR (75.5 MHz; $CDCl_3$): **Alkyl unit:** δ 29.2 (C-1, C-2); **Neu5Ac unit**: δ 20.97, 21.00, 21.02, 21.3 (8 × OC(O)*Me*), 23.4 (2 × NC(O)*Me*), 38.2 (2 × C-3), 49.6 (2 × C-5), 53.3 (2 × CO_2*Me*), 62.4 (2 × C-9), 67.6 (2 × C-7), 69.1 (2 × C-8), 69.7 (2 × C-4), 74.6 (2 × C-6), 83.4 (2 × C-2), 168.8 (2 × C-1), 170.3, 170.4, 170.8, 170.9, 171.1 (4 × O*C*(O)Me, N*C*(O)Me);

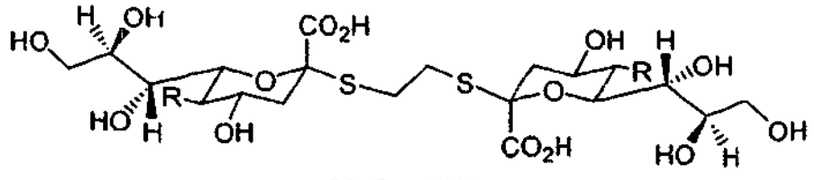
LRFABMS: *m/z* 1041 [$(M + H_2O)^+$, 7%], 966(26), 474(100).

$C_{42}H_{60}N_2O_{24}S_2$ (1040.2977)

Methyl 2-*S*-ethyl (5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic) 5-acetamido-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (52)



64 R = NHAc



52 R = NHAc

Compound **64** (0.112 g, 0.108 mmol) was treated with a solution of NaOMe (0.054 mmol) in anhydrous MeOH (5 mL) at room temperature under N_2. After stirring for 2 h, the MeOH was removed under reduced pressure, H_2O (5 mL) was added and the pH adjusted to 12 with 0.1 M NaOH. After stirring overnight, the pH of the reaction mixture was adjusted to 7.0-7.5 with Dowex-50W×8 (H^+) resin. After filtration, the filtrate was lyophilised to afford the target compound **52** as an amorphous cream coloured solid which was in a pure state according to ^{1}H NMR spectroscopy (72 mg, quantitative).

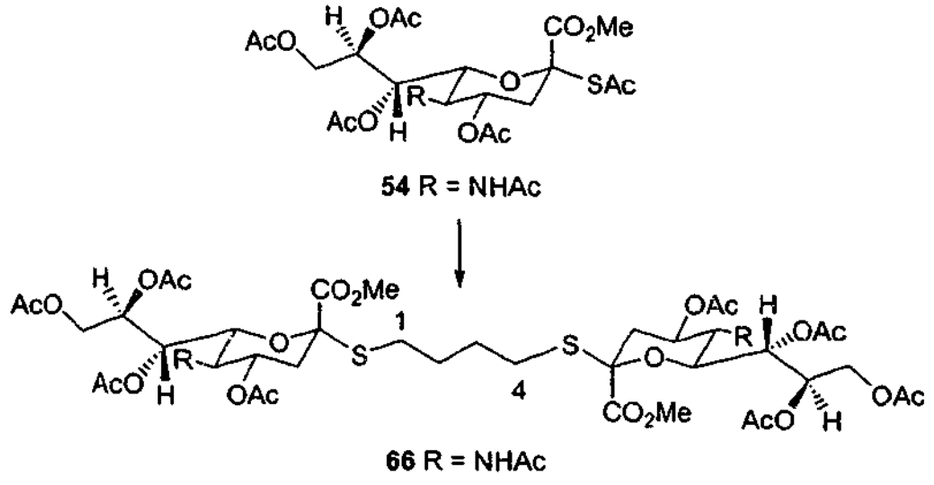
Mp 210 °C (dec); R_f 0.32 (EtOAc:*i*-PrOH:H_2O; 3:2:1).

^{1}H NMR (300 MHz; D_2O): **Alkyl unit:** δ 2.94-3.12 (4 H, m, H-1, 2); **Neu5Ac unit:**δ 1.82 (2 H, t, $J_{3ax,3eq} = J_{3ax,4}$ = 12.0 Hz, H-3ax), 2.10 (6 H, s, AcN), 2.86 (2 H, dd, $J_{3ax,3eq}$ 12.0, $J_{3eq,4}$ 4.7 Hz, H-3eq), 3.47-3.64 (8 H, m, H-4, 6, 7*, 9a), 3.70-3.83 (6 H, m, H-5, 8*, 9b).

^{13}C NMR (75.5 MHz; D_2O): **Alkyl unit:** δ 31.4 (C-1, C-2); **Neu5Ac unit:**δ 23.1 (2 × NC(O)*Me*), 42.2 (2 × C-3), 52.9 (2 × C-5), 63.7 (2 × C-9), 69.4, 69.7 (2 × C-4, C-7*), 73.1 (2 × C-6*), 76.0 (2 × C-8), 87.3 (2 × C-2), 175.1 (2 × C-1), 176.0 (2 × NH*C*(O)Me).

$C_{24}H_{40}N_2O_{16}S_2$ (676.7266)

Methyl 2-*S*-butyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate) 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (66)



Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (**54**) (0.2 g, 0.36 mmol) and 1,4-dibromobutane (0.08 g, 0.36 mmol) were dissolved in dry *N,N*-DMF (6 mL) at room temperature under N_2. Dry Et_2NH (1.8 mL) was added to the reaction and after stirring for 3 h at ambient temperature, a further amount of **54** (0.2 g, 0.36 mmol) was added. With subsequent stirring for 3 h, the reaction mixture was concentrated under reduced pressure and the residue diluted with EtOAc (20 mL) and washed with 0.1 N HCl (15 mL), H_2O (2 × 15 mL), dried (Na_2SO_4) and evaporated to dryness under reduced pressure. Column chromatography on silica gel (EtOAc:hexane; 9:1) gave **66** (0.15 g, 39%) as a yellow coloured amorphous mass.

Mp 106-108 °C; R_f 0.52 (EtOAc:*i*-PrOH; 9:1)

^{1}H NMR (500 MHz; $CDCl_3$): **Alkyl unit:** δ 1.52-1.59 (4H, m, J_{gem} 7.9, H-2, 3), 2.52-2.56 (4H, m, J_{gem} 12.6, H-1, 4); **Neu5Ac unit**: δ 1.88 (6H, s, 2 × AcNH), 1.97 (2H, t, $J_{3ax,3eq}$ = $J_{3ax,4}$ = 12.3 Hz, H-3ax), 2.03 (6H, s, 2 × AcO), 2.05 (6H, s, 2 × AcO), 2.14 (6H, s, 2

× AcO), 2.16 (6H, s, 2 × AcO), 2.70(2H, dd, $J_{3eq,3ax}$12.3, $J_{3eq,4}$ 4.6 Hz, H-3eq), 3.78-3.80 (8H, m, CO_2Me, H-6), 4.05 (2H, q, $J_{5,4}$ =$J_{5,6}$ =$J_{5,NH}$ =10.5 Hz, H-5), 4.09 (2H, d, $J_{9a,9b}$ 12.6 $J_{9a,8}$ 4.2Hz, H-9a), 4.26 (2H, d, $J_{9b,9a}$ 12.6, $J_{9b,8}$ 1.6 Hz, H-9b), 4.84 (2H, ddd, $J_{4,3ax}$ 12.3, $J_{4,5}$ 10.5, $J_{4,3eq}$ 4.6 Hz, H-4), 5.14 (2H, d, $J_{NH,5}$ 10.2 Hz, NH), 5.31-5.35 (4H, m, H-7, 8).

^{1}H assignments were confirmed using ^{1}H -^{1}H COSY NMR spectroscopy.

^{13}C NMR (75.5 MHz; $CDCl_3$): **Alkyl unit:** δ 28.39 (C-2/C-3), 28.43 (C-1, C-4); **Neu5Ac unit**: δ 20.66, 20.69, 20.8, 21.0 (8 × OC(O)*Me*), 22.7 (2 × NC(O)*Me*), 38.0 (2 × C-3), 53.0 (2 × CO_2*Me*), 48.9 (2 × C-5), 62.3 (2 × C-9), 67.6 (2 × C-7), 69.2 (2 × C-8), 70.0 (2 × C-4), 74.1 (2 × C-6), 83.2 (2 × C-2), 168.7 (2 × C-1), 170.4, 171.0, 171.0, 171.2 (4 × O*C*(O)Me, N*C*(O)Me).

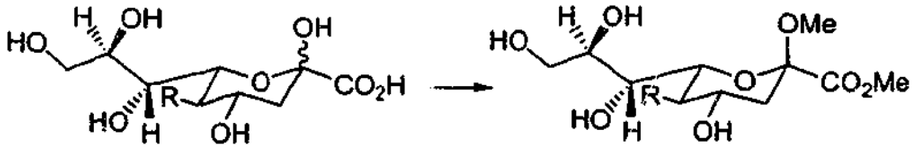
^{1}H assignments were confirmed using ^{1}H -^{13}C HMQC NMR spectroscopy.

LRFABMS: *m/z* 1086.8 [$(M + H_2O)^+$, 25%], 1069.8 [$(M + 1)^+$, 32%], 414(100), 252(55).

$C_{44}H_{64}N_2O_{24}S_2$ (1068.35188)

5.3.2 9-Tag 8-OMe Synthesis

Methyl (Methyl 5-Acetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosid) onate (36)



Compound **2** (2.0 g, 6.5 mmol) was treated with acidic resin (Dowex 50x450) (5.0 g) in anhydrous MeOH (400 mL) under a N_2 atmosphere for 20 h. After heating to reflux for 20 h, the resin was filtered and MeOH was removed under reduced pressure. The crude reaction mixture was purified by column chromatography on silica (EtOAc:MeOH; 8:2)

and recrystallized from MeOH to afford the target compound **36** (1.80 g, 83%).

Mp 138-140 °C; *Rf* 0.52 (EtOAc:MeOH:H_2O; 6:3:0.5).

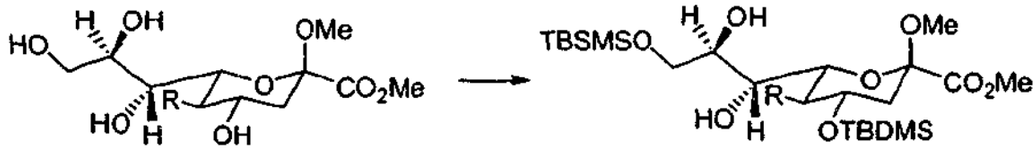
^{1}H NMR (d_4-MeOH): δ 1.61 (1H, t, $J_{3ax,3eq}$ 12.9, $J_{3ax,4}$ 11.2 Hz, H-3ax), 1.98 (3H, s, AcNH), 2.31 (1H, dd, $J_{3eq,3ax}$ 12.9, $J_{3eq,4}$ 4.9 Hz, H-3eq), 3.23 (3H, s, OMe), 3.28 (1H, m, H-5*), 3.47 (1H, m, H-OH*), 3.62 (1H, dd, $J_{9a,9b}$ 12.0, $J_{9a,8}$ 6.0 Hz, H-9a), 3.77 (3H, s, CO_2Me), 3.75-3.84 (4H, m, H-6*, 7*, 8*, 9b*), 3.95 (1H, ddd, $J_{4,3ax}$ 11.2, $J_{4,5}$ 10.8, $J_{4,3eq}$ 4.9 Hz, H-4) ;

^{13}C NMR (75.5 MHz; d_4-MeOH): δ 23.0 (NC(O)*Me*), 41.6 (C-3), 51.9 (O*Me*), 53.7 (CO_2Me), 53.9 (C-5), 65.3 (C-9), 67.8, 70.2 (C-4*/C-7*), 71.5 (C-6*), 72.3 (C-8*), 100.6 (C-2), 171.4 (C-1), 175.3 (NH*C*(O)Me).

$C_{13}H_{23}NO_4$ (337.1374)

LRESIMS (70V): *m/z* 360 [$(M + Na)^+$, 100%], 121 (40);

Methyl (Methyl 5-Acetamido-4,9-di-*O*-*tert*-butyldimethylsilyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosid) onate (89)



36 R = NHAc **89** R = NHAc

Compound **36** (0.5 g, 1.48 mmol), imidazole (0.44 g, 6.53 mmol) and 3Å molecular sieves (0.5 g) were dried under high vacuum overnight. Dry DMF (5 mL) was added to the flask which was then cooled to 0°C under a nitrogen atmosphere. *tert*-Butylchlorodimethyl silane (0.49 g, 3.26 mmol) was added to the solution. After stirring for 5 min at 0°C, the reaction mixture was warmed to room temperature and then stirred overnight. DMF was removed *in vacuo* and the crude product purified *via* column chromatography on silica gel

(EtOAc:Hexane; 8:2) to afford **89** (0.25 g, 30 %) with recovered starting material (0.365 g).

Rf 0.26 (EtOAc:Hexane; 4:1)

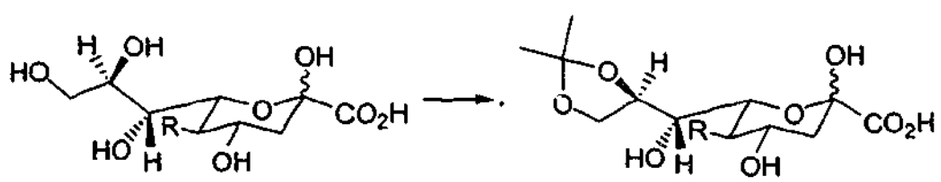
^{1}H NMR ($CDCl_3$): δ 0.08, 0.09 (2 × 6H, 2 × s, $Si(CH_3)_2$), 0.87, 0.90 (2 × 9H, 2 × s, $SiC(CH_3)_3$), 1.78 (1H, dd, $J_{3ax,3eq}$ 13.0, $J_{3ax,4}$ 10.7 Hz, H-3ax), 2.01 (3H, s, AcNH), 2.52 (1H, dd, $J_{3eq,3ax}$ 13.0, $J_{3eq,4}$ 5.0 Hz, H-3eq), 2.53 (1H, d, $J_{OH,X}$ 5.6 Hz, OH), 3.32 (3H, s, OMe), 3.46 (1H, dd, H-7*), 3.74-3.94 (5H, m, H-5*, 8*, 6*, 9*, 9b'*), 3.79 (3H, s, CO_2Me), 4.05 (1H, ddd, $J_{4,5} = J_{4,3ax}$ 10.7, $J_{4,3eq}$ 4.9 Hz, H-4), 4.26 (1H, d, $J_{OH,X}$ 5.6 Hz, OH), 5.24 (1H, d, $J_{NH,5}$ 8.1 Hz, NH).

^{13}C NMR (75.5 Hz): δ -5.2, -4.5, -3.9 (4 × Si*Me*), 18.0, 18.5 (2 × $SiCMe_3$), 23.3 (NC(O)*Me*), 25.8, 26.1 (2 × $SiCMe_3$), 41.6 (C-3), 51.1 (C-5*), 52.7 (CO_2Me*), 53.5 (O*Me*), 64.7 (C-9), 68.2 (C-4*), 69.1 (C-6*), 69.6 (C-7*), 71.5 (C-8*), 99.3 (C-2), 168.9 (C-1), 172.3 (NH*C*(O)Me);

LRFABMS: *m/z* 566 [(M + 1)$^+$, 14], 534 (10%), 402 (40%), 229 (68%), 201 (86%), 185 (100%).

$C_{25}H_{51}NO_9Si_2$ (565.86)

5-Acetamido-3,5-dideoxy-8,9-*O*-isopropylidene-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (92)



2 R = NHAc **92** R = NHAc

To a solution of Neu5Ac (**2**) (2.5g, 8.1 mmol) in DMF (25 mL) was added 2,2-dimethoxypropane (DMP) (10 mL) and a catalytic amount of Amberlyst 15 H^+ resin. The

reaction was stirred for 12 h at ambient temperature under N_2 atmosphere. The solution was adjusted to pH 9 with 1 M NaOH. The resin was filtered and the DMF and excess DMP removed *in vacuo*. Toluene (2 × 50 mL) was added to azeotrope any residual water. Compound **92** was isolated as a white amorphous solid which was in a pure state according to ^{1}H NMR spectroscopy (quantitative).

Mp 155-159 °C; R_f 0.25 (EtOAc:*i*-PrOH:H_2O; 3:2:1).

^{1}H NMR (300 MHz; D_2O): δ 1.46 (3 H, s, C*Me*), 1.51 (3 H, s, ' *e*), 1.92 (1 H, t, $J_{3ax,3eq}$ 12.9, $J_{3ax,4}$ 12.0 Hz, H-3ax), 2.13 (3 H, s, AcN), 2.27 (1H, dd, $J_{3eq,3ax}$ 12.9, $J_{3eq,4}$ 4.7 Hz, H-3eq), 3.68 (1 H, d, $J_{7,8}$ 7.4 Hz, H-7), 3.96-4.14 (4 H, m, H-4, 5, 6, 9a), 4.20(1 H, dd, $J_{9b,9a}$ 8.8, $J_{9b,8}$ 5.0 Hz, H-9b), 4.31 (1 H, dd, $J_{8,9b}$ 5.2 Hz, H-8);

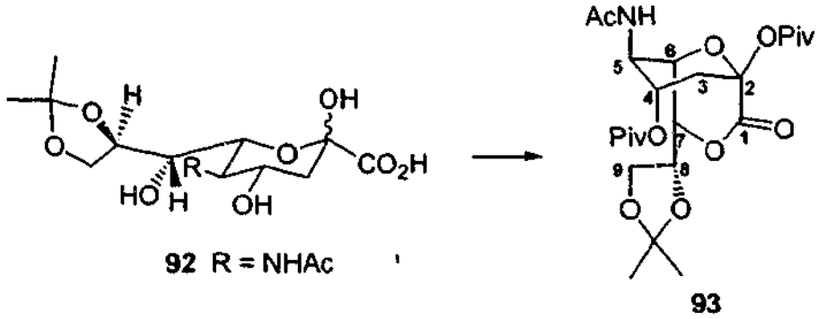
^{1}H assignments were confirmed by ^{1}H -^{1}H COSY NMR spectroscopy.

^{13}C NMR (75.5 MHz; D_2O): δ 22.1 (NC(O)*Me*), 24.3, 25.8 (2 × C(CH_3)$_2$), 39.3 (C-3), 52.2 (C-5), 65.5 (C-9), 67.1, 69.1 (C-7*, C-8*), 70.7 (C-4*), 75.1 (C-6*), 100.5 (*C*(CH_3)$_2$), 109.6 (C-2), 173.8 (C-1), 174.6 (NH*C*(O)Me)

LRESIMS: *m/z* 372 [$(M + Na)^+$, 100%], 350[$(M + 1)^+$, 70%], 332(20).

$C_{14}H_{23}NO_9$ (349.34)

5-Acetamido-3,5-dideoxy-8,9-*O*-isopropylidene-2,4-di-*O*-pivaloyl-D-*glycero*-β-D-*galacto*-2-nonulopyranoson-1,7-lactone (93)



92 R = NHAc **93**

Pivaloyl chloride (9.87 mL, 8.0 mmol) was added dropwise to a stirred suspension of compound **92** (5.6g, 1.6 mmol) in dried pyridine (50 mL) at 0°C, and the reaction mixture

was warmed to room temperature and stirred for 6 days. Pyridine was removed *in vacuo*. The crude mixture was purified on silica gel (EtOAc/hexane 9:1) and recrystallized from EtOAc/hexane to give compound **93** as white lustrous crystals (5.99 g, 75 %).

Mp 182-183°C; *Rf* 0.75 (EtOAc:Hexane; 9:1);

^{1}H NMR (500 MHz; $CDCl_3$): δ 1.18 (9 H, s, $C(CH_3)_3$), 1.21 (9 H, s, $C(CH_3)_3$), 1.34 (3 H, s, CH_3), 1.42 (3 H, s, CH_3), 2.06 (3 H, s, AcNH), 2.19 (1 H, dd, $J_{3ax,3eq}$ 14.9, $J_{3ax,4}$ 4.0 Hz, H-3ax), 2.28 (1 H, d, $J_{3eq,3ax}$ 14.9 Hz, H-3eq), 4.11-4.19 (3 H, m, H-5, 7, 9a), 4.25 (1 H, dd, $J_{9b,9a}$ 9.2, $J_{9b,8}$ 6.8 Hz, H-9b), 4.53 (1H, br s, H-6), 4.60 (1 H, ddd, $J_{8,7}$ 9.4 Hz, H-8), 5.12 (1 H, m, H-4), 6.22 (1 H, d, $J_{NH,5}$ 8.7 Hz, NH);

^{1}H assignments were confirmed by ^{1}H -^{1}H COSY NMR spectroscopy.

^{13}C NMR (75.5 MHz; $CDCl_3$): δ 23.2 (NC(O)*Me*), 25.0 ($C(CH_3)_2$), 26.6, 26.7 (2 × $C(CH_3)_3$), 27.1 ($C(CH_3)_2$), 33.2 (C-3), 38.6, 38.8 (2 × $C(CH_3)_3$), 48.5 (C-5), 67.0 (C-9), 67.4 (C-4), 72.3 (C-6), 73.5 (C-8), 79.2 (C-7), 91.1 (C-2), 110.1 ($C(CH_3)_2$), 164.2 (NC(O)Me), 169.0 (C-1), 175.5, 176.7 (2 × $OC(O)C(CH_3)_3$);

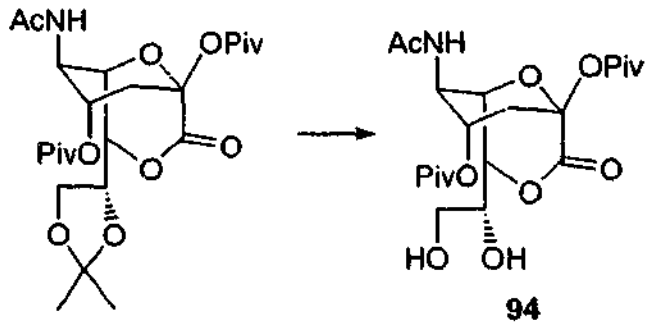
^{13}C assignments were confirmed by ^{1}H -^{13}C HMQC NMR spectroscopy.

LRESIMS: *m/z* 522 [$(M + Na)^+$, 20%], 500 [$(M + 1)^+$, 100%], 442(25).

Analytical data calculated for $C_{24}H_{37}NO_{10}$: C, 57.69; H, 7.47; N, 2.80. Found: C, 57.76; H, 7.69; N, 2.72%.

$C_{24}H_{37}NO_{10}$ (499.241)

5-Acetamido-3,5-dideoxy-2,4-di-*O*-pivaloyl-D-*glycero*-β-D-*galacto*-2-nonulopyranoson-1,7-lactone (94)



Compound **93** (2.6 g, 5.2 mmol) was dissolved in 80 % acetic acid (26 mL). The reaction mixture was heated to 60°C and stirred for 3 h under an N_2 atmosphere. Excess solvent was removed *in vacuo*, initially, followed by azeotroping with toluene. Column chromatography on silica gel (EtOAc) and subsequent recrystallization (EtOAc/Hex), afforded the title compound **94** (2.23g, 93%) as a white solid.

Mp 138-140°C; *Rf* 0.23 ($CHCl_3$:MeOH; 21:1).

^{1}H NMR (500 MHz; $CDCl_3$): δ 1.19 (9 H, s, C(CH_3)$_3$), 1.21 (9 H, s, C(CH_3)$_3$), 2.07 (3 H, s, AcNH), 2.21 (1 H, dd, $J_{3ax,3eq}$ 14.9, $J_{3ax,4}$ 3.9 Hz, H-3ax), 2.31 (1 H, d, $J_{3eq,3ax}$ 14.9 Hz, H-3eq), 3.92 (2 H, m, H-9a, 9b), 4.13-4.17 (2 H, m, H-8, 5), 4.45 (1H, d, $J_{7,8}$ 7.2 Hz, H-7), 4.67 (1 H, s, H-6), 5.14 (1 H, br s, H-4), 6.15 (1 H, br d, $J_{NH,5}$ 8.3 Hz, NH).

^{1}H assignments were confirmed by ^{1}H -^{1}H COSY NMR spectroscopy.

^{13}C NMR (75.5 MHz; $CDCl_3$): δ 23.3 (NC(O)*Me*), 27.0 (2 × C(CH_3)$_3$), 33.6 (C-3), 39.2 (2 × *C*(CH_3)$_3$), 49.4 (C-5), 62.7 (C-9), 68.0 (C-4), 71.0 (C-8), 71.9 (C-6), 78.8 (C-7), 91.7 (C-2), 165.2 (N*C*(O)Me), 170.8 (C-1), 176.4, 177.5 (2 × O*C*(O)C(CH_3)$_3$);

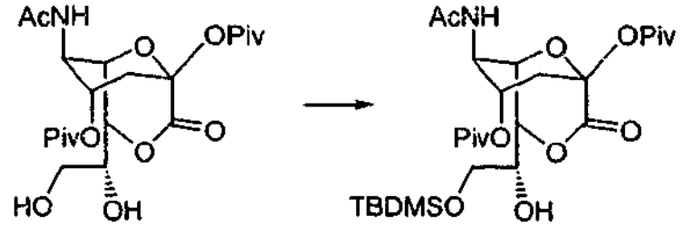
^{13}C assignments were confirmed by ^{1}H -^{13}C HMQC NMR spectroscopy.

LRESIMS: *m/z* 482 [(M + Na)$^+$, 13%], 477[(M + H_2O)$^+$, 25%], 460 [(M + 1)$^+$, 100%], 358(35).

Analytical data calculated for $C_{21}H_{33}NO_{10}\cdot {}^{1}/_{2}H_2O$: C, 53.85; H, 7.26; N, 2.99. Found: C, 53.73; H, 7.30; N, 2.76%.

$C_{21}H_{33}NO_{10}$ (459.2104)

5-Acetamido-9-*O*-*tert*-butyldimethylsilyl-3,5-dideoxy-2,4-di-*O*-pivaloyl-D-*glycero*-β-D-*galacto*-2-nonulopyranoson-1,7-lactone (96)



94 **96**

tert-Butylchlorodimethyl silane (0.27g, 1.81mmol) was added to a solution of the diol, **94** (0.8g, 1.74mmol), imidazole (0.54g, 7.97mmol) and dry 3Å sieves (0.8 g) in dry DMF (8mL) at 0°C under an N_2 atmosphere. The reaction was warmed to room temperature. After stirring for 3 h the reaction mixture was diluted with EtOAc, washed with water (3 × 15mL) and dried (Na_2SO_4). Column chromatography on silica gel (EtOAc:Hexane; 6:4) to afforded compound **96** (0.63g, 65%) as a creamy coloured amorphous mass.

Mp 183-185°C; *Rf* 0.31 (EtOAc:Hexane; 6:4);

^{1}H NMR (300 MHz; $CDCl_3$): δ 0.11 (6H, s, $Si(CH_3)_2$), 0.91 (9H, s, $SiC(CH_3)_3$), 1.19 (9 H, s, $C(CH_3)_3$), 1.22 (9 H, s, $C(CH_3)_3$), 2.06 (3 H, s, AcNH), 2.19 (1 H, dd, $J_{3ax,3eq}$ 14.8, $J_{3ax,4}$ 3.9 Hz, H-3ax), 2.31 (1 H, d, $J_{3eq,3ax}$ 14.8 Hz, H-3eq), 2.78 (1 H, d, $J_{OH,8}$ 8.2 Hz, OH), 3.83 (1 H, dd, $J_{9a,8}$ 3.7, $J_{9a,9b}$ 10.7 Hz, H-9a), 4.05 (1 H, dd, $J_{9b,8}$ 2.3, $J_{9b,9a}$ 10.7 Hz, H-9b), 4.19 (2H,m, H-8, 5), 4.37 (1 H, d, $J_{7,8}$ 8.6 Hz, H-7), 4.68 (1 H, s, H-6), 5.11 (1 H, m, H-4), 6.06 (1 H, d, $J_{NH,5}$ 8.2 Hz, NH);

^{1}H assignments were confirmed by ^{1}H -^{1}H COSY NMR spectroscopy.

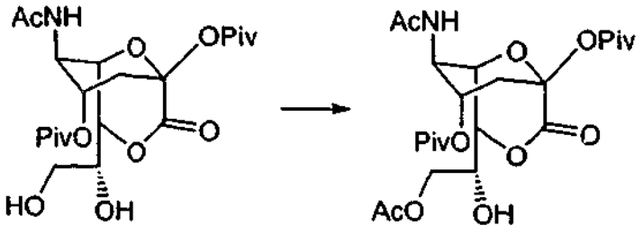
^{13}C NMR (75.5 MHz; $CDCl_3$): δ -5.36, -1.35 (Si(CH_3)$_2$), 18.4 (SiC(CH$_3$)$_3$), 23.3 (NC(O)*Me*), 26.0 (SiC(CH_3)$_3$), 26.9, 27.0 (2 × C(CH_3)$_3$), 33.4 (C-3), 38.8, 39.0 (2 × C(CH$_3$)$_3$), 49.0 (C-5), 62.1 (C-9), 67.8 (C-4), 70.0 (C-8), 72.0 (C-6), 77.9 (C-7), 91.4 (C-2), 164.8 (NC(O)Me), 169.4 (C-1), 176.0, 176.9 (2 × OC(O)C(CH$_3$)$_3$);

LRMS (ESI): *m/z* 596 [(M + Na)$^+$, 30%], 574 [(M + 1)$^+$, 100%], 472(30), 442(20).

HRMS (ESI) calculated for $C_{27}H_{48}NO_{10}Si$ [M + 1] 574.30483, found 574.30398.

$C_{27}H_{47}NO_{10}Si$ (573.29693)

5-Acetamido-9-*O*-acetyl-3,5-dideoxy-2,4-di-*O*-pivaloyl-D-*glycero*-β-D-*galacto*-2-nonulopyranoson-1,7-lactone (97)



94 **97**

To a solution of the diol, **94** (242 mg, 0.53 mmol) in dry pyridine (2 mL) and dichloromethane (DCM) (4 mL) was added a solution of acetyl chloride (49 μL, 0.69 mmol) in DCM (2 mL) at -40°C under an N_2 atmosphere. After the reaction mixture was stirred for 2 h at -40°C, MeOH (0.1 mL) was added to quench the reaction. The solvents were removed *in vacuo*. Column chromatography on silica gel (EtOAc:Hexane; 1:1) afforded compound **97** (0.194g, 73%) as a white amorphous mass.

Mp 202-205°C; R_f 0.21 (EtOAc:Hexane; 4:1).

^{1}H NMR (300 MHz; $CDCl_3$): δ 1.20 (9 H, s, C(CH$_3$)$_3$), 1.23 (9 H, s, C(CH$_3$)$_3$), 2.07 (3 H, s, AcNH), 2.11 (3 H, s, AcO), 2.20 (1 H, dd, $J_{3ax,3eq}$ 14.9, $J_{3ax,4}$ 4.0 Hz, H-3ax), 2.31 (1 H, d, H-3eq), 4.18 (1 H, d, $J_{5,NH}$ 8.1 Hz, H-5), 4.32-4.41 (3 H, m, H-9a, 8, 7), 4.50 (1 H, dd,

$J_{9b,8}$ 2.0, $J_{9b,9a}$ 11.3 Hz, H-9b), 4.64 (1 H, s, H-6), 5.15 (1 H, m, H-4), 6.18 (1 H, d, $J_{NH,5}$ 8.1 Hz, NH);

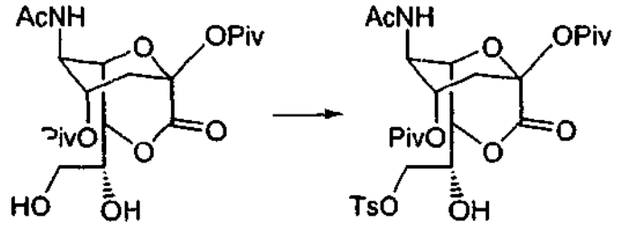
^{1}H assignments were confirmed by ^{1}H -^{1}H COSY NMR spectroscopy.

^{13}C NMR (75.5 MHz; $CDCl_3$): δ 20.6 (OAc), 22.4 (NC(O)*Me*), 26.6 (2 × C(*C*H$_3$)$_3$), 33.0 (C-3), 38.7, 38.9 (2 × *C*(CH$_3$)$_3$), 49.0 (C-5), 65.1 (C-9), 67.9 (C-4), 68.3 (C-8), 71.5 (C-6), 78.2 (C-7), 91.3 (C-2), 165.1 (N*C*(O)Me), 171.0 (O*C*(O)Me), 171.6 (C-1), 176.0, 177.1 (2 × OC(O)C(CH$_3$)$_3$);

LRESIMS: *m/z* 519 [(M + H_2O)$^+$, 20%], 502 [(M + 1)$^+$, 100], 484 (30), 400 (20).

$C_{23}H_{35}NO_{11}$ (502.5296)

5-Acetamido-3,5-dideoxy-2,4-di-*O*-pivaloyl-9-*O*-*p*-toluenesulfonyl-D-*glycero*-β-D-*galacto*-2-nonulopyranoson-1,7-lactone (100)



94 **100**

The diol **94** (1.35 g, 2.9 mmol), was dissolved in dry pyridine (13 mL) and cooled to 0°C under an N_2 atmosphere. *p*-Toluenesulfonyl chloride (0.84 g, 4.4 mmol) was added to the reaction mixture. After stirring for 3 h, MeOH (4 mL) was added to quench the reaction. The solvent was removed *in vacuo* initially, and then azeotroped with toluene. Column chromatography on silica gel ($CHCl_3$:MeOH; 50:1) afforded compound **100** (1.0 g, 55%) as a white solid with recovered starting material (0.55 g, 40%);

Mp 117-119°C; *Rf* 0.29 ($CHCl_3$:MeOH; 50:1).

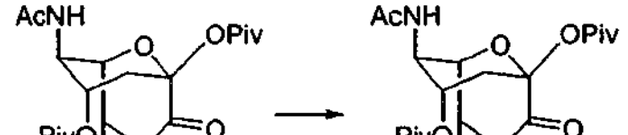
^{1}H NMR (300 MHz; $CDCl_3$): **Tosyl unit:** δ 2.45 ($C_6H_4CH_3$), 7.38 (2 H, d, $J_{2,3}$ 7.6, H-2, 6*), 7.81 (2 H, d, $J_{5,6}$ 8.3, H-3, 5*); **Neu5Ac unit:** δ 1.16 (9 H, s, $C(CH_3)_3$), 1.19 (9 H, s, $C(CH_3)_3$), 2.05 (3 H, s, AcNH), 2.18 (1 H, dd, $J_{3ax,3eq}$ 14.9, $J_{3ax,4}$ 3.9 Hz, H-3ax), 2.28 (1 H, d, $J_{3eq,3ax}$ 14.9, Hz, H-3eq), 4.19 (1 H, d, $J_{5,NH}$ 8.3 Hz, H-5), 4.35-4.45 (4 H, m, H-9a, 9b, 8, 7), 4.62 (1 H, s, H-6), 5.12 (1 H, m, H-4), 6.04 (1 H, m, NH);

^{13}C NMR (75.5 MHz; $CDCl_3$): **Tosyl unit:** δ 21.8 ($C_6H_4CH_3$), 128.1 (C-2, C-6*), 130.3 (C-3, C-5*), 132.5 (C-4*), 145.6 (C-1*); **Neu5Ac unit:** δ 23.4 (NC(O)*Me*), 26.8, 26.9 (2 × $C(CH_3)_3$), 33.5 (C-3), 38.8, 39.0 (2 × $C(CH_3)_3$), 49.0 (C-5), 67.7 (C-4), 68.5 (C-8*), 70.6 (C-9), 71.5 (C-6*), 77.7 (C-7*), 91.4 (C-2), 164.5 (NC(O)Me), 170.3 (C-1), 176.0, 177.1 (2 × $OC(O)C(CH_3)_3$);

LRFABMS: *m/z* 614.6 [$(M + 1)^+$, 100%], 478 (20).

$C_{28}H_{39}NO_{12}S$ (613.6756)

5-Acetamido-9-*O*-*p*-bromotoluenesulfonyl-3,5-dideoxy-2,4-di-*O*-pivaloyl-D-*glycero*-β-D-*galacto*-2-nonulopyranoson-1,7-lactone (103)



94 **103**

The diol compound, **94** (2.114 g, 4.6 mmol) was dissolved in dry pyridine (21 mL) and cooled to 0°C under an N_2 atmosphere. To the reaction mixture was added *p*-bromobenzenesulfonyl chloride (0.823 g, 3.2 mmol). The mixture was stirred for 3 h before a further amount of *p*-bromobenzenesulfonyl chloride (0.353 g, 1.4 mmol) was added. The resulting solution was stirred for a further 1 h at 0°C, H_2O (15 mL) was added

to quench the reaction. The solvent was removed *in vacuo*. Any residual solvent was azeotroped with toluene. Column chromatography on silica gel (EtOAc/Hexane; 1:1) afforded compound **103** (1.211 g, 39%) as a white amorphous mass with recovered starting material (1.09 g, 50%). Compound **103** was further purified by recrystallization (EtOAc/Hexane) to obtain white crystals.

Mp 188-190°C; *Rf* 0.66 (EtOAc/Hexane; 9:1).

^{1}H NMR (300 MHz; $CDCl_3$): **Brosyl unit:** δ 7.73 (2 H, d, $J_{2,3}$ 8.7, H-2, 6*), 7.81 (2 H, d, $J_{5,6}$ 8.7, H-3, 5*); **Neu5Ac unit:** δ 1.17 (9 H, s, $C(CH_3)_3$), 1.21 (9 H, s, $C(CH_3)_3$), 2.07 (3 H, s, AcNH), 2.20 (1 H, dd, $J_{3ax,3eq}$ 14.9, $J_{3ax,4}$ 3.8 Hz, H-3ax), 2.30 (1 H, d, $J_{3eq,3ax}$ 14.8 Hz, H-3eq), 4.18 (1 H, d, $J_{5,NH}$ 8.0 Hz, H-5), 4.38-4.42 (4 H, m, H-9a, 9b, 8, 7), 4.62 (1 H, s, H-6), 5.14 (1 H, br s, H-4), 6.06 (1 H, d, $J_{NH,5}$ 8.0 Hz, NH);

^{1}H assignments were confirmed by ^{1}H -^{1}H COSY NMR spectroscopy.

^{13}C NMR (75.5 MHz; $CDCl_3$): **Brosyl unit:** δ 129.7 (C-1*), 129.8 (C-2, C-6*), 133.1 (C-3, C-5*), 134.8 (C-4*); **Neu5Ac unit:** δ 23.4 (NC(O)*Me*), 27.1 (2 × $C(CH_3)_3$), 33.5 (C-3), 39.0, 39.2 (2 × $C(CH_3)_3$), 49.1 (C-5), 67.9 (C-4), 68.6 (C-8), 71.5 (C-9), 71.7 (C-6), 77.9 (C-7), 91.6 (C-2), 164.6 (N*C*(O)Me), 170.7 (C-1), 176.3, 177.4 (2 × O*C*(O)C$(CH_3)_3$);

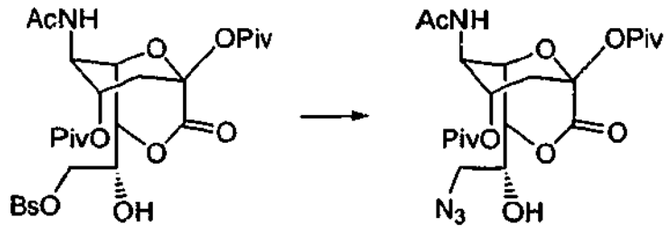
^{13}C assignments were confirmed by ^{1}H -^{13}C HMQC NMR spectroscopy.

LRESIMS: *m/z* 680 [(^{81}Br M + 1)$^+$, 60%], 678 [(^{79}Br M + 1)$^+$, 65%], 578 (40), 576(35), 494(40), 492(35).

Analytical data calculated for $C_{27}H_{36}NO_{12}SBr$: C, 47.78; H, 5.35; N, 2.06. Found: C, 47.87; H, 5.36; N, 1.93%.

$C_{27}H_{36}NO_{12}$ SBr (678.5449)

5-Acetamido-9-azido-3,5,9-trideoxy-2,4-di-*O*-pivaloyl-D-*glycero*-β-D-*galacto*-2-nonulopyranoson-1,7-lactone (102)



103 **102**

A suspension of brosylate compound **103** (0.5296g, 0.78mmol) and sodium azide (0.5076g, 7.8mmol) in DMF (10mL) was stirred at 60°C under an N_2 atmosphere. After 6 h, the reaction mixture was cooled to room temperature and DMF was removed *in vacuo*. The crude product was purified by column chromatography on silica gel ($CHCl_3$:MeOH 100:1) to isolate the target compound **102** as a white amorphous mass in quantitative yield (0.4003g).

Mp 112-114°C; *Rf* 0.46 ($CHCl_3$:MeOH 21:1); ν_{max} (KBr) 3363, 2974, 2105, 1741, 1656, 1535, 1277, 1120 cm^{-1};

^1H NMR (500 MHz; $CDCl_3$): δ 1.18 (9 H, s, $C(CH_3)_3$), 1.20 (9 H, s, $C(CH_3)_3$), 2.05 (3 H, s, AcNH), 2.21 (1 H, dd, $J_{3ax,3eq}$ 14.0, $J_{3ax,4}$ 3.5 Hz, H-3ax), 2.26 (1 H, d, $J_{3eq,3ax}$ 14.0 Hz, H-3eq), 3.70 (2 H, ddd, $J_{9a,8}$ 8.0, $J_{9a,9b}$ 13, $J_{9b,8}$ 4.0 Hz, H-9a, 9b), 4.10 (1 H, d, $J_{5,NH}$ 8.0 Hz, H-5), 4.26 (1H, q, $J_{8,9a}$ 8.0, $J_{8,9b}$ 4.0 Hz, H-8), 4.37 (1H, $J_{7,8}$ 8.5 Hz, H-7), 4.62 (1 H, s, H-6), 5.13 (1 H, s, H-4), 6.51 (1 H, d, $J_{NH,5}$ 8.0 Hz, NH);

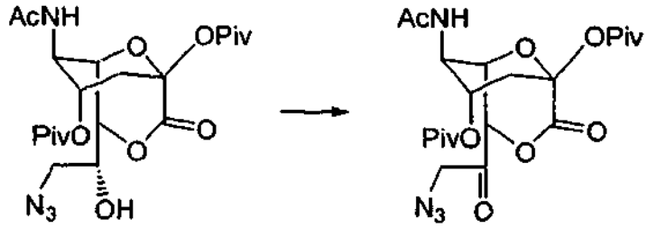
^1H assignments were confirmed by ^1H -^1H COSY NMR spectroscopy.

^{13}C NMR (75.5 MHz; $CDCl_3$): δ 23.2 (NC(O)*Me*), 26.8 (2 × C(*C*H_3)$_3$), 33.3 (C-3), 38.8, 39.0 (2 × *C*(CH_3)$_3$), 49.1 (C-5), 53.3 (C-9), 67.6 (C-4*), 69.5 (C-8*), 71.5 (C-6*), 78.5 (C-7), 91.3 (C-2), 165.6 (N*C*(O)Me), 170.5 (C-1), 176.1; 177.4 (2 × O*C*(O)C(CH_3)$_3$);

LRFABMS: *m/z* 523 [$(M + K)^+$, 30%], 507 [$(M + Na)^+$, 50%], 485 [$(M + 1)^+$, 100%].

$C_{21}H_{32}N_4O_9$ (484.5052)

5-Acetamido-9-azido-3,5,9-trideoxy-2,4-di-*O*-pivaloyl-D-*glycero*-β-D-*galacto*-2,8-nondiulopyranoson-1,7-lactone (105)



102 **105**

To a suspension of **102** (0.10 g, 0.21 mmol) and ground pyridinium dichromate (0.71 g, 0.19 mmol) with silica (0.71 g) in dry dichloromethane (2.5 mL) was added Ac_2O ($8.4x10^{-5}$ g, $0.8x10^{-3}$ mmol) under an N_2 atmosphere. After the reaction mixture was stirred at room temperature for 4 h, *iso*-propanol (1.0mL) was added. The crude reaction mixture was filtered through a plug of silica with a solution of 10% MeOH in EtOAc followed by purification on silica gel chromatography to give the ketone **105** (0.059g, 59%) (as a white amorphous solid) together with recovered starting material (0.022 g, 22%).

Mp 122-125°C; *Rf* 0.63 ($CHCl_3$:MeOH 21:1); ν_{max} (KBr) 3366, 2977, 2109, 1743, 1664, 1536, 1279, 1119 cm^{-1};

^1H NMR (300 MHz; $CDCl_3$): δ 1.17 (9 H, s, $C(CH_3)_3$), 1.19 (9 H, s, $C(CH_3)_3$), 2.07 (3 H, s, AcNH), 2.23 (1 H, dd, $J_{3ax,3eq}$ 14.0, $J_{3ax,4}$ 3.5 Hz, H-3ax), 2.35 (1 H, d, $J_{3eq,3ax}$ 15.0 Hz, H-3eq), 4.29 (1 H, d, $J_{5,NH}$ 7.3 Hz, H-5), 4.44 (1 H, d, $J_{9a,9b}$ 19.9 Hz, H-9a), 4.79-4.93 (3 H, m, H-6, 7, 9b), 5.14 (1 H, m, H-4), 6.00 (1 H, d, $J_{NH,5}$ 8.0 Hz, NH);

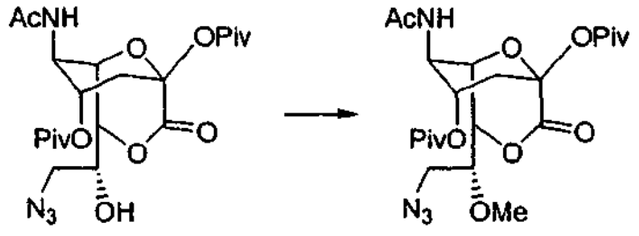
^1H assignments were confirmed by ^1H -^1H COSY NMR spectroscopy.

^{13}C NMR (75.5 MHz; $CDCl_3$): δ 23.1 (NC(O)*Me*), 26.8, 26.9 (2 × C(*C*H$_3$)$_3$), 33.3 (C-3), 38.8, 39.0 (2 × *C*(CH$_3$)$_3$), 48.5 (C-5), 56.4 (C-9), 67.6 (C-4*), 73.4 (C-6*), 82.6 (C-7*), 91.5 (C-2), 163.3 (N*C*(O)Me), 169.7 (C-1), 1705.9, 176.9 (2 × O*C*(O)C(CH$_3$)$_3$), 201.7 (C-8);

LRFABMS: *m/z* 521 [(M + K)$^+$, 80%], 505 [(M + Na)$^+$, 100%], 483 [(M + 1)$^+$, 30%].

$C_{21}H_{30}N_4O_9$ (482.4894)

5-Acetamido-9-azido-3,5,9-trideoxy-8-*O*-methyl-2,4-di-*O*-pivaloyl-D-*glycero*-β-D-*galacto*-2-nonulopyranoson-1,7-lactone (106)



102 **106**

Compound **102** (0.8636 g, 1.8 mmol), MeI (12 mL), Ag_2O (1.65 g, 7.1 mmol) and $CaSO_4$ were heated to reflux for 48 h under a nitrogen atmosphere. The crude reaction mixture was filtered through a plug of celite followed by purification by silica gel chromatography ($CHCl_3$:MeOH 100:1) to give the methylated substrate **106** (0.6784 g, 84 %) (as a white coloured amorphous solid) with recovered starting material (0.077 g, 9 %).

Mp 97-100°C; R_f 0.65 ($CHCl_3$:MeOH 21:1); ν_{max} (KBr) 3390, 2975, 2104, 1751, 1656, 1542, 1277, 1118 cm^{-1};

^{1}H NMR (300 MHz; $CDCl_3$): δ 1.21 (9 H, s, C(CH$_3$)$_3$), 1.24 (9 H, s, C(CH$_3$)$_3$), 2.08 (3 H, s, AcNH), 2.20 (1 H, dd, $J_{3ax,3eq}$ 11.1, $J_{3ax,4}$ 4.0 Hz, H-3ax), 2.27 (1 H, dd, $J_{3eq,4}$ 1.6 Hz, H-3eq), 3.48 (1H, s, OMe), 3.60 (1 H, dd, $J_{9a,9b}$ 14.2, $J_{9a,8}$ 3.2 Hz, H-9a), 3.84-3.90 (2 H, m,

H-8, 9b), 4.22 (1 H, d, $J_{5,NH}$ 8.2 Hz, H-5), 4.48-4.53 (2H, m, H-6, 7), 5.14 (1 H, m, H-4), 5.99 (1 H, d, NH);

^{1}H assignments were confirmed by ^{1}H -^{1}H COSY NMR spectroscopy.

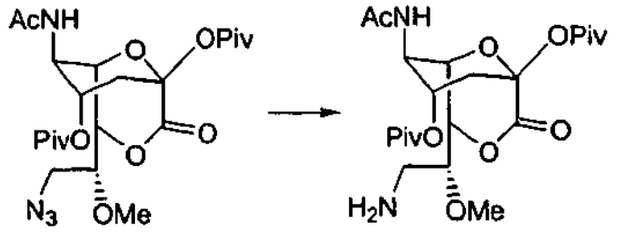
^{13}C NMR (75.5 MHz; $CDCl_3$): δ 23.1 (NC(O)*Me*), 26.6 (2 × C(CH_3)$_3$), 33.2 (C-3), 38.6, 38.7 (2 × *C*(CH_3)$_3$), 48.2 (C-9), 48.4 (C-5), 57.5 (OMe), 67.4 (C-4), 71.7 (C-6), 76.5 (C-7), 78.0 (C-8), 91.2 (C-2), 164.1 (N*C*(O)Me), 169.0 (C-1), 175.6, 176.7 (2 × O*C*(O)C(CH_3)$_3$);

^{13}C assignments were confirmed by ^{1}H -^{13}C HMQC NMR spectroscopy.

LRESIMS: *m/z* 537 [$(M + K)^+$, 10%], 521 [$(M + Na)^+$, 50%], 499 [$(M + 1)^+$, 100%].

$C_{22}H_{34}N_4O_9$ (498.532)

5-Acetamido-9-amino-3,5,9-trideoxy-8-*O*-methyl-2,4-di-*O*-pivaloyl-D-*glycero*-β-D-*galacto*-2-nonulopyranoson-1,7-lactone (110)



106 **110**

Compound **106** (100mg, 0.20 mmol), anhydrous MeOH (20 mL), CH_3COOH (2 mL) and a catalytic amount of palladium on carbon (Pd/C 10%) were stirred in a Parr hydogenator at 30Psi over-night. The crude reaction mixture was filtered through a plug of celite, concentrated, and the purified by silica gel chromatography (EtOAc:*i*-PrOH:H_2O; 3:1:0.5) to give the substrate **110** (65.8 mg, 70 %) as a cream coloured amorphous solid.

Mp 183-185°C; R_f 0.48 (EtOAc:*i*-PrOH:H_2O; 3:2:1); ν_{max} (KBr) 3367, 2974, 2361, 1742, 1655, 1542, 1282, 1124 cm^{-1};

^{1}H NMR (500 MHz; $CDCl_3$): δ 1.95 (18 H, s, $C(CH_3)_3$), 2.07 (3 H, s, AcNH), 2.34 (1 H, d, $J_{3ax,3eq}$ 13.5 Hz, H-3ax), 3.03 (1 H, t, $J_{3eq,3ax}$ 13.5 Hz, H-3eq), 3.19 (1 H, dd, $J_{9a,9b}$ 15.0, $J_{9a,8}$ 6.5 Hz, H-9a), 3.33 (1H, s, H-8), 3.45 (1H, s, OMe), 4.17 (1 H, s, H-6), 4.39 (1 H, m, H-5), 4.65 (1H, s, H-7), 4.87 (1H, m, H-9b), 5.08 (1H, m, H-4), 6.41 (1H, d, $J_{NH,5}$ 4 Hz, NH);

^{1}H assignments were confirmed by ^{1}H -^{1}H COSY NMR spectroscopy.

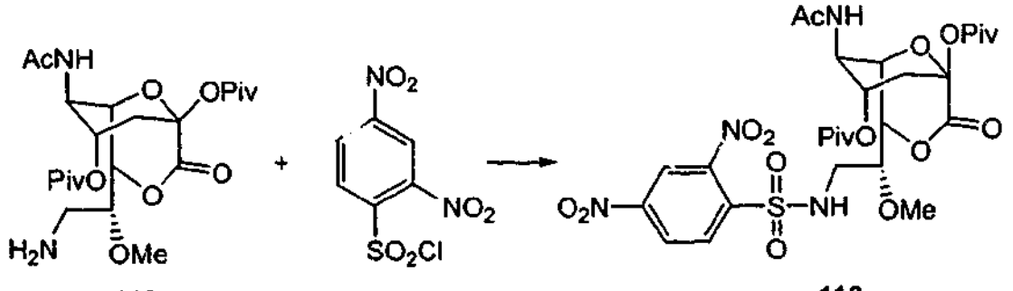
^{13}C NMR (75.5 MHz; $CDCl_3$): δ 23.2 (NC(O)*Me*), 27.1, 27.2 (2 × C(*CH*$_3$)$_3$), 35.6 (C-3), 39.0, 39.2 (2 × *C*(CH$_3$)$_3$), 46.0 (C-9), 53.7 (C-5), 56.9 (OMe), 68.2 (C-4), 70.9 (C-7), 79.6 (C-8), 81.4 (C-6), 100.5 (C-2), 171.0, 171.8 (2 × O*C*(O)C(CH$_3$)$_3$), 177.0 (N*C*(O)Me*), 180.0 (C-1*);

^{13}C assignments were confirmed by ^{1}H -^{13}C HMQC NMR spectroscopy.

LRFABMS: *m/z* 473.5 [(M + 1)$^+$, 100%], 371(15).

$C_{22}H_{36}N_2O_9$ (472.5344)

5-Acetamido-3,5,9-trideoxy-9-[2,4-dinitrobenzene sulfonamide]-8-*O*-methyl-2,4-di-*O*-pivaloyl-D-*glycero*-β-D-*galacto*-2-nonulopyranoson-1,7-lactone (113)



2,4-Dinitrobenzene sulfonyl chloride (0.102 g, 0.38 mmol) was added to a solution of the free amine, **110** (79 mg, 0.16 mmol) in dry pyridine (5 mL) and was stirred at room temperature under an N_2 atmosphere. After stirring for 2 days, a further amount of *2,4*-dinitrobenzene sulfonyl chloride (0.102 g, 0.38 mmol) was added to the reaction mixture.

A total of 3 days was required before tlc showed no more starting material. The pyridine was removed by azeotroping with toluene from the reaction mixture which was then purified by column chromatography on silica gel (Chloroform; 100 %) to afford compound **113** (71.7 mg, 61%) as a yellow coloured amorphous mass.

Mp 133-138°C; *Rf* 0.65 (Chloroform/MeOH; 20:1);

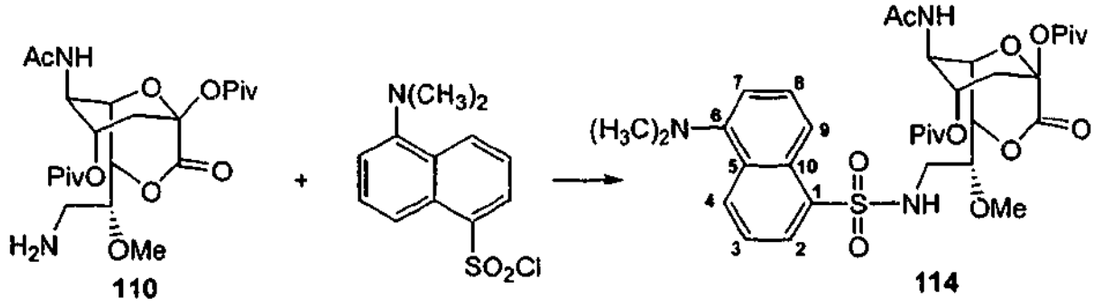
^{1}H NMR (500 MHz; $CDCl_3$): **Aromatic unit:** δ 8.38 (1 H, d, $J_{6',5'}$ 8.5 Hz, H-6), 8.54 (1 H, dd, $J_{5',3'}$ 2.0 Hz, H-5), 8.69 (1H, d, H-3); **Neu5Ac unit:** δ 1.18 (9 H, s, $C(CH_3)_3$), 1.21 (9 H, s, $C(CH_3)_3$), 2.05 (3 H, s, AcNH), 2.23 (1 H, dd, $J_{3ax,3eq}$ 13.0, $J_{3ax,4}$ 4.0 Hz, H-3ax), 2.28 (1 H, d, H-3eq), 3.39 (3H, s, OMe), 3.70-3.78 (3 H, m, H-8, 9a, 9b), 4.15 (1 H, d, $J_{5,NH}$ 8.0 Hz, H-5), 4.30 (1H, s, H-6), 4.38 (1 H, d, $J_{7,8}$ 8.0 Hz, H-7), 5.11 (1 H, m, H-4), 5.79 (1 H, m, NH), 6.08 (1 H, d, NH);

^{1}H assignments were confirmed by ^{1}H -^{1}H COSY NMR spectroscopy.

^{13}C NMR (75.5 MHz; $CDCl_3$): **Aromatic unit:** δ 120.8 (C-6*), 126.9 (C-5*), 132.5 (C-3*), 139.3 (C-4*), 148.1 (C-2*), 149.7 (C-1*); **Neu5Ac unit:** δ 23.1 (NC(O)*Me*), 26.6 (2 × C(*CH*$_3$)$_3$), 33.3 (C-3), 38.6 (2 × *C*(CH$_3$)$_3$), 48.4 (C-5*), 57.6 (OMe), 67.0 (C-9), 67.3 (C-4*), 72.2 (C-6*), 76.9 (C-7*), 78.5 (C-8*), 91.3 (C-2), 163.8 (N*C*(O)Me), 169.1 (C-1), 175.9, 176.8 (2 × O*C*(O)C(CH$_3$)$_3$);

$C_{28}H_{38}N_4O_{15}S$ (702.686)

5-Acetamido-3,5-dideoxy-9-[(*N*-5-dimethylamino)-1-napthalene sulfonyl]-8-*O*-methyl-2,4-di-*O*-pivaloyl-D-*glycero*-β-D-*galacto*-2-nonulopyranoson-1,7-lactone (114)



Dansyl chloride (62.6 mg, 0.23 mmol) was added to a solution of the free amine, **110** (54.8 mg, 0.12 mmol) in dry pyridine (2.5 mL) and was stirred at room temperature under an N_2 atmosphere for 24 h. The pyridine was removed by azeotroping with toluene from the reaction mixture which was then purified by column chromatography on silica gel (Chloroform; 100 %) to afford compound **114** (31.5 mg, 38%) as a yellow coloured amorphous mass;

Mp 125-130°C; *Rf* 0.61 (Chloroform/MeOH; 20:1);

^{1}H NMR (300 MHz; $CDCl_3$): **Aromatic unit:** δ 2.91 (6H, s, $N(CH_3)_2$), 7.23 (2 H, d, $J_{7,8}$ 7.5 Hz, H-7), 7.56 (2 H, q, $J_{3,4}$ 8.1 Hz, H-3, 8), 8.30 (2H, m, H-2, 9), 8.58 (1H, d, $J_{4,3}$ 8.1 Hz, H-4); **Neu5Ac unit:** δ 1.16 (9 H, s, $C(CH_3)_3$), 1.21 (9 H, s, $C(CH_3)_3$), 2.06 (3 H, s, AcNH), 2.16 (1 H, dd, $J_{3ax,3eq}$ 15.0, $J_{3ax,4}$ 3.6 Hz, H-3ax), 2.29 (1 H, d, H-3eq), 3.17 (1H, s, OMe), 3.29-3.36 (2 H, m, H-9a, 9b), 3.69 (1 H, dd, 1H, q, $J_{8,7}$ 7.8, $J_{8,9b}$ 3.8 Hz, H-8), 4.12 (1 H, d, $J_{5,NH}$ 8.4 Hz, H-5), 4.37 (1H, s, H-6), 4.42 (1 H, d, $J_{7,8}$ 7.8 Hz, H-7), 5.00 (1 H, m, NH), 5.10 (1 H, s, H-4), 5.97 (1 H, d, $J_{NH,5}$ 8.4 Hz, NH);

^{1}H assignments were confirmed by ^{1}H -^{1}H COSY NMR spectroscopy.

^{13}C NMR (75.5 MHz; $CDCl_3$): **Aromatic unit:** δ 45.9 ($N(CH_3)_2$), 115.9 (C-7), 124.0 (C-3), 128.5 (C-8), 129.9 (C-5*), 130.0 (C-2/C-9), 130.4 (C-4), 134.9 (C-1*/C-10*), 150 (C-6*); **Neu5Ac unit:** δ 23.5 (NC(O)*Me*), 26.9, 27.0 (2 × $C(CH_3)_3$), 33.4 (C-3), 38.9, 39.1 (2

× $C(CH_3)_3$), 40.8 (C-9), 48.7 (C-5), 57.6 (OMe), 67.7 (C-4), 72.3 (C-6), 77.1 (C-7), 78.6 (C-8), 91.5 (C-2), 164.3 (N*C*(O)Me), 169.3 (C-1), 176.1, 177.0 (2 × O*C*(O)C$(CH_3)_3$);

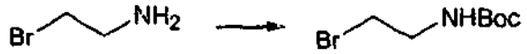
^{13}C assignments were confirmed by ^{1}H -^{13}C HMQC NMR spectroscopy.

LRFABMS: *m/z* 728.5 [$(M + Na)^+$, 18%], 706.5 [$(M + 1)^+$, 100%], 622.5(19), 520(16), 170(33).

$C_{34}H_{47}N_3O_{11}S$ (705.8188)

5.3.3 Synthesis of affinity ligands.

2-Bromo-*N*-(*tert*-Butyloxycarbonyl)-ethylamine (133)[279]



133

2-Bromoethylamine hydrobromide (2.16 g, 11 mmol) was dissolved in water (10 mL), and the pH of the solution was adjusted to 9 with Et_3N. To this solution at 0 °C was added di-*tert*-butyl dicarbonate (2.26 g, 11 mmol) in CH_3CN (10 mL) and the pH of the solution monitored and kept at 9 by the addition of Et_3N. The reaction was allowed to stir at room temperature for 24 h, before being concentrated under reduced pressure. The crude product was diluted with CH_2Cl_2 (50 mL), washed with 1 M HCl (50 mL), dried (Na_2SO_4) and concentrated under reduced pressure. Column chromatography on silica gel (EtOAc:hexane; 1:3) gave the title compound **133** (1.72 g, 73%) as an amorphous mass;

mp 25-26°C (dec); *Rf* 0.75 (EtOAc:hexane; 1:3);

ν_{max} (NaCl) 3360, 2984, 2940, 1694, 774 cm^{-1};

^{1}H NMR (300 MHz; $CDCl_3$): δ 1.43 (9H, s, $C(CH_3)_3$), 3.41-3.52 (4 H, m, H-1, 2), 5.03, (1 H, bs, N*H*);

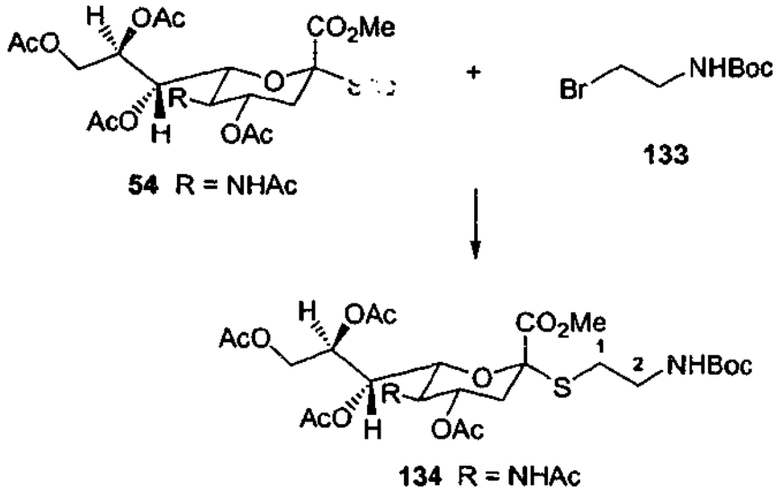
^{13}C NMR (75.5 MHz; $CDCl_3$): δ 28.5 ($C(CH_3)_3$), 32.6 (C-2), 42.5 (C-1), 79.9 ($C(CH_3)_3$), 155.8 (NC(O)O);

LRFABMS: *m/z* 226 [(^{81}Br M + 1)$^+$, 38%], 224 [(^{79}Br M + 1)$^+$, 39], 198 (43), 196 (47), 170 (100), 168 (100), 126 (34), 124 (37).

HRFABMS (positive-ion): Calcd for $C_7H_{15}NO_2{}^{79}Br$ 224.02861; Found: 224.02929.

$C_7H_{14}NO_2Br$ (224.0971)

Methyl [2-*S*-(2′-*N*-*tert*-butyloxycarbonyl-aminoethyl) 5-acetamido-4,7,8,9-tetra-*O*-acetyl 3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (134)[279]



Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (**54**) (0.7 g, 1.27 mmol) and **133** (0.27 g, 1.27 mmol) were dissolved in dry *N,N*-DMF (8 mL) at room temperature under N_2, Et_2NH (3.2 mL) was added and the reaction stirred for 3 h at room temperature. The mixture was concentrated under reduced pressure and the residue diluted with EtOAc (50 mL) and washed with pH 4 buffer (50 mL), H_2O (2 × 50 mL), dried (Na_2SO_4) and evaporated to dryness under reduced pressure. Column chromatography on silica gel (EtOAc) afforded **134** (0.64 g, 77%) as an amorphous mass.

Mp 69-70°C; *Rf* 0.31 (EtOAc); $[\alpha]_D^{28}$ +13.5° (*c* 1, $CHCl_3$); ν_{max} (KBr) 3456, 1744, 1690, 1608, 1226, 1032 cm^{-1};

^1H NMR (300 MHz; $CDCl_3$): **Alkyl unit:** δ 1.45 (9 H, s, $C(CH_3)_3$), 2.64-2.82 (2 H, m, H-2), 3.25-3.32 (2 H, m, H-1); **Neu5Ac unit:** δ 1.88 (3 H, s, AcN), 2.03, 2.05, 2.16, 2.19 (12 H, 4 × s, 4 × AcO), 2.64-2.82 (2 H, m, H-3eq, 3ax), 3.80 (3 H, s, CO_2Me), 3.83 (1H, dd, $J_{6,7}$ 1.9 Hz, H-6), 4.02 (1 H, ddd, $J_{5,4} = J_{5,6} = J_{5,NH}$ = 10.2 Hz, H-5), 4.05 (1 H, dd, $J_{9a,9b}$ 12.2, $J_{9a,8}$ 5.4 Hz, H-9a), 4.44 (1 H, dd, $J_{9b,8}$ 2.7 Hz, H-9b), 4.87 (1 H, ddd, $J_{4,3ax}$ 11.2, $J_{4,3eq}$ 4.7Hz, H-4), 5.13 (1 H, d, NH), 5.30 (1 H, dd, $J_{7,8}$ 9.1 Hz, H-7), 5.37 (1 H, ddd, H-8);

^{13}C NMR (75.5 MHz; $CDCl_3$): **Alkyl unit:** δ 28.4 ($C(CH_3)_3$), 28.9 (C-2), 41.1 (C-1), 79.2 ($C(CH_3)_3$), 155.9 (NC(O)O); **Neu5Ac unit:** δ 20.8, 20.9, 21.3 (4 × OC(O)*Me*), 23.2 (NC(O)*Me*), 38.2 (C-3), 49.3 (C-5), 53.1 (CO_2Me), 62.6 (C-9), 67.2, 68.3 (C-7/C-8), 69.7 (C-4), 74.2 (C-6), 83.1 (C-2), 168.8 (C-1), 170.2, 170.3, 170.4, 170.9 (4 × OC(O)Me, NC(O)Me);

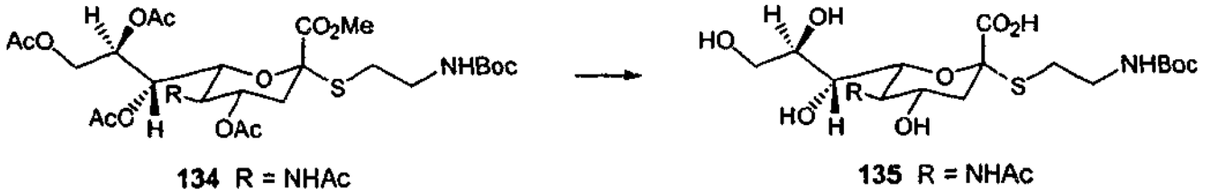
^{13}C assignments were confirmed by ^1H -^{13}C HMQC NMR spectroscopy.

LRFABMS: *m/z* 651 [$(M + 1)^+$, 33%], 551(100), 414(100).

Analytical data. Calculated for $C_{27}H_{42}N_2O_{14}S{\cdot}H_2O$: C, 48.50; H, 6.63; N, 4.19. Found: C, 48.14; H, 6.51; N, 3.76%.

$C_{27}H_{42}N_2O_{14}S$ (650.23567)

2-*S*-(2'-*N*-*tert*-butyloxycarbonyl-aminoethyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (135)[279]



134 R = NHAc **135** R = NHAc

Compound **134** (0.2 g, 0.32 mmol) was treated with a solution of NaOMe (0.16 mmol) in anhydrous MeOH (10 mL) at room temperature under N_2. After stirring for 2 h, the MeOH was removed under reduced pressure, H_2O (10 mL) added and the pH adjusted to 12 with 0.1 M NaOH. After stirring overnight, the pH of the reaction mixture was adjusted to 7.0-7.5 with Dowex-50W×8 (H^+) resin. After filtration, the filtrate was lyophilised to afford the target compound **135** as an amorphous white solid that was in a pure state according to NMR spectroscopy (0.15 g, 99%);

Mp 175°C (dec); *Rf* 0.62 (EtOAc:^{i}PrOH:H_2O; 2:3:1); $[\alpha]_D^{28}$ -5.0° (*c* 0.6, H_2O); ν_{max} (KBr) 3416, 1692, 1276, 1030 cm^{-1};

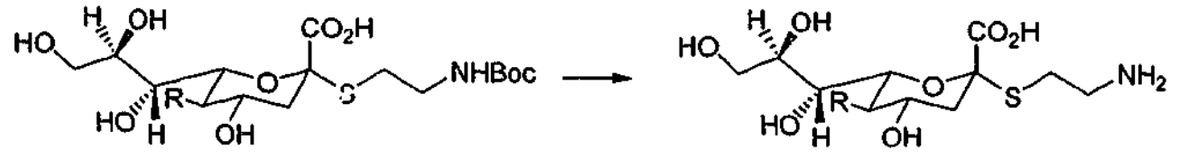
^{1}H NMR (300 MHz; D_2O): **Alkyl unit:** δ 1.56 (9 H, s, $C(CH_3)_3$), 2.87-3.03 (2 H, m, H-2), 3.39-3.45 (2 H, m, H-1); **Neu5Ac unit:** δ 1.89 (1 H, dd, $J_{3ax,3eq}$ 12.3, $J_{3ax,4}$ 11.9 Hz, H-3ax), 2.18 (3 H, s, AcN), 2.87-3.03 (1 H, m, H-3eq), 3.69-3.85 (4 H, m, H-4, 6, 7, 9a), 3.91-4.01 (3 H, m, H-5, 8, 9b);

^{13}C NMR (75.5 MHz; D_2O): **Alkyl unit:** δ 30.3 ($C(CH_3)_3$), 32.3 (C-2), 43.6 (C-1), 160.9 (N*C*(O)O); **Neu5Ac unit:** δ 24.7 (NC(O)*Me*), 42.7, (C-3), 54.4 (C-5), 65.2 (C-9), 70.7 (C-7*), 71.2 (C-8*), 74.5 (C-4), 77.5 (C-6*), 83.9 ($C(CH_3)_3$), 88.7 (C-2), 176.8 (C-1), 177.7 (N*C*(O)Me);

LRFABMS: *m/z* 491 [$(M + Na)^+$, 38%], 469 [$(M + 1)^+$, 15], 369(31), 201(100).

$C_{18}H_{32}N_2O_{10}S$ (468.1778)

2-*S*-(2′-Aminoethyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulo-pyranosidonic acid (127)[279]



135 R = NHAc

127 R = NHAc

To a solution of **135** (0.15 g, 0.32 mmol) in CH_3COOH (0.9 mL, 15.7 mmol) was added $BF_3{\cdot}OEt_2$ (10 μl, 0.79 mmol), and the mixture stirred for 4 h at room temperature under N_2. The solution was diluted with H_2O (20 mL) and the pH was adjusted to 7 with Et_3N. The solution was then lyophilised and purified by reverse phase HPLC (H_2O as eluent) to afford the target compound **127** (76 mg, 64%) as a white amorphous mass;

Mp 210°C (dec); *Rf* 0.12 (EtOAc:^{i}PrOH:H_2O; 2:3:1); $[\alpha]_D^{28}$ +52.6° (*c* 0.5, H_2O); ν_{max} (KBr) 3404, 1620, 1374, 1078 cm^{-1};

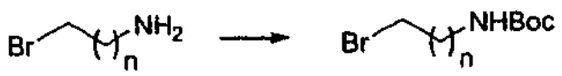
^{1}H NMR (300 MHz; D_2O): **Alkyl unit:** δ 2.88-2.97 (2 H, m, H-1), 3.13-3.23 (2 H, m, H-2); **Neu5Ac unit:** δ 1.75 (1 H, t, $J_{3ax,3eq} = J_{3ax,4}$ = 12.0 Hz, H-3ax), 1.98 (3 H, s, AcN), 2.76 (1 H, dd, $J_{3eq,4}$ 5.0 Hz, H-3eq), 3.52-3.56 (2 H, m, H-6, 7), 3.58 (1 H, dd, $J_{9a,9b}$ 12.0, $J_{9a,8}$ 6.5 Hz, H-9a), 3.65 (1 H, ddd, $J_{4,5}$ 10.5 Hz, H-4), 3.72 (1 H, ddd, $J_{8,7}$ 7.4, $J_{8,9b}$ 1.5 Hz, H-8), 3.77 (1 H, t, $J_{5,6}$ 10.5 Hz, H-5), 3.81 (1 H, dd, H-9b);

^{13}C NMR (75.5 MHz; D_2O): **Alkyl unit:** δ 31.2 (C-2), 43.5 (C-1); **Neu5Ac unit:** δ 25.8 (NC(O)*Me*), 45.1 (C-3), 55.9 (C-5), 66.8 (C-9), 72.2 (C-4/C-7*), 76.1 (C-6*), 79.2 (C-8), 90.7 (C-2), 177.4 (C-1), 178.9 (NHC(O)Me);

LRFABMS: *m/z* 391 [$(M + Na)^+$, 4%], 369 [$(M + 1)^+$, 32], 338 (17), 312 (29); HRFABMS: $C_{13}H_{25}N_2O_8S$ requires 369.13315, found 369.13564.

Analytical data calculated for $C_{13}H_{24}N_2O_8SNa{\cdot}4H_2O$: C, 33.76; H, 6.76; N, 6.06. Found: C, 33.82; H, 6.64; N, 6.04%; $C_{13}H_{24}N_2O_8S$ (368.125)

3-Bromo-*N*-(*tert*-butyloxycarbonyl)-propylamine (137)



n = 2 **137** n = 2

3-Bromopropylamine hydrobromide (2.41 g, 11 mmol) was dissolved in water (10 mL), and the pH of the solution was adjusted to 9 with Et_3N. To this solution at 0°C was added di-*tert*-butyl dicarbonate (2.26 g, 11 mmol) in CH_3CN (10 mL) and the pH of the solution monitored and kept at 9 by the addition of Et_3N. The reaction was allowed to stir at room temperature for 24 h, before being concentrated under reduced pressure. The crude product was diluted with EtOAc (50 mL), washed with 1 M HCl (50 mL), dried (Na_2SO_4) and concentrated under reduced pressure to afford **137**, an amorphous mass (2.0 g, 76 %) which was in a pure state according to ^{1}H NMR spectroscopy.

Mp 35-37°C;

^{1}H NMR (300 MHz; $CDCl_3$): δ 1.44 (9H, s, $C(CH_3)_3$), 2.05 (2 H, quin, $J_{2,1}$ 6.5 Hz, H-2), 3.25 (2 H, q , $J_{1,2}$ 6.5 Hz, H-1), 3.44 (2 H, t, , $J_{3,2}$ 6.5 Hz, H-3), 4.88, (1 H, bs, N*H*);

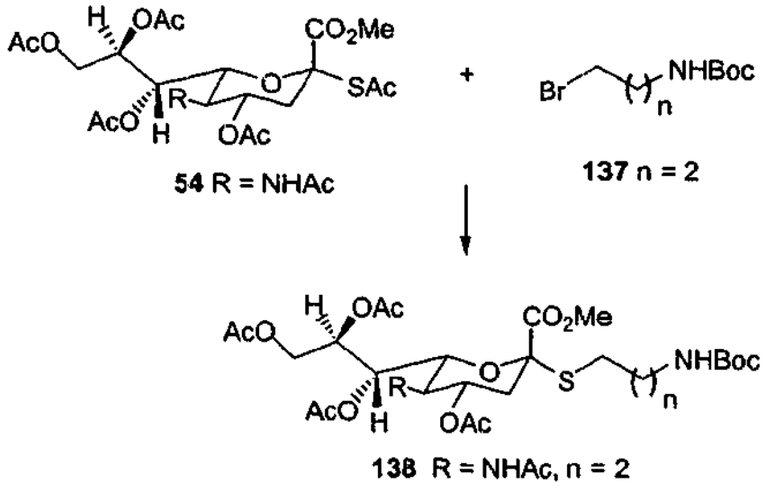
^{13}C NMR (75.5 MHz; $CDCl_3$): δ 28.5 ($C(CH_3)_3$), 30.9 (C-2), 33.0 (C-3), 39.2 (C-1), 79.6 ($C(CH_3)_3$), 156.1 (NC(O)O);

LRFABMS: *m/z* 240 [(^{81}Br M + 1)$^+$, 46%], 238 [(^{79}Br M + 1)$^+$, 59], 184 (100), 182 (100), 138 (70), 140 (53).

HRFABMS (positive-ion): Calcd for $C_8H_{17}NO_2{}^{79}Br$ 238.04427; Found: 238.04449.

$C_8H_{16}NO_2Br$ (238.1131)

Methyl [2-*S*-(3'-*N*-*tert*-butyloxycarbonyl-aminopropyl) 5-acetamido-4,7,8,9-tetra-*O*-acetyl 3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (138)



Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (**54**) (0.7 g, 1.27 mmol) and **137** (0.30 g, 1.27 mmol) were dissolved in dry *N,N*-DMF (9 mL) which was cooled in an ice-bath under N_2, Et_2NH (2.7 mL) was added and the reaction stirred for 15 min in an ice-bath and then warmed to room temperature and stirred for a further 3 h. The mixture was concentrated under reduced pressure and the residue diluted with EtOAc (50 mL) and washed with pH 4 buffer (50 mL), H_2O (2 × 50 mL), dried (Na_2SO_4) and evaporated to dryness under reduced pressure. Column chromatography on silica gel (EtOAc:Hex 9:1) gave **138** (0.74 g, 87%) as a white amorphous mass;

Mp 69-71°C; *Rf* 0.30 (EtOAc:Hex 9:1); $[\alpha]_D^{28}$ +19.86° (*c* 1, $CHCl_3$); ν_{max} (KBr) 3392 (NHBoc), 1738 (COOMe), 1686 (CO), 1226 (AcO), 1030 (C-O-C) cm^{-1};

^1H NMR (300 MHz; $CDCl_3$): **Alkyl unit:** δ 1.38 (9 H, s, $C(CH_3)_3$), 1.65 (2 H, m, H-2), 2.45-2.68 (2 H, m, H-3), 3.12 (2 H, m, H-1); **Neu5Ac unit:** δ 1.81 (3 H, s, AcN), 1.85-2.13 (13 H, m, H-3ax, 4 × AcO), 2.45-2.68 (1 H, m, H-3eq), 3.72-3.78 (4 H, m, CO_2Me, H-6), 3.93-4.09 (2 H, m, H-5, 9a), 4.24 (1 H, dd, $J_{9b,8}$ 2.6, $J_{9b,9a}$ 12.4Hz, H-9b), 4.79 (1 H, ddd, $J_{4,3ax} = J_{4,5}$ 11.0, $J_{4,3eq}$ 4.6Hz, H-4), 5.24-5.34 (2 H, m, H-7, 8), 5.56 (1 H, d, $J_{NH,5}$ 10.0Hz,

NH);

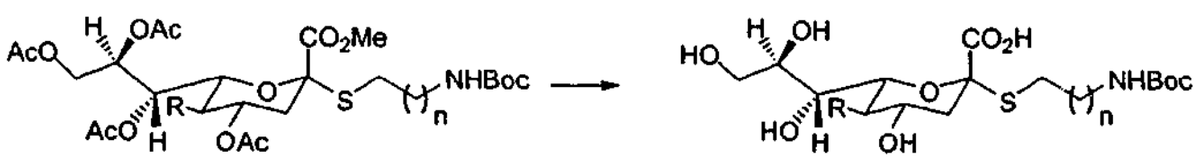
^{13}C NMR (75.5 MHz; $CDCl_3$): **Alkyl unit:** δ 26.7 (C-2), 28.6 ($C(CH_3)_3$), 30.2 (C-3), 39.9 (C-1), 79.0 ($C(CH_3)_3$), 156.2 (NC(O)O); **Neu5Ac unit:** δ 20.9, 21.3 (4 × OC(O)*Me*), 23.2 (NC(O)*Me*), 38.2 (C-3), 49.4 (C-5), 53.0 (CO_2Me), 62.5 (C-9), 67.3, 68.5 (C-7, C-8), 69.8 (C-4), 74.1 (C-6), 83.4 (C-2), 168.6 (C-1), 170.3, 170.5, 170.9, 171.0 (4 × OC(O)Me, NC(O)Me);

LRFABMS: *m/z* 665 [$(M + 1)^+$, 17%], 565(100), 549(78).

Analytical data. Calculated for $C_{28}H_{44}N_2O_{14}S{\cdot}H_2O$: C, 49.27; H, 6.74. Found: C, 49.39; H, 6.68%.

$C_{28}H_{44}N_2O_{14}S$ (664.7206)

2-*S*-(3'-*N*-*tert*-butyloxycarbonyl-aminopropyl) 5-acetamido-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (139)



138 R = NHAc, n = 2 **139** R = NHAc, n = 2

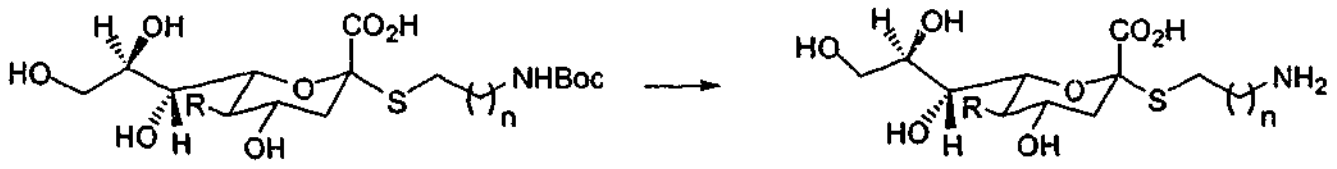
Compound **138** (0.57 g, 0.86 mmol) was treated with a solution of NaOMe (0.43 mmol) in anhydrous MeOH (11 mL) at room temperature under N_2. After stirring for 2 h, the MeOH was removed under reduced pressure, H_2O (10 mL) added and the pH adjusted to 12 with 0.1 M NaOH. After stirring overnight, the pH of the reaction mixture was adjusted to 7.0-7.5 with Dowex-50W×8 (H^+) resin. After filtration, the filtrate was lyophilised to afford compound **139** as an amorphous white solid that was in a pure state according to NMR spectroscopy (0.414 g, 99%);

Mp 176-180 °C; R_f 0.57 (EtOAc:^{i}PrOH:H_2O; 2:3:1);

^{1}H NMR (300 MHz; D_2O): **Alkyl unit:** δ 1.49 (9 H, s, $C(CH_3)_3$), 1.77-1.96 (2 H, m, H-2), 2.59-2.89 (2 H, m, H-3), 3.23 (2 H, m, H-1), **Neu5Ac unit:** δ 1.77-1.96 (1 H, m, H-3ax), 2.11 (3 H, s, AcN), 2.59-2.89 (1 H, m, H-3eq), 3.60-3.94 (7 H, m, H-4, 5, 6, 7, 8, 9a, 9b); ^{13}C NMR (75.5 MHz; D_2O): **Alkyl unit:** δ 29.8 (C-2), 30.9 ($C(CH_3)_3$), 32.6 (C-1), 43.4 (C-3*), 83.7 ($C(CH_3)_3$), 161.0 (NC(O)O); **Neu5Ac unit:** δ 25.2 (NC(O)*Me*), 44.3 (C-3*), 55.0 (C-5), 65.8 (C-9), 71.4 (C-7*), 71.6 (C-8*), 75.0 (C-4), 77.9 (C-6*), 89.2 (C-2), 177.1(C-1), 178.1 (NC(O)Me); LRFABMS: *m/z* 505 [$(M + Na)^+$, 77%], 519(42).

$C_{19}H_{34}N_2O_{10}S$ (482.545)

2-*S*-(3′-Aminopropyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulo-pyranosidonic acid (128)



139 R = NHAc, n = 2 **128** R = NHAc, n = 2

A solution of compound **139** (0.3g 0.62 mmol) and TFA (20 mL) were stirred at room temperature for 10 min under N_2 atmosphere. The solution was then diluted with toluene (50 mL) and then concentrated under reduced pressure. The crude product was slightly red in colour and was purified by reverse phase HPLC (H_2O as eluent) to afford the target compound **128** (170 mg, 71%) as a cream coloured amorphous mass.

Mp 178-180°C (dec); R_f 0.10 (EtOAc:^{i}PrOH:H_2O; 2:3:1); ν_{max} (KBr) 3428, 1626, 1378, 1122 cm^{-1};

^{1}H NMR (300 MHz; D_2O): **Alkyl unit:** δ 1.90 (2H , m, H-2), 2.66-2.81 (2 H, m, H-1), 3.00 (2 H, t, $J_{3,2}$ 6.5 Hz, H-3); **Neu5Ac unit:** δ 1.73 (1 H, t, $J_{3ax,3eq} = J_{3ax,4} = 11.9$ Hz, H-3ax), 1.98 (3 H, s, AcN), 2.66-2.81 (1 H, m, H-3eq), 3.51-3.83 (7 H, m, H-4, 5, 6, 7, 8, 9a, 9b);

^{13}C NMR (75.5 MHz; D_2O): **Alkyl unit:** δ 27.9 (C-2), 29.2 (C-1), 42.4 (C-3); **Neu5Ac unit:** δ 25.1 (NC(O)*Me*), 44.1 (C-3), 54.8 (C-5), 65.8 (C-9), 71.3, 71.5 (C-4/C-7*), 75.1 (C-6*), 77.9 (C-8), 89.2 (C-2), 177.1 (C-1), 178.1 (NH*C*(O)Me);

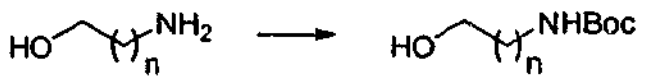
LREIMS: *m/z* 383 [$(M + 1)^+$, 20], 237 (35);

Analytical data; Calculated for $C_{14}H_{26}N_2O_8SNa{\cdot}H_2O$: C, 39.80; H, 6.45. Found: C, 39.75; H, 6.81%;

HRFABMS: $C_{14}H_{27}N_2O_8S$ requires 383.14576, found 383.14880.

$C_{14}H_{26}N_2O_8S$ (382.5486)

4-*N*-(*tert*-Butyloxycarbonyl)aminobutanol (140)



n = 3 **140** n = 3

4-Aminobutanol (1.10 g, 12.3 mmol) was dissolved in water (6 mL), and was cooled to 0°C. To this solution was added di-*tert*-butyl dicarbonate (2.60 g, 12.3 mmol) in CH_3CN (6 mL) and the pH of the solution monitored and kept at 12 by the addition of Et_3N. The reaction was allowed to stir at room temperature for 24 h and then was concentrated under reduced pressure. The crude product was diluted with EtOAc (25 mL), washed with 1 M HCl (25 mL), dried (Na_2SO_4) and concentrated under reduced pressure to afford compound **140** as a white amorphous mass (1.79 g, 77%) which was in a pure state according to ^{1}H NMR spectroscopy.

Mp 38-49°C; *Rf* 0.57 (EtOAc);

^{1}H NMR (300 MHz; $CDCl_3$): 1.42 (9H, s, $C(CH_3)_3$), 1.53-1.60 (4 H, m, H-2, 3), 3.13 (2 H, t, $J_{4,3}$ 6.5 Hz, H-4), 3.64 (2H, t, $J_{1,2}$ 5.9 Hz, H-1), ;

^{13}C NMR (75.5 MHz; $CDCl_3$): 26.9 (C-3), 28.6 ($C(CH_3)_3$), 29.9 (C-2), 40.7 (C-4), 62.5 (C-1), 79.5 ($C(CH_3)_3$), 156.4 (NC(O)O);

LRFABMS: *m/z* 190 [$(M + 1)^+$, 100%], 134(100), 116(40).

HRFABMS (positive-ion): Calcd for $C_9H_{20}NO_3$ 190.14432; Found: 190.14363.

$C_9H_{19}NO_3$ (189.13642)

4-Bromo-*N*-(*tert*-butyloxycarbonyl)-butylamine (142)

140 n = 3 **142** n = 3

A solution of triphenylphosphine (1.98 g, 7.41 mmol) in dichloromethane (12 mL) was added to a stirred suspension of *N*-bromosuccinimide (1.41 g, 7.92 mmol) in dichloromethane (12 mL). After stirring for 5 min at room temperature, pyridine (0.26 mL, 3.16 mmol) was added dropwise, followed by slow addition of the alcohol **140** (0.5 g, 2.64 mmol) in dichloromethane (6 mL). After stirring for 12 h at room temperature, the reaction mixture was poured onto saturated $NaHCO_3$ solution (25 mL), washed with water (25 mL), washed with brine (25 mL), dried (Na_2SO_4) and concentrated under reduced pressure. Column chromatography on silica gel (EtOAc:hexane 1:2) gave **142** (0.55 g, 83%) as an amorphous white crystalline product.

Mp 31-34°C; *Rf* 0.60 (EtOAc:hexane 1:2);

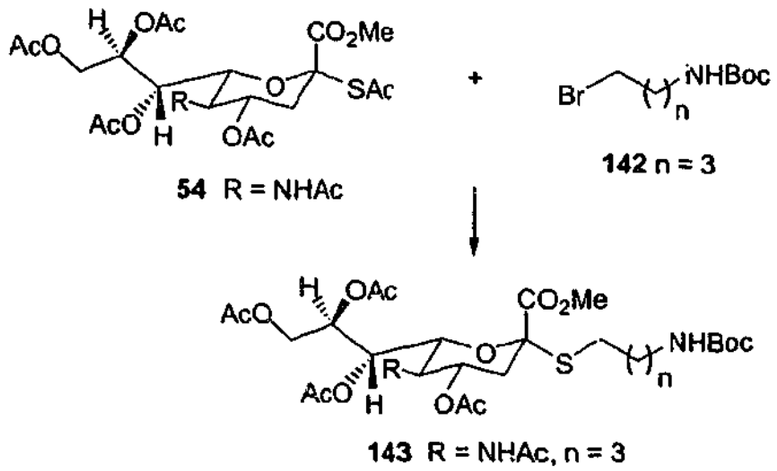
^{1}H NMR (300 MHz; $CDCl_3$): δ 1.35 (9H, s, $C(CH_3)_3$), 1.55 (2 H, m, H-2), 1.78 (2 H, m, H-3), 3.05 (2 H, q, $J_{1,2}$ 6.3 Hz, H-1), 3.33 (2 H, t, $J_{5,4}$ 6.7 Hz, H-5), 4.80 (1 H, bs, N*H*);

^{13}C NMR (75.5 MHz; $CDCl_3$): δ 28.5 ($C(CH_3)_3$), 28.7 (C-3), 29.9 (C-2), 33.1 (C-4), 39.6 (C-1), 78.9 ($C(CH_3)_3$), 156.0 (NC(O)O);

LRFABMS: *m/z* 254 [(^{81}Br M + 1)$^+$, 35%], 252 [(^{79}Br M + 1)$^+$, 45%], 198 (100), 195 (100). HRFABMS (positive-ion): Calcd for $C_9H_{19}NO_3{}^{79}Br$ 252.05848; Found: 252.05992.

$C_9H_{18}NO_2Br$ (252.1507)

Methyl [2-*S*-(4'-*N*-*tert*-butyloxycarbonyl-aminobutyl) 5-acetamido-4,7,8,9-tetra-*O*-acetyl 3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (143)



Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (**54**) (0.58 g, 0.69 mmol) and **142** (0.175 g, 0.69 mmol) were dissolved in dry *N,N*-DMF (6 mL) at 0°C under N_2, Et_2NH (2.4 mL) was added and the reaction stirred for 15 min and warmed to room temperature and stirred for a further 3 h. The mixture was concentrated under reduced pressure and the residue diluted with EtOAc (35 mL) and washed with pH 4 buffer (35 mL), H_2O (2 × 35 mL), dried (Na_2SO_4) and evaporated to dryness under reduced pressure. Column chromatography on silica gel (EtOAc:Hex 4:1) isolated **143** (0.45 g, 99%) as an amorphous mass;

Mp 65-68°C; *Rf* 0.33 (EtOAc:Hex 4:1); $[\alpha]_D^{28}$ +24.6° (*c* 1, $CHCl_3$); ν_{max} (KBr) 3450 (NHBoc), 1744 (COOMe), 1686 (CO), 1224 (AcO), 1032 (C-O-C) cm^{-1};

^1H NMR (300 MHz; $CDCl_3$): **Alkyl unit:** δ 1.43 (9 H, s, $C(CH_3)_3$), 1.48-1.59 (4H, m, H-2, 3), 2.56 (1 H, m, H-1a), 2.66-2.75 (1 H, m, H-1b), 3.14 (2 H, m, H-4); **Neu5Ac unit:** δ 1.88 (3 H, s, AcN), 1.96 (1 H, m, H-3ax), 2.03, 2.04, 2.15, 2.18 (12 H, 4 × s, 4 × AcO), 2.66-2.75 (1H, m, H3eq), 3.80 (3 H, s, CO_2Me), 3.83 (1 H, dd, $J_{6,7}$ 1.9 Hz, H-6), 3.98-4.10 (2 H, m, H-5, 9a), 4.31 (1 H, dd, $J_{9b,9a}$ 12.5, $J_{9b,8}$ 2.5 Hz, H-9b), 4.86 (1 H, ddd, $J_{4,3ax}$ 11.3,

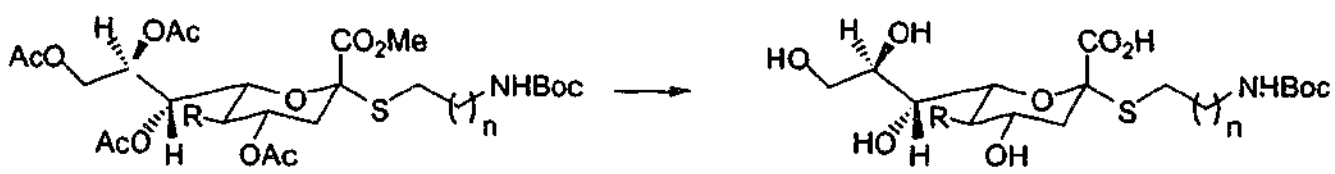
$J_{4,5}$ 11.0, $J_{4,3eq}$ 4.7 Hz, H-4), 5.14 (1 H, d, $J_{NH,5}$ 9.4 Hz, NH), 5.32 (1 H, dd, $J_{7,8}$ 8.9, $J_{7,8}$ 1.9 Hz, H-7), 5.38 (1 H, ddd, $J_{8,9a}$ 5.1 Hz, H-8);

^{13}C NMR (75.5 MHz; $CDCl_3$): **Alkyl unit:** δ 26.8 (C-3), 28.6 (C(CH_3)$_3$), 29.1 (C-2), 29.5 (C-1), 40.0 (C-4), 79.1 (*C*(CH_3)$_3$), 156.2 (NC(O)O); **Neu5Ac unit:** δ 20.8, 20.9, 21.3 (4 × OC(O)*Me*), 23.2 (NC(O)*Me*), 38.3 (C-3), 49.5 (C-5), 53.0 (CO_2*Me*), 62.5 (C-9), 67.5, 68.7 (C-7/C-8), 69.9 (C-4), 74.2 (C-6), 83.3 (C-2), 168.7 (C-1), 170.3, 170.8, 171.0 (4 × O*C*(O)Me, N*C*(O)Me);

LRFABMS: *m/z* 679 [$(M + 1)^+$, 11%], 578(100), 414(100).

$C_{29}H_{46}N_2O_{14}S$ (678.7474)

2-*S*-(4′-*N*-*tert*-butyloxycarbonyl-aminobutyl) 5-acetamido-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (144)



143 R = NHAc, n = 3 **144** R = NHAc, n = 3

Compound **143** (0.20 g, 0.36 mmol) was treated with a solution of NaOMe (0.19 mmol) in anhydrous MeOH (5 mL) at room temperature under N_2. After stirring for 2 h, the MeOH was removed under reduced pressure, H_2O (5 mL) added and the pH adjusted to 12 with 0.1 M NaOH. After stirring overnight, the pH of the reaction mixture was adjusted to 7.0-7.5 with Dowex-50W×8 (H^+) resin. After filtration, the filtrate was lyophilised to afford the target compound **144** as an amorphous solid that was in a pure state according to NMR spectroscopy (0.13 g. 87%);

Mp 168-170°C;

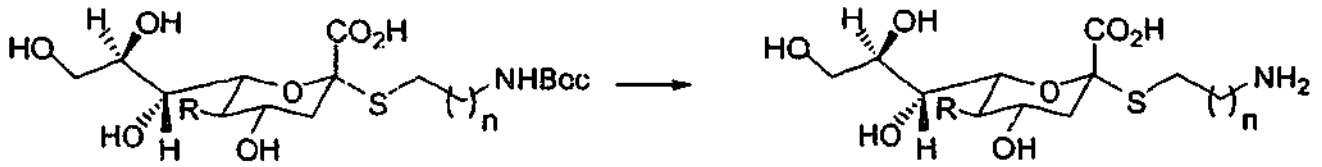
^{1}H NMR (300 MHz; D_2O): **Alkyl unit:** δ 1.35 (9 H, s, $C(CH_3)_3$), 1.40-1.60 (4 H, m, H-3/H-2), 2.51-2.67 (2 H, m, H-1), 2.98 (t, 2 H, $J_{4,3}$ 6.6 Hz, H-4); **Neu5Ac unit:** δ 1.81 (1 H, t, $J_{3ax,3eq}$ 12.0, $J_{3ax,4}$ 11.4 Hz, H-3ax), 1.95 (3 H, s, AcN), 2.72 (1 H, dd, $J_{3eq,4}$ 4.7 Hz, H-3eq), 3.47-3.64 (4 H, m, H-4, 6, 7, 9a), 3.69-3.88 (3 H, m, H-5, 8, 9b);

^{13}C NMR (75.5 MHz; D_2O): **Alkyl unit:** δ 29.6 (C-3), 30.7 ($C(CH_3)_3$), 31.5 (C-2), 32.1 (C-1), 42.6, 44.2 (C-3/C-4), 160.9 (NC(O)O); **Neu5Ac unit:** δ 24.9 (NC(O)*Me*), 54.9 (C-5), 65.7 (C-9), 71.2 (C-7*), 71.4 (C-8*), 74.9 (C-4), 77.8 (C-6*), 83.3 ($C(CH_3)_3$), 89.1 (C-2), 177.0 (C-1), 177.9 (NC(O)Me);

LRFABMS: *m/z* 540 [$(M + 2Na)^+$, 92%], 518 [$(M + Na)^+$, 72%], 441(28).

$C_{20}H_{36}N_2O_{10}S$ (496.5718)

2-*S*-(4′-*N*-*tert*-butyloxycarbonyl-aminobutyl) 5-acetamido-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (129)



144 R = NHAc, n = 3 **129** R = NHAc, n = 3

Compound **144** (0.10 g, 0.21 mmol) in trifluoroacetic acid (6 mL) was stirred at room temperature for 10 min. The reaction mixture was diluted with toluene and concentrated under reduced pressure. The crude product was taken up in toluene a further two times to remove any residual trifluoroacetic acid. The crude product was purified by reverse phase HPLC (3% acetonitrile in H_2O as eluent) to afford the target compound **129** (63.2 mg, 78%) as a white amorphous mass;

Mp 196-200°C (dec); ν_{max} (KBr) 3450, 1626, 1380, 1126 cm^{-1};

^{1}H NMR (500 MHz; D_2O): **Alkyl unit:** δ 1.66-1.80 (4 H, m, H-3, 2), 2.68-2.80 (2 H, m, H-1), 3.03 (2 H, t, $J_{4,3}$ 7.2 Hz, H-4); **Neu5Ac unit:** δ 1.66-1.80 (1 H, m, H-3ax), 2.06 (3 H, s, AcN), 2.83 (1 H, dd, $J_{3eq,3ax}$ 13.0, $J_{3eq,4}$ 4.8 Hz, H-3eq), 3.59-3.73 (4 H, m, H-4, 6, 7, 9a), 3.81-3.86 (2 H, m, H-5, 8), 3.88 (1H, dd, $J_{9b,9a}$ 11.8, $J_{9b,8}$ 2.3 Hz, H-9b);

^{13}C NMR (75.5 MHz; D_2O): **Alkyl unit:** δ 29.0, 29.3 (C-3, C-2), 31.8 (C-1), 44.2 (C-4); **Neu5Ac unit:** δ 25.1 (NC(O)*Me*), 42.1 (C-3*), 54.3 (C-5), 65.8 (C-9), 71.3, 71.5 (C-4/C-7*), 75.2 (C-6*), 77.9 (C-8), 89.3 (C-2), 177.2 (C-1), 178.2 (NH*C*(O)Me);

LRFABMS: *m/z* 419 [$(M + Na)^+$, 20%], 397 [$(M + 1)^+$, 100], 237 (30); HRFABMS (positive-ion): Calcd for $C_{15}H_{29}N_2O_8S$ 397.16446; Found: 397.16409.

Analytical data. Calculated for $C_{15}H_{28}N_2O_8S{\cdot}2H_2ONa$: C, 38.87; H, 6.96; N, 6.04. Found: C, 38.88; H, 6.72; N, 5.89%.

$C_{15}H_{28}N_2O_8S$ (396.4548)

5-Hydroxy-*N*-(*tert*-Butyloxycarbonyl)-pentylamine (147)

n = 4 **147** n = 4

5-Aminopentanol (1.00 g, 9.7 mmol) was dissolved in water (6 mL), and was cooled to 0°C. To this solution was added di-*tert*-butyl dicarbonate (2.04 g, 9.7 mmol) in CH_3CN (6 mL) and the pH of the solution monitored and kept at 12 by the addition of Et_3N. The reaction was allowed to stir at room temperature for 12 h, then a further amount of di-*tert*-butyl dicarbonate (2.04 g, 9.7 mmol) was added. After stirring for a further 4 h at room temperature, the reaction mixture was concentrated under reduced pressure. The crude product was diluted with CH_2Cl_2 (25 mL), washed with 1 M HCl (25 mL), dried (Na_2SO_4) and concentrated under reduced pressure to give compound **147** as a clear oil (1.95 g, 99%) which was in a pure state according to ^{1}H NMR spectroscopy.

Rf 0.68 (EtOAc); ν_{max} (NaCl) 3376, 2984, 2944, 1690 cm^{-1};

^1H NMR (300 MHz; $CDCl_3$): 1.37 (9H, s, $C(CH_3)_3$), 1.26-1.58 (6 H, m, H-2, 3, 4), 3.04, (2 H, t, $J_{5,4}$ 6.9 Hz H-5), 3.55 (2H, t, $J_{1,2}$ 6.5 Hz, H-1) ;

^{13}C NMR (75.5 MHz; $CDCl_3$): 23.0 (C-3), 28.5 ($C(CH_3)_3$), 29.8 (C-4), 32.2 (C-2), 40.7 (C-5), 62.4 (C-1), 79.2 ($C(CH_3)_3$), 156.3 (NC(O)O);

LRFABMS: *m/z* 204 [$(M + 1)^+$, 39%], 148(100), 104(100).

HRFABMS (positive-ion): Calcd for $C_{10}H_{22}NO_3$ 204.15997; Found: 204.15894.

$C_{10}H_{21}NO_3$ (203.2808)

5-Bromo-*N*-(*tert*-butyloxycarbonyl)-pentylamine (145).

147 n = 4 **145** n = 4

A solution of triphenylphosphine (1.84 g, 6.89 mmol) in dichloromethane (12 mL) was added to a stirred suspension of *N*-bromosuccinimide (1.31 g, 7.38 mmol) in dichloromethane (12 mL). After stirring for 5 min at room temperature, pyridine (0.24 mL, 2.95 mmol) was added dropwise, followed by slow addition of the alcohol **147** (0.5 g, 2.46 mmol). After stirring for 8 h at room temperature, the reaction mixture was poured onto saturated $NaHCO_3$ solution (25 mL), washed with water (25 mL), washed with brine (25 mL), dried (Na_2SO_4) and concentrated under reduced pressure. Column chromatography on silica gel (EtOAc:hexane 1:2) afforded **145** (0.34 g, 52%) as an amorphous pink-coloured mass;

Mp 26-27°C (dec); *Rf* 0.63 (EtOAc:hexane 1:2); ν_{max} (NaCl)3368, 2984, 2940, 1688cm^{-1};

^1H NMR (300 MHz; $CDCl_3$): δ 1.41 (9H, s, $C(CH_3)_3$), 1.53-1.41 (4 H, m, H-4, 3), 1.84 (2 H, p, $J_{2,1}$ 6.5 Hz, H-2), 3.09 (2 H, q, $J_{1,2}$ 6.5 Hz, H-1), 3.38 (2 H, t, $J_{5,4}$ 6.8 Hz, H-5), 4.63 (1 H, bs, N*H*);

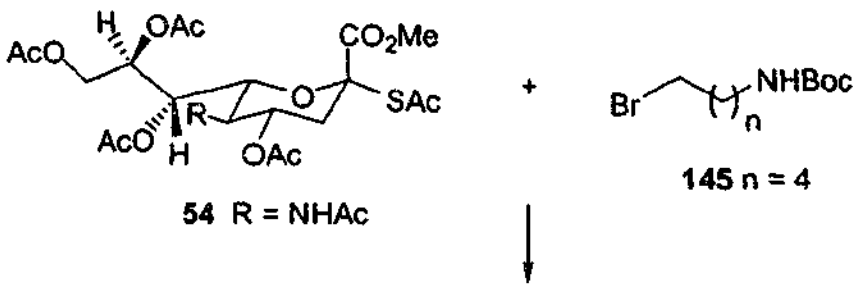
^{13}C NMR (75.5 MHz; $CDCl_3$): δ 25.5 (C-3), 28.5 ($C(CH_3)_3$), 29.4 (C-4), 32.4 (C-2), 33.8 (C-5), 40.4 (C-1), 79.2 ($C(CH_3)_3$), 156.1 (NC(O)O);

LRFABMS: *m/z* 268 [(^{81}Br M + 1)$^+$, 38%], 266 [(^{79}Br M + 1)$^+$, 39%], 212 (43), 210 (47), 192 (32), 168 (15).

HRFABMS (positive-ion): Calcd for $C_{10}H_{21}NO_3{}^{79}Br$ 266.07556; Found: 266.07574.

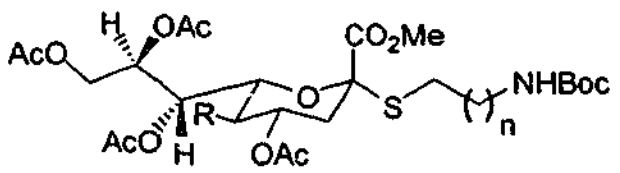
$C_{10}H_{20}NO_2Br$ (266.1775)

Methyl [2-*S*-(5′-*N*-*tert*-butyloxycarbonyl-aminopentyl) 5-acetamido-4,7,8,9-tetra-*O*-acetyl 3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (148)[280]



54 R = NHAc

145 n = 4



148 R = NHAc, n = 4

Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosonate (**54**) (0.7 g, 1.27 mmol) and **145** (0.34 g, 1.27 mmol) were dissolved in dry *N,N*-DMF (8 mL) at room temperature under N_2, Et_2NH (3.2 mL) was added and the reaction stirred for 3 h at room temperature. The mixture was concentrated under reduced pressure and the residue diluted with EtOAc (50 mL) and washed with pH 4 buffer (50 mL), H_2O (2 × 50 mL), dried (Na_2SO_4) and evaporated to dryness under reduced pressure. Column chromatography on silica gel (EtOAc) gave **148** (0.71 g, 80%) as an amorphous mass;

Mp 61-64°C; *Rf* 0.57 (EtOAc); $[\alpha]_D^{28}$ +23.5° (*c* 1, $CHCl_3$); ν_{max} (KBr) 3408 (NHBoc), 1740 (COOMe), 1688 (CO), 1228 (AcO), 1030 (C-O-C) cm^{-1};

^1H NMR (300 MHz; $CDCl_3$): **Alkyl unit:** δ 1.39-1.59 (6H, m, H-2, 3, 4), 1.44 (9 H, s, $C(CH_3)_3$), 2.49-2.59 (1 H, m, H-1a), 2.67-2.79 (1 H, m, H-1b), 3.11 (2 H, q, $J_{5,4}$ 6.2 Hz, H-5), 4.76 (1 H, bs, NHC(O)O); **Neu5Ac unit:** δ 1.88 (3 H, s, AcN), 1.89-2.03 (1 H, m, H-3ax), 2.03, 2.04, 2.15, 2.17 (12 H, 4 × s, 4 × AcO), 2.67-2.79 (2 H, m, H-3eq), 3.80 (3 H, s, CO_2Me), 3.83 (1 H, dd, $J_{6,7}$ 2.0 Hz, H-6), 4.02-4.34 (2 H, m, H-5, 9a), 4.32 (1 H, dd, $J_{9b,9a}$ 9.9, $J_{9b,9a}$ 2.7 Hz, H-9b), 4.87 (1 H, ddd, $J_{4,3ax}$ 11.0, $J_{4,5}$ 10.5, $J_{4,3eq}$ 4.6 Hz, H-4), 5.11 (1 H, d, $J_{NH,5}$ 10.0 Hz, NH), 5.31 (1 H, dd, $J_{7,8}$ 8.6 Hz, H-7), 5.37 (1 H, ddd, $J_{8,9a}$ 5.2 Hz, H-8);

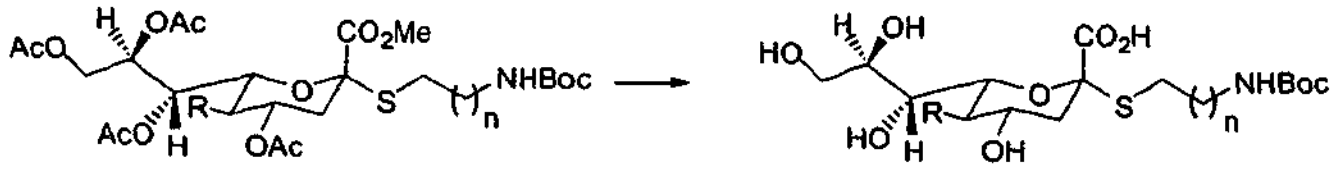
^{13}C NMR (75.5 MHz; $CDCl_3$): **Alkyl unit:** δ 26.1 (C-3), 28.6 ($C(CH_3)_3$), 28.8 (C-4), 29.1 (C-2), 29.5 (C-1), 41.1 (C-5), 83.4 ($C(CH_3)_3$*), 156.2 (NC(O)O); **Neu5Ac unit:** δ 20.9, 21.0, 21.3 (4 × OC(O)*Me*), 23.3 (NC(O)*Me*), 38.3 (C-3), 49.6 (C-5), 53.1 (CO_2Me), 62.5 (C-9), 67.7, 68.9 (C-7/C-8), 69.9 (C-4), 74.3 (C-6), 83.4 (C-2), 168.7 (C-1), 170.4, 170.8, 171.1 (4 × O*C*(O)Me, N*C*(O)Me);

LRFABMS: *m/z* 693 [$(M + 1)^+$, 10%], 593(40), 414(44).

Analytical data. Calculated for $C_{30}H_{48}N_2O_{14}S \cdot H_2O$: C, 50.69; H, 7.09; N, 3.94. Found: C, 50.26; H, 6.76; N, 3.94%.

$C_{30}H_{48}N_2O_{14}S$ (692.7742)

2-*S*-(5'-*N*-*tert*-butyloxycarbonyl-aminopentyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (149)[280]



148 R = NHAc, n = 4 **149** R = NHAc, n = 4

Compound **148** (0.61 g, 0.88 mmol) was treated with a solution of NaOMe (0.44 mmol) in anhydrous MeOH (12 mL) at room temperature under N_2. After stirring for 2 h, the MeOH was removed under reduced pressure, H_2O (12 mL) added and the pH adjusted to 12 with 0.1 M NaOH. After stirring overnight, the pH of the reaction mixture was adjusted to 7.0-7.5 with Dowex-50W×8 (H^+) resin. After filtration, the filtrate was lyophilised to afford the target compound **149** as an amorphous cream colored solid which was in a pure state according to NMR spectroscopy (0.37 g, 83%);

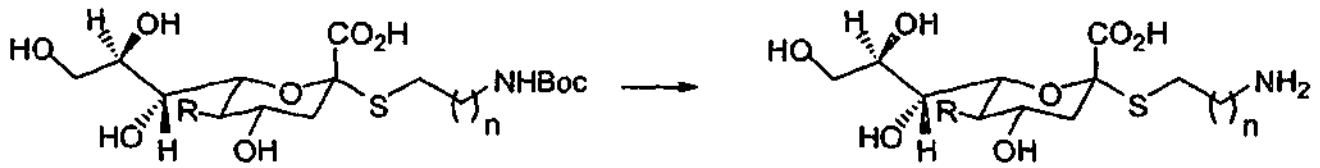
Mp 148°C (dec); *Rf* 0.72 (EtOAc:^{i}PrOH:H_2O; 2:3:1); $[\alpha]_D^{28}$ +21.7 ° (*c* 0.5, H_2O); ν_{max} (KBr) 3448, 1616, 1366, 1032 cm^{-1};

^{1}H NMR (300 MHz; D_2O): **Alkyl unit:** δ 1.41-1.58 (4 H, m, H-3, 4), 1.50 (9 H, s, $C(CH_3)_3$), 1.63-1.74 (2H, m, H-2), 2.64-2.82 (2 H, m, H-1), 3.10-3.14 (2 H, t, $J_{5,4}$ 6.6 Hz, H-5); **Neu5Ac unit:** δ 1.81 (1 H, dd, $J_{3ax,3eq}$ 12.0, $J_{3ax,4}$ 11.9 Hz, H-3ax), 2.11 (3 H, s, AcN), 2.87 (1 H, dd, $J_{3eq,4}$ 4.7 Hz, H-3eq), 3.60-3.81 (4 H, m, H-4, 6, 7, 9a), 3.84-3.95 (3 H, m, H-5, 8, 9b);

^{13}C NMR (75.5 MHz; D_2O): **Alkyl unit:** δ 28.5 (C-3), 31.0 ($C(CH_3)_3$), 31.6 (C-4), 32.1 (C-2), 32.5 (C-1), 43.1 or 44.3 (C-5), 83.8 ($C(CH_3)_3$), 161.0 (NC(O)O); **Neu5Ac unit:** δ 25.2 (NC(O)*Me*), 43.1 or 44.3 (C-3), 54.9 (C-5), 65.7 (C-9), 71.3 (C-7*), 71.6 (C-8*), 74.1 (C-4), 77:9 (C-6*), 89.4 (C-2), 177.2 (C-1), 178.1 (NC(O)Me);

LRFABMS: *m/z* 533 [$(M + Na)^+$, 33%], 455(31); $C_{21}H_{38}N_2O_{10}S$ (510.5986)

2-*S*-(5'-aminopentyl) 5-acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulo-pyranosidonic acid (130)[280]



149 R = NHAc, n = 4 **130** R = NHAc, n = 4

Compound **149** (0.25 g, 0.49 mmol) in trifluoroacetic acid (8 mL) was strirred at room temperature for 10 min. The reaction mixture was diluted with toluene and concentrated under reduced pressure. The crude product was taken up in toluene a further two times to remove any residual trifluoroacetic acid. The crude product was purified by reverse phase HPLC (3% acetonitrile in H_2O as eluent) to afford the target compound **130** (170.5 mg, 85%) as a white amorphous mass.

Mp 192-194°C (dec); (^{i}PrOH:H_2O; 3:1, R_f 0.43); $[\alpha]_D^{28}$ +44.8° (*c* 0.56, H_2O); ν_{max} (KBr) 3412, 1626, 1374, 1124 cm^{-1};

^{1}H NMR (500 MHz; D_2O): **Alkyl unit:** δ 1.41-1.52 (2 H, m, H-3), 1.61-1.79 (4 H, m, H-2, 4), 2.63-2.69 (1 H, m, H-1a), 2.73-2.79 (1 H, m, H-1b), 3.0 (2 H, t, $J_{5,4}$ 7.5 Hz, H-5); **Neu5Ac unit:** δ 1.61-1.79 (1 H, m, H-3ax), 2.1 (3 H, s, AcN), 2.82 (1 H, dd, $J_{3eq,3ax}$ 13.0, $J_{3eq,4}$ 12.8 Hz, H-3eq), 3.58-3.72 (4 H, m, H-4, 6, 7, 9a), 3.80-3.85 (3 H, m, H-5, 8, 9b);

^{13}C NMR (75.5 MHz; D_2O): **Alkyl unit:** δ 27.2 (C-3, C-4), 38.3 (C-2), 40.4 (C-5), 41.9 (C-1); **Neu5Ac unit:** δ 23.1 (NC(O)*Me*), 39.6 (C-3), 52.8 (C-5), 64.8 (C-9), 69.2, 69.4 (C-4/C-7*), 73.0 (C-6*), 75.9 (C-8), 86.9 (C-2), 174.9 (C-1), 176.1 (NH*C*(O)Me);

LRFABMS: *m/z* 433 [$(M + Na)^+$, 10%], 411 [$(M + 1)^+$, 23], 383 (100), 237 (38);

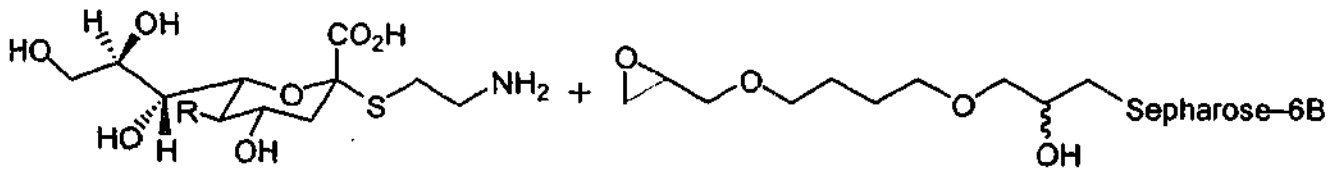
HRFABMS (positive-ion): Calcd for $C_{16}H_{31}N_2O_8S$ 411.18011; Found: 411.18095.

$C_{16}H_{30}N_2O_8S$ (410.4916)

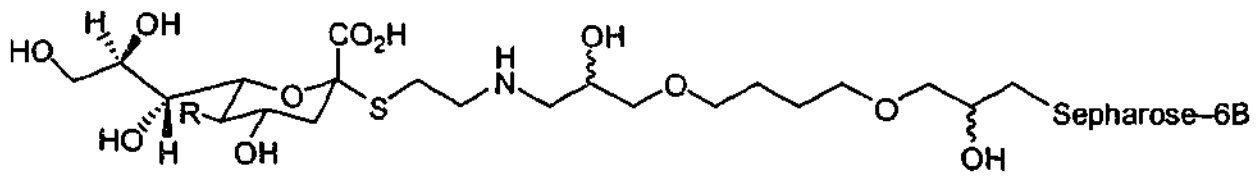
* Tentative assignments

5.4 Biochemistry

5.4.1 Preparation of epoxy-activated Sepharose 6B affinity matrices (150)[279]



127 R = NHAc



150 R = NHAc

Epoxy-activated Sepharose 6B was hydrated and washed with distilled H_2O as per manufacturer's instructions. The ligand **127** (40 mg for each 1 ml of swollen matrix) was dissolved in enough 0.4 M Na_2CO_3 (pH 13.0) to allow adequate mixing and then combined with the swollen matrix. The pH was checked and adjusted if needed. The suspension was mixed at 21± 2 °C for 24 h on a rotating platform. After coupling, the Sepharose was collected by filtration and uncoupled **127** was washed away with 0.4 M Na_2CO_3 (pH 13.0) followed by distilled H_2O. The binding of **127** onto the Sepharose 6B was determined by measuring the absorbance at 220 nm against a standard curve of known amounts of **127** in 0.4 M Na_2CO_3 (pH 13.0), and was estimated to be 15%. The remaining active groups on the Sepharose were blocked with ethanolamine (1.0 M, pH 8.0) for 16 h at 35 °C, and then the matrix washed with three cycles of 0.1 M Tris (pH = 8 by adding HCl) containing 0.5 M NaCl followed by 0.1 M NaOAc (pH 4.0) containing 0.5 M NaCl. It was then equilibrated with 50 mM MES buffer (pH 5.6) containing 0.02 % aq. NaN_3 and stored at 4 °C until use. (This cycle of washing was also used to regenerate the column after use in the purification of *V.c.* sialidase.)

5.4.2 Expression of *Vibrio cholerae* sialidase

The *nanH* gene of *V.c.* sialidase, kindly supplied by E. Vimr (University of Illinois, Urbana, Illinois), was expressed in *E. coli* as described by Vimr *et al*[288] and Taylor *et al.*[258] Briefly, the *E. coli* cell line HB101 transformed with pCVD364 was grown overnight in 2YT culture at 37 °C with vigorous shaking. The expressed sialidase was extracted by the osmotic shock procedure. The final pellet, containing the sialidase activity, was resuspended in 50 mM NaOAc (pH 5.2, 4 ml), $CaCl_2$ (9 mM), NaCl (154 mM) and 0.05% NaN_3 for storage at 4 °C.

5.4.3 Affinity Chromatography with epoxy-activated Sepharose 6B

All purification procedures were carried out at 4 °C. The *V.c.* sialidase was exchanged into 50 mM MES buffer (pH 5.6) and $CaCl_2$ (6 mM) and loaded onto affinity column **150** which was equilibrated with the same buffer. Unbound proteins were eluted with 50 mM MES buffer (pH 5.6) + $CaCl_2$ (6 mM) until the protein concentration came down to base line. The bound sialidase was then eluted with 50 mM MES (pH 5.6) + 1.0 M NaCl + 0.01% Triton X-100 + 1 mM EDTA. Five ml fractions were collected at the rate of 0.75 ml $minute^{-1}$. The protein concentration was followed by absorbance at 280 nm and the sialidase activity was assayed using the fluorescence substrate MUN as described below. Fractions were pooled, concentrated and run on 12.5 % SDS-PAGE. Proteins were visualised by silver staining. A parallel experiment was performed without adding $CaCl_2$ to the loading buffer.

5.4.4 Sialidase Assay

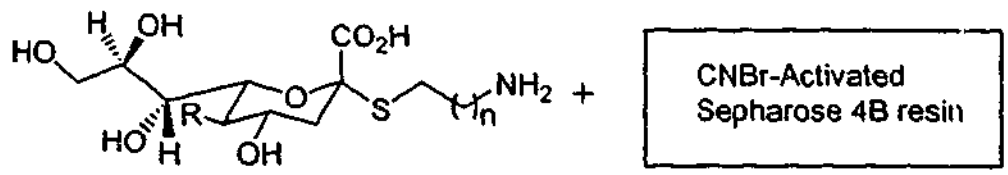
Sialidase activity was assayed using a modification[291] of a fluorometric method previously described[292] and MUN was used as substrate in NaOAc buffer (pH 5.6). $CaCl_2$ at 24 mM was added when the buffer system contained EDTA (1 mM), and at 6 mM when the buffer

system did not contain EDTA. Results were calculated from a 4-methylumbelliferyl (MU) standard curve. 1 unit of activity was defined as the amount of enzyme which catalyses 1 μmol of MU cleaved/hour.

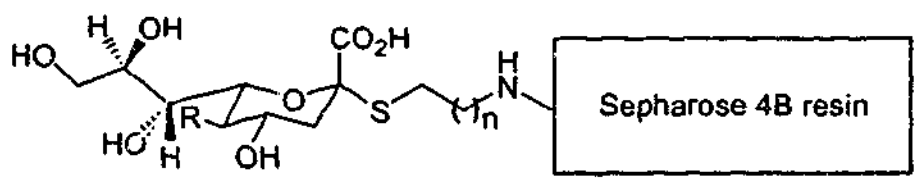
5.4.5 K_m Determination:

The K_m of *V.c.* sialidase for the substrate MUN (4-methylumbelliferyl α-D-*N*-acetylneuraminic acid) was determined using the sialidase assay mentioned above with a varying amount of substrate. Substrate concentrations ranged from 0 to 150 mM MUN. The K_m was calculated using a double reciprocal Lineweaver-Burk plot. The inhibition constant (K_i) for **127** against *V.c.* sialidase was determined by Dixon plot at two different MUN concentrations, 100 mM and 350 mM MUN and various inhibitor concentrations .[119]

5.4.6 Preparation of affinity matrix with CNBr-activated Sepharose 4B[280].



127 n = 1, R = NHAc
130 n = 4, R = NHAc



151 n = 1, R = NHAc
152 n = 4, R = NHAc

CNBr Sepharose 4B was hydrated and swollen in 1 mM HCl as per manufacturer's instructions. The ligand **127** or **130** (2.2 or 2.5 mg, respectively, for each 1 mL of swollen matrix) was dissolved in enough coupling buffer, 0.1 M $NaHCO_3$ (pH 9) to allow adequate mixing and then combined with the swollen matrix. The pH was checked and adjusted if

needed. The suspension was mixed at room temperature for 2-16 h on a rotating platform. After coupling, the Sepharose was collected by filtration and uncoupled **127** or **130** was washed away with 0.1 M $NaHCO_3$ (pH 9.0). The residual active groups on the Sepharose were blocked with a large excess of 0.1 M Tris-HCl (pH 8.0) for 2 h at room temperature, and then the matrix washed with three cycles of 0.1 M Tris (pH 8) containing 0.5 M NaCl followed by 0.1 M NaOAc (pH 4.0) and 0.5 M NaCl. The affinity matrix **151** or **152** was then equilibrated with a suitable buffer containing 0.02 % aq. NaN_3 and stored at 4 °C until use. (This cycle of washing was also used to regenerate the column after use in the purification of any SARPs). The amount of **127** or **130** bound onto the Sepharose was determined by measuring the absorbance at 220 nm against a standard curve of known amounts of **127** or **130** in 0.1 M $NaHCO_3$ (pH 9), and was estimated to be over 75%.

5.4.6.1 Affinity Chromatography with CNBr-activated Sepharose 4B

All purification procedures were carried out at 4 °C. The *nanH* gene of *V.c.* sialidase, was overexpressed and purified under similar conditions as described by Vimr *et al* [288] and Taylor *et al.*[258] The recombinant sialidase-L was exchanged into 20 mM sodium phosphate buffer pH 6.8 containing 1 mM EDTA before being loaded onto the affinity column previously equilibrated with the same buffer. The enzyme was eluted with the same buffer containing 1 M NaCl. *Trans*-sialidase was exchanged onto 50 mM cacodylic acid (Na salt) pH 6.9 then loaded onto the affinity column equilibrated with same buffer. The enzyme was then eluted with the equilibration buffer in the presence of 1 M NaCl. Rat liver was used as the source for sialyltransferases where Golgi-enriched fractions were prepared according to Leelavathi *et al.*[293] This crude enzyme preparation, as well as the affinity column were equilibrated in 50 mM cacodylic acid (Na salt) pH 6.5. A discontinous gradient of varying concentrations (0.1 - 1 M) of NaCl was used to elute the enzyme activity. For all the above mentioned enzymes, 4 mL fractions were collected at the rate of

0.75 ml./minute. The protein concentration was followed by absorbance at 280 nm and the enzymes activities were assayed as described below. Fractions were pooled, concentrated and run on 12.5 % SDS-PAGE. Proteins were visualised by silver staining.

5.4.6.2 Enzymes activity assays

V.c. sialidase was assayed as described in section 5.4.4 (*vide supra*) using MUN as substrate. MUN was also the substrate for Sialidase-L following a published assay.[294] For both enzymes, activities were calculated from a MU standard curve. One unit of activity was defined as the amount of enzyme which catalyses 1 μ mol of MU cleaved/hour. *Trans*-sialidase was assayed following the published method[289] using [^{14}C] *N*-acetyllactosamine (Sigma) as acceptor substrate and 2,3-sialyllactose as the specific donor substrate for this enzyme. Sialyltransferases were assayed following the general procedure published by Harvey and Thomas.[205] The donor substrate for the assay was [^{14}C] CMPNeu5Ac (NEN Chemicals). Asialofetuin was used as a general acceptor substrate for sialyltransferases.

Appendix A

Minimisation profile for all of the protein and ligand

INPUT FILE FOR DISCOVER GENERATED BY INSIGHT

```
Overlap = 0.01

 begin simulation
*        add-automatic bond torsion valence out-of-plane
 reduce

 set dielectric = 1.000000*r
 cutoff = 15.0
 swtdis = 1.5
 cutdis = 11.5
 nrneib = 20.0

 fixed atom list generation; brief
 * add heavy atoms molecules 1

 minimize using steepest descents for 1000 cycles
*        until the maximum derivative is less than 10.0
*        with no cross terms and no morse functions

 fixed atom list generation; brief
 * clear

 fixed atom list generation; brief
 * add main
 * molecule 1

 minimize using steepest descents for 1000 cycles
*        until the maximum derivative is less than 10.0
*        with no cross terms and no morse functions

 fixed atom list generation; brief
* clear

 tethered atoms list generation
* add main
* molecule 1

 template force for 500 cycles
* using steepest descents
* with no more functions and
* no cross terms
* and a forcing constant of 500.0 kcal/angstrom

 template force for 500 cycles
* using steepest descents
```

```
* with no more functions and
* no cross terms
* and a forcing constant of 100.0 kcal/angstrom

 template force for 500 cycles
* using steepest descents
* with no more functions and
* no cross terms
* and a forcing constant of 50.0 kcal/angstrom

 tethered atoms list generation
* clear

 minimize using steepest descents for 500 cycles
* until the maximum derivative is less than 5.0 kcal/angstrom
* with no cross terms and no morse functions

 minimize using conjugate gradient for 5000 cycles
* until the maximum derivative is less than 0.1 kcal/angstrom
* with no cross terms and no morse functions

end
```

Appendix B

Molecular dynamics on protein and ligand

INPUT FILE FOR DISCOVER GENERATED BY INSIGHT

```
  overlap = 0.01

 begin simulation
*          add-automatic bond torsion valence out-of-plane
 reduce

  set dielectric = 1.000000*r
  cutoff = 15.0
  swtdis = 1.5
  cutdis = 1.5
  nrneib = 20.0


 fix residues
* molecule 1 residues LEUn A42 to ASN A96
* molecule 1 residues TYR A100 to GLN A132
* molecule 1 residues GLY A142 to PRO A143
* molecule 1 residues PHE A142 to ASN A152
* molecule 1 residues LEU A154
* molecule 1 residues LYS+ A156 to ILE A182
* molecule 1 residues ASN A188
* molecule 1 residues THR A192
* molecule 1 residues VAL A196 to PRO A221
* molecule 1 residues SER A231 to LEU A251
* molecule 1 residues ALA A253 to LYS A310

 minimize
*          no cross terms
*          no morse
*          for 500 iterations
*          using steepest descents
*          until the maximum derivative is less than 1.0 kcal/A

 minimize
*          no cross terms
*          no morse
*          for 5000 iterations
*          using conjugated gradients
*          until the maximum derivative is less than 0.01 kcal/A

 archive as file number 1

  restrain heavy atoms in molecule 3 to 273
* using 0.005
* with a maximum force of 0.05
```

```
      ifile = 2

lop1    retrieve file number 1

      iloop = 0

      initialize dynamics at 350 K for 2000 steps of 1.0 fs
     * no cross terms
     * no morse
     * write history every 500 steps
lop2    resume dynamics at 350 K for 2000 steps of 2.0 fs
     * no cross terms
     * no morse

        iloop = iloop + 1

        minimize
     * no cross terms
     * no morse
     * for 500 iterations
     * using steepest descents
     * until the maximum derivative is less than 1.0 kcal/A

      minimize
     * no cross terms
     * no morse
     * for 5000 iterations
     * using conjugate gradients
     * until the maximum derivative is less than 0.010 kcal/A

        archive as file number ifile
        ifile = ifile + 1
        if iloop.eq.1 then lop2
        if ifile.le.14 then lop1

        end
```

Bibliography

(1) Sharon, N.; Lis, H. *Scientific America* **1993**, 74-81.

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

(3) Borman, S. *Chem. Eng. News* **1993**, *71*, 27-34.

(4) Faillard, H. In *Proceedings of the Japanese-German Symposium on Sialic Acids*, 1988, pp 6-18.

(5) Blix, F. G.; Gottschalk, A.; Klenk, A. *Nature* **1957**, *179*, 1088.

(6) von Itzstein, M.; Thomson, R. *Curr. Med. Chem.* **1997**, *4*, 185-210.

(7) Angata, T.; Varki, A. *Chem. Rev.* **2002**, *102*, 439-469.

(8) Schauer, R.; Kamerling, J. P. In *Glycoproteins II*; Montreuil, J., Vliegenthart, J. F. G., Schachter, H., Eds.; Elseiver Science, 1997, pp 243-402.

(9) Schauer, R. *Arch. Biochem. Biophys.* **2004**, *426*, 132-141.

(10) Traving, C.; Schauer, R. *CMLS, Cell. Mol. Life Sci.* **1998**, *54*, 1330-1349.

(11) Whitesides, G.; Martichonock, V. *J. Am. Chem. Soc.* **1996**, *35*, 8187-8191.

(12) Mühlenhoff, M.; Eckhardt, M.; Gerardy-Schahn, R. *Curr. Opin. Struct. Biol.* **1998**, *8*, 558-564.

(13) Reuter, G.; Gabius, H.-j. *Biol. Chem. Hoppe-Sevier* **1996**, *377*, 325-342.

(14) Schauer, R. *Adv. Carbohydr. Chem. Biochem* **1982**, *40*, 131-234.

(15) Yu, R. K.; Ledeen, R. *J. Biol. Chem.* **1969**, *244*, 1306-1313.

(16) Haverkamp, j.; Spoormaker, T.; Dorland, L.; Vliegenthart, J. F. G.; Schauer, R. *J. Am. Chem. Soc.* **1979**, *101*, 4851-4853.

(17) Chäppell, M. D.; Halcomb, R. L. *J. Am. Chem. Soc.* **1997**, *119*, 3393-3394.

(18) Kitazume, S.; Kitajima, K.; Inoue, S.; Troy II, F. A.; Cho, J.-W.; Lennarz, W. J.; Inoue, Y. *J. Biol. Chem.* **1994**, *269*, 22712-22718.

(19) Kitazume, S.; Kitajima, K.; Inoue, S.; Haslam, S. M.; Morris, H. R.; Dell, A.; Lennarz, W. J.; Inoue, Y. *J. Biol. Chem.* **1996**, *271*, 6694-6701.

(20) Dall'Olio, F.; Chiricolo, M. *Glycoconjugate J.* **2001**, *18*, 841-850.

(21) Schauer, R.; Kelm, S.; Reuter, G.; Roggentin, P.; Shaw, L. In *Biology of the Sialic Acids*; Rosenberg, A., Ed.; Plenum Press: New York, 1995, pp 7-67.

(22) Varki, A. *Glycobiology* **1992**, *2*, 25-40.

(23) Schauer, R. In *Carbohydrates. Synthetic Methods and Applications in Medicinal Chemistry*; Ogura, H., Hasegawa, A., Suami, T., Eds.; VCH: Weinheim, 1992, pp 340-354.

(24) Ledeen, R. W.; Yu, R. K. In *Biological Roles of Sialic Acids*; Rosenburg, A., Schengrund, C.-L., Eds.; Plenum Press: New York, 1976, pp 1-58.

(25) DeNinno, M. P. *Synthesis* **1991**, 583-593 and references within.

(26) Corfield, A. P.; Schauer, R. In *Cell Biology Monographs*; Schauer, R., Ed.; Springer-Verlag Wien, NY, 1982; Vol. 10.

(27) Musser, J. H.; Fügedi, P.; Anderson, M. B.; 5th ed.; John Wiley & Sons, Inc., 1995; Vol. 1, pp 901-947.

(28) Kim, Y., -J.; Kim, K.-S.; Do, S.; Kim, C., -H.; Kim, S.-K.; Lee, Y.-C. *Biochem. Biophys. Res. Comm.* **1997**, *235*, 327-330.

(29) Karlsson, K.-A. *Trends Pharmacol. Sci.* **1991**, *12*, 265-272.

(30) Reutter, W.; Kottgen, E.; Bauer, C.; Gerok, W. In *Sialic Acids. Chemistry, Metabolism and Function.*; Shauer, R., Ed.; Springer-Verlag Wien., 1982, pp 263-305.

(31) Beuth, J.; Ko, H. L.; Pulverer, G.; Uhlenbruck, G.; Pichlmaier, H. *Glycoconjugate J.* **1995**, *12*, 1-6.

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

(33) Phillips, M. L.; Nudelman, E.; Gaeta, F. C.; Perez, M.; Singhal, A. K.; Hakomori, S.; Paulson, J. C. *Science* **1990**, *250*, 1130-1132.

(34) Feizi, T. *Curr. Opin. Struct. Biol.* **1993**, *3*, 701-710.

(35) Levy, D. E.; Tang, P. C.; Musser, J. H. In *Annual Reports in Medicinal Chemistry*; Academic Press, 1994; Vol. 29, pp 215-224.

(36) Lasky, L. A. *Annu. Rev. Biochem.* **1995**, *64*, 113-139.

(37) Roy, R. In *Carbohydrates in Drug Design*; Witczak, Z. J., Nieforth, K. A., Eds.; Marcel Dekker Inc., 1997, pp 83-135.

(38) Simanek, E. E.; McGarvey, G. J.; Jablonowski, J. A.; Wong, C.-H. *Chem. Rev.* **1998**, *98*, 833-862.

(39) Barnes, Y. C.; Skelton, T. P.; Stamaenkovic, I.; Sgroi, D. C. *Blood* **1999**, *93*, 1245-1252.

(40) Angata, T.; Varki, A. *Glycobiology* **2000**, *10*, 431-438.

(41) Brinkman-Van der Linden, E. C. M.; Varki, A. In *Methods Enzymol.*; Academic Press, 2003; Vol. 363, pp 113-120.

(42) Bhunia, A.; Jayalakshmi, V.; Benie, A. J.; Schuster, O.; Kelm, S.; Krishna, N. R.; Peters, T. *Carbohydr. Res.* **2004**, *339*, 259-267.

(43) von Itzstein, M.; Thomson, R. J. *Top. Curr. Chem.* **1997**, *186*, 119-170.

(44) Jacobs, C. L.; Goon, S.; Yarema, K. J.; Hinderlich, S.; Hang, H. C.; Chai, D. H.; Bertozzi, C. R. *Biochemistry* **2001**, *40*, 12864-12874.

(45) Collins, B. E.; Fralich, T. J.; Itonori, S.; Ichikawa, Y.; Schnaar, R. *Glycobiology* **2000**, *10*, 11-20.

(46) Luchansky, S. J.; Yarema, K. J.; Takahashi, S.; Bertozzi, C. R. *J. Biol. Chem.* **2003**, *278*, 8035-8042.

(47) Kleineidam, R. G.; Schmelter, T.; Schwarz, R. T.; Schauer, R. *Glycoconjugate J.* **1997**, *14*, 57-66.

(48) Oritz, A. I.; Reglero, A.; Rodriguez-Aparicio, L. B.; Luengo, J. M. *Eur. J. Biochem.* **1989**, *178*, 741-749.

(49) Saito, M.; Yu, R. K. In *Biology of the Sialic Acids*; Rosenburg, A., Ed.; Plenum Press: New York, 1995, pp 261-313.

(50) Friebolin, H.; Baumann, W.; Brossmer, R.; Keilich, G.; Supp, M.; Ziegler, D.; von Nicolai, H. *Biochem. Int.* **1981**, *3*, 321.

(51) Deijl, C. M.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1984**, *126*, 338.

(52) Chong, A. J.; Pegg, M. S.; Taylor, N. R.; von Itzstein, M. *Eur. J. of Biochem.* **1992**, *207*, 335.

(53) Wilson, J. C.; Angus, D. I.; von Itzstein, M. *J. Am. Chem. Soc.* **1995**, *117*, 4214.

(54) Corfield, T. *Glycobiology* **1992**, *2*, 509-521.

(55) Herrler, G.; Hausmann, J.; Klenk, H.-D. In *Biology of the Sialic Acids*; Rosenberg, A., Ed.; Plenum Press: New York, 1995, pp 315-331.

(56) Liu, C.; Eichelberger, M. C.; Compans, R. W.; Air, G. M. *Virology* **1995**, *69*, 1099-1106.

(57) Schauer, R.; Vliegenthart, J. F. G. In *Cell Biology Monographs*; Schauer, R., Ed.; Springer-Verlag:Wien, 1982; Vol. 10.

(58) Martin, J. E.; Tanenbaum, S. W.; Flashner, M. *Carbohydr. Res.* **1977**, *56*, 423-425.

(59) Koketsu, M.; Juneja, L. R.; Kawanami, H.; Kim, M.; Yamamoto, T. *Glycoconjugate J.* **1992**, *9*, 70-74.

(60) Zbiral, E. In *Carbohydrates-Synthetic Methods and Applications in Medicinal Chemistry.*; Ogura, H., Hasegawa, A., Suami, T., Eds., 1992, pp 304-339.

(61) von Itzstein, M.; Kiefel, M. J. In *Carbohydrates in Drug Design*; Witczak, Z. J., Nieforth, K. A., Eds.; Marcel Dekker Inc., 1997, pp 39-82.

(62) Kiefel, M. J.; von Itzstein, M. *Chem. Rev.* **2002**, *102*, 471-490.

(63) Wong, C.-H.; Whitesides, G. M. *Enzymes in Synthetic Organic Chemistry.Tetrahedron Organic Chemistry Series.*; Elsevier Science: Oxford, 1994; Vol. 12.

(64) Fitz, W.; Schwark, J.-R.; Wong, C.-H. *J. Org. Chem.* **1995**, *60*, 3663-3670.

(65) Lin, C.-C.; Lin, C.-H.; Wong, C.-H. *Tetrahedron Lett.* **1997**, *38*, 2649-2652.

(66) Simon, E. S.; Bednarski, M. D.; Whitesides, G. M. *J. Am. Chem. Soc.* **1988**, *110*, 7159-7163.

(67) Augé, C.; David, S.; Gautheron, C. *Tetrahedron Lett.* **1984**, *25*, 4663-4664.

(68) Kragl, U.; Gygax, D.; Ghisalba, O.; Wandrey, C. *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 827-828.

(69) Maru, I.; Ohnishi, J.; Ohta, Y.; Tsukada, Y. *Carbohydr. Res.* **1998**, *306*, 572-578.

(70) Augé, C.; David, S.; Gautheron, C.; Malleron, A.; Cavyé, B. *New J. Chem* **1988**, *12*, 733.

(71) Sparks, M. A.; Williams, K. W.; Lukacs, C.; Schrell, A.; Priebe, G.; Spaltenstein, A.; Whitesides, G. M. *Tetrahedron* **1993**, *49*, 1-12.

(72) Augé, C.; Gautheron, C. *J. Chem. Soc., Chem. Comm.* **1987**, 859.

(73) Kim, M.-J.; Hennen, W. J.; Sweers, H. M.; Wong, C.-H. *J Am Chem Soc* **1988**, *110*, 6481-6486.

(74) David, S.; Augé, C.; Gautheron, C. *Adv. Carbohydr. Chem. Biochem.* **1991**, *49*, 175-237.

(75) David, S.; Malleron, A.; Cavayé, B. *New. J. Chem.* **1992**, *16*, 751-755.

(76) Luchansky, S. J.; Goon, S.: Bertozzi, C. R. *Chem. Bio. Chem.* **2004**, *5*, 371-374.

(77) Cornforth, J. W.; Firth, M. E.; Gottschalk, A. *J. Biol. Chem.* **1958**, *68*, 57-61.

(78) Carroll, P. M.; Cornforth, J. W. *Biochim. Biophys. Acta* **1960**, *39*, 161-.

(79) (220)Danishefsky, S. J.; DeNinno, M. P. *J. Org. Chem.* **1986**, *51*, 2615-2617.

(80) Danishefsky, S. J.; DeNinno, M. P.; Chen, S.-H. *J Am Chem Soc* **1988**, *110*, 3929-3940.

(81) Banwell, M.; De Savi, C.; Watson, K. *Perkins Trans 1* **1998**, 2251-2252.

(82) Gordon, D. M.; Whitesides, G. M. *J. Org. Chem.* **1993**, *58*, 7937-7938.

(83) Chan, T.-H.; Lee, M.-C. *J. Org. Chem.* **1995**, *60*, 4228-4232.

(84) Choi, S.-K.; Lee, S.; Whitesides, G. M. *J. Org. Chem.* **1996**, *61*, 8739-8745.

(85) Li, L.-S.; Wu, Y.-L.; Wu, Y. *Org. Lett.* **2000**, *2*, 891-894.

(86) Takahashi, T.; Tsukamoto, H.; Kurosaki, M.; Yamada, H. *Synlett* **1997**, 1065-1066.

(87) Dondoni, A.; Marra, A.; Merino, P. *J. Am. Chem. Soc.* **1994**, *116*, 3324-3336.

(88) Liu, K.-G.; Yan, S.; Wu, Y.-L.; Yao, Z.-J. *J. Org. Chem.* **2002**, *67*, 6758-6763.

(89) Kiefel, M. J.; von Itzstein, M. *Prog. Med. Chem.* **1999**, *36*, 1-28.

(90) Roy, R. *J. Carbohydr. Chem.* **2002**, *21*, 769-798.

(91) Kaplan, M. M.; Webster, R. G. *Scientific American* **1977**, *237*, 88-106.

(92) Crosby, A. W. *America's Forgotten Pandemic. The Influenza of 1918.*; Cambridge University Press: Cambridge, 1989.

(93) Bramford, M. J. *J. Enzyme Inhibition* **1995**, *10*, 1-16.

(94) Wade, R. C. *Structure* **1997**, *5*, 1139-1145.

(95) WHO; www.who.int/csr/disease/avian_influenza/country/cases_table_2004_09_28/en/ ed., 2004.

(96) WHO; WEBB, 2004, p http://www.who.int/csr/don/2004_2002_2018/en/.

(97) WHO; http://www.who.int/csr/disease/avian_influenza/update/en/ ed.; Webb, 2004.

(98) In *Catalyst*; Australian Broadcasting C, 19-02-2004.

(99) Smith, P. W.; Cherry, P. C.; Howes; Sollis, S. L.; Taylor, N. R. In *Anti-infectives: Recent Advances in Chemistry and Structure-Activity Relationships.*; Bentley, P. H., O'Hanlon, P. J., Eds.; The Royal Society of Chemistry, 1997, pp 269-287.

(100) von Itzstein, M.; Barry, J. G.; Chong, A. K. J. *Curr. Opin. Thera. Patents* **1993**, *3*, 1755-1762.

(101) Webster, R. G.; Laver, W. G.; Air, G. M.; Schild, G. C. *Nature* **1982**, *296*, 115-121.

(102) Whittington, A.; Bethell, R. *Exp. Opin. Ther. Patents* **1995**, *5*, 793-803.

(103) WHO; http://www.who.int/car/disease/influenza/pandemic/en/ ed.; Webb, 2004; Vol. 2004.

(104) Verhoeyen, M.; Fang, R.; Jou, W. M.; Devos, R.; Huylebroeck, D.; Saman, E.; Fiers, W. *Nature* **1980**, *286*, 771-776.

(105) Porter, A. G.; Barber, C.; Carey, N. H.; Hallewell, R. A.; Threlfall, G.; Emtage, J. S. *Nature* **1979**, *282*, 471-477.

(106) Skehel, J. J.; Wiley, D. C. *Annu. Rev. Biochem.* **2000**, *69*, 531-569.

(107) Ha, Y.; Stevens, D. J.; Skehel, J. J.; Wiley, D. C. *EMBO* **2002**, *21*, 865-875.

(108) Meanwell, N. A.; Krystal, M. *DDT* **1996**, *1*, 316-324.

(109) Klenk, H.; Rott, R. *Advances in Virus Research* **1988**, *34*, 247-281.

(110) Suarez-Lopez, P.; Orrin, J. *J. Gen. Virol.* **1994**, *75*, 389-393.

(111) Steinhauer, D. A.; Holland, J. J. *Ann. Rev. Microbiol.* **1987**, *41*, 409-433.

(112) Choi, S.-K.; Mammen, M.; Whitesides, G. M. *Chem. Biol.* **1996**, *3*, 97-104.

(113) Skehel, J. J.; Wiley, D. C. *Vaccine* **2002**, *20*, S51-S54.

(114) Wiley, D. C.; Skehel, J. J. *Ann. Rev. Biochem.* **1987**, *56*, 365-394.

(115) Weis, W.; Brown, J. H.; Cusak, S.; Paulson, J. C.; Skehel, J. J.; Wiley, D. C. *Nature* **1988**, *333*, 426-431.

(116) Gubareva, L. V.; Kaiser, L.; Hayden, F. G. *The Lancet* **2000**, *355*, 827-835.

(117) von Itzstein, M.; Wu, W.-Y.; Kok, G. B.; Pegg, M. S.; Dyason, J. C.; Jin, B.; Phan, T. V.; Smythe, M. L.; White, H. F.; Oliver, S. W.; Colman, P. M.; Varghese, J. N.; Ryan, M. D.; Woods, J. M.; Bethell, R. C.; Hotham, V. J.; Cameron, J. M.; Penn, C. R. *Nature* **1993**, *363*, 418-423.

(118) Varghese, J. N.; Laver, W. G.; Colman, P. M. *Nature* **1983**, *303*, 247-281.

(119) Holzer, C. T.; von Itzstein, M.; Jin, B.; Pegg, M. S.; Stewart, W. P.; Wu, W.-Y. *Glycoconjugate J.* **1993**, *10*, 40-44.

(120) Colman, P. M. *Protein Sci.* **1994**, *3*, 1687-1696.

(121) McKimm-Breschkin, J. L. *Antiviral Research* **2000**, *47*, 1-17.

(122) Palese, P.; Tobita, K.; Ueda, M.; Compans, R. W. *Virology* **1974**, *61*, 397-410.

(123) Bullough, P. A.; Hughson, F. M.; Treharne, A. C.; Ruigrok, R. W. H.; Skehel, J. J.; Wiley, D. C. *J. Mol. Biol.* **1994**, *236*, 1262-1265.

(124) Bullough, P. A.; Hughson, F. M.; Skehel, J. J.; Wiley, D. C. *Nature* **1994**, *371*, 37-43.

(125) Borredo-Diaz, E.; Peeples, M. E.; Markosyan, R. M.; Melikyan, G. B.; Cohen, F. S. *Virology* **2003**, *316*, 234-244.

(126) Wilson, I. A.; Skehel, J. J.; Wiley, D. C. *Nature* **1981**, *289*, 366-373.

(127) Takemoto, D. K.; Skehel, J. J.; Wiley, D. C. *Virology* **1996**, *217*, 452-458.

(128) Sauter, N. K.; Bednarski, M. D.; Wurzburg, B. A.; Hanson, J. E.; Whitesides, G. M.; Skehel, J. J.; Wiley, D. C. *Biochemistry* **1989**, *28*, 8388-8396.

(129) Kraulis, P. *J. of Applied Crystallography* **1991**, *24*, 946-950.

(130) Pritchett, T. J.; Brossmer, R.; Rose, U.; Paulson, J. C. *Virology* **1987**, *160*, 502-506.

(131) Ha, Y.; Stevens, D. J.; Skehel, J. J.; Wiley, D. C. *Proc. Natl. Acad. Sci.* **2001**, *98*, 11181-11186.

(132) Matrosovich, M. N.; Gamaryan, A. S.; Tuzikov, A. B.; Byramova, N. E.; Mochalova, A. A.; Golbraikh, A. A.; Shenderovich, M. D.; Finne, J.; Bovin, N. V. *Virology* **1993**, *196*, 111-121.

(133) Ito, T.; Suzuki, Y.; Mitnaul, L.; Vines, A.; Kida, H.; Kawaoka, Y. *Virology* **1997**, *227*, 493-499.

(134) Watowich, S. J.; Skehel, J. J.; Wiley, D. C. *Structure* **1994**, *2*, 719-731.

(135) Merritt, E. A.; Bacon, D. J. *Methods Enzymol.* **1997**, *277*, 505-524.

(136) Taylor, N. R.; von Itzstein, M. *Journal of Computer-Aided Molecular Design* **1996**, *10*, 233-246.

(137) Kelm, S.; Paulson, J. C.; Rose, U.; Brossmer, R.; Schmid, W.; Bandgar, B. P.; Schreiner, E.; Hartmann, M.; Zbiral, E. *Eur. J. Biochem* **1992**, *205*, 147-153.

(138) Toogood, P. J.; Galliker, P. K.; Glick, G. D.; Knowles, J. R. *J. Med. Chem.* **1991**, *34*, 3138-3140.

(139) Weinhold, E. G.; Knowles, J. R. *J. Am. Chem. Soc.* **1992**, *114*, 9270-9275.

(140) Matrosovich, M. N.; Mochalova, L. V.; Marinina, V. P.; Byramova, N. E.; Bovin, N. V. *FEBS Letters* **1990**, *272*, 209-212.

(141) Sigal, G. B.; Mammen, M.; Dahmann, G.; Whitesides, G. M. *J. Am. Chem. Soc.* **1996**, *118*, 3789-3800.

(142) Mammen, M.; Dahmann, G.; Whitesides, G. M. *J. Med.Chem* **1995**, *38*, 4179-4190.

(143) Roy, R. *Curr. Opin. Struct. Biol.* **1996**, *6*, 692-702.

(144) Univerzagt, C.; Kelm, S.; Paulson, J. C. *Carbohydr. Res.* **1994**, *251*, 285-301.

(145) Gestwicki, J. E.; Cairo, C. W.; Strong, L. E.; Oetjen, K. A.; Kiessling, L. L. *J. Am. Chem. Soc.* **2002**, *124*, 14922-14933.

(146) Roy, R.; Zanini, D.; Meunier, S. J.; Romanowska, A. *J. Chem. Soc., Chem. Commun.* **1993**, 1869-1872.

(147) Reuter, J. D.; Myc, A.; Hayes, M. M.; Gan, Z.; Roy, R.; Qin, D.; Yin, R.; Piehler, L. T.; Esfand, R.; Tomalia, D. A.; Baker Jr., J. R. *Bioconjugate Chem.* **1999**, *10*, 271-278.

(148) Zanini, D.; Roy, R. *J. Org. Chem.* **1996**, *61*, 7348-7354.

(149) Zanini, D.; Roy, R. *J. Am. Chem. Soc.* **1997**, *119*, 2088-2095S.

(150) Zanini, D.; Park, W. K. C.; Roy, R. *Tetrahedron Lett.* **1995**, *36*, 7383-7386.

(151) Glick, G.; Knowles, J. R. *J. Am. Chem. Soc.* **1991**, *113*, 4701-4703.

(152) Sabesan, S.; Duus, J.; Domaille, P. *J. Am. Chem. Soc.* **1991**, *113*, 5865-5866.

(153) Ogura, H.; Furuhata, K.; Itoh, M.; Shitori, Y. *Carbohydr. Res.* **1986**, *158*, 37-51.

(154) Sharma, M.; Petrie III[rd], C. R.; Korytnyk, W. *Carbohydr. Res.* **1988**, *175*, 25-34.

(155) Kok, G. B.; Groves, D. R.; von Itzstein, M. *Chem. Commun.* **1996**, 2017-2018.

(156) Schreiner, E.; Zbiral, E.; Kleineidam, R. G.; Schauer, R. *Liebigs Ann. Chem.* **1991**, 129-134.

(157) von Itzstein, M.; Jin, B.; Wu, W.-Y.; Chandler, M. *Carbohydr. Res.* **1993**, *244*, 181-185.

(158) Smith, P. W.; Starkey, I. D.; Howes, P. D.; Sollis, S. L.; Keeling, S. P.; Cherry, P. C.; von Itzstein, M.; Wu, W. Y.; Jin, B. *Eur. J. Med. Chem.* **1996**, *31*, 143-150.

(159) Zbiral, E.; Schreiner, E.; Christian, R. *Carbohydr. Res.* **1989**, *194*, C15-C18.

(160) Okamoto, K.; Kondo, T.; Goto, T. *Bull. Chem. Soc. Jpn.* **1987**, *60*, 631-636.

(161) von Itzstein, M.; Wu, W.-Y.; Jin, B. *Carbohydr. Res.* **1994**, *259*, 301-305.

(162) Barnford, M. J.; Castro Pichel, J.; Husman, W.; Patel, B.; Storer, R.; Weir, N. G. *J. Chem. Soc. Perkin Trans. 1* **1995**, 1181-1187.

(163) Chandler, M.; Conroy, R.; Cooper, A. W. J.; Lamont, R. B.; Scicinski, J. J.; Smart, J. E.; Storer, R.; Weir, N. G.; Wilson, R. D.; Wyatt, P. G. *J. Chem. Soc. Perkin Trans. 1* **1995**, 1189-1197.

(164) Bernatowicz, M. S.; Wu, Y.; Matsueda, G. R. *Tetrahedron Lett.* **1993**, *34*, 3389-3392.

(165) Ciccotosto, S.; von Itzstein, M. *Tetrahedron Lett.* **1995**, *36*, 5405-5408.

(166) Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. In *Spectrometric Identification of Organic Compounds*; 5th ed.; John Wiley & Sons, Inc., 1991.

(167) Hasegawa, A.; Nakamura, J.; Kiso, M. *J. Carbohydr. Chem.* **1986**, *5*, 11-19.

(168) Suzuki, Y., Binding Studies for Hemagglutinin (personal communication).

(169) Eisen, M. B.; Sabesan, S.; Skehel, J. J.; Wiley, D. C. *Virology* **1997**, *232*, 19-31.

(170) Glick, G. D.; Toogood, P. L.; Wiley, D. C.; Skehel, J. J.; Knowles, J. R. *J. Biol. Chem.* **1991**, *266*, 23660-23669.

(171) Pritchett, T. J.; Paulson, J. C. *J. Biol. Chem.* **1989**, *264*, 9850-9858.

(172) Spaltenstein, A.; Whitesides, G. M. *J. Am. Chem. Soc.* **1991**, *113*, 686-687.

(173) Llinares, M.; Roy, R. *Chem. Commun.* **1997**, 2119-2120.

(174) Zanini, D.; Roy, R. *J. Org. Chem.* **1998**, *63*, 3486-3491.

(175) Guo, C.-T.; Sun, X.-L.; Kanie, O.; Shortridge, K. F.; Suzuki, T.; Miyamoto, D.; Hidari, K. I.-P. J.; Wong, C.-H.; Suzuki, Y. *Glycobiology* **2002**, *12*, 183-190.

(176) Suzuki, Y.; Sato, K.; Kiso, M.; Hasegawa, A. *Glycoconjugate J.* **1990**, *7*, 349-356.

(177) Wilson, J. C.; Kiefel, M. J.; Angus, D. I.; von Itzstein, M. *Organic Lett.* **1999**, *1*, 443-446.

(178) Eschenfelder, V.; Brossmer, R. *Carbohydr. Res.* **1987**, *162*, 294-297.

(179) Lubineau, A.; Le Gallic, J. *J. Carbohydr. Chem.* **1991**, *10*, 263-268.

(180) Warner, T. G.; Lee, L. A. *Carbohydr. Res.* **1988**, *176*, 211-218.

(181) Kanie, O.; Nakamura, J.; Kiso, M.; Hasegawa, A. *J. Carbohydr. Chem.* **1987**, *6*, 105-115.

(182) Kanie, O.; Nakamura, J.; Itoh, Y.; Kiso, M.; Hasegawa, A. *J. Carbohydr. Chem.* **1987**, *6*, 117-128.

(183) Defaye, J.; Guillot, J.-M. *Carbohydr. Res.* **1994**, *253*, 185-194.

(184) Blanc-Muesser, A.; Defaye, J.; Driguez, H. *Carbohydr. Res.* **1978**, *67*, 305-328.

(185) Bennett, S.; von Itzstein, M.; Kiefel, M. J. *Carbohydr. Res.* **1994**, *259*, 293-299.

(186) Kiefel, M. J.; Beisner, B.; Bennett, S.; Holmes, I. D.; von Itzstein, M. *J. Med. Chem.* **1996**, *39*, 1314-1320.

(187) Angus, D. I.; von Itzstein, M. *Carbohydr. Res.* **1995**, *274*, 279-283.

(188) Smalec, B.; von Itzstein, M. *Carbohydr. Res.* **1995**, *266*, 269-272.

(189) Nagarajan, S.; Ganem, B. *J. Org. Chem.* **1987**, *52*, 5044-5046.

(190) Ichikawa, Y.; Look, G. C.; Wong, C.-H. *Anal. Biochem.* **1992**, *202*, 215-238.

(191) Kitazume, S.; Kitajima, K.; S., I.; Inoue, Y.; Troy II, F. A. *J. Biol. Chem.* **1994**, *269*, 10330-10340.

(192) Wagner, H. E.; Thomas, P.; Wclf, B. C.; Rapoza, A.; Steele, G. *Arch Surg* **1990**, *125*, 351-354.

(193) Beyer, T. A.; Sadler, J. E.; Rearick, J. I.; Paulson, J. C.; Hill, R. L. In *Adv. Enzymol Areas Mol Biol*, 1981; Vol. 5, pp 23-175.

(194) Paulson, J. C.; Colley, K. J. *J. Biol. Chem.* **1989**, *264*, 17615-17618.

(195) Brossmer, R.; Gross, H. J. *Methods Enzymol.* **1994**, *247*, 177-193.

(196) Harvey, B. E.; Toth, C. A.; Wagner, H. E.; Steele Jr, G. D.; Thomas, P. *Cancer Research* **1992**, *52*, 1775-1779.

(197) Basu, S.; Basu, M.; Basu, S. S. In *Biology of Sialic Acids*; Rosenberg, A., Ed.; Plenum Press: New York and London, 1995, pp 69-94.

(198) Livingston, B. D.; Paulson, J. C. *J. Biol. Chem.* **1993**, *268*, 11504-11507.

(199) Harduin-Lepers, A.; Vallejo-Ruiz, V.; Krewinski-Recchi, M.-A.; Samyn-Petit, B.; Julien, S.; Delannoy, P. *Biochimie* **2001**, *83*, 727-737.

(200) Lloyd, K. O.; Furukawa, K. *Glycoconjugate J.* **1998**, *15*, 627-636.

(201) Gross, H. J.; Brossmer, R. *Clin. Chim. Acta* **1991**, *197*, 237-248.

(202) Yogeeswaran, G.; Stalk, P. L. *Science* **1981**, *212*, 1514-1516.

(203) Schaub, C.; Müller, B.; Schmidt, R. R. *Glycoconjugate J.* **1998**, *15*, 345-354.

(204) Schaub, C.; Müller, B.; Schmidt, R. R. *Eur J. Org. Chem.* **2000**, 1745-1758.

(205) Harvey, B. E.; Thomas, P. *Biochem. Biophys. Res. Comm.* **1993**, *190*, 571-575.

(206) Petrick, A. T.; Meterissian, S.; Steele Jr, G.; Thomas, P. *Clin. Exp. Metastasis* **1994**, *12*, 108-106.

(207) Miura, S.; Kim, Y. S. *Cancer* **1986**, *38*, 197-205.

(208) Humphries, M. J.; Kazue, M.; White, S. L.; Olden, K. *Proc. Nat. Acad. Sci., USA* **1986**, *83*, 1752-1756.

(209) Irimura, S.; Gonzalez, R.; Nicolson, G. L. *Cancer Res.* **1981**, *41*, 3411-3418.

(210) Troy II, F. A. In *Biology of the Sialic Acids*; Rosenberg, A., Ed.; Plenum Press: New York, 1995, pp 95-144.

(211) Easton, E. W.; Schiphorst, W. E. C. M.; Koeleman, C. A. M.; Michalides, R. J. A. M.; Van Den Eijnden, D. H. *Glycoconjugate J.* **1995**, *12*, 829-837.

(212) Halberstadt, J. B.; Flowers, H.; Glick, M. C. *Anal. Biochem.* **1993**, *209*, 136-142.

(213) Stoykova, L. I.; Glick, M. C. *Biochem. Biophys. Res. Comm.* **1995**, *217*, 777-783.

(214) Gross, H. J.; Sticher, U.; Brossmer, R. *Anal. Biochem.* **1990**, *186*, 127-134.

(215) Groß, H. J.; Brossmer, R. *Glycoconjugate J.* **1995**, *12*, 739-746.

(216) Gross, H. J.; Rose, U.; Krause, J. M.; Paulson, J. C.; Schmid, K.; Feeney, R. E.; Brossmer, R. *Biochemistry* **1989**, *28*, 7386-7392.

(217) Gross, H. J.; Brossmer, R. *European Journal of Biochemistry* **1988**, *177*, 583-589.

(218) Limberg, G.; Slim, G. C.; Compston, C. A.; Stangier, P.; Palcic, M. M.; Furneaux, R. H. *Liebigs. Ann.* **1996**, 1773-1784.

(219) Brandstetter, H. H.; Zbiral, E.; Schulz, G. *Liebigs Ann. Chem.* **1982**, 1-13.

(220) Tsvetkov, Y. E.; Schmidt, R. R. *Tetrahedron Lett.* **1994**, *35*, 8583-8586.

(221) Castro-Palomino, J. C.; Tsvetkov, Y. E.; Schneider, R.; Schmidt, R. R. *Tetrahedron Lett.* **1997**, *38*, 6837-6840.

(222) Castro-Palomino, J. C.; Tsvetkov, Y. E.; Schmidt, R. R. *J. Am. Chem. Soc.* **1998**, *120*, 5434-5440.

(223) Khorlin, A. Y.; Privalova, I. M. *Carbohydr. Res.* **1970**, *13*, 373-377.

(224) Dufner, G.; Schwörer, R.; Müller, B.; Schmidt, R. R. *Eur. J. Org. Chem.* **2000**, 1467-1482.

(225) Tsvetkov, Y. E.; Schmidt, R. R. In *Biology and Chemistry of Sialic Acids International Conference*: Moscow, 1994, p 100.

(226) Purdie, T.; Irvine, J. C. *J. Chem. Soc.* **1903**, *83*, 1021.

(227) Clinton, E.; Ballou, C. E.; Pizer, L. I. *J. Am. Chem. Soc.* **1960**, *82*, 3333-3335.

(228) Jones, T. K.; Reamer, R. A.; Desmond, R.; Mills, S. G. *J. Am. Chem. Soc.* **1990**, *112*, 2998-3017.

(229) Boons, G.-J.; Demchenko, A. V. In *Carbohydrate-based Drug Discovery.*; Wong, C.-H., Ed.; Wiley-VCH, 2003; Vol. 1, pp 55-102.

(230) Gervay, J.; Ramamoorthy, P. S.; Mamuya, N. N. *Tetrahedron* **1997**, *53*, 11039-11048.

(231) Berry, J. M.; Hall, L. D. *Carbohydr. Res.* **1976**, *47*, 307-310.

(232) Lemieux, R. U.; Kondo, T. *Carbohydr. Res.* **1974**, *35*, C4-C6.

(233) Alais, J.; Veyrières, A. *J. Chem. Soc. Perkin I* **1981**, 377-381.

(234) Cruzado, C.; Bernabé, M.; Martin-Lomas, M. *J. Org. Chem.* **1989**, *54*, 465-469.

(235) Reinhard, B.; Becker, A.; Rothermel, J.; Faillard, H. *Biol. Chem. Hoppe-Seyler* **1992**, *373*, 63-68.

(236) Fitz, W.; Wong, C.-H. *J. Org. Chem.* **1994**, *59*, 8279-8280.

(237) Nashed, M. *Carbohydr. Res.* **1979**, *71*, 299-304.

(238) Hartmann, M.; Christian, R.; Zbiral, E. *Liebigs Ann. Chem.* **1990**, 83-91.

(239) Omura, R.; Swern, D. *Tetrahedron* **1978**, *34*, 1651.

(240) Mancuso, A. J.; Huang, S.-L.; Swern, D. *J. Org. Chem.* **1978**, *43*, 2480.

(241) Adams, L. L.; Luzzio, F. A. *J. Org. Chem.* **1989**, *22*, 5387-5390.

(242) Lorette, N. B.; Howard, W. L.; Brown Jr, J. H. *J. Org. Chem.* **1959**, *24*, 1731-1733.

(243) Pearlman, B. A. *J. Am. Chem. Soc.* **1979**, *101*, 6404-6408.

(244) Martin, T. J.; Brescello, R.; Toepfer, A.; Schmidt, R. R. *Glycoconjugate J.* **1993**, *10*, 16-25.

(245) Martin, T. J.; Schmidt, R. R. *Tetrahedron Lett.* **1993**, *34*, 1765-1768.

(246) Martin, T. J.; Schmidt, R. R. *Tetrahedron Lett.* **1992**, *33*, 6123-6126.

(247) Muller, B.; Martin, T. J.; Schaub, C.; Schmidt, R. R. *Tetrahedron Lett.* **1998**, *39*, 509-512.

(248) Amann, F.; Schaub, C.; Müller, B.; Schmidt, R. R. *Chem. Eur. J.* **1998**, *4*, 1106-1115.

(249) Yoshino, T.; Schmidt, R. R. *Carbohydrate Letters* **1995**, *1*, 329-334.

(250) Varki, A. *The FASEB Journal* **1997**, *11*, 248-255.

(251) Graves, B. J.; Crowther, R. L.; Chandran, C.; Rumberger, J. M.; Li, S.; Huang, K.-S.; Presky, D. H.; Familletti, P. C.; Wolitzky, B. A.; Burns, D. K. *Nature* **1994**, *367*, 532-538.

(252) Taylor, G. *Curr. Opin. Struct. Biol* **1996**, *6*, 830-837.

(253) Crennel, S.; Garmen, E.; Laver, W. G.; Vimr, E.; Taylor, G. *Structure* **1994**, *2*, 535-544.

(254) Merritt, E. A.; Sarfaty, S.; van den Akker, F.; L'Hoir, C.; Martial, J. A.; Hol, W. G. J. *Protein Sci.* **1994**, *3*, 166-175.

(255) Amaya, F. M.; Buschiazzo, A.; Nguyen, T.; Alzari, P. *J. Mol. Biol.* **2003**, *325*, 773-784.

(256) Buschiazzo, A.; Amaya, M. F.; Cremano, M. L.; Frash, A. C. C.; Alzari, P. M. *Mol. Cell.* **2002**, *10*, 757-768.

(257) Galen, J. E.; Ketley, J. M.; Fasano, A.; Richardsón, S. H.; Wasserman, S. S.; Kaper, J. B. *Infect. Immun.* **1992**, *60*, 406-415.

(258) Taylor, G.; Vimr, E.; Garmen, E.; Laver, W. G. *J. Mol. Biol.* **1992**, *226*, 1287-1290.

(259) Chou, M.-Y.; Li, S.-C.; Li, Y.-T. *J. Biol. Chem.* **1996**, *271*, 19219-19224.

(260) Sinnot, M. L.; Guo, X.; Li, S.-C.; Li, Y.-T. *J. Am. Chem. Soc.* **1993**, *115*, 3334-3335.

(261) Buschiazzo, A.; Tavares, G. A.; Campetella, O.; Spinelli, S.; Cremona, M. L.; París, G.; Amaya, M. F.; Frash, A. C. C.; Alzari, P. M. *EMBO* **2000**, *19*, 16-24.

(262) Wilson, J. C.; Kiefel, M. J.; Albouz-Abo, S.; von Itzstein, M. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2791-2794.

(263) Suzuki, J.; Murakami, K.; Nishimura, Y. *J. Carbohydr. Chem.* **1993**, *12*, 201-208.

(264) Reinhard, B.; Götz, C.; Faillard, H. *Biological Chemistry Hoppe-Seyler* **1992**, *373*, 1243-1248.

(265) Schauer, R.; Wember, M. *Biological Chemistry Hoppe-Seyler* **1989**, *370*, 183-190.

(266) Glasgow, L. R.; Paulson, J. C.; Hill, R. L. *J. Biol. Chem.* **1977**, *252*, 8615-8623.

(267) Crampen, M.; von Nicolai, H.; Zilliken, F. *Hoppe-Seyler's Z Physiol. Chem.* **1979**, *360*, 1703-1712.

(268) Ohta, Y.; Tsukada, Y.; Sugimori, T. *J. Biochem.* **1989**, *106*, 1086-1089.

(269) Narayanan, S. R. *J. Chromat. A* **1994**, *658*, 237-258.

(270) Holmquist, L. *Acta Chemica Scandinavica B* **1974**, *28*, 1065-1068.

(271) Holmquist, L.; Östman, B. *FEBS Letters* **1975**, *60*, 327-330.

(272) Van Dessel, G.; De Wolf, M.; Lagrou, A.; Hilderson, H.; Dierick, W. *J. Biochem.* **1984**, *96*, 937-947.

(273) Michalski, J.-C.; Corfield, A. P.; Schauer, R. *Hoppe-Seyler's Z Physiol. Chem.* **1982**, *363*, 1097-1102.

(274) Geisow, M. J. *J. Biochem.* **1975**, *151*, 181-183.

(275) Corfield, A. P.; Parker, T. L.; Schauer, R. *Anal. Biochem.* **1979**, *100*, 221-232.

(276) Brossmer, R.; Ziegler, D.; Keilich, G. *Hoppe-Seyler's Z Physiol. Chem.* **1977**, *358*, 397-400.

(277) Cuatrecasas, P.; Illiano, G. *Biochem. Biophys. Res. Comm.* **1971**, *44*, 178-184.

(278) Suzuki, Y.; Sato, K.; Kiso, M.; Hasegawa, A.; *Glycoconjugate J.* **1990**, *7*, 349-356.

(279) Ciccotosto, S.; Kiefel, M. J.; Abo, S.; Stewart, W.; Quelch, K.; von Itzstein, M. *Glycoconjugate J.* **1998**, *15*, 663-669.

(280) Abo, S.; Ciccotosto, S.; Alafaci, A.; von Itzstein, M. *Carbohydr. Res.* **1999**, *322*, 201-208.

(281) Schnabel, E.; Klostermeyer, H.; Berndt, H. *Liebigs Ann. Chem.* **1971**, *749*, 90-108.

(282) Tius, M. A.; Fauq, A. H. *J. Am. Chem. Soc.* **1986**, *108*, 1035-1039.

(283) Weinstein, J.; de Souza-e-Silva; Paulson, J. C. *J. Biol. Chem.* **1982**, *257*, 13835-13844.

(284) Chou, M.-Y.; Li, S.-C.; Kiso, M.; Hasegawa, A.; Li, Y.-T. *J. Biol. Chem.* **1994**, *269*, 18821-18826.

(285) Perrin, D. D.; Armarego, W. L. F. *Purification of Laboratory Chemicals*; 3rd ed.; Pergamon Press, 1988.

(286) Myers, R. W.; Lee, R. T.; Lee, Y. C.; Thomas, G. H.; Reynolds, L. W.; Uchida, Y. *Anal. Biochem.* **1980**, *101*, 166-174.

(287) Meindl, P.; Tuppy, H. *Monatsh. Chem* **1969**, *100*, 1295-1306.

(288) Vimr, E. R.; Lawrisuk, L., Galen, J.,; Kaper, J. B. *J. Bacteriol.* **1988**, *170*, 1495-1504.

(289) Scudder, P.; Doom, J. P.; Chuenkova, M.; Manger, I. D.; Pereira, M. E. A. *J. Biol. Chem.* **1993**, *268*, 9886-9891.

(290) Sauter, N. K.; Hanson, J. E.; Glick, G. D.; Brown, J. H.; Crowther, R. L.; Park, S.-J.; Skehel, J. J.; Wiley, D. C. *Biochemistry* **1992**, *31*, 9609-9621.

(291) Chong, A. K. J.; Pegg, M. S.; von Itzstein, M. *Biochim. Biophys. Acta* **1991**, *1077*, 65-71.

(292) Potier, M.; Mameli, L.; Bélisle, M.; Dallaire, L.; Melancon, S. B. *Anal. Biochem.* **1979**, *94*, 287-296.

(293) Leelavathi, D. S.; Estes, L. W.; Feingold, D. S.; Lombardi, B. *Biochem. Biophys. Acta.* **1970**, *211*, 124-138.

(294) Li, Y.-T.; Nakagawa, H.; Ross, S. A.; Hansson, G. C.; Li, S.-C. *J. Biol. Chem.* **1990**, *265*, 21629-21633.