

Synthesis and biological evaluation of ganglioside GM3 analogues

Changping Zheng

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THESE

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Par M Changping ZHENG

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DOCTEUR de SORBONNE UNIVERSITÉ

Spécialité: Chimie Organique

Synthesis and biological evaluation of

ganglioside GM3 analogues

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May 2019

List of abbreviations

Ac	acetyl
AgOTf	silver trifluoromethanesulfonate
BF ₃ •Et ₂ O	boron trifluoride diethyl etherate
Bz	benzoyl
calcd	calculated value
Су	cyclohexane
d	doublet
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
dd	doublet of doublet
DMF	N,N-dimethylformamide
DMSO	dimethyl sulfoxide
DTBP	2,6-di(<i>tert</i> -butyl)pyridine
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
eq.	equivalent
Et	ethyl
GFR	growth factor receptor
h	hour
HRMS	high-resolution mass spectrometry
Hz	hertz
J	coupling constant (in Hz)
<i>m</i> -CPBA	meta-chloroperoxybenzoic acid
min	minute
NIS	N-iodosuccinimide
NMR	nuclear magnetic resonance
Me	methyl
PDMP	l-phenyl-2-decanolyamlno-3-morpholino-l-propanol
Ph	phenyl
PhSCl	benzenesulfenyl chloride
Phth	phthalimido
ppm	parts per million
PTSA	<i>p</i> -toluenesulfonic acid
q	quadruplet

quant.	quantitative
r.t.	room temperature
s	singlet
t	triplet
TBDMSCl	tert-butyldimethylsilyl chloride
TE	2-(trimethylsilyl)ethyl
Tf ₂ O	trifluoromethanesulfonic anhydride or triflic anhydride
TfOH	trifluoromethanesulfonic acid or triflic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
δ	chemical shift

Synthèse et évaluation biologique d'analogues

du ganglioside GM3

Résumé

Glycosphingolipides (GSLs) sont des composants omniprésents des membranes cellulaires animales et impliqués dans de nombreuses fonctions cellulaires, y compris la prolifération, l'adhérence, la motilité et la différenciation. Ganglioside GM3 (NeuAcα3Galβ4Glcβ1Cer), le premier et le plus simple membre de la série métabolique d'une famille de GSLs, contenant de l'acide sialique, du lactose et du céramide. Il a été rapporté que GM3 est impliqué dans certaines maladies graves humaines, telles que les cancers et les maladies nerveuses. La recherche au cours des dernières décennies a démontré que GM3 et ses analogues jouent des rôles importants dans de nombreux processus physiologiques et pathologiques. Il est bien connu que GM3 est un antigène glucide associé à la tumeur sur plusieurs types de tumeur.

Dans la présente étude, une série d'analogues avec des sialosides α -2, 6 contenant des sucres différents, y compris le galactose, le mannose, le lactose et la glucosamine, ont été entièrement synthétisés sur la base d'un protocole concis provenant de substrats et de réactifs disponibles dans le commerce. Et les blocs de construction de sucre avec un 6-OH libre ont été préparés par hydrolyse enzymatique, qui peut éviter de multiples procédures chimiques. En particulier, nous avons développé une nouvelle méthode chimioenzymatique pour synthétiser les dérivés glucosamine sialylée ou galactosamine sialylée, puis en utilisant cette approche, des analogues GM3 contenant de la glucosamine ont été construits avec succès. Tous les nouveaux composés ont été purifiés par chromatographie et ont été caractérisés par les spectres RMN et HRMS.

Les activités biologiques de ces analogues synthétiques de GM3 ont été évaluées. Par des essais de cytotoxicité, les analogues GM3 ont été déterminés contre la cellule du cancer du côlon humain HCT116 et la cellule de leucémie K562. Il est intéressant de noter que, par le

test de guérison des plaies, certains analogues ont montré des effets significatifs sur le mouvement des cellules tumorales, qui est censé représenter l'un des facteurs pertinents dans l'évaluation de la malignité des cancers. En outre, nous avons employé le modèle de cellule PC12 pour évaluer les effets de certains analogues de GM3 sur l'excroissance de neurites.

Cette étude peut fournir des informations précieuses pour synthétiser divers analogues de GM3 et leurs études biologiques.

Mots-clés: glycosphingolipide, ganglioside GM3, acide sialique, synthèse, tumeur, l'excroissance neurites, évaluation biologique.

Synthesis and biological evaluation of ganglioside GM3 analogues

Abstract

Glycosphingolipids (GSLs) are ubiquitous components on animal cell membranes and involved in many cellular functions, such as cell proliferation, adhesion, motility and differentiation. Ganglioside GM3 (NeuAc α 3Gal β 4Glc β 1Cer), the first and simplest member in the metabolic series of a GSLs family, containing sialic acid, lactose and ceramide. It has been reported that GM3 is implicated in some human serious diseases, such as cancers and nervous diseases. Studies over the past decades have demonstrated that GM3 and its analogues play important roles in many physiological and pathological processes. It is well known that GM3 is a tumor-associated carbohydrate antigen on several types of cancer.

In the present study, a series of GM3 analogues with α -2,6 sialosides containing different sugars, including galactose, mannose, lactose and glucosamine, were totally synthesized based on a concise protocol from commercially available substrates and reagents. And the sugar building blocks with a free 6-OH were prepared by regioselective enzymatic hydrolysis, which can avoid multiple chemical procedures. In particular, we developed a new chemoenzymatic method to synthesize sialylated glucosamine or galactosamine derivatives, then using this approach, glucosamine-containing GM3 analogues were constructed successfully. All the new compounds were purified by chromatography and characterized by NMR and HRMS spectra.

Furthermore, the biological activities of these synthetic GM3 analogues were evaluated. By cytotoxicity assays, synthesized GM3 analogues were determined against human colon cancer cell HCT116 and leukemia cell K562. Interestingly, by wound healing test, some analogues showed significant effects on tumor cell movement, which is believed to represent one of the relevant factors in assessing the malignancy of cancers. In addition, we employed the PC12 cell model to evaluate the effects of some GM3 analogues on neurite outgrowth.

This study can provide valuable information for synthesizing various GM3 analogues and their biological studies.

Keywords: glycosphingolipid, ganglioside GM3, sialic acid, synthesis, tumor, neurite outgrowth, biological evaluation.

Chapter I Introduction

Carbohydrates, along with proteins, nucleic acids and lipids, are the most important organic natural products on earth. It has been demonstrated that carbohydrates and their derivatives play important roles in many biological processes, such as protein structure modulation or cell-cell recognition. The biological implications of carbohydrates are strongly related with serious human diseases, such as cancers or diabetes. Carbohydrates are becoming highly attractive targets for biological study, including drug discovery ¹.

Carbohydrates usually expose on the outer surface of cell membranes, and exist as glycoconjugates, including glycolipids and glycoproteins as shown in **Figure I-1**. The glycolipids are composed of hydrophobic lipid moieties with carbohydrate attached, including acylglycerol, ceramide and prenyl phosphate, and the glycoproteins contain proteins with carbohydrate attached.



Figure I-1 Carbohydrates exist as glycoconjugates on the cell membranes.

Moreover, glycolipids can be divided into two categories, glycosphingolipids (GSLs) and glycoglycerolipids, depending on the different structures of lipids as shown in **Figure I-2**. The former has an alkaline-stable sphingoid base backbone. However, the later has an alkaline-labile glycerol backbone. Ceramide is the most important lipid anchor for animal glycolipids, it is composed of sphingosine that is substituted at its

¹ C. H. Wong, *Carbohydrate-based drug discovery*, Wiley-VCH, Weinheim, 2003.

amino group with different chain lengths of fatty acids, and ceramide can be glycosylated at its primary hydroxyl.



Figure I-2 Structures of GSL and glycoglycerolipid. The structures of glucosylceramide and seminolipid are illustrated as an example of GSL and glycoglycerolipid, respectively.

1.1 Glycosphingolipids

1.1.1 Structures of GSLs

The discovery of GSLs is generally attributed to Johan L. W. Thudichum, who reported on the chemical composition of brain in 1884.

GSLs exhibit a huge diversity of structures, and so far, over 60 different sphingoid bases and more than 300 different oligosaccharide chains have been characterized ². In general, GSLs can be categorized into two groups: neutral and negatively charged (sialic acid or sulfate group) GSLs, and the four most common ones are globo-, lacto-, neolacto- and ganglio- series. The typical examples of GSLs are listed in **Figure I-3**.

² S. Degroote, J. Wolthoorn, G. van Meer, Semin. Cell Dev. Biol., 2004, 15, 375-387.



Figure I-3 Structures of typical GSLs.

1.1.2 Biological functions of GSLs

In various tissues, GSLs exhibit different distribution and the specific pattern which indicate that they may play important and specific functions in these tissues. At the cellular level, the organizing potential of GSLs plays important roles in the lateral build-up of membranes. In addition, the self-organizing principles of GSLs in combination with their structural specificity have been confirmed to play crucial roles in signaling pathways at the cell surface. Studies over the past decades have demonstrated that GSLs are involved in many physiological and pathological processes ^{3,4}. **Figure I-4** describes some of the biological functions that are associated with GSLs.

³ S. Roseman, J. Biol. Chem., 2001, 276, 41527-41542.

⁴ T. Yamakawa, *Glycoconj. J.*, **1996**, *13*, 123-126.



Figure I-4 Some of the biological functions that are associated with GSLs. GSLs associate with oncogenesis (process 1). GSLs interact with bacterial receptors (process 2), with GFRs (process 3), with integrin receptor (process 4), or with integrin/TSP complex (process 5). Cells recognition is mediated by GSL-to-GSL interaction in microdomains (process 6). GSLs interact with signal transducers in signal pathways (process 7).

Due to the tightly packed backbone of GSLs, they are able to interact with themselves or with sphingomyelin (SM) and cholesterol in a phospholipid environment, therefore forming rafts (**Figure I-5**) ^{5,6}. It is believed that raft formation is necessary for many properties of GSLs. They are thought to be enriched in both specific proteins in their lumenal leaflet ^{7,8} and peripheral proteins carrying myristoyl and palmitoyl chains in their cytoplasmic leaflet, such as Src-family kinases related to signal pathway ^{9,10}. Then the concept of glycosynapse has emerged, defined as a microdomain involved in glycosylation-dependent adhesion, recognition and signaling at the cell surface.

⁵ K. Simons, D. Toomre, Nat. Rev. Mol. Cell Biol., 2000, 1, 31-39.

⁶ K. Simons, E. Ikonen, *Nature*, **1997**, *387*, 569-572.

⁷ D. A. Brown, J. K. Rose, *Cell*, **1992**, *68*, 533-544.

⁸ W. Rodgers, B. Crise, J. K. Rose, *Mol. Cell Biol.*, **1994**, *14*, 5384-5391.

⁹ S. Hakomori, K. Handa, K, Iwabuchi, S. Yamamura, A. Prinetti, *Glycobiology*, **1998**, *8*, xi–xix.

¹⁰ S. Hakomori, K. Handa, *FEBS Lett.*, **2002**, *531*, 88–92.



Figure I-5 Scheme of a sphingolipid/cholesterol raft. GSLs can form a hydrogen-bonded network with sphingomyelin and cholesterol in a phospholipid environment, forming rafts on the noncytosolic side of the plasma membrane. GPI-anchored proteins (\mathbf{G}) are enriched in these rafts. Acylated proteins (\mathbf{A}) are thought to associate with the cytosolic leaflet of rafts and prenylated proteins (\mathbf{P}) are excluded.

Cell-cell interactions are involved in many biological functions, and the results displayed that carbohydrates play an important role as recognition molecules in such processes. Cell adhesion can be mediated by carbohydrate-to-carbohydrate interaction (CCI). CCI can mediate cell adhesion based on both interfacing glycosyl groups carried by clustered GSLs and GSLs interaction with other glycosyl residues presenting in the same membrane microdomain, as illustrated in **Figure I-6**¹¹.

¹¹ A. R. Todeschini, S. Hakomori, *Biochim. Biophys. Acta*, 2008, 1780 (3), 421-433.



Figure I-6 Carbohydrate-to-carbohydrate interactions controlling glycosylation-dependent cell motility and growth. GFR, TSP and integrin subunits α and β can be mediated by N-linked glycans and surrounding GSLs. GSLs associate with Src family kinases and other signal transducers. (a) GFR and its interaction with surrounding GSLs. (b) TSP interaction with GSLs.

GSLs are ubiquitous components on animal cell membranes. Some studies have revealed that they are involved in tumor cells proliferation, adhesion, invasion and apoptosis ^{12,13,14}. Usually, the specific types of GSLs are expressed more highly in tumors than in normal tissues, which are known as tumor-associated antigens. The expression of GSLs on some kinds of human cancers is shown in **Table I-1**. It has been concluded that most cancer cells exhibit altered GSLs patterns on their surface, especially the aberrant sialylation of GSLs, abnormal GSLs signaling and biosynthesis, which together play a major role in tumor progression ^{15,16,17}.

¹² M. S. Toledo, E. Suzuki, K. Handa, S. Hakomori, J. Bio. Chem., **2004**, 279 (33), 34655-34664.

¹³ M. Ono, K. Handa, S. Sonnino, D. A. Withers, H. Nagai, S. Hakomori, *Biochem.*, **2001**, *40*, 6414-6421.

¹⁴ D. Zhuo, X. Li, F. Guan, Front. Physiol., 2018, 9, 466.

¹⁵ J. L. Daniotti, A. A. Vilcaes, V. T. Demichelis, F. M. Ruggiero, M. Rodriguez-Walker, *Front. Oncol.*, **2013**, *3*, 306.

¹⁶ B. Ogretmen, Y. A. Hannun, *Nat. Rev. Cancer*, **2004**, *4*, 604-616.

¹⁷ S. Hakomori, *Cancer Res.*, **1996**, *56*, 5309-5318.

GSLs	Tumor types	
GM3	melanoma, medulloblastoma, superficial bladder cancer	
de-N-acetyl GM3	melanoma	
Neu5Gc GM3	melanoma, colon and breast cancers, retinoblastoma	
9-O-acetyl GM3	melanoma, glioblastoma	
GM2	melanoma, neuroblastoma, medulloblastoma	
GM1	small cell lung carcinoma, renal cell carcinoma	
Fucosyl-GM1	small cell lung carcinoma	
9-O-acetyl GD3	melanoma, neuroblastoma, breast cancer	
GD2	melanoma, neuroblastoma, retinoblastoma	
9-O-acetyl GD2	ovarian cancer, neuroblastoma, small cell lung cancer	
Gb3	ovarian cancer, burkitt lymphoma	
Globo H	ovarian and breast cancers, teratocarcinoma	
Disialosyl galactosyl globoside	renal cell carcinoma	
Gb5	teratocarcinoma	
Monosialyl Gb5	renal cell carcinoma	
Disialyl Gb5	renal cell carcinoma	
Disialyl Lc4	colon cancer	
Le ^a -Le ^a	gastric, colon, bladder and breast cancers	
Sialyl Le ^a	colon, pancreatic and gastric cancers	
Disialyl Le ^a	colon cancer	
Le ^x	gastric, breast and colorectal cancers	
Le ^x -Le ^x	colon, gastric, breast, lung and liver cancers	
Sialyl Le ^x	colon and bladder cancers, T cell leukemia	
Sialyl Le ^x -Le ^x	colon, gastrointestinal, colorectal, lung and breast cancers	
Le ^y	colon, gastric and lung cancers	
Le ^y -Le ^x	x colon, liver, lung, colorectal and pancreatic cancers	

Table I-1 The expression of GSLs on some types of cancers

Tumor-associated GSL antigens have been used in the development of anticancer vaccines. The abnormal expression of GSLs on different cancers would be a promising target for cancer treatment and can provide the valuable sight to develop the new strategies for cancer therapy.

1.2 Gangliosides

Gangliosides are distinguished from other GSLs that contain sialic acid, and can be divided into several subclasses based on the structure of the oligosaccharide backbones.

1.2.1 Sialic acid

Sialic acids are nine-carbon monosaccharides implicated in a wide range of biological processes. Their unique structural characterizations include a carboxylic group, which is ionized at physiological pH, deoxygenated C-3, the presence of glycerol chain at C-6 and differently functionalized C-5.

So far, among the 50 derivatives, *N*-acetylneuraminic acid (Neu5Ac) is the most widespread, and *N*-glycolylneuraminic acid (Neu5Gc) and 2-keto-3-deoxy-nonulosonic acid (KDN) are also usually found in biological systems (**Figure I-7**).



Figure I-7 Three types of naturally occurring sialic acids.

Usually, sialic acids were found as $(\alpha 2 \rightarrow 3)$ - or $(\alpha 2 \rightarrow 6)$ - linked to galactosides or

 $(\alpha 2 \rightarrow 6)$ - linked to *N*-acetyl galactosaminides. In addition, the disialosyl structures $(\alpha 2 \rightarrow 8)$ and $(\alpha 2 \rightarrow 9)$ have been found as constituents of glycoconjugates. Polysialic acids of Neu5Ac and Neu5Gc, having $(\alpha 2 \rightarrow 8)$, $(\alpha 2 \rightarrow 9)$, or alternating $(\alpha 2 \rightarrow 8)/(\alpha 2 \rightarrow 9)$ glycosidic linkages, have also been found. Other sialosides, such as $(\alpha 2 \rightarrow 4)$ -linked homopolymer and $(\alpha 2 \rightarrow 5)$ -linked derivatives, have also been reported ^{18,19,20}.

At the terminal of natural glycoconjugates on the cell surface, sialic acids can mediate carbohydrate-protein interactions in cell-cell recognition processes. For example, sialic acids are involved in the sialyl Lewis^x-selectin binding which occurs in the recruitment of leukocytes during the inflammation process. In addition, sialic acids can act as receptors for some toxins, viruses and bacteria, such as the interaction between heamaglutinin and Neu5Ac that is the first stage of infection by influenza virus. Sialic acids are also implicated in preventing biological recognition, and the cell interaction can be interfered by modifications of sialic acids. For example, O-acetylation or N-acetyl hydroxylation can result in a longer lifespan for rat erythrocytes due to hindering the action of sialidases 21,22,23 .

1.2.2 Gangliosides and tumors

Some evidences have already demonstrated that tumor cellular function and phenotype are strongly influenced by the presence of gangliosides. The research results have indicated that tumor-associated gangliosides play a key role in invasion and metastasis of cancer development. The role of gangliosides in tumor development

¹⁸ K. Yamada, Y. Harada, Y. Nagaregawa, T. Miyamoto, R. Isobe, R. Higuchi, *Eur. J. Org. Chem.*, **1998**, 2519-2525.

¹⁹ S. Kitazume, K. Kitajima, S. Inoue, S. M. Haslam, H. R. Morris, A. Dell, W. J. Lennarz, Y. Inoue, J. *Biol. Chem.*, **1996**, 271, 6694-6701.

²⁰ M. Inagaki, R. Isobe, R. Higuchi, Eur. J. Org. Chem., 1999, 771-774.

²¹ A. Varki, *Glycobiology*, **1993**, *3*, 97-130.

²² G. Herrler, J. Hausmann, H. D. Klenk, in *Biology of the sialic acids* (Ed. A. Rosenberg), Plenum Press, New York and London, **1995**, 315-336.

²³ W. Reutter, E. Kottgen, C. Bauer, W. Gerok, in *Sialic acids. chemistry, metabolism and function* (Ed. R. Schauer), Springer Vienna, New York, **1982**, 263-305.

is crucially important for cancer research ²⁴.



Figure I-8 Malignancy enhancement by ganglioside GD3 in melanomas. GD3 can activate FAK, p130Cas and paxillin, thus promoting cells proliferation and invasion.

For example, in human melanomas, ganglioside GD3 is the predominant ganglioside component ^{25,26}. Activation of natural killer T (NKT) cells produces cytokines to influence immune responses against cancers, and GD3 can induce NKT cells response in melanoma. In addition, some studies showed that GD3 can enhance malignancy of melanomas as shown in **Figure I-8**. The molecules involved in GD3-mediated signaling pathway include focal adhesion kinase (FAK), p130Cas and paxillin. Further, siRNA can block p130Cas and paxillin, which can obviously suppress melanoma growth ²⁶. In GD3-positive, not GD3-negative, human melanoma SK-MEL-28-N1 cells, the proliferation and apoptosis resistance were enhanced through hepatocyte growth factor (HGF) or adhesion to collagen type I via MAPK (Mitogen-activated protein kinases) and Akt (Protein kinase B) signaling pathways. Increased GD3 expression can promote melanoma cells adhesion to surrounding tissues and

²⁴ S. Birklé, G. Zeng, L. Gao, R. K. Yu, J. Aubry, *Biochim.*, **2003**, *85*, 455-463.

²⁵ T. Tsuchida, R. E. Saxton, D. L. Morton, R. F. Irie, J. Natl. Cancer Inst., 1987, 78, 45-54.

²⁶ Y. Makino, K. Hamamura, Y. Takei, R. H. Bhuiyan, Y. Ohkawa, Y. Ohmi, H. Nakashima, K. Furukawa, *Biochim. Biophys. Acta*, **2016**, *1860*, 1753-1763.

susceptibility to HGF presented in the tumor microenvironment ^{27,28}. Other results further showed that the melanoma malignancy is enhanced by GD3 due to g-secretase recruiting to rafts and facilitating efficient cleavage of neogenin ²⁹.



Figure I-9 Activation of c-Met by ganglioside GD2. (a) Breast cancer cell line MDA-MB-231 mainly expresses GM3 and GM2. (b) GD3 synthase induces b- and c- series gangliosides accumulation, mainly GD2, which can activate c-Met in the absence of HGF, then increase proliferation and migration via PI3K/Akt and MEK/Erk pathways. (c) In the competition assays, anti-GD2 mAb was used, which can inhibit c-Met phosphorylation and cells proliferation.

In a MDA-MB-231 breast cancer cell line, it was reported that ganglioside GD2 can enhance proliferation and tumorigenicity by ganglioside-mediated activation of c-Met receptor ³⁰ as shown in **Figure I-9**. GD2 has been considered as a breast cancer marker ³¹. The expression of GSLs in CD44^{hi}/CD24^{lo} breast cancer stem cells (CSCs)

²⁷ K. Furukawa, M. Kambe, M. Miyata, Y. Ohkawa, O. Tajima, K. Furukawa, *Cancer Sci.*, 2014, 105, 52-63.

²⁸ K. Hamamura, K. Furukawa, *Cancer Transl. Med.*, **2017**, *3* (6), 209-213.

²⁹ K. Kaneko, Y. Ohkawa, N. Hashimoto, Y. Ohmi, N. Kotani, K. Honke, M. Ogawa, T. Okajima, K. Furukawa, K. Furukawa, J. Biol. Chem., 2016, 291, 16630-16643.

³⁰ S. Groux-Degroote, Y. Guérardel, S. Julien, P. Delannoy, *Biochemistry (Mosc)*, **2015**, *80 (7)*, 808-819.

³¹ V. L. Battula, Y. Shi, K. W. Evans, R. Y. Wang, E. L. Spaeth, R. O. Jacamo, R. Guerra, A. A. Sahin, F. C. Marini, G. Hortobagyi, S. A. Mani, *J. Clin. Invest.*, **2012**, *122*, 2066-2078.

and non-CSCs was also studied by flow cytometry, and up-regulation of GD2, GD3, GM2, and GD1a in CSCs were detected ³².

It has been demonstrated that gangliosides are shed from tumor to their microenvironment in the form of micelles, monomers, or membrane vesicles ^{33,34}. Shed gangliosides can bind and interact with a wide variety of proteins, including signaling molecules presenting in the tumor microenvironment ³⁵. In addition, shed gangliosides can be incorporated into the membrane of neighboring host cells, and it is possible that they can modulate tumor–host cell interactions ^{36,37}.

1.2.3 Gangliosides and nerves

1.2.3.1 Distribution of gangliosides in brain and nervous tissues

It is well known that gangliosides are widely expressed in mammalian tissues, particularly in brain and nervous tissues ³⁸. Gangliosides express differently at different time period during neurodevelopment, brain maturation or aging, and their expression patterns alter obviously during these processes ^{39,40,41}.

There is a specific regional distribution about gangliosides in the adult human brain. In general, a-series gangliosides, such as GD1a and GM1, were found in parietal, frontal and temporal cortical areas, while b-series gangliosides, including GQ1b, GT1b, and GD1b, were found in occipital cortex or structures related to visual system. In addition, in cerebellum, b-series gangliosides were found as the main gangliosides,

³² Y. J. Liang, Y. Ding, S. B. Levery, M. Lobaton, K. Handa, S. Hakomori, *Proc. Natl. Acad. Sci. USA*, 2013, *110*, 4968-4973.

³³ Y. Kong, R. Li, S. Ladisch, *Biochim. Biophys. Acta*, **1998**, *1394*, 43–56.

³⁴ V. Dolo, R. Li, M. Dillinger, S. Flati, J. Manel, B. J. Taylor, A. Pavan, S. Ladisch, *Biochim. Biophys. Acta*, 2000, 1486, 265–274.

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³⁶ F. Chang, R. Li, S. Ladisch, *Exp. Cell Res.*, 1997, 234, 341–346.

³⁷ R. X. Li, S. Ladisch, *Biochim. Biophys. Acta*, **1991**, *1083*, 57–64.

³⁸ Y. T. Li, S. C. Li, Adv. Carbohydr. Chem. Biochem., **1982**, 40, 235-288.

³⁹ L. Svennerholm, K. Bostrom, B. Jungbjer, L. Olsson, J. Neurochem., **1994**, 63, 1802-1811.

⁴⁰ H. Rosner, *Prog. Mol. Subcell. Biol.*, **2003**, *32*, 49-73.

⁴¹ I. Kracun, H. Rosner, V. Drnovsek, Z. Vukelic, C. Cosovic, M. Trbojevic-Cepe, M. Kubat, *Neurochem. Int.*, **1992**, *20*, 421-431.

and in hippocampal archicortex and the amygdala ⁴², a-series gangliosides are the predominant species. The c-series gangliosides are biosynthesized during the early neurodevelopment, and found in modest quantities in adult brain. GM1, GD1b and GT1b are abundant during neurogenesis, neuronal migration and neuritogenesis. GD1a is in much higher proportion during synaptogenesis. It was reported that gangliosides are enriched in terminal axons and synaptic endings ⁴³.

Gangliosides expression differed in the brain samples of Alzheimer's disease from the parietal, frontal and temporal cortices, displaying decreased ganglioside GD1a, GD1b and GT1b, and increased ganglioside GM2, GM3 and GM4. The expression of gangliosides in brain is highly region-specific and strongly associated with neurodevelopment, including neural tube formation, neuritogenesis, synaptogenesis, axonogenesis and myelination ^{44,45,46,47}.

1.2.3.2 Effect of gangliosides on nerve growth

The effects of gangliosides on nerve growth and development have already been studied for a long time. It has been revealed that ganglioside GD3 plays a key role in maintaining the self-renewal capacity for neural stem cells, and hence neurogenesis, through sustaining EFG-induced EFGR signaling ⁴⁸. The study also displayed that GM1a clustering in lipid rafts is involved in neurite outgrowth ⁴⁹. In addition, in the developing human brain, GM1a is implicated in glia-neuronal contacts during neuroblast migration ⁵⁰. Gangliosides also have been confirmed to be neuroprotective against toxic nutrients insult ⁵¹. Anti-apoptotic during neural cell differentiation ⁵²

⁴² I. Kracun, H. Rosner, C. Cosovic, A. Stavljenic, J. Neurochem., 1984, 43, 979-989.

⁴³ K. Palmano, A. Rowan, R. Guillermo, J. Guan, P. McJarrow, *Nutrients*, **2015**, *7*, 3891-3913.

⁴⁴ P. B. Crino, M. D. Ullman, B. A. Vogt, E. D. Bird, L. Volicer, Arch. Neurol., **1989**, 46, 398-401.

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⁴⁸ J. Wang, R. K. Yu, Proc. Nat. Acad. Sci. USA, 2013, 110, 19137-19142.

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⁵⁰ M. Stojiljkovic, T. Blagojevic, S. Vukosavic, N. Zvezdina, S. Pekovic, G. Nikezic, L. Rakic, Int. J. Dev. Neurosci., 1996, 14, 35-44.

⁵¹ I. Mochetti, Cell Mol. Life Sci., 2005, 62, 2283-2294.

and pro-apoptotic during neural cell proliferation ⁵³ were also observed, respectively.

The 9-*O*-acetyl GD3 is implicated in glial-guided neuronal migration and neurite outgrowth for both the developing and adult rat nervous system. Additionally, during the development and regeneration of mouse peripheral nerve, 9-*O*-acetyl GD3 is also involved in facilitation of axonal growth and Schwann cell-induced myelination ^{54,55}. The 9-*O*-acetyl GD3 involved in neuronal motility has also been studied as shown in **Figure I-10** ⁵⁶.



Figure I-10 Mechanism for 9-*O*-acetyl GD3 on neuronal motility. The 9-*O*-acetyl GD3 may modulate the integrin receptor through a Ca^{2+} -dependent mechanism or by a direct interaction between the ceramide moiety and the integrin transmembrane helix. Gangliosides in the adjacent membrane are laterally packed around the integrin receptor, affording an optimal Ca^{2+} -enriched microenvironment for the activation and recognition of extracellular matrix proteins by this receptor.

Despite exogenously added gangliosides are known to promote neurite outgrowth in a variety of cell lines, including some neuroblastoma cell lines, a study in SH-SY5Y human neuroblastoma cell line demonstrated that gangliosides can inhibit neurite outgrowth. Further results revealed that gangliosides have inhibitory effects on cell

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⁵⁵ V. T. Ribeiro-Resende, T. A. Gomes, S. de Lima, M. Nascimento-Lima, M. Bargas-Rega, M. F. Santiago, R. A. de Melo Reis, F. G. de Mello, *PLos One*, **2014**, *9*, e108919.

⁵⁶ R. Mendez-Otero, M. F. Santigo, Brazil J. Med. Biol. Res., 2003, 36, 1003-1013.

growth through the regulation of lipid rafts ^{57,58,59}.

Myelin-associated glycoprotein (MAG) can bind to the nerve cell surface resulting in inhibiting nerve regeneration. Gangliosides GD1a and GT1b can be as ligands for MAG-mediated inhibition of neurite outgrowth from primary rat cerebellar granule neurons. Other results showed that MAG–gangliosides binding can not only ensure optimal axon–myelin cell–cell interactions, but also enhance long-term axon–myelin stability, and further inhibit axon outgrowth after injury. It is encouraging that studying brain gangliosides interactions can provide new chances to enhance recovery after nerve injury ^{60,61}.

1.2.3.3 Neural tumors

It has been observed that anti-GD2 mAbs addition can significantly improve event-free survival and overall survival in patients with high-risk neuroblastoma ⁶². The GSLs composition in an F-11 neuroblastoma cell line has also been studied, and the results showed that major neutral GSLs are Gb4, Gb3, LacCer, and GlcCer, and major gangliosides are GM3, GD3, *O*-acetylated GD3, and GD1a with trace amounts of GD2. Further studies indicated that the alteration of gangliosides, especially GD3 and *O*-acetylated GD3, is associated with neuroblastoma behavior, and the specific ganglioside expression may be as a neural cancer marker ^{63,64}. A ratio between GD3 and 9-*O*-acetylated GD3 on glioblastoma promotes tumor survival, and restoring GD3 through removing acetyl group can reduce tumor cell viability through inducing

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⁶⁰ G. Mukhopadhyay, P. Doherty, F. S. Walsh, P. R. Crocker, M. T. Filbin, *Neuron*, **1994**, *13*, 757-767.

⁶¹ A. A. Vyas, H. V. Patel, S. E. Fromholt, M. Heffer-Lauc, K. A. Vyas, J. Dang, M. Schachner, R. L. Schnaar, Proc. Natl. Acad. Sci. USA, 2002, 19, 8412-8417.

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⁶⁴ T. Kaneko, H. Okita, H. Nakajima, K. Iijima, N. Ogasawara, Y. Miyagawa, Y. U. Katagiri, A. Nakagawa, N. Kiyokawa, T. Sato, J. Int. J. Oncol., 2010, 37 (5), 1279-1288.

mitochondrial-mediated apoptosis ^{65,66}. By adding PDMP, an effective inhibitor of ceramide glucosyltransferase, GSLs biosynthesis in murine neuroblastoma cell line was inhibited, and the inhibitory effect on neurite outgrowth was also observed ⁶⁷.

1.3 Ganglioside GM3 and cancers

Ganglioside GM3, the first and simplest member in the metabolic series of a GSLs family, contains a single terminal sialic acid, lactose and ceramide (**Figure I-11**).



Figure I-11 Structure of ganglioside GM3.

Ganglioside GM3 is the main ganglioside in the majority of extraneural tissues of vertebrata with varied contents. Ganglioside GM3 is one of the essential components on the plasma membranes. It has been reported that ganglioside GM3 is implicated in some human serious diseases, such as cancers, nervous diseases and diabetes ^{68,69}.

The obvious influence of GM3 on the proliferation, invasion and metastasis of cancer cells has been observed ⁷⁰. GM3 is over expressed on some cancers, and it is well-known as a tumor-associated carbohydrate antigen (TACA). Until now, many types of cancers associated with GM3 have been studied as shown in **Table I-2**. GM3 can modulate the receptors related to cancers, such as EGFR and VEGF-2. GM3 can

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⁶⁹ S. Hakomori, K. Handa, *Glycoconj. J.*, **2015**, *32*, 1-8.

⁷⁰ T. W. Chung, H. J. Choi, S. J. Kim, C. H. Kwak, K. H. Song, U. H. Jin, Y. C. Chang, H. W. Chang, Y. C. Lee, K. T. Ha, C. H. Kim, *Plos One*, **2014**, *9* (5), e92786.

also inhibit tumor cell migration. In particular, GM3 can modulate functions of other molecules co-localized in membrane microdomain.

Tumor types	Mode of changing cell GM3 content	Effect on tumor
KB and A431 human epidemoid carcinoma cells	Exogenous addition	Suppression of proliferation
Human glioblastoma cells	The same	The same
Rat 9L gliosarcoma cells into mice	The same	Increase in symptom-free survival of mice
Primary brain tumors Ependymomas Combined gliomas Astrocytomas Oligodendrogliomas Gangliogliomas	The same	Suppression of proliferation
Human urinary bladder cancer cells T24 and KK-47	The same	Decrease the invasive potential
The SCC12 human epidermoid carcinoma cells	Increase in GM3 degradation by transfecting with cDNA of neuraminidase Neu3	Activation the EGFR-induced proliferation
The same	Increase in GM3 content by inhibition of higher ganglioside synthesis by treatment with antisense oligodeoxy nucleotides	Activation the urokinase-caused proliferation
Meningeal gliomatosis of rats	Intrathecal addition	Increase in animal lifespan
Cells of urinary bladder cancer	Treatment of the cells with PPPP, exogenous addition	Decrease the cell mobility, invasiveness, metastasizing

Table I-2 Cancers related with ganglioside GM3

1.3.1 Expression on tumor cells

GM3 expression is strongly related to human cancers, and change of GM3 expression is associated with the growth and invasion of tumor cells ^{71,72}. It has been reported that GM3 is expressed with a much higher proportion in the primary and metastatic melanomas than GD2, and that GM3 and GD3 expressions are strongly related to each other. GM3 can not be found in normal skin or on skin neighboring primary melanoma, which is in contrast with GD3 ^{73,74}. GM3 is more exposed in the strongly metastatic line than the weakly metastatic line on the melanomas. It is clear that GM3 expression on the tumor cell surface is an important factor in determining the metastatic phenotype in cancer cells ⁷⁵.

Cancer cell growth can be stimulated with GM3 depletion, whereas increasing GM3 can lead to suppression. In the murine MBT-2 bladder tumors, the exogenous GM3 can inhibit tumor growth and invasion. It is obvious that GM3 has a significant antitumor effect on bladder cancer ^{76,77}. And a large quantity of GM3 is accumulated in nonmuscle-invasive bladder cancer, but a small quantity of GM3 is accumulated in muscle-invasive bladder tumor.

The expression of GM3 on the invasive and metastatic properties in four mouse melanoma Bl6 variants has been studied. The results showed that GM3 expression is the highest in B16, and it is the most invasive and metastatic type, followed by F10, F1 and WA4. It has been reported that GM3 can be regarded as an adhesion molecule for another two GSLs, gangliotriaosylceramide (Gg3Cer) and lactosylceramide (LacCer). The metastasis of Bl6 is triggered through the recognition between GM3 and Gg3Cer or LacCer on endothelial cells, which is GSL-GSL interaction, and the

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interaction initiates the signaling pathway ^{78,79}.

N-glycolyl-GM3 contains N-glycolylneuraminic acid instead of N-acetylneuraminic acid, and it is widely expressed in most mammals⁸⁰. However, it has been detected in many human cancers, such as colon carcinomas and breast cancers, not in normal human cells, and it can be considered as one of potential target molecules for cancer therapy⁸¹.

1.3.2 Effect on tumor EGFR

Over activation of epidermal growth factor receptor (EGFR) is well known to be associated with cancer development and progression. Many studies have demonstrated that GM3 has an inhibitory effect on EGFR. In human epidermoid carcinoma A431 cells, exogenous addition of GM3 inhibited EGF-induced autophosphorylation of EGFR⁸². GM3 has been known to inhibit EGFR tyrosine kinase since 1986^{83,84}. Further research results showed that GM3 inhibits EGFR tyrosine kinase through binding to N-linked glycan having multivalent GlcNAc termini on EGFR. It is important to realize that GM3 susceptibility for EGFR is strongly dependent on the presence of N-glycosylation, and it is carbohydrate to carbohydrate interaction in the same membrane plane with presumably within the same glyco synaptic microdomain ^{85,86}.

The studies also indicated that receptor dimer formation is not strongly blocked by GM3, but tyrosine phosphorylation on both monomeric and dimeric EGFR is

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inhibited by GM3⁸⁷. Besides, other studies further determined the effect of GM3 on the Tyr-1173 and Tyr-1086 residues phosphorylation of EGFR. The results indicated that GM3 suppressed the phosphorylation of Tyr-1173 residue, and enhanced the phosphorylation of Tyr-1086 residue. Both Tyr-1173 and Tyr-1086 residues, which located at EGFR's C-terminal, are important EGFR autophosphorylation sites ^{88,89}.

The importance of sialosyl group in GM3 for its EGFR inhibitory effect was also studied. In A431 cells, they were transfected with a gene that encodes for a soluble Mr 42,000 sialidase. Compared with control cells, despite having similar level of EGF binding, the transfected cells could decrease sialic acid expression, promote cells growth, and enhance EGFR autophosphorylation ⁹⁰. Miyagi's group demonstrated that the enhanced NEU3 expression can lead to suppression of apoptosis and decreased NEU3 expression can inhibit Ras activation. The involvement of NEU3 up regulation in cancer development can also be confirmed from increased EGFR phosphorylation detected in NEU3 transgenic mice ^{91,92,93,94}.

Previous study revealed the order of binding intensity for some gangliosides, and it is clear that GM3 exhibits the strongest effect on inhibiting EGFR among ganglioside family ⁹⁵.

GM3 is the main ganglioside for Hepa1-6 cells. Research found that GM3 can suppress the EGF-induced autophosphorylation of EGFR at tyr1173, and subsequently,

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down regulates the PI-3K/Akt signaling pathway ⁹⁶. However, GM3 can enhance the HGF-induced autophosphorylation of HGFR at Tyr-1313 and Tyr-1365, and subsequently, up regulates the PI-3K/Akt signaling pathway ⁹⁷. It is demonstrated that GM3 has an opposite effect on the EGF and HGF-induced Hepa1-6 cells motility and migration ⁹⁸. Actually, GM3 is implicated in tumor progression through different ways, especially for EGFR activity. Firstly, GM3 can directly influence the EGFR activity by interaction with N-linked GlcNAc termini on the receptor. Secondly, GM3 can indirectly regulate EGFR activity not only through the interaction between EGFR and membrane proteins such as integrins and CD9, but also influencing the intracellular Src kinase and protein tyrosine phosphatase activity. Therefore, it seems that the mechanism of GM3 modulating EGFR activity is rather complex.

The co-expression of EGFR and ganglioside Neu5Gc GM3 in different human tumors has been reported ⁹⁹. Neu5Gc GM3 and EGFR can coordinately contribute to the tumor progression. Combinations against these two targets will provide a valuable insight for cancer therapy.

1.3.3 Effect on tumor angiogenesis

The ratio of GM3 to a variety of gangliosides, such as GM1, GD3, GD1a and GT1b, can influence the angiogenic properties on many brain tumor types. The ratio of GM3 to the proangiogenic gangliosides GD3 and GD1a (GM3/GD3; GM3/GD1a) is lower in more metastatic and aggressive tumors than that in less metastatic tumors ^{100,101,102}. In mouse or most cultured human brain tumor cells, GM3 expression serves to regulate or counteract the proangiogenic action of other gangliosides ¹⁰³. It has been

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¹⁰³ T. N. Seyfried, P. Mukherjee, J. Oncol., **2010**, 961243.
reported that brain tumors with high GM3 expression are less angiogenic. Other studies showed that removing GM3 expression gene in the ependymoblastoma tumor that contains GM3 as the only ganglioside, increased angiogenesis was observed ^{104,105}. And in the highly angiogenic CT-2A astrocytoma, decreased angiogenesis was observed when GM3 expression was up regulated ¹⁰⁶.

It has been reported that urokinase plasminogen activator receptor (μ PAR) localization in caveolar lipid-rafts (LRs) is necessary for an efficient angiogenesis program in endothelial progenitor cells or endothelial colony forming cells, EPCs/ECFCs, usually considered as the main source of vasculogenesis in human cancers ^{107,108}. Further studies found that μ PAR is present on the ECFC surface in at least three compartments, one associated to GM1, another associated to GM3, a third one related to caveolar-LRs. Following the exogenous GM1 addition, μ PAR is depleted in the GM3 compartment which is recruited within caveolar-LRs compartment. Therefore, angiogenesis-related signaling pathways are triggered, which result in enhanced ECFC invasion and capillary morphogenesis ¹⁰⁹.

Some evidences also indicated that GM3 can reduce the ability of endothelial cell membrane to transfer growth signals present in the microenvironment into the endothelial cell itself, leading to block the growth stimulation of vascular endothelium ¹¹⁰. Some cancer cells, such as melanomas, shed GM3 or other gangliosides into the microenvironment. Then gangliosides can act as modulators of the angiogenic response by promoting the angiogenic capacity of PGEl (Prostaglandin E1) and bFGF (Basic fibroblast growth factor), normally present in the tissue microenvironment.

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¹⁰⁵ J. A. Ecsedy, K. A. Holthaus, H. C. Yohe, T. N. Seyfried, *J. Neurochem.*, **1999**, *73*, 254-259.

¹⁰⁶ M. G. Manfredi, S. Lim, K. P. Claffey, T. N. Seyfried, *Cancer Res.*, **1999**, *59*, 5392-5397.

¹⁰⁷ F. Margheri, A. Chillà, A. Laurenzana, S. Serratì, B. Mazzanti, R. Saccardi, M. Santosuosso, G. Danza, N. Sturli, F. Rosati, L. Magnelli, L. Papucci, L. Calorini, F. Bianchini, M. Del Rosso, G. Fibbi, *Blood*, **2011**, *118*, 3743-55.

¹⁰⁸ A. Chillà, F. Magherini, F. Margheri, A. Laurenzana, T. Gamberi, L. Bini, L. Bianchi, G. Danza, B. Mazzanti, S. Serratì, A. Modesti, M. Del Rosso, G. Fibbi, *Mol. Cell Proteomics*, **2013**, *12*, 1926-1938.

¹⁰⁹ F. Margheri, L. Papucci, N. Schiavone, R. D'Agostino, S. Trigari, S. Serrat, A. Laurenzana, A. Biagioni, C. Luciani, A. Chill, E. Andreucci, T. Del Rosso, G. Margheri, M. Del Rosso, G. Fibbi, J. Cell Mol. Med., 2015, 19, 113-123.

¹¹⁰ G. Alessandri, P. Cornaglia-Ferraris, P. M. Gullino, Acta Oncol., 1997, 36, 383-387.

Perhaps their modulatory effects on angiogenesis play roles in metastatic or primary tumor growth ¹¹¹.

Vascular endothelial growth factor receptor-2 (VEGFR-2) is also involved in tumor angiogenesis. The various downstream signaling pathways can be regulated by GM3 through the inhibitory effect on VEGF-induced VEGFR-2 activation ^{112,113}. GM3 can inhibit the VEGF-induced activation of PI-3K/Akt, extracellular signal–regulated kinases (ERK), vascular endothelial cadherin and FAK/paxillin, then suppress proliferation, migration and the tube formation caused by VEGF.

1.3.4 Effect on membrane microdomain

The concept of membrane microdomains is that GSLs (GM3), growth factor receptors (GFRs), and other signaling molecules, such as TSs and integrins, are localized and functionalized as clusters in the membrane. Further studies indicated that membrane microdomains are involved in tumor cells interaction, adhesion or other related signal transduction.

Studies of melanoma B16 cells revealed the presence of "GSL signaling domains", which is different from caveolin-containing membrane domains ¹¹⁴. In mouse neuroblastoma Neuro2a cells, studies found that GM3 enriched microdomains were involved in signal transduction during neurite formation ¹¹⁵. Moreover, Hakomori's group indicated that clustered GSLs associated with c-Src, small G-protein (Rho A) and FAK are involved in GSL-dependent signaling pathways ^{116,117}. A typical example is GM3 clustering at the cell surface of B16, forming a "glycosignaling

¹¹¹ P. M. Gullino, Acta Oncol., **1995**, 34, 439-441.

¹¹² T. W. Chung, S. J. Kim, H. J. Choi, K. J. Kim, M. J. Kim, S. H. Kim, H. J. Lee, J. H. Ko, Y. C. Lee, A. Suzuki, C. H. Kim, *Glycobiology*, **2009**, *19* (3), 229-239.

¹¹³ P. Mukherjee, A. C. Faber, L. M. Shelton, R. C. Baek, T. C. Chiles, T. N. Seyfried, J. Lipid Res., 2008, 49 (5), 929-938.

¹¹⁴ K. Iwabuchi, K. Handa, S. Hakomori, J. Biol. Chem., **1998**, 273, 33766-33773.

¹¹⁵ A. Prinetti, K. Iwabuchi, S. Hakomori, J. Biol. Chem., **1999**, 274, 20916-20924.

¹¹⁶ K. Iwabuchi, S. Yamamura, A. Prinetti, K. Handa, S. Hakomori, *J. Biol. Chem.*, **1998**, *273*, 9130-9138.

¹¹⁷ K. Iwabuchi, Y. Zhang, K. Handa, D. A. Withers, P. Sinay, S. Hakomori, *J. Biol. Chem.*, **2000**, *275*, 15174-15181.

domain" (GSD) ¹¹⁸. GM3 attributes to initiate the adhesion of melanoma to endothelial cells, which is the first step in metastasis ¹¹⁹. Antigenicity, adhesion mediation and signaling initiation by GM3 are believed to be maintained by GM3 clustering in GSD.

The "glycosynapse" is highly enriched in TSs, hydrophobic proteolipids, integrins, and a wider range of signaling molecules and GFRs ^{120,121}. The studies demonstrated that glycosynapses are involved in various cellular functions, such as cell growth and motility, which are enhanced during tumor progression ¹²². Studies also showed that increased GM3 expression can enhance the anti-metastatic functions of TSs, CD82 and CD9 ¹²³. In a human colon cancer cell line HRT18 that highly expresses CD9, the increased GM3 expression resulted in increased interaction between CD9 and integrin α 3 and decreased cell migration through laminin-5-coated membranes ¹²⁴.

1.3.5 Effect on tumor cell movement

Tumor cell adhesion, motility and migration are very important in the development and progression of cancers. Studies have demonstrated that GM3 has a clear effect on cell adhesion, motility and migration, and change of GM3 expression plays multiple roles in the tumor cell motility, invasiveness and survival ¹²⁵.

On ovarian cancer, the effect of GM3 synthase over expression on cell motility is due to the change of ganglioside content. The cell motility can be reduced by the exogenous addition of GM3, GM2 or GM1 (not GD1a) as well as pharmacologically

¹¹⁸ Y. Zhang, K. Iwabuchi, S. Nunomura, S. Hakomori, *Biochem.*, **2000**, *39*, 2459-2468.

¹¹⁹ N. Kojima, M. Shiota, Y. Sadahira, K. Handa, S. Hakomori, J. Biol. Chem., **1992**, 267, 17264-17270.

¹²⁰ S. K. Bromley, W. R. Burack, K. G. Johnson, K. Somersalo, T. N. Sims, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, M. L. Dustin, *Ann. Rev. Immun.*, 2001, *19*, 375-396.

¹²¹ A. Viola, S. Schroeder, Y. Sakakibara, A. Lanzavecchia, *Sci.*, **1999**, *283*, 680-682.

¹²² S. Hakomori, Proc. Natl. Acad. Sci. USA, 2002, 99 (16), 10231-10233.

¹²³ M. Ono, K. Handa, D. A. Withers, S. Hakomori, *Cancer Res.*, **1999**, *59*, 2335-2339.

¹²⁴ Y. Kawakami, K. Kawakami, W. F. Steelant, M. Ono, R. C. Baek, K. Handa, D. A. Withers, S. Hakomori, J. Biol. Chem., 2002, 277 (37), 34349-34358.

¹²⁵ S. Ueno, S. Saito, T. Wada, K. Yamaguchi, M. Satoh, Y. Arai, T. Miyagi, J. Bio. Chem., 2006, 281 (12), 7756-7764.

achieved higher ganglioside levels in human ovarian cancer cell line A2780^{126,127}. Further results demonstrated that the reduced cell motility is associated with modulating caveolin expression and regulating c-Src kinase activity in the glycolipid-enriched microdomains.

GM3 has already been confirmed as a cofactor of CD9, which is a motility-regulatory membrane receptor. In regulating cancer cell motility, CD9 complexed with GM3 can inhibit motility. The enhanced synthesis of GM3 caused by brefeldin A can lead to decreased invasiveness in some tumors, including bladder cancer and colonic cancer, due to the capability of GM3 to interconnect integrin with CD9¹²⁸.

GM2/GM3 complex fixed on silica nanospheres strongly inhibits cell motility through CD82/c-Met-mediated pathway has been reported ¹²⁹. Other report also explained that HGFR/c-Met is involved in the regulation of cell motility and migration ⁴⁷. Further studies showed that GM3 and GM2, independently or through the formation of GM3/GM2 dimers, can negatively regulate the motility through inhibiting c-Src or c-Met tyrosine kinase signaling pathways in the tetraspanin-expressing normal and tumor cells ¹³⁰.

GM3-enriched microdomain plays an important role in modulating tumor cell motility, and three kinds of bladder cancer cell lines have been studied, and show different cell motility and GM3 expression ¹³¹. Through the study, the scheme of the inhibitory or suppressive effects for GM3 on cell motility and growth was established (**Figure I-12**). Firstly, GM3 can enhance interaction between CD9 and integrin α 3 β 1 and suppresses c-Src activation by the Ln-induced integrin activation. Secondly, GM2 alone or GM2/GM3 complex binds with CD82. However, the former binding is

¹²⁶ A. Prinetti, T. Cao, G. Illuzzi, S. Prioni, M. Aureli, N. Gagliano, G. Tredici, V. Rodriguez-Menendez, V. Chigorno, S. Sonnino, J. Biol. Chem., 2011, 286 (47), 40900-40910.

¹²⁷ A. Prinetti, M. Aureli, G. Illuzzi, S. Prioni, V. Nocco, F. Scandroglio, N. Gagliano, G. Tredici, V. Rodriguez-Menendez, V. Chigorno, S. Sonnino, *Glycobiology*, **2010**, *20* (1), 62-77.

¹²⁸ M. Ono, S. Sonnino, D. A. Withers, H. Nagai, S. Hakomori, *Biochem.*, **2001**, 40, 6414-6421.

 ¹²⁹ A. R. Todeschini, J. N. Dos Santos, K. Handa, S. Hakomori, *Proc. Natl. Acad. Sci. USA*, **2008**, *105* (6), 1925-1930.

¹³⁰ K. Mitsuzuka, K. Handa, M. Satoh, Y. Arai, S. Hakomori, J. Bio. Chem., **2005**, 280 (42), 35545-35553.

¹³¹ H. Wang, T. Isaji, M. Satoh, D. Li, Y. Arai, J. Gu, Urology, **2013**, 81 (1), 210 e11-e15.

weaker, and the complex can inhibit HGF-induced activation of c-Met and the cross-talk between c-Met and integrin $\alpha 3\beta 1$. Further, various signal transducer molecules, such as FAK, Src, Ras, Raf and MAPK (mitogen-activated protein kinase), are implicated. Finally, the cell adhesion, motility and growth are mediated ¹³². These results demonstrated that GM3 inhibits cell motility through modulating functions of other molecules co-localized in membrane microdomains.



Figure I-12 Scheme of the inhibitory or suppressive effects for GM3 on cell motility and growth through modulating functions of other molecules co-localized in membrane microdomains.

1.4 Synthetic strategies towards gangliosides

Several important concepts can be applied to the synthesis of almost all gangliosides. At first, the lipid moiety should be incorporated into the oligosaccharide chain after complete construction of the sugar chain. For example, to synthesize ganglioside GM3, it has been found that when the lipid precursor is coupled with glucose that is the reducing terminal of the oligosaccharide chain, due to the steric hindrance, the reactivity of the resulting glycolipid intermediate is too low to serve as a glycosyl acceptor for further sugar chain elongation. In addition, it is popular that azidosphingosine is employed as a ceramide precursor, a sphingosine equivalent.

¹³² A. R. Todeschini, J. N. Dos Santos, K. Handa, S. Hakomori, J. Biol. Chem., 2007, 282 (11), 8123-8133.

Despite the formed glycosyl azidosphingosine needs further transformation, the good coupling yield and the versatility to accept different fatty acids as components of ceramide can be achieved. Finally, the sialic acid should be introduced at an early stage during the synthetic route due to its structural feature. Sialic acid can afford the corresponding glycoside in high selectivity with good yield only when it is coupled with reactive acceptors.

1.4.1 Synthesis of sialosides

The classical approach to chemical *O*-sialylation involves the coupling between a sialosyl donor having an appropriate leaving group (LG) at the anomeric center (C-2) and a glycosyl acceptor bearing at least one free hydroxyl (ROH, **Scheme I-1**). The sialic acid donors usually are sialyl halides, thiosialosides or phosphite derivatives ^{133,134,135,136,137,138,139}.



Scheme I-1 Classical approach for O-sialylation.

The synthesis of sialosides from 2-chloride derivatives was the main method through

¹³³ G. J. Boons, A. V. Demchenko, *Chem. Rev.*, **2000**, *100*, 4539-4565.

¹³⁴ K. Okamoto, T. Goto, *Tetrahedron*, **1990**, *46*, 5835-5857.

¹³⁵ M. P. De Ninno, *Synthesis*, **1991**, 583-593.

¹³⁶ P. J. Garegg, Adv. Carbohydr. Chem. Biochem., **1997**, 52, 179-205.

¹³⁷ J. D. C. Codee, R. E. J. N. Litjens, L. J. van den Bos, H. S. Overkleeft, G. A. van der Marel, *Chem. Soc. Rev.*, **2005**, *34*, 769-782.

¹³⁸ H. Kondo, Y. Ichikawa, C. H. Wong, J. Am. Chem. Soc., **1992**, 114, 8748-8750.

¹³⁹ T. J. Martin, R. R. Schmidt, *Tetrahedron Lett.*, **1992**, *33*, 6123-6126.

1980. However, these derivatives are limited to be used for the glycosylation of simple alcohols or as precursors for other building blocks synthesis, such as thiosialosides. Usually, for halo-derivatives activation, silver (i) and mercury (ii) salts are used, based on Koenigs-Knoor and Helferich methods, respectively. Silver salts are less reactive than mercury salts, allowing a slightly higher stereoselectivity ^{140,141}.



Scheme I-2 Synthesis of Neu5Ac2→6Gal derivatives.

For example, a Neu5Ac2 \rightarrow 6Gal derivative was synthesized in an excellent yield with 84% as a 3/4 mixture of α/β -anomers, using Hg(CN)₂/HgBr₂ as promoter (**Scheme I-2**). When using relatively unreactive Ag₂CO₃ as promoter, although complete stereoselectivity, poor yield was obtained. But still in the presence of Ag₂CO₃, when partially protected diol acceptor was used, higher yield with good stereoselectivity can

 ¹⁴⁰ X. L. Sun, Y. Kanie, C. T. Guo, O. Kanie, Y. Suzuki, C. H. Wong, *Eur. J. Org. Chem.*, 2000, 2643-2653.
 ¹⁴¹ T. O. G. M. T. G. M. H. C. Chem. 2004, 60 (1001) 1000.

¹⁴¹ T. Q. Gregar, T. Gervay-Hague, J. Org. Chem., 2004, 69, 1001-1009.

be obtained. This approach is principally effective for the sialylation of primary acceptors ^{142,143}.

In particular, thioglycosides have been used as versatile building blocks for sugar synthesis, and for its activation, many different conditions have been developed ^{144,145}. When synthesizing Neu5Aca2 \rightarrow 3Gal derivative, in the presence of promoter dimethyl(methylthio)sulfonium trifluoromethanesulfonate (DMTST, **Scheme I-3**), good yield with high α -anomeric selectivity was achieved. Especially, the best results were obtained when glycosyl acceptor was only protected at C-1 and C-6 positions ^{146,147,148,149}. For this sialylation, the complete regioselectivity is attributed to the greater reactivity of the 3-OH and a considerable steric bulkiness of the sialosyl donor.

When still using DMTST as promoter for the sialylation with a lactosyl acceptor bearing a free secondary 3',4'-diol, the yield accompanied with the stereoselectivity was reduced, 38% yield of a mixture $\alpha/\beta - 4/1$ (Scheme I-3). However, the yield and the stereoselectivity (69% yield of a mixture $\alpha/\beta - 6/1$) were improved using highly reactive promoter system NIS/TfOH. It has been confirmed that NIS/TfOH promoter is especially useful for glycosylations of sterically more hindered hydroxyls ¹⁵⁰.

¹⁴² H. Paulsen, H. Tietz, Angew. Chem. Int. Ed. Engl., **1982**, 21, 927-928.

¹⁴³ G. Pazynina, A. Tuzikov, A. Chinarev, P. Obukhova, N. Bovin, *Tetrahedron Lett.*, **2002**, *43*, 8011-8013.

¹⁴⁴ S. Oscarson, in *Carbohydrates in chemistry and biology*, Vol. 1 (Eds. B. Ernst, G. W. Hart, P. Sinay), Wiley-VCH, Weinheim, New York, **2000**, 93-116.

¹⁴⁵ S. Oscarson, in *Glycoscience: chemistry and chemical biology*, Vol. 1 (Eds. B. Fraser-Reid, K. Tatsuta, J. Thiem), Springer, Berlin-Heidelberg-New York, **2001**, 643-671.

¹⁴⁶ O. Kanie, M. Kiso, A. Hasegawa, J. Carbohydr. Chem., **1988**, 7, 501-506.

¹⁴⁷ T. Murase, H. Ishida, M. Kiso, A. Hasegawa, *Carbohydr. Res.*, **1988**, 184, c1-c4.

¹⁴⁸ T. Murase, H. Ishida, M. Kiso, A. Hasegawa, *Carbohydr. Res.*, **1989**, *188*, 71-80.

¹⁴⁹ T. Murase, A. Kameyama, K. P. R. Kartha, H. Ishida, M. Kiso, A. Hasegawa, J. Carbohydr. Chem., **1989**, *8*, 265-283.

¹⁵⁰ A. Hasegawa, T. Nagahama, H. Ohki, K. Hotta, H. Ishida, M. Kiso, *J. Carbohydr. Chem.*, **1991**, *10*, 493-498.



Scheme I-3 Synthesis of Neu5Ac2→3Gal derivatives.

Good yield with high stereoselectivity has also been achieved in the presence of phenyl sulfenyl trifluoromethanesulfonate (PhSOTf) obtained from benzenesulfenyl chloride (PhSCl) and silver trifluoromethanesulfonate (AgOTf), and 2,6-di(*tert*-butyl)pyridine (DTBP) at low temperature (-70 °C) ¹⁵¹. As shown in **Scheme I-4**, the trisaccharide was synthesized in a good yield of 74% mainly as the α -anomer (α/β - 19/1).

It has been reported that thiosialosides can be selectively activated in the presence of the other thioglycosides, an example was shown in **Scheme I-5**. In a number of applications, thiosialosides outperformed their other *S*-alkyl counterparts in direct sialylation reactions¹⁵².

¹⁵¹ V. Martichonok, G. M. Whitesides, J. Org. Chem., **1996**, 61, 1702-1706.

¹⁵² C. De Meo, O. Parker, *Tetrahedron: Asymmetry*, **2005**, *16*, 303-307.



Scheme I-4 PhSOTf as promoter in the sialylation reaction.



Scheme I-5 Sialylation that thiosialoside outperforming other S-alkyl counterpart.

Until now, a lot of efforts have been exerted in the investigation of positional modifications for sialic acids, including the introduction of participating auxiliaries at

the C-1 and C-3 positions ^{153,154,155,156,157}. In addition, C-5 acetamido group modifications have also been reported that have effects on glycosylation reactions. Furthermore, it has been shown that the nature of the protecting group at the C-5 position plays an important role in influencing the reactivity of both sialosyl donors and acceptors ^{158,159,160,161,162,163}.

1.4.2 Glycosylation of azidosphingosines

Usually, β -glucosyl- or β -galactosyl- ceramides are synthesized by coupling between azidosphingosine ¹⁶⁴ and glucose or galactose trichloroacetimidates protected with participating groups, including acetyl, benzoyl or pivaloyl esters. Then the obtained glycosyl azidosphingosines can be further converted into the amino derivatives to construct the ceramide moieties. Finally, goal products are synthesized. As shown in **Scheme I-6**, glucosyl azidosphingosine **L-4** was prepared in 56% yield through reaction between **L-1** and lipid precursor **L-3** using BF₃·Et₂O as promoter ¹⁶⁵. Galactosyl azidosphingosine **L-5a** was synthesized in 55% yield by reaction of **L-2a** with **L-3** using triethylsilyl trifluoromethanesulfonate as promoter ¹⁶⁶. From galactose trichloroacetimidate **L-2b** using BF₃·Et₂O as promoter, **L-5b** was obtained in an excellent 88% yield ¹⁶⁷.

¹⁵³ T. Takahashi, H. Tsukamoto, H. Yamada, *Tetrahedron Lett.*, **1997**, *38*, 8223-8226.

¹⁵⁴ K. Okamoto, T. Kondo, T. Goto, *Chem. Lett.*, **1986**, *9*, 1449-1452.

¹⁵⁵ Y. Ito, T. Ogawa, *Tetrahedron*, **1990**, *46*, 89-102.

¹⁵⁶ J. M. Haberman, D. Y. Gin, Org. Lett., 2001, 3, 1665-1668.

¹⁵⁷ A. Ishiwata, Y. Ito, *Synlett*, **2003**, *9*, 1339-1343.

¹⁵⁸ A. V. Demchenko, G. J. Boons, *Tetrahedron Lett.*, **1998**, *39*, 3065-3068.

¹⁵⁹ A. V. Demchenko, G. J. Boons, *Chem. Eur. J.*, **1999**, *5*, 1278-1283.

¹⁶⁰ E. Schreiner, E. Zbiral, *Liebigs Ann. Chem.*, **1990**, 581-586.

¹⁶¹ C. S. Yu, K. Niikura, C. C. Lin, C. H. Wong, Angew. Chem. Int. Ed., 2001, 40, 2900-2903.

¹⁶² C. De Meo, A. V. Demchenko, G. J. Boons, J. Org. Chem., 2001, 66, 5490-5497.

¹⁶³ H. Ando, Y. Koike, S. Koizumi, H. Ishida, M. Kiso, Angew. Chem. Int. Ed., 2005, 44, 2-7.

¹⁶⁴ R. R. Schmidt, P. Zimmermann, Angew. Chem. Int. Ed. Engl., 1986, 25, 725-726.

¹⁶⁵ R. I. Duclos, *Chem. Phys. Lipids*, **2001**, *111*, 111-138.

¹⁶⁶ L. Franchini, F. Compostella, A. Donda, L. Mori, D. Colombo, G. De Libero, P. Matto, F. Ronchetti, L. Panza, *Eur. J. Org. Chem.*, 2004, 4755-4761.

¹⁶⁷ F. Compostella, L. Franchini, G. De Libero, G. Palmisano, F. Ronchetti, L. Panza, *Tetrahedron*, **2002**, *58*, 8703-8708.



Scheme I-6 Synthesis of β -glucosyl and β -galactosyl azidosphingosines.



Scheme I-7 Synthesis of ganglioside GD3.

For synthesizing ganglioside GD3, the coupling between the oligosaccharide trichloroacetimidate **L-6** and azidosphingosine **L-3** was also conducted with BF₃·Et₂O as promoter. In this case, glycosyl sphingosine **L-7** was obtained with the yield of 82% as only β isomer (Scheme I-7)¹⁶⁸. At last, the intermediate L-7 was transformed into target compound L-8 after several standard steps.

Using glycal epoxides for the construction of glycosyl azidosphingosine was also examined. A typical example is involved in using galactal-derived epoxide. The use of a cyclic carbonate protecting group at C3 and C4 OH can lead to favored β -galactosylation. In this situation, coupling between **L-9** and **L-10** resulted in forming **L-11** with a high selectivity for β isomer (**Scheme I-8**) ^{169,170}.



Scheme I-8 Construction of galactosyl azidosphingosine using glycal epoxide.

Another example is glycal L-12 which was directly used for sialylation reaction to form sialoside L-13. Further, epoxidation of L-13 with dimethyldioxirane (DMDO) and glycosylation with ceramide precursor L-10 using ZnCl₂ produced glycoside L-14. Actually, compound L-14 was converted to the pure sodium salt of ganglioside GM4 (Scheme I-9).

¹⁶⁸ J. C. Castro-Palomino, B. Simon, O. Speer, M. Leist, R. R. Schmidt, *Chem. Eur. J.*, **2001**, *7*, 2178-2184.

¹⁶⁹ S. J. Ddnishefsky, M. T. Bilodeau, Angew. Chem. Int. Ed. Engl., 1996, 35, 1380-1419.

¹⁷⁰ J. Gervay, J. M. Peterson, T. Oriyama. S. J. Danishefsky, J. Org. Chem., **1993**. 58,5465-5468.



Scheme I-9 Synthesis of sialylated galactosyl-azidosphingosine using glycal epoxide.

1.5 Previous studies on ganglioside GM3 analogues

Based on the previous presentation, it is encouraging that studying ganglioside GM3 and its analogues appear to be a logical matter of research, and until now, some achievements have been obtained. In this chapter, some typical examples about GM3 analogues are described.

A stable GM3-lactone (between carboxyl group of sialic aicd and C2-OH of galactose) analogue has been synthesized, which can be as hapten for a possible immunization against cancer ¹⁷¹.

It has been reported that de-*N*-acetyl-GM3 derivative can enhance EGFR activity and EGF-induced cell growth, including A431, Swiss 3T3 and B16 cells. GM3 can inhibit tyrosine phosphorylation, but de-*N*-acetyl-GM3 enhances serine phosphorylation of EGFR ¹⁷².

Monochloro-GM3 and dichloro-GM3 analogues have been synthesized (**Figure I-13**)¹⁷³. In analyses of EGFR autophosphorylation and cell proliferation in A431 cells, two chloro-derivatives exhibited stronger inhibitory effects than GM3 on EGFR activity.

¹⁷¹ L. T. Tietze, H. Keim, Angew. Chem. Int. Ed. Engl., **1997**, 36, 1615-1616.

¹⁷² Q. Zhou, S. Hakomori, K. Kitamuras, Y. Igarashi, J. Biol. Chem., **1994**, 269, 1950-1965.

¹⁷³ N. Kawashimai, H. Qu, M. Lobaton, Z. Zhu, M. Sollogoub, W. K. Cavenee, K. Handa, S. Hakomori, Y. Zhang, Oncol. Lett., 2014, 7, 933-940.

In particular, monochloro-GM3, not GM3 or dichloro-GM3, showed a significant inhibitory effect on Δ EGFR.



Figure I-13 Monochloro-GM3 and dichloro-GM3 analogues.

A GM3 position isomer was also synthesized, which is a α -2,6 sialoside instead of α -2,3 sialoside (Figure I-14)¹⁷⁴.



Figure I-14 GM3 position isomer analogue.

Sialylated glucosamine or galactosamine core plays important roles in many biological processes, and the GM3 anglogues containing sialylated glucosamine and galactosamine were already synthesized, respectively (**Figure I-15**)¹⁷⁵.

¹⁷⁴ A. Hasegawa, M. Ogawa, M. Ki, *Biosci. Biotechnol. Biochem.*, **1992**, *56*, 535-536.

¹⁷⁵ A. Hasegawa, Y. Kojima, M. Ogawa, M. Kiso, J. Carbohydr. Chem., **1992**, 11, 343-357.



Figure I-15 Glucosamine- and galactosamine- containing GM3 analogues.



Figure I-16 Glucose- and galactose- containing GM3 analogues.

In the previous studies of our lab, glucose-containing analogues with α -2,6 sialoside and galactose-containing analogues with α -2,3 sialoside were synthesized (**Figure I-16**). These analogues showed inhibitory effects on human colon cancer cell line HCT116 and leukemia cell line K562. In particular, the former glucose-containing analogues with α -2,6 sialoside showed much better results than the latter ones ¹⁷⁶.

Multivalent interactions are important in a variety of biological processes. In these cases, a multivalent ligand can bind to one or more receptors with enhanced functional affinity ^{177,178,179}. The lyso-GM3, its dimer and multimers (**Figure I-17**) have been synthesized. The results revealed that lyso-GM3 or lyso-GM3 dimer strongly inhibited EGFR kinase activity, while lyso-GM3 trimer and tetramer did not. When the concentration was above 50 μ M, lyso-GM3 was cytolytic, while lyso-GM3 dimer was not cytolytic, yet still inhibited EGFR kinase strongly ¹⁸⁰.



Figure I-17 Lyso-GM3 and its multimers.

The lipid mimetic of lyso-GM3 dimer (Figure I-18) was synthesized, and the results

¹⁷⁶ H. Qu, J. Li, J. Wdzieczak-Bakala, D. Lu, X. He, W. Sun, M. Sollogoub, Y. Zhang, *Eur. J. Med. Chem.*, **2014**, 75, 247-257.

¹⁷⁷ L. L. Kiessling, J. E. Gestwicki, L. E. Strong, Curr. Opin. Chem. Biol., 2000, 4, 696-703.

¹⁷⁸ M. Mammen, S. K. Choi, G. M. Whitesides, Angew. Chem. Int. Ed. Engl., 1998, 37, 2755-2794.

¹⁷⁹ J. J. Ludquist, E. J. Toone, Chem. Rev., 2002, 102, 555-578.

¹⁸⁰ Y. Murozuka, N. Watanabe, K. Hatanaka, S. Hakomori, *Glycoconj. J.*, **2007**, *24*, 551-563.

showed that the lipid mimetic of lyso-GM3 dimer and authentic lyso-GM3 dimer showed similar inhibitory effects on EGFR tyrosine kinase. Akt kinase can be activated by EGF, and its activity was also inhibited by the lipid mimetic of lyso-GM3 dimer in A431 cells. The lipid mimetic of lyso-GM3 dimer inhibiting EGFR tyrosine kinase resulted in inhibition of mitogenesis in A431 cells¹⁸¹.



Figure I-18 Lipid mimetic of lyso-GM3 dimer analogue.

1.6 Objective of this project

As previous description, ganglioside GM3 is over expressed on some cancers, and it is well-known as a tumor-associated carbohydrate antigen on several types of cancers. Furthermore, GM3 can modulate the receptors or signaling pathways related to tumors, such as EGFR signaling. In addition, GM3 can inhibit tumor cell growth and migration by anti-angiogenesis or motility. In particular, GM3 can modulate functions of other molecules co-localized in the membrane microdomains. Taking these into consideration, in order to find the agents with better biological activities than GM3, the study of GM3 analogues with antitumor properties appears to be a logical matter of research. In this study, a series of GM3 analogues, including galactose-, mannose-, lactose- and glucosamine- containing analogues with α -2,6 sialoside instead of α -2,3 sialoside, will be designed and synthesized from commercially available substrates and reagents by enzymatic hydrolysis and chemical synthesis. Then the antitumor

¹⁸¹ Y. Haga, K. Hatanaka, S. Hakomori, *Biochim. Biophys. Acta*, **2008**, *1780 (3)*, 393-404.

activities of these analogues will be studied.

In addition, the important role of ganglioside in neuritogenesis has been revealed. Neurite outgrowth is a key process in the development of functional neuronal circuits and regeneration of the nervous system after injury. However, the study of ganglioside GM3 on neurite outgrowth is very limited. In the present study, the effect of GM3 analogues on neurite outgrowth will also be studied.

Chapter II Synthesis of galactose- and mannosecontaining ganglioside GM3 analogues

2.1 Introduction

The total synthesis of galactose- and mannose- containing ganglioside GM3 analogues was described in this chapter. These four analogues as shown in **Figure II-1** include:



Figure II-1 Target galactose- and mannose- containing ganglioside GM3 analogues.

 ${\it O-}(5\mbox{-}acetamido-3,5\mbox{-}dideoxy-D\mbox{-}glycero-\alpha-D\mbox{-}galacto-2\mbox{-}nonulopyranosylonic}$

acid)- $(2\rightarrow 6)$ -O- $(\beta$ -D-galactopyranosyl)- $(1\rightarrow 1)$ -(2S, 3R, 4E)-2-octadecanamido-4-octad ecene-1,3-diol.

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-(β-D-galactopyranosyl)-(1→1)-(2S,3R,4E)-2-amino-4-octadecene-1,3-diol.

O-(5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-(α -D-mannopyranosyl)-(1→1)-(2S,3R,4E)-2-octadecanamido-4-octad ecene-1,3-diol.

O-(5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-(α -D-mannopyranosyl)-(1→1)-(2S,3R,4E)-2-amino-4-octadecene-1,3diol.

These analogues can be divided into three units as shown in **Figure II-1**. The synthetic strategy is as follows: Firstly, preparation of three key building blocks. Secondly, conduction of the sialylation reaction with sialic acid acceptors to construct the sialo-oligosaccharides. Finally, incorporation the lipid precursor into the sugar chains.

2.2 Preparation of sialic acid block

In this study, the sialic acid donor was activated as sialyl xanthate or sialyl chloride form. The synthesis of sialic acid donors 2 and 3 can be achieved as shown in **Scheme II-1**.

Firstly, methyl ester 1 was formed by reaction of sialic acid with methanol under acidic conditions. After 6 h at 45 °C, the reaction solution became clear. For this reaction, we adopted Amblyst-H⁺ resin as acidic catalyst to avoid liquid acid, which was easy to remove by filtration. The obtained compound 1 in good yield was pure enough to be used in the subsequent reaction without further purification. Then, the crude methyl ester 1 was treated with acetyl chloride for 3 days resulting in formation

of the fully acetylated chloroneuraminic acid methyl ester 2, which was one of our target sialic acid donors. Although the long time was needed for this reaction, the yield was excellent.



Scheme II-1 Reagents and conditions: (a) Amblyst-H⁺, MeOH, 45 °C, 6 h, 90%; (b) AcCl, 0 °C, MeOH, r.t., 72 h, 90%; (c) EtOH, 0 °C, KS₂COEt, 12 h, 0 °C - r.t., 12 h, 76% (for **3**), 19% (for **4**).

Finally, when the sialyl chloride **2** was reacted with *O*-ethyl *S*-potassium dithiocarbonate in dry EtOH, a mixture of α - and β -sialyl xanthate **3** (16:1) and glycal **4** were obtained. Chromatographic separation gave our target sialosyl donor **3**, which was contaminated with sialyl glycal **4**, probably due to their partial decomposition on silica gel. The sialic acid donor, α/β -**3** mixture can be directly used in the sialylation reaction without separation of anomers.

2.3 Preparation of azidosphingosine block

D-*erythro*-sphingosine is an important moiety in the GSLs. Previous studies have already shown that azidosphingosine is the preferable glycosyl acceptor for preparation of these glycolipids ^{182,183,184}. A number of synthetic routes about

¹⁸² R. R. Schmidt, P. Zimmermann, Angew. Chem. Int. Ed. Engl., 1986, 25, 725-726.

¹⁸³ T. Baer, R. R. Schmidt, *Liebigs Ann. Chem.*, **1988**, 7, 669-674.

¹⁸⁴ Y. D. Vankar, R. R. Schmidt, Chem. Soc. Rev., 2000, 29, 201-206.

azidosphingosine have been reported 185,186,187,188,189,190 . Herein we adopted a short and practical route to synthesize azidosphingosine from the commercially available **D-(+)-galactose (Scheme II-2)** ¹⁹¹.



Scheme II-2 Reagents and conditions: (a) α,α -dimethoxytoluene, PTSA, DMF, 40 °C, 1.5 h, 80%; (b) NaIO₄, phosphate buffer (pH = 7.6, 76 mL, 0.1 M), r.t., 2 h, 75%; (c) tetradecyltriphenylphosphonium bromide 7, *n*-butyllithium, THF, -30 °C, 2 h, 45% (*trans-8*) and 18% (*cis-8*); (d) diphenyldisulfide, cyclohexane, dioxane, high-pressure mercury lamp, 5 h, 45%; (e) Tf₂O, CH₂Cl₂, pyridine, -15 °C, 15 min; NaN₃, DMF, r.t., 3 h, 75% (two steps); (f) PTSA, MeOH, CH₂Cl₂, r.t., 24 h, 90%; (g) TBDMSCl, CH₂Cl₂, pyridine, r.t., 3 h, 90%; (h) BzCl, pyridine, r.t., 1 h, quant.; (i) BF₃·Et₂O, CH₂Cl₂, 0 °C - r.t., 4 h, 80%.

At the beginning, the introduction of 4,6-*O*-benzylidene to **D**-(+)-galactose was performed using α,α -dimethoxytoluene in the presence of *p*-toluenesulfonic acid (PTSA) in dry DMF. The methanol formed during the reaction was evaporated at 40 °C progressively to promote the reaction. Compound **5** was obtained in high yield,

¹⁸⁵ P. Zimmermann, R. R. Schmidt, *Liebigs Ann. Chem.*, **1988**, 7, 663-667.

¹⁸⁶ Y. Ito, M. Kiso, A. Hasegawa, J. Carbohydr. Chem., **1989**, 8, 285-294.

¹⁸⁷ P. Kumar, R. R. Schmidt, Synthesis, **1998**, 1, 33-35.

¹⁸⁸ P. M. Koskinen, A. M. P. Koskinen, *Synthesis*, **1998**, *8*, 1075-1091.

¹⁸⁹ F. Compostella, L. Franchini, G. B. Giovenzana, L. Panza, D. Prosperi, F. Ronchetti, *Tetrahedron: Asymmetry*, **2002**, *13*, 867-872.

¹⁹⁰ Y. Gao, X. He, F. Ding, Y. Zhang, Synthesis, 2016, 48, 4017-4037.

¹⁹¹ R. R. Schmidt, P. Zimmermann, *Tetrahedron Lett.*, **1986**, *27*, 481-484.

further treated with NaIO₄ in phosphate buffer at room temperature for 2 h to give aldehyde **6**. Then tetradecyltriphenylphosphonium bromide **7** was prepared using 1-bromotetradecane and PPh₃ at 140 °C for 7 h⁻¹⁹². The wittig reaction was conducted with *n*-butyllithium in THF at low temperature to provide a mixture of isomers in the radio of E/Z = 3/1. After careful purification, the desired *trans*-**8** was obtained in 45% yield. The Z-isomer can be transformed to the desired *E*-isomer through an illumination reaction in the presence of diphenyldisulfide. Finally, the introduction of azide group was carefully carried out from *trans*-**8** via the corresponding triflate intermediate by the reported method ^{193,194}. It is important to notice that the extraction and concentration of reaction mixtures containing sodium azide have been reported to be potentially explosive, so this procedure should be performed with caution ^{195,196}.

Removing of 4,6-*O*-benzylidene group from compound **9** by PTSA catalysis with an Et₃N quench and non-aqueous workup can give a higher yield to azidosphingosine **10**. Next, for the diol **10**, the primary hydroxyl was selectively protected using TBDMSCl to obtain **11** in high yield. Then in the compound **11**, the secondary hydroxyl was protected with benzoyl group to get compound **12** quantitatively. At last, removing TBDMS group, the desired 3-*O*-benzoyl azidosphingosine derivative **13** was synthesized, which can be used for glycosidic coupling reactions.

2.4 Galactose-containing analogues

2.4.1 Preparation of galactose with a free 6-OH block

Using regioselective enzymatic hydrolysis of per-acetylated sugar has been proposed as an important tool for developing simple and efficient synthesis of sugar building

¹⁹² O. H. Abdelmageed, R. I. Duclos Jr, R. G. Griffin, D. J. Siminovitch, M. J. Ruocco, A. Makriyannis, *Chem. Phys. Lipids*, **1989**, 50, 163-169.

¹⁹³ H. S. Byun, R. Bittman, in *Phospholipids handbook* (Ed. G. Cevc), Marcel Dekker, New York, **1993**, 97-140.

¹⁹⁴ K. H Jung, R. R. Schmidt, *Methods Enzymol.*, **2000**, *311*, 441-457.

¹⁹⁵ V. J. Hruby, L. W. Boteju, G. Li. Chem. Eng. News, **1993**, 71 (41), 2.

¹⁹⁶ N. P. Peet, P. M. Weintraub, *Chem. Eng. News*, **1993**, *71 (16)*, 4.

blocks ¹⁹⁷. The most used bio-catalyst for regioselective deacetylation is the lipase from *Candida rugosa* (CRL). The galactose bearing a free 6-OH was prepared by enzymatic hydrolysis using CRL ¹⁹⁸. The enzyme is specific to remove the C-6 acetyl group from per-acetylated sugars. In particular, when the per-acetylated sugar is α and β mixture, the enzyme can be completely selective to hydrolyze α anomer, while β anomer remains completely unreacted.

As shown in Scheme II-3, the galactose residue 15 having a free 6-OH can be synthesized in high yield starting with commercially available D-(+)-galactose. At first, the D-(+)-galactose was acetylated using Ac₂O and pyridine to obtain the per-acetylated sugar 14. Then compound 14 was dissolved in the phosphate buffer with 20% of CH₃CN. After adding the enzyme CRL, the enzymatic hydrolysis was started and went on for 4 h. When the compound 14 was completely disappeared, filtration to recover the enzyme, the compound 15 was obtained.



Scheme II-3 Reagents and conditions: (a) Ac₂O, pyridine, r.t., 24 h, 70%; (b) enzyme CRL, phosphate buffer, CH₃CN, r.t., 4 h, 80%.

Actually, studying complex molecules including glycoconjugated products is some time limited due to the complex procedure required for the preparation of oligosaccharides by the classical orthogonal synthetic strategy. In particular, the preparation of different sugar building blocks having only one free hydroxyl group in the desired position for glycosylation is the most time consuming task. In this study, the enzymatic hydrolysis is introduced to prepare GM3 building block. By enzymatic hydrolysis, we can avoid multiple chemical procedures.

¹⁹⁷ M. Filice, J. M. Guisan, M. Terreni, J. M. Palomo, *Nat. Protoc.*, **2012**, *7*, 1783-1796.

¹⁹⁸ M. Filice, T. Bavaro, R. Fernandez-Lafuente, M. Pregnolato, J. M. Guisan, J. M. Palomo, M. Terreni, *Catal. Today*, **2009**, *140*, 11-18.

2.4.2 Synthesis of galactose-containing analogues

For synthesizing galactose-containing analogues, based on retrosynthetic analysis, three key building blocks were needed (**Figure II-2**). Firstly, the sialic acid was activated as previous sialyl xanthate **3**. Then the galactose residue **15**, which was prepared by enzymatic hydrolysis. Finally, 3-*O*-benzoyl azidosphingosine block **13** was used as a lipid precursor.



Figure II-2 Three key building blocks: sialyl xanthate block **3**, galactose residue **15** and 3-*O*-benzoyl azidosphingosine block **13**.

Sialylation is the key step for the total synthesis of our target analogues. In spite of extensive efforts and notable progress, the chemical synthesis of sialosides in high yield with complete stereoselectivity remains a significant challenge ^{199,200,201,202}. The presence of a destabilizing electron-withdrawing carboxylic group along with a tertiary anomeric center and the lack of a participating auxiliary often drive glycosylation reactions toward competitive elimination reactions resulting in poor stereoselectivity (β -anomer) and in the formation of a 2,3-dehydro derivative ²⁰³. To overcome these problems, different methods have developed. A variety of leaving groups and activation conditions have been used.

From our previous studies, we tried to optimize the α -sialylation reaction between acceptors and the sialic acid donors. The reaction in the presence of PhSCl and

¹⁹⁹ C. De Meo, B. T. Jones, Adv. Carbohydr. Chem. Biochem., 2018, 75, 215-316.

²⁰⁰ M. Kiso, H. Ishida, H. Ito, in *Carbohydrates in chemistry and biology*, Vol. 1 (Eds. B. Ernst, G. W. Hart, P. Sinay), Wiley-VCH, Weinheim, New York, **2000**, 345-366.

²⁰¹ M. von Itzstein, R. J. Thomson, *Topics Curr. Chem.*, **1997**, *186*, 119-170.

²⁰² D. K. Ress, R. J. Linhardt, Curr. Org. Synt., 2004, 1, 31-46.

²⁰³ C. De Meo, G. J. Boons, A. V. Demchenko, in *Comprehensive glycoscience*, Vol. 1 (Ed. J. P. Kamerling), Elsevier Ltd. 2007, 583-604.

AgOTf as promoters in a 2:1 mixture of CH₃CN/CH₂Cl₂ at low temperature afforded α sialoside in significantly higher yield ¹⁷⁶. So for the sialylation of galactose residue with a free 6-OH **15**, we directly selected this Martichonok and Whitesides method. As shown in **Scheme II-4**, the α -sialylation reaction was carried out at -68 °C with PhSCl and AgOTf as promoters in the mixture of CH₂Cl₂:CH₃CN (1:2) for 3 h. PhSCl was freshly prepared each time using *S*-Phenyl thioacetate and SO₂Cl₂¹⁵¹. In fact, the PhSCl and AgOTf formed PhSOTf *in situ* as a activator in this reaction. At last, the sialylation product **16** was obtained as absolute α isomer in the yield of 70%.

Empirical rules have been used for confirmation of the anomeric configuration due to the absence of an anomeric hydrogen (C-2) for sialic acid, including δ H-3eq, δ H-4, $J_{H-7,H-8}$, $\Delta\delta$ {H-9a-H-9b} and $J_{C-1,H-3ax}$. It was observed that the chemical shifts for H-3eq are different, and α isomer is shifted downfield (δ 2.67-2.72 ppm) compared to β isomer (δ 2.25-2.40 ppm). In addition, the chemical shift for H-4 of an α -anomer appears at higher field (δ 4.89-4.93 ppm) than the corresponding β -anomer (δ 5.68-5.81 ppm). The $J_{H-7,H-8}$ of α -glycosides are larger (6.2-8.5 Hz) than β -anomers (1.5-2.6 Hz), which suggests different conformations of the side chain. Furthermore, the anomeric configuration of the sialosides can also be determined from the chemical shift difference between two vicinal protons at C-9, such as $\Delta\delta$ {H-9a-H-9b} value, which is 0.5 ppm for α -glycosides and around 1.0 ppm for β -anomers. The anomeric configuration can also be determined through the long-range $J_{C,H}$. It was demonstrated that sialic acid residues within α -anomers show a larger $J_{C-1,H-3ax}$ (5.8-7.5 Hz) than the β-anomers (1.0-1.7 Hz). Moreover, the $J_{C-2,H-3ax}$ values were found to be around 8.0 whereas about 4.0 Hz for the Hz for the α -anomers, β-anomers 204,205,206,207,208,209,210,211

²⁰⁴ H. Paulsen, H. Tietz, Angew. Chem. Int. Ed. Engl., 1982, 21, 927-928.

²⁰⁵ D. J. M. van der Vleugel, W. A. R. van Heeswijk, J. F. G. Vliegenthart, *Carbohydr. Res.*, **1982**, *102*, 121-130.

²⁰⁶ K. Okamoto, T. Kondo, T. Goto, *Bull. Chem. Soc. Jpn.*, **1987**, *60*, 637-643.

²⁰⁷ U. Dabrowski, H. Friebolin, R. Brossmer, M. Supp, *Tetrahedron Lett.*, **1979**, *20*, 4637-4640.

²⁰⁸ H. van Halbeek, L. Dorland, J. F. G. Vliegenthart, R. Pfeil, R. Schauer, *Eur. J. Biochem.*, **1982**, *122*, 305-311.

²⁰⁹ H. Hori, T. Nakajima, Y. Nishida, H. Ohrui, H. Meguro, *Tetrahedron Lett.*, **1988**, *29*, 6317-6320.

²¹⁰ R. Christian, G. Schulz, *Carbohydr. Res.*, **1997**, *162*, 1-11.



Figure II-3 Proposed mechanism for α -sialylation reaction.

From the literature ¹⁵¹, the mechanism was proposed as **Figure II-3**. At first, sialyl xanthate reacting with PhSOTf gives the oxocarbenium cation **M-2** through intermediate **M-1**. Then oxocarbenium cation **M-2** is stabilized by reaction with CH₃CN to form nitrilium cations **M-3** and **M-4**. Nitrilium cations have been evoked to explain the stereoselectivities of glycosylation in CH₃CN solvent. Probably the nitrilium cation **M-3** is formed first because of kinetic and thermodynamic control. The attack of CH₃CN from the *re* side of **M-2** to form **M-4** is hindered by interactions with protons at C-4 and C-6 of the sialic acid. Both **M-3** and **M-4** are probably

²¹¹ S. Prytulla, J. Lambert, J. Lauterwein, M. Klessinger, J. Thiem, *Magn. Reson. Chem.*, **1990**, *28*, 888-901.

formed in this step, and the nitrilium cations M-3 and M-4 are in equilibrium. Finally, acceptor ROH reacts with the cation M-4 to give α -product M-5. Attack on cation M-3 is very hindered due to the protons at C-4 and C-6 and the CO₂CH₃ group.



Scheme II-4 Reagents and conditions: (a) CH₃CN, CH₂Cl₂, molecular sieves, r.t., 1 h, AgOTf, DTBP, -68 °C, PhSCl, 3 h, 70%; (b) PhCH₂NH₂, THF, r.t., 24 h; (c) CCl₃CN, DBU, CH₂Cl₂, -5 °C, 5 h, 70% (two steps from **16**).

Next, for the α -sialosides 16, the acetyl group at the anomeric center was selectively

removed by PhCH₂NH₂ in the dry THF at room temperature for 24 h to provide the hemiacetal **17** as α and β mixture. This crude product **17** was not separated and could be directly used for the next step. This hemiacetal was then treated with trichloroacetonitrile in the presence o f DBU to give the trichloroacetimidate **18** in 70% yield for two steps as shown in **Scheme II-4**. The ¹H NMR spectrum showed that compound **18** is the α -trichloroacetimidate on the basis of the H-1' and H-2' coupling constant ($J_{1',2'} = 3.7$ Hz) of the galactose residue.

Conjugation of trichloroacetimidate **18** with 3-*O*-benzoyl azidosphingosine **13** was performed using BF₃·Et₂O as promoter to provide the desired glycolipid **19** in 65% yield, as shown in **Scheme II-5**. The β configuration of the newly introduced glycosidic linkage was confirmed from the ¹H NMR spectrum ($J_{1',2'} = 8.0$ Hz) due to the neighboring participation effect of acetyl group at C-2 position of galactose.

Then the azide group of **19** was reduced by triphenylphosphine in a mixture of toluene and water at 50 °C for 12 h to give an amino free derivative **20**. Compound **20** was not separated and directly reacted with stearic acid in the presence of EDC in dry CH_2Cl_2 to give the glycosyl ceramide **21** in 60% yield for two steps from **19**. The protected groups of compound **21** were subsequently removed by NaOMe/MeOH at room temperature overnight. After adding several drops of water, new ganglioside GM3 analogue **22** was obtained with the yield of 95%.



Scheme II-5 Reagents and conditions: (a) BF₃·Et₂O, CH₂Cl₂, molecular sieves, -15 °C, 3 h, 65%;
(b) PPh₃, toluene, H₂O, 50 °C, 12 h; (c) Stearic acid, EDC, CH₂Cl₂, r.t., 24 h, 60% (two steps from 19); (d) NaOMe, MeOH, r.t., overnight; H₂O, 0 °C - r.t., 2 h, 95%.

On the other hand, as shown in **Scheme II-6**, all the protected groups of compound **19** were removed by NaOMe/MeOH at room temperature overnight. After adding several drops of water, compound **23** was obtained. Then the azide in the **23** was reduced with

propanedithiol/triethylamine to afford the new analogue 24 in 85% yield.



Scheme II-6 Reagents and conditions: (a) NaOMe, MeOH, r.t., overnight; H_2O , 0 °C - r.t., 2 h; (b) $HS(CH_2)_3SH$, Et_3N , MeOH, r.t., 4 days, 85% (two steps from **19**).

2.5 Mannose-containing analogues

2.5.1 Preparation of mannose with a free 6-OH block

As previous method, the enzymatic regioselective hydrolysis of per-acetylated sugar was used to prepare the mannose building block with a free 6-OH ²¹². The enzymatic hydrolysis was conducted with the lipase from *Candida rugosa* (CRL), and the enzyme can be specific to remove the C-6 acetyl group from per-acetylated mannose. As shown in **Scheme II-7**, the mannose residue **26** having a free 6-OH can be obtained in high yield.

²¹² T. Bavaro, M. Filice, C. Temporini, S. Tengattini, I. Serra, C. Morelli, G. Massolini, M. Terreni, *RSC Adv.*, **2014**, *4*, 56455-56465.



Scheme II-7 Reagents and conditions: (a) Ac_2O , pyridine, r.t., 24 h, 70%; (b) enzyme CRL, phosphate buffer, CH₃CN, r.t., 4 h, 80%.

2.5.2 Synthesis of mannose-containing analogues

For synthesizing mannose-containing analogues, according to the retrosynthetic analysis, three building blocks are needed as shown in **Figure II-4**. They are the sialyl xanthate block **3**, the mannose bearing a free 6-OH residue **26** and the 3-*O*-benzoyl azidosphingosine block **13**.



Figure II-4 Three key building blocks: sialyl xanthate block **3**, mannose residue **26** and 3-*O*-benzoyl azidosphingosine block **13**.

Next, the key step was also stereoselective α -sialylation of sialyl xanthate to free 6-OH of all acetylated mannose residue. And for this step, Martichonok and Whitesides method was used. As shown in **Scheme II-8**, the α -sialylation reaction was carried out at -68 °C with PhSCl and AgOTf as promoters in the mixture of CH₂Cl₂:CH₃CN (1:2) for 3 h. PhSCl was freshly prepared each time. At last, the sialylation product **27** was obtained as α isomer in the yield of 75%. In this step, it needs the careful chromatography to purify the crude product. Especially, adding DTBP to scavenge the proton can improve the yield.

Then for the α -sialoside 27, the acetyl group at the anomeric center was selectively removed by reaction of PhCH₂NH₂ in the dry THF at room temperature for 5 h to provide the hemiacetal **28** as an α and β mixture as shown in **Scheme II-8**. This crude product **28** was not separated at this stage and was used directly for the next step. The hemiacetal was then treated with trichloroacetonitrile in the presence of DBU to give the trichloroacetimidate **29** in 70% yield for two steps as shown in **Scheme II-8**.



Scheme II-8 Reagents and conditions: (a) CH₃CN, CH₂Cl₂, molecular sieves, r.t., 1 h, AgOTf, DTBP, -68 °C, PhSCl, 3 h, 75%; (b) PhCH₂NH₂, THF, r.t., 5 h; (c) CCl₃CN, DBU, CH₂Cl₂, -5 °C, 3 h, 70% (two steps from **27**).


Scheme II-9 Reagents and conditions: (a) $BF_3 \cdot Et_2O$, CH_2Cl_2 , molecular sieves, -15 °C, 2.5 h, 60%; (b) PPh₃, toluene, H₂O, 50 °C, 12 h; (c) Stearic acid, EDC, CH_2Cl_2 , r.t., 20 h, 62% (two steps from **30**); (d) NaOMe, MeOH, r.t., 14 h; H₂O, 0 °C - r.t., 2 h, 95%.

Glycosylation of 3-*O*-benzoyl azidosphingosine with trichloroacetimidate **29** was performed by $BF_3 \cdot Et_2O$ as promoter to provide the desired glycolipid **30** in 60% yield, as shown in **Scheme II-9**, and the α configuration of the newly introduced glycosidic linkage was obtained due to the acetyl group at C-2 position of mannose is axial and neighboring participation effect of C-2 acetyl group. Then the azide group of **30** was reduced by triphenylphosphine in a mixture of toluene and water at 50 °C for 12 h to give an amino free derivative **31**. Compound **31** was not purified and directly reacted with stearic acid in the presence of EDC in the dry CH_2Cl_2 to give the glycosylated ceramide **32** in 62% yield for two steps. At last, all the protected groups of compound **32** were removed by Zemplén transesterification (NaOMe/MeOH). After adding several drops of water, new analogue **33** was obtained in 95% yield.



Scheme II-10 Reagents and conditions: (a) NaOMe, MeOH, r.t., 14 h; H₂O, 0 °C - r.t., 2 h; (b) $HS(CH_2)_3SH$, Et₃N, MeOH, r.t., 4 days, 80% (two steps from **30**).

On the other hand, as shown in **Scheme II-10**, the protected groups of compound **30** were removed by Zemplén transesterification, followed by adding several drops of water, product **34** was obtained. Subsequently, without further purification, the azide group in **34** was reduced with propanedithiol/triethylamine to afford the new analogue **35** in 80% yield.

Chapter III Synthesis of lactose-containing ganglioside GM3 analogues

3.1 Introduction

The total synthesis of lactose-containing ganglioside GM3 analogues was described in this chapter. These two analogues as shown in **Figure III-1** include:

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2S,3R, 4E)-2-octadecanamido-4-octadecene-1,3-diol.

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*, 4*E*)-2-amino-4-octadecene-1,3-diol.



Figure III-1 Target lactose-containing ganglioside GM3 analogues.

3.2 Retrosynthetic analysis

The retrosynthetic analysis is shown in **Figure III-2**. The synthetic strategy is as follows: Firstly, preparation of three key building blocks, including sialic acid block, lactal block and lipid precursor moiety. Secondly, performing the sialylation reaction between sialyl xanthate and acceptor to construct the sugar chain. Finally,

incorporation the lipid precursor into the sialo-oligosaccharide.



Figure III-2 Retrosynthetic analysis of lactose-containing analogues.

3.3 Preparation of lactal block with a free 6-OH at galactose

Acetyl xylan esterase from *B. pumiluswas* (AXE) is an enzyme that in nature hydrolyzes esters of xylose implicated in polysaccharide structure such as hemicellulose. In these natural substrates, the acetyl esters are present only in the C-2

and C-3 positions of the xylan moiety. It was reported to be poorly regioselective in the hydrolysis of various acetylated pyranosides. However, the behavior of this enzyme in the hydrolysis of per-acetylated disaccharides and related compounds were very different depending on the substrates. For lactose octaacetate, AXE did not exhibit good activity (the substrate was not completely hydrolyzed), but it appeared to be completely regioselective towards the anomeric position. A significant change for the properties of the enzyme was observed when the substrates without acetyl groups at the C-1 position or both the C-1 and C-2 positions were used ^{213,214,215}.

In this study, the enzyme AXE was used to regioselectively remove the C-6 acetyl group of galactose at lactal ²¹⁶. By this enzymatic hydrolysis, we can obtain the lactal with a free 6-OH at galactose **36** from per-acetylated lactal in high yield. As shown in **Scheme III-1**, per-acetylated lactal was dissolved in the phosphate buffer with 20% CH₃CN kept pH 5. After adding the enzyme AXE, the reaction went on for 4 h at the ambient temperature. Finally, the block **36** was obtained as the major product with minor byproduct **37**.



Scheme III-1 Reagents and conditions: enzyme AXE, phosphate buffer, CH₃CN, pH 5, r.t., 4 h, 69% (**36**) and 28% (**37**).

²¹³ G. Degrassi, B. C. Okeke, C. V. Bruschi, V. Venturi, Appl. Environ. Microbiol., 1998, 64, 789–792.

²¹⁴ F. Vincent, S. J. Charnock, K. H. G. Verschueren, J. P. Turken-burg, D. J. Scott, W. A. Offen, S. Roberts, G. Pell, H. J. Gilbert, G. J. Davies, J. A. Brannigan, *J. Mol. Biol.*, **2003**, *330*, 593–606.

²¹⁵ A. Riise-Moen, T. Anthonsen, *Biocatal. Biotransform.*, 2009, 27, 226–236.

²¹⁶ T. Bavaro, M. Filice, P. Bonomi, Q. Abu alassal, G. Speranza, J. Guisan, M. Terreni, *Eur. J. Org. Chem.*, **2011**, 6181-6185.

3.4 Synthesis of lactose-containing analogues

For synthesizing lactose-containing analogues, based on retrosynthetic analysis, three building blocks are needed as shown in **Figure III-3**. They are the sialyl xanthate block **3**, the lactal bearing a free 6-OH at galactose **36** and the 3-*O*-benzoyl azidosphingosine block **13**.



Figure III-3 Three key building blocks: sialyl xanthate block **3**, lactal residue **36** and 3-*O*-benzoyl azidosphingosine block **13**.

As discussed in chapter II, sialylation is the key step of the total synthesis of our target compounds. Herein, for the sialylation of free 6-OH at galactose of lactal, we also gave priority to use the Martichonok and Whitesides method which can afford α -sialoside in significantly higher yield.



Scheme III-2 Reagents and conditions: CH₃CN, CH₂Cl₂, molecular sieves, r.t., 1 h, AgOTf, DTBP, -68 °C, PhSCl, 3 h, 65%.

As shown in **Scheme III-2**, the α -sialylation reaction was carried out at -68 °C with PhSCl and AgOTf as promoters in the mixture of CH₂Cl₂:CH₃CN (1:2) for 3 h. PhSCl was freshly prepared each time using previous method. At last, the sialylation product **38** was obtained as α isomer in the yield of 65%. The stereoselectivity of this glycosylation was confirmed by ¹H NMR, for H-3eq of sialic acid residue, the chemical shift was smaller for β -glycosides than for α -glycosides. In this step, it also needs the careful chromatography to purify the crude product. Especially, adding DTBP to scavenge the proton can improve the yield obviously.

Epoxides of glycals are important intermediates in the synthesis of carbohydrate derivatives ^{169,170}. Next, for trisaccharide **38**, we designed to form the epoxide using the double bond through oxidation. Further, the epoxide ring could be opened and conjugated with lipid precursor under mild acidic condition.

AcO AcO Act	OAc HN AcO AcO AcO AcO AcO AcO AcO AcO	AcO 39	O O O O O O O O O O O O O O O O O O O
Entry	Conditions	Yield (%)	Gluco: Manno
1	1.5 eq <i>m</i> -CPBA, CH ₂ Cl ₂ , r.t., 5 h.	20	9: 1
2	2.5 eq <i>m</i> -CPBA, CH ₂ Cl ₂ , r.t., 2 h.	40	8:1
3	2.5 eq <i>m</i> -CPBA, CH ₂ Cl ₂ , 0 °C - r.t., 3 h.	50	7:3
4	2 eq oxone, H ₂ O, NaHCO ₃ , acetone, CH ₂ Cl ₂ , 0 °C - r.t., 3 h.	50	4:1
5	2 eq oxone, H ₂ O, NaHCO ₃ , TBAHS, acetone, CH ₂ Cl ₂ , 0 °C - r.t., 2.5 h.	75	9:1

Table III-1 Optimization conditions for forming epoxides

We tried to optimize the conditions for forming the epoxide, and the results are shown in **Table III-1**. At first, we used *m*-CPBA as the oxidant, but the yield was not good (entry 1). Then we increased the equivalent of *m*-CPBA, and the yield was improved (entry 2). Further, we tried to reduce the reaction temperature, the decent yield was obtained (entry 3). The high reactivity of formed oxirane can be rapidly opened by the acids during the course of the epoxidation. Next, we selected oxone as the oxidant in the biphasic solution of H₂O and CH₂Cl₂, and decent yield was also obtained (entry 4). In this case, DMDO generated *in situ* from oxone/acetone under biphasic conditions ²¹⁷. Finally, adding the phase transfer catalyst tetrabutylammonium hydrogen sulfate (TBAHS), gave a good yield of **39** (entry **5**). For this step, the major gluco- and minor manno- isomers were obtained, and this mixture was used for the next step without further purification due to 1,2-anhydro sugar was unstable.



Scheme III-3 Reagents and conditions: (a) ZnCl₂, THF, -45 °C - r.t., 3 h; (b) Ac₂O, pyridine, r.t., 24 h, 53% (two steps from **39**).

Then the epoxide ring in **39** was opened and conjugated with the lipid precursor **13** using $ZnCl_2$ in dry THF, resulting in the formation of glycolipid intermediate **40** as shown in **Scheme III-3**. At first, we conducted this reaction at 0 °C, but the yield was poor. So we further reduced the reaction temperature to -45 °C, and the yield was improved. Next, as the equivalent of acceptor **13** was increased from 2 eq. to 3 eq. at -45 °C, the decent yield of **40** was obtained. Then the free C-2 OH in **40** was protected by acetyl group using Ac₂O and pyridine to give **41**. In fact, although the mixture **39**

²¹⁷ D. Lafont, J. D'Attoma, R. Gomez, P. G. Goekjian, *Tetrahedron: Asymmetry*, 2011, 22, 1197-1204.

was used for conjugating with lipid precursor 13, in this step, after careful purification, pure 41 could be obtained, and the newly introduced β configuration was confirmed.



Scheme III-4 Reagents and conditions: (a) PPh₃, toluene, H₂O, 50 °C, 24 h; (b) Stearic acid, EDC, CH₂Cl₂, r.t., 20 h, 62% (two steps from 41); (c) NaOMe, MeOH, r.t., 14 h; H₂O, 0 °C, 2 h, 90%.

As shown in Scheme III-4, the azide group of 41 was reduced by Staudinger reaction using triphenylphosphine in a mixture of toluene and water at 50 °C for 24 h to give an amino free derivative 42. Again, compound 42 was not purified and directly reacted with stearic acid in the presence of EDC in the dry CH_2Cl_2 to give the glycosylated ceramide 43 in 62% yield for two steps from 41. At last, all the protected groups of compound 43 were removed by Zemplén transesterification (NaOMe/MeOH), followed by adding several drops of water, known analogue 44 was obtained in 90% yield. For synthesizing goal analogue **44**, compared with the synthetic route in the literature ¹⁷⁴, ours is different, which is much shorter, more convenient and economical, avoiding redundant procedures.

On the other hand, the acetyl and benzoyl groups of compound **41** were also removed by Zemplén transesterification as shown in **Scheme III-5**. After adding several drops of water, product **45** was obtained. Subsequently, without further purification, the azide group in **45** was reduced with propanedithiol/triethylamine to afford the new analogue **46** in 80% yield.



Scheme III-5 Reagents and conditions: (a) NaOMe, MeOH, r.t., 14 h; H₂O, 0 °C, 2 h; (b) $HS(CH_2)_3SH$, Et₃N, MeOH, r.t., 4 days, 80% (two steps from 41).

Chapter IV Synthesis of glucosamine-containing ganglioside GM3 analogues by developing new chemoenzymatic method

4.1 Introduction

Sialylated oligosaccharides are implicated in cell-cell adhesion processes ²¹⁸ and many of these oligosaccharides are considered as TACAs because over expressed in cancer cells. GSLs can be detectable in normal cells, but they are more highly expressed in cancers. The over expression on the surface of tumor cells causes an antibody response towards these GSLs, which are therefore considered as targets for cancer therapy ²¹⁹. Similarly, sialyl-Le^X, sialyl- α -1-6-galactosamine (Sialyl-Tn antigen), and other sialylated TACAs with a core of *N*-acetyl glucosamine or galactosamine, are over expressed in a wide variety of human cancers ²²⁰.

However, studying carbohydrates is limited by the complex procedures required for preparation of protected building blocks bearing free hydroxyl groups in the desired positions. In the presence of glucosamine or galactosamine, the synthesis of oligosaccharides is currently performed protecting the amino as acetyl-amide, as naturally existing in the TACAs structure ^{221,222} or as phthalimide ²²³. At the anomeric position of the acceptors, different leaving groups can be used due to after glycosylation of the free hydroxyl group, these products can be used as sugar donors for a further glycosylation reaction ²²⁴.

In this chapter, we described a chemoenzymatic method to synthesize sialylated glucosamine or galactosamine derivatives. The results demonstrated that this approach is versatile and can be conveniently used for the preparation of different building blocks bearing only a free hydroxyl group at C-6 with presenting different leaving groups at anomeric position. Then these intermediates can be glycosylated with sialic acid donors, allowing the preparation of sialylated sugars, which can be

²¹⁸ T. M. Altamore, C. Fernandez-Garcia, A. H. Gordon, T. Hubscher, N. Promsawan, M. G. Ryadnov, A. J. Doig, D. N. Woolfson, T. Gallagher, *Angew. Chem. Int. Ed.*, **2011**, *50*, 11167-11171.

²¹⁹ S. Hakomori, Y. Zhang, Chemistry & Biology, **1997**, *4*, 97-104.

²²⁰ Z. Yin, X. Huang, J. Carbohydr. Chem., 2012, 31, 143-186.

²²¹ S. I. van Kasteren, S. J. Campbell, S. Serres, D. C. Anthony, N. R. Sibson, B. G. Davis, Proc. Natl. Acad. Sci. USA, 2009, 106, 18-23.

²²² D. P. Gamblin, E. M. Scanlan, B. G. Davis, *Chem. Rev.*, **2009**, *109*, 131-163.

²²³ D. Lu, Y. Hu, M. Sollogoub, Y. Zhang, *Carbohydr. Res.*, **2014**, *383*, 89-96.

²²⁴ X. Huang, L. Huang, H. Wang, X. S. Ye, Angew. Chem. Int. Ed., 2004, 43, 5221-5224.

used for the synthesis of glycoconjugated derivatives.

Finally, using the sialylated glucosamine derivative, two glucosamine-containing analogues were synthesized. These two analogues are (**Figure IV-1**):

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-(2-acetamido-2-deoxy-β-glucopyranosyl)-(1→1)-(2S,3R,4E)-2-octade canamido-4-octadecene-1,3-diol.

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-(2-acetamido-2-deoxy-β-glucopyranosyl)-(1→1)-(2S,3R,4E)-2-amino -4-octadecene-1,3-diol.



Figure IV-1 Target glucosamine-containing ganglioside GM3 analogues.

4.2 Regioselective hydrolysis of 2-amino pyranose derivatives by

enzyme

4.2.1 Preparation of different substrates for enzymatic hydrolysis

Firstly, we prepared different 2-acetamido pyranose derivatives as the substrates for enzymatic hydrolysis, including per-acetylated glucosamine with OAc or SCH_2CN group at C-1 position and per-acetylated galactosamine with OAc group at the anomeric center as shown in **Figure IV-2**.

2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-α-D-glucopyranose(47),cyanomethyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-1-thio-β-glucopyranoside(48)and2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-α-D-galactopyranose(49)wereprepared by classical chemical methods.



Figure IV-2 2-acetamido pyranose substrates.

Secondly, we prepared different 2-phthalimido pyranose derivatives as the substrates for enzymatic hydrolysis, containing glucosamine with different leaving groups, including OAc, OMe, SEt, SPh, OCH2CH2N3, OCH2CH2Si(CH3)3 at the anomeric and galactosamine with SEt C-1 position. center group at the 1,3,4,6-tetra-*O*-acetyl-2-phthalimido-2-deoxy-α-D-glucopyranose (50), methyl 3,4,6-tri-O-acetyl-2-phthalimido-2-deoxy-β-D-glucopyranoside (52), ethyl 3,4,6-tri-*O*-acetyl-2-phthalimido-2-deoxy-1-thio- β -D-glucopyranoside (53), phenyl 3,4,6-tri-*O*-acetyl-2-phthalimido-2-deoxy-1-thio- β -D-glucopyranoside (54), 2-azido ethyl 3,4,6-tri-O-acetyl-2-phthalimido-2-deoxy-β-D-glucopyranoside (55), 2-trimethylsilyl ethyl 3,4,6-tri-O-acetyl-2-phthalimido-2-deoxy-β-D-glucopyranoside (56), ethyl 3,4,6-tri-O-acetyl-2-phthalimido-2-deoxy-1-thio-β-D-galactopyranoside (58) were synthesized using classical chemical approaches as shown in Scheme IV-1.



Scheme IV-1 Reagents and conditions: (a) phthalic anhydride, NaOH, H₂O, MeOH, 0 °C - r.t., overnight; Ac₂O, pyridine, r.t., overnight, 70% (α), 17% (β); (b) 33% HBr in AcOH, CH₂Cl₂, 0 °C - r.t, 89%; (c) CH₂Cl₂, MeOH, HgBr₂, HgO, r.t., overnight, 70%; (d) BF₃·Et₂O, CH₃CH₂SH, CH₂Cl₂, 0 °C - r.t., overnight, 75%; (e) BF₃·Et₂O, PhSH, CH₂Cl₂, 0 °C - r.t., overnight, 80%; (f) CH₂Cl₂, HOCH₂CH₂N₃, HgBr₂, HgO, r.t., overnight, 68%; (g) CH₂Cl₂, HOCH₂CH₂Si(CH₃)₃, HgBr₂, HgO, r.t., overnight, 72%; (h) phthalic anhydride, NaOH, H₂O, MeOH, 0 °C - r.t., overnight; Ac₂O, pyridine, r.t., overnight, 22% (α), 68% (β); (i) BF₃·Et₂O, CH₃CH₂SH, CH₂Cl₂, 0 °C - r.t., overnight, 66%.

4.2.2 Enzymatic hydrolysis

Despite the lipase from *Candida rugosa* (CRL) is the most used bio-catalyst for regioselective deacetylation, other enzymes were also used. In particular, acetyl xylan esterase from *Bacillus pumilus* (AXE), has been successfully employed in the preparation of advanced building blocks by regioselective deacetylation of acetylated disaccharides previously ²¹⁶. The use of this chemoenzymatic approach has never been investigated for the preparation of glyco-derivatives using TACAs with a core of glucosamine or galactosamine, such as the STn antigen. Consequently, we have studied the regioselective enzymatic hydrolysis of 2-amino-pyranoside derivatives

(**Table IV-1**), exploring the effect on the enzyme activity and selectivity induced by the presence of different reactive groups at anomeric position and by the protection of the 2-amino function.

Table IV-1 Regioselective hydrolysis of acetylated glucosamine and galactosamine

 derivatives catalyzed by immobilized CRL and AXE.



Entry	Substrates	CRL: yield (h)	AXE: yield (h)
1	47: R,R ₄ =OAc; R ₁ ,R ₃ =H; R ₂ =NHAc	94% (24)	10% (3)
2	48: R,R ₃ =H; R ₁ =SCH ₂ CN; R ₂ =NHAc; R ₄ =OAc	68% (48)	90% (24)
3	49: R,R ₃ =OAc; R ₁ ,R ₄ =H; R ₂ =NHAc	70% (30)	-
4	50: R,R ₄ =OAc; R ₁ ,R ₃ =H; R ₂ =NPhth	76% (6)	<10% (24)
5	52: R,R ₃ =H; R ₁ =OMe; R ₂ =NPhth; R ₄ =OAc	85% (4)	12% (8) ^a
6	53: R,R ₃ =H; R ₁ =SEt; R ₂ =NPhth; R ₄ =OAc	64% (24)	30% (96) ^a
7	54: R,R ₃ =H; R ₁ =SPh; R ₂ =NPhth; R ₄ =OAc	38% (24)	<10% (194)
8	55: R,R ₃ =H; R ₁ =OCH ₂ CH ₂ N ₃ ; R ₂ =NPhth; R ₄ =OAc	25% (168 h)	30% (108h)
9	56: R,R ₃ =H; R ₁ =OCH ₂ CH ₂ Si(CH ₃) ₃ ; R ₂ =NPhth; R ₄ =OAc	5% (144h)	24% (168h)
10	58: R,R ₄ =H; R ₁ =SEt; R ₂ =NPhth; R ₃ =OAc	80% (5)	-

In the present work, two immobilized enzymes have been compared (CRL and AXE) for preparation of various glucosamine and galactosamine building blocks. A

screening has been performed at low substrate concentration (10 mM) with the presence of 20% CH₃CN in order to ensure the complete solubility and hydrolysis of the substrates (**Table IV-1**). The esterase AXE was covalently attached on epoxy carrier, and in the case of CRL, the enzyme was immobilized by adsorption on the hydrophobic carriers.



Figure IV-3 Enzymatic hydrolysis products.

Actually, the pure α -anomer of 47, with the anomeric acetyl leaving group, can be used as substrate for immobilized CRL according to the procedure previously reported ²²⁵. In this work, we used CRL immobilized on Sepabeads-OD (a C18 hydrophobic carrier) and the obtained results (Table IV-1) were similar (94% of 2-deoxy-2-acetamido-1,3,4-tri-*O*-acetyl-α-D-glucopyranose **59**) to previously reported using CRL immobilized on octyl-agarose (a C8 hydrophobic carrier). However, CRL-Sepabeads-OD allowed much higher reaction rate providing almost quantitative hydrolysis of 47 after 24 h. When the same reaction condition was used, the CRL immobilized on C8 agarose provided only 75% of the substrate hydrolyzed after 48 h. In addition, the hydrolysis of 47 with CRL was also performed using the α and β mixture (5/1) avoiding the separation of the two anomers after the chemical

²²⁵ M. Filice, J. M. Palomo, P. Bonomi, T. Bavaro, R. Fernandez-Lafuente, J. M. Guisan, M. Terreni, *Tetrahedron*, **2008**, *64*, 9286-9292.

synthesis. The enzyme can be completely selective to provide the pure α anomer of **59**, corresponding to almost complete hydrolysis of the α -form of **47**. In this case, the β anomer remains completely unreacted and can be easily separated from the α -**59**.

Cyanomethyl 2-deoxy-2-acetamido-3,4-di-O-acetyl-1-thio-β-D-glucopyranoside 60 90% was prepared in yield using AXE. The hydrolysis of 2-deoxy-2-acetamido-1,3,4,6-tetra-O-acetyl-α-D-galactopyranose 49 has been performed, providing 2-deoxy-2-acetamido-1,3,4-tri-O-acetyl-α-D-galactopyranose 61 with 70% yield using CRL.

In the hydrolysis of 2-deoxy-2-phthalimido glucopyranoside derivatives, good yields could be obtained, but in these cases, CRL provided the best results. Starting from the 2-deoxy-2-phthalimido-1,3,4,6-tetra-O-acetyl- α -D-glucopyranose **50**, 76% yield of 2-deoxy-2-phthalimido-1,3,4-tri-O-acetyl- α -D-glucopyranose **62** was obtained after 6 h (**Table IV-1**). In this case, it was also observed that the reaction catalyzed by CRL immobilized on octyl-agarose proceeded much slower compared with the CRL-Sepabeads-OD.

Methyl 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl- β -D-glucopyranoside **52** (85% of **63**) and ethyl 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl-1-thio- β -D-glucopyranoside **53** (64% of **64**) were also conducted by enzymatic hydrolysis. About 90% of these two substrates, characterized by a little *O*- or *S*-alkyl anomeric group, were hydrolyzed in 4 and 24 h, respectively. Lower yield was obtained in the hydrolysis of phenyl 2-deoxy-2-phthalimido-3,4,6-tri-*O*-acetyl-1-thio- β -D-glucopyranoside **54**, because this reaction proceeded slowly (38% of **65** in 24 h). Furthermore, for the substrates **55** and **56**, the regioselective enzymatic hydrolysis was also carried out, but the yields were a little poor for products **66** and **67**.

Ethyl 2-deoxy-2-phthalimido-3,4,6-tri-O-acetyl-1-thio- β -D-galactopyranoside **58** proceeded much faster compared with the fully acetylated galactosamine **49**, and ethyl 2-deoxy-2-phthalimido-3,4-di-O-acetyl-1-thio- β -D-galactopyranoside **68** was obtained in very good yield after only 5 h.

4.3 Synthesis of sialylated glucosamine or galactosamine derivatives

Then the different building blocks prepared by enzymatic hydrolysis have been used as acceptors for the preparation of sialylated disaccharides. However, when the building block **59** was used for glycosylation with sialyl xanthate, the product **69** (60% yield) was produced in 2/1 α/β mixture (**Scheme IV-2**). Similarly, sialylation of building blocks **60** and **61**, also allowed preparation of the disaccharides **70** and **71** as a mixture of α/β anomers. In addition, the sialylation of **61** was investigated using sialyl chloride as donor and Ag₂CO₃ as promoter, but the similar yield and anomeric mixtures of **71** were obtained.

The intrinsic difficulties in the stereo-controlled synthesis of α -sialosides have been largely reported and complex procedures are required in order to obtain pure or almost pure anomers ²²⁶. In fact, α/β mixtures are obtained in different ratio using mono-deprotected sugar acceptors, regardless the protection used for the C-2 amino group of the pyranose acceptor or using precursors with the 2-azido group ^{227,228}. In this work, this effect was observed regardless the kind of sialyl-donor used in the glycosylation of 2-deoxy-2-acetamido pyranose building block.

²²⁶ C. Navuluri, D. Crich, Angew. Chem. Int. Ed., 2013, 52, 11339-11342.

²²⁷ S. Cai, B. Yu, Org. Lett., **2003**, *5*, 3827-3830.

²²⁸ S. Sahabuddin, T. C. Chang, C. C. Lin, F. D. Jan, H. Y. Hsiao, K. T. Huang, J. H. Chen, J. C. Horng, J. A. Ho, *Tetrahedron*, **2010**, *66*, 7510-7519.



Scheme IV-2 Reagents and conditions: (a) DTBP, AgOTf, PhSCl, CH₃CN/CH₂Cl₂ (2/1), -68 °C, 3 h, 60% (α/β 2/1); (b) NIS, TfOH, CH₃CN, -40 °C, 3 h, 55% (α/β 3/2); (c) DTBP, AgOTf, PhSCl, CH₃CN/CH₂Cl₂ (2/1), -68 °C, 3 h, 64% (α/β 1/1); (d) Ag₂CO₃, CH₂Cl₂, r.t., overnight, 67% (α/β 1/1).

High stereo-selectivity was obtained when *N*-phthalimido glucosamine or galactosamine intermediates were used for the sialylation reaction after enzymatic

deprotection at C-6. Accordingly, it was possible to perform the preparation of sialyl-disaccharides with different leaving groups at C-1 in pure or almost pure α -form (Scheme IV-3). Product 72 (with 1- α -acetoxy) was obtained in 70% yield as almost pure α anomer, allowing results much better compared with previously reported ²²⁷. Similarly, pure α anomer of 73 (with 1- β -methoxy) was also obtained in 64% yield.



Scheme IV-3 Reagents and conditions: (a) sialyl xanthate, DTBP, AgOTf, PhSCl, CH₃CN/CH₂Cl₂ (2/1), -68°C, 3 h; 72 yield 70% (98%); 73 yield 64% (98%); 74 yield 32% (98%); 75 yield 30% (98%); (b) sialyl chloride, AgOTf, CH₂Cl₂, -40 °C - r.t., 14 h; 74 yield 65% (95%); 75 yield 70% (95%).

Much poorer yields were obtained when sialyl xanthate was used as donor for sialylation of *N*-phthalimido- glucosamine **64** and galactosamine **68** (with 1- β -thioethyl), probably due to the activation of the anomeric *S*-alkyl group in the reaction. Starting from *N*-phthalyl-glucosamine **64**, pure **74** was obtained only in 32% yield, and the sialyl-Tn derivative **75** was produced in 30% yield from *N*-phthalyl-galactosamine **68**. For improving the yield for the sialylation of *N*-phthalimido building blocks, using the AgOTf as promoter, the sialyl chloride was investigated as donor in the glycosylation of intermediates **64** and **68** (Scheme IV-3). In this condition, 65% and 70% yields were obtained respectively for product **74** and sialyl-Tn precursor **75** (in both cases as 95/5 mixture of α/β isomers).

These results suggest that the presence of an acetamido group at position C-2, unlike

the 2-phthalimido group, could be responsible for the formation of the β -anomer during sialylation. In particular, the 2-acetamido group could be involved in the formation of a hydrogen bond with the carboxy-ester of the sialic acid donor. Consequently, the use of 2-phthalimido pyranoses should be preferred for the synthesis of sialyl disaccharides.

4.4 Synthesis of glucosamine-containing GM3 analogues

From the previous studies, we synthesized a series of sialyl disaccharides with glucosamine or galactosamine. Next, we started with sialylated glucosamine having acetyl group at C-1 72 to synthesize glucosamine-containing GM3 analogues.

For α -sialoside **72**, the acetyl group at the anomeric center was selectively removed. At first, we tried to use PhCH₂NH₂ as promoter, but after optimizing the reaction conditions, including solvents and reaction temperature, the yield still was so poor. Next, we tried to use hydrazine acetate to remove the C-1 position acetyl group, but no reaction happened. At last, we used NH₂CH₂CH₂NH₂ and CH₃COOH forming salts as promoter in the dry THF at room temperature for 3 h to provide the hemiacetal **76** as an α and β mixture as shown in **Scheme IV-4**. After extraction, this crude product **76** was not separated at this stage and was used directly for the next step in spite of a little impurities. The hemiacetal **76** was then treated with trichloroacetonitrile in the presence of DBU to give the trichloroacetimidate **77** in 60% yield as an α and β mixture after purification by chromatography as shown in **Scheme IV-4**.

Glycosylation of 3-*O*-benzoyl azidosphingosine with trichloroacetimidate 77 was performed by $BF_3 \cdot Et_2O$ as promoter at low temperature to provide the desired glycolipid 78 in 61% yield, as shown in Scheme IV-4, and the β configuration of newly introduced glycosidic linkage was obtained.



Scheme IV-4 Reagents and conditions: (a) $NH_2CH_2CH_2NH_2$, CH_3COOH , THF, r.t., 3 h, 75% (crude product); (b) CCl_3CN , DBU, CH_2Cl_2 , -5 °C, 2 h, 60%; (c) $BF_3 \cdot Et_2O$, CH_2Cl_2 , -30 °C, 1.5 h, 61%.

Then the azide group of **78** was reduced by triphenylphosphine in a mixture of toluene and water at 50 °C for 24 h to give an amino free derivative **79**. Compound **79** was not purified at this stage and was directly reacted with stearic acid in the presence of EDC in the dry CH_2Cl_2 to give the glycosylated ceramide **80** in 60% yield for two steps from **78**. At last, the protected groups of compound **80** were removed using MeHNNH₂ in EtOH, including phthalimido, acetyl and benzoyl groups. Then the methyl ester was hydrolyzed. Further for the obtained free NH_2 group, it was selectively acetylated using Ac₂O in CH_2Cl_2 and MeOH. The known analogue **81** was obtained in 75% yield as shown in **Scheme IV-5**.



Scheme IV-5 Reagents and conditions: (a) PPh₃, toluene, H₂O, 50 °C, 24 h; (b) Stearic acid, EDC, CH₂Cl₂, r.t., 20 h, 60% (two steps from **78**); (c) MeNHNH₂, EtOH, 80 °C, 12 h; NaOH, H₂O, r.t., 1 h; Ac₂O, CH₂Cl₂, MeOH, r.t., overnight, 75% (three steps from **80**).

On the other hand, as shown in Scheme IV-6, the protected groups of compound 78

were removed using MeHNNH₂ in EtOH. Also, adding NaOH aqueous solution, the methyl ester was removed. And for the free NH₂ group, it was selectively acetylated using Ac₂O in CH₂Cl₂ and MeOH. Subsequently, the azide group of **82** was reduced with propanedithiol/triethylamine to afford the new analogue **83** in 70% yield.



Scheme IV-6 Reagents and conditions: (a) MeNHNH₂, EtOH, 80 °C, 12 h; NaOH, H₂O, r.t., 1 h; Ac₂O, CH₂Cl₂, MeOH, r.t., overnight; (b) HS(CH₂)₃SH, Et₃N, MeOH, r.t., 4 days, 70% (four steps from **78**).

Chapter V Biological evaluation of synthetic ganglioside GM3 analogues

5.1 Introduction

Glycoconjugates including glycoproteins and glycolipids are involved in various cell functions. Among them, GSLs are ubiquitous components on mammalian cell membranes, and studies demonstrated that they are implicated in many cellular functions, such as cell proliferation, adhesion, motility and differentiation. The changes of GSLs structures associated with biological functions have been a matter of research recently because structural change is the starting point for understanding biological significance, and enzymatic or genetic mechanisms. Ganglioside GM3 is the first and simplest member in the metabolic series of a GSLs family. It has been reported that ganglioside GM3 is implicated in some human serious diseases, such as cancers, nervous diseases and diabetes ^{3,4,68,69,70}.

In the previous chapters, we reported the preparation of GM3 analogues based on a concise protocol that can provide a series of gangliosides. Herein, the biological activities of these synthetic GM3 analogues, including galactose-, mannose-, lactose- and glucosamine- containing analogues, were delineated through cooperation with other labs.

5.2 Inhibitory effects on tumor cell growth and migration

Ganglioside GM3 is over expressed on some cancers, and it is well known as the tumor-associated carbohydrate antigen on several types of tumor, such as melanomas and bladder cancers. The obvious influence of GM3 on the proliferation, invasion and metastasis of cancer cells has been observed. GM3 can not only modulate the receptors, such as EGFR and VEGFR-2, but also glycolipid-enriched membrane microdomain signaling pathway. Besides, some studies have revealed that GM3 has a clear effect on tumor cell movement, such as motility and migration. It has been confirmed that cell movement in tumor growth is crucial because it is not only believed to have effects on the tumor shape, but also cell to cell adhesive properties,

and it represents one of the relevant factors in assessing the cancer malignancy ^{229,230}. In this section, the effects of synthesized ganglioside GM3 analogues on tumor cell growth and migration will be studied.

5.2.1 Experimental methods

5.2.1.1 Cell culture

Human colon cancer HCT116 cells, human leukemia K562 cells, highly metastatic human liver cancer HCCLM3 cells, murine melanoma B16 cells and highly metastatic murine melanoma B16-F10 cells were originally obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (China), and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), as well as penicillin and streptomycin (100 μg/ml each), at 37 °C in a humidified atmosphere of 5% CO₂.

5.2.1.2 Cytotoxicity assay

Cell viability was assessed using MTT method. Briefly, the cells (8 × 10³/well) were seeded into 96-well plate, and treated with GM3 analogues at 20, 200, 400, 1000 μ M or vehicle DMSO for 48 h. At the end of the incubation period, 20 μ L (5 mg/mL) MTT solution was added into each well of the 96-well plate. Following 4 h of incubation at 37 °C with 5% CO₂, absorbance was detected at a wavelength of 570 nm. The results are presented as means ± SD from the three independent experiments.

5.2.1.3 Wound healing test

We used wound healing test to evaluate the effect of GM3 analogues on tumor cell

²²⁹ M, Yilmaz, G. Christofori, *Mol. Cancer Res.*, 2010, 8, 629-642.

 ²³⁰ A. Wells, J. Grahovac, S. Wheeler, B. Ma, D. Lauffenburger, *Trends Pharmacol. Sci.*, 2013, 34, 283-289.

motility by B16, B16-F10 and HCCLM3 tumor cell lines. The cells $(1.5 \times 10^5 \text{ cells/24} \text{ wells})$ were cultured into 24-well plates and grown in RPMI-1640 medium containing 10% FBS to nearly confluent cell monolayer. A 10 µL plastic pipette tip was used to draw a linear "wound" in the cell monolayer of each well. The monolayer was then washed twice with serum free RPMI-1640 medium to remove debris or detached cells, and GM3 analogues were added at different concentrations in fresh medium with FBS, RPMI-1640 medium containing DMSO added to the control well as the solvent control, and subsequently cultured for 12 h, 24 h or 48 h depending on the different ability of the cell migration. The wound healing of the scratched cells was photographed under a DMIL LED AE2000 inverted microscope (Leica, Wetzlar, Germany) and the effect of GM3 analogues on tumor cell motility was expressed as migration % of 0 hour (wound width at exposure time point/wound width at 0 hour). The experiments were performed in triplicate.

5.2.2 Results and discussion

5.2.2.1 Galactose- and mannose- containing analogues

For synthesized galactose- and mannose- containing analogues (Figure V-1), their antitumor activities were evaluated by cytotoxicity assay and wound healing test.

At first, the effect of GM3 analogues 22, 24, 33 and 35 on the proliferation of human colon cancer HCT116 cells and chronic myeloid leukemia K562 cells was evaluated by MTT assay after treating with different concentrations of GM3 analogues for 48 h (Figure V-2). The IC₅₀ of analogue 33 for HCT116 and K562 was lower than that of 22, indicated that 33 had more potent antiproliferative activity than 22. Similarly, the IC₅₀ of analogue 35 for K562 was lower than 24, therefore 35 possessed stronger cytotoxicity than 24. The results showed that mannose-containing analogues 33 and 35 could have more potent antiproliferative activity than galactose-containing analogues 22 and 24.



Figure V-1 Galactose- and mannose- containing analogues for anticancer tests.



Figure V-2 GM3 analogues inhibited cancer cell growth: HCT116 and K562 cells were treated with 0-1000 μ M GM3 analogues for 48 h. Cell viability was measured by MTT assay and expressed related to vehicle-treated control. Results are presented as means \pm SD of three independent experiments.

Wound healing test, also called scratch assay, is a common tool to study cell migration, which makes a scratch on a cell monolayer. In general, for cancer cells, the scratch wound can be healed automatically after some time (**Figure V-3A**). If this process can be inhibited by exogenous compounds, it means that the compounds have an inhibitory effect on cell movement (**Figure V-3B**).



Figure V-3 Mode of wound healing test.

Then, cell migration was investigated by a wound healing assay *in vitro*, in which cells migrate bidirectionally from the edges of a scratch wound, and the effect of GM3 analogues on tumor cell migration was expressed as percentage of scratch wound healing. We found that GM3 analogues could inhibit the migration of B16 (**Figure V-4**), B16-F10 (**Figure V-5**) and HCCLM3 cells (**Figure V-6**), and the concentration of GM3 analogues on migration inhibition was $6.25 - 50 \mu$ M, much lower than their antiproliferative concentration, suggesting that compared with inhibiting tumor cell proliferation, these analogues have a stronger effect on tumor cell migration.


Figure V-4 Galactose- and mannose- containing analogues inhibited cancer cell migration: B16 cells after 48 h of exposure. Significant inhibition in migration of cancer cells was observed upon treatment with GM3 analogues. Values were calculated as a percentage of the 0 hour (wound width at exposure time point/wound width at 0 h %).



Figure V-5 Galactose- and mannose- containing analogues inhibited cancer cell migration: B16-F10 cells after 12 h of exposure. Significant inhibition in migration of cancer cells was observed upon treatment with GM3 analogues. Values were calculated as a percentage of the 0 hour (wound width at exposure time point/wound width at 0 h %).



Figure V-6 Galactose- and mannose- containing analogues inhibited cancer cell migration: HCCLM3 cells after 48 h of exposure. Values were calculated as a percentage of the 0 hour (wound width at exposure time point/wound width at 0 h %).

Figure V-4 and **Figure V-5** revealed that these GM3 analogues at concentration of 6.25 μ M and 12.5 μ M resulted in a suppressive effect on migration of murine melanoma cells, as if the anti-migration activity of analogues **35** and **24** was stronger than **33** and **22**. However, except for murine melanoma, whether GM3 analogues also had similar effect on human cancer cell migration. Hence we used the highly metastatic liver cancer cell HCCLM3 to observe their effects on cell migration, and the results showed that only GM3 analogues **35** and **24** at high concentration of 50 μ M produced inhibitory effects for HCCLM3 cells, whereas GM3 analogues **33** and **22** had no effect on cell migration even at 50 μ M concentration (**Figure V-6**).



Figure V-7 Lactose- and glucosamine- containing analogues for anticancer tests.

5.2.2.2 Lactose- and glucosamine- containing analogues

For synthesized lactose- and glucosamine- containing analogues (Figure V-7), their

antitumor activities were also evaluated by cytotoxicity assay and wound healing test.

At first, the effect of GM3 analogues **44**, **46**, **81** and **83** on the proliferation of human colon cancer HCT116 cells and chronic myeloid leukemia K562 cells was evaluated. As shown in **Table V-1**, the IC₅₀ of analogue **81** for HCT116 and K562 was lower than that of **44**, indicated that **81** had more potent antiproliferative activity than **44**. Similarly, the IC₅₀ of analogue **83** for K562 was lower than **46**, therefore **83** possessed stronger cytotoxicity than **46**. The results showed that glucosamine-containing analogues have more potent antiproliferative activity than lactose-containing analogues.

Table V-1 IC₅₀ (μ M) values for 48 h

Tumor cell lines	44	46	81	83
HCT116	161.1	108.9	147.8	126.8
K562	161.9	104.9	71.5	66.7

Figure V-8 and Figure V-9 revealed that these GM3 analogues at concentration of 12.5 μ M and 25 μ M resulted in a suppressive effect on migration of murine melanoma cells, as if the anti-migration activity of analogue 46 was stronger than 44, and analogue 81 was similar to 83.

Little is known on the mechanisms which explain how ganglioside GM3 inhibits tumor cell motility. Previous studies suggest that the suppressive effect of ganglioside GM3 on cell motility and growth associated with modulating functions of other molecules co-localized in membrane microdomains ²³¹.

Along with some old analogues that synthesized previously in our lab 176 , for a series of GM3 analogues containing different sugars with α -2,6 sialosides, the structure-activity relationships were discussed. We found that different glycosyl residues can influence antiproliferation and antimigration activities. Whether it's

²³¹ C. Tabolacci, A. Lentini, P. Mattioli, B. Provenzano, S. Oliverio, F. Carlomosti, S. Beninati, *Life Sciences*, 2010, 87, 316-324.

ceramide or sphingosine moiety, it's more likely to influence the antimigration activities.

At first, for antiproliferation ability: glucosamine-containing analogue > lactose-, mannose- containing analogues > galactose-containing analogue > glucose-containing analogue. Compared with glucose-containing analogue, galactose-containing analogue is much stronger than it. However, the mannose- and lactose- containing analogues are similar, but both of them are stronger than galactose-containing analogue. Among them, glucosamine-containing analogue is the strongest.

Then for antimigration ability: glucosamine-, lactose-containing analogues > mannose-containing analogue > galactose-containing analogue. We found that mannose-containing analogue is stronger than galactose-containing analogue. And lactose- and glucosamine- containing analogues are similar, but it seems that both of them are a little stronger than mannose-containing analogue. Especially, for antimigration ability, it also showed that analogues with sphingosine are stronger than the corresponding analogues with ceramide moiety.

These are preliminary results from our analogues, and we think that more analogues are needed for obtaining more information on structure-activity relationships.



Figure V-8 Lactose- and glucosamine- containing analogues inhibited cancer cell migration: B16-F1 cells after 24 h of exposure. Significant inhibition in migration of cancer cells was observed upon treatment with GM3 analogues. Values were calculated as a percentage of the 0 hour (wound width at exposure time point/wound width at 0 h %).



Figure V-9 Lactose- and glucosamine- containing analogues inhibited cancer cell migration: B16-F10 cells after 24 h of exposure. Significant inhibition in migration of cancer cells was observed upon treatment with GM3 analogues. Values were calculated as a percentage of the 0 hour (wound width at exposure time point/wound width at 0 h %).

5.3 Inducing neurite outgrowth activity



Figure V-10 Structures of seven analogues for neurite outgrowth tests.

Neurite outgrowth is a key process in the development of functional neuronal circuits

and regeneration of the nervous system after injury ²³². The addition of neurotrophic factors such as nerve growth factor (NGF) to rat PC12 pheochromocytoma cells leads to cell cycle arrest and differentiate into neuron-like cells with elongated neurites ²³³. The phenotypic changes associated with NGF-induced differentiation include the biosynthesis of neurotransmitters, the acquisition of electrical excitability and the growth of axon-like extensions named neuritis through a process known as neuritogenesis ²³⁴.

In the present study, we employed PC12 cells as a model to study the effects of ganglioside GM3 analogues on neurite outgrowth, including galactose-containing novel analogues 22 and 24 along with other analogues 84-88 prepared previously in our lab (Figure V-10).

5.3.1 Experimental methods

PC12 cells were obtained from the ATCC (Manassas, VA, USA). The cells were maintained in DMEM (Dulbecco's modified Eagle's medium; HyClone, USA), supplemented with 10% (v/v) heat-inactivated horse serum (Gibco, USA) and 5% (v/v) FBS (Gibco, USA). The cells were cultured in a humidified incubator at 37 °C with 5% CO₂ atmosphere.

The experiments were conducted as the previous study ²³⁵. Briefly, PC12 cells were seeded into a 12-well plate pre-coated with 50 ng/mL poly-L-lysine (Sigma–Aldrich, USA) at a density of 2×10^4 cells/well. The cells were allowed to attach for 24 h, then treated with DMEM containing 1% horse serum and 0.5% FBS for 24 h. In the non-NGF-induced experiments, PC12 cells were treated by the indicated compounds at the concentration of 10 μ M or treated by NGF (25 ng/mL) alone, subsequently cultured for another 72 h. In the NGF-induced assay, NGF (25 ng/mL) was added into

²³² S. Higgins, J. S. Lee, L. Ha, J. Y. Lim, *Biores OPEN ACCESS*, 2013, 2, 212-216.

²³³ L. A. Greene, A. S. Tischler, Proc. Natl. Acad. Sci. USA, **1976**, 73, 2424-2428.

²³⁴ A. T. Jocavina, F. Zhong, E. Khazanova, E. Lev, A. B. Deora, K. A. Hajjar, J. Biol. Chem., 2001, 276, 49350-49358.

²³⁵ J. Li, J. Fang, Y. Qin, W. Liao, H. Liu, Y. Zhou, K. Ding, *Biochem.*, **2014**, 459, 405-415.

the culture medium accompanied with the compounds, then maintained for further 72 h. PC12 cell morphology was observed by the inverted microscope (Olympus, Japan) and determined by counting the proportion of cells containing at least one neurite twice as long as the cell body diameter, at least over 200 cells per sample were scored (Image J software, NIH, USA).

5.3.2 Results and discussion

Firstly, we employed no NGF induced model, and in this model, NGF was used as a positive control. Generally, the cells maintained in low-serum medium were treated with NGF (25 ng/mL), compound **84**, **85**, **24**, **22**, **86**, **87** and **88** (10 μM). After 72 h, the morphology of the cells was photographed under an inverted microscope and the representative micrographs were exhibited in Figure V-11A, and the number of neurite bearing cells was counted and shown in Figure V-11B. Interestingly, neuritogenesis was augmented robustly by compound **84** comparing to the blank group (Figure V-11A-c), the percentage of neurite-bearing cells reached 26%, even higher than that of the NGF group (Figure V-11B). The compound **24** (Figure V-11A-e), **86** (Figure V-11A-g) and **88** (Figure V-11A-i) could significantly promote neurite outgrowth, and the percentage of neurite-bearing cells was 9%, 6% and 8%, respectively (Figure V-11B). While the compound **85**, **22** and **87** had no obvious effects on neuritogenesis, as the proportion of neurite-bearing cells was closed to the blank group (Figure V-11B).

The NGF-induced PC12 cell model was a common tool to study neuritogenesis, we then investigated the effects of these analogues on neurite outgrowth in the NGF-induced model. In this model, NGF (at the concentration of 25 ng/mL) was added into the cell culture medium along with the compounds. The results showed that the proportion of neurite-bearing cells in 84 is more than 30% (Figure V-12A-b and Figure V-12B), and the 86 (Figure V-12A-f and Figure V-12B) also could significantly accelerate neuritogenesis comparing to NGF group. It seemed that 85

(Figure V-12A-c), 24 (Figure V-12A-d), 22 (Figure V-12A-e), 87 (Figure V-12A-g) and 88 (Figure V-12A-h) had no obvious effects on neurite outgrowth comparing to NGF group. These data indicated that GM3 analogues 84 and 86 potently induce neurite outgrowth in PC12 cells both in NGF free or NGF-induced systems.

Several literatures had emphasized the important role of GM3 and glycolipids in neuritogenesis. For example, in mouse neuroblastoma Neuro2a cells, studies found that GM3 enriched microdomains were involved in signal transduction during neurite formation. PC12 cell line has been widely used as a model system for studying neuronal differentiation and neurosecretion. In the present study, we firstly employed PC12 cells as a model to evaluate the effects of GM3 analogues on neuritogenesis ^{236,237,238}. Our results demonstrated that some GM3 analogues could induce neurite outgrowth. From the preliminary results, it seems that GM3 analogues having sphingosine moiety are more prone to induce neurite outgrowth, despite of different sugar composition or linkages.

It has been reported that nervous system injuries affect many people every year, and due to its high incidence, nerve regeneration or repair, is becoming a rapidly growing field for discovery of new ways to recover nerve functionality after injury ^{239,240}. Neurite outgrowth is a key process in the development of functional neuronal circuits and regeneration of the nervous system after injury. The ability of GM3 analogues to induce neurite outgrowth can provide a valuable sight for developing potential therapeutic agents to recover nervous functionality after injury.

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²³⁸ R. H. S. Westerink, A. G. Ewing, Acta Physiol. (Oxf), 2009, 192, 273-285.

²³⁹ S. E. Stabenfeldt, A. J. García, M. C. La Placa, J. Biomed. Mater. Res. A, 2006, 77, 18-25.

²⁴⁰ P. Prang, R. Müller, A. Eljaouhari, K. Heckmann, W. Kunz, T. Weber, C. Faber, M. Vroemen, U. Bogdahn, N. Weidner, *Biomaterials*, **2006**, *27*, 3560-3569.



Figure V-11 Compound 84, 24, 86 and 88 could induce the neuritogenesis of PC12 cells. (A): PC12 cells were seeded in a 12-well plate, incubated overnight and then were maintained in a low-serum medium (DMEM supplemented with 1.0% horse serum and 0.5% FBS) for 24 h, followed by the treatment with the compound 84 (c), 85 (d), 24 (e), 22 (f), 86 (g), 87 (h), 88 (i) at the concentration of 10 μ M or 25 ng/mL NGF (b) or without (a) for an additional 72 h. The cells were photographed by a phase contrast microscope, and the representative micrographs of each group were exhibited. (B): Quantification of PC12 cells bearing one neurite at least twice the length of the cell body diameter in each group were shown. Results are presented as means ± S.D., scale bar, 5 μ m, and experiments were repeated at least three times. ***, p < 0.001; *, p < 0.05.





Chapter VI Experimental section

6.1 General methods

All chemical reactions were carried out under N_2 atmosphere and in the anhydrous conditions with fresh solvents, unless otherwise noted. The solvents were evaporated under reduced pressure and below 40 °C (water bath), followed by removal of residual solvent at high vacuum (< 0.2 mbar).

¹H NMR and ¹³C NMR spectra were recorded at Bruker AVANCE DRX 400 spectrometer. 2D COSY, HSQC and HMBC experiments were used to assist NMR assignments. The chemical shifts were referenced to the solvent peaks, $\delta = 7.26$ ppm (¹H) and $\delta = 77.16$ ppm (¹³C) for CDCl₃, $\delta = 3.31$ ppm (¹H) and $\delta = 49.00$ ppm (¹³C) for CDCl₃, $\delta = 39.52$ ppm (¹H) and $\delta = 49.00$ ppm (¹³C) for CD₃OD, $\delta = 2.50$ ppm (¹H) and $\delta = 39.52$ ppm (¹³C) for DMSO, at 25 °C, and the coupling constants were given in Hz.

High-resolution mass spectra (HRMS) were recorded on a Bruker micrOTOF spectrometer in electrospray ionization (ESI) mode, using Tuning-Mix as a reference. Optical rotations were measured at 589 nm (Na line) at 20 °C with a Perkin-Elmer Model 343 digital polarimeter using a 10 cm, 1 mL cell.

Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of silica gel 60 F254 (Merck) and detected by visualization through UV light and charring with 5% H₂SO₄/EtOH or Hanessian's Stain. Column chromatography was generally performed using Kieselgel 60 silica gel (230-400 mesh), typically using a 30:1 to 100:1 weight ratio of silica gel to crude product.

Reagents were purchased from Sigma-Aldrich, Fluka, Alfa Aesar or Acros Organics. Dry solvents used in the reaction as following: tetrahydrofuran (THF) was refluxed with sodium and benzophenone each time. CH_2Cl_2 was freshly distilled each time over CaH₂. DMF, pyridine, methanol, acetonitrile and Et₃N were stored for several days over a bed of molecular sieves (4 Å, beads, 8-12 mesh) before use. All the mixture of solvents is presented in v/v.

6.2 Molecule numbering

In this chapter, for the assignment of NMR spectra, the numbering of complex molecules is shown in **FigureVI-1**.



FigureVI-1 Molecule numbering.

6.3 Preparation of sialic acid block

Methyl 5-acetamido-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulopyranosonate (1)
241



A solution of *N*-acetyl neuraminic acid (6.0 g, 19.40 mmol) in methanol (192 mL) containing Amblyst-H⁺ resin (4.5 g) was stirred for 6 h at 45 °C. The progress of the reaction was monitored by TLC. Finally, the solution was filtered and the resin was washed several times with methanol. The filtrate was then evaporated. The methyl ester **1** was obtained as a white solid (5.64 g, 90%).

 $R_{\rm f} = 0.33$ (CH₂Cl₂-MeOH 3:1).

¹**H NMR (400 MHz, CD₃OD):** δ 4.11-3.99 (m, 2H, H-6, H-4), 3.86-3.81 (m, 2H, H-5, Ha-9), 3.80 (s, 3H, COOCH₃), 3.72 (dd, J = 9.0, 2.9 Hz, H-8), 3.64 (dd, J = 11.2, 5.7 Hz, 1H, Hb-9), 3.50 (dd, J = 9.1, 1.3 Hz, 1H, H-7), 2.24 (dd, J = 12.9, 4.9 Hz, 1H, H-3eq), 2.03 (d, J = 5.7 Hz, 3H, NAc), 1.91 (dd, J = 12.8, 11.4 Hz, 1H, H-3ax).

Methyl 5-acetamido-2-chloro-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-β-Dgalacto-2-nonulopyranosonate (2) ²⁴²



²⁴¹ R. Kuhn, P. Lutz, D. L. MacDonald, *Chem. Ber.*, **1966**, *99*, 611-617.

²⁴² I. Carlescu, H. M. I. Osborn, J. Desbrieres, D. Popa, *Carbohydr. Res.*, **2010**, *345*, 33-40.

To a suspension of sialic acid methyl ester 1 (2.7 g, 8.35 mmol) in acetyl chloride (150 mL) cooled to 0 °C was slowly added absolute MeOH (1.8 mL). The mixture was allowed to warm to room temperature, and stirring was continued for 3 days, during which time a clear solution was obtained. The reaction mixture was concentrated, and purified by silica gel column, eluting with EtOAc. Concentration *in vacuo* afforded sialyl chloride **2** as a white foam (3.83 g, 90%).

 $R_{\rm f} = 0.38$ (CHCl₃-EtOAc 1:5).

¹**H NMR (400 MHz, CDCl₃):** δ 5.47 (d, J = 5.9 Hz, 1H, H-7), 5.38 (dd, J = 10.5, 4.4 Hz, 1H, H-4), 5.17 (t, J = 4.9 Hz, 1H, H-8), 4.42 (dd, J = 12.5, 2.3 Hz, 1H, Ha-9), 4.35 (d, J = 10.3 Hz, 1H, H-6), 4.20 (dd, J = 10.5, 10.3 Hz, 1H, H-5), 4.06 (dd, J = 12.5, 5.9 Hz, 1H, Hb-9), 3.87 (s, 3H, COOCH₃), 2.78 (dd, J = 13.9, 4.5 Hz, 1H, H-3eq), 2.27 (dd, J = 13.7, 11.3 Hz, 1H, H-3ax). 2.11 (s, 3H, OAc), 2.07 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.04 (s, 3H, OAc), 1.90 (s, 3H, NAc).

O-ethyl *S*-[methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero- α/β -D-galacto-2-nonulopyranosyl)onate] dithiocarbonate (α/β -3) ¹⁵¹



To a solution of compound **2** (3.8 g, 7.48 mmol) in dry EtOH (152 mL) was added solid *O*-ethyl *S*-potassium dithiocarbonate (1.8 g, 11.23 mmol) in small portions within 10 min. The mixture was stirred at 0 °C for 12 h and then at room temperature for additional 12 h. The reaction mixture was diluted with pentane, filtered, and concentrated under reduced pressure. The residue was dissolved in CH₂Cl₂ and washed with H₂O, saturated NaHCO₃ solution and brine. After drying over MgSO₄ and concentration, the residue was purified by silica gel column, eluting with CH₂Cl₂-EtOAc 1:4. The eluent was concentrated to afford a mixture of α -3 and β -3 in molar ratio 16:1 contaminated with known sialyl glycal **4** (4.06 g, 76% of α/β -**3**, 19% of **4**). This mixture was used for the sialylations.

β-3

 $R_{f} = 0.29$ (CH₂Cl₂-EtOAc 1:4).

¹**H NMR (400 MHz, CDCl₃):** δ 5.58 (bd, J = 10.1 Hz, 1H, NH), 5.33 (dd, J = 4.1, 2.1 Hz, 1H, H-7), 5.21 (ddd, J = 8.0, 4.1, 2.8 Hz, 1H, H-8), 4.83 (dt, J = 12.7, 4.6 Hz, 1H, H-4), 4.77 (m, 1H, OCH_aCH₃), 4.63 (dd, J = 10.7, 2.1 Hz, 1H, H-6), 4.53 (m, 1H, OCH_bCH₃), 4.01 (q, J = 10.4 Hz, 1H, H-5), 4.01 (dd, J = 12.1, 2.8 Hz, 1H, Ha-9), 3.79 (s, 3H, COOCH₃), 3.51 (dd, J = 12.2, 8.0 Hz, 1H, Hb-9), 2.57 (dd, J = 12.9, 4.6 Hz, 1H, H-3eq), 2.13 (s, 3H, OAc), 2.11 (s, 6H, 2 × OAc), 2.00 (s, 3H, OAc), 1.94 (t, J = 12.6 Hz, 1H, H-3ax), 1.85 (s, 3H, NAc), 1.36 (t, J = 7.1 Hz, 3H, CH₃).

¹³C NMR (100 MHz, CDCl₃): δ 206.92 (C=S), 170.77 (C=O), 170.21 (C=O), 170.03 (C=O), 168.72 (C=O), 86.41 (C-2), 73.41, 70.89 (OCH₂CH₃), 68.61, 53.37 (COOCH₃), 49.05 (C-5), 36.89 (C-3), 23.08 (CH₃, NAc), 20.89 (CH₃, OAc), 20.83 (CH₃, OAc), 20.75 (CH₃, OAc), 20.68 (CH₃, OAc), 13.26 (OCH₂CH₃).

α-3

 $R_{\rm f} = 0.22$ (CH₂Cl₂-EtOAc 1:4).

¹**H NMR (400 MHz, CDCl₃):** δ 5.33-5.29 (m, 2H, H-7, H-8), 5.24 (d, J = 9.5 Hz, 1H, NH), 4.89 (td, J = 11.4, 4.6 Hz, 1H, H-4), 4.84-4.76 (m, 1H, OCH_aCH₃), 4.60-4.51 (m, 2H, OCH_bCH₃, H-6), 4.33 (dd, J = 12.4, 2.3 Hz, 1H, Ha-9), 4.19 (dd, J = 12.4, 5.1 Hz, 1H, Hb-9), 4.02 (dd, J = 20.7, 10.4 Hz, 1H, H-5), 3.80 (s, 3H, COOCH₃), 2.63 (dd, J = 13.0, 4.7 Hz, 1H, H-3eq), 2.14 (s, 3H, OAc), 2.12 (s, 3H, OAc), 2.08-2.05 (m, 1H, H-3ax), 2.03 (t, J = 3.1 Hz, 6H, 2 × OAc), 1.88 (d, J = 5.6 Hz, 3H, NAc), 1.37 (t, J = 7.1 Hz, 3H, CH₃).

¹³C NMR (100 MHz, CDCl₃): δ 207.12 (C=S), 170.74 (C=O), 170.45 (C=O), 170.19 (C=O), 170.16 (C=O), 169.99 (C=O), 168.60 (C=O), 86.44 (C-2), 75.08, 70.15, 68.77, 67.67 (C-4,6,7,8), 70.37 (OCH₂CH₃), 61.98 (C-9), 53.21 (COOCH₃), 49.02 (C-5),

37.03 (C-3), 23.03 (CH₃, NAc), 20.98 (CH₃, OAc), 20.72 (CH₃, OAc), 20.69 (CH₃, OAc), 20.66 (CH₃, OAc), 13.23 (OCH₂CH₃).

6.4 Preparation of azidosphingosine block

4,6-*O*-benzylidene- α/β -D-galactose (α/β -5) ²⁴³



D-(+)-galactose (4.0 g, 22.20 mmol) was dissolved in dry DMF (60 mL). Then α,α -dimethoxytoluene (4 mL, 26.55 mmol) and PTSA (140 mg, 0.736 mmol) were added. The methanol formed was evaporated at 40 °C during the reaction. After 1.5 h, the mixture was neutralized by Et₃N, then concentrated under reduced pressure. The residue was purified by silica gel column (EtOAc-MeOH 95:5). Compound **5** (4.77 g, 80%) was obtained as a white amorphous solid.

 $R_{\rm f} = 0.39$ (CH₂Cl₂-MeOH 9:1).

α-5

¹**H NMR (400 MHz, DMSO-d₆):** δ 7.50-7.30 (m, 5H, Ar-H), 5.53 (s, 1H, CHPh), 5.05 (dd, 1H, J = 4.2, 3.4 Hz, H-1), 6.26 (d, J = 4.2 Hz, 1H, 1-OH), 4.59 (d, J = 6.2 Hz, 1H, 3-OH), 4.41 (d, J = 6.7 Hz, 1H, 2-OH), 4.11 (d, J = 2.6 Hz, 1H, H-4), 4.05 (d, J = 12.1 Hz, 1H, Hb-6), 3.96 (d, J = 12.1 Hz, 1H, Ha-6), 3.77 (s, 1H, H-5), 3.76-3.74 (m, 1H, H-3), 3.62 (ddd, J = 9.4, 6.7, 3.4 Hz, 1H, H-2).

β-5

¹**H** NMR (400 MHz, DMSO-d₆): δ 7.50-7.30 (m, 5H, Ar-H), 5.55 (s, 1H, C*H*Ph), 6.56 (d, J = 6.9 Hz, 1H, 1-OH), 4.77 (d, J = 5.2 Hz, 1H, 2-OH), 4.76 (d, J = 6.4 Hz, 1H, 3-OH), 4.34 (dd, J = 7.5, 6.9 Hz, 1H, H-1), 4.10-4.00 (m, 3H, H-4, H₂-6), 3.46-3.40 (m, 1H, H-3), 3.45 (br s, 1H, H-5).3.34-3.28 (m, 1H, H-2).

²⁴³ R. I. Duclos, Chem. Phys. Lipids, 2001, 111, 111-138.

4,6-O-benzylidene-D-threose (6) ²⁴³



A solution of **5** (3.0 g, 11.18 mmol) in phosphate buffer (pH = 7.6, 128 mL, 0.1 M) was stirred vigorously and NaIO₄ (5.55 g, 25.70 mmol) was added at room temperature. The pH was maintained above 7.6 though addition of the saturated solution of NaHCO₃. After 2 h, the mixture was concentrated under reduced pressure. The off-white solid residue was dissolved in the frozen THF, filtered, concentrated, and the residue dried under vacuum to give crude intermediate **6** (1.75 g, 75%) as a yellow foam. This crude intermediate, which was not separated and further characterized at this stage, was directly engaged in the following step.

 $R_{\rm f} = 0.51$ (CH₂Cl₂-MeOH 9:1).

Tetradecyltriphenylphosphonium bromide (7)¹⁹²



A mixture of tetradecylbromide (7.2 mL, 26.38 mmol) and triphenylphosphine (6.2 g, 23.76 mmol) were heated at 140 °C for 7 h, then dissolved in 15 mL of warm Me₂CO and the product crystallized by the addition of 1000 mL of anhydrous Et₂O. A second crystallization from Me₂CO/Et₂O gave 7 (10.8 g, 85%) as a white crystalline solid, which was free of alkyl halide and triphenylphosphine.

 $R_{\rm f} = 0.35$ (Me₂CO).

¹**H** NMR (400 MHz, CDCl₃): δ 7.90-7.70 (m, 15H, Ar-H), 3.74 (m, 2H, H₂-1), 1.68-1.55 (m, 4H, H₂-2, H₂-3), 1.42-1.25 (m, 20H, 10 × CH₂), 0.87 (t, *J* = 6.5 Hz, 3H, CH₃).

(2R,3R,4Z)-1,3-O-benzylidene-4-octadecene-1,2,3-triol (cis-8) ²⁴⁴



A mechanically stirred mixture of tetradecyltriphenylphosphonium bromide 7 (7.0 g, 12.97 mmol) in 32 mL of anhydrous THF was cooled to -30 °C and then *n*-butyllithium solution (5.6 mL, 2.5 M in hexanes) was added dropwise over 15 min. The reaction mixture was stirred for an additional 30 min, then a solution of **6** (2.0 g, 9.68 mmol) in 10 mL of anhydrous THF was added over 10 min. The reaction mixture was stirred for an additional 2 h at -30 °C, further gradually warmed to room temperature and stirred overnight. Then the reaction was quenched with MeOH and H₂O and allowed to stir at ambient temperature for 1 h. After that, extracted by CH₂Cl₂ and dried over MgSO₄, filtered and concentrated under reduced pressure. The *trans* and *cis* isomers were separated by silica gel column (gradient elution: Cy-CH₂Cl₂ 1:3 to 1:6) to give *trans*-**8** (1.68 g, 45%) and *cis*-**8** (670 mg, 18%) as a white powder, respectively.

cis-7

 $R_{\rm f} = 0.58$ (CH₂Cl₂-EtOAc 25:1).

²⁴⁴ K. Ohashi, S. Kosai, M. Arizuka, T. Watanabe, Y. Yamagiwa, T. Kamikawa, M. Kates, *Tetrahedron*, **1989**, *45*, 2557-2570.

¹**H NMR (300 MHz, CDCl₃)**: δ 7.52 (dd, J = 6.7, 3.0 Hz, 2H, Ar-H), 7.37 (dd, J = 5.0, 1.9 Hz, 3H, Ar-H), 5.69 (dd, J = 5.3, 3.3 Hz, 2H, H-5, H-4), 5.66 (s, 1H, CHPh), 4.73 (d, J = 5.0 Hz, 1H, H-3), 4.25 (dd, J = 11.9, 1.8 Hz, 1H, Ha-1), 4.11 (d, J = 11.8 Hz, 1H, Hb-1), 3.49 (d, J = 11.2 Hz, 1H, H-2), 2.75 (d, J = 11.2 Hz, 1H, OH), 2.25-2.01 (m, 2H, H₂-6), 1.27 (m, 22H, 11 × CH₂), 0.89 (t, J = 6.3 Hz, 3H, CH₃).

(2R,3R,4E)-1,3-O-benzylidene-4-octadecene-1,2,3-triol (trans-8)²⁴⁴



A solution of the *cis*-8 (575 mg, 1.48 mmol) and diphenyldisulfide (35.6 g, 0.163 mmol) in a mixture of cyclohexane (7.5 mL) and dioxane (1.9 mL) was irradiated with a 100 W high-pressure mercury lamp for 5 h. After removal of the solvents, the residue was purified by silica gel column (gradient elution: Cy-CH₂Cl₂ 1:3 to 1:6) to yield *trans*-8 (259 mg, 45%) as a white powder.

trans-8

 $R_{f} = 0.60 (CH_{2}Cl_{2}-EtOAc 25:1).$

¹**H NMR (400 MHz, CDCl₃):** δ 7.57-7.50 (m, 2H, Ar-H), 7.42-7.33 (m, 3H, Ar-H), 5.88 (dtd, J = 15.4, 6.7, 1.0 Hz, 1H, H-5), 5.72-5.64 (m, 1H, H-4), 5.62 (s, 1H, CHPh), 4.41 (d, J = 6.1 Hz, 1H, H-3), 4.25 (dd, J = 11.8, 1.9 Hz, 1H, Ha-1), 4.08 (dd, J = 11.8, 1.3 Hz, 1H, Hb-1), 3.53 (dd, J = 10.4, 1.3 Hz, 1H, H-2), 2.66 (d, J = 10.4 Hz, 1H, OH), 2.09 (q, J = 7.1 Hz, 2H, H₂-6), 1.44-1.36 (m, 2H, H₂-7), 1.27 (s, 20H, 10 × CH₂), 0.89 (t, J = 6.9 Hz, 3H, CH₃).

(2S,3R,4E)-2-azido-1,3-benzylidene-4-octadecene-1,3-diol (9) ²⁴⁴



Trifluoromethanesulfonic anhydride (1.23 mL, 6.94 mmol) was added to a solution of *trans-8* (1.9 g, 4.89 mmol) in dry CH_2Cl_2 (24 mL) and pyridine (1.2 mL) at -15 °C. After 15 min, the DMF (27 mL) and sodium azide (1.61 g, 24.61 mmol) were added, stirring at -15 °C for 3 h. Further, gradually warmed to room temperature and stirred overnight. Finally, water was added and then extracted with CH_2Cl_2 , washed with brine, dried over MgSO₄, filtered and concentrated under reduced pressure. The residue was purified by silica gel column (Cy-CH₂Cl₂ 3:1) to yield **9** (1.58 g, 75%) as a white solid.

 $R_{\rm f} = 0.34$ (Cy-CH₂Cl₂ 3:2).

¹**H NMR (400 MHz, CDCl₃):** δ 7.49 (dd, J = 5.5, 2.5 Hz, 2H, Ar-H), 7.40-7.34 (m, 3H, Ar-H), 6.05-5.96 (m, 1H, H-5), 5.60 (dd, J = 15.5, 7.4 Hz, 1H, H-4), 5.50 (s, 1H, C*H*Ph), 4.34 (dd, J = 11.0, 5.1 Hz, 1H, Ha-1), 4.10-4.03 (m, 1H, H-3), 3.62 (t, J = 10.9 Hz, 1H, Hb-1), 3.47 (td, J = 10.1, 5.1 Hz, 1H, H-2), 2.12 (dd, J = 14.4, 7.1 Hz, 2H, H₂-6), 1.43 (dt, J = 14.4, 7.2 Hz, 2H, H₂-7), 1.27 (s, 20H, 10 × CH₂), 0.89 (t, J = 6.7 Hz, 3H, CH₃).

(2S,3R,4E)-2-azido-4-octadecene-1,3-diol (10) ²⁴⁴



A solution of benzylidene azidosphingosine **9** (1.5 g, 3.65 mmol) in 40 mL of 1:2 CH₂Cl₂/MeOH was stirred vigorously at room temperature and a solution of PTSA (70 mg, 0.36 mmol) in 13 mL of MeOH was added dropwise. The solution was stirred at room temperature overnight, then quenched with Et₃N. The mixture was concentrated and purified by column chromatography (Cy-EtOAc 2:1) to give azidosphingosine **10** (1.06 g, 90%) as a pale yellow solid.

 $R_{\rm f} = 0.35$ (Cy-EtOAc 2:1).

¹**H NMR (400 MHz, CDCl₃):** δ 5.85-5.75 (m, 1H, H-5), 5.51 (ddd, J = 15.4, 7.3, 1.2 Hz, 1H, H-4), 4.23 (t, J = 6.3 Hz, 1H, H-3), 3.83-3.71 (m, 2H, H₂-1), 3.48 (q, J = 5.2 Hz, 1H, H-2), 2.16-2.01 (m, 4H, H₂-6, 2 × OH), 1.41-1.32 (m, 2H, H₂-7), 1.24 (s, 20H, 10 × CH₂), 0.86 (t, J = 6.6 Hz, 3H, CH₃).

(2S,3R,4E)-2-azido-1-O-tertbutyldimethylsilyl-4-octadecene-1,3-diol (11)¹⁸⁶



TBDMSCl (720 mg, 4.82 mmol) was added at 0 °C to a solution of compound **10** (785 mg, 2.41 mmol) in dry pyridine (9.2 mL). The mixture was stirred at room temperature for 3 h, quenched with methanol and then concentrated under reduced pressure. The residue was purified by silica gel chromatography (Cy-CH₂Cl₂ 2:3) to yield **11** (950 mg, 90%) as colorless oil.

 $R_{\rm f} = 0.58$ (Cy-EtOAc 4:1).

¹**H NMR (400 MHz, CDCl₃):** δ 5.82-5.72 (m, 1H, H-5), 5.54-5.44 (m, 1H, H-4), 4.21 (dd, J = 11.7, 5.3 Hz, 1H, H-3), 3.82-3.77 (m, 2H, H₂-1), 3.41 (dd, J = 10.7, 5.3 Hz, 1H, H-2), 2.36 (d, J = 5.0 Hz, 1H, OH), 2.06 (q, J = 6.9 Hz, 2H, H₂-6), 1.42-1.34 (m, 2H, H₂-7), 1.26 (s, 20H, 10 × CH₂), 0.92-0.85 (m, 12H, (CH₃)₃C, CH₃), 0.11-0.07 (m, 6H, (CH₃)₂Si).

(2S,3R,4E)-2-azido-3-O-benzoyl-1-O-tertbutyldimethylsilyl-4-octadecene-1,3-diol (12) ¹⁸⁶



The compound **11** (950 mg, 2.15 mmo1) in the dry pyridine (13 mL) was cooled to 0 $^{\circ}$ C, and benzoyl chloride (0.5 mL, 4.30 mmol) was added. After stirring at room temperature overnight, the mixture was diluted with CH₂Cl₂ and washed successively with saturated aqueous NaHCO₃ solution, and H₂O, then dried over MgSO₄, filtered and concentrated under reduced pressure. The residue was chromatographed (Cy-EtOAc 40:1) to give **12** (1.17 g, quantitative) as colorless oil.

 $R_{\rm f} = 0.35$ (Cy-EtOAc 40:1).

¹**H NMR (400 MHz, CDCl₃):** δ 8.09-8.05 (m, 2H, Ar-H), 7.60-7.54 (m, 1H, Ar-H), 7.49-7.43 (m, 2H, Ar-H), 5.92 (dt, J = 14.7, 6.8 Hz, 1H, H-5), 5.59 (ddd, J = 23.0, 11.5, 6.2 Hz, 2H, H-4, H-3), 3.80 (dt, J = 7.0, 4.9 Hz, 1H, H-2), 3.71 (qd, J = 10.3, 6.1 Hz, 2H, H₂-1), 2.07 (dd, J = 14.4, 7.0 Hz, 2H, H₂-6), 1.41-1.20 (m, 22H, 11 × CH₂), 0.94-0.85 (m, 12H, (CH₃)₃C, CH₃), 0.07 (d, J = 0.6 Hz, 6H, (CH₃)₂Si).

(2S,3R,4E)-2-azido-3-O-benzoyl-4-octadecene-1,3-diol (13) ¹⁸⁵



To a stirred solution of **12** (1.1 g, 2.03 mmol) in CH_2Cl_2 (17 mL) was added BF₃·Et₂O (0.6 mL, 4.73 mmol) dropwise over 5 min at 0 °C. After 4 h at room temperature, the reaction was quenched and neutralized by the addition of saturated aqueous NaHCO₃ followed by adding CH_2Cl_2 . The organic phase was washed with H₂O, dried over

MgSO₄, filtered and concentrated under reduced pressure. The residue was chromatographed (Cy-EtOAc 5:1) to give 3-*O*-benzoyl azidosphingosine **13** (722 mg, 80%) as colorless oil.

 $R_{f} = 0.28$ (Cy-EtOAc 5:1).

¹**H NMR (400 MHz, CDCl₃):** δ 8.14-8.04 (m, 2H, Ar-H), 7.64-7.56 (m, 1H, Ar-H), 7.49-7.43 (m, 2H, Ar-H), 6.03-5.90 (m, 1H, H-5), 5.68-5.55 (m, 2H, H-4, H-3), 3.86-3.73 (m, 2H, H-2, Ha-1), 3.64 (dd, J = 11.6, 7.1 Hz, 1H, Hb-1), 2.15-2.01 (m, 2H, H₂-6), 1.41-1.21 (m, 22H, 11 × CH₂), 0.88 (t, J = 6.9 Hz, 3H, CH₃).

¹³C NMR (100 MHz, CDCl₃): δ 165.52 (C=O), 133.37 (Ar-C), 129.84 (3Ar-C), 128.53 (2Ar-C), 138.83 (C-5), 123.38 (C-4), 74.62 (C-3), 66.26 (C-2), 62.05 (C-1), 32.39 (C-6), 31.95-28.72 (10 × CH₂), 22.71 (C-17), 14.13 (CH₃).

6.5 Synthesis of galactose-containing analogues

6.5.1 Preparation of galactose with a free 6-OH block

1,2,3,4,6-penta-*O***-acetyl-** α **-D-galactopyranose** (14) ²⁴⁵



D-(+)-galactose (5.0 g) was dissolved in the pyridine (100 mL), and acetic anhydride (100 mL) was added slowly. Further, the mixture was stirred at room temperature overnight. To the resulting reaction mixture, CH_2Cl_2 was added, and extracted from a chilled solution of HCl (1.0 M). The combined organic phases were then washed with saturated NaHCO₃ solution and brine, dried over MgSO₄, and concentrated under reduced pressure. The residue was purified by column, eluted with Cy-EtOAc 1:1 to give 14 (7.9 g, 70%) as thick colourless oil.

 $R_{f} = 0.55$ (Cy-EtOAc 1:1).

¹**H NMR (400 MHz, CDCl₃)**: δ 6.38 (d, J = 2.8 Hz, 1H, H-1), 5.50 (t, J = 8.7 Hz, 1H, H-4), 5.34 (m, 2H, H-2, H-3), 4.43-4.01 (m, 3H, H-5, H₂-6), 2.27-1.92 (5s, 15H, 5 × OAc).

1,2,3,4-tetra-O-acetyl-α-D-galactopyranose (15)¹⁹⁸



Substrate 14 (720 mg, 1.84 mmol) was added to 100 mL solution of 50 mM phosphate buffer with 20% CH₃CN kept pH 5 at room temperature, and the reaction was

²⁴⁵ M. A. Flos, M. I. G. Moreno, C. O. Mellet, J. M. G. Fernndez, J. Nierengarten, S. P. Vincent, *Chem. Eur. J.*, **2016**, *22*, 11450-11460.

initialized by adding 3 g of biocatalyst CRL. The hydrolytic reaction was carried out under mechanical stirring and the pH value was controlled by automatic titration. After 4 h, the reaction was stopped by filtering off the enzyme. The mixture was extracted by EtOAc, washed with water, dried over MgSO₄, and concentrated under reduced pressure. The residue was separated by column, eluted with Cy-EtOAc 1:1, to obtain the compound **15** (514 mg, 80%) as a white foam.

 $R_{\rm f} = 0.30$ (Cy-EtOAc 1:1).

¹**H NMR (400 Hz, CDCl₃)**: δ 6.37 (d, J = 2.8 Hz, 1H, H-1). 5.49 (t, J = 8.7 Hz, 1H, H-4), 5.32 (m, 2H, H-2, H-3), 4.33 (t, J = 6.5 Hz, 1H, H-5), 4.04-4.16 (2m, ABX system, 2H, H₂-6), 2.15-1.98 (4s, 12H, 4 × OAc).

6.5.2 Sialylation of galactoside with sialic acid donor

Benzenesulfenyl chloride 151



To a solution of phenyl thioacetate (25g, 164 mmol) in CCl₄ (10 mL) cooled to 0 °C was added SO₂Cl₂ (13.3 mL, 164 mmol) within 10 min. The mixture was stirred at room temperature for 30 min, concentrated, and distilled using Kugelrohr. Distillation with a Vigreux column afforded benzenesulfenyl chloride as a red liquid (19.8 g, 83%), bp 50-53 °C/3 mmHg, d 1.25 g/cm³ (lit. : bp 61.5 °C/5 mmHg; lit. : bp 51-53 °C/3 mmHg).

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero- α -D-galacto-2nonulopyranosylonate)-(2 \rightarrow 6)-1,2,3,4-tetra-*O*-acetyl- α -D-galactopyranoside (16)



A mixture of sialyl xanthate **3** (1.03g, 1.73 mmol) and compound **15** (400 mg, 1.15 mmol) with 4Å powdered molecular sieves (2.2 g) were dissolved in the dry CH₃CN (24 mL) and CH₂Cl₂ (12 mL) stirring at room temperature for 1 h. Further, AgOTf (488 mg, 1.90 mmol) and DTBP (0.45 mL, 2.00 mmol) were added, and the mixture was cooled to -68 °C and kept protected from light. Then PhSCl (0.23 mL, 2.00 mmol) in dry CH₂Cl₂ (1.2 mL) was added by running the solution down the cold wall of the reaction flask. Finally, the mixture was stirred for 3 h at -68 °C. After that, the mixture was diluted with a suspension of silica gel (6 g) in EtOAc (36 mL), filtered through celite, washed with saturated aqueous NaHCO₃ and water, dried with MgSO₄, and concentrated under reduced pressure. The residue was chromatographed (Cy-EtOAc 1:3) to give the compound **16** as a white foam (659.4 mg, 70%).

 $R_{\rm f} = 0.29$ (Cy-EtOAc 1:3, twice).

 $[\alpha]_D^{20} = -11.9$ (*c* 1.0 in CHCl₃).

¹**H NMR (400 MHz, CDCl₃)**: δ 6.29 (d, J = 3.7 Hz, 1H, H-1'), 5.42 (t, J = 9.8 Hz, 1H, H-3'), 5.32-5.27 (m, 3H, H-7, H-8, NH), 5.19 (d, J = 9.3 Hz, 1H, H-5'), 5.06 (dd, J = 10.2, 3.6 Hz, 1H, H-2'), 4.88-4.81 (m, 1H, H-4), 4.25 (dd, J = 11.3, 3.2 Hz, 1H, Ha-9), 4.07-4.00 (m, 4H, H-4', H-5, Ha-6', H-6), 3.93 (dd, J = 11.2, 3.3 Hz, 1H, Hb-9), 3.78 (s, 3H, COOCH₃), 3.36 (dd, J = 11.4, 1.9 Hz, 1H, Hb-6'), 2.61 (dd, J = 13.4, 4.7 Hz, 1H, H-3eq), 2.15 (s, 3H, OAc), 2.14 (s, 6H, 2 × OAc), 2.07 (s, 3H, OAc), 2.02 (s, 9H, 3 × OAc), 1.98 (s, 3H, OAc), 1.92 (t, 1H, J = 9.8 Hz, H-3ax), 1.86 (s, 3H, NAc).

¹³C NMR (100 MHz, CDCl₃): δ 173.49 (C=O), 172.40 (C=O), 171.89 (C=O), 171.80 (C=O), 171.67 (C=O), 171.61 (C=O), 171.52 (C=O), 171.48 (C=O), 170.96 (C=O), 169.18 (C=O), 100.09 (C-2), 90.82 (C-1'), 73.46, 71.06, 70.56, 69.32, 69.18, 68.95, 68.54, 68.15 (C-4, C-6, C-7, C-8, C-2', C-3', C-4', C-5'), 63.93 (C-9), 63.53 (C-6'),

53.51 (COOCH₃), 50.01 (C-5), 38.91 (C-3), 22.73 (CH₃, NAc), 21.22 (CH₃, OAc), 20.91 (CH₃, OAc), 20.80 (CH₃, OAc), 20.73 (CH₃, OAc), 20.70 (CH₃, OAc), 20.68 (CH₃, OAc), 20.61 (CH₃, OAc), 20.43 (CH₃, OAc).

ESI-HRMS (*m*/*z*) calcd for C₃₄H₄₇NO₂₂Na [M+Na]⁺: 844.2487, found: 844.2469.

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2nonulopyranosylonate)- $(2\rightarrow 6)$ -2,3,4-tri-*O*-acetyl-α/β-D-galactopyranoside (17)



The solution of compound **16** (610 mg, 0.74 mmol) in 3 mL of dry THF was added PhCH₂NH₂ (122 μ L, 1.12 mmol). The mixture was stirred at room temperature for 24 h. The solvent was evaporated and the residue was taken into CH₂Cl₂ and washed progressively with 1.0 M HCl, saturated aqueous NaHCO₃ and water. The organic layer was dried over MgSO₄ and concentrated to give the crude intermediate **17**. The crude intermediate was directly engaged in the following step without further purification.

 $R_{\rm f} = 0.43$ (EtOAc).

O-(methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2nonulopyranosylonate)-(2→6)-2,3,4-tri-O-acetyl-α-D-galactopyranosyl trichloroacetimidate (18)



The intermediate **17** was dissolved in the dry CH_2Cl_2 (19.6 mL), and 2 mL of trichloroacetonitrile was added to the solution. After that, 192 µL of DBU was added dropwise at -5 °C, and the mixture was stirred at -5 °C for 5 h. After concentration, the residue was purified by flash column chromatography (Cy-EtOAc 1:4) to yield compound **18** as a pale yellow foam (514.1 mg, 70% for two steps from **16**).

 $R_{\rm f} = 0.48$ (EtOAc).

 $[\alpha]_D^{20} = +7.8$ (*c* 1.0 in CHCl₃).

¹**H NMR (400 MHz, CDCl₃)**: δ 8.74 (s, 1H, NH), 6.40 (d, J = 3.7 Hz, 1H, H-1'), 5.40 (t, J = 9.7 Hz, 1H, H-3'), 5.30-5.27 (m, 3H, H-7, H-8, H-5'), 5.05 (dd, J = 10.1, 3.5 Hz, 1H, H-2'), 4.88-4.79 (m, 1H, H-4), 4.24 (dd, J = 11.2, 3.2 Hz, 1H, Ha-9), 4.07-3.99 (m, 4H, H-4', H-5, Ha-6', H-6), 3.92 (dd, J = 11.2, 3.4 Hz, 1H, Hb-9), 3.78 (s, 3H, COOCH₃), 3.36 (dd, J = 11.4, 2.0 Hz, 1H, Hb-6'), 2.62 (dd, J = 13.5, 4.6 Hz, 1H, H-3eq), 2.15 (s, 3H, OAc), 2.14 (s, 6H, 2 × OAc), 2.07 (s, 3H, OAc), 2.01 (s, 6H, 2 × OAc), 1.98 (s, 3H, OAc), 1.89 (t, 1H, J = 9.6 Hz, H-3ax), 1.86 (s, 3H, NAc).

¹³C NMR (100 MHz, CDCl₃): δ 172.39 (C=O), 171.90 (C=O), 171.79 (C=O), 171.68 (C=O), 171.61 (C=O), 171.54 (C=O), 171.48 (C=O), 170.95 (C=O), 169.20 (C=O), 161.17 (C=NH), 100.10 (C-2), 92.49 (C-1'), 90.00 (CCl₃), 73.48, 71.05, 70.56, 69.34, 69.18, 68.93, 68.53, 68.15 (C-4, C-6, C-7, C-8, C-2', C-3', C-4', C-5'), 63.94 (C-9), 63.55 (C-6'), 53.52 (COOCH₃), 49.99 (C-5), 38.90 (C-3), 22.75 (CH₃, NAc), 20.91 (CH₃, OAc), 20.81 (CH₃, OAc), 20.75 (CH₃, OAc), 20.70 (CH₃, OAc), 20.67 (CH₃, OAc), 20.61 (CH₃, OAc), 20.45 (CH₃, OAc).

ESI-HRMS (*m*/*z*) calcd for C₃₄H₄₅N₂Cl₃O₂₁Na [M+Na]⁺: 945.1478, found: 945.1459.
6.5.3 Conjugation of lipid part with the sialo-oligosaccharide

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto -2-nonulopyranosylonate)-(2→6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 →1)-(2*S*,3*R*,4*E*)-2-azido-3-*O*-benzoyl-4-octadecene-l,3-diol (19)



The compound **18** (114.6 mg, 0.124 mmol) and 3-*O*-benzoyl azidosphingosine (90.2 mg, 0.21 mmol) in 6 mL of dry CH₂Cl₂ was stirred with 4Å powdered molecular sieves (500 mg) at room temperature for 1 h. The mixture was then cooled to -15 °C, and BF₃·Et₂O (78 μ L, 0.62 mmol) was added dropwise, stirred for 3 h at -15 °C, and then filtered through celite. The filtrate was washed with saturated aqueous NaHCO₃ and water, dried over MgSO₄ and concentrated under reduced pressure. The residue was applied to a flash chromatography eluted with Cy-EtOAc 1:5 to give the product **19** (96.0 mg, 65%) as an amorphous solid.

 $R_{\rm f} = 0.58$ (EtOAc).

 $[\alpha]_D^{20} = -14.5$ (*c* 1.0 in CHCl₃).

¹**H NMR (400 MHz, CDCl₃)**: δ 8.02-7.99 (m, 2H, Ar-H), 7.58-7.54 (m, 1H, Ar-H), 7.46-7.42 (m, 2H, Ar-H), 5.85 (dd, J = 14.2, 7.3 Hz, 1H, H-5''), 5.59-5.48 (m, 2H, H-3'', H-4''), 5.36-5.23 (m, 5H, H-4, H-7, H-8, NH, H-3'), 5.12 (d, J = 9.59, 1H, H-2'), 4.92-4.84 (m, 1H, H-4'), 4.73 (d, J = 8.0 Hz, 1H, H-1'), 4.50 (m, 1H, Hb-9), 4.27 (dd, J = 12.4, 2.7 Hz, 1H, Ha-9), 4.08-3.99 (m, 3H, Ha-6', H-2'', H-5), 3.94-3.87 (m, 2H, H-5', Hb-6'), 3.79 (m, 1H, Ha-1''), 3.76 (s, 3H, COOCH₃), 3.65 (dd, J = 10.8, 4.3 Hz, 1H, Hb-1''), 3.40 (d, J = 9.1 Hz, 1H, H-6), 2.59 (dd, J = 12.7, 4.6 Hz, 1H, H-3eq), 2.20 (m, 2H, H₂-6''), 2.13 (s, 3H, OAc), 2.12 (d, J = 2.2 Hz, 6H, 2 × OAc), 2.05 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.94 (t, J = 2.55 (d, J = 10.8, 0.05 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.94 (t, J = 1.5).

= 11.2 Hz, 1H, H-3ax), 1.86 (s, 3H, NAc), 1.36 (m, 2H, H₂-7"), 1.24 (s, 20H, $10 \times CH_2$), 0.86 (t, J = 6.8 Hz, 3H, CH₃).

¹³C NMR (100 MHz, CDCl₃): δ 172.39 (C=O), 171.88 (C=O), 171.78 (C=O), 171.65 (C=O), 171.61 (C=O), 171.50 (C=O), 171.47 (C=O), 170.95 (C=O), 169.20 (C=O), 166.51 (Ph*C*=O), 138.24 (C-5''), 133.53 (C, CH aromatic), 130.48 (C aromatic), 130.15 (2C, CH aromatic), 128.90 (2C, CH aromatic), 125.08 (C-4''), 100.09 (C-1'), 97.53 (C-2), 75.05 (C-3''), 73.46 (C-3'), 71.08 (C-4'), 70.55 (C-6), 69.81 (C-2'), 69.30 (C-4), 69.19 (C-5'), 68.93 (C-1''), 68.55 (C-8), 68.15 (C-7), 64.05 (C-2''), 63.93 (C-6'), 63.53 (C-9), 53.50 (COOCH₃), 50.02 (C-5), 38.94 (C-3), 32.38 (C-6''), 30.17, 30.16, 30.12, 29.82 (11 × CH₂), 22.73 (CH₃, NAc), 20.91 (CH₃, OAc), 20.80 (CH₃, OAc), 20.72 (CH₃, OAc), 20.70 (CH₃, OAc), 20.69 (CH₃, OAc), 20.59 (CH₃, OAc), 20.44 (CH₃, OAc), 14.57 (CH₃).

ESI-HRMS (*m*/*z*) calcd for C₅₇H₈₂N₄O₂₃Na [M+Na]⁺: 1213.5268, found: 1213.5286.

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto -2-nonulopyranosylonate)-(2→6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 →1)-(2*S*,3*R*,4*E*)-2-amido-3-*O*-benzoyl-4-octadecene-l,3-diol (20)



To a solution of compound **19** (40 mg, 0.034 mmol) in 3.9 mL of toluene and 0.16 mL of water was added 22.0 mg of triphenylphosphine. The mixture was stirred at 50 °C for 12 h. After concentration, the crude residue **20** was directly used for the next step.

$$R_{\rm f} = 0.26$$
 (EtOAc).

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto -2-nonulopyranosylonate)-(2→6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 →1)-(2*S*,3*R*,4*E*)-2-octadecanamido-3-*O*-benzoyl-4-octadecene-l,3-diol (21)



The intermediate **20**, stearic acid (32.9 mg, 0.12 mmol), EDC·HCl (34.2 mg, 0.18 mmol) in 5.4 mL of CH_2Cl_2 were stirred at room temperature for 24 h. Then the mixture was washed with water, dried over MgSO₄ and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography (Cy-EtOAc 1:3) to afford compound **21** (37.2 mg, 60% for two steps from **19**) as an amorphous solid.

 $R_{\rm f} = 0.33$ (Cy-EtOAc 1:3).

 $[\alpha]_D^{20} = -17.5$ (*c* 1.0 in CHCl₃).

¹**H NMR (400 MHz, CDCl₃)**: δ 8.03-7.98 (m, 2H, Ar-H), 7.57-7.54 (m, H, Ar-H), 7.45-7.42 (m, 2H, Ar-H), 5.93-5.83 (m, 2H, NH, H-5''), 5.57-5.48 (m, 2H, H-3'', H-4''), 5.34-5.22 (m, 4H, H-8, H-7, H-3', NH), 5.11 (d, J = 9.4 Hz, H-5'), 4.94-4.83 (m, 1H, H-2'), 4.73 (d, J = 9.4 Hz, 1H, H-1'), 4.54-4.47 (m, 1H, H-4), 4.26 (dd, J = 12.4, 2.5 Hz, 1H, Ha-9), 4.07–4.01 (m, 3H, Hb-9, H-5, H-6), 3.94-3.89 (m, 3H, H-2'', H₂-6'), 3.81 (dd, J = 10.4, 3.2 Hz, , Ha-1''), 3.77 (s, 3H, COOCH₃), 3.66 (dd, J = 5.7, 2.4 Hz, 1H, H-4'), 3.40 (d, J = 9.0 Hz, 1H, Hb-1''), 2.60 (dd, J = 12.8, 4.7 Hz, 1H, H-3eq), 2.25-2.18 (m, 2H, COCH₂), 2.14 (s, 3H, OAc), 2.13 (d, J = 1.6 Hz, 6H, 2 × OAc), 2.05 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.94 (t, J = 7.4 Hz, 1H, H-3ax), 1.86 (s, 3H, NAc), 1.64-1.59 (m, 2H, H₂-6''), 1.42 (m, 2H, H₂-7''), 1.24 (s, 50H, 25 × CH₂), 0.87 (t, J = 7.0 Hz, 6H, 2 × CH₃).

¹³C NMR (100 MHz, CDCl₃): δ 173.49 (C=O), 172.41 (C=O), 171.89 (C=O), 171.81

(C=O), 171.66 (C=O), 171.60 (C=O), 171.50 (C=O), 171.49 (C=O), 170.95 (C=O), 169.19 (C=O), 166.49 (PhC=O), 138.23 (C-5''), 133.51 (C, CH aromatic), 130.48 (C aromatic), 130.14 (2C, CH aromatic), 128.88 (2C, CH aromatic), 125.07 (C-4''), 100.10 (C-1'), 97.50 (C-2), 75.08 (C-3''), 73.48 (C-3'), 71.04 (C-4'), 70.58 (C-6), 69.82 (C-2'), 69.34 (C-4), 69.19 (C-5'), 68.93 (C-1''), 68.53 (C-8), 68.14 (C-7), 64.36 (C-2''), 63.92 (C-6'), 63.53 (C-9), 53.50 (COOCH₃), 50.02 (C-5), 38.92 (C-3), 31.38, 30.16, 30.15, 30.11, 29.81 (CH₂), 22.73 (CH₃, NAc), 20.91 (CH₃, OAc), 20.80 (CH₃, OAc), 20.73 (CH₃, OAc), 20.70 (CH₃, OAc), 20.68 (CH₃, OAc), 20.61 (CH₃, OAc), 20.43 (CH₃, OAc), 14.57 (2 × CH₃).

ESI-HRMS (*m*/*z*) calcd for C₇₅H₁₁₈N₂O₂₄Na [M+Na]⁺: 1453.7972, found: 1453.7988.

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2 →6)-O-(β-D-galactopyranosyl)-(1→1)-(2S,3R,4E)-2-octadecanamido-4-octadecene-l,3-diol (22)



A solution of compound **21** (30 mg, 0.021 mmol) in 4.1 mL of NaOMe/MeOH (0.04 M) was stirred at room temperature for 14 h. A few drops of water were added at 0 °C. Then the mixture was stirred at room temperature for another 2 h, and further neutralized by Amberlite IR $120/H^+$ ion exchange resin. After filtration and concentration, the residue obtained was flash-chromatographed, eluting with CHCl₃-MeOH 3:1 to yield **22** (20.2 mg, 95%) as a white amorphous solid.

 $R_{f} = 0.36$ (EtOAc-*i*PrOH-H₂O 4:2:1).

 $[\alpha]_D^{20} = +6.1$ (*c* 1.0 in CHCl₃-MeOH 1:1).

¹**H NMR (400 MHz, CD₃OD)**: δ 5.74-5.67 (m, 1H, H-5''), 5.49-5.43 (m, 1H, H-4''), 4.41 (d, *J*=9.8 Hz, 1H, H-1'), 4.09-4.00 (m, 2H, Ha-1'', H-3''), 3.99-3.95 (m, 1H, H-3'), 3.87-3.80 (m, 5H, H-4, H-5, H-4', Ha-6', Hb-1''), 3.70-3.51 (m, 9H, H-6, H-7, H-8, H₂-9, H-2', H-5', Hb-6', H-2''), 3.35 (s, 1H, NH), 2.71 (dd, *J* = 12.1, 4.9 Hz, 1H, H-3eq), 2.18 (t, *J* = 6.8 Hz, 2H, CH₂C(O)), 2.06-2.01 (m, 2H, H₂-6''), 2.00 (s, 3H, NAc), 1.79 (t, *J* = 12.1 Hz, 1H, H-3ax), 1.58 (m, 2H, CH₂CH₂C(O)), 1.29 (s, 50H, 25 × CH₂), 0.90 (t, *J* = 7.4 Hz, 6H, 2 × CH₃).

¹³C NMR (100 MHz, CD₃OD): δ 174.16 (C=O), 169.18 (C=O), 168.65 (C=O), 134.19 (C-5''), 128.45 (C-4''), 100.09 (C-1'), 97.53 (C-2), 75.03 (C-3''), 73.48 (C-3'), 71.05 (C-4'), 70.54 (C-6), 69.79 (C-2'), 69.30 (C-4), 69.20 (C-5'), 68.94 (C-1''), 68.55 (C-8), 68.17 (C-7), 64.35 (C-2''), 63.95 (C-6'), 63.55 (C-9), 50.97 (C-5), 40.12 (C-3), 30.17, 30.16, 30.11, 29.80 (CH₂), 22.71 (CH₃, NAc), 15.47 (2 × CH₃).

ESI-HRMS (*m*/*z*) calcd for C₅₃H₉₇N₂O₁₆ [M-H]⁻: 1017.6844, found: 1017.6824.

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2 \rightarrow 6)-*O*-(β-D-galactopyranosyl)-(1 \rightarrow 1)-(2S,3R,4E)-2-azido-4-octadecene-l,3-diol (23)



The compound **19** (40.0 mg, 0.034 mmol) in 6.7 mL of NaOMe/MeOH (0.04 M) was stirred at room temperature for 14 h. Then a few drops of water were added at 0 °C. After stirring at room temperature for another 2 h, the mixture was neutralized by Amberlite IR $120/H^+$ ion exchange resin. After filtration and concentration, the residue was dried in *vacuo* to afford crude intermediate **23**.

$$R_{f} = 0.39$$
 (EtOAc-*i*PrOH-H₂O 4:2:1).

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2 →6)-*O*-(β-D-galactopyranosyl)-(1→1)-(2*S*,3*R*,4*E*)-2-amino-4-octadecene-l,3-diol (24)



The crude intermediate **23** in the anhydrous MeOH (1.8 mL) was added propane-1,3-dithiol (0.18 mL) and triethylamine (0.18 mL), and the mixture was stirred at room temperature for 4 days. A white precipitate was formed. After filtration and washing with MeOH, the filtrate was concentrated under reduced pressure. The residue obtained was flash-chromatographed, eluting with CHCl₃-MeOH 3:1 to yield **24** (21.5 mg, 85% for two steps from **19**) as a white amorphous solid.

 $R_{f} = 0.33$ (EtOAc-*i*PrOH-H₂O 4:2:1).

 $[\alpha]_D^{20} = -2.1$ (*c* 1.0 in CHCl₃-MeOH 1:1).

¹**H NMR** (400 MHz, **DMSO-d**₆): δ 8.48 (s, 1H, NH), 5.75-5.67 (m, 1H, H-5''), 5.50-5.43 (m, 1H, H-4''), 4.45 (d, J = 7.4 Hz, 2H, H-3'', H-1'), 4.12 (d, J = 9.1 Hz, 1H, Ha-1''), 3.90 (dt, J = 10.5, 9.1 Hz, 3H, H₂-9, Hb-1''), 3.70 (d, J = 9.2 Hz, 3H, Ha-6', H-4, H-4'), 3.58-3.45 (m, 3H, H-3', H-5, Hb-6'), 3.35 (d, J = 8.6 Hz, 2H, H-8, H-5'), 3.30-3.21 (m, 2H, H-6, H-2''), 3.10 (dd, J = 16.4, 8.5 Hz, 2H, H-2', H-7), 2.73 (d, J = 7.4, 3.8 Hz, 1H, H-3eq), 2.06-2.01 (m, 2H, H₂-6''), 2.00 (s, 3H, NAc), 1.61-1.56 (m, 3H, H-3ax, H₂-7''), 1.29 (s, 20H, 10 × CH₂), 0.90 (t, J = 6.8 Hz, 3H, CH₃).

¹³C NMR (100 MHz, DMSO-d₆): δ 169.19 (C=O), 168.66 (C=O), 134.17 (C-5''), 128.42 (C-4''), 100.07 (C-1'), 97.52 (C-2), 75.07 (C-3''), 73.44 (C-3'), 71.03 (C-4'), 70.55 (C-6), 69.83 (C-2'), 69.34 (C-4), 69.20 (C-5'), 68.93 (C-1''), 68.56 (C-8), 68.14 (C-7), 64.37 (C-2''), 63.95 (C-6'), 63.50 (C-9), 50.99 (C-5), 40.10 (C-3), 30.16, 30.15, 30.11, 29.83 (CH₂), 22.73 (CH₃, NAc), 15.48 (CH₃).

ESI-HRMS (*m*/*z*) calcd for C₃₅H₆₃N₂O₁₅ [M-H]⁻: 751.4234, found: 751.4218.

6.6 Synthesis of mannose-containing analogues

6.6.1 Preparation of mannose with a free 6-OH block

1,2,3,4,6-penta-*O***-acetyl-***α***-D-mannopyranose** (**25**) ²⁴⁶



D-(+)-**Mannose** (5.0 g) was dissolved in the pyridine (100 mL), and acetic anhydride (100 mL) was added slowly. Further, the mixture was stirred at room temperature overnight. To the resulting reaction mixture, CH_2Cl_2 was added, and extracted from a chilled solution of HCl (1.0 M). The combined organic phases were then washed with saturated NaHCO₃ solution and brine, dried over MgSO₄, and concentrated under reduced pressure. The residue was purified by column, eluted with Cy-EtOAc 1:1 to give **25** (7.9 g, 70%) as thick colourless oil.

 $R_{f} = 0.55$ (Cy-EtOAc 1:1).

¹**H NMR (400 MHz, CDCl₃)**: δ 6.06 (d, J = 1.8 Hz, 1H, H-1), 5.33 (m, 2H, H-3, H-4), 5.23 (d, J = 2.3 Hz, 1H, H-2), 4.24 (m, 1H, Ha-6), 4.09-4.05 (m, 2H, Hb-6, H-5), 2.15 (s, 3H, OAc), 2.15 (s, 3H, OAc), 2.07 (s, 3H, OAc), 2.03 (s, 3H, OAc), 1.98 (s, 3H, OAc). OAc).

1,2,3,4-tetra-*O*-acetyl-α-D-mannopyranose (26)²¹²



²⁴⁶ I. Kalograiaki, M. Abellan-Flos, L. Fernandez, M. Menendez, S. P. Vincent, D. Solís, *Anal. Chem.*, 2018, 90, 12314-12321.

Substrate **25** (1.84 mmol, 720 mg) was added to 100 mL solution of 50 mM phosphate buffer with 20% CH₃CN kept pH 5 at room temperature, and the reaction was initialized by adding 3 g of biocatalyst CRL. The hydrolytic reaction was carried out under mechanical stirring and the pH value was controlled by automatic titration. After 4 h, the reaction was stopped by filtering off the enzyme. The mixture was extracted by EtOAc, washed with water, dried over MgSO₄, and concentrated under reduced pressure. The residue was separated by column, eluted with Cy-EtOAc 1:1, to obtain the compound **26** (514 mg, 80%) as a white foam.

 $R_{\rm f} = 0.30$ (Cy-EtOAc 1:1).

¹**H NMR (400 Hz, CDCl₃)**: δ 6.10 (d, J = 1.7 Hz, 1H, H-1), 5.40 (dd, J = 3.3, 1.7 Hz, 1H, H-2), 5.30 (dd, J = 3.4, 1.4 Hz, 1H, H-3), 5.27 (m, 1H, H-4), 3.83 (m, 1H, H-5), 3.70 (dd, J = 11.9, 6.5 Hz, 1H, Ha-6), 3.61 (dd, J = 11.6, 6.4 Hz, 1H, Hb-6), 2.15 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.00 (s, 3H, OAc).

6.6.2 Sialylation of mannoside with sialic acid donor

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2nonulopyranosylonate)- $(2\rightarrow 6)$ -1,2,3,4-tetra-*O*-acetyl-α-D-mannopyranoside (27)



A mixture of sialyl xanthate **3** (1.29 g, 2.12 mmol) and compound **26** (500 mg, 1.44 mmol) with 4Å powdered molecular sieves (2.8 g) were dissolved in the dry CH₃CN (30 mL) and CH₂Cl₂ (15 mL) stirring at room temperature for 1 h. Further, AgOTf (610 mg, 2.34 mmol) and DTBP (0.56 ml, 2.50 mmol) were added, and the mixture was cooled to -68 °C and kept protected from light. Then PhSCl (0.29 mL, 2.50 mmol)

in the dry CH_2Cl_2 (1.5 mL) was added by running the solution down the cold wall of the reaction flask. Finally, the mixture was stirred for 2.5 h at -68 °C. After that, the mixture was diluted with a suspension of silica gel (7.5 g) in EtOAc (45 mL), filtered through celite, washed with saturated aqueous NaHCO₃ and water, dried with MgSO₄, and concentrated under reduced pressure. The residue was chromatographed (Cy-EtOAc 1:3) to give the compound **27** as a white foam (883.9 mg, 75%).

 $R_{f} = 0.31$ (Cy-EtOAc 1:3, twice).

 $[\alpha]_D^{20} = -10.8 \ (c \ 1.0 \ in \ CHCl_3).$

¹**H NMR (400 MHz, CDCl₃)**: δ 6.05 (d, J = 1.8 Hz, 1H, H-1'), 5.46 (t, J = 9.5 Hz, 1H, H-3'), 5.34-5.29 (m, 4H, H7, H8, H-5', NH), 5.23-5.21 (m, 1H, H-2'), 4.91-4.85 (m, 1H, H-4), 4.36 (dd, J = 11.3, 2.7 Hz, 1H, Ha-9), 4.07-3.97 (m, 5H, H-6, Hb-9, H-4', H-5, Ha-6'), 3.79 (s, 3H, COOCH₃), 3.43 (dd, J = 11.2, 2.6 Hz, 1H, Hb-6'), 2.64 (dd, J = 11.4, 3.7 Hz, 1H, H-3eq), 2.18 (s, 3H, OAc), 2.15 (s, 6H, 2 × OAc), 2.14 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.89 (t, J = 9.8 Hz, 1H, H-3ax), 1.87 (s, 3H, NAc).

¹³C NMR (100 MHz, CDCl₃): δ 170.97 (C=O), 170.57 (C=O), 170.17 (C=O), 170.09 (C=O), 170.04 (C=O), 170.02 (C=O), 169.88 (C=O), 168.94 (C=O), 168.16 (C=O), 167.53 (C=O), 98.42 (C-2), 90.74 (C-1'), 72.31, 71.13, 69.22, 69.09, 68.56, 67.99, 67.21, 65.39 (C-4, C-6, C-7, C-8, C-2', C-3', C-4', C-5'), 62.62 (C-9), 62.37 (C-6'), 52.84 (COOCH₃), 49.41 (C-5), 37.66 (C-3), 23.21 (CH₃, NAc), 21.13 (CH₃, OAc), 20.91 (CH₃, OAc), 20.83 (CH₃, OAc), 20.82 (CH₃, OAc), 20.78 (CH₃, OAc), 20.71 (CH₃, OAc), 20.69 (CH₃, OAc), 20.67 (CH₃, OAc).

ESI-HRMS (*m*/*z*) calcd for C₃₄H₄₇NO₂₂Na [M+Na]⁺: 844.2487, found: 844.2495.

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2nonulopyranosylonate)- $(2\rightarrow 6)$ -2,3,4-tri-*O*-acetyl-α/β-D-mannopyranoside (28)



The solution of compound **27** (610 mg, 0.74 mmol) in 3 mL of dry THF was added PhCH₂NH₂ (122 μ L, 1.12 mmol) and the mixture was stirred at room temperature for 5 h. The solvent was evaporated and the residue was taken into CH₂Cl₂ and washed progressively with 1.0 M HCl, saturated aqueous NaHCO₃ and water. The organic layer was dried over MgSO₄ and concentrated, obtained the crude intermediate **28**. The crude intermediate was directly engaged in the following step without further purification.

 $R_{\rm f}$ = 0.29 (EtOAc).

O-(methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2nonulopyranosylonate)-(2→6)-2,3,4-tri-O-acetyl-α-D-mannopyranosyl trichloroacetimidate (29)



The intermediate **28** was dissolved in the dry CH_2Cl_2 (19.6 mL), and 2 mL of trichloroacetonitrile was added to the solution. After that, 192 µL of DBU was added dropwise at -5 °C, and the mixture was stirred at -5 °C for 3 h. After concentration, the residue was purified by column chromatography (Cy-EtOAc 1:4) to yield compound **29** as a pale yellow foam (514 mg, 70% for two steps from **27**).

 $R_{\rm f} = 0.33$ (EtOAc).

 $[\alpha]_D^{20} = +2.8 \ (c \ 1.0 \ in \ CHCl_3).$

¹**H NMR (400 MHz, CDCl₃)**: δ 8.74 (s, 1H, NH), 6.25 (d, J = 1.9 Hz, 1H, H-1'), 5.47 (t, J = 11.8 Hz, 1H, H-3'), 5.42 (m, 1H, H-5'), 5.36 (dd, J = 10.2, 3.6 Hz, 1H, H-2'), 5.32-5.29 (m, 3H, H-7, H-8, NH), 4.91-4.85 (m, 1H, H-4), 4.26 (dd, J = 11.3, 3.2 Hz, 1H, Ha-9), 4.05-3.93 (m, 5H, H-5, H-6, Hb-9, H-4', Ha-6'), 3.78 (s, 3H, COOCH₃), 3.47 (dd, J = 11.4, 1.9 Hz, 1H, Hb-6'), 2.60 (dd, J = 11.4, 4.7 Hz, 1H, H-3eq), 2.19 (s, 3H, OAc), 2.14 (s, 3H, OAc), 2.12 (s, 3H, OAc), 2.07 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.94 (t, J = 9.8 Hz, 1H, H-3ax), 1.86 (s, 3H, NAc).

¹³C NMR (100 MHz, CDCl₃): δ 170.79 (C=O), 170.39 (C=O), 170.02 (C=O), 169.88 (C=O), 169.77 (C=O), 169.74 (C=O), 169.62 (C=O), 168.82 (C=O), 167.48 (C=O), 159.63 (C=NH), 98.23 (C-2), 94.65 (C-1'), 90.38 (CCl₃), 72.20, 71.69, 69.04, 68.88, 68.03, 67.78, 67.06, 65.05 (C-4, C-6, C-7, C-8, C-2', C-3', C-4', C-5'), 62.46 (C-9), 62.13 (C-6'), 52.64 (COOCH₃), 49.24 (C-5), 37.47 (C-3), 23.02 (CH₃, NAc), 20.92 (CH₃, OAc), 20.86 (CH₃, OAc), 20.67 (CH₃, OAc), 20.65 (CH₃, OAc), 20.62 (CH₃, OAc), 20.46 (CH₃, OAc).

ESI-HRMS (*m*/*z*) calcd for C₃₄H₄₅N₂Cl₃O₂₁Na [M+Na]⁺: 945.1478, found: 945.1460.

6.6.3 Conjugation of lipid part with the sialo-oligosaccharide

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→6)-*O*-(2,3,4-tri-*O*-acetyl-α-D-mannosyl)-(1→1)-(2*S*, 3*R*,4*E*)-2-azido-3-*O*-benzoyl-4-octadecene-l,3-diol (30)



The compound **29** (229 mg, 0.25 mmol) and 3-*O*-benzoyl azidosphingosine (180 mg, 0.42 mmol) in 12 mL of dry CH₂Cl₂ was stirred with 4Å powdered molecular sieves (1 g) at room temperature for 0.5 h. The mixture was then cooled to -15 °C, and BF₃·Et₂O (156 μ L, 1.24 mmol) was added dropwise, stirred for 2.5 h at -15 °C, and then filtered through celite. The filtrate was washed with saturated aqueous NaHCO₃ and water, dried over MgSO₄ and concentrated under reduced pressure. The residue was applied to a chromatography eluted with Cy-EtOAc 1:5 to give the product **30** (177 mg, 60%) as an amorphous solid.

 $R_{\rm f} = 0.47$ (EtOAc).

 $[\alpha]_D^{20} = -24.5$ (*c* 1.0 in CHCl₃).

¹**H NMR (400 MHz, CDCl₃)**: δ 8.06-8.03 (m, 5H, Ar-H), 5.82-5.75 (m, 1H, H-5''), 5.53-5.48 (m, 2H, H-3'', H-4''), 5.38-5.31 (m, 6H, H-4, H-7, H-8, NH, H-2', H-3'), 4.91-4.87 (m, 1H, H-4'), 4.86 (d, J = 2.0 Hz, 1H, H-1'), 4.36 (m, 1H, Ha-9), 4.28 (dd, J = 12.4, 2.7 Hz, 1H, Hb-9), 3.98-3.92 (m, 5H, H-5, H-5', H-6', H-2''), 3.86 (dd, J = 10.4, 3.6 Hz, 1H, Ha-1''), 3.76 (s, 3H, COOCH₃), 3.52-3.44 (m, 2H, H-6, Hb-1''), 2.59 (dd, J = 12.7, 4.6 Hz, 1H, H-3eq), 2.17-2.15 (m, 2H, H₂-6''), 2.14 (s, 3H, OAc), 2.13 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.98 (s, 3H, OAc), 1.90 (t, J = 11.2 Hz, 1H, H-3ax), 1.85 (s, 3H, NAc), 1.39-1.36 (m, 2H, H₂-7''), 1.24 (s, 20H, 10 × CH₂), 0.87 (t, J = 6.8 Hz, 3H, CH₃).

¹³C NMR (100 MHz, CDCl₃): δ 170.86 (C=O), 170.57 (C=O), 170.22 (C=O), 170.07 (C=O), 169.99 (C=O), 169.86 (C=O), 169.79 (C=O), 169.14 (C=O), 167.83 (C=O), 165.13 (Ph*C*=O), 138.67 (C-5''), 133.29 (C, CH aromatic), 133.25 (C aromatic),

129.81 (C, CH aromatic), 129.78 (C, CH aromatic), 129.76 (C, CH aromatic), 123.29 (C-4''), 98.45 (C-1'), 98.36 (C-2), 74.55 (C-3''), 72.45 (C-6), 69.64 (C-4'), 69.49 (C-3'), 69.19 (C-2'), 69.06 (C-4), 68.22 (C-8), 67.88 (C-1''), 67.29 (C-5'), 66.76 (C-7), 63.81 (C-2''), 62.36 (C-6'), 61.93 (C-9), 52.73 (COOCH₃), 49.37 (C-5), 37.89 (C-3), 32.36 (C-6''), 29.63, 29.58, 29.41, 29.34 (CH₂), 22.68 (CH₃, NAc), 21.09 (CH₃, OAc), 20.89 (CH₃, OAc), 20.85 (CH₃, OAc), 20.79 (CH₃, 2 × OAc), 20.71 (CH₃, 2 × OAc), 20.67 (CH₃, OAc), 14.11 (CH₃).

ESI-HRMS (*m*/*z*) calcd for C₅₇H₈₂N₄O₂₃Na [M+Na]⁺: 1213.5268, found: 1213.5259.

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→6)-*O*-(2,3,4-tri-*O*-acetyl-α-D-mannosyl)-(1→1)-(2*S*, 3*R*,4*E*)-2-amido-3-*O*-benzoyl-4-octadecene-l,3-diol (31)



The compound **30** (80 mg, 0.068 mmol) in 7.8 mL of toluene and 0.32 mL of water was added 44.0 mg of triphenylphosphine. The mixture was stirred at 50 °C for 12 h. After concentration, the crude residue **31**, was directly used for the next step.

 $R_{\rm f} = 0.23$ (EtOAc).

O-(methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→6)-O-(2,3,4-tri-O-acetyl-α-D-mannosyl)-(1→1)-(2S, 3R,4E)-2-octadecanamido-3-O-benzoyl-4-octadecene-l,3-diol (32)



The intermediate **31**, stearic acid (66 mg, 0.24 mmol), EDC (56 mg, 0.36 mmol) in 10.8 mL of CH_2Cl_2 were stirred at room temperature for 20 h. Then the mixture was washed with water, dried over MgSO₄ and concentrated under reduced pressure. The resulting residue was purified by column chromatography (Cy-EtOAc 1:3) to afford compound **32** (76 mg, 62% for two steps from **30**) as an amorphous solid.

 $R_{\rm f} = 0.39$ (Cy-EtOAc 1:3).

 $[\alpha]_D^{20} = -10.5$ (*c* 1.0 in CHCl₃).

¹**H NMR** (400 MHz, CDCl₃): δ 8.00-7.98 (m, 2H, Ar-H), 7.58-7.53 (m, H, Ar-H), 7.44-7.42 (m, 2H, Ar-H), 5.92-5.83 (m, 2H, NH, H-5''), 5.60-5.48 (m, 2H, H-3'', H-4''), 5.35-5.24 (m, 4H, H-8, H-7, H-3', NH), 5.10 (d, J = 9.4 Hz, 1H, H-5'), 4.95-4.85 (m, 1H, H-2'), 4.73 (d, J = 1.8 Hz, 1H, H-1'), 4.55-4.47 (m, 1H, H-4), 4.26 (dd, J = 12.5, 2.6 Hz, 1H, Ha-9), 4.07-4.00 (m, 3H, H-5, H-6, Hb-9), 3.94-3.90 (m, 3H, H₂-6', H-2''), 3.80 (dd, J = 10.6, 3.1 Hz, 1H, Ha-1''), 3.77 (s, 3H, COOCH₃), 3.65 (dd, J = 5.6, 2.6 Hz, 1H, H-4'), 3.40 (d, J = 9.0 Hz, 1H, Hb-1''), 2.60 (dd, J = 12.8, 4.5 Hz, 1H, H-3eq), 2.25-2.20 (m, 2H, COCH₂), 2.13 (s, 3H, OAc), 2.12 (d, J = 1.4 Hz, 6H, 2 × OAc), 2.05 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.95 (t, J = 7.3 Hz, 1H, H-3ax), 1.86 (s, 3H, NAc), 1.64-1.60 (m, 2H, H₂-6''), 1.25 (s, 52H, 26 × CH₂), 0.87 (t, J = 6.9 Hz, 6H, 2 × CH₃).

¹³C NMR (100 MHz, CDCl₃): δ 173.34 (C=O), 171.41 (C=O), 171.03 (C=O), 170.65 (C=O), 170.54 (C=O), 170.44 (C=O), 170.30 (C=O), 170.27 (C=O), 169.59 (C=O), 168.22 (C=O), 165.73 (Ph*C*=O), 138.22 (C-5"), 133.57 (C, CH aromatic), 130.46 (C aromatic), 130.15 (2C, CH aromatic), 128.90 (2C, CH aromatic), 125.07 (C-4"),

98.98 (C-1'), 98.90 (C-2), 74.51 (C-3''), 72.90 (C-6), 69.96 (C-4'), 69.78 (C-3'), 69.74 (C-2'), 69.47 (C-4), 68.61 (C-8), 67.70 (C-1''), 67.38 (C-5'), 66.60 (C-7), 63.51 (C-6'), 62.80 (C-9), 53.25 (COOCH₃), 51.37 (C-2''), 49.97 (C-5), 37.34 (C-3), 32.38, 30.17, 30.16, 30.12, 29.82 (CH₂), 23.66 (CH₃, NAc), 21.33 (CH₃, OAc), 21.30 (CH₃, OAc), 21.26 (CH₃, $2 \times OAc$), 21.21 (CH₃, OAc), 21.17 (CH₃, $2 \times OAc$), 14.57 (2 × CH₃).

ESI-HRMS (*m*/*z*) calcd for C₇₅H₁₁₈N₂O₂₄Na [M+Na]⁺: 1453.7972, found: 1453.7982.

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2 →6)-O-(α-D-mannosyl)-(1→1)-(2S,3R,4E)-2-octadecanamido-4-octadecene-l,3diol (33)



A solution of compound **32** (60 mg, 0.042 mmol) in 8.2 mL of NaOMe/MeOH (0.04 M) was stirred at room temperature for 14 h. A few drops of water were added at 0 °C. Then the mixture was stirred at room temperature for another 2 h, and further neutralized by Amberlite IR $120/H^+$ ion exchange resin. After filtration and concentration, the residue obtained was flash-chromatographed, eluting with CHCl₃-MeOH 3:1 to yield the analogue **33** (40 mg, 95%) as a white amorphous solid.

 $R_{f} = 0.33$ (EtOAc-*i*PrOH-H₂O 3:2:1).

 $[\alpha]_D^{20} = +10.1$ (*c* 1.0 in CHCl₃-MeOH 1:1).

¹**H NMR (400 MHz, CD₃OD)**: δ 5.75-5.66 (m, 1H, H-5''), 5.50-5.43 (m, 1H, H-4''), 4.72 (d, *J* = 1.8 Hz, 1H, H-1'), 4.10-4.01 (m, 2H, Ha-1'', H-3''), 4.00-3.95 (m, 1H, H-3'), 3.87-3.81 (m, 5H, H-4, H-5, H-4', Ha-6', Hb-1''), 3.69-3.52 (m, 9H, H-6, H-7, H-8, H-9, H-2', H-5', Hb-6', H-2''), 2.70 (dd, J = 12.0, 5.0 Hz, 1H, H-3eq), 2.19 (t, J = 6.8 Hz, 2H, CH₂C(O)), 2.05-2.00 (m, 2H, H₂-6''), 1.99 (s, 3H, NAc), 1.80 (t, J = 12.1 Hz, 1H, H-3ax), 1.59 (m, 2H, CH₂CH₂C(O)), 1.30 (s, 50H, 25 × CH₂), 0.90 (t, J = 7.5 Hz, 6H, 2 × CH₃).

¹³C NMR (100 MHz, CD₃OD): δ 174.15 (C=O), 169.20 (C=O), 168.66 (C=O), 134.20 (C-5''), 128.44 (C-4''), 100.10 (C-1'), 97.52 (C-2), 75.05 (C-3''), 73.46 (C-3'), 71.06 (C-4'), 70.56 (C-6), 69.81 (C-2'), 69.32 (C-4), 69.18 (C-5'), 68.95 (C-1''), 68.54 (C-8), 68.15 (C-7), 63.93 (C-6'), 63.53 (C-9), 55.01 (C-2''), 50.95 (C-5), 40.11 (C-3), 30.18, 30.16, 30.12, 29.82 (CH₂), 22.70 (CH₃, NAc), 15.47 (2 × CH₃).

ESI-HRMS (*m*/*z*) calcd for C₅₃H₉₇N₂O₁₆ [M-H]⁻: 1017.6844, found: 1017.6822.

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2 →6)-O-(α-D-mannosyl)-(1→1)-(2S,3R,4E)-2-azido-4-octadecene-l,3-diol (34)



The compound **30** (40.0 mg, 0.034 mmol) in 6.7 mL of NaOMe/MeOH (0.04 M) was stirred at room temperature for 14 h. Then a few drops of water were added at 0 °C. After stirring at room temperature for additional 2 h, the mixture was neutralized by Amberlite IR 120/H⁺ ion exchange resin. After filtration and concentration, the residue was dried in *vacuo* to afford crude intermediate **34**.

 $R_{f} = 0.43$ (EtOAc-*i*PrOH-H₂O 3:2:1).

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2 →6)-O-(α-D-mannosyl)-(1→1)-(2S,3R,4E)-2-amino-4-octadecene-l,3-diol (35)



The crude intermediate **34** in the anhydrous MeOH (1.8 mL) were added propane-1,3-dithiol (0.18 mL) and triethylamine (0.18 mL), and the mixture was stirred at room temperature for 4 days. A white precipitate was formed. After filtration and washing with MeOH, the filtrate was concentrated under reduced pressure. The residue obtained was chromatographed, eluting with CHCl₃-MeOH 3:1 to yield the analogue **35** (20.2 mg, 80% for two steps from **30**) as a white amorphous solid.

 $R_{f} = 0.28$ (EtOAc-*i*PrOH-H₂O 3:2:1).

 $[\alpha]_D^{20} = -3.1$ (*c* 1.0 in CHCl₃-MeOH 1:1).

¹**H NMR (400 MHz, CD₃OD)**: δ 5.90-5.85 (m, 1H, H-5''), 5.53-5.48 (m, 1H, H-4''), 4.76 (d, J = 1.8 Hz, 1H, H-1'), 4.01-3.78 (m, 8H, H-4, H-5, H-3', H-4', Ha-6', H-1'', H-3''), 3.72-3.6 (m, 7H, H-6, H-8, H₂-9, H-2', Hb-6', H-2''), 3.52 (dd, J = 9.1, 1.5 Hz, 1H, H-7), 3.44-3.40 (m, 1H, H-5'), 2.83 (dd, J = 12.1, 4.9 Hz, 1H, H-3eq), 2.15-2.08 (m, 2H, H₂-6''), 2.00 (s, 3H, NAc), 1.79 (t, J = 12.1 Hz, 1H, H-3ax), 1.43-1.41 (m, 2H, H₂-7''), 1.30 (s, 20H, 10 × CH₂), 0.90 (t, J = 7.4 Hz, 3H, CH₃).

¹³C NMR (100 MHz, CD₃OD): δ 175.56 (C=O), 175.53 (C=O), 136.32 (C-5''), 128.71 (C-4''), 102.59 (C-1'), 101.46 (C-2), 74.42, 73.48, 72.96, 72.42, 71.66, 71.35, 71.20, 70.30, 69.45, 68.33 (C-4, C-6, C-7, C-8, C-2', C-3', C-4', C-5', C-1'', C-3''), 64.74 (C-6'), 64.51 (C-9), 58.46 (C-2''), 54.20 (C-5), 42.45 (C-3), 33.06, 30.81, 30.78, 30.73, 30.45 (CH₂), 23.72 (CH₃, NAc), 14.42 (CH₃).

ESI-HRMS (*m*/*z*) calcd for C₃₅H₆₃N₂O₁₅ [M-H]⁻: 751.4234, found: 751.4209.

6.7 Synthesis of lactose-containing analogues

6.7.1 Preparation of lactal block with a free 6-OH at galactose

2,3,4-tri-O-acetyl-β-D-galactopyranoside-(1→4)-3,6-di-O-acetyl-D-glucal (36)²¹⁶



Per-acetylated lactal (1.84 mmol, 1.03 g) was added to 100 mL solution of 50 mM phosphate buffer with 20% acetonitrile kept pH 5 at room temperature, and the reaction was initialized by adding 3 g of biocatalyst AXE. The hydrolytic reaction was also carried out under mechanical stirring and the pH value was controlled by automatic titration. After 4 h, the reaction was stopped by filtering off the enzyme. The mixture was extracted by EtOAc, washed with H₂O, dried over MgSO₄, and concentrated under reduced pressure. The residue was separated by column, eluted with CH₂Cl₂-EtOAc 7:3, to obtain the compound **36** (657 mg, 69%) and **37** (245 mg, 28%) as a white foam, respectively.

 $R_{\rm f} = 0.30 \ (CH_2Cl_2-EtOAc \ 7:3).$

¹**H NMR (400 Hz, CDCl₃)**: δ 6.47 (dd, J = 6.2, 0.9 Hz, 1H, H-1), 5.46 (t, J = 4.2 Hz, 1H, H-3), 5.37 (d, J = 3.2 Hz, 1H, H-4'), 5.23 (dd, J = 10.5, 7.9 Hz, 1H, H-2'), 5.05 (dd, J = 10.5, 3.5 Hz, 1H, H-3'), 4.83 (dd, J = 6.2, 4.2 Hz, 1 H, H-2), 4.68 (d, J = 7.9 Hz, 1H, H-1'), 4.40 (dd, J = 11.2, 2.8 Hz, 1H, Ha-6), 4.28-4.19 (m, 2 H, H-5, Hb-6), 3.98 (t, J = 5.2 Hz, 1H, H-4), 3.79-3.71 (m, 2H, H-5', Ha-6'), 3.53 (dd, J = 10.4, 3.8 Hz, 1H, Hb-6'), 2.18, 2.14, 2.10, 2.09, 2.01 (5s, 15H, 5 × OAc).

6.7.2 Sialylation of lactal with sialic acid donor

O-(methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-

nonulopyranosylonate)-(2→6)-2,3,4-tri-*O*-acetyl-β-D-galactopyranoside-(1→4)-3, 6-di-*O*-acetyl-D-glucal (38)



A mixture of sialyl xanthate **3** (483 mg, 0.81 mmol) and acceptor **36** (230 mg, 0.44 mmol) with 4Å powdered molecular sieves (1.5 g) in the dry CH₃CN (7 mL) and CH₂Cl₂ (3.5 mL) were stirred at room temperature for 1 h. Then AgOTf (208 mg, 0.81 mmol) and DTBP (184 μ L, 0.82 mmol) were added, and the mixture was cooled to -68 °C and kept protected from light. PhSCl (97 μ L, 0.84 mmol) in dry CH₂Cl₂ (0.51 mL) was added by running the solution down the cold wall of the reaction flask, and the stirring was continued for 3 h at -68 °C. The mixture was diluted with a suspension of silica gel (3.5 g) in EtOAc (21 mL), filtered through celite, washed with saturated aqueous NaHCO₃ and water, dried over MgSO₄ and concentrated under reduced pressure. The residue was chromatographed (Cy-EtOAc 1:3) to give **38** as a white foam (286 mg, 65%).

 $R_f = 0.35$ (Cy-EtOAc 1:3, twice).

 $[\alpha]^{20}_{D} = -19.7 \ (c \ 1.0 \ in \ CHCl_3).$

¹**H** NMR (400 MHz, CDCl₃): δ 6.38 (d, J = 6.2 Hz, 1H, H-1"), 5.43 (d, J = 3.4 Hz, 1H), 5.37-5.25 (m, 5H), 5.18-5.13 (m, 3H), 5.03 (dd, J = 9.7, 4.0 Hz, 1H), 4.85-4.83 (m, 2H), 4.70 (d, J = 5.9 Hz, 1H, H-1"), 4.33-4.21 (2m, 2H), 4.10-4.04 (m, 4H), 3.78 (s, 3H, CH₃), 3.38 (dd, J = 7.3, 4.0 Hz, 1H), 2.51 (dd, J = 7.4, 3.2 Hz, 1H, H-3eq), 2.17 (s, 3H, OAc), 2.12 (s, 3H, OAc), 2.10 (2s, 6H, 2 × OAc), 2.06 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.96 (s, 3H, OAc), 1.89 (m, 1H, H-3ax), 1.87 (s, 3H, NAc).

¹³C NMR (100 MHz, CDCl₃): δ 170.91 (C=O), 170.65 (C=O), 170.47 (C=O), 170.34

(C=O), 170.15 (C=O), 169.94 (C=O), 169.89 (C=O), 169.68 (C=O), 169.39 (C=O), 167.87 (C=O), 145.21 (C-1"), 100.46 (C-1"), 99.15 (C-2"), 99.09 (C-2), 74.11, 72.56, 71.75, 71.05, 69.09, 68.64, 68.42, 68.04, 67.10, 67.07, 62.94, 62.51, 61.97, 52.92 (COOCH₃), 49.48 (C-5), 37.87 (C-3), 23.19 (CH₃, NAc), 21.08 (CH₃, OAc), 20.99 (CH₃, OAc), 20.82 (CH₃, OAc), 20.76 (CH₃, OAc), 20.72 (CH₃, OAc), 20.70 (CH₃, OAc), 20.66 (CH₃, OAc), 20.56 (CH₃, OAc).

ESI-HRMS (m/z) calcd for C₄₂H₅₇NNaO₂₆ [M+Na]⁺: 1014.3067, found: 1014.3052.

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2nonulopyranosylonate)-(2 \rightarrow 6)-2,3,4-tri-*O*-acetyl-β-D-galactopyranoside-(1 \rightarrow 4)-3, 6-di-*O*-acetyl-α-D-glucopyranoside/β-D-mannopyranoside (39)



Method (A): The **38** (30 mg, 0.03 mmol) was dissolved in dry CH_2Cl_2 (1 mL), then cooled to 0 °C. *m*-CPBA (17 mg, 0.075 mmol) was added, and gradually warmed to room temperature. Finally, the mixture was stirred at room temperature for 3 h. After that, the reaction was quenched by adding Na₂S₂O₃ solution, further washed by saturated aqueous NaHCO₃ and water, dried over MgSO₄, and concentrated under reduced pressure, to obtain the **39** mixture (15 mg, 50%) as a white foam.

Method (B): A solution of oxone (246 mg, 0.40 mmol) in water (1 mL) was added dropwise at 0 °C for 15 min to a vigorously stirred biphasic mixture of **38** (200 mg, 0.20 mmol), tetrabutylammonium hydrogen sulfate (23 mg, 0.07 mmol), acetone (83 μ L), CH₂Cl₂ (1.7 mL) and saturated NaHCO₃ solution (2.8 mL). Stirring was maintained for 30 min at 0 °C, and 2.5 h at room temperature. The aqueous phase was extracted with CH₂Cl₂ and the combined organic phases were washed with water and

dried over MgSO₄, and concentrated under reduced pressure, to obtain the **39** mixture (152 mg, 75%) as a white foam. Due to 1,2-anhydro sugar was unstable, and the mixture was directly used for the next step without further purification.

 $R_f = 0.30$ (CH₂Cl₂-MeOH 10:1, twice).

6.7.3 Conjugation of lipid part with the sialo-oligosaccharide

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 →4)-*O*-(3,6-di-*O*-acetyl-β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*,4*E*)-2-azido-3-*O*-benzoyl-4-octadecene-l,3-diol (40)



The crude mixture **39** (90 mg, 0.09 mmol) and 3-*O*-benzoyl azidosphingosine (116 mg, 0.27 mmol) were dissolved in 0.45 mL of dry THF. The solution was cooled to -45 °C and ZnCl₂ (45 μ L of a 1 M solution in diethyl ether, 0.045 mmol) was added dropwise. The reaction was allowed to warm to room temperature and was stirred for another 3 h. The reaction was quenched by filtration through celite, concentrated, and subjected to the silica gel column using Cy-acetone 1:1 as eluent, to obtain the crude intermediate **40**.

 $R_{\rm f} = 0.40$ (EtOAc).

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)- $(2\rightarrow 6)$ -*O*-(2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 \rightarrow 4)-*O*-(2,3,6-tri-*O*-acetyl- β -D-glucopyranosyl)-(1 \rightarrow 1)-(2*S*,3*R*,4*E*)-2-azido-3-*O*-benzoyl-4-octadecene-l,3-diol (41) ¹⁷⁴



The crude **40** (60 mg) was dissolved in the pyridine (5 mL), adding 0.5 mL Ac₂O, and then stirred at room temperature for 24 h. After concentration, the mixture was subjected to the silica gel column using CH_2Cl_2 -MeOH 20:1 as eluent to afford pure **41** (57 mg, 53% for two steps) as a white solid.

 $R_{\rm f} = 0.45$ (EtOAc).

¹**H NMR (400 MHz, CDCl₃)**: δ 8.20-7.45 (m, 5H, Ph), 5.95 (dt, J = 15.1, 6.8 Hz, 1H, H-5cer), 5.59-5.55 (m, 1H, H-4cer), 5.42 (dd, J = 9.3, 2.7 Hz, 1H, H-7), 5.0 l (d, J = 10.2 Hz, 1H, H-4'), 4.71 (d, J = 7.8 Hz, 1H, H-1'), 4.53 (d, J = 7.8 Hz, 1H, H-1''), 4.07-3.87 (m, 2H, H-5, Ha-1cer), 3.87 (s, 3H, COOCH₃), 3.66 (dd, J = 10.7, 2.7 Hz, H-2cer), 3.62-3.57 (m, 1H, Hb-1cer), 2.62 (dd, J = 12.7, 4.6 Hz, 1H, H-3eq), 2.27, 2.19, 2.12, 2.11, 2.10, 2.08, 2.06 (7s, 33H, 11 × OAc), 1.71 (t, J = 12.4 Hz, 1H, H-3ax), 1.42-1.40 (m, 2H, H₂-6cer), 1.27 (s, 22H, 11 × CH₂), 0.91 (t, J = 6.8 Hz, 3H, CH₃).

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2→6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 →4)-*O*-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*,4*E*)-2-amino-3-*O*benzoyl-4-octadecene-l,3-diol (42)



To a solution of compound **41** (30 mg, 0.02 mmol) in 2 mL of toluene and 0.08 mL of water was added 13 mg of triphenylphosphine. The mixture was stirred at 45 °C for 12 h. After concentration, the crude product **42** was obtained. The crude product could be directly used for the next step without further purification.

 $R_{\rm f} = 0.35$ (CH₂Cl₂-MeOH 20:1).

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)-(2 \rightarrow 6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl)-(1 \rightarrow 4)-*O*-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1 \rightarrow 1)-(2*S*,3*R*,4*E*)-2-octadecanamido-3-*O*-benzoyl-4-octadecene-l,3-diol (43) ¹⁷⁴



The mixture of **42**, stearic acid (25 mg, 0.088 mmol), EDC (32 mg, 0.166 mmol) in 2.1 mL of CH_2Cl_2 was stirred at room temperature for 20 h. Then the mixture was washed with water, dried over MgSO₄ and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography (Cy-EtOAc 1:3) to afford **43** (22 mg, 62% for two steps from **41**) as an amorphous solid.

 $R_f = 0.39$ (CH₂Cl₂-MeOH 20:1).

¹H NMR (400 MHz, CDCl₃): δ 8.07-7.26 (m, 5H, Ar-H), 5.85 (dd, J = 14.1, 7.1 Hz,

1H, H-5cer), 5.78 (s, 1H, NH), 5.21 (dd, J = 9.5, 2.9 Hz, 1H, H-7), 5.06 (m, 1H, H-2'), 4.97 (dd, J = 10.5, 3.4 Hz, 1H, H-3'), 4.53 (d, J = 7.3 Hz, 1H, H-1'), 4.45 (d, J = 7.7Hz, 1H, H-1''), 3.75 (s, 3H, COOCH₃), 2.55 (dd, J = 12.8, 4.4 Hz, 1H, H-3eq), 1.89 (s, 3H, NAc), 1.25 (s, 50H, 25 × CH₂), 0.88 (t, J = 6.2 Hz, 6H, 2 × CH₃).

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2 →6)-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*,4*E*)-2 -octadecanamido-4-octadecene-l,3-diol (44) ¹⁷⁴



A solution of product **43** (20 mg, 0.011 mmol) in 2 mL of NaOMe/MeOH (0.04 M) was stirred at room temperature for 14 h. A few drops of water were added at 0 °C. After stirring at room temperature for another 1 h, the mixture was neutralized by Amberlite IR 120/H⁺ ion exchange resin. After filtration and concentration, the residue obtained was flash-chromatographed, eluting with CHCl₃-MeOH 3:1 to yield **44** (12 mg, 90%) as a white amorphous solid.

 $R_{f} = 0.29$ (EtOAc-*i*PrOH-H₂O 3:2:1).

¹**H NMR (400 MHz, CDOD₃):** δ 5.61 (d, J = 9.1 Hz, 1H, NH), 5.54 (dt, J = 15.0, 7.5 Hz, 1H, H-5cer), 5.44 (dd, J = 15.4, 6.7 Hz, 1H, H-4cer), 4.19 (d, J = 7.6 Hz, 1H, H-1'), 4.17 (d, J = 7.7 Hz, 1H, H-1''), 4.00 (dd, J = 10.2, 4.1 Hz, 1H, Ha-1cer), 3.41 (dd, J = 9.7, 4.0 Hz, 1H, Hb-1cer), 3.04 (dd, J = 10.5, 8.0 Hz, 1H, H-2''), 2.73 (dd, J = 12.0, 4.4 Hz, 1H, H-3eq), 1.89 (s, 3H, NAc), 1.39-1.37 (m, 1H, H-3ax), 1.28 (s, 54H, 27 × CH₂), 0.87 (t, J = 6.9 Hz, 6H, 2 × CH₃).

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2 →6)-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2S,3R,4E)-2 -azido-4-octadecene-l,3-diol (45)



The compound **41** (25 mg, 0.017 mmol) in 3.3 mL of NaOMe/MeOH (0.04 M) was stirred at room temperature for 14 h. Then a few drops of water were added at 0 °C. After stirring at room temperature for another 2 h, the mixture was neutralized by Amberlite IR $120/H^+$ ion exchange resin. After filtration and concentration, the residue was dried to afford crude intermediate **45**.

 $R_{f} = 0.25$ (EtOAc-*i*PrOH-H₂O 3:2:1).

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2 →6)-O-(β-D-galactopyranosyl)-(1→4)-O-(β-D-glucopyranosyl)-(1→1)-(2S,3R,4E)-2 - amino-4-octadecene-1,3-diol (46)



The crude intermediate **45** in the anhydrous MeOH (2.1 mL) were added propane-1,3-dithiol (0.16 mL) and triethylamine (0.16 mL), and the mixture was stirred at ambient temperature for 4 days. A white precipitate was formed. After filtration and washing with MeOH, the filtrate was concentrated under reduced

pressure. The residue was flash-chromatographed, eluted with CHCl₃-MeOH 3:1 to yield the analogue **46** (12 mg, 80% for two steps from **41**) as a white amorphous solid.

 $R_f = 0.20$ (EtOAc-*i*PrOH-H₂O 3:2:1).

 $[\alpha]^{20}_{D} = +12.1$ (*c* 1.0 in CHCl₃-MeOH 1:1).

¹H NMR (400 MHz, CD₃OD): δ 5.92-5.86 (m, 1H, H-5cer), 5.50-5.34 (m, 1H, H-4cer), 4.35-4.30 (m, 2H), 3.91-3.67 (m, 21H), 3.35-3.23 (m, 2H), 2.80 (dd, J = 7.4, 3.2 Hz, 1H, H-3eq), 2.03 (s, 3H, NAc), 1.71-1.65 (m, 2H, H₂-6cer), 1.47-1.42 (m, 3H, H-3ax, H₂-7cer), 1.31 (s, 20H, 10 × CH₂), 0.92 (t, J = 6.8 Hz, 3H, CH₃).

¹³C NMR (100 MHz, CD₃OD): δ 167.86 (C=O), 167.00 (C=O), 136.82 (5cer), 128.73 (4cer), 100.35 (C-1'), 99.14 (C-1''), 99.10 (C-2), 73.99, 72.65, 71.84, 71.05, 70.76, 69.35, 69.10, 68.65, 68.42, 68.02, 67.78, 66.59, 62.95, 62.65, 62.50, 61.90, 57.58 (2cer), 51.69 (C-5), 37.89 (C-3), 31.27 (CH₂), 30.13 (CH₂), 29.63 (CH₂), 23.19 (CH₃, NAc), 14.92 (CH₃).

ESI-HRMS (m/z) calcd for C₄₁H₇₃N₂O₂₀ [M-H]⁻: 913.4762, found: 913.4750.

6.8 Chemoenzymatic synthesis of sialylated glucosamine or galactosamine derivatives

6.8.1 Synthesis of substrates for enzymatic hydrolysis

2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-α-D-glucopyranose (47)¹⁹⁷



To *N*-acetyl-D-glucosamine (6 g, 27.3 mmol) in dry CH_2Cl_2 (25 mL), was added pyridine (11 mL) followed by DMAP (cat.) and Ac₂O (13 mL, 136 mmol) at 0 °C. The reaction was stirred at 0 °C for 30 min, and then at room temperature for 24 h. The reaction was cooled to 0 °C, water was added. The aqueous layer was then extracted with EtOAc, dried over MgSO₄, filtered and concentrated under reduced pressure. The crude product was purified by crystallization from EtOAc/hexane to give the per-acetylated compound **47** (9.66 g, 90%) as a white solid.

 $R_f = 0.39$ (MeOH-CH₂Cl₂ 1:15).

¹**H NMR (400 MHz, CDCl3):** δ 6.14 (d, J = 3.6 Hz, 1H, H-1), 5.56 (d, J = 9.0 Hz, 1H, NH), 5.24-5.14 (m, 2H, H-3, H-4), 4.49-4.41 (m, 1H, H-2), 4.22 (dd, J = 12.6, 4.2 Hz, 1H, Ha-6), 4.03 (dd, J = 12.3, 2.4 Hz, 1H, Hb-6), 3.98-3.93 (m, 1H, H-5), 2.16 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.90 (s, 3H, NAc).

Cyanomethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-β-glucopyranoside (48) ²⁴⁷

²⁴⁷ C. Temporini, T. Bavaroa, S. Tengattini, I. Serraa, G. Marrubini, E. Calleri, F. Fasanella, L. Piubelli, F. Marinelli, L. Pollegioni, G. Speranza, G. Massolini, M. Terreni, *J. Chromatogr. A*, **2014**, *1367*, 57-67.



N-acetyl-D-glucosamine (2 g, 9.041 mmol) was suspended in acetyl chloride (20 mL) in the presence of molecular sieves 4 Å, and was stirred for 48 h at room temperature. The solution was diluted with CH₂Cl₂ and poured into ice cold water. The aqueous phase was extracted with CH₂Cl₂ and the organic phases were combined and washed with saturated NaHCO₃ solution, dried over MgSO₄, filtered and concentrated in vacuo. The crude solid was purified by flash chromatography (Cy-EtOAc, 3:7) to give a white solid with a yield of 68%. $R_f = 0.71$ (CH₂Cl₂-MeOH, 95:5). Next, this white solid 1-chloro-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-glucopyranoside (1 g, 2.73 mmol) and thiourea (0.38 g, 5 mmol) were dissolved in anhydrous acetone (10 ml) with molecular sieves 4 Å. The reaction mixture was stirred at room temperature for 3 h. A white precipitate was formed, further filtered, washed with ethanol and concentrated in vacuo to give pure product with a yield of 90%. Finally, the product 1-thiourea-2-acetamido-3,4,6,-tri-O-acetyl-2-deoxy-α-D-glucopyranoside (245 mg, 0.554 mmol) was dissolved in 1:1 H₂O/acetone mixture (2.6 mL) and sodium metabisulphite (212 mg, 1.115 mmol), potassium carbonate (93 mg, 0.672 mmol) and chloroacetonitrile (712 µL) were added, stirring at room temperature. Upon completion, 8 mL of ice water was added to the solution that was stirred for another 45 min. The reaction mixture was extracted with CH₂Cl₂ and the combined organic extracts were washed with brine and dried over MgSO4 and concentrated in vacuo, to give 48 (230 mg, 98%).

 $R_f = 0.71$ (CH₂Cl₂-MeOH 95:5).

¹**H NMR (400 MHz, CDCl₃)**: δ 5.80 (d, J = 9.5 Hz, 1H, NH), 5.20 (t, J = 9.5 Hz, 1H, H-3), 5.13 (t, J = 9.5 Hz, 1H, H-4), 4.74 (d, J = 10.3 Hz, 1H, H-1), 4.20 (td, J = 10.3, 9.5 Hz, 1H, H-2), 3.74 (d, J = 17.3 Hz, 1H, CH₂CN), 3.80-3.71 (m, 1H, Ha-6), 3.66-3.61 (m, 1H, H-5), 3.60-3.58 (m, 1H, Hb-6), 3.32 (d, J = 17.3 Hz, 1H, CH₂CN), 2.07 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.97 (s, 3H, NAc).

2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-α-D-galactopyranose (49) ²⁴⁸



To a stirred solution of *N*-acetyl D-galactosamine (2.0 g, 9.04 mmol) in pyridine (40 mL) was added Ac₂O (40 mL) followed by addition of DMAP (19 mg, 0.16 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and then at room temperature for 24 h. After that, the reaction was cooled to 0 °C, water was added. The aqueous layer was then extracted with EtOAc, dried over MgSO₄, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using Cy-EtOAc, 2:3 to give **49** (3.32 g, 95 %) as a crystalline solid.

 $R_{\rm f} = 0.40$ (Cy-EtOAc 2:3).

¹**H NMR (400 MHz, CDCl₃)**: δ 6.28 (d, J = 3.6 Hz, 1H, H-1), 5.45-5.40 (m, 2H, H-4, NH), 5.26 (dd, J = 3.4, 11.8 Hz, 1H, H-3), 4.80-4.71 (m, 1H, H-2), 4.26-4.10 (m, 3H, H-5, H₂-6), 2.20 (s, 6H, 2 × OAc), 2.08 (s, 6H, 2 × OAc), 1.98 (s, 3H, NAc).

2-phthalimido-1,3,4,6-tetra-O-acetyl-2-deoxy-α-D-glucopyranose (50) ²⁴⁹



The **glucosamine hydrochloride** (5.0 g, 23.20 mmol) in methanol (10 mL) and water (20 mL) was added NaOH (1.2 g) at 0 °C. Then, phthalic anhydride (5.0 g, 33.76 mmol) dissolved in acetone (40 mL) was added while maintaining the temperature

 ²⁴⁸ K. Agarwal, R. Kaul, M. Garg, A. Shajahan, S. K. Jha, S. G. Sampathkumar J. Am. Chem. Soc., 2013, 135, 14189-14197.

²⁴⁹ R. P. McGeary, K. Wright, I. Toth, J. Org. Chem., 2001, 66, 5102-5105.

below 15 °C. More phthalic anhydride (2.1 g, 14.18 mmol) and NaHCO₃ (5.0 g) were added, and the solution was subsequently stirred at room temperature overnight. The mixture was then brought to pH 1 with concentrated HCl, concentrated *in vacuo* and left at 2 °C in the refrigerator overnight. The resulting precipitate was filtered off, washed with cold water and dried under vacuum. The crude product was dissolved in pyridine (70 mL) and treated with acetic anhydride (52 mL), and the mixture was stirred at room temperature overnight. The solvents were evaporated, and the residue was separated by column (Toluene-EtOAc, 3:2) yielding substrate **50** as a colourless solid (7.7 g, 70%) with α isomer and 2-phthalimido-2-deoxy- β -D-glucopyranoside tetraacetate (1.9 g, 17%) was also obtained.

 $R_f = 0.44$ (Toluene-EtOAc 3:2).

¹**H NMR (400 Hz, CDCl₃)**: δ 7.87-7.72 (m, 4H, Ar-H), 6.55 (dd, J = 11.5, 9.1 Hz, 1H, H-3), 6.27 (d, J = 3.3 Hz, 1H, H-1), 5.15 (t, J = 9.6 Hz, 1H, H-4), 4.71 (dd, J = 11.6, 3.3 Hz, 1H, H-2), 4.38-4.28 (m, 2H, H₂-6), 4.14-4.09 (m, 1H, H-5), 2.11 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.05 (s, 3H, OAc), 1.86 (s, 3H, OAc).

1-bromide-2-phthalimido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranoside (51)



To a stirred ice-cold solution of substrate **50** (4 g, 8.36 mmol) in CH₂Cl₂ (35.6 mL) was added dropwise HBr in acetic acid (33 wt%, 14.4 mL, 83.4 mmol). The mixture was allowed to gradually warm to room temperature and stirred for 3.5 h. The reaction mixture was neutralized by careful addition of NaHCO₃ and iced-water, and the aqueous solution was extracted with CH₂Cl₂. The combined organic extracts were dried over MgSO₄ and concentrated to give crude intermediate **51** (3.7g, 89%) as a

yellow amorphous solid. This crude intermediate, which was not separated and further characterized at this stage, was directly engaged in the following step.

 $R_{\rm f} = 0.40$ (Cy-EtOAc 1:1).

Methyl 3,4,6-tri-O-acetyl-2-phthalimido-2-deoxy-β-D-glucopyranoside (52)²⁵⁰



The crude intermediate **51** (0.9 g, 1.96 mmol) was dissolved in dry CH₂Cl₂ (9.2 mL), and methanol (0.66 mL, 16.23 mmol), mercury (II) oxide (426.0 mg, 2.16 mmol) and mercury (II) bromide (114.0 mg, 0.34 mmol) were added. The resulting suspension was stirred for 18 h at room temperature. The reaction mixture was then diluted with CH₂Cl₂, stirred with KI and NaHCO₃ solution for another 2 h at room temperature, and filtered through a pad of celite. The aqueous eluate was extracted with CH₂Cl₂, and the combined organic extracts were washed with brine, dried over MgSO₄ and concentrated under reduced pressure. The resulting residue was purified by column chromatography (Cy-EtOAc, 1:1). Substrate **52** was obtained (567.0 mg, 70%) as a white amorphous solid with β isomer.

 $R_{f} = 0.61$ (Cy-EtOAc 1:1).

¹**H NMR (400 Hz, CDCl₃)**: δ 7.87-7.84 (m, 2H, Ar-H), 7.75-7.72 (m, 2H, Ar-H), 5.78 (dd, J = 9.8, 1.5 Hz, 1H, H-3), 5.31 (d, J = 9.4 Hz, 1H, H-1), 5.18 (t, J = 9.0 Hz, 1H, H-4), 4.37-4.28 (m, 2H, H-2, Ha-6), 4.21-4.16 (m, 1H, Hb-6), 3.90-3.85 (m, 1H, H-5), 3.44 (s, 3H, OCH₃), 2.12, 2.04, 1.86 (3s, 9H, 3 × OAc).

Ethyl 3,4,6-tri-O-acetyl-2-phthalimido-2-deoxy-1-thio-β-D-glucopyranoside (53)

²⁵⁰ D. A. Schwartz, H. H. Lee, J. P. Carver, J. J. Prekinsky, Can. J. Chem., **1985**, 63, 1073-1079.



The 2-phthalimido-2-deoxy- β -D-glucopyranoside tetraacetate (0.8 g, 1.68 mmol) and CH₃CH₂SH (0.33 mL) were in the dry CH₂Cl₂ (3.3 mL), and cooled to 0°C, and BF₃·Et₂O (0.16 mL) was added. The mixture was stirred at 0°C for 1 h, then at room temperature for 48 h. The reaction was quenched by saturated aqueous NaHCO₃ solution, stirred at room temperature for another 1 h. The aqueous layer was extracted with CH₂Cl₂. The combined organic layers were dried over MgSO₄, filtered, and evaporated. The mixture was separated by column (Cy-EtOAc, 3:2) to obtain substrate **53** as a pale yellow foam (600 mg, 75%) with β isomer.

 $R_{f} = 0.54$ (Cy-EtOAc 1:1)

¹**H NMR (400 Hz, CDCl₃)**: δ 7.86, 7.75 (2m, 4H, phthalimido protons), 5.82 (t, J = 9.9 Hz, 1H, H-3), 5.48 (d, J = 10.5 Hz, 1H, H-1), 5.17 (t, J = 9.6 Hz, 1H, H-4), 4.39 (t, J = 10.5 Hz, 1H, H-2), 4.30, 4.18 (2m, 2H, H₂-6), 3.90 (m, 1H, H-5), 2.67 (m, 2H, SCH₂CH₃), 2.09, 2.03, 1.85 (3s, 9H, 3 × OAc), 1.21 (t, J = 7.2 Hz, 3H, SCH₂CH₃).

Phenyl 3,4,6-tri-*O*-acetyl-2-phthalimido-2-deoxy-1-thio-β-D-glucopyranoside (54)



The 2-phthalimido-2-deoxy-\beta-D-glucopyranoside tetraacetate (0.8 g, 2.05 mmol) and

²⁵¹ J. Landström, M. Bergström, C. Hamark, S. Ohlson, G. Widmalm, *Org. Biomol. Chem.*, **2012**, *10*, 3019-3032.

²⁵² U. S. Chowdhury, *Tetrahedron*, **1996**, *52*, 12775-12782.

PhSH (0.32 mL) were in the dry CH₂Cl₂ (4.8 mL), and cooled to 0°C, and BF₃·Et₂O (1.31 mL) was added. The mixture was stirred at 0°C for 1 h, then at room temperature overnight. The reaction was quenched by saturated aqueous NaHCO₃ solution, and the aqueous layer was extracted with CH₂Cl₂. The combined organic layers were dried over MgSO₄, filtered, and evaporated. The mixture was separated by column (Cy-EtOAc, 3:2) to obtain substrate **54** as a pale yellow foam (722 mg, 80%) with β isomer.

 $R_{\rm f} = 0.50$ (Cy-EtOAc 1:1).

¹**H NMR (400 Hz, CDCl₃)**: δ 7.96-7.72 (m, 4H, phthalimido protons), 7.62-7.20 (m, 5H, SPh), 5.82 (t, *J* = 9.9 Hz, 1H, H-3), 5.73 (d, *J* = 10.5 Hz, 1H, H-1), 5.17 (t, *J* = 9.6 Hz, 1H, H-4), 4.39 (t, *J* = 10.5 Hz, 1H, H-2), 4.30, 4.18 (2m, 2H, H₂-6), 3.85-3.95 (m, 1H, H-5), 2.09, 2.03, 1.85 (3s, 9H, 3 × OAc).

O-(2-azido ethyl) 3,4,6-tri-*O*-acetyl-2-phthalimido-2-deoxy-1-thio-β-D-glucopyranoside (55) ²⁵³



The **51** (1.0 g, 2.00 mmol) was dissolved in dry CH_2Cl_2 (10.0 mL), and 2-azidoethanol (522.0 mg, 6.00 mmol), mercury (II) oxide (473.0 mg, 2.20 mmol) and mercury (II) bromide (125.0 mg, 0.34 mmol) were added. The resulting suspension was stirred for 18 h at room temperature. The reaction mixture was then diluted with CH_2Cl_2 , stirred with KI and NaHCO₃ solution for another 2 h at room temperature, and filtered through a pad of celite. The aqueous eluate was extracted with CH_2Cl_2 , and the combined organic extracts were washed with brine, dried over MgSO₄ and concentrated under reduced pressure. The resulting residue was purified by flash

²⁵³ Y. X. Chen, W. Zhao, L. Zhao, Y. F. Zhao, Y. M. Li, *Carbohydr. Res.*, **2008**, *343*, 607-614.

column chromatography (Cy-EtOAc 3:2). Substrate **55** was obtained (680 mg, 68%) as a white amorphous solid.

 $R_{f} = 0.28$ (Cy-EtOAc 1:1).

¹**H NMR (400 Hz, CDCl₃)**: δ 7.84, 7.72 (2m, 4H, Ar-H), 5.77 (dd, J = 10.8, 9.0 Hz, 1H, H-3), 5.46 (d, J = 8.4 Hz, 1H, H-1), 5.18 (t, J = 9.6 Hz, 1H, H-4), 4.34-4.31 (m, 2H, H-2, Ha-6), 4.19 (dd, J = 12.6, 2.4 Hz, 1H, Hb-6), 4.00 (ddd, J = 10.2, 4.8, 3.6 Hz, 1H), 3.88 (ddd, J = 10.2, 4.8, 2.4 Hz, 1H, H-5), 3.64 (ddd, J = 11.4, 8.4, 3.6 Hz, 1H), 3.38 (ddd, J = 13.2, 8.4, 3.6 Hz, 1H), 3.16 (ddd, J = 13.2, 4.8, 3.6 Hz, 1H), 2.11 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.85 (s, 3H, OAc).

O-(2-trimethylsilyl ethyl) 3,4,6-tri-*O*-acetyl-2-phthalimido-2-deoxy-1-thio-β-D-glucopyranoside (56) ²⁵⁴



The **51** (0.9 g, 1.81 mmol) was dissolved in dry CH_2Cl_2 (9.0 mL), and 2-(trimethylsilyl)ethanol (0.8 mL, 5.43 mmol), mercury (II) oxide (430.0 mg, 2.00 mmol) and mercury (II) bromide (112.0 mg, 0.31 mmol) were added. The resulting suspension was stirred at room temperature for 18 h. The reaction mixture was then diluted with CH_2Cl_2 , stirred with KI and NaHCO₃ solution at room temperature for another 2 h, and filtered through a pad of celite. The aqueous eluate was extracted with CH_2Cl_2 , and the combined organic extracts were washed with brine, dried over MgSO₄ and concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography (Cy-EtOAc 3:2). Substrate **56** was obtained (696 mg, 72%) as a white amorphous solid.

 $R_{\rm f} = 0.50$ (Cy-EtOAc 3:2).

²⁵⁴ K. Jansson, S. Ahlfors, T. Frejd, J. Kihlberg, G. Magnusson, J. Org. Chem., **1988**, 53, 5629-5647.
¹**H NMR (400 Hz, CDCl₃)**: δ 7.86, 7.75 (2m, 4H, phthalimido protons), 5.77 (dd, J = 10.7, 9.0 Hz, 1H, H-3), 5.38 (d, J = 8.6 Hz, 1H, H-1), 5.17 (t, J = 10.3 Hz, 1H, H-4), 4.39 (m, 2H, H-2, Ha-6), 4.18 (dd, J = 12.1, 2.4 Hz, 1H, Hb-6), 3.96-3.84 (m, 2H, H-5, OC*H*₂CH₂Si(CH₃)₃), 3.53 (m, 1H, OC*H*₂CH₂Si(CH₃)₃), 2.10, 2.02, 1.85 (3s, 9H, 3 × OAc), 0.84 (m, 2H, OCH₂CH₂Si(CH₃)₃), -0.13 (s, 9H, Si(CH₃)₃).

2-phthalimido-1,3,4,6-tetra-O-acetyl-2-deoxy-β-D-galactopyranose (57)²⁵⁵



To a stirred solution of **galactosamine hydrochloride** (500 mg, 2.32 mmol) in MeOH (5 mL) and water (10 mL) was added NaOH (650 mg) at 0 °C. Then, phthalic anhydride (2.5 g, 16.88 mmol) dissolved in acetone (20 mL) was added while maintaining the temperature below 15 °C. More phthalic anhydride (1.1 g, 7.43 mmol) and NaHCO₃ (2.6 g) were added, and the solution was subsequently stirred at room temperature for 4 h. The mixture was then evaporated and dried under vacuum. The crude product was dissolved in pyridine (7 mL) and treated with acetic anhydride (4 mL), and the mixture was stirred at room temperature overnight. The solvents were evaporated, and the residue was separated by column (Cy-EtOAc, 3:2) yielding **57** as a colourless solid (750 mg, 68%).

 $R_{f} = 0.48$ (Cy-EtOAc 1:1).

¹**H NMR (400 Hz, CDCl₃)**: δ 7.90-7.80 (m, 2H, Ar-H), 7.78-7.71 (m, 2H, Ar-H), 6.52 (dd, J = 11.3, 3.2 Hz, 1H, H-3), 6.34 (d, J = 6.8 Hz, 1H, H-1), 5.66 (d, J = 3.2 Hz, 1H, H-4), 4.90 (dd, J = 11.3, 3.2 Hz, 1H, H-2), 4.49 (t, J = 8.1 Hz, 1H, H-5), 4.17-4.11 (m, 2H, H₂-6), 2.18 (s, 3 H, OAc), 2.05 (s, 6 H, 2 × OAc), 1.88 (s, 3 H, OAc).

²⁵⁵ S. S. Mandal, G. Liaoa, Z. Guo, *RSC Adv.*, **2015**, *5*, 23311-23319.

Ethyl 3,4,6-tri-*O*-acetyl-2-phthalimido-2-deoxy-1-thio-β-D-galactopyranoside (58)



The compound **57** (1.0 g, 2.10 mmol) and CH₃CH₂SH (0.41 mL) were in the dry CH₂Cl₂ (4.1 mL), and cooled to 0 °C, then BF₃·Et₂O (0.21 mL) was added. The mixture was stirred at 0 °C for 1 h, then at room temperature for 24 h. The reaction was quenched by saturated aqueous NaHCO₃ solution, stirred at room temperature for another 1 h. The aqueous layer was extracted with CH₂Cl₂. The combined organic layers were dried over MgSO₄, filtered, and evaporated. The mixture was separated by column (Cy-EtOAc, 3:2) to obtain substrate **58** as a pale yellow foam (660 mg, 66%) with β isomer.

 $R_{\rm f} = 0.53$ (Cy-EtOAc 1:1).

¹**H NMR (400 Hz, CDCl₃)**: *δ* 7.87-7.83 (m, 2H, phthalimido protons), 7.76-7.72 (m, 2H, phthalimido protons), 5.83 (dd, *J* = 10.6, 3.2 Hz, 1H, H-3), 5.51 (d, *J* = 3.3 Hz, 1H, H-4), 5.46 (d, *J* = 10.4 Hz, 1H, H-1), 4.60 (t, *J* = 10.5 Hz, 1H, H-2), 4.23-4.10 (m, 3H, H-5, H₂-6), 2.74-2.63 (m, 2H, SCH₂CH₃), 2.19, 2.05, 1.85 (3s, 9H, 3 × OAc), 1.22 (t, *J* = 7.2 Hz, 3H, SCH₂CH₃).

6.8.2 Enzymatic hydrolysis

6.8.2.1 Immobilization of Candida rugosa lipase (CRL)

Immobilization of CRL was performed slightly modifying the procedure previously reported ²⁵⁷. Briefly, the crude extract of CRL (2 g) was diluted in 25 mM phosphate

²⁵⁶ U. Ellervik, G. Magnusson, *Carbohydr. Res.*, **1996**, *280*, 251-260.

²⁵⁷ T. Bavaro, D. Ubiali, S. Brocca, S. Rocchietti, I. Nieto, M. Pregnolato, M. Lotti, M. Terreni, *Biocatal. Biotransfor.*, **2010**, *28*, 108-116.

buffer pH 7 (32 mL) and kept under stirring for 30 min. 5 g of octadecyl-SepabeadsTM, previously washed with the same buffer, was added and the suspension was stirred at room temperature for 3 h. The enzyme derivative (CRL-OD) was then filtered and washed with distilled water.

The activity of the enzyme was measured at pH 7.0 and room temperature in the hydrolysis of tripropioninn according to the literature ²⁵⁸.

6.8.2.2 Enzymatic hydrolysis

The enzymatic hydrolysis of substrates (10 mM) was carried out in 50 mM phosphate buffer (15 mL) containing 20% acetonitrile under mechanical stirring. The reaction started after the addition of 1 g of immobilized enzyme CRL-OD (1200 UI/g) or AXE (90 UI/g). During the reaction, the pH of the solution was maintained by automatic titration. The course of the hydrolysis reaction was monitored by HPLC (HPLC analysis: 30-50% acetonitrile in phosphate buffer (10 mM) at pH 4, flow rate 1.0 mL/min, $\lambda = 210$ nm) and TLC. After complete or almost-complete consumption of the substrate, the reaction was stopped by biocatalyst filtration. The obtained mixtures were extracted by EtOAc, dried over MgSO₄ and filtered, and concentrated under the reduced pressure. The crude product was purified by silica gel chromatography (product **59** and **60**: CH₂Cl₂-MeOH 95:5, product **61** and **63**: Hexane-EtOAc 1:1, product **62**: Toluene-EtOAc 3:2, product **64**, **65**, **67** and **68**: Hexane-EtOAc 3:2, product **66**: Hexane-EtOAc 1:2) and further characterized by NMR spectra and HRMS spectrometry.

6.8.2.3 Data analysis of enzymatic hydrolysis products

1,3,4-tri-O-acetyl-2-acetamido-2-deoxy-α-D-glucopyranose (59)¹⁹⁸

²⁵⁸ P. Torres-Salas, A. Pedrali, T. Bavaro, S. Ambrosini, G. Marrubini, V. M. Pappalardo, G. Massolini, M. Terreni, D. Ubiali, *Eur. J. Lipid Sci. Technol.*, **2014**, *116*, 1496-1504.



 $R_{\rm f} = 0.36$ (CH₂Cl₂-MeOH 95:5).

¹**H NMR (400 MHz, CDCl₃)**: δ 6.30 (d, J = 3.3 Hz, 1H, H-1), 5.64 (d, J = 8.2 Hz, 1H, NH), 5.32 (t, J = 9.8 Hz, 1H, H-3), 5.18 (t, J = 9.6 Hz, 1H, H-4), 4.49 (m, 1H, H-2), 3.84 (m, 1H, H-5), 3.74, 3.60 (dd, 2H, H₂-6), 2.22 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.05 (s, 3H, OAc), 1.96 (s, 3H, NAc).

Cyanomethyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-1-thio-β-D-glucopyranoside (60) ²⁵⁹



 $R_{\rm f} = 0.28$ (CH₂Cl₂-MeOH 95:5).

¹**H NMR (400 MHz, CDCl₃)**: δ 5.80 (d, J = 9.5 Hz, 1H, NH), 5.20 (t, J = 9.5 Hz, 1H, H-3), 5.13 (t, J = 9.5 Hz, 1H, H-4), 4.74 (d, J = 10.3 Hz, 1H, H-1), 4.20 (td, J = 10.3, 9.5 Hz, 1H, H-2), 3.74 (d, J = 17.3 Hz, 1H, CH₂CN), 3.80-3.71 (m, 1H, Ha-6), 3.66-3.61 (m, 1H, H-5), 3.60-3.58 (m, 1H, Hb-6), 3.32 (d, J = 17.3 Hz, 1H, CH₂CN), 2.07 (s, 3H, OAc), 2.06 (s, 3H, OAc), 1.97 (s, 3H, NAc).

2-acetamido-1,3,4-tri-O-acetyl-2-deoxy-α-D-galactopyranose (61)¹⁹⁸

²⁵⁹ Q. Abualassal, K. M. Al Azzam, J. A. Jilani, *Biomed. Chromatogr.*, **2016**, *30*, 1416-1422.



 $R_{\rm f} = 0.30$ (EtOAc-Hexane 1:1).

¹**H NMR (400 MHz, CDCl₃)**: δ 6.25 (d, J = 3.6 Hz, 1H, H-1), 5.58 (bd, 1H, NH), 5.45 (bdd, 1H, H-4), 5.30 (dd, J = 8.7, 3.1 Hz, 1H, H-3), 4.80 (ddd, J = 11.0, 9.8, 3.6 Hz, 1H, H-2), 4.08 (t, J = 12.8 Hz, 1H, H-5), 3.64-3.50 (m, 2H, H₂-6), 2.23 (s, 3H, OAc), 2.18 (s, 3H, OAc), 2.08 (s, 3H, OAc), 1.96 (s, 3H, NAc).

1,3,4-tri-O-acetyl-2-phthalimido-2-deoxy-α-D-glucopyranose (62)²⁶⁰



 $R_{\rm f} = 0.36$ (Toluene-EtOAc 3:2).

¹**H NMR (400 Hz, CDCl₃)**: δ 7.87-7.83 (m, 2H, phthalimido protons), 7.75-7.72 (m, 2H, phthalimido protons), 6.60 (dd, J = 11.5, 9.1 Hz, 1H, H-3), 6.29 (d, J = 3.3 Hz, 1H, H-1), 5.15 (t, J = 9.6 Hz, 1H, H-4), 4.71 (dd, J = 11.6, 3.3 Hz, 1H, H-2), 4.15-4.08 (m, 1H, H-5), 3.82-3.62 (2m, 2H, H₂-6), 2.09 (s, 3H, OAc), 2.08 (s, 3H, OAc), 1.88 (s, 3H, OAc).

Methyl 3,4-di-O-acetyl-2-phthalimido-2-deoxy-β-D-glucopyranoside (63)

²⁶⁰ F. Yang, Y. Du, *Carbohydr. Res.*, **2003**, *338*, 495-502.



 $R_f = 0.40$ (Hexane-EtOAc 1:1).

¹**H NMR (400 Hz, CDCl₃)**: δ 7.88-7.84 (m, 2H, phthalimido protons H), 7.75-7.73 (m, 2H, phthalimido protons), 5.83 (dd, *J* = 9.8, 1.6 Hz, 1H, H-3), 5.34 (d, *J* = 9.6 Hz, 1H, H-1), 5.12 (t, *J* = 9.2 Hz, 1H, H-4), 4.28 (dd, *J* = 8.8, 2.6 Hz, 1H, H-2), 3.84-3.81 (m, 1H, H-5), 3.71-3.64 (m, 2H, H₂-6), 3.45 (s, 3H, OCH₃), 2.07 (s, 3H, OAc), 1.87 (s, 3H, OAc).

¹³C NMR (100 Hz, CDCl₃): δ 171.29-167.60 (C=O), 134.99, 124.07 (aromatic C),
90.63 (C-1), 72.80 (C-5), 70.17 (C-3), 66.45 (C-4), 61.21 (C-6), 60.52 (OCH₃), 50.65 (C-2), 20.92 (CH₃, OAc), 20.67 (CH₃, OAc).

ESI-HRMS (m/z) calcd for C₁₉H₂₁NNaO₉ [M+Na]⁺: 430.1114, found: 430.1126.

Ethyl 3,4-di-O-acetyl-2-phthalimido-2-deoxy-1-thio-β-D-glucopyranoside (64)²⁶¹



 $R_f = 0.41$ (Hexane-EtOAc 3:2).

¹**H NMR** (400 MHz, CDCl₃): δ 7.95-7.75 (m, 4H, phthalimido protons), 5.90 (t, *J*=8.8 Hz, 1H, H-3), 5.52 (d, *J* = 10.5 Hz, 1H, H-1), 5.16 (t, *J* = 8.6 Hz, 1H, H-4), 4.40 (t, *J* = 9.5 Hz, 1H, H-2), 4.31 (dd, *J* = 11.7, 5.1 Hz, 1H, H-5), 3.72 (m, 1H, Ha-6), 3.68 (m, 1H, Hb-6), 2.82-2.60 (m, 2H, SCH₂CH₃), 2.15 (s, 3H, OAc), 1.89 (s, 3H, OAc), 1.22 (t, *J* = 7.4 Hz, 3H, SCH₂CH₃).

²⁶¹ T. Peters, T. Weimar, *Liebigs Annalen der Chemie*, **1991**, *3*, 237-242.

Phenyl3,4-di-O-acetyl-2-phthalimido-2-deoxy-1-thio-β-D-glucopyranoside(65)261



 $R_{\rm f} = 0.38$ (Hexane-EtOAc 3:2).

¹**H NMR (400 MHz, CDCl**₃): δ 7.94-7.75 (m, 4H, phthalimido protons), 7.48-7.26 (m, 5H, Ph), 5.85 (dd, *J* = 9.5 Hz, 1H, H-3), 5.79 (d, *J* = 10.6 Hz, 1H, H-1), 5.14 (t, *J* = 9.6 Hz, 1H, H-4), 4.39 (t, *J* = 10.6 Hz, 1H, H-2), 3.84-3.61 (m, 3H, H-5, H₂-6), 2.05 (s, 3H, OAc), 1.87 (s, 3H, OAc).

O-(2-azido ethyl) 3,4-di-*O*-acetyl-2-phthalimido-2-deoxy-1-β-D-glucopyranoside (66)



 $R_f = 0.38$ (Hexane-EtOAc 1:2).

¹**H NMR (400 Hz, CDCl₃)**: δ 7.87-7.85 (m, 2H, phthalimido protons), 7.75-7.73 (m, 2H, phthalimido protons), 5.84 (dd, *J* = 10.4, 9.2 Hz, 1H, H-3), 5.50 (d, *J* = 8.6 Hz, 1H, H-1), 5.15 (t, *J* = 9.8 Hz, 1H, H-4), 4.34 (dd, *J* = 12.4, 2.6 Hz, 1H, H-2), 4.12-4.02 (m, 1H, CH₂), 3.84-3.65 (m, 4H, H-5, CH₂, H₂-6), 3.38 (ddd, *J* = 13.2, 8.2, 3.6 Hz, 1H, CH₂), 3.21 (ddd, *J* = 13.4, 4.8, 3.6 Hz, 1H, CH₂), 2.08 (s, 3H, OAc), 1.88 (s, 3H, OAc).

¹³C NMR (100 Hz, CDCl₃): δ 170.69 (C=O), 170.49 (C=O), 170.42 (C=O), 170.29 (C=O), 134.77, 134.47, 123.70 (aromatic C), 97.45 (C-1), 73.85 (C-5), 69.98 (C-3), 67.87 (C-4), 66.59 (C-6), 61.73 (*C*H₂CH₂N₃), 54.74, 53.67 (CH₂CH₂N₃, C-2), 20.75

(CH₃, OAc), 20.40 (CH₃, OAc).

ESI-HRMS (m/z) calcd for C₂₀H₂₂N₄NaO₉ [M+Na]⁺: 485.1284, found: 485.1261.

O-(2-trimethylsilyl ethyl) 3,4-di-*O*-acetyl-2-phthalimido-2-deoxy-1-β-D-glucopyranoside (67)



 $R_f = 0.48$ (Hexane-EtOAc 3:2).

¹**H NMR (400 Hz, CDCl₃)**: δ 7.88-7.86 (m, 2H, phthalimido protons), 7.76-7.74 (m, 2H, phthalimido protons), 5.84 (dd, J = 10.6, 9.0 Hz, 1H, H-3), 5.43 (d, J = 8.6 Hz, 1H, H-1), 5.13 (t, J = 10.4 Hz, 1H, H-4), 4.37-4.27 (m, 1H, H-2), 4.18 (dd, J = 12.1, 2.4 Hz, 1H, Ha-6), 3.98-3.93 (m, 1H, OC*H*₂CH₂SiMe₃), 3.84-3.69 (m, 3H, H-5, Hb-6, OC*H*₂CH₂Si(CH₃)₃), 2.08 (s, 3H, OAc), 1.88 (s, 3H, OAc), 0.81 (m, 2H, OCH₂CH₂Si(CH₃)₃), -0.11 (s, 9H, Si(CH₃)₃).

¹³C NMR (100 Hz, CDCl₃): δ 171.80 (C=O), 171.77 (C=O), 171.22 (C=O), 170.19 (C=O), 134.17, 123.44 (aromatic C), 97.45 (C-1), 73.89 (C-5), 69.57 (C-3), 67.23 (C-4), 65.00 (C-6), 54.49 (C-2), 20.81 (CH₃, OAc), 20.79 (CH₃, OAc), 17.74 (OCH₂CH₂Si(CH₃)₃), -1.69 (Si(CH₃)₃).

ESI-HRMS (m/z) calcd for C₂₃H₃₁NNaO₉Si [M+Na]⁺: 516.1666, found: 516.1643.

Ethyl 3,4-di-O-acetyl-2-phthalimido-2-deoxy-1-thio-β-D-galactopyranoside (68)



 $R_{\rm f} = 0.35$ (Hexane-EtOAc 3:2).

¹**H NMR (400 Hz, CDCl₃)**: δ 7.87-7.85 (m, 2H, phthalimido protons), 7.76-7.74 (m, 2H, phthalimido protons), 5.85 (dd, *J* = 10.6, 3.2 Hz, 1H, H-3), 5.50 (d, *J* = 3.0 Hz, 1H, H-4), 5.48 (d, *J* = 10.4 Hz, 1H, H-1), 4.63 (t, *J* = 10.5 Hz, 1H, H-2), 3.98-3.95 (m, 1H, H-5), 3.78, 3.55 (2m, 2H, H₂-6), 2.75-2.67 (m, 2H, SCH₂CH₃), 2.22 (s, 3H, OAc), 1.86 (s, 3H, OAc), 1.21 (t, *J* = 7.2 Hz, 3H, SCH₂CH₃).

¹³C NMR (100 Hz, CDCl₃): δ 171.29 (C=O), 169.80 (C=O), 168.11 (C=O), 167.60 (C=O), 134.54, 134.47, 134.42, 123.86, 123.77 (aromatic C), 81.85 (C-1), 77.69 (C-5), 69.07 (C-3), 67.97 (C-4), 61.21 (C-6), 50.65 (C-2), 24.71 (SCH₂CH₃), 20.92 (CH₃, OAc), 20.67 (CH₃, OAc), 15.11 (SCH₂CH₃).

ESI-HRMS (m/z) calcd for C₂₀H₂₃NO₈NaS [M+Na]⁺: 460.1042, found: 460.1025.

6.8.3 Synthesis of sialyl glucosamine or galactosamine disaccharides

O-(methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α/β-D-galacto -2-nonulopyranosylonate)-(2→6)-2-acetamido-1,3,4-tri-O-acetyl-2-deoxy-α-D-glucopyranoside (69)



A mixture of sialyl xanthate **3** (137 mg, 0.23 mmol) and compound **59** (40 mg, 0.12 mmol) with 4Å powdered molecular sieves were dissolved in the dry CH₃CN (2.4 mL) and CH₂Cl₂ (1.2 mL) stirring at room temperature for 1 h. Further, AgOTf (50 mg, 0.19 mmol) and DTBP (46 uL, 0.21 mmol) were added, and the mixture was cooled to -68 °C and kept protected from light. Then PhSCl (22 uL, 0.19 mmol) in the dry

CH₂Cl₂ (0.12 mL) was added by running the solution down the cold wall of the reaction flask. Finally, the mixture was stirred for 2.5 h at -68 °C. After that, the mixture was diluted with a suspension of silica gel (0.5 g) in EtOAc (3 mL), filtered through celite, washed with saturated aqueous NaHCO₃ and water, dried with MgSO₄, and concentrated under reduced pressure. The residue was chromatographed (MeOH-EtOAc 1:20 to 1:5) to give disaccharide **69** (58 mg, 60%) as α and β isomer in the ratio of 2:1 from ¹H NMR.

 $R_{\rm f} = 0.29$ (MeOH-EtOAc 1:10).

¹**H NMR (400 MHz, CDCl₃)**: δ 6.22, 6.16, H-1'; 3.81, 3.79, COOCH₃; 2.63, 2.47, H-3eq; 2.20-2.05, OAc; 1.94-1.89, NAc.

¹³C NMR (100 MHz, CDCl₃): δ 171.93-167.49, C=O; 98.42, 98.32, C-2; 90.82, 90.68, C-1'; 52.94, 52.77, COOCH₃; 51.21, 51.12, C-2'; 49.33, C-5; 37.59, C-3; 23.22-23.08, CH₃, NAc; 21.18-20.61, CH₃, OAc.

ESI-HRMS (m/z) calcd for C₃₄H₄₈N₂O₂₁Na [M+Na]⁺: 843.2647, found: 843.2637.

Cyanomethyl *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero- α/β -D-galacto-2-nonulopyranosylonate)-(2 \rightarrow 6)-2-acetamido-3,4-di-*O*-acetyl-2-de-oxy- β -D-glucopyranoside (70)



The sialyl xanthate **3** (248 mg, 0.42 mmol) and compound **60** (100 mg, 0.28 mmol) were dissolved in the CH₃CN (4.5 mL) stirred for 1 h with 4Å powdered molecular sieves. Then it cooled to -40 °C, and NIS (187 mg, 0.83 mmol) and TfOH (36 uL, 0.46 mmol) were added. After 2 h, the reaction was quenched with Et₃N. The mixture

was diluted with CH₂Cl₂, filtered through celite, washed with 20% aqueous Na₂S₂O₃ solution, dried over MgSO₄, and concentrated under reduced pressure. The residue was purified by column (Cy-EtOAc, 1:4) to obtain the disaccharide **70** (127 mg, 55%) as α and β isomer in the ratio of 3:2 from ¹H NMR.

 $R_{\rm f} = 0.35$ (Cy-EtOAc 1:4, twice).

¹H NMR (400 MHz, CDCl₃): δ 4.63, 4.62, H-1'; 3.81, 3.79, COOCH₃; 3.51, 3.25, SCH₂CN; 2.64, 2.48, H-3eq; 2.20-1.89, OAc, NAc.

¹³C NMR (100 MHz, CDCl₃): δ 171.93-167.49, C=O; 115.18, CN; 98.42, 98.32, C-2; 83.53, 83.50, C-1'; 52.94, 52.77, COOCH₃; 51.12, C-2'; 49.33, C-5; 37.59, C-3; 23.22-20.61, CH₃, NAc, OAc; 14.20, SCH₂CN.

ESI-HRMS (m/z) calcd for C₃₄H₄₇N₃O₁₉NaS [M+Na]⁺: 856.2422, found: 856.2402.

O-(methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α/β-D-galacto -2-nonulopyranosylonate)-(2→6)-2-acetamido-1,3,4-tri-O-acetyl-2-deoxy-α-D-galactopyranoside (71)



Method A: A mixture of sialyl xanthate **3** (343 mg, 0.58 mmol) and compound **61** (40 mg, 0.29 mmol) with 4Å powdered molecular sieves were dissolved in the dry CH₃CN (6.0 mL) and CH₂Cl₂ (3.0 mL) stirring at room temperature for 1 h. Further, AgOTf (123 mg, 0.47 mmol) and DTBP (115 uL, 0.53 mmol) were added, and the mixture was cooled to -68 °C and kept protected from light. Then PhSCl (54 uL, 0.47 mmol) in the dry CH₂Cl₂ (0.3 mL) was added by running the solution down the cold wall of the reaction flask. Finally, the mixture was stirred for 3 h at -68 °C. After that,

the mixture was diluted with a suspension of silica gel (1.25 g) in EtOAc (7.5 mL), filtered through celite, washed with saturated aqueous NaHCO₃ and water, dried with MgSO₄, and concentrated under reduced pressure. The residue was chromatographed (MeOH-EtOAc 0:1 to 1:9) to give disaccharide **71** (150 mg, 64%) as α and β isomer in the ratio of 1:1 from ¹H NMR.

Method B: The acceptor 61 (20 mg, 0.058 mmol) and Ag₂CO₃ (112 mg, 0.406 mmol) in the dry CH₂Cl₂ (2.9 ml) were stirred with 4Å powdered molecular sieves protected from light for 0.5 h at room temperature. Then acetochloroneuraminate methyl ester 2 (88 mg, 0.173 mmol) in the dry CH₂Cl₂ (2.9 ml) was added dropwise. The mixture was then stirred at room temperature overnight, and gave disaccharide 27 (32 mg, 67%) as α and β mixture of 1:1.

 $R_{\rm f} = 0.26$ (EtOAc, twice).

¹H NMR (400 MHz, CDCl₃): δ 6.18, H-1'; 3.78, 3.76, COOCH₃; 2.50, 2.43, H-3eq; 2.18-1.85, OAc, NAc.

¹³C NMR (100 MHz, CDCl₃): δ 172.06-167.15, C=O; 98.84, 98.58, C-2; 90.32, C-1';
53.12, 52.03, COOCH₃; 49.33, C-2'; 48.00, C-5; 37.88, 36.57, C-3; 23.26-20.81, CH₃, NAc, OAc.

ESI-HRMS (m/z) calcd for C₃₄H₄₈N₂O₂₁Na [M+Na]⁺: 843.2647, found: 843.2627.

O-(methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2nonulopyranosylonate)-(2→6)-1,3,4-tri-O-acetyl-2-phthalimido-2-deoxy-α-D-glucopyranoside (72)



A mixture of sialyl xanthate **3** (912 mg, 1.54 mmol) and acceptor **62** (370 mg, 0.85 mmol) with 4Å powdered molecular sieves were dissolved in the dry CH₃CN (17.2 mL) and CH₂Cl₂ (8.6 mL) stirring at room temperature for 1 h. Further, AgOTf (360 mg, 1.38 mmol) and DTBP (337 uL, 1.55 mmol) were added, and the mixture was cooled to -68 °C and kept protected from light. Then PhSCl (337 uL, 1.38 mmol) in the dry CH₂Cl₂ (0.86 mL) was added by running the solution down the cold wall of the reaction flask. Finally, the mixture was stirred for 3 h at -68 °C. After that, the mixture was diluted with a suspension of silica gel (3.6 g) in EtOAc (22.5 mL), filtered through celite, washed with saturated aqueous NaHCO₃ and water, dried with MgSO₄, and concentrated under reduced pressure. The residue was chromatographed (Cy-EtOAc 1:3) to give disaccharide **72** (540 mg, 70%).

 $R_{f} = 0.31$ (Cy-EtOAc 1:3, twice).

¹**H NMR (400 MHz, CDCl₃)**: δ 7.84-7.81 (m, 2H, phthalimido protons), 7.73-7.71 (m, 2H, phthalimido protons), 6.52 (dd, J = 9.2, 2.2 Hz, 1H, H-3'), 6.24 (d, J = 3.9 Hz, 1H, H-1'), 5.35-5.15 (m, 3H, H-4', H-7, H-8), 4.89-4.82 (m, 1H, H-4), 4.68 (dd, J = 8.2, 3.6 Hz, 1H, H-2'), 4.28-4.23 (m, 2H, H-5', Ha-9), 4.03-3.99 (m, 4H, Ha-6', H-5, H-6, Hb-9), 3.80 (s, 3H, COOCH₃), 3.40 (dd, J = 11.4, 1.6 Hz, 1H, Hb-6'), 2.65 (dd, J = 12.7, 4.4 Hz, 1H, H-3eq), 2.14 (s, 3H, OAc), 2.13 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.96 (t, J = 3.6 Hz, 1H, H-3ax), 1.85 (s, 3H, NAc).

¹³C NMR (100 MHz, CDCl₃): δ 171.17 (C=O), 170.73 (C=O), 170.40 (C=O), 170.32 (C=O), 170.22 (C=O), 170.18 (C=O), 169.83 (C=O), 169.56 (C=O), 169.52 (C=O), 167.79 (C=O), 134.51, 123.81 (aromatic C), 98.46 (C-2), 90.80 (C-1'), 72.47, 70.74, 69.26, 69.14, 68.01, 67.50, 67.33 (C-4, C-6, C-7, C-8, C-3', C-4', C-5'), 62.60 (C-9), 62.30 (C-6'), 53.67 (C-2'), 53.05 (COOCH₃), 49.50 (C-5), 37.89 (C-3), 23.33 (CH₃, NAc), 21.29 (CH₃, OAc), 21.15 (CH₃, OAc), 20.99 (CH₃, OAc), 20.96 (CH₃, OAc), 20.92 (CH₃, OAc), 20.85 (CH₃, OAc), 20.81 (CH₃, OAc).

ESI-HRMS (m/z) calcd for C₄₀H₄₈N₂O₂₂Na [M+Na]⁺: 931.2596, found: 931.2590.

Methyl *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero- α -D-g-alacto-2-nonulopyranosylonate)-(2 \rightarrow 6)-3,4-di-*O*-acetyl-2-phthalimido-2-deoxy- β -D-glucopyranoside (73)



A mixture of sialyl xanthate **3** (117 mg, 0.20 mmol) and acceptor **63** (40 mg, 0.10 mmol) with 4Å powdered molecular sieves were dissolved in the dry CH₃CN (1.66 mL) and CH₂Cl₂ (0.83 mL) stirring at room temperature for 1 h. Further, AgOTf (50 mg, 0.20 mmol) and DTBP (45 uL, 0.20 mmol) were added, and the mixture was cooled to -68 °C and kept protected from light. Then PhSCl (24 uL, 0.21 mmol) in the dry CH₂Cl₂ (0.13 mL) was added by running the solution down the cold wall of the reaction flask. Finally, the mixture was stirred for 3 h at -68 °C. After that, the mixture was diluted with a suspension of silica gel (0.42 g) in EtOAc (2.5 mL), filtered through celite, washed with saturated aqueous NaHCO₃ and water, dried with MgSO₄, and concentrated under reduced pressure. The residue was chromatographed (Cy-EtOAc 1:3) to give disaccharide **73** (55 mg, 64%).

 $R_{\rm f} = 0.47$ (Cy-EtOAc 1:3, twice).

¹**H NMR (400 MHz, CDCl₃)**: δ 7.88-7.86 (m, 2H, phthalimido protons), 7.76-7.74 (m, 2H, phthalimido protons), 5.78 (dd, J = 9.0, 1.3 Hz, 1H, H-3'), 5.44 (d, J = 11.0 Hz, 1H, H-1'), 5.32-5.17 (m, 3H, H-4', H-7, H-8), 4.94-4.85 (m, 1H, H-4), 4.69 (dd, J = 9.3, 3.5 Hz, 1H, H-2'), 4.29-4.24 (m, 2H, H-5', H-9a), 4.06-4.01 (m, 4H, Ha-6', H-5, H-6, H-9b), 3.80 (s, 3H, COOCH₃), 3.41 (dd, J = 11.4, 1.5 Hz, 1H, Hb-6'), 3.31 (s, 3H, COOCH₃), 2.64 (dd, J = 12.6, 4.4 Hz, 1H, H-3eq), 2.12 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.96 (t, J = 3.5 Hz, 1H, H-3ax), 1.86 (s, 3H, NAc).

¹³C NMR (100 MHz, CDCl₃): δ 171.16, 170.72, 170.39, 170.33, 170.22, 170.18, 169.82, 169.55, 169.51, 167.80 (C=O), 134.52, 123.80 (aromatic C), 98.45 (C-2), 91.91 (C-1'), 72.48, 70.76, 69.27, 69.15, 68.03, 67.52, 67.31 (C-4, C-6, C-7, C-8, C-3', C-4', C-5'), 62.59 (C-9), 62.29 (C-6'), 60.53 (OCH₃), 53.65 (COOCH₃), 53.05 (C-2'), 49.51 (C-5), 37.90 (C-3), 23.33 (CH₃, NAc), 21.30, 21.14, 20.98, 20.94, 20.91, 20.85 (CH₃, OAc).

ESI-HRMS (m/z) calcd for C₃₉H₄₈N₂O₂₁Na [M+Na]⁺: 903.2647, found: 903.2658.

Ethyl *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero- α -D-gal-acto-2-nonulopyranosylonate)-(2 \rightarrow 6)-3,4-di-*O*-acetyl-2-phthalimido-2-deoxy-1-thio- β -D-glucopyranoside (74)



Method A: The acceptor 64 (20 mg, 0.046 mmol) and acetochloroneuraminate methyl ester 2 (70 mg, 0.138 mmol) in the dry CH_2Cl_2 (4.6 ml) were stirred with 4Å powdered molecular sieves for 1 h at room temperature. Then it was cooled to -45°C and kept protected from light, and AgOTf (83 mg, 0.322 mmol) was added. The mixture was gradually warmed to room temperature during 12 h, and further stirred at room temperature for 3 h. At last, filtered by celite and concentrated under reduced pressure. The residue was chromatographed (DCM-MeOH 9:1) to give disaccharide 74 (27 mg, 65%).

Method B: A mixture of sialyl xanthate **3** (109 mg, 0.18 mmol) and acceptor **64** (40 mg, 0.09 mmol) with 4Å powdered molecular sieves were dissolved in the dry CH₃CN (1.53 mL) and CH₂Cl₂ (0.77 mL) stirring at room temperature for 1 h. Then it

cooled to -68 °C, AgOTf (47 mg, 0.18 mmol) and DTBP (42 uL, 0.19 mmol) were added and kept protected from light. Further, PhSCl (22 uL, 0.19 mmol) in the dry CH₂Cl₂ (0.12 mL) was added dropwise. Finally, the mixture was stirred for 3 h at -68 °C. After that, the mixture was diluted with a suspension of silica gel (0.42 g) in EtOAc (2.5 mL), filtered through celite, washed with saturated aqueous NaHCO₃ and water, dried with MgSO₄, and concentrated under reduced pressure. The residue was chromatographed (Cy-EtOAc 1:3) to give disaccharide **74** (27 mg, 32%).

 $R_{f} = 0.41$ (Cy-EtOAc 1:3, twice).

¹**H NMR (400 MHz, CDCl₃)**: δ 7.88-7.85 (m, 2H, phthalimido protons), 7.76-7.74 (m, 2H, phthalimido protons), 5.81 (dd, J = 9.1, 1.2 Hz, 1H, H-3'), 5.45 (d, J = 10.9 Hz, 1H, H-1'), 5.30-5.17 (m, 3H, H-4', H-7, H-8), 4.94-4.85 (m, 1H, H-4), 4.51 (dd, J = 9.2, 3.6 Hz, 1H, H-2'), 4.36-4.30 (m, 2H, H-5', Ha-9), 4.17-4.02 (m, 5H, H₂-6', H-5, H-6, Hb-9), 3.80 (s, 3H, COOCH₃), 2.73-2.55 (m, 3H, H-3eq, SCH₂CH₃), 2.13 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.96 (t, J = 3.6 Hz, 1H, H-3ax), 1.85 (s, 3H, NAc), 1.23 (t, J = 1.9 Hz, 3H, SCH₂CH₃).

¹³C NMR (100 MHz, CDCl₃): δ 172.06 (C=O), 172.00 (C=O), 170.99 (C=O), 170.56 (C=O), 170.36 (C=O), 170.33 (C=O), 170.09 (C=O), 170.06 (C=O), 169.61 (C=O), 167.61 (C=O), 134.66, 123.08 (aromatic C), 98.49 (C-2), 81.12 (C-1'), 76.19, 71.24, 70.15, 69.37, 68.96, 68.28, 67.34 (C-4, C-6, C-7, C-8, C-3', C-4', C-5'), 62.24 (C-9), 62.07 (C-6'), 53.81 (COOCH₃), 51.89 (C-2'), 49.14 (C-5), 37.88 (C-3), 23.97 (SCH₂CH₃), 21.31 (CH₃, NAc), 19.34 (CH₃, OAc), 19.31 (CH₃, OAc), 19.29 (CH₃, OAc), 19.25 (CH₃, OAc), 19.21 (CH₃, OAc), 14.16 (SCH₂CH₃).

ESI-HRMS (m/z) calcd for C₄₀H₅₀N₂O₂₀NaS [M+Na]⁺: 933.2575, found: 933.2585.

Ethyl *O*-(methyl-5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate)- $(2\rightarrow 6)$ -3,4-di-*O*-acetyl-2-phthalimido-2-deoxy-1-thio-β-D-galactopyranoside (75)



Method A: The acceptor **68** (20 mg, 0.046 mmol) and acetochloroneuraminate methyl ester **2** (70 mg, 0.138 mmol) in the dry CH_2Cl_2 (4.6 ml) were stirred with 4Å powdered molecular sieves for 1 h at room temperature. Then it was cooled to -45 °C and kept protected from light, and AgOTf (83 mg, 0.322 mmol) was added. The mixture was gradually warmed to room temperature during 12 h, and further stirred at room temperature for 3 h. At last, filtered by celite and concentrated under reduced pressure. The residue was chromatographed (DCM-MeOH 9:1) to give disaccharide **75** (29 mg, 70%).

Method B: A mixture of sialyl xanthate **3** (136 mg, 0.23 mmol) and acceptor **68** (50 mg, 0.11 mmol) with 4Å powdered molecular sieves were dissolved in the dry CH₃CN (1.92 mL) and CH₂Cl₂ (0.96 mL) stirring at room temperature for 1 h. Further, AgOTf (59 mg, 0.23 mmol) and DTBP (53 uL, 0.24 mmol) were added, and the mixture was cooled to -68 °C and kept protected from light. Then PhSCl (28 uL, 0.24 mmol) in the dry CH₂Cl₂ (0.15 mL) was added by running the solution down the cold wall of the reaction flask. Finally, the mixture was stirred for 1.5 h at -68 °C. After that, the mixture was diluted with a suspension of silica gel (0.51 g) in EtOAc (3.1 mL), filtered through celite, washed with saturated aqueous NaHCO₃ and water, dried with MgSO₄, and concentrated under reduced pressure. The residue was chromatographed (Cy-EtOAc 1:3) to give disaccharide **75** (31 mg, 30%).

Method C: (pre-activation method): A mixture of sialyl xanthate **3** (65 mg, 0.110 mmol) and 4Å powdered molecular sieves were dissolved in the dry CH₃CN (2.4 mL) and CH₂Cl₂ (1.2 mL) stirring at room temperature for 1 h. Then it cooled to -68 °C, AgOTf (38 mg, 0.148 mmol) was added and kept protected from light. After 15 minutes, PhSCl (18 uL, 0.154 mmol) was added quickly, and the mixture was stirred

for another 15 minutes. After that, acceptor **68** (32 mg, 0.073 mmol) and DTBP (34 uL, 0.154 mmol) in the dry CH_2Cl_2 (0.4 mL) were added. Finally, the mixture was stirred for 3 h at -68 °C, but no product was formed.

Method D: The acceptor **68** (20 mg, 0.046 mmol) and Ag_2CO_3 (89 mg, 0.322 mmol) in the dry CH_2Cl_2 (2.3 ml) were stirred with 4Å powdered molecular sieves protected from light for 0.5 h at room temperature. Then acetochloroneuraminate methyl ester **2** (70 mg, 0.138 mmol) in the dry CH_2Cl_2 (2.3 ml) was added dropwise. The mixture was then stirred at room temperature overnight, and no desired product was formed.

Method E: The acceptor 68 (20 mg, 0.046 mmol) and acetochloroneuraminate methyl ester 2 (70 mg, 0.138 mmol) in the dry CH_2Cl_2 (4.6 ml) were stirred with 4Å powdered molecular sieves for 1 h at room temperature. Then AgOTf (83 mg, 0.322 mmol) was added protected from light. The mixture was stirred at room temperature overnight. At last, filtered by celite and concentrated under reduced pressure. The residue was chromatographed (DCM-MeOH 9:1) to give disaccharide 75 (27 mg, 65%) as α and β mixture of 3:2.

 $R_{f} = 0.38$ (Cy-EtOAc 1:3, twice).

¹**H NMR (400 MHz, CDCl₃)**: δ 7.87-7.85 (m, 2H, phthalimido protons), 7.76-7.73 (m, 2H, phthalimido protons), 5.87 (dd, J = 9.4, 2.9 Hz, 1H, H-3'), 5.72-5.70 (m, 2H, NH, H-4'), 5.52 (d, J = 10.9 Hz, 1H, H-1'), 5.37-5.35 (m, 1H, H-8), 5.27-5.20 (m, 1H, H-4), 5.14-5.10 (m, 1H, H-7), 4.72 (dd, J = 12.2, 3.6 Hz, 1H, H-6), 4.60 (t, J = 10.2 Hz, 1H, H-2'), 4.19-4.05 (m, 3H, H-5, H-5', Ha-9), 3.83 (s, 3H, COOCH₃), 3.66-3.59 (m, 2H, Ha-6', Hb-9), 3.44 (t, J = 7.2 Hz, 1H, Hb-6'), 2.74-2.63 (m, 1H, SCH₂CH₃), 2.47 (dd, J = 12.7, 4.4 Hz, 1H, H-3eq), 2.35 (s, 3H, OAc), 2.16 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.89 (s, 3H, OAc), 1.86 (s, 3H, NAc), 1.83 (t, J = 10.4 Hz, 1H, H-3ax), 1.20 (t, J = 9.6 Hz, 3H, SCH₂CH₃).

¹³C NMR (100 MHz, CDCl₃): δ 172.13 (C=O), 170.71 (C=O), 170.67 (C=O), 170.65 (C=O), 170.43 (C=O), 170.17 (C=O), 169.56 (C=O), 168.06 (C=O), 167.61 (C=O), 167.06 (C=O), 134.57, 134.40, 131.76, 131.39, 123.83 (aromatic C), 98.70 (C-2),

81.94 (C-1'), 75.10, 72.18, 72.08, 69.04, 69.01, 68.15, 67.70 (C-4, C-6, C-7, C-8, C-3', C-4', C-5'), 62.50 (C-9), 60.55 (C-6'), 53.13 (COOCH₃), 50.76 (C-2'), 48.32 (C-5), 37.06 (C-3), 24.66 (SCH₂CH₃), 23.33 (CH₃, NAc), 21.43 (CH₃, OAc), 21.23 (CH₃, OAc), 21.07 (CH₃, OAc), 20.95 (CH₃, OAc), 20.65 (CH₃, OAc), 15.03 (SCH₂CH₃).

ESI-HRMS (m/z) calcd for C₄₀H₅₀N₂O₂₀NaS [M+Na]⁺: 933.2575, found: 933.2580.

6.9 Synthesis of glucosamine-containing analogues

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto -2-nonulopyranosylonate)-(2 \rightarrow 6)-3,4-di-*O*-acetyl-2-phthalimido-2-deoxy-α/β-D-glucopyranoside (76)



The NH₂CH₂CH₂NH₂ (18 uL) in the dry THF (5.2 mL) was added AcOH (18 uL) dropwise. Then compound **72** (200 mg, 0.22 mmol) was added, and the mixture was further stirred at room temperature for 3 h. The solvent was evaporated and the residue was taken into CH₂Cl₂ and washed progressively with 1.0 M HCl, saturated aqueous NaHCO₃ and water. The organic layer was dried over MgSO₄ and concentrated to give the crude intermediate **76** (143 mg, 75%).

 $R_{\rm f} = 0.40$ (EtOAc).

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto -2-nonulopyranosylonate)-(2 \rightarrow 6)-3,4-di-*O*-acetyl-2-phthalimido-2-deoxy-α/β-Dglucopyranosyl trichloroacetimidate (77)



The intermediate **76** (110 mg, 0.13 mmol) was dissolved in the dry CH₂Cl₂ (4.2 mL), cooled to -5 °C, and trichloroacetonitrile (420 uL) and 42 μ L of DBU were added at -5 °C, and the mixture was stirred at -5 °C for 2 h. After concentration, the residue was purified by flash column chromatography (Cy-EtOAc 1:4) to yield the trichloracetimidate **77** with α and β (4:1) mixture as a pale yellow foam (77 mg, 60%).

 $R_{\rm f} = 0.45$ (EtOAc).

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto -2-nonulopyranosylonate)-(2 \rightarrow 6)-3,4-di-*O*-acetyl-2-phthalimido-2-deoxy-β-D-glucopyranoside(1 \rightarrow 1)-(2S,3R,4E)-2-azido-3-*O*-benzoyl-4-octadecene-l,3-diol (78)



The 77 (60 mg, 0.059 mmol) and 3-*O*-benzoyl azidosphingosine (50 mg, 0.12 mmol) in 2.8 mL of dry CH₂Cl₂ were stirred with 4Å powdered molecular sieves (500 mg) at room temperature for 1 h. The mixture was then cooled to -30 °C, and BF₃·Et₂O (60 μ L, 0.48 mmol) was added dropwise, stirred for another 1.5 h at -30 °C, and then filtered through celite. The filtrate was washed with saturated aqueous NaHCO₃ and water, dried over MgSO₄ and concentrated under reduced pressure. The residue was applied to a flash chromatography eluted with Cy-EtOAc 1:5 to give **78** (46 mg, 61%) as an amorphous solid.

 $R_{\rm f} = 0.58$ (EtOAc).

 $[\alpha]_D^{20} = -30.9 \ (c \ 1.0 \ in \ CHCl_3).$

¹**H NMR (400 MHz, CDCl₃)**: δ 7.98-7.95 (m, 2H, Ar-H), 7.86-7.82 (m, 2H, Ar-H), 7.73-7.71 (m, 2H, Ar-H), 7.57-7.53 (m, 1H, Ar-H), 7.44-7.40 (m, 2H, Ar-H),

5.83-5.64 (m, 1H, H-5"), 5.58-5.46 (m, 2H, H-3", H-4"), 5.41-5.30 (m, 6H, H-8, H-7, H-5', H-3', NH, H-2'), 4.87-4.84 (m, 1H, H-4), 4.34-4.28 (m, 2H, H-1', Ha-9), 4.12-4.01 (m, 6H, Hb-9, H-5, H-6, H-2", Ha-6', Ha-1"), 3.84-3.81 (m, 2H, Hb-1", Hb-6'), 3.79 (s, 3H, COOCH₃), 3.58 (dd, J = 5.7, 2.5 Hz, 1H, H-4'), 2.57 (dd, J = 12.8, 4.6 Hz, 1H, H-3eq), 2.14 (t, J = 1.5 Hz, 9H, 3 × OAc), 2.04 (s, 6H, 2 × OAc), 2.03 (d, J = 2.1 Hz, 2H, H₂-6"), 2.02 (s, 3H, OAc), 1.93 (t, J = 5.9 Hz, 1H, H-3ax), 1.87 (s, 3H, NAc), 1.24 (m, 22H, 11 × CH₂), 0.87 (t, J = 6.9 Hz, 3H, CH₃).

¹³C NMR (100 MHz, CDCl₃): δ 171.02 (C=O), 170.67 (C=O), 170.28 (C=O), 170.24 (C=O), 170.17 (2C=O), 170.15 (C=O), 170.03 (C=O), 168.55 (C=O), 167.70 (C=O), 164.87 (C=O), 138.92 (C-5"), 134.22 (C, CH aromatic), 133.13 (C, CH aromatic), 131.44 (C, CH aromatic), 129.70 (C, CH aromatic), 128.39 (C, CH aromatic), 123.60 (C-4"), 98.76 (C-1'), 98.49 (C-2), 74.78, 72.61, 72.44, 70.96, 69.13, 69.03, 68.58, 68.12, 67.37 (C-4, C-6, C-7, C-8, C-3', C-4', C-5', C-1", C-3"), 63.35 (C-2"), 62.38 (C-6'), 60.79 (C-9), 54.46 (C-2'), 52.63 (COOCH₃), 49.51 (C-5), 38.19 (C-3), 31.91 (CH₂, C-6"), 29.68 (CH₂), 29.66 (CH₂), 29.64 (CH₂), 29.57 (CH₂), 29.39 (CH₂), 29.34 (CH₂), 29.15 (CH₂), 28.62 (CH₂), 23.20 (CH₃, NAc), 21.09 (CH₃, OAc), 20.85 (CH₃, OAc), 20.84 (CH₃, OAc), 20.82 (CH₃, OAc), 20.76 (CH₃, OAc), 20.70 (CH₃, OAc), 14.10 (CH₃).

ESI-HRMS (m/z) calcd for C₆₃H₈₃N₅O₂₃Na [M+Na]⁺: 1300.5377, found: 1300.5365.

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto -2-nonulopyranosylonate)-(2 \rightarrow 6)-3,4-di-*O*-acetyl-2-phthalimido-2-deoxy-β-D-glucopyranoside(1 \rightarrow 1)-(2S,3R,4E)-2-amino-3-*O*-benzoyl-4-octadecene-l,3-diol (79)



The compound **78** (30 mg, 0.023 mmol) in 3 mL of toluene and 0.12 mL of water was added 17 mg of triphenylphosphine. The mixture was stirred at 50 °C for 24 h. After concentration, the crude residue **79** was directly used for the next step.

 $R_{\rm f} = 0.12$ (EtOAc).

O-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto -2-nonulopyranosylonate)-(2 \rightarrow 6)-3,4-di-*O*-acetyl-2-phthalimido-2-deoxy-β-D-glucopyranoside(1 \rightarrow 1)-(2S,3R,4E)-2-octadecanamido-3-*O*-benzoyl-4-octadecene-1,3diol (80)



The intermediate **79**, stearic acid (19 mg, 0.069 mmol), EDC (7 mg, 0.046 mmol) in 6 mL of CH_2Cl_2 were stirred at room temperature for 24 h. Then the mixture was washed with water, dried over MgSO₄ and concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography (Cy-EtOAc 1:3) to afford compound **80** (23 mg, 60% for two steps from **78**) as an amorphous solid.

 $R_{\rm f} = 0.39$ (Cy-EtOAc 1:3).

 $[\alpha]^{20}_{D} = -20.7$ (*c* 1.0 in CHCl₃).

¹**H NMR** (400 MHz, CDCl₃): δ 7.97-7.95 (m, 1H, Ar-H), 7.83 (m, 2H, Ar-H), 7.75-7.65 (m, 6H, Ar-H), 5.95-5.91 (m, 1H, H-5"), 5.66-5.61 (m, 1H, H-4"), 5.37-5.15 (m, 5H), 4.85 (m, 1H, H-4), 4.28-4.25 (m, 2H), 4.04-4.01 (m, 4H), 3.82-3.78 (m, 3H), 3.76 (s, 3H, COOCH₃), 3.64 (dd, J = 10.7, 4.6 Hz, 1H), 3.54 (d, J = 11.9 Hz, 1H), 2.59 (dd, J = 7.4, 3.2 Hz, 1H, H-3eq), 2.14 (s, 3H, OAc), 2.12 (s, 3H, OAc), 2.09 (m, 2H, CH₂CO), 2.07 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.03 (s, 3H, OAc), 1.94 (m, 2H, H₂-6"), 1.87 (s, 3H, OAc), 1.86 (s, 3H, NAc), 1.65-1.59 (m, 3H, H-3ax, H₂-7"), 1.26 (s, 50H, 25 × CH₂), 0.88 (t, *J* = 6.8 Hz, 6H, 2 × CH₃).

¹³C NMR (100 MHz, CDCl₃): δ 170.99, 170.65, 170.57, 170.35, 170.28, 170.17, 170.15, 169.99, 168.57, 167.72, 164.89 (C=O), 138.89 (C-5''), 134.25, 133.12, 131.45, 129.50, 128.40 (C, CH aromatic), 123.72 (C-4''), 99.64 (C-1'), 98.57 (C-2), 74.87, 72.59, 72.45, 71.00, 69.15, 69.00, 68.60, 68.11, 67.35, 63.24 (C-2''), 62.28 (C-6'), 60.90 (C-9), 54.37 (C-2'), 52.60 (COOCH₃), 49.49 (C-5), 38.18 (C-3), 31.99, 29.75, 29.65, 29.63, 29.58, 29.40, 29.33, 29.14, 28.58 (CH₂), 23.05 (CH₃, NAc), 21.07, 20.84, 20.83, 20.82, 20.78, 20.69 (CH₃, OAc), 15.22 (CH₃).

ESI-HRMS (m/z) calcd for C₈₁H₁₁₉N₃NaO₂₄ [M+Na]⁺: 1540.8081, found: 1540.8068.

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-β-D-glucopyranoside-(1→1)-(2*S*,3*R*,4*E*)-2-octadecanamido-4-octadecene-l,3-diol (81) ¹²⁵



The compound **80** (19 mg, 0.013 mmol) was dissolved in the dry EtOH (9 mL), and MeHNNH₂ (4.5 mL) was added. The mixture was refluxed at 80 °C for 18 h. After concentration, the residue was purified by column Sephadex HL-20, eluted with MeOH-CH₂Cl₂ 1:1, to obtain the intermediate. Then the intermediate was dissolved in 2 ml MeOH, and a few drops of 0.5 M NaOH aqueous solution were added. When the hydrolysis was completed after 1 h, the mixture was neutralized by Amberlite IR 120/H⁺ ion exchange resin. After filtration and concentration, the residue was dried in vacuo to afford crude intermediate. Further it was dissolved in the MeOH (1.5 mL)

and CH_2Cl_2 (1.5 mL), to which 0.3 mL of acetic anhydride was introduced. The mixture was stirred at room temperature overnight. After concentration, the crude product was flash-chromatographed, eluted with EtOAc-*i*PrOH-H₂O 2:2:1 to yield the analogue **81** (9 mg, 75% for three steps) as a white amorphous solid.

 $R_{f} = 0.35$ (EtOAc-*i*PrOH-H₂O 2:2:1).

¹**H NMR (400 MHz, CD₃OD)**: δ 5.58-5.45 (m, 1H, H-5''), 5.40-5.27 (m, 1H, H-4''), 4.07 (d, J = 7.3 Hz, 1H, H-1'), 3.85 (m, 1H, H-3''), 2.65 (d, J = 8.4 Hz, 1H, H-3eq), 2.02 (d, J = 6.3 Hz, 2H, CH₂C(O)), 1.97-1.81 (m, 2H, H₂-6''), 2.01, 1.88 (2s, 6H, 2 × NAc), 1.50-1.37 (dd, J = 14.3, 6.8 Hz, 3H, H-3ax, CH₂CH₂C(O)), 1.23 (s, 50H, 25 × CH₂), 0.89 (d, J = 6.8 Hz, 6H, 2 × CH₃).

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-β-D-glucopyranoside-(1→1)-(*2S*,3*R*,4*E*)-2-azido-4octadecene-l,3-diol (82)



The compound **78** (38 mg, 0.03 mmol) was dissolved in the dry EtOH (26 mL), and MeHNNH₂ (13 mL) was added. The mixture was refluxed at 80 °C for 18 h. After concentration, the residue was purified by column Sephadex HL-20, eluted with MeOH-CH₂Cl₂ 1:1, to obtain the intermediate. Then the intermediate was dissolved in 4 ml MeOH, and a few drops of 0.5 M NaOH aqueous solution were added. After 1 h, the mixture was neutralized by Amberlite IR 120/H⁺ ion exchange resin. Further filtration and concentration, the residue was dissolved in the MeOH and CH₂Cl₂ 7 mL

(1:1), to which 0.5 mL of acetic anhydride was added. The mixture was stirred at room temperature overnight. After concentration, the crude product was purified by column Sephadex HL-20, eluted with MeOH-CH₂Cl₂ 1:1, to obtain **82**.

 $R_{f} = 0.32$ (EtOAc-*i*PrOH-H₂O 2:2:1).

O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-β-D-glucopyranoside-(1→1)-(2*S*,3*R*,4*E*)-2-amino-4octadecene-l,3-diol (83)



The **82** was dissolved in the anhydrous MeOH (2.1 mL) were added propane-1,3-dithiol (0.16 mL) and triethylamine (0.16 mL), and the mixture was stirred at room temperature for 4 days. A white precipitate was formed. After filtration and washing with MeOH, the filtrate was concentrated under reduced pressure. The residue was flash-chromatographed, eluted with CHCl₃-MeOH 3:1 to yield the analogue **83** (16 mg, 70% for four steps) as a white amorphous solid.

 $R_{f} = 0.25$ (EtOAc-*i*PrOH-H₂O 2:2:1).

 $[\alpha]^{20}_{D} = -26.4 (c \ 1.0 \text{ in } 1:1 \text{ CHCl}_3\text{-MeOH}).$

¹**H NMR (400 MHz, CD₃OD)**: δ 5.90-5.87 (m, 1H, H-5''), 5.54-5.48 (m, 1H, H-4''), 4.01-3.61 (m, 18H), 2.60 (dd, J = 7.4, 3.2 Hz, 1H, H-3eq), 2.13-2.11 (m, 2H, H₂-6''), 2.04 (s, 3H, NAc), 2.03 (s, 3H, NAc), 1.44-1.40 (m, 3H, H-3ax, H₂-7''), 1.31 (s, 20H, 10 × CH₂), 0.90 (t, J = 6.8 Hz, 3H, CH₃).

¹³C NMR (100 MHz, CD₃OD): δ 175.60 (C=O), 175.55 (C=O), 175.53 (C=O),

136.48 (C-5''), 128.80 (C-4''), 101.59 (C-1'), 101.36 (C-2), 73.56, 73.00, 72.42, 71.66, 71.35, 71.20, 70.25, 69.56, 68.25, 63.74, 63.50, 57.45 (C-2''), 56.48 (C-2'), 53.20 (C-5), 42.50 (C-3), 33.00 (CH₂), 30.81 (CH₂), 30.79 (CH₂), 30.45 (CH₂), 30.43 (CH₂), 23.70 (CH₃, NAc), 22.65 (CH₃, NAc), 14.42 (CH₃).

ESI-HRMS (m/z) calcd for C₃₇H₆₆N₃O₁₅ [M-H]⁻: 792.4499, found: 792.4486.

Summary and perspectives

Studies over the past decades have proven that GSLs are involved in a variety of physiological and pathological processes, and tremendous progresses have been made in this field. GSLs and their structural changes associated with biological functions have been the focus of research, because structural change is the starting point for understanding biological significance, and enzymatic/genetic mechanisms. Moreover, most cancer cells exhibit altered GSLs patterns on their surface, abnormal GSLs signaling and increased GSLs biosynthesis, which together play roles in tumor growth, angiogenesis, migration and metastasis.

Ganglioside GM3, one of the most abundant tumor-associated carbohydrate antigens on several types of tumors, is implicated in many cellular functions, such as proliferation, adhesion, motility, and differentiation. It has been demonstrated that ganglioside GM3 is involved in many human diseases, such as cancers, diabetes or nervous diseases. Study on ganglioside GM3 and its analogues with biological activities appears to be a logical matter of research.

In this thesis, we reported the preparation of GM3 analogues based on a concise protocol that can provide a series of gangliosides from the commercially available substrates and reagents by enzymatic hydrolysis and chemical procedures. We introduced enzymatic hydrolysis to prepare ganglioside GM3 building blocks, which can avoid multiple chemical procedures. In addition, we developed new chemoenzymatic method to synthesize sialylated glucosamine or galactosamine derivatives, and it was successfully applied to synthesize ganglioside GM3 analogues containing glucosamine. The key step is a highly regioselective and stereoselective α -sialylation which was established by the previous method. At last, eight analogues with α -2,6 sialosides instead of α -2,3 sialosides containing different sugars, including galactose, mannose, lactose and glucosamine, were successfully synthesized.

The biological activities of these synthetic GM3 analogues were evaluated. By cytotoxicity assays, synthesized GM3 analogues were determined to be against human HCT116 and K562 cancer cells. Interestingly, by wound healing test, some analogues showed significant effects on tumor cell movement, which is believed to represent one of the relevant factors in assessing the malignancy of cancers. In addition, we employed the PC12 cell model to show that some GM3 analogues can induce neurite outgrowth with or without NGF induction.

We believe that the present work can facilitate to construct various GM3 analogues with improved biological activities and provide the useful information for exploring novel carbohydrate agents for biological studies associated with human diseases.



Synthesized six novel and two known ganglioside GM3 analogues

Publications

1. <u>Changping Zheng</u>, Huimin Guan, Yan Liu, Zhihao Li, Teodora Bavaro, Marco Terreni, Matthieu Sollogoub, Jianhua Xu, Yongmin Zhang. Chemoenzymatically synthesized ganglioside GM3 analogues with inhibitory effects on tumor cell growth and migration. *Eur. J. Med. Chem.*, **2019**, *165*, 107-114.

2. <u>Changping Zheng</u>, Huanhuan Qu, Wenfeng Liao, Teodora Bavaro, Marco Terreni, Matthieu Sollogoub, Kan Ding, Yongmin Zhang. Chemoenzymatically synthesized GM3 analogues as potential therapeutic agents to recover nervous functionality after injury by inducing neurite outgrowth. *Eur. J. Med. Chem.*, **2018**, *146*, 613-620.

3. <u>Changping Zheng</u>, Teodora Bavaro, Sara Tengattini, Andrea Gualla Mascherpa, Matthieu Sollogoub, Yongmin Zhang, Marco Terreni. Chemoenzymatic synthesis of glycoconjugates mediated by regioselective enzymatic hydrolysis of acetylated 2-amino pyranose derivatives. *Eur. J. Org. Chem.*, **2019**, *22*, 3622-3631.

4. <u>Changping Zheng</u>, Marco Terreni, Matthieu Sollogoub, Yongmin Zhang. Ganglioside GM3 and its role in cancer. *Curr. Med. Chem.*, **2018**, in press, doi: 10.2174/0929867325666180129100619.

 Zhihao Li, <u>Changping Zheng</u>, Marco Terreni, Lisa Tanzi, Matthieu Sollogoub, Yongmin Zhang. Novel vaccine candidates against tuberculosis. *Curr. Med. Chem.*, 2018, in press, doi: 10.2174/0929867326666181126112124.

SCIENTIFIC COMMUNICATIONS

1. <u>Changping Zheng</u>, Marco Terreni, Matthieu Sollogoub, Yongmin Zhang. Synthesis of ganglioside GM3 analogues with biological activities. *Rencontre des Facultés de Biologie et de Chimie 07/11/2016*, in Paris, France. **Poster communication**.

2. <u>Changping Zheng</u>, Marco Terreni, Matthieu Sollogoub, Yongmin Zhang. Synthesis of ganglioside GM3 analogues with biological activities. *The 24th Young Research Fellows Meeting in Medicinal Chemistry 08/02/2017-10/02/2017*, in Châtenay-Malabry, France. **Poster communication**.

3. <u>Changping Zheng</u>, Marco Terreni, Matthieu Sollogoub, Yongmin Zhang. Synthesis of ganglioside GM3 analogues with antitumor activities. 21^{ème} Journée de Chimie Organique et Chimie Organique Biologique de la Montagne Sainte-Geneviève 30/05/2017, in Paris, France. **Poster communication**.

4. <u>Changping Zheng</u>, Jianhua Xu, Marco Terreni, Matthieu Sollogoub, Yongmin Zhang. Chemoenzymatically synthesized ganglioside GM3 analogues with inhibitory effects on tumor cells growth. *16^{es} Rencontres de Chimie Organique 05/04/2018*, in Paris, France. Poster communication.

5. <u>Changping Zheng</u>, Jianhua Xu, Marco Terreni, Matthieu Sollogoub, Yongmin Zhang. Chemoenzymatically synthesized ganglioside GM3 analogues with inhibitory effects on tumor cells growth. *Carbohydrate and Fluorine Symposium* 18/10/2018-19/10/2018, in Potiers, France. **Oral communication**.

6. <u>Changping Zheng</u>, Marco Terreni, Matthieu Sollogoub, Yongmin Zhang. Chemoenzymatic synthesis of ganglioside GM3 analogues with antitumor activities. *The 26th Young Research Fellows Meeting in Medicinal Chemistry* 20/02/2019-22/02/2019, in Paris, France. **Poster communication**.