Synthetic strategies for modified glycosphingolipids and their design as probes

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Table of Contents

Abstract 4
Introduction 4
1.1 Glycolipid structure, classification, and nomenclature 5
1.1.1 Vertebrate glycolipid structures 5
1.1.2 Classification and nomenclature 6
1.2 Physical properties and analytical methods for GSLs 15
1.3 Biological roles of glycolipids and gangliosides 17
1.4 Scope of this review 19
2 Chemical synthesis of glycolipids 20
2.1 Chemical synthesis of sphingoid bases and ceramides 21
2.2 Chemical synthesis of cerebrosides, sulfatides, and derivatives. 24
2.3 Chemical synthesis of gangliosides 26
2.4 Neoglycolipids 31
2.5 Chemical degradation methods for semisynthesis 33
2.5.1 Removal of the fatty acid chain 34
2.5.2 Sphingosine degradation 35
2.5.3 Glycan degradation 36
3 Chemoenzymatic synthesis of glycolipids 37
3.1 Chemoenzymatic synthesis using glycosyltransferases 37
3.1.1 Synthesis of ganglio-series glycosphingolipids 39
3.1.2 Synthesis of globo/isoglobo and lacto/neolacto series glycosphingolipids 41
3.1.3 Engineered microorganisms 46
3.2 Chemoenzymatic synthesis using glycosidases and glycosynthases 48
3.3 Degradation enzymes for semisynthesis 52
3.3.1 Endoglycoceramidase (EGCase) 53
3.3.2 Sphingolipid ceramide deacylase (SCDase) 54
3.3.3 Galactose-6-oxidase 55
3.3.4 Exoglycosidases 56
Table 4. Enzyme abbreviations 58
4 Labelled glycolipid analogs 60
4.1 Radiolabelled glycolipids 64
4.1.1 Metabolic radiolabelling 64

4.1.2 Semisynthetic radiolabeling approaches ... 65
4.2 Fluorescent glycolipids ... 66
4.2.1 Lipid labelling ... 67
4.2.2 Glycan labeling ... 69
4.3 FRET-based glycolipid probes ... 71
4.3.1 Intermolecular FRET assays ... 71
4.3.2 Intramolecular FRET assays ... 74
4.4 Bioorthogonal-tagged glycolipids ... 75
4.4.1 Tagging the glycan ... 76
4.4.2 Tagging the lipid ... 78
4.5 Photoresponsive glycolipids ... 79
4.5.1 Glycan photocrosslinkers ... 80
4.5.2 Lipid photocrosslinkers ... 81
4.5.3 Caged glycolipids ... 82
4.5.4 Photoswitchable glycolipids ... 84
4.6 Glycolipid microarrays ... 84
4.6.1 Non-covalent attachment ... 85
4.6.2 Covalent attachment ... 86
5 Conclusions and outlook ... 87
ORCID ... 88
Author Biographies ... 88
Acknowledgements ... 89
Abbreviations ... 90
References ... 93

Abstract

The plasma membrane of cells contains a diverse array of lipids that provide important structural and biological features. Glycolipids are typically a minor component of the cell membrane and consist primarily of glycosphingolipids (GSLs). GSLs in vertebrates contain a multifarious assortment of glycan headgroups, which can be important to biological functions based on lipid-lipid and lipid-protein interactions. The design of probes to study these complex targets requires advanced synthetic methodologies. In this review, we will discuss recent advances in chemical and chemoenzymatic synthesis of GSLs in conjunction with the use of these approaches to design new probes. Examples using either chemical or enzymatic semisynthesis methods starting from isolated GSLs will also be reviewed. Focusing primarily on vertebrate glycolipids, we will highlight examples of radionuclide, fluorophore, photoresponsive, and bioorthogonal tagged GSL probes.

Introduction

The lipid bilayers of vertebrate cells contain a surprising diversity of glycolipids which play a number of important roles in cell biology. While the bilayers of the plasma membrane and organelles are more abundant in phospholipids;[1] glycolipids provide a rich source of binding epitopes and physical properties distinct from those of phospholipids and glycoproteins.[2] Similar to phospholipids in these cells, glycolipids contain a bifurcated lipid tail. However, vertebrate glycolipids are largely built on a ceramide (Cer) core, containing a sphingosine (Sph) chain and a fatty acid linked via an amide, as opposed to the diacyl chains attached as esters in phospholipids. These features make glycosphingolipids (GSLs) more akin to sphingolipids, which have received renewed attention in recent decades as their roles in signaling have been more fully elucidated.[3] The simplest GSLs, such as glucosylceramide (GlcCer), contain only a single carbohydrate residue in the headgroup; while larger structures may feature nine or more carbohydrate residues (e.g. GP1c). The amount of GSLs found in specific membranes is highly variable, with the highest

levels found in neuronal tissues where GSLs can act as the major carrier of sialosides.[4] The complexity of GSL structures, coupled with their low abundance makes investigation of their biological functions challenging. The biological function, nomenclature, and structures of GSLs are well reviewed elsewhere.[2,5,6] This review will focus on describing strategies for the synthesis of GSL probes that can be used to investigate their function in biological systems (see **Sec 1.4**). We include a short introduction to the biological roles of GSLs before providing a detailed review of the synthesis of glycolipids and analogs which can be used as probes.

1.1 Glycolipid structure, classification, and nomenclature

Several book chapters[2,5,7] and reviews[8,9] provide detailed descriptions of glycolipid structure, classification, and nomenclature.[10-12] As such, we will only briefly discuss the topic here to provide essential context.

1.1.1 Vertebrate glycolipid structures

Glycolipids are amphiphilic molecules with a polar head group containing a mono- or oligo-saccharide attached to a nonpolar lipid tail via a glycosidic bond.[13] In bacteria and plants, the lipid tail is most commonly a diacyl glycerol, whereas in insects and vertebrates, it is predominantly an acylated Sph.[8] The most common form of Sph is D-*erythro*-sphingosine d18:1 (**Figure 1**), which is biosynthesized from palmitoyl-CoA and L-serine,[7,8] although 16- and 20-carbon sphingosines are found in mammalian cells.[2,7] Cer has a fatty acid attached via an amide bond to sphingosine, and fatty acids from 14 to 32 carbon atoms have been observed in mammalian systems (**Figure 1**).[7] There are several ways of classifying GSLs; for example, those with a single carbohydrate residue in the headgroup are known as cerebrosides, and those containing at least one sialic acid (Sia) residue are named as gangliosides (**Table 2**). Other amphiphilic glycoconjugates are found

in vertebrates, including glycosylphosphatidylinostitol-linked (GPI-linked) proteins and lipopolysaccharides or saccharolipids.[14]

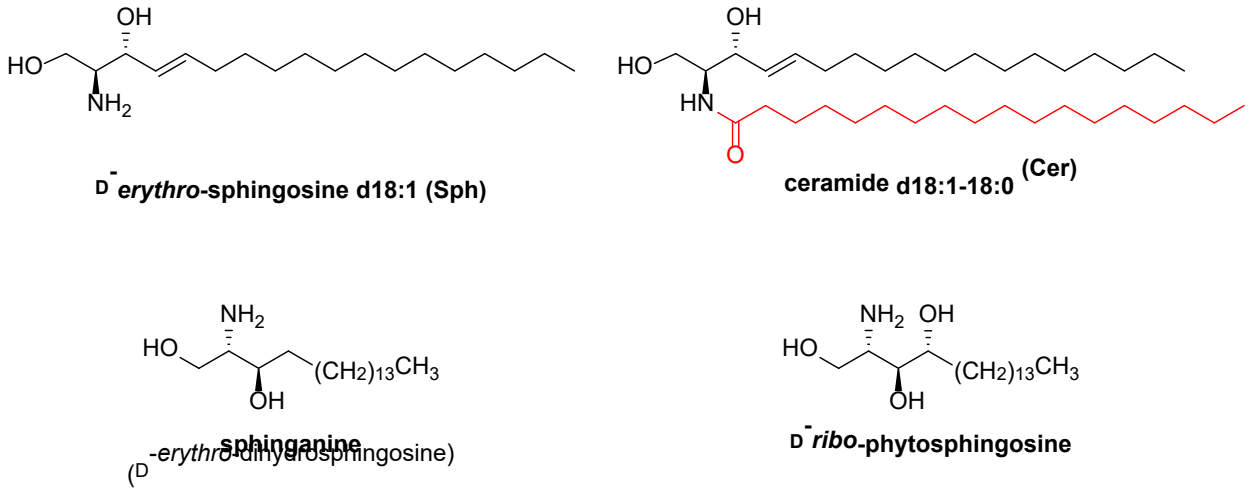


Figure 1. The structures of Sph and Cer, sphinganine, and an example of a phytosphingosine. For Cer, the stearate (C-18 fatty acid) is shown in red. The length of the alkyl chains in Sph and Cer are indicated for each chain as "d18:1" or "d18:1-18:0", respectively, where the first number denotes the number of carbon atoms in the chain and the second number indicates the number of unsaturated bonds. The chain of the sphingosine base (a 1,3-diol) is indicated by a preceding "d".[11]

1.1.2 Classification and nomenclature

The complexity of lipids is a result of variations in their polar head groups and nonpolar tails. With the rise of lipidomics, a systematic classification and database structure has been developed to complement those for nucleic acids and proteins.[15] The LIPID MAPS (Lipid Metabolites and Pathways Strategy) consortium has developed a system and tools; specifically, online tools[12,16-18] and a searchable, structural database.[19] The classification system divides lipids into eight categories based on their lipid tails – fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterol lipids, and prenol lipids. Each category is then broken into subclasses, and the individual lipids are given a 12- or 14-character identifier with the

first six or eight characters identifying the lipid class and subclass.[15] For example, gangliosides would all start with the characters "LMSP0601".[12]

It is important to note that the lipid tails of GSLs are not always determined,[12] so they are often categorized solely by their glycan structure, which can vary from a simple monosaccharide up to 50 carbohydrate residues.[8,14] The glycan structures of the GSLs, as well as their abbreviated chemical names, reflect their complexity and diversity. To simplify this complexity, glycolipids can be represented using abbreviated symbol nomenclature,[20-22] in addition to traditional chemical structures (**Figure 2, Table 1**).[10] Additionally, several methods of classification have been developed, including an abbreviated systematic convention[8,13,14] and a series of trivial names based partly on the chromatographic separation of gangliosides introduced by Svennerholm (**Table 2**).[23,24] We will mainly use the latter in this review. Svennerholm's system begins with a G to indicate a ganglioside structure, where the second letter refers to the number of sialic acid residues (M, mono; D, di; T, tri; Q, quad; P, penta). Finally, the name ends with a number to indicate the relative R_f value during normal phase chromatographic separation, for example in the monosialo-series, GM1 has the lowest R_f value, and GM3 the highest (**Table 2**).[23]

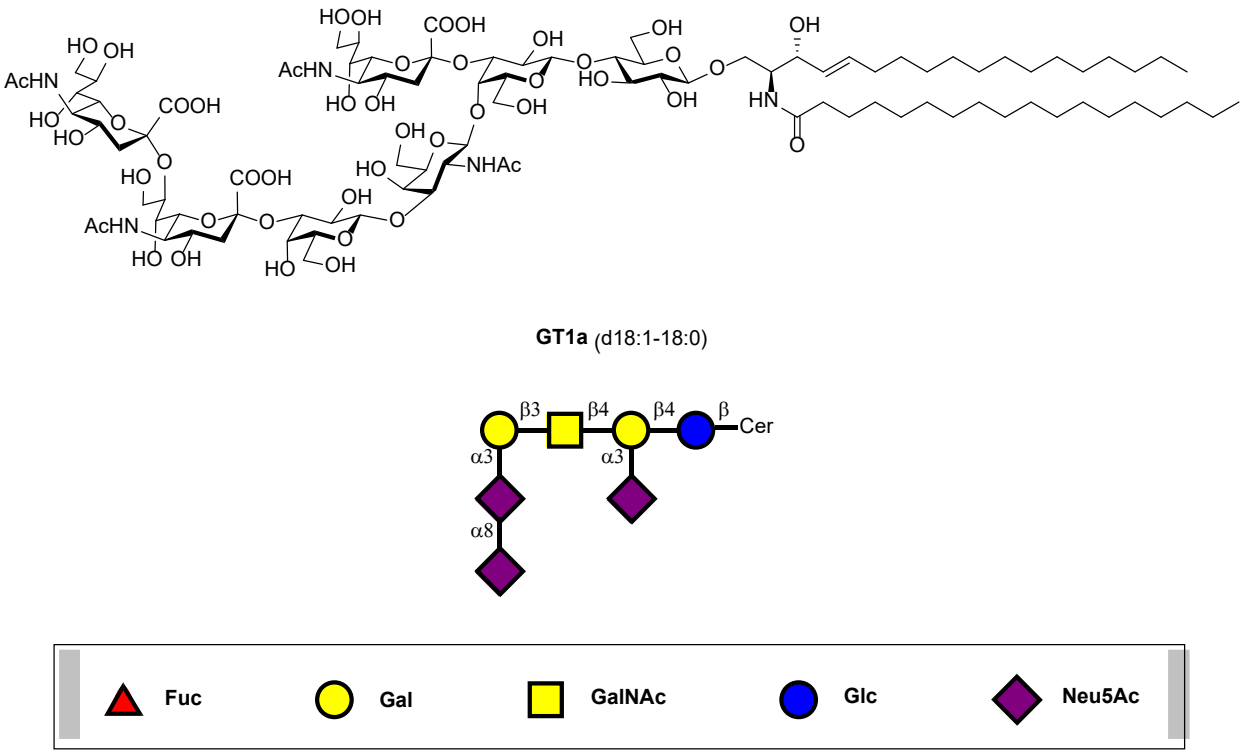
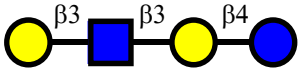
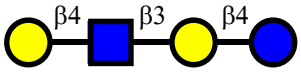
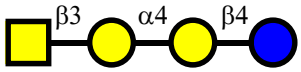
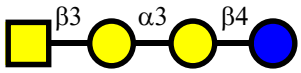


Figure 2. Bond-line structure and Symbol Nomenclature for Glycans (SNFG)[20] for GT1a. For clarity, sialic acids are drawn in neutral form throughout. *N*-acetylneuraminic acid has a pKa of 2.6 and is anionic at neutral pH.[25]

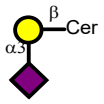
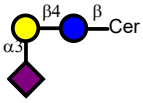
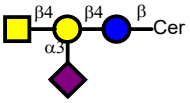
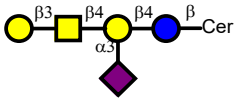
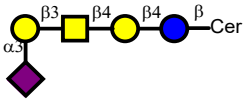
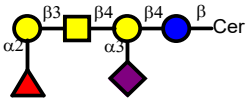
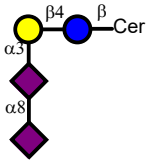
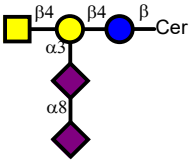
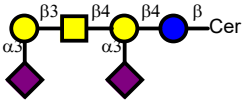
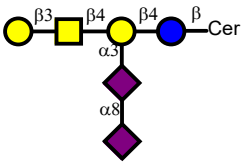
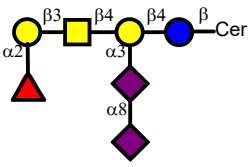
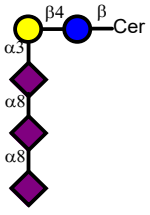
As mentioned above, the majority of glycolipids in mammalian systems are GSLs, with galactosyl ceramide (GalCer) making up 16% of the total lipid composition in the brain.[8] If GalCer contains a 3-*O*-sulfo modification, the lipid is known as sulfatide. More complex GSLs are generally based on lactosyl ceramide (LacCer), and the five common tetrasaccharide cores present in mammalian systems are listed in **Table 1**.[14] Inspired by a recent review of ganglioside syntheses from the Kiso group,[26] we have correlated the nomenclature for these structures with references to chemical and chemoenzymatic total syntheses discussed in this review in **Table 2** (gangliosides) and **Table 3** (other vertebrate glycolipids).

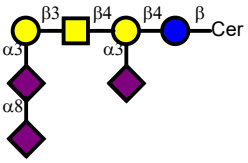
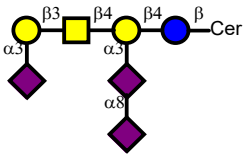
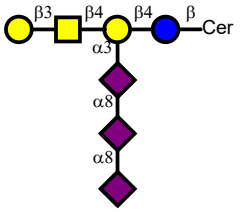
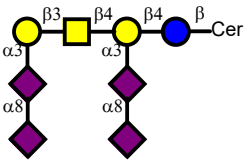
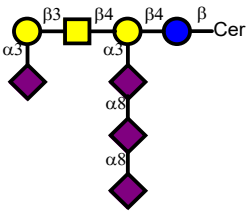
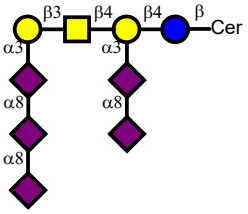
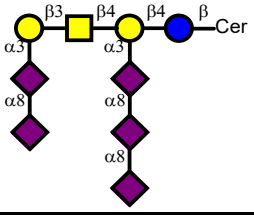
Table 1. Major glycolipids found in mammalian systems [a]

Root	Symbol	Root structure (abbreviations)	Symbol nomenclature[20]
ganglio	Gg	Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc	
lacto	Lc	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	
neolacto	nLc	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	
globo	Gb	GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc	
isoglobo	iGb	GalNAc(β1-3)Gal(α1-3)-Gal(β1-4)Glc	

[a] Nomenclature from Chester, M. A. *Eur. J. Biochem.* **1998**, *257*, 293–298.

Table 2. Svennerholm's ganglioside nomenclature [a]

Name	Structure (condensed form)	Symbol nomenclature [b,20]	Total syntheses [c]
GM4	Neu5Ac(α2-3)Gal(β1-1')Cer		[27] Reviewed in [26]
GM3	Neu5Ac(α2-3)Gal(β1-4)Glc(β1-1')Cer		[28-36] Reviewed in [26,37,38]
GM2	GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		[39,40] Reviewed in [26,37]
GM1[d]	Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		[41-43] Reviewed in [26]
GM1b[e]	Neu5Ac(α2-3)Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1')Cer		Reviewed in [26]
Fuc-GM1	Fuc(α1-2)Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		Reviewed in [37]
GD3	Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc(β1-1')Cer		[44-47] Reviewed in [26]
GD2	GalNAc(β1-4)[Neu5Ac(α2-8)Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		Reviewed in [26]
GD1a	Neu5Ac(α2-3)Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		[42] Reviewed in [26,37]
GD1b	Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-8)Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		Reviewed in [26]
Fuc-GD1b	Fuc(α1-2)Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-8)Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		
GT3	Neu5Ac(α2-8)Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc(β1-1')Cer		Reviewed in [26]

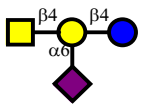
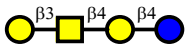
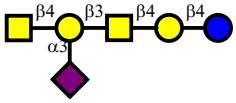
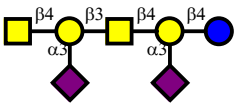
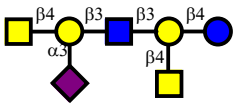
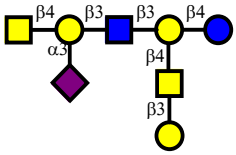
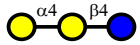
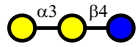
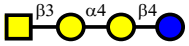
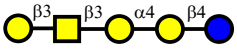
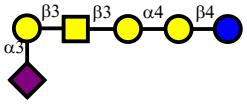
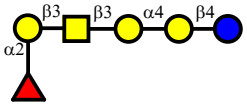
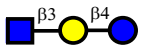
Name	Structure (condensed form)	Symbol nomenclature [b,20]	Total syntheses [c]
GT1a	Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		[42] Reviewed in [26,37]
GT1b	Neu5Ac(α2-3)Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-8)Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		Reviewed in [26]
GT1c	Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-8)Neu5Ac(α2-8)Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		
GQ1b	Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-8)Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		[48] Reviewed in [26 37,38]
GQ1c	Neu5Ac(α2-3)Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-8)Neu5Ac(α2-8)Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		
GP1b	Neu5Ac(α2-8)Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-8)Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		
GP1c	Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-8)Neu5Ac(α2-8)Neu5Ac(α2-3)]Gal(β1-4)Glc(β1-1')Cer		[49]

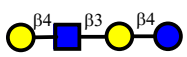
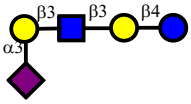
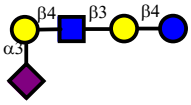
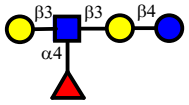
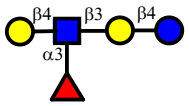
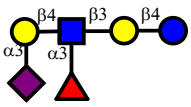
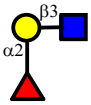
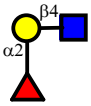
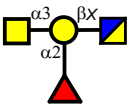
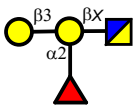
[a] Nomenclature from Svennerholm, L. *Progress in Brain Research* **1994**, *101*, xi–xiv.
[b] The key for the Symbol Nomenclature for Glycans (SNFG) is provided in Figure 2.
[c] References for the synthesis of each lipid, or reviews thereof.
[d] Also called GM1a
[e] Also called *cis*GM1.[13,24]

While gangliosides are defined by the presence of at least one sialic acid residue, there are multiple forms of sialic acid (Neu5Ac) that may occur in vertebrates. The most predominant of

these are Neu5Ac and Neu5Gc, and *O*-acetylated forms thereof. Several *O*-acetylated forms of GSLs are thought to be important in disease.[9,50] Humans lack the ability to express a functional cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (CMAH) enzyme, preventing native biosynthesis of CMP-Neu5Gc from CMP-Neu5Ac and, therefore, the incorporation of Neu5Gc in glycoproteins and glycolipids. Human ganglioside structures that incorporate Neu5Gc are likely acquired from dietary sources.[51,52] Interestingly, gangliosides of vertebrate brains in organisms which express functional CMAH also have remarkably low amounts of Neu5Gc gangliosides despite its occurrence in other tissues.[53] Gangliosides found in dietary sources have been recently analyzed by high resolution mass spectrometry.[54] Bovine milk is a useful source of GD3 and GM3 gangliosides,[55,56] which tend to be composed primarily of Neu5Ac with minor amounts of Neu5Gc (2%).[57]

Table 3. Oligosaccharide portions of other glycolipids discussed in this review

Name	Structure (condensed form)	Symbol nomenclature [a,20]	Total syntheses [b]
6'-Neu5Ac-GM2	GalNAc(β1-4)[Neu5Ac(α2-6)]Gal(β1-4)Glc(β)		40
GA1	Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β)		58
GalNAc-GM1b	GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β)		59 Reviewed in [26]
GalNAc-GD1a	GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-3)GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-4)Glc(β)		32,60 Reviewed in [26,38]
Ganglioside X1	GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-3)GlcNAc(β1-3)[GalNAc(β1-4)]Gal(β1-4)Glc(β)		61,62 Reviewed in [26]
Ganglioside X2	GalNAc(β1-4)[Neu5Ac(α2-3)]Gal(β1-3)GlcNAc(β1-3)[GalNAc(β1-4)Gal(β1-3)]Gal(β1-4)Glc(β)		62 Reviewed in [26]
Gb3	Gal(α1-4)Gal(β1-4)Glc(β)		34,63-72
iGb3	Gal(α1-3)Gal(β1-4)Glc(β)		63,70,72,73
Gb4	GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β)		63,64,69,74
Gb5	Gal(β1-3)GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β)		63,64,74
Sialyl-Gb5 (SSEA4)	Neu5Ac(α2-3)Gal(β1-3)GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β)		63,64,66,75
Globo-H	Fuc(α1-2)Gal(β1-3)GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β)		64,66,75
Lc3	GlcNAc(β1-3)Gal(β1-4)Glc(β)		63,65,76
Lc4	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc(β)		76,77

Name	Structure (condensed form)	Symbol nomenclature [a,20]	Total syntheses [b]
nLc4	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(β)		63,65,78-80
Sialyl Lc4	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc(β)		63
Sialyl nLc4	Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(β)		63,78
Lewis[a]	Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Gal(β1-4)Glc(β)		81
Lewis[x]	Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc(β)		63,78,81-84
Sialyl Lewis[x]	Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc(β)		63,82,85-88 Reviewed in [26,37]
H type I antigen	Fuc(α1-2)Gal(β1-3)GlcNAc(β)		89
H type II antigen	Fuc(α1-2)Gal(β1-4)GlcNAc(β)		89-91
A antigen	GalNAc(α1-3)[Fuc(α1-2)]Gal(β1-X)HexNAc(β) [c]		89,90
B antigen	Gal(β1-3)[Fuc(α1-2)]Gal(β1-X)HexNAc(β) [c]		89

[a] The key for the Symbol Nomenclature for Glycans (SNFG)is provided in Figure 2.
[b] References for the synthesis of each lipid.
[c] The reducing end monosaccharide may be a GlcNAc or a GalNAc linked (β1-3) or (β1-4) to the neighboring Gal, depending on blood group subtype.[92]

1.2 Physical properties and analytical methods for GSLs

GSLs which do not contain sialic acids or modified residues are generally neutral, while gangliosides and sulfatides are negatively charged (acidic). The amphipathic nature of glycolipids is critical for their function and their formation of aggregates.[93] Glycolipids, much like phospholipids,[94] tend to self-associate in aqueous solution forming micelles.[95] The minimum concentration at which gangliosides will form micelles (the critical micelle concentration, or CMC) is remarkably low as compared with other lipids.[95] Additionally, gangliosides form clusters or domains within their native membrane environments.[96,97] When studying GSLs in vitro, they are often prepared in mixtures of other phospholipids to mimic their low abundance in biological membranes.[98] Lipids can be taken up by cell membranes, providing a tool commonly used for investigation of their roles in cells.[99,100] Spontaneous transfer of gangliosides between membranes is temperature and lipid dependent.[101,102] The uptake of exogenous gangliosides into the plasma membrane is complex, and can involve multiple mechanisms, including non-specific association and protein-mediated binding.[103] The shedding and uptake of gangliosides between cells is a known biological mechanism that is likely mediated by transfer proteins.[104] The mixed polarity of GSLs also presents challenges for purification of these compounds from complex mixtures or in large scale. The most common forms of analytical separation are high performance thin layer chromatography (HP-TLC)[105-107] and liquid-chromatography mass spectrometry (LC-MS).[108]

Common models for study of GSLs in vitro include supported bilayers and unilamellar vesicles. Bilayers are prepared from liposomes which can interact with a glass surface to provide a supported bilayer.[109] Artificial bilayers (also called supported bilayers) are commonly used to provide an oriented, and freely-diffusing, model of the lipid bilayer. Membrane proteins can be incorporated for binding and diffusion measurements,[110-112] and micropatterning can be used to study mechanisms of diffusion.[113] The lateral mobility of GSLs is affected by their immediate environment. Measurements of GSL or spectator lipid diffusion have found reductions in lateral

mobility for GSLs that participate in microdomains or protein binding.[114-116] Membrane sheets are excised sections of lipid bilayer attached to poly-lysine coated surfaces and have been found to retain the activity of integral membrane proteins as well as the fluidity in both leaflets of the membrane.[117] The method has been applied to the study of membrane proteins,[118] and localization of gangliosides.[119-121] Giant unilamellar vesicles (GUV) have provided a model system for examining the lateral partitioning of GSLs within complex mixtures.[116,122,123] Scaffold proteins can be used to generate bicellar GSL-protein complexes, referred to as nanodiscs[124,125] or picodiscs.[126-128] Methods used to investigate protein-GSL interactions have included mass spectrometry,[129,130] ESR,[131] NMR,[132,133] neutron scattering,[134,135] DSC,[136] microarrays,[137] and computational methods.[138-141] Mass spectrometry-based methods have the potential to provide data on binding interactions in the context of a bilayer-like model, including lipid-lipid or lipid-protein affinities.[129]

The molecular size and complexity of GSLs present specific challenges for the development of probes to study these molecules in their native environment. Unlike proteins, which generally contain a number of ancillary groups that can be used for chemical modification, GSLs have only a few sites to choose for attachment points. Furthermore, one has to consider that any new chemical appendage introduced to a GSL structure may have a large influence on its physical properties and associations.[142] This is perhaps nowhere more critical than in the use of synthetic fluorophores on GSLs. If one considers the relative molecular mass of a typical GSL, such as GM1, and one of the smallest fluorochromes, 7-nitro-1,2,3-benzoxadiazole (NBD) – the ratio of masses is approximately 10:1 (GSL:Fluor). Compare this to a typical membrane protein of even moderate size (~25 kDa), and that ratio drops to 150:1 (Protein:Fluor), or even smaller for larger proteins. As a result, successful probes must consider the molecular mass, polarity, and attachment point in their design (**see Sec 4**).

1.3 Biological roles of glycolipids and gangliosides

Gangliosides are known to play important roles in membrane structure and function,[143] most notably in myelination of nerve tissue.[144] Early murine models which disrupted the biosynthesis of more complex GSLs had suggested that gangliosides might not be essential for neuronal function.[145] However, more recent animal models which ablate the biosynthesis of all ganglio-series GSLs show "rapid and profound neurodegeneration", leaving no doubt that GSLs are critical components of a functioning nervous system.[146,147] GSLs may carry important binding epitopes, such as ABH blood group antigens (**Table 3**),[92,148,149] or ligands for the selectins.[150,151] The presentation of GSL binding epitopes may be complex, as heterogenous mixtures can produce novel epitopes.[152]

Interactions of GSLs with intracellular and exogenous proteins are critical for membrane organization, signaling, and recognition events. Direct interactions of gangliosides with important signaling receptors, such as the endothelial growth factor receptor (EGFR) are well known.[153,154] A number of membrane proteins are known to have ganglioside-binding domains.[155,156] Caveolin proteins (Cav) are known to associate with GSL-rich microdomains and are likely important to their organization.[157,158] Cav is known to interact directly with GM1, leading to enrichment of GM1 in Cav domains.[159] Photocrosslinking of GM1 (containing an aryl-azide in the acyl chain) has been used to capture Cav-1,[160] a finding supportive of direct ganglioside-protein interactions within the membrane environment. GSL interactions with flotillin-1 and flotillin-2 are required for GSL trafficking and endocytosis.[161] Flotillins have also been captured through photocrosslinking of fatty acid chains, supporting a direct ganglioside-protein interaction.[162] Exogeneous proteins, such as bacterial toxins, specifically bind to glycolipids as cellular receptors.[163,164] The specificity of bacterial toxins may be used for the detection of GSLs by fluorescence imaging.[165] It should be noted, however, that methods for detection of GM1 that exploit the binding of cholera toxins may

be confounded by the mixed binding specificity for this lectin.[166] Other proteins or peptides with high affinity for specific GSLs have been developed as probes.[167-169]

Glycosphingolipids likely play central roles in the heterogenous organization of lipids and proteins within the membrane environment.[170,171] Membrane domains are enriched in particular lipids and proteins, and these structures are generally referred to as lipid rafts or microdomains.[172-174] The enrichment of GSLs in lipid rafts (and detergent-resistant microdomains) has been observed in numerous systems.[175-179] Partitioning of GSL into microdomains is influenced by structural features of the GSL.[180-185] Lipid raft organization is proposed to be critical for signal transduction,[186] and membrane microdomains where glycan-binding domains are enriched have been termed a glycosynapse.[187,188] Glycan-glycan interactions may play a role in lateral interactions of GSLs within the membrane.[189] Glycans of the ABH antigens, and other sialosides, have been proposed to form "saccharide patches" on erythrocyte membranes.[149] As the structures of GSLs are important for inter-molecular interactions, enzymes which modify GSL in the membrane environment may influence membrane organization.[190,191]

As membrane components, glycosphingolipids traffic into and out of the cell through various mechanisms. Incorporation of exogenous gangliosides into cellular membranes can occur through endocytosis and redistribution of gangliosides to the plasma membrane.[99] Cholesterol (Chol) interactions with GSLs may be important for endocytosis.[192] In addition, gangliosides can traffic along endocytic and retrograde pathways[193] and can be found in a specialized structure of the endoplasmic reticulum that is associated with mitochondria (mitochondria-associated membranes or MAM).[194,195] Exosomes have been reported to contain gangliosides;[196,197] however, extensive analyses of exosomes for ganglioside content have been limited.[198,199] Glycosphingolipids and flotillins are implicated in regulating the release of exosomes from cells.[200]

Gangliosides and other GSLs are implicated in a range of human diseases.[9,147,201,202] Gangliosides have emerged as important biomarkers in cancers,[203] and are even used as therapeutic

targets.[204,205] Variations in the structures of gangliosides have been correlated with metabolic diseases.[206] GSLs have been found to play roles in transmembrane signaling and can modulate the function of membrane proteins through a variety of proposed mechanisms, primarily protein-GSL and lipid-GSL interactions.[143,207] Deficiencies in enzymes that process GSLs can lead to excess storage of these lipids, known as gangliosidosis,[208,209] and are also responsible for Tay-Sachs disease.[210] Therapeutic strategies for forms of these diseases which result from misfolded proteins include enzyme replacement and pharmacological chaperones, or substrate reduction through inhibiting biosynthesis of lipids.[211-215] Amyloid protein undergoes increased aggregation in the presence of exosomes containing GSLs and is sensitive to the glycan structure of these lipids.[216]

1.4 Scope of this review

GSLs possess a unique set of molecular properties which allow them to participate in diverse roles in cell biology. Furthermore, the remarkable complexity of these structures and their low abundance have made them extremely challenging to study as components of the cell membrane. Chemists have found synthesis of analogs to be a powerful strategy in the examination of protein and phospholipid biochemistry. GSLs present substantial synthetic challenges that have slowed progress in the development of new chemical tools for these systems. Chemical and chemoenzymatic approaches towards GSL targets have matured in recent years, opening the door to more elaborate strategies for the development of glycolipid probes. This review will begin with a survey of recent developments in chemical and chemoenzymatic strategies towards GSL structures, with selected formative examples provided for context. In general, we have restricted our discussion to vertebrate GSLs as these are most central to understanding human physiology and disease. These two approaches form the foundation of any successful probe design by enabling access to the required complex intermediates. Subsequently, we discuss examples of synthetic strategies used to design chemically modified GSL probes. These probes feature tags which can

be used to report on the fate or interactions of GSLs within a biological system. In comparison to other biomolecules, GSLs have not been as thoroughly explored and the development of new and more versatile probes is likely to improve our understanding of their function. It is our hope that collecting the examples here alongside advances in synthetic methodology will inspire new strategies and tools for glycolipid research.

2 Chemical synthesis of glycolipids

The chemical synthesis of GSLs and other vertebrate glycolipids is a fundamental tool for the development of new GSL probes. A successful strategy for the synthesis of a native GSL can typically be adapted to introduce modifications into the target with precise control of regio- and stereochemistry. Over the last decade, reliable chemical methodologies have been established for the total synthesis of GSLs, particularly gangliosides and their analogues. These methods have been highlighted by the Kiso group[26,38,217] and by Kulkarni.[37] Thorough reviews of GPI-anchors and GPI-linked proteins are available elsewhere,[218-220] along with the synthesis and biological applications of inositols.[221] General synthetic strategies towards GSLs are outlined in **Figure 3**. The most intuitive disconnection begins with glycosylation of Cer with an appropriate oligosaccharide donor, and the synthesis of these respective components. More recent methodologies begin from disconnecting the glycan to GlcCer plus the non-reducing end residue, in what is referred to as a "cassette strategy" pioneered by Kiso and coworkers.[26] This approach provides improved yields for more complex GSL structures.[48]

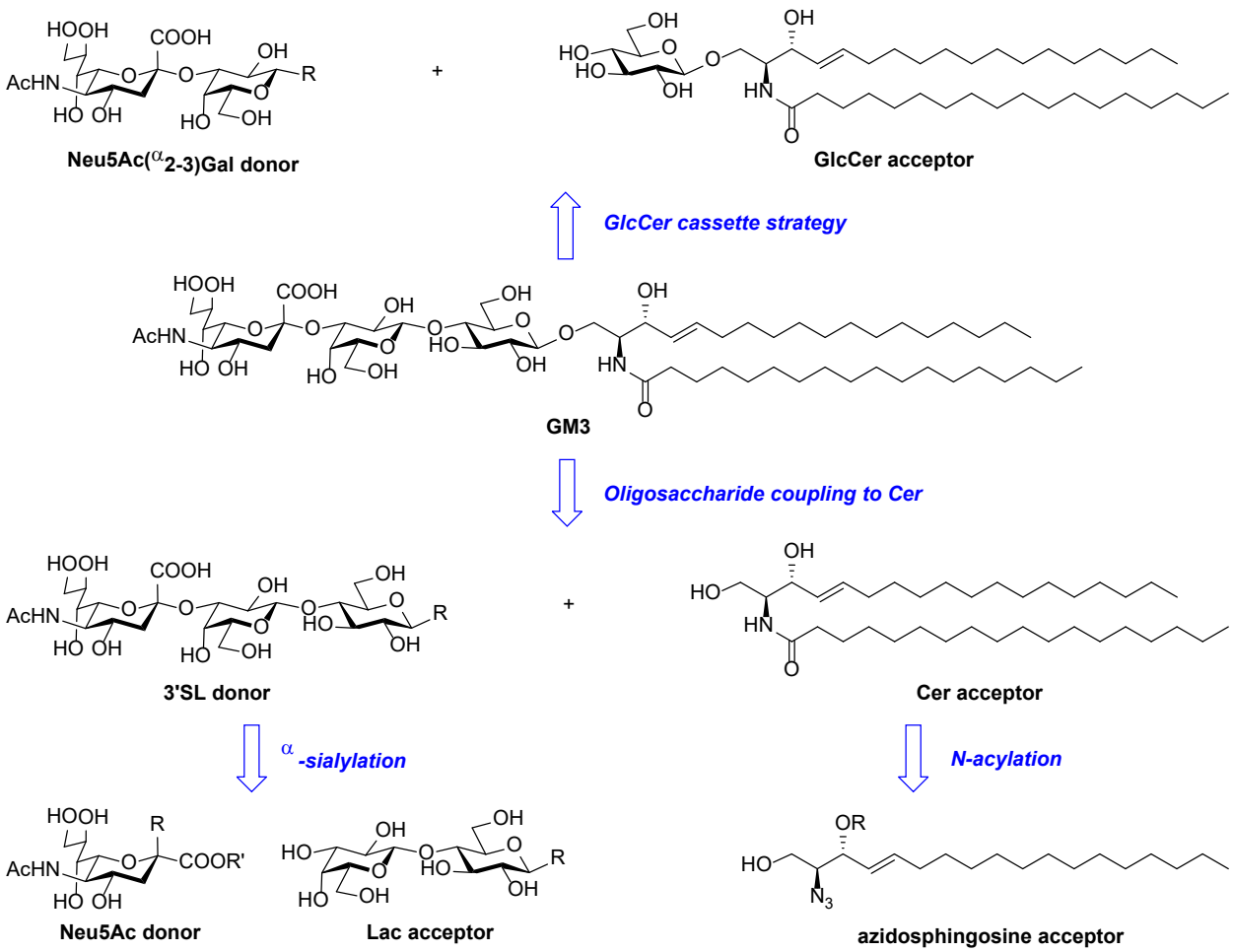


Figure 3. General synthetic approaches toward gangliosides, with GM3 shown as an example.

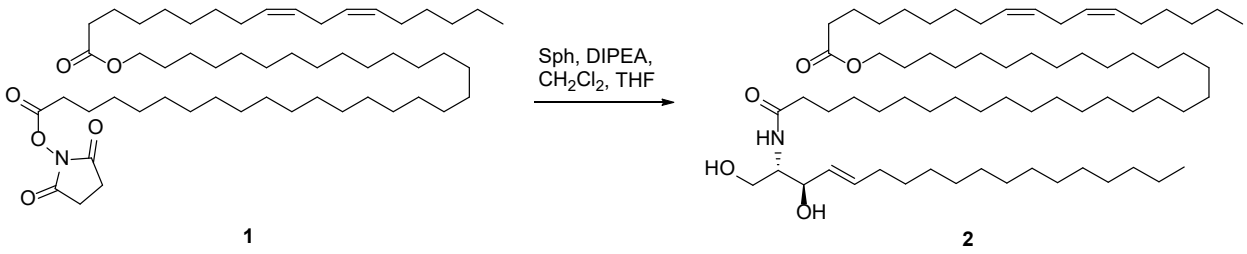
2.1 Chemical synthesis of sphingoid bases and ceramides

GSLs consist of a polar oligosaccharide head group, and an apolar tail, which is made up of a sphingoid base linked to a fatty acid via an amide bond (**Figure 1**). In vertebrate systems, the sphingoid base is D-*erythro*-sphingosine (2*S*,3*R* configuration), and synthesis of the Sph represents one of the major challenges in the construction of GSLs.[37,222] Sphingoid bases fall into three major classes: Sph, sphinganine, and the four diastereomers of phytosphingosine (**Figure 1**). The major challenges associated with the synthesis of sphingoid bases involve setting the *erythro* stereochemistry, and for sphingosine in particular, setting the *trans* stereochemistry of the alkene.[222] In general, *erythro* stereochemistry has been achieved from stereochemical starting

materials such as L-serine[223-233] or carbohydrates,[234-243] or from racemic intermediates using various asymmetric strategies.[244-256] Synthetic methods to access sphingoid bases have been thoroughly reviewed elsewhere.[257-260]

Once sphingosine has been synthesized, *N*-acylation with a fatty acid provides Cer (**Figure 3**). Cer syntheses tend to start from Sph, or a synthetic equivalent as a precursor.[261,262] However, because of its poor solubility and low reactivity, synthesis of Cer remains challenging. In general, the *N*-acylation reaction is carried out between a sphingosine and an activated fatty acid. One example is the synthesis of the unusual, human skin ceramides via coupling Sph with *N*-hydroxysuccinimide (NHS) fatty acid esters (e.g. **1**) to provide ultralong Cer analogs (e.g. **2**, **Scheme 1**).[263] The NHS-ester was used to form the amide of the target and also enhanced the solubility of the fatty acid.

Scheme 1. Synthesis of ultralong human skin ceramide.



1 **2**

Cross-metathesis (CM) strategies have been explored for the synthesis of ceramides.[264,265] Basu and Rai reported a four step synthesis of D-*erythro*-ceramide using truncated, Fmoc-protected, sphingosine analogues as precursors, which could be extended by a stereoselective olefin CM reaction.[266] Overkleeft and coworkers employed a similar strategy to prepare 6-hydroxyceramide analogue **3** (**Figure 4**).[264] Katsumura and coworkers synthesized ceramide **4** via a CM reaction.[265]

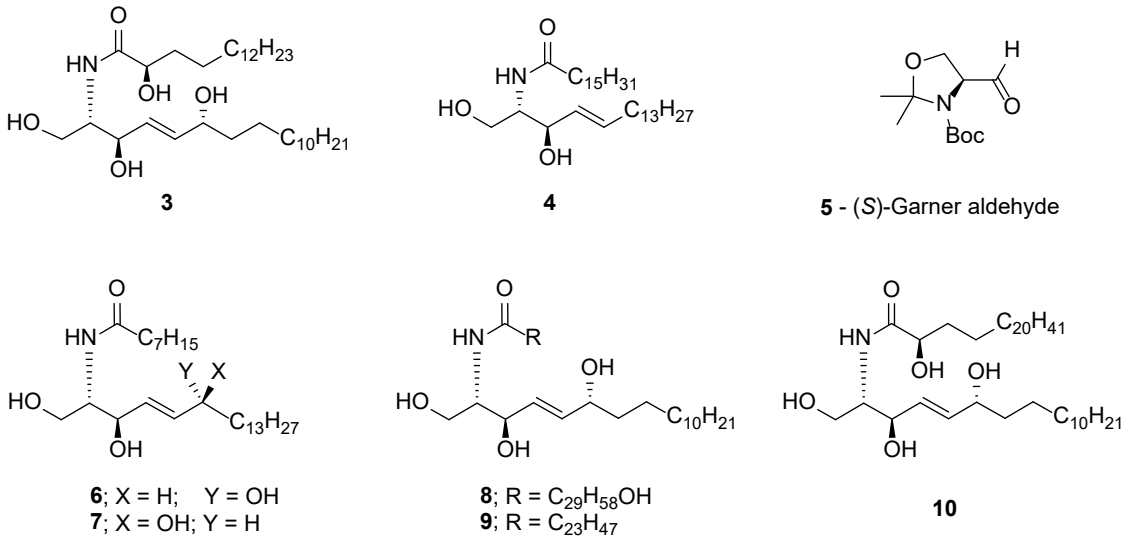


Figure 4. Ceramides prepared by cross-metathesis strategies.

The presence of additional modifications of the sphingosine, such as those in 6-hydroxyceramide analogue **3**, complicate the syntheses of sphingolipids further, and various approaches have appeared for their stereoselective synthesis.[228-231,264,267] Many strategies rely on the utilization of the L-serine-derived (*S*)-Garner aldehyde (**5**) as the central precursor. Bitmann and coworkers reported the asymmetric synthesis of **6** and **7** starting from **5**,[228] as well as the 5*S* and 5*R* diastereoisomers of *N*-acyl 5-hydroxy-3-sphingenines.[228] Masuda and Mori also employed a similar strategy to make a TBS-protected sphingenine **7**. The 6-hydroxylated ceramides (**8–10**) were prepared by *N*-acylation, followed by deprotection.[230] More recently, Vávrová and coworkers used a ruthenium catalyst to selectively reduce an alkyne as a key reaction to provide **9**.[267] Yadav and coworkers employed a double elimination strategy for the total synthesis of the (6*R*)- and (6*S*)-diastereomers of 6-hydroxysphingosine.[231] Sharpless asymmetric epoxidation of an allylic alcohol followed by reductive ring opening of the chiral epoxy chloride were key reactions.

Recently, Iga et al. developed two complementary methods for the synthesis of ceramide and sphingosine.[268] The strategy used sequential periodate oxidation, sodium borohydride mediated reduction, and acidic hydrolysis of galacto- or gluco-cerebrosides to form Cer analogues.

The major difficulty associated with this method is that periodate oxidation can destroy phytosphingosine-derived ceramides. Alternatively, sphingolipids isolated from natural sources could be converted into ceramides via sequential acidic hydrolysis followed by isolation of the sphingosine, and subsequent *N*-acylation with a suitable fatty acid (see **Sec 4**).

2.2 Chemical synthesis of cerebrosides, sulfatides, and derivatives.

Cerebrosides are GSLs containing only a monosaccharide head group, and as mentioned in **Sec 1.1.2**, GalCer is a common cerebroside found in the human brain, along with the 3-*O*-sulfated GalCer, sulfatide.[8] Several examples of GalCer, differing in the Cer moiety, are shown in **Figure 5** (**11**, **15–17**), and their syntheses from 1969 to 2003 have been reviewed.[269] The synthesis of sulfatides (e.g. **12** and **14**), have been reviewed by Compostella, et al.[270]

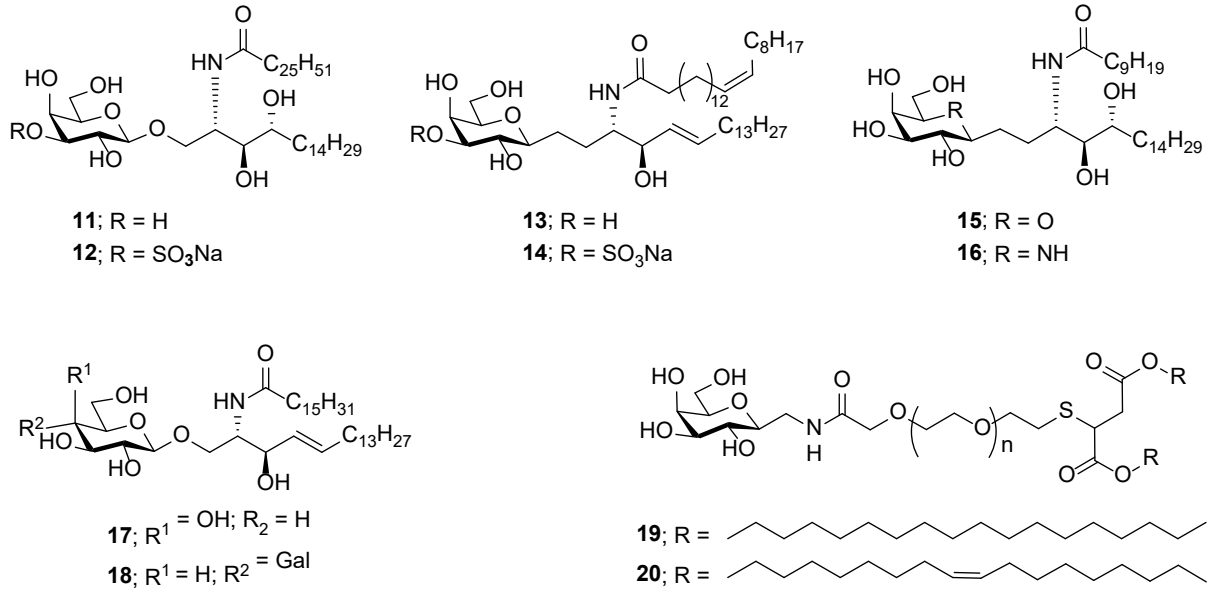


Figure 5. Structure of GalCer analogues

Various β-*C*- and β-*O*-glycoside GalCer analogues have been synthesized; many of which were for tested as inhibitors of HIV infection.[271-279] For example, sulfatide **12** was synthesized in five steps via glycosylation of azidosphingosine with a trichloroacetimidate donor.[271] Both the β-

C- and β-*O*-glycosides of sulfatide **14** have been synthesized.[272] A Wittig sigmatropic rearrangement was followed by a Horner-Wadsworth-Emmons olefination (HWE) to install the Sph backbone for the stereoselective synthesis of derivative **13** over thirteen steps. This was followed by regioselective sulfation of Gal to provide **14**. Lankalapalli and Gorantla also employed the HWE reaction to synthesize β-*C*-GalCer **15**, as well as aza-*C*-GalCer **16**.[275] Stannyl Cer was directly glycosylated with an α-iodo galactoside donor in a chemo- and stereo-selective route to access GalCer **17**.[273,274] Disarmed glycosyl donor-promoter pairs provided excellent yields and stereoselectivity when glycosylated with a broad range of stannylated glycosyl acceptors.[274] Recently, Taylor and coworkers developed a concise, convergent route to glycosyl ceramides using diphenylboronic acid-promoted β-selective glycosylation of unprotected ceramide with a glycosyl methansulfonate.[277] Selective activation of the ceramide 1,3-diol with diphenylborinic anhydride, followed by glycosylation with the corresponding glycosyl mesylates, and subsequent cleavage of the protecting groups afforded GalCer **17** and LacCer **18** in two steps. This synthetic route could have applications to the GlcCer cassette strategy for more complex GSLs (see **Sec 2.3**).[26] Thota, et al., synthesized the C-glycoside derivative of **17** as a potential HIV inhibitor, over sixteen steps using a Sharpless dihydroxylation, followed by a CM reaction, and subsequent manipulations to install the ceramide moiety.[278] To test variations in fatty acid chains, LaBell, et al., synthesized glycolipids **19** and **20** with unnatural saturated and unsaturated lipid tails.[279] Synthesis was achieved through a convergent coupling between methylene amine functionalized β-galactoside and pentafluorophenyl activated lipids.

Isolation of marine-derived α-linked galactosyl ceramides from *Agelas mauritianus* was first reported by researchers at Kirin Pharma, and these compounds, called agelasphins, were found to act as stimulants of human leukocytes.[280-282] Since then, the synthesis of α-linked GalCer KRN7000 and analogues has been an active area of research and is reviewed in detail elsewhere.[283,284] Synthesis of α-GalCer has primarily relied on three strategies: direct glycosylation

of functionalized ceramide with an activated sugar;[73,285-289] coupling of the azidosphingosine with an activated sugar followed by attachment of the fatty acid;[236,238,290-295] or construction of the glycosyl ceramide from a disaccharide precursor.[296-299] Of these methods, a one-pot strategy developed by Gervay-Hague and co-workers has also been applied to the synthesis the vertebrate β-GalCer.[300]

2.3 Chemical synthesis of gangliosides

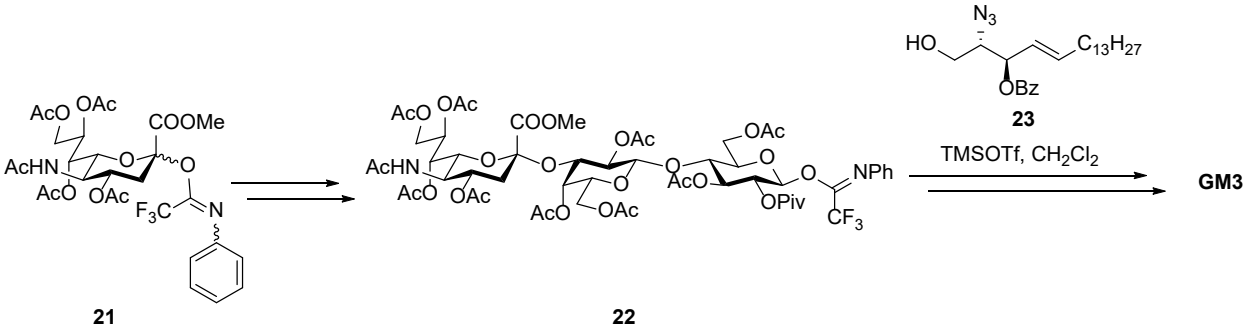
Many synthetic strategies to construct gangliosides focus on regio- and stereo-selective α-sialylation of the oligosaccharide, and Ando et al. have recently catalogued synthetic methods for α-sialylation with modified sialic acids.[38] Subsequent glycosylation of the ceramide, a protected sphingosine (like azidosphingosine, **Figure 3**),[222] or GlcCer provides the target. As mentioned earlier, the latter is often referred to as the GlcCer "cassette strategy".[26] This section summarizes the synthesis of a mammalian ganglio-, lacto- and neolacto-series of GSL structures, focusing on those not previously reviewed elsewhere.

Various syntheses of GM3[28-30] and derivatives[30,301-305] have been reported. Synthetic equivalents derived from the ganglioside GM3 have been used as a precursor for synthesis of other ganglio-series gangliosides such as GM1[41-43] and GD3.[44] Other chemical methods for the synthesis of gangliosides including GM2[39,40] and GP1c[49] have been reported, along with analogues of GM4[27] and GD3.[45-47,306] In particular, the GlcCer cassette strategy has been exploited for the synthesis of a series of natural gangliosides including GM3,[32] GM2,[32] GM1,[32] GQ1b,[48] *N*-acetylgalactosamine (GalNAc)-GD1a,[32] and gangliosides X1[61,62] and X2.[62]

Duclos reported one of the first total syntheses of GM3,[29] in which a GM3 trichloroacetimidate donor was directly coupled with a protected Cer acceptor using a convergent strategy. Selective α-sialylation was obtained with a β-phosphite donor of Neu5Ac. Kurosu et al. employed a similar procedure in synthesis of GM3 derivatives with truncated Cer moieties.[30] In

their synthesis of GD3, Schmidt and coworkers also used a β-sialyl phosphite donor.[44] Li and coworkers used a sialyl *N*-phenyltrifluoroacetimidate donor (**21, Scheme 2**) to synthesize an (α2-3)-sialoside intermediate that was subsequently converted to the GM3 trisaccharide *N*-phenyltrifluoroacetimidate (**22**).[31] The trisaccharide donor was then coupled with an azidosphingosine acceptor (**23**), followed by further manipulations to provide the target. Derivatives of GM3 have been synthesized including those with different acyl moieties either at the *N*-5 of Neu5Ac, *N*-3 of the Cer,[301] or with a phytoceramide.[302]

Scheme 2. Synthesis of GM3 using sialyl *N*-phenyltrifluoroacetimidate donor **21**

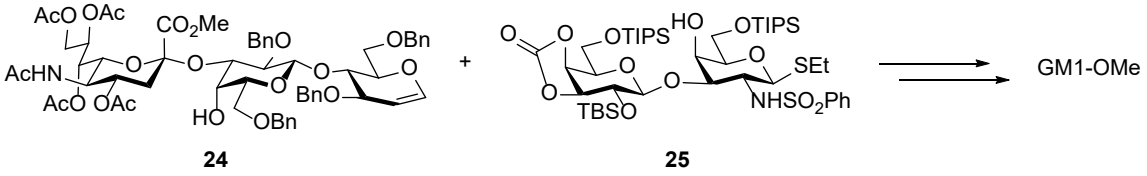


Kiso and coworkers synthesized GM2 analogs with various glycosyl lipid units using a strategy of adding the lipid acceptors to GM2-core tetrasaccharide donors.[39] The same group extended their studies with a more general route to construct both GM2 and its isomer 6'-Neu5Ac-GM2.[40] The route was a sequential process with the key step involving an *N*-Troc-protected sialyl donor reacting with the appropriate Lac acceptors in a regio- and α-selective manner to form the 3′- or 6′-sialylated trisaccharide intermediates. Addition of a protected GalNAc and an azidosphingosine, followed by further manipulations, provided GM2, as well as its 6'-Neu5Ac-GM2 isomer. Kiso and coworkers later used the Troc-protected GM2 acceptor intermediate for synthesis of GM1, GD1a, and GT1a.[42]

To evaluate the influence of the core carbohydrate domain of GM1 in binding to *P. aeruginosa*, Danishefsky and coworkers reported a total synthesis of the methyl glycoside of the

GM1 oligosaccharide (GM1-OMe) using a [3+2] glycosylation strategy.[41] The key step was azaglycosidation of sialylated glycal acceptor **24** with thioethyl glycoside **25**. The resulting protected pentasaccharide was transformed to GM1-OMe over six steps (**Scheme 3**). A similar strategy was followed by Huang and coworkers to produce a GM1 glycoside functionalized with an aminopropyl linker moiety.[43]

Scheme 3. Synthesis of GM1-OMe

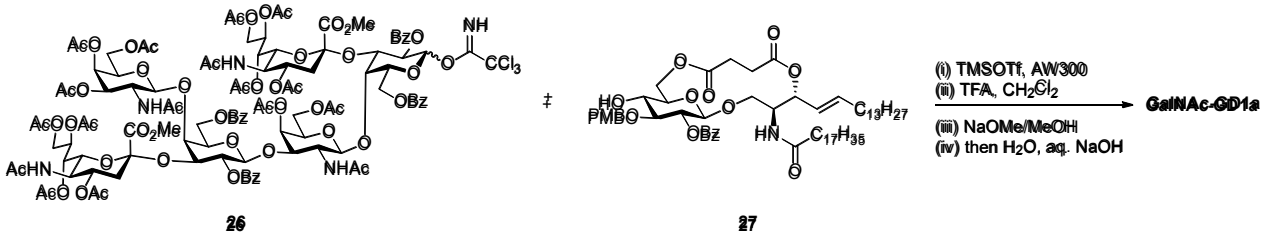


In the synthesis of the methyl glycoside of the GD3 oligosaccharide (GD3-OMe), De Meo, et al., formed the α-disialoside from a 2-thioglycoside of Neu5Ac and a suitably protected sialyl acceptor.[46] The disialoside was then converted into a thiosialoside and reacted with 3'- and 4'-unprotected Lac to give the GD3 tetrasaccharide. Tsvetkov and coworkers reported an improved synthesis of GD3-OMe using an *N*-trifluoroacetyl sialoside as an efficient sialyl donor.[47] Electron withdrawing protecting groups enhanced the reactivity and steoreoselectivity of sialyl donors in (α2-8)-sialylation with various sialyl and glycosyl acceptors.

The first total synthesis of a hybrid-type ganglioside structure, ganglioside X1,[61] was achieved by Kiso and coworkers using a convergent strategy based on direct coupling between the oligosaccharide and Cer. An α-sialylated heptasaccharide trichloroacetimidate donor with a *C*-2 pivaloate on the reducing-end Glc was employed to increase efficiency of glycosylation to the Cer. Next, the same group reported the total synthesis of GQ1b[48] based on a GlcCer cassette approach. This strategy was further expanded to use a cyclic GlcCer cassette as an acceptor for the total syntheses of highly branched gangliosides, like GalNAc-GD1a (**Scheme 4**).[32,60] The macrocyclic acceptor **27** was obtained via an intramolecular glycosylation between a glucosyl imidate donor

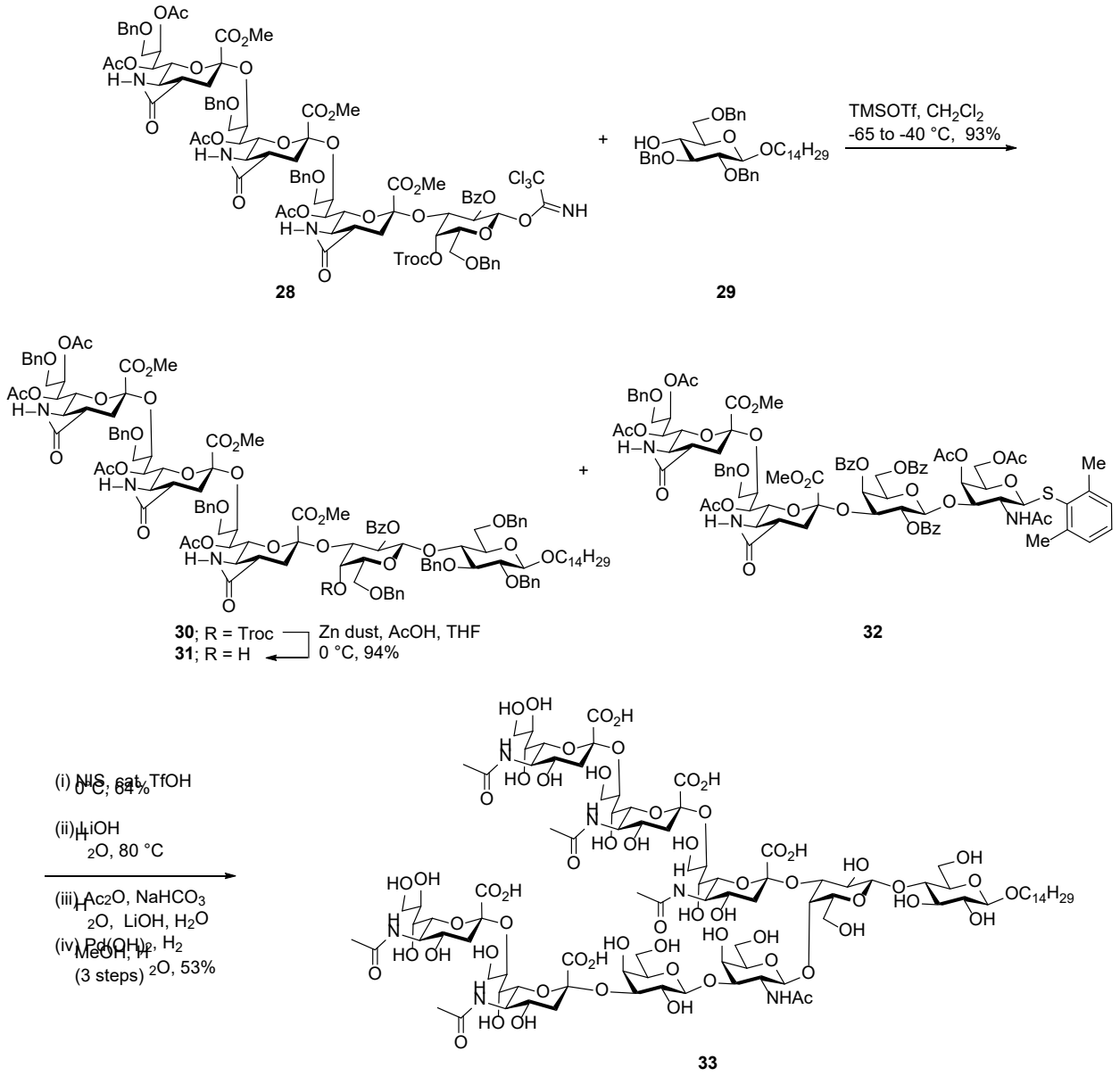
and a ceramide acceptor as a critical step.[307] Acceptor **27** was then reacted with the GM2-core imidate donor **26**, followed by global deprotection to give GalNAc-GD1a.[60] A similar approach was used for the synthesis of GalNAc-GM1b,[59] as well as gangliosides X1[61,62] and X2.[62]

Scheme 4. Synthesis of ganglioside GalNAc-GD1a



GP1c is one of the most complex gangliosides known, and its synthesis has been realized by Takahashi and coworkers (**Scheme 5**).[49] A convergent synthesis of ganglioside GP1c oligosaccharide **33** was achieved via coupling of the tetrasaccharide donor **28** with tetradecyl glucoside **29** in the presence of TMSOTf to provide pentasaccharide **30**. Removal of the Troc-protecting group provided acceptor **31** in excellent yield. Condensation of acceptor **31** with two equivalents of the protected thioglycoside donor **32** followed by global deprotection and *N*-acylation of the *C*5 amino groups provided nonasaccharide **33**.

Scheme 5. Synthesis of tetradecyl GP1c glycoside.



Hydrolytically stable derivatives of GM3, GM4, and GD3 have been synthesized using a number of strategies. Hossain et al. described synthesis of the GM3-tetrasaccharide lactam **34** (**Figure 6**).[306] Tietze et al. synthesized a cyclic-ether containing stable analogue of GM3 **35**.[303] Bundle and coworkers have used two different strategies to synthesize a glycosidase-resistant *S*-linked Neu5Ac-analogues of GM3 (**36–37**).[304,305] Sodeoka and coworkers developed a stereoselective route using an Ireland-Claisen rearrangement to synthesize a nonhydrolyzable

GM4 analogue (**38**).[27] Elleruik and Meijer used an interhalogen-promoted α-sialylation of a disialoside donor to synthesize GD3 bislactams **39** and **40**.[45]

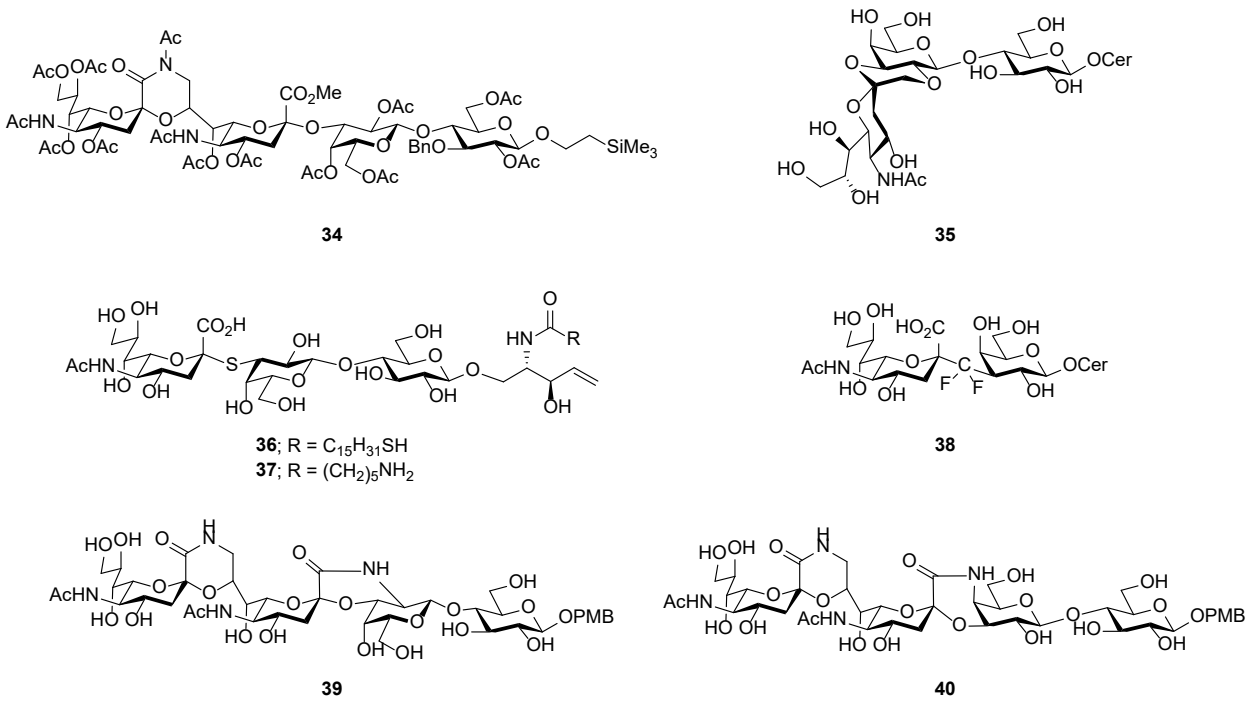


Figure 6. Nonhydrolyzable derivatives of GM3, GM4, and GD3

2.4 Neoglycolipids

Various syntheses of neoglycolipids have been reported to investigate their physical properties,[308] as well as to develop novel compounds that mimic their natural analogues.[309,310] Kiso and coworkers synthesized sialyl Lewis X (sLe^x) neoglycolipids containing a novel, *N*-deacetylated and lactamized sialic acid **41**.[311] They also synthesized a neoglycolipid containing sLe^x on the mucin GlcNAc(β1-6)GalNAc(α) core structure.[85] Other sLe^x neoglycolipids with different spacer lengths and structures (e.g. **42**, **Figure 7**) have been synthesized to determine their effects on leukocyte rolling[86] and sLe^x presentation.[312] Simplified sLe^x neoglycolipids embedded in liposomes were recognized by E-selectin.[313] These derivatives were more selective for E-selectin than native sLe^x, suggesting that simplified glycolipid derivatives could be a fruitful approach to targeting adhesion receptors.

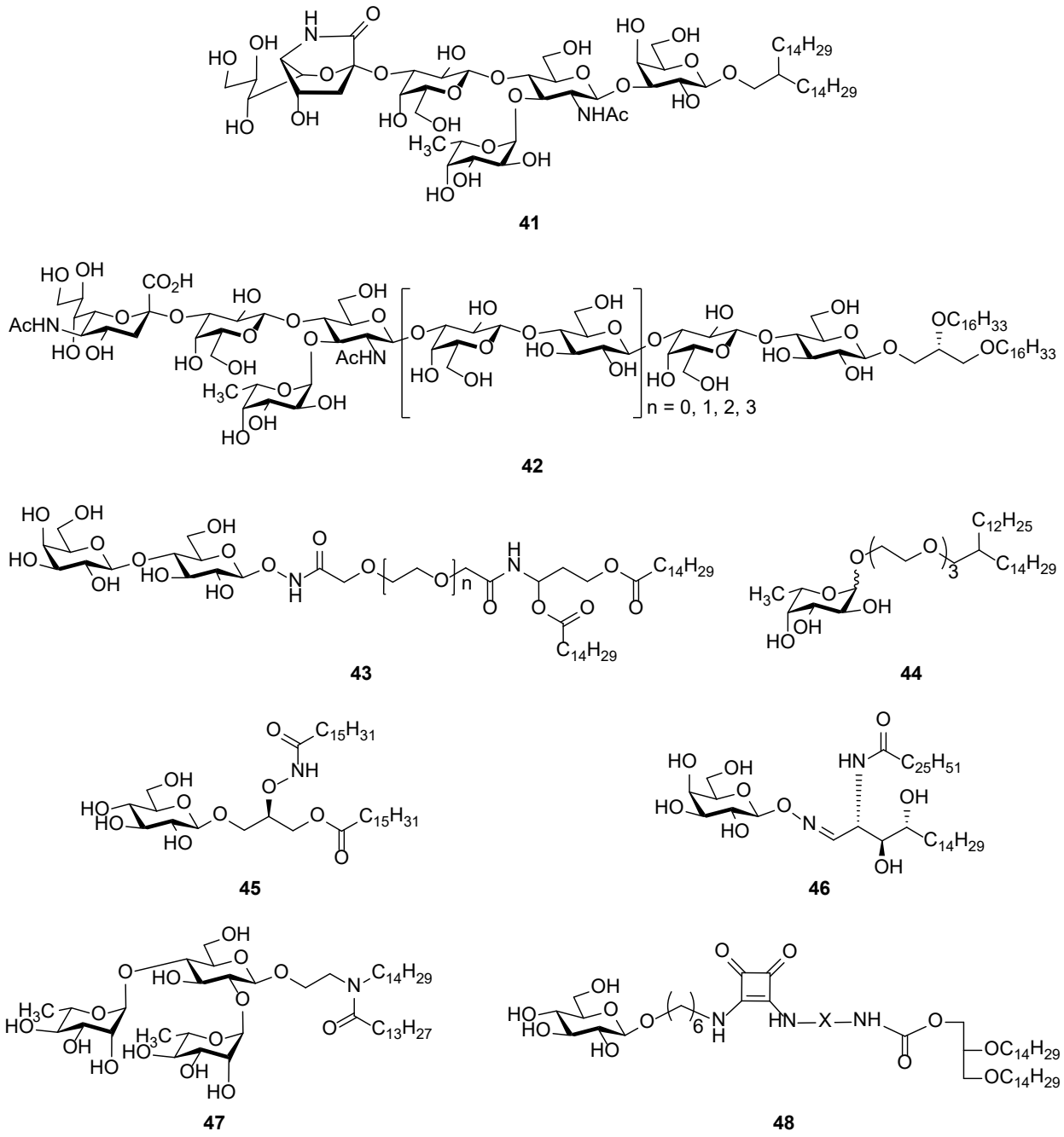


Figure 7. Structures of neoglycolipids discussed in this section.

Amphiphilic Lac-based neoglycolipids differing in the length of the spacer fragment, or the aliphatic chain have served as components of targeted drug delivery systems.[314-316] A series of Lac-based neoglycolipids with varying ethylene-glycol aglycones were prepared, and a representative, **43**, is shown in **Figure 7**.[315,316] Fuc derivatives with ethylene glycol linkers, such

as **44**, were synthesized by Faivre et al., and assessed for their potential as amphiphilic drug carriers. The anomeric configuration of Fuc influenced the organization of monolayers but not the fluidity of liposomes.[317]

Various *N*-oxyamide-linked neoglycolipids (**45**) were prepared as a way to improve metabolic stability and hydrogen bonding properties of the glycolipid,[318] as oxime linkages are hydrolytically stable under physiological conditions.[319] Other synthetic neoglycolipid probes have been generated via oxime ligation of the reducing end with an aminooxy-functionalized lipid.[320] Wang et al. developed a concise route to synthesize GalCer analogues (**46**) via oxime ligation from β-D-galactose pentaacetate-derived galactosyl oxyamines.[321] GM3 neoglycolipid analogs with *N*-methylaminooxy-modified ceramide lipid tails have been evaluated for activity against matrix metalloprotein-9 as inhibitors of tumor migration.[322]

Nohara and coworkers reported a coupling of a chacotrioside (L-rhamnosyl-(α1-4)-[L-rhamnosyl-(α1-2)]-D-Glc)-based neoglycolipids exemplified by **47**.[323] Maslov et al. used a bivalent squaramide linker to connect a galactose derivatives with the glycerol moieties of neoglycolipids like **48**.[324] A multicomponent strategy for the synthesis of ceramide analogues and their application for the synthesis of glycolipids was established using an Ugi four-component reaction.[325] Recently, a report of using boronic acids as phase transfer reagents has allowed for the direct coupling of unprotected monosaccharides to sterol-derived aglycones.[326]

2.5 Chemical degradation methods for semisynthesis

Advanced intermediates for GSL syntheses are commonly obtained by partial degradation of glycolipids isolated from natural sources. Semisynthetic methods for glycolipid synthesis involve the controlled degradation of isolated GSLs and can be essential for generating targets that incorporate modified groups, such as tags or labels. Both chemical and enzymatic methods have

found use in semisynthesis and analysis of glycolipids. This section introduces some common chemical strategies for semisynthetic degradation available for the synthesis of GSL targets.

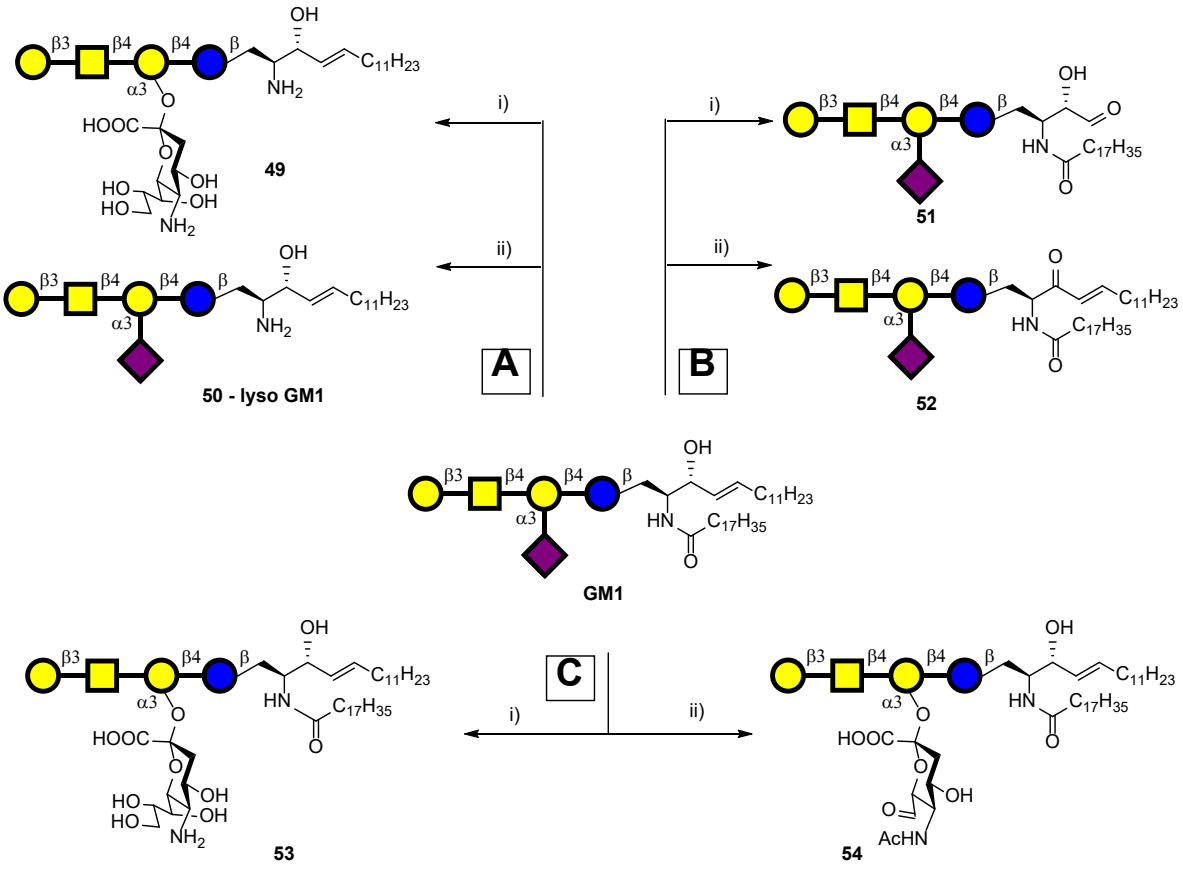


Figure 8. Common methods for the selective chemical degradation of glycolipids, using GM1 as an example. A) Removal of the fatty acid by alkaline hydrolysis, as detailed in **Sec 2.5.1**; general conditions: i) KOH to give amine **49** (**49**), ii) to produce lyso GM1(**50**). B) Oxidation of the sphingosine alcohol or alkene, as detailed in **Sec 2.5.2**; general conditions: i) O_3, or $NaIO_4$ with OsO_4 to yield an aldehyde (**51**), ii) DDQ-mediated oxidation to provide the α,β-unsaturated ketone **52**. C) Chemical degradation of the glycan; general conditions: i) selective sialic acid de-*N*-acetylation to give amine **53**, ii) $NaIO_4$ oxidation to produce aldehyde **54**.

2.5.1 Removal of the fatty acid chain

Perhaps the most common semisynthetic approach to generate glycolipid probes involves alkaline hydrolysis of the fatty acid chain to produce a lyso-glycolipid that contains a free amine for further functionalization. A discussion of the re-acetylation of lyso-glycolipids with labeled fatty acids to

generate glycolipid probes can be found throughout **Sec 4**. Early hydrolysis conditions used 1 M KOH in 90 % butanol (**49, Figure 8 A.i.**),[327,328] but modifications include changing the solvent to aqueous methanol[329] or mild basic conditions, such as 0.3 M KOH[330] or 1 M NaOH.[331] Even under these milder conditions, the sialic acid *N*-acetamido group is typically cleaved, while *N*-acetylgalactosamine residues tend to be stable. Lyso-gangliosides have been obtained after selective sialic acid *N*-acylation of deacetyl-deacyl gangliosides.[329] Changing the base to $(CH_3)_4NOH$ has improved yields of the lyso-gangliosides.[332] Sodium *tert*-butoxide in anhydrous methanol is reported to selectively remove the *N*-acyl fatty acid and generate lyso-GM3 (**Figure 8 A.ii.**)[333]

2.5.2 Sphingosine degradation

Selective oxidation can be used to introduce a variety of functional groups into the sphingosine portion of GSLs. Methods have typically targeted either the *C*-3 alcohol or the *C*-4 alkene of the sphingosine moiety. Ozonolysis of the sphingosine *C*-4 alkene generated an aldehyde-containing GSL[334,335] (**51, Figure 8 B.i.**), which can be further functionalized. The resulting α-hydroxyaldehyde GSLs are sensitive to alkaline conditions, which can release the carbohydrate moiety through a β-elimination mechanism.[335] The glycan of ozone-treated GSLs can be released in near-neutral conditions, allowing more reliable analysis of glycan composition.[336] Further treatment of aldehyde-containing GSLs with H_2O_2 generated a *C*-4 carboxylic acid, which could then be used for amide bond-forming reactions.[337] Quenching of the ozonolysis reaction with Me_2S[338,339] or PPh_3[340] allowed for the isolation of the aldehyde-containing GSLs, which could be further derivatized via the aldehyde. Ozonolysis of peracetylated GSLs, followed by reduction with $NaBH_4$, generated GSLs with a truncated sphingosine containing a *C*-4 primary alcohol.[341]

Oxidation of the GSL alkene with periodate is an alternative to ozonolysis but requires protection of the glycan. After peracetylation of the GSL, OsO_4 was used to catalyze oxidation by

sodium periodate to generate a GSL containing a *C*-4 sphingosine aldehyde (**51, Figure 8 B.i.**) Subsequent hydrolysis under alkaline conditions released the carbohydrate moiety.[342] Using periodic acid instead of sodium periodate improved the yields of the GSL aldehyde **51**, which could then be used to release the glycan (under alkaline conditions), or could be oxidized to a carboxylic acid using performic acid.[343] Treatment of peracetylated GSLs with $KMnO_4$ in the presence of dicyclohexyl-18-crown-6 or acetone generated carboxylic acid-containing GSLs directly.[343,344] This method has been further modified to use less potassium permanganate and an excess of sodium periodate.[345,346] Oxidation under basic conditions produced the further truncated serine acids.[346]

Methods targeting the alcohol at *C*-3 of sphingosine for oxidation have primarily employed 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) as the oxidizing agent. DDQ specifically oxidized the *C*-3 alcohol to a ketone in neutral glycosphingolipids[347,348] and gangliosides (**52, Figure 8 B.ii.**)[349] Although this degradation method has primarily been used for radiolabeling of glycosphingolipids through subsequent reduction of the ketone with NaB^3H_4 (see **Section 4.1.1**), treatment of the 3-keto-GSLs, like **52**, with triethylamine has been used to promote β-elimination release of the carbohydrate moiety.[350,351] Recently, a new approach to oxidative degradation was reported in which NaClO was used to release glycan nitriles from GSLs, which could be reduced with $NiCl_2$-$NaBH_4$ to generate an ethylamine aglycone. The proposed oxidative mechanism does not involve the alkene or the alcohol at *C*-3, but rather the oxidative elimination of the amide.[352]

2.5.3 Glycan degradation

Residue-selective degradation methods for the glycan of GSLs typically target sialic acid residues. The selective removal of the *N*-acyl group of sialic acid was applied to the semisynthesis of Neu5Gc GM1.[332,353] Hydrazinolysis was also used to selectively remove the sialic acid *N*-acyl group (**53, Figure 8 C.i.**)[354] A milder and more versatile sialic acid degradation method has been

periodate oxidation of the glycerol side chain to generate an aldehyde at *C*-7 of sialic acid (**54, Figure 8 C.ii.**)[355,356] The first application of sialic acid oxidation with sodium periodate for semisynthetic labeling was on a sialoglycoprotein substrate;[357] however, the method was expanded to a range of ganglioside substrates[358,359] and is still used for the controlled degradation of sialic acids on ganglioside substrates.[161,360 (refs therein) 195,361] Mild periodate oxidation conditions are compatible with sialic acid labeling on live cells (see **Sec 4.4.1**).[362-365]

3 Chemoenzymatic synthesis of glycolipids

A major challenge to the synthesis of glycolipids lies in generating the glycan moiety with regio- and stereo- selectivity. Chemical approaches, such as those described in **Sec 2** of this review, typically involve careful manipulation of protecting groups and glycosylation strategies that may require long synthetic routes. In contrast, chemoenzymatic approaches exploit the selectivity of enzymes to catalyze glycosylation without the need for protecting groups. The advantages of using enzymes for glycan synthesis were recognized as soon as biosynthetic enzymes were identified;[366] however, due to limited access to the enzymes and sugar nucleotide donors,[367] the application of enzymatic approaches were initially limited. Excellent reviews pertaining to the biosynthesis of glycosphingolipids are available.[6,368,369] An improved understanding of glycan anabolism, catabolism, enzyme mechanisms, and the development of protein engineering techniques have led to improved efficiency and reduced costs for enzymatic approaches. Several recent reviews are available on this topic.[370-376] This section will give a brief overview of the development and principles of enzymatic synthesis as applied to GSLs and glycolipids.

3.1 Chemoenzymatic synthesis using glycosyltransferases

Most mammalian glycosyltransferases are membrane-associated and difficult to purify, making them incompatible with large scale preparations. This limitation, along with the high cost of sugar

nucleotides, was a major limitation for early chemoenzymatic approaches to the synthesis of simple oligosaccharide fragments.[367,377] A major breakthrough for chemoenzymatic approaches to glycan synthesis was the finding that many pathogenic bacteria expressed oligosaccharide epitopes that mimic mammalian carbohydrate structures,[378] which led to the discovery of bacterial glycosyltransferases with similar functions to mammalian enzymes.[379] Bacterial glycosyltransferases are more easily expressed as soluble recombinant proteins and often have broad substrate scope for glycosyl acceptors.[379] The substrate scope, physical properties, and efficiencies of these enzymes may be further improved through protein engineering.[374]

Ceramides tend to be too hydrophobic to act as acceptors in glycosyltransferase-mediated glycosylation.[380] The chemoenzymatic synthesis of GSLs has instead employed sphingosine or 2-azidosphingosine as the glycosyl acceptor, with the fatty acid introduced in the last step of the synthesis, similar to many chemical synthetic methods.[29,35,36,380-382] This approach is still relevant for the total synthesis of glycolipids using chemoenzymatic methods.[33,383] The glycans of glycolipids can be synthesized in a combinatorial manner analogous to their biosynthetic pathways.[384,385] The high cost of sugar nucleotides used as donors in enzymatic glycosylation was lowered by the development of sugar nucleotide generating systems, where the donors were generated in situ from cheap starting materials. Sugar nucleotides such as UDP-GlcNAc, UDP-GalNAc, UDP-Gal, and GDP-Fuc can be generated from monosaccharides using glycokinase and sugar nucleotide pyrophosphorylase (or nucleotidyltransferase).[375] CMP-Sia can be generated from sialic acids and CTP using the CMP-sialic acid synthetases (CSS). Sialic acids may also be generated in situ from mannose analogues and pyruvate using aldolase enzymes.[386] These sugar donor-generating systems have been used in combination with different bacterial glycosyltransferases in glycolipid synthesis.[375] In practice, the generation of sugar donors and glycosyltransferase-catalyzed glycosylation can be conducted in one pot, and this strategy is known as a one-pot multienzyme (OPME) system.[375,387] The OPME systems reduced the

purification steps needed for chemoenzymatic syntheses, leading to more straightforward synthetic procedures and higher product yields.[375] Strategies to simplify this approach have included immobilization of the panel of enzymes onto solid-support, known as "super beads". [388,389] A summary of enzymes relevant for chemoenzymatic synthesis of glycolipids discussed in this review is provided in **Table 4**.

3.1.1 Synthesis of ganglio-series glycosphingolipids

For ganglio-series glycolipids, chemoenzymatic strategies have typically started from Lac or lactosides (**Figure 9**). Selective sialylation of lactoside was achieved using three (α2-3)-sialyltransferases (SiaT) identified from *P. multocida:* PmST1^{M144D}[33,63], PmST2[390], or PmST3.[391] PmST1^{M144D} is a mutant form of the multifunctional enzyme PmST1. The M144D mutation has decreased (α2-3)-sialidase and donor hydrolysis activities without impeding (α2-3)-SiaT activity.[392] PmST1 preferred oligosaccharide acceptors, so Lac was primarily used as the acceptor, and the lipid moiety was incorporated afterwards using chemical synthesis.[33,63] PmST2 and PmST3 are both monofunctional (α2-3)-SiaTs, which can use lactosyl lipids as acceptors. PmST2 preferred glycolipids and PmST3 accepted both oligosaccharides and glycolipids.[390,391]

Several SiaTs responsible for the biosynthesis of lipo-oligosaccharides in *C. jejuni* have found wide application in the synthesis of ganglio-series glycans.[393] The (α2-3)-SiaT CjCstI was used to sialylate a lactosyl lipid,[79] and has been applied to the synthesis of fluorescent GM3 analogues as FRET probes for ganglioside-processing enzymes.[394] Another multifunctional (α2-3/8)-SiaT, CjCstII, was used to sialylate the GM3 trisaccharide.[63,395] The enzyme could also use Lac and GD3 oligosaccharides as acceptors. Starting from lactoside and an excess of CMP-Neu5Ac, CjCstII caltalyzed multiple sialylations with the yields of GD3 and GT3 corresponding to the equivalents of CMP-Neu5Ac in the reaction.[396] Many SiaTs can tolerate sialic acid modifications. Using PmST1^{M144D}, GM3 trisaccharides with Neu5Gc, Kdn, and Neu5Ac8OMe

sialic acids have been synthesized.[63] A mutant form of CjCstII, CjCstIIΔ32^{I53S} was used to make GD3 oligosaccharide analogues with *C*5 or *C*9 modifications on the terminal or penultimate sialyl groups.[397] In the synthesis of several Neu4,5Ac$_2$-containing glycans, including GM3, only PmST3 could accommodate CMP-Neu4,5Ac$_2$ and CMP-Neu4Ac5Gc donors.[398] Glycosphingolipid analogs have also been tolerated. The chemoenzymatic synthesis of GM3 and GM2 analogs with a truncated ceramide,[399] and GM3 analogs with alkyl aglycones have been reported.[400,401] Recently, soluble recombinant enzymes have been used to modify glycolipid substrates embedded within a liposome.[402] An *N*-acetylglucolipid was inserted into a phospholipid liposome and was extended by the sequential action of a (β1-4)-GalT and a trans-sialidase to make liposome-embedded GM3 analogues.[402]

The glycans of a number of complex gangliosides have been synthesized using chemoenzymatic strategies. The (β1-4)-GalNAcT CjCgtA from *C. jejuni* introduced a GalNAc to GM3/GD3/GT3 to generate the corresponding GM2/GD2/GT2 glycans in high yields. However, the enzyme did not tolerate asialo substrates.[403] GM2 and GD2 oligosaccharides were extended to generate GM1 and GD1b using the (β1-3)-GalT CjCgtB (*C. jejuni*).[63,396] A mammalian (α2-3)-SiaT (ST3GalI)[404] was used to sialylate GM1 to generate GD1a in high yields.[396] The synthesis of gangliosides containing disialylated glycans where the two sialic acids are linked in different configurations is challenging. The SiaTs PmST1 and Pd2,6ST (an (α2-6)-SiaT) were employed to generate the non-reducing end tetrasaccharide of GT1aα and GQ1bα in a mixed synthetic and enzymatic approach.[405] The Neu5Ac(α2-3)Gal linkage was generated with PmST1, followed by chemical 1,4-lactonization between the *C*1''-carboxyl group of Neu5Ac and the *C*4'-hydroxyl group of the adjacent Gal, which enabled the selective formation of the Neu5Ac(α2-6)GalNAc linkage using Pd2,6ST.[405] Thio-oligosaccharides have been used as substrates for glycosyltransferases. The CjCgtA and CjCstII enzymes recognized S-linked GM3 as an acceptor

to synthesize S-linked GM2 and GD3, which were conjugated to proteins and used as immunogens.[305]

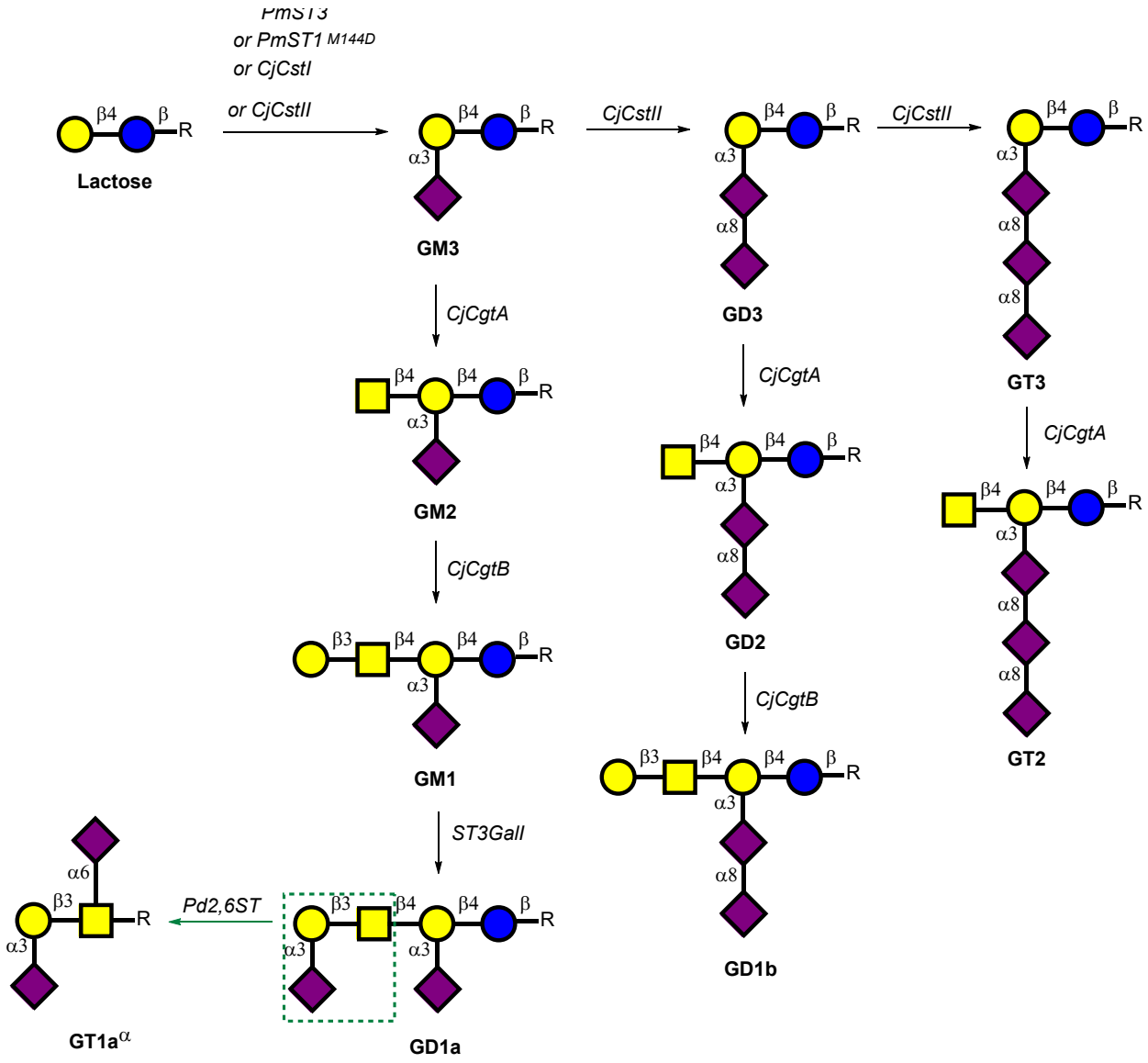


Figure 9. Chemoenzymatic synthesis of ganglio-series oligosaccharides. For clarity, glycolipid names are used to describe only the glycan moiety, where R can be OH in the oligosaccharides or Cer for glycolipids. The green box indicates the partial structure that was used in the transformation described by the green arrow.

3.1.2 Synthesis of globo/isoglobo and lacto/neolacto series glycosphingolipids

Chemoenzymatic syntheses of globo/isoglobo and lacto/neolacto series oligosaccharides have been reported (**Figure 10**). An (α1-4)-GalT, NmLgtC,[71] was used to construct the (α1-4) linkage

between the two galactose residues in the globotriose Gb3.[63-66] A bovine (α1-3)-GalT (Bα1-3GalT) catalyzed the formation of the (α1-3) linkage in isoglobotriose iGb3. The (β1-3)-GalNAcT activity of HiLgtD was used to convert Gb3/iGb3 to Gb4/iGb4. HiLgtD was also used to synthesize Gb5 (stage-specific embryonic antigen-3, SSEA-3) and iGb5 pentasaccharides in moderate yields.[64] Improved yields of Gb5 were obtained with the (β1-3)-GalNAcT, CjCgtB.[63] A sialyl group, with or without modifications at the C8 position, could be introduced to Gb5/iGb5 using CjCstI[75] or PMST1^{M144D} [63] to generate sialyl-Gb5 (also called stage-specific embryonic antigen-4, SSEA-4) or sialyl-iGb5. Another sialyltransferase with a broader acceptor scope, JT-FAJ-16 (*Vibrio sp*.),[406] catalyzed this step.[64] A large scale chemoenzymatic synthesis of Gb3 and iGb3 has been developed using a chemoenzymatic strategy which exploited an epimerase to convert UDP-Glc to UDP-Gal (GalE, from *E. coli*).[72] The total synthesis of Gb3 and iGb3 using a combination of chemoenzymatic and synthetic methods has been reported.[70]

The discovery of bacterial fucosyltrasferases has allowed for the enzymatic syntheses of more complex globo/isoglobo series oligosaccharides. The (α1-2)-FucT, EcWbsJ[407], and HpFutC[408] enzymes were used to fucosylate Gb5 for the synthesis of Globo H.[64,75] Using the fucosyltransferase HpFutC, sialyltransferase JT-FAJ-16, and the NTP recycling system, allyl derivatives of Globo H and SSEA4 glycans were made at multi-gram scale.[64,66]

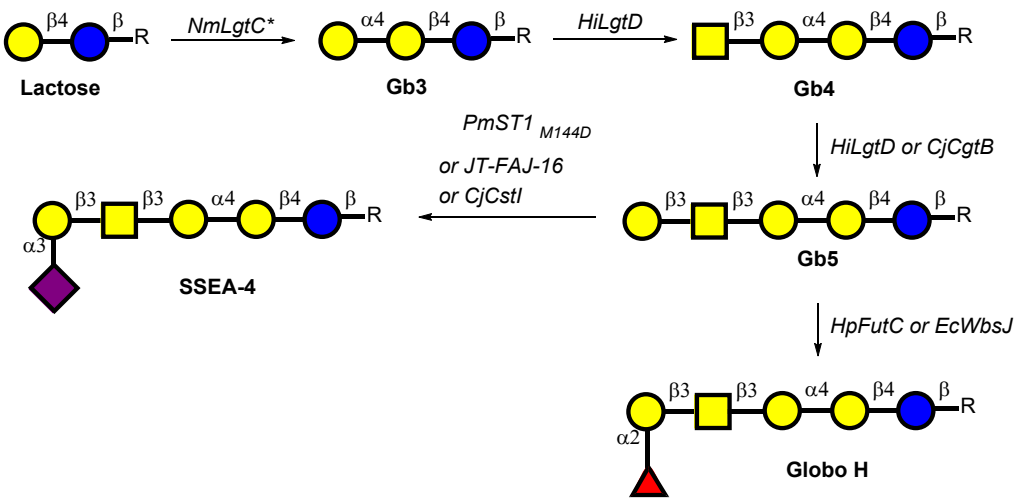


Figure 10. Chemoenzymatic synthesis of globo/isoglobo series oligosaccharides. Steps indicated with a * indicates that if an (α1-3)-GalT was used in the first transformation, instead of an (α1-4)-GalT, iGb3 was generated. Additionally, the same enzymes used in the synthesis of Gb4/Gb5/sialyl-Gb5 can be used for iGb4/iGb5/sialyl-iGb5.

The lacto and neolacto-series of glycolipids have been prepared using lactose or lactosides as a starting material (**Figure 11**), and Lc4 and nLc4 share epitopes with the human milk oligosaccharides, lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT), respectively. Two (β1-3)-GlcNAcTs, HpLgtA[65] and NmLgtA,[63] catalyzed the formation of Lc3 from lactoside in good yields. The enzymatic conversion of Lc3 to Lc4 was reported using the (β1-3)-GalT Ecwbgo,[77] two (β1-4)-GalTs – NmLgtB,[63,65,78] and Hp1,4GalT[79] – catalyzed the conversion from Lc3 to nLc4. Both Lc4 and nLc4 were sialylated by PmST1^{M144D} to form sialyl-Lc4 and sialyl-nLc4, which could also be prepared with modifications to the sialic acid.[63,78] Different isomers of disialylated Lc4 and nLc4 with (α2-3) or (α2-6)-linked sialyl groups on the terminal Gal residue were made using the (α2-6)-SiaT Pd2,6ST, and PMST1 mutants including PMST1^{M144D} and PMST1$^{P34H/M144L}$.[409-411]

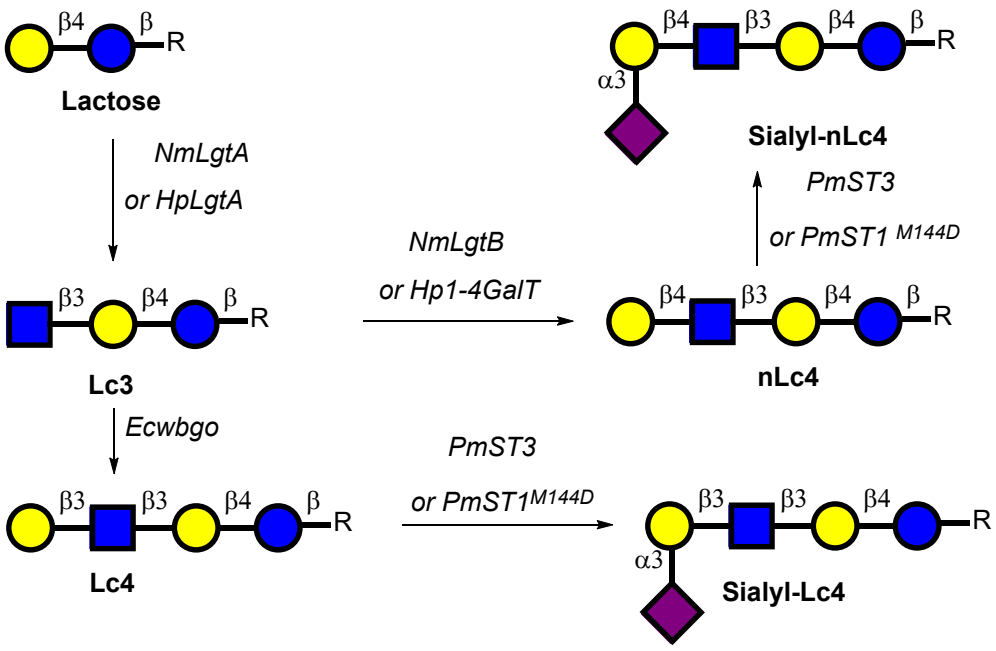


Figure 11. Chemoenzymatic synthesis of lacto/neolacto series oligosaccharides.

Some members of the globo/isoglobo and lacto/neolacto oligosaccharide series share glycan structures with blood group antigens, particularly the ABH, Lewis, and the P^1P^K blood group antigens. For instance, Gb3 shares a triose with the P^K antigen, and Gb4 with the P antigen. These fragments are widely distributed on glycolipids, glycoproteins, and oligosaccharides.[412] Lewis blood group antigens could be synthesized from lacto/neo-lacto series precursors (**Figure 12**). A glycosynthase (see **Sec 3.2**) from *B. bifidum* ($BbAfcB^{D703S}$) was used to fucosylate the Lc4 tetrasaccharide to generate the Lewis^a (Le^a) pentasaccharide.[81] Fucosylation of *N*-acetyllactoseamine (LacNAc) generated the nonreducing-end trisaccharide of the Lewis^x (Le^x) antigen.[81] The full Le^x pentasaccharide was synthesized from nLc4 through fucosylation by Hpα1,3FT[82,83] or its mutant form Hpα1,3FTΔ66,[78,82] and sLe^x was generated through sialylation of Le^x by $PMST1^{M144D}$.[87] Alternatively, the sLe^x tetrasaccharide was synthesized through fucosylation of Neu5Ac(α2-3)Galβ(β1-4)GlcNAc(β)ProN_3 with Hpα1,3FTΔ66.[82] A rat liver (α2-3)-SiaT and milk (α1-3)-FucT were used for the synthesis of a trimeric sLe^x.[88] Fucosylation of the

nonreducing-end disaccharide of Lc4 using (α1-2)-FucT HpFutC generated the H type I antigen; and the same enzyme was used to generate H type III and type IV antigens from their disaccharide precursors.[89] The H type IV antigen shares an epitope with the terminal trisaccharide of Globo H. From the non-reducing end disaccharide of nLc4, fucosylation with (α1-2)-FucT EcWbgL generated the H type II antigen; and the H type VI antigen was generated using the same enzyme.[89] Starting from the H antigen, the (α1-3)-GalNAcT HmBgtA enzyme was used to introduce a terminal GalNAc to generate the blood group A antigen tetrasaccharides. Alternatively, a recombinant human blood group B glycosyltransferase (GTB) introduced the (α1-3)-galactosyl linkage for the blood group B antigens (**Figure 12**). Together, this approach provides access to the 15 naturally occurring human ABH antigen determinants.[89] A combination of glycosyltransferase and glycosynthase (*vide infra*) activity has also been applied to the synthesis of the A type II antigen. Starting from 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (MU-βGlcNAc), the (β1-4)-galactosynthase Abg^{2F6} catalyzed the addition of (β1-4)Gal. Subsequent (α1-2)-fucosylation and (α1-3)-*N*-acetylgalactosamination, catalyzed by EcWbgL and HmBgtA respectively, could be performed in one pot.[90]

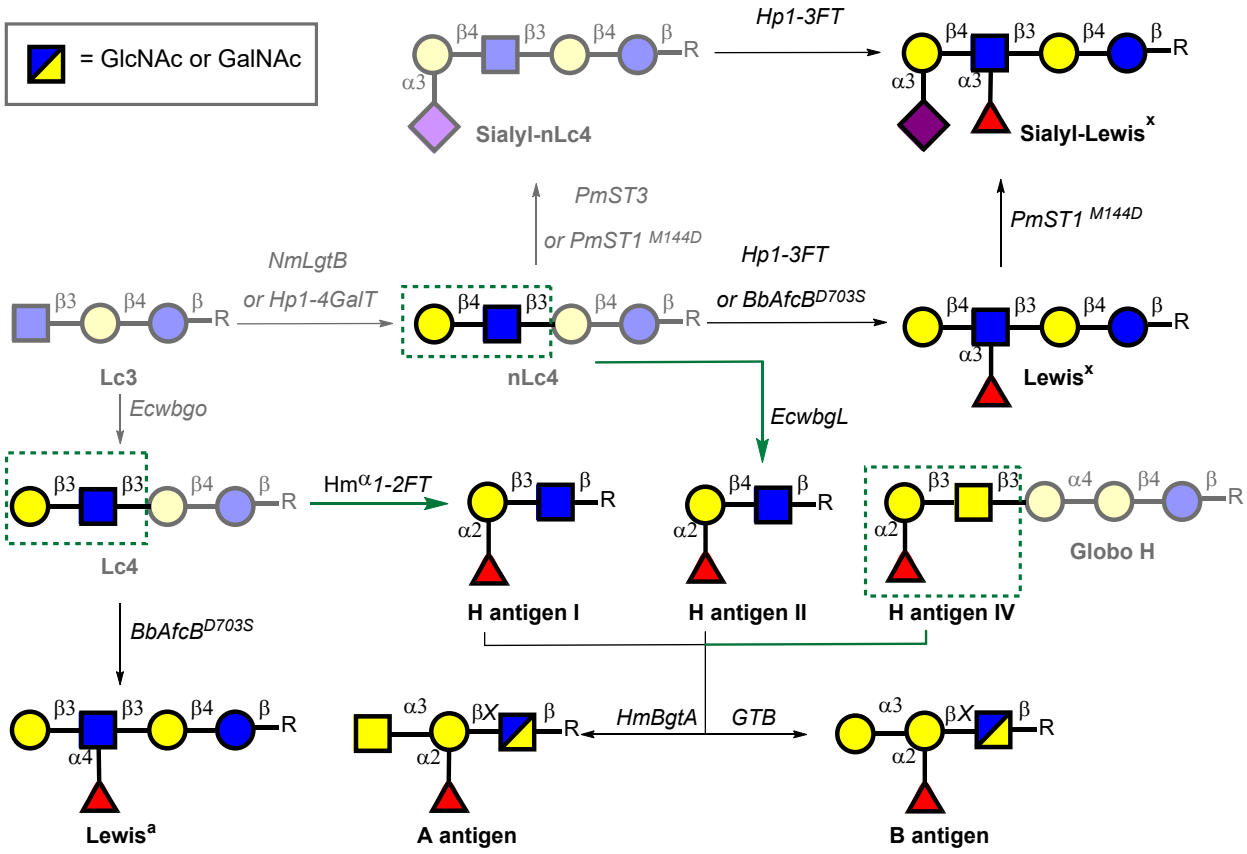


Figure 12. Chemoenzymatic synthesis Lewis and ABH antigen oligosaccharides from lacto/neolacto and globo/isoglobo series oligosaccharides. For clarity, R can represent OH in the case of the oligosaccharide, or an unspecified aglycone. Faded structures indicate glycan structures reproduced from Figure 11. Green boxes indicate the partial structures that were used in the transformations described by the green arrows.

3.1.3 Engineered microorganisms

Although widely used in the assembly of oligosaccharides, the use of OPME systems is limited by its requirement for purified enzymes. Enzyme expression systems, such as engineered microorganisms, with the ability to synthesize glycans without enzyme purification overcome this limitation.[413,414] Substantial progress to this end has been made using two strategies: bacterial coupling, where the enzymes needed for synthesis are expressed by several bacterial strains,[67] and single bacteria "superbugs",[413,415] where all the enzymes required for synthesis of the target are expressed in a single bacterial strain.[80,416-418] Engineered bacteria used for this purpose will also

have genes encoding sugar degrading enzymes knocked out to avoid hydrolysis of starting materials or products. Additionally, permeases allow uptake of the sugar precursors by the bacteria.[413,414] Both strategies have been applied to the synthesis of the oligosaccharide moieties of glycolipids, particularly the ganglio-series, as discussed below.

Bacterial coupling was used to generate a UDP-Gal expression system from three *E. coli* strains transfected with UDP-Gal biosynthetic genes (galT, galK and galU) and one *C. ammoniagenes* strain (for production of UTP). The system generated UDP-Gal upon addition of orotic acid, galactose, and xylene. Adding an α-GalT gene (*N. gonorhoeae*) and Lac to the system produced Gb3.[67] Using the same strategy, a LacNAc expression system[419] and a CMP-Sia/Sialyllactose-expression system[420] was also developed.

The single bacteria "superbug" strategy has found more applications in the synthesis of glycolipid oligosaccharides and has the potential to be more scalable and cost-effective than other strategies.[421] An engineered bacterial strain that overexpressed an (α2-3)-SiaT gene and a CSS gene from *N. meningitidis* produced the GM3 oligosaccharide from exogenous Neu5Ac and lactose.[80] Additional overexpression of CjCstII generated a strain that produced GD3 and GT3 oligosaccharides. The proportion of GD3 or GT3 produced could be adjusted by varying the lactose:Neu5Ac ratio provided to the bacteria.[422] From an engineered GM2 oligosaccharide-producing strain, addition of a (β1-3)-GalT (CjCgtB), resulted in a GM1 oligosaccharide-producing strain.[423] Using modified Neu5Ac precursors, modified GM2 analogues were produced.[424] Modifications to the lactoside were also tolerated, generating saccharide moieties of GM2 and GM3 which could be further conjugated.[425] Encoding Neu5Ac synthesizing enzymes avoided the need for exogeneous Neu5Ac, which should further reduce the cost of production for ganglioside oligosaccharides;[426] however, complications may include the possibility of excess sialylation.[427] Controlled polysialylation of lactosides has been realized in engineered bacteria by overexpressing polysialyltransferases.[428]

The globo and lacto/neolacto series oligosaccharides have been made using engineered organisms. Gb3 synthesis was achieved using a bacterial coupling approach[67] as well as using a single engineered *E. coli* "superbug" strain.[68,69] Engineered bacterial strains were produced for the synthesis of Gb4[69] and Gb5.[74] Interestingly, Gb4 and Gb5 could be produced from the same engineered *E. coli* "superbug" where HiLgtD demonstrated (β1-3)-GalNAcT activity specific for Gb3 acceptors and (β1-3)-GalT activity specific for Gb4 acceptors.[74] The Lc3 oligosaccharide was produced by providing lactose to a (β1-3)-GlcNAcT overexpressing strain. Additional overexpression of a (β1-3)-GalT in the Lc3-producing strain gave an Lc4-producing strain,[76] while overexpression of a (β1-4)-GalT gave an nLc4-producing strain.[84] Fucosylated nLc4 products, including Le^x analogues, were obtained by the overexpression of an (α1-3)-FucT to the nLc4 producing strain,[84] whereas overexpression of an α1,2-FucT produced the H type II antigen.[91] Interestingly, the (α1-2)-FucT used (HpFutC) was only able to fucosylate Lc4 scaffolds *in vitro*,[89] indicating that glycosyltransferases may have different properties *in vitro* and *in vivo*.

3.2 Chemoenzymatic synthesis using glycosidases and glycosynthases

Glycosidases (glycoside hydrolases) are defined by the CAZy database (Carbohydrate-Active enZYmes Database) as enzymes that hydrolyze or rearrange glycosidic bonds.[429] They are classified as exo- or endo-glycosidases based on the glycosidic bond they cleave (terminal vs internal). The stereochemical outcomes of the anomeric center could be the same as (retaining glycosidases) or opposite to (inverting glycosidases) the substrate. Both catalytic mechanisms have been thoroughly reviewed elsewhere.[430-432] Some retaining glycosidases can catalyze transglycosylation reactions, in which a second sugar molecule acts as the nucleophile in place of water.[433] A small portion of glycosidases adopt unusual mechanisms[431] such as substrate-assistance in *N*-acetylhexosaminidases,[434] and β-elimination in bacterial lyases.[435]

The hydrolysis of glycans mediated by retaining glycosidases is reversible, which offers the potential for using retaining glycosidases to catalyze the formation of glycosidic bonds by optimizing reaction conditions. Glycosidic bond formation by glycosidases has been achieved via thermodynamic control (reverse hydrolysis) or kinetic control (transglycosidation). The formation of glycosidic bonds is thermodynamically unfavorable (~4 kcal mol^{-1}),[436] so reverse hydrolysis strategies tend to require a high concentration of sugars, organic co-solvents, and elevated reaction temperatures to shift the equilibrium towards bond formation.[437] Transglycosylation reactions have employed activated glycosides (usually glycosyl fluorides[438] or glycosyl phenols with the correct donor configuration) to set the stereochemistry of the products, which could also act as substrates for enzymatic hydrolysis. Control of reaction times has been used to avoid product hydrolysis, however, yields may still be low.[433,439]

Despite the challenges of glycosidase-mediated glycosylation, glycosidases have been used in the synthesis of some simple glycolipid components. Promiscuous regioselectivity was a problem for some early transglycosylation efforts,[440-443] although others afforded an (α1-3)Fuc linkage[444,445] and a (β1-4)GlcNAc linkage selectively.[446] The latter study generated a series of sulfated disaccharides that may be useful in the synthesis of sulfated Le^x and Le^a.[446] A neoglycolipid component was synthesized using reverse hydrolysis of a crude cellulase preparation from *T. reesei.* Lac and a large excess of glycerol were condensed to form 1-*O*-β-lactosyl-(*R,S*)-glycerol and 2-*O*-β-lactosyl glycerol in a ratio of 7:3.[447] The same cellulase also condensed Lac or LacNAc with excess 1,6-hexanediol to form 6-hydroxylhexyl β-lactoside (Lacβ-HD) and 6-hydroxylhexyl β-*N*-acetyllactoside (LacNAcβ-HD). These products were conjugated with dipalmitoylphosphatidyl choline (DPPC) using phospholipase D (*Streptomyces sp.*), to form Lac-DPPA (dipalmitoylphosphatidic acid) and LacNAc-DPPA. Introduction of an NBD group into the lipid moiety markedly decreased the yield of transphosphophatidylation. The same strategy was employed to make two other neoglycolipid analogues from allyl β-lactose.[314]

A major impediment to glycosidase-mediated glycosylation is the competing hydrolysis reaction, which can severely limit product yields. A clear understanding of the mechanisms of glycosidases has enabled the mutation of these enzymes into efficient transglycoslases, known as glycosynthases.[448,449] In a pioneering study, Withers and coworkers mutated the active-site nucleophile of *Agrobacterium sp.* β-glucosidase (Abg) to alanine, abolishing its hydrolase activity. Transglycosylation activity could be rescued using activated glycosyl donors that mimicked the glycosyl enzyme intermediate and could be trapped by an acceptor.[450] Random mutagenesis of this enzyme identified additional glycosynthases.[451] The concept of glycosynthases has since been widely adopted.[372] This concept has been applied to endoglycosidases to generate an inverting β-glucansythase from a retaining (1-3)-(1-4)-β-glucanase (*B. licheniformis,* EC 3.2.1.73).[436] This engineered glycosynthase used the activated dissacharide α-laminaribiosyl fluoride as donor, broadening the application of glycosynthases in oligosaccharide synthesis.[373,436] Other enzymes which have been used to produce glycosynthases include α-retaining glycosidases,[81,452-454] inverting glycosidases,[455,456] and *N*-acetylhexosaminidases.[373] Glycosynthases have been widely used in the synthesis of oligosaccharides, glycopeptides, glycoproteins, and glycosylated natural products.[373]

The enzymatic synthesis of galactosylceramides was made possible by the development of a galactosynthase to catalyze the formation of lyso-β-GalCer. A β-galactosidase from *T. thermophilus,* TtbGly, was converted to a glycosynthase and applied in the stereoselective β-galactosylation of 2-amino-3-thiazolylpropan-1,3-diol, a precursor for sphingosine synthesis.[457] Recently, another galactosynthase was generated from *B. circulans* ($BgaC^{E233G}$), and was found to selectively catalyze the formation of a β1,3-galactosyl linkage in LacNAc, galacto-*N*-biose analogues,[458,459] and poly-LacNAc oligomers (in combination with glycosyltransferases).[460]

A glycosynthase was engineered from endoglycoceramidase II (EGCase II, *Rhodococcus*) and used for glycosylation of ceramide precursors. Using D-erythro-sphingosine as the acceptor,

EGCase II^{E351S} catalyzed the formation of lyso-Lac, -GM3, -GM1, -Gb3, and cellobiosyl sphingosines with excellent yields (>90%). Modifications to the sphingosine moiety were moderately tolerated. Intact gangliosides were obtained by fatty acid acylation of their lyso analogues.[34] Screening for EGCase II glycosynthases identified a mutant with improved efficacy towards different sphingosine acceptors.[461]

Combining glycosyltransferases with EGCase II glycosynthases to build the carbohydrate moiety on sphingosine could be a powerful approach to glycosphingolipid synthesis. This strategy was adopted for the synthesis of lyso-GM3 analogues and two neolacto series GSLs, starting from lactosyl fluoride. The GSLs were constructed either through immediate glycosylation of sphinsosine using an EGCase II glycosynthase followed by elaboration of the carbohydrate moiety with glycosyltransferases, or by first expanding the carbohydrate moiety and finishing with EGCase II-mediated glycosylation of Sph.[79]

Although glycosynthases are overwhelmingly made from retaining glycosidases, inverting glycosidases have also been explored. An understanding of the "Hehre resynthesis-hydrolysis mechanism" was credited with inspiring the invention of glycosynthases.[438,455,462] Glycosynthases generated from inverting glycosidases were obtained by mutating the active site base, rather than the active site nucleophile.[455,456] The (α1-2) fucosynthase made from an inverting FucT can be used to generate H-antigens[463] and will directly fucosylate GM1.[464] Mutation of the catalytic acid/base residues has been applied to retaining glycosidases on the principle that once a covalent glycosyl enzyme intermediate was formed, acceptors with a strong nucleophile could compete with water to trap the intermediate despite the absence of a general base. The strategy was first applied to the synthesis of thioglycosides to take advantage of the nucleophilicity of the thiol group, and the mutated enzymes were called thioglycoligases.[465] The approach was later expanded to *O*-glycoligases.[466] Glycoligases differ from glycosynthases in that they produce a retaining product from an activated glycoside.

In summary, enzymatic approaches have been used widely in glycolipid synthesis, and the combination of glycosyltransferases and glycosynthases have proven to be powerful tools for the total synthesis of glycolipids. Many GSL analogues, including the ganglio-/globo-/isoglobo-/lacto-/neolacto-series have been successfully synthesized via enzymatic approaches alone, or in combination with, chemical approaches (**Figure 9 – Figure 12**). While glycosyltransferases are primarily applied in the construction of oligosaccharide fragments of glycolipids; the glycosynthase EGCase II^{E351X} shows great promise in glycosylation of sphingosine. Additionally, glycosynthases complement glycosyltransferases by building linkages (such as the (α1-4)-fucosyl linkage) that have not yet been accomplished using glycosyltransferases.

3.3 Degradation enzymes for semisynthesis

Enzymatic degradation of GSLs (**Figure 13**) provides alternative and complimentary methods to chemical degradation strategies discussed in **Sec 2.5**.[467] Often, enzymatic methods are preferred as a way to avoid harsh conditions or to provide increased specificity. Enzymes that degrade the glycan of GSLs may not be strongly influenced by the identity of the aglycone; thus, when using them to target glycolipids in crude mixtures, it is prudent to consider sources of contaminating glycans. Enzymes that degrade the lipid moiety and the carbohydrate moiety have been used as tools for the selective degradation of GSLs in analysis and semisynthesis, as detailed below.

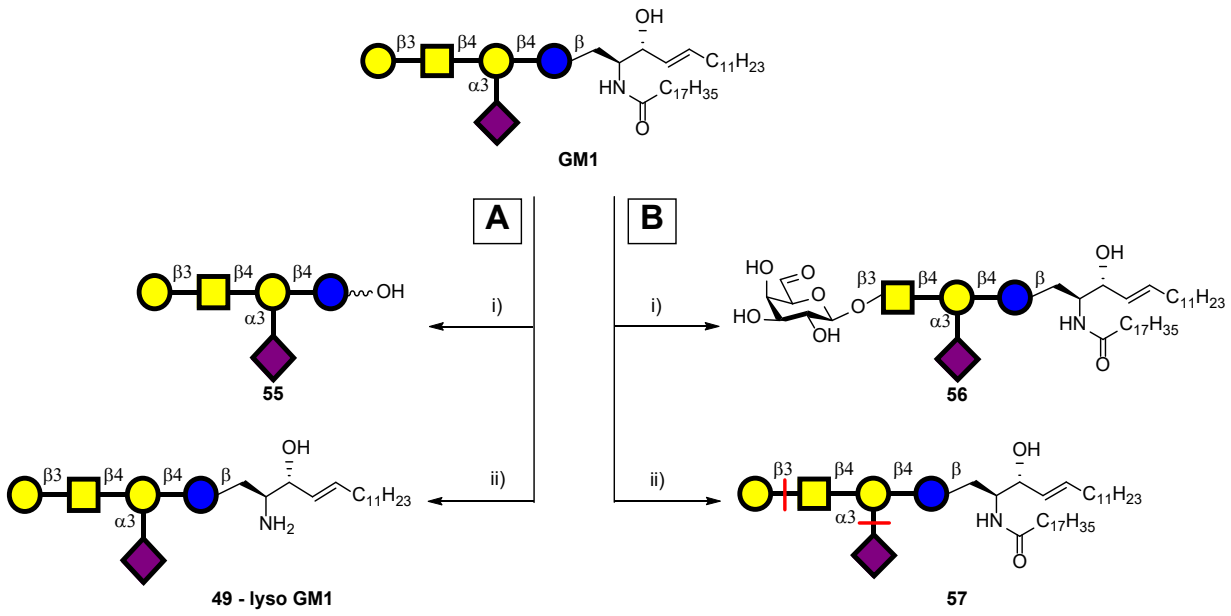


Figure 13. Common methods for the selective enzymatic degradation of glycolipids, using GM1 as an example. A) Enzymatic degradation of the lipid moiety; conditions: i) EGCase to give the oligosaccharide **55**, ii) SCDase to provide lysoGM1 (**49**). B) Enzymatic degradation of the glycan; conditions: i) oxidation of terminal galactose residues by galactose-6-oxidase to yield aldehyde **56**, ii) hydrolysis by exoglycosidases generating hydrolyzed forms of **57**.

3.3.1 Endoglycoceramidase (EGCase)

Enzymes that hydrolyze the glycosidic bond between ceramide and the carbohydrate moiety of GSLs (**55**, **Figure 13 A.i.**) are referred to as endoglycoceramidases or ceramide glycanases. The enzymes were first identified nearly simultaneously in *Rhodoccocus*[468] and a species of leech.[469] For consistency, we will refer to this class of enzymes as endoglycoceramidase (EGCase) throughout the remainder of the section. Although all EGCases exhibit glycosidase activity to release the glycan from ceramide in GSLs, they may have different specificity for mono- (EC 3.2.1.45, 3.2.1.46) or oligosaccharide-containing (EC 3.2.1.123) GSLs; or may exhibit transglycosidase or reverse hydrolysis activity.[373]

EGCases have been found in numerous organisms;[470-473] however, the EGCase from *Rhodococcus* has been the most popular in the study of glycolipids. There have been three molecular species of the enzyme identified and cloned:[474] EGCase I,[475] EGCase II,[476] and EGCase III (also referred to as endogalactosylceramidase, EGALC);[477] where the numerals indicate the order in which they elute from an anion-exchange column.[474] EGCase I has the broadest substrate scope.[475] Recently, a new EGCase I was cloned with significantly higher expression levels in *E. coli*, enhancing its utility for GSL analysis and semisynthesis.[478] Crystal structures of EGCase I[478] and EGCase II[479] have enabled efforts towards the rational design of EGCases with tailored substrate specificities.[478]

EGCases have been used both for GSL analysis and semisynthesis. The application of EGCase enzymes for the analysis of GSLs and their functions is reviewed elsewhere.[480] EGCase I[475] and EGCase III[481,482] have transglycosylation activity, which has been exploited for the synthesis of glycolipid probes. EGCase II has been converted to a glycosynthase by mutation of the catalytic nucleophile (see **Sec 3.2** for more details or **Sec 4.3.2** for applications).[34,461] The transglycosylation capacity of EGCase enzymes from other organisms has been explored.[483] Notably, EGCase from the jellyfish *Cyanea nozakii* demonstrated both transglycosylation and reverse hydrolysis activity,[484] condensing Lac and Cer to form LacCer.[485] The leech EGCase was able to glycosylate acceptors with a GM1 glycan[486] and recognized the GM3 glycan immobilized on polyacrylamide for the synthesis of GM3[487] and lyso-GM3.[488] The EGCase enzyme from *Corynebacterium* has also demonstrated transglycosylation activity.[489]

3.3.2 Sphingolipid ceramide deacylase (SCDase)

Enzymes that hydrolyze the amide bond between the fatty acid and Sph of GSLs are known as sphingolipid ceramide deacylase (SCDase) enzymes (EC 3.5.1.69; **Figure 13 A.ii.**).[490] The products of the hydrolysis reaction are lyso-GSLs (**49**), which are often key intermediates in the

semisynthesis of labeled GSLs. Examples using lyso-GSLs as starting material for labeled glycolipids are discussed throughout **Sec 4**. The synthesis of lyso-GSLs by SCDase enzymes has been impeded by competition between hydrolysis and reverse hydrolysis activities,[491,492] which has limited the yield of lyso-GSLs.[493] Historically, the SCDase from *Pseudomonas sp.* has been the most widely used in semisynthesis of glycolipids.[494] Competing enzyme activities have been controlled by altering reaction conditions,[493] and an aqueous/organic biphasic system has been used to favor hydrolysis.[495] Alternatively, SCDase has been used for transacylation reactions to generate GSLs containing modified fatty acids with various lengths and degrees of unsaturation,[496] ω-amines,[497] diazirines,[498] and NBD fluorophores.[499] The immobilization of SCDase has been used to facilitate product purification.[500]

Recently, the SCDase from *Shewanella* has gained attention for the semisynthesis of glycolipid probes. Unlike the SCDase from *Pseudomonas*,[494] the SCDase from *Shewanella* could be overexpressed in *E. coli*,[501] making its production more practical. Further comparisons between the two enzymes indicated that the SCDase from *Shewanella* had a broader substrate specificity towards the fatty acid group, allowing for versatility in condensation reactions with modified fatty acids.[502] The enzyme accommodated 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY)-labeled fatty acid in a condensation reaction for the synthesis of a ganglioside-based FRET probe (see **Sec 4.3.2**).[394] Recent improvements to the hydrolysis conditions for glycolipids by SCDase have identified calcium chloride and taurodeoxycholate as additives which drive the hydrolysis towards lyso-glycolipid targets.[503]

3.3.3 Galactose-6-oxidase

The most popular enzyme for selective modification of the glycan of GSLs is galactose-6-oxidase (EC 1.1.3.9; Gal-6-ox), which oxidizes the *C*-6 hydroxy group of galactose to an aldehyde (**56**, **Figure 13 B.i.**). The enzyme is not specific for glycolipids – it has also been widely used to oxidize

terminal galactose residues on glycoproteins[504,505] and free sugars.[506] Gal-6-ox was isolated from what was thought to be *Polyporus circinatus* in 1961.[506,507] (The fungus was misidentified[508] and has since been identified as *Fusarium graminearum*.)[509] Soon after, Gal-6-ox was applied to the oxidation of galactoside glycolipids.[510] The mechanism of Gal-6-ox action has been reviewed.[511] Historically, the most common application of Gal-6-ox in the synthesis of glycolipid probes was the selective radiolabeling of galactose through reduction of the aldehyde product with NaB^3H_4.[512,513] More recently it has become popular for conjugation of fluorophores through oxime ligation (see **Sec 4.4.1**).

The substrate specificity of Gal-6-ox has been studied extensively. While GalNAc was cleaved with comparable efficiency to unmodified Gal,[507] other Gal modifications were not accommodated by the enzyme.[514,515] Gal-6-ox is specific for terminal Gal residues;[504,516] for instance, gangliosides GM1 and GM2 were substrates for Gal-6-ox, while GD1a was not.[517,518] Efforts to expand the substrate scope of Gal-6-ox through mutagenesis have made modest progress.[519,520]

3.3.4 Exoglycosidases

A less common strategy to generate modified glycolipids is through the degradation of GSL to less complex glycolipids using exoglycosidases (**57**, **Figure 13 B.ii.**) The exoglycosidases pertinent to the degradation of many vertebrate glycolipids are exo-β-galactosidases, β-*N*-acetylhexosaminidases, and neuraminidases (NEU, also called sialidases), which cleave non-reducing end Gal, GalNAc or GlcNAc, and Neu5Ac residues, respectively.[521] Exoglycosidases typically have strict substrate specificities towards the monosaccharide that they hydrolyze; the remainder of the glycan chain or aglycone is often more relaxed but still requires scrutiny. Consider the NEU enzymes as an example: NEU from *A. urafaciens* was approximately 100-fold more efficient at cleaving ganglioside GM1 than NEU from *C. perfringens*.[522] Furthermore, of the four

human NEU isoenzymes, NEU3 was reported to primarily cleave glycolipid substrates,[210,401,523] whereas NEU1 did not act on glycolipid substrates.[523]

Exoglycosidases have been employed to generate target glycolipids which are naturally present in low abundance. A crude mixture of polysialogangliosides was extracted from rat brains and degraded to GM1 using NEU from *V. cholera*, followed by β-galactosidase to degrade GM1 to GM2.[524-526] NEU from *C. perfringens* has been used to convert a crude polysialoganglioside mixture to GM1; however, under certain conditions it was able to degrade GM1 to GA1.[58] Using a similar strategy, GD1b was degraded to GD2[527] and GM2.[528] GM3 could be generated from GM2 with a β-*N*-acetylhexosaminidase.[529] Exoglycosidase degradation strategies have been combined with metabolic radiolabeling (see **Sec 4.1.1**) to obtain radiolabeled gangliosides.[524,525]

Table 4. Enzyme abbreviations

Subclass	*CAZy*	*Abbreviation*	*Full Name*
GlcNAcT	GT8	HpLgtA	*Helicobacter pylori* β1,3-*N*-acetylglucosaminyltransferase[65]
	GT2	NmLgtA	*Neisseria meningitidis* β1,3-N-acetylglucosaminyltransferase[78]
GalT	GT6	Bovine α1,3GalT	Recombinant bovine α1,3-galactosyltransferase[63,530]
	GT2	CjCgtB	*Campylobacter jejuni* β1,3-galactosyltransferase[63]
	GT 2	Ecwgbo	*Escherichia coli* O55:H7 β1,3-galactosyltransferase[77]
	GT25	Hp1-4GalT	*Helicobacter pylori* β1,4-galactosyltransferase[79,531,532]
	GT6	Human GTB	Human blood group B glycosyltransferase (GTB)[89,533]
	GT25	NmLgtB	*Neisseria meningitidis* β1,4-galactosyltransferase[63,65,78]
	GT8	NmLgtC	*Neisseria meningitidis* α1,4-galactosyltransferase[63-65]
GalNAcT	GT82	CjCgtA	*Campylobacter jejuni* β1,4-N-acetylgalactosaminyltransferases[63]
	GT2	HiLgtD	*Haemophilus influenza* β1,3-N-acetylgalactosaminyltransferase[63,64]
	GT6	HmBgtA	*Helicobacter mustelae* α1,3-N-acetylgalactosaminyltransferase [33,90,534]
SiaT	GT42	CjCstI	*Campylobacter jejuni* α2,3-sialyltransferase[75,79,394,403]
	GT42	CjCstII	*Campylobacter jejuni* α2,3/8-sialyltransferase[63,395,396]
	GT42	CjCstIIΔ32^{I53S}	A mutant of CjCstII[397]
	GT80	JT-FAJ-16	Marine α2,3-sialyltransferase[64]
	GT80	PMST1^{M144D}	*Pasteurella multocida* α2,3-sialyltransferase 1 M144D mutant[33,63]
	GT52	PMST2	*Pasteurella multocida* α2,3-sialyltransferase 2[390]
	GT42	PMST3	*Pasteurella multocida* α2,3-sialyltransferase 3[391,398]
	GT80	Pd2,6ST	*Photobacterium damselae* α2,6-sialyltransferase[405,409,411]
	GT29	ST3GalI	Mammalian α2,3-sialyltransferase[404]
FucT	GT11	EcwbgL	*Escherichia coli* O126 α1,2-fucosyltransferase[89,90]
	GT11	HpFutC	*Helicobacter pylori* α1,2-fucosyltransferase[64,89]
	GT10	Hp1-3FT	*Helicobacter pylori* α1,3-fucosyltransferase[63,82]
	GT10	Human α1-3FucT	Human milk α1,3-fucosyltransferase[88]

Subclass	*CAZy*	*Abbreviation*	*Full Name*
Glycosidase	GH1	Abg	*Agrobacterium sp.* β-glucosidase[450]
	GH29	BbAfcB^{D703S}	Glycosynthase derived from (α1-3)-(α1-4)-L-fucosidase of *Bifidobacterium bifidum*[81]
	GH35	BgaC^{E233G}	Galalactosynthase from *B. circulans*[458,459]
	GH5	EGALC	Endogalactosylceramidase (another name for EGCaseIII)[477]
	GH5	EGCase	Endoglycoceramidase[468]/ceramide glycanase[469]
	GH5	EGCase II ^{E351X}	Glycosynthases derived from endoglycoceramidase II (EGCase II) from *Rhodococcus* strain M-777[34,79,461]
	GH1	TtbGly	B-galactosidase from *T. thermophilus*[457]
	GH33	NEU	Neuraminidase/sialidase[401,523]
Sugar Donor Synthesizing Enzymes		EcGalE	*Escherichia coli* UDP-galactose-4-epimerase[78]
		EcGalK	*Escherichia coli* K-12 galactose kinase[63-65,89,535,536]
		AGX1	Human UDP-GalNAc pyrophosphorylase[33]
		AtUSP	*Arabidopsis thaliana* UDP-sugar pyrophosphorylase[64]
		BiNahK	*Bifidobacterium infantis* N-acetylhexosamine-1-kinase[33,64]
		BlNahK	*Bifidobacterium longum* N-acetylhexosamine-1-kinase[63,89]
		BLUSP	*Bifidobacterium longum* UDP-sugar pyrophosphorylase[63,65,89]
		BfFKP	Bifunctional enzyme from *Bacteroides fragilis* that has both L-fucokinase and GDP-fucose pyrophosphorylase activities [33,63,64,82,89]
		EcGlmU	*Escherichia coli* N-acetylglucosamine 1-phosphate uridylyltransferase[64,65,89]
		Gal-6-ox	Galactose-6-oxidase[506-509]
		PmCSS	*Pasteurella multocida* CMP-sialic acid synthetase[64,398]
		PmGlmU	*Pasteurella multocida* N-acetylglucosamine 1-phosphate uridylyltransferase[63]
Other		CMAH	Cytidine monophosphate *N*-acetylneuraminic acid hydroxylase[53]
		Gal-6-ox	Galactose-6-oxidase[506-509]
		SCDase	Sphingolipid ceramide deacylase[490,494,501]

4 Labelled glycolipid analogs

Labeled GSL analogues are important probes to investigate the location, fate, and function of glycolipids in biological systems. These compounds have been developed for in vitro enzyme assays, labeling in cells, and investigating the fate of glycolipids in live animals.[369,537-541] Early elucidation of GSL biosynthesis and metabolic fate relied heavily on the use of radionuclide glycolipid probes. Glycolipid probes have also been crucial for detecting glycolipid partitioning both *within* and *across* membranes or into specific cellular compartments. In this section, we will discuss the synthesis and applications of labeled GSLs used as biological probes.

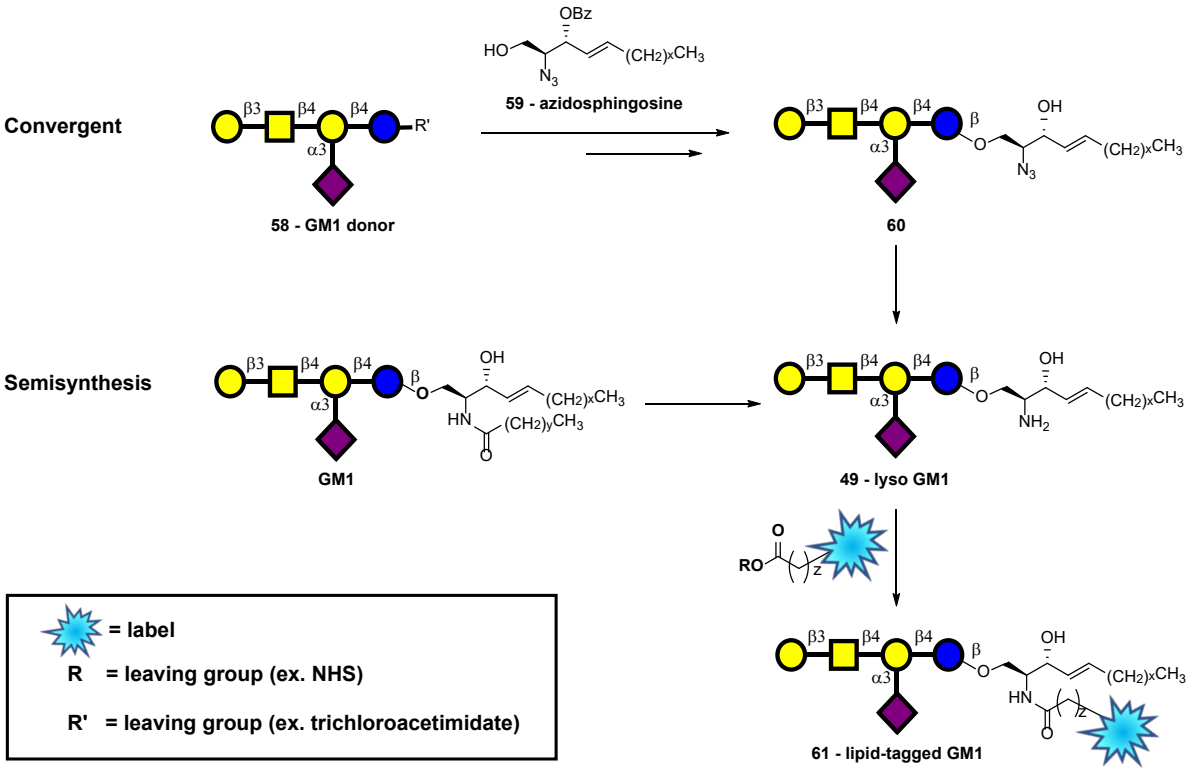


Figure 14. General routes towards glycolipids tagged at the lipid moiety, using GM1 as an example. Lipid chain lengths may vary, and are denoted by x, y, and z subscripts, referring to the sphingosine and fatty acid chains, respectively.

The synthesis of labeled glycolipids is generally accomplished through one of three primary strategies: semisynthesis, convergent synthesis, or metabolic labeling. The basis of most semisynthetic strategies is discussed in **Secs 2.5** and **3.3**. The choice of strategy will often depend on the intended label and the required location within the GSL itself. Labels can be incorporated into in the glycan or lipid moieties, or both. Modifications to the lipid are most often accomplished through the introduction of a modified fatty acid through either a semisynthetic or convergent synthesis (**Figure 14**). In semisynthetic routes, *N*-deacylation of the sphingolipid yields the corresponding lyso-derivative **49**. Re-acylation with a modified fatty acid is typically done through an activated ester or mixed anhydride. Convergent syntheses usually start from D-*erythro*-sphingosine and are most practical for glycolipids with small glycans.

Carbohydrate labels are typically introduced at sites where selective chemical reactivity can be easily achieved (**Figure 15**). Semisynthetic approaches targeting the glycan are almost exclusively applied to glycolipids with *N*-acyl-containing carbohydrates, such as amido sugars or sialic acid, where the revealed amino group can be used as a chemical handle (e.g. **53**). Alternative semisynthetic strategies take advantage of selective oxidation strategies that may target individual terminal residues, such as galactose or sialic acid (e.g. **54** or **56**). Convergent synthetic approaches may also exploit either an *N*-acyl group or the reactivity of the primary alcohol at *C*-6 of hexoses or *C*-9 of Sia.

Metabolic labeling is an alternative to convergent and semisynthetic methods that exploits cellular machinery to develop synthetic targets. A modified precursor (e.g. a monosaccharide, sphingosine, or fatty acid) is provided to cells or whole organisms, where it is taken up into the biosynthetic pathway and incorporated into the glycolipid. This method has been termed "Metabolic Oligosaccharide Engineering" or "Metabolic Glycoengineering" when applied to labeling of glycans, and is used extensively to study the composition of glycans in organisms or to probe the effects of carbohydrate modifications in living systems.[542-547] Potential applications for

this technology also include cancer immunotherapy by generating highly immunogenic gangliosides from modified ManNAc precursors.[548,549]

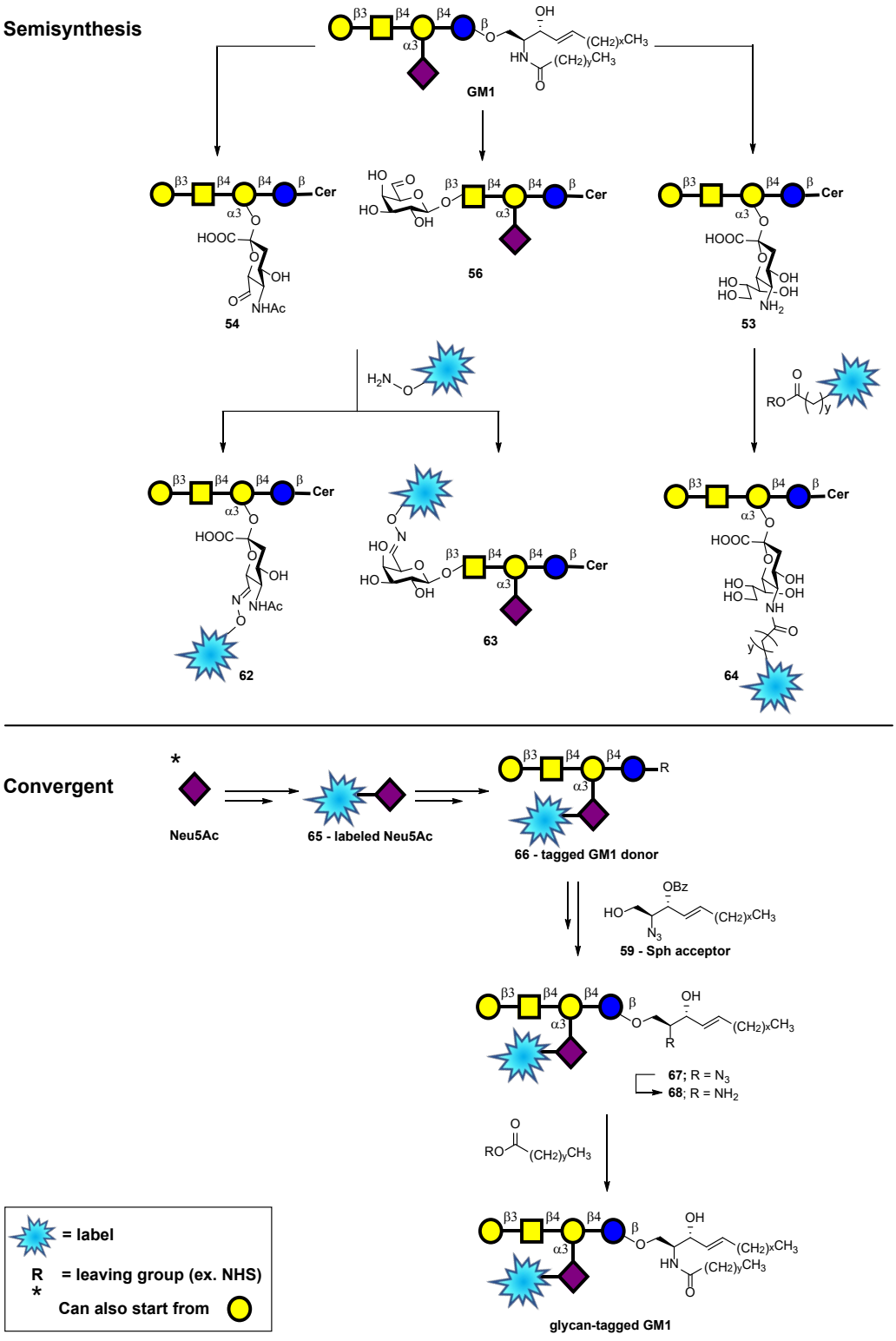


Figure 15: General routes towards glycolipids tagged at the glycan moiety, using GM1 as an example. For clarity, the convergent synthesis only shows an example with Sia labeling; however, labeling the terminal Gal residue is also common.

In this section, we will discuss different approaches to generate labelled GSLs for use as probes in chemical biology. We note that some of the strategies used for GSL have built on methods for labeling of phospholipids or sphingolipids, which are reviewed elsewhere.[550-552] However, the unique requirements of the glycan present additional challenges for the design of GSL probes. As above, we will restrict the discussion to vertebrate GSLs or those that interact specifically with vertebrate systems (e.g. α-GalCer). Some strategies which may be relevant for GSL probes have been applied to the construction of probes based on GPI-anchors.[553-557] Our focus will be on how synthetic, semisynthetic, and metabolic labeling methods were used to generate GSL probes with labels in the glycan or the lipid moiety. We have placed more emphasis on publications from the year 2000 onward.

4.1 Radiolabelled glycolipids

Perhaps the most established method of generating labelled glycolipid analogues is through radiolabelling. The development of fluorescent probes and the cost associated with radionuclide use has led to a shift away from radiolabeling in more recent years. Methods that employ radiolabeled substrates may also be incompatible with real-time analyses, be labor intensive, or require specialized equipment. However, radiolabeling produces a probe with minimal structural difference to the native substrate and can provide for sensitive detection. As such, radiolabeled glycolipids maintain their relevance as important probes in modern glycolipid research.

4.1.1 Metabolic radiolabelling

Radiolabelling was the earliest method applied to metabolically labelled glycolipids[558-560] and has been reviewed by Diaz and Varki.[561] Studies employing radioactive serine, Sph, palmitic acid, and Gal have been key to the elucidation of GSL biosynthesis.[538] Radiolabelling has been used extensively to study ganglioside composition and perturbations to glycolipid and ganglioside

biosynthetic pathways. To this end, ^{3}H or ^{14}C labeled glucosamine and Gal,[562-574] mannosamine,[575] serine,[576-578] or Sph[105,567,579-586] were provided to cells, and their incorporation into glycolipids was tracked. GPI has been metabolically labeled with ^{3}H mannose,[587-589] ^{3}H glucosamine,[590] ^{3}H inositol,[590-592] and ^{3}H myristate.[593] Radiolabeled monosaccharides such as Glc or Gal are also used in enzyme activity assays.[594,595] Although the biosynthetic generation of radiolabeled glycolipids is still commonly used for many purposes, this approach generates mixtures of radiolabeled glycolipids from which the glycolipid of interest must be separated and may have low specific radioactivity.[596] Efforts towards methods that generate homogeneous radiolabelled GSLs with high specific radioactivity are discussed below.

4.1.2 Semisynthetic radiolabeling approaches

Semisynthetic methods to introduce labels into gangliosides have been reviewed extensively.[596-598] There are three general semisynthetic approaches used to generate homogeneous radiolabeled glycosphingolipids. Oxidation and reduction with a ^{3}H source ($NaB^{3}H_4$) at the *C*-6 of Gal or *C*-3 of Sph is commonly used. Reduction of alkenes with $NaB^{3}H_4$ allows for selective labeling of Sph, but destroys the alkene functionality. Deacetylation and re-acetylation of the *N*-acetyl group of Neu5Ac or the fatty acid chain, or both, with ^{3}H or ^{14}C labeled groups employs the harshest conditions of the three methods.[596,597]

The methods developed from the 1960s to the 1990s for the selective radiolabeling of gangliosides are still used routinely.[597] Recently, oxidation of the *C*-3 position of Sph was used to generate an ^{3}H labelled GM3,[599] GD1a,[600-604] and GM1.[605] The Neu5Ac moieties of GD3 were deacetylated and re-acetylated with $^{2}H_6$-acetic anhydride before feeding to rats and detection of labeled GD3 in plasma using LC-MS.[606] Radiolabelled Sph has been used to perform imaging by electron microscopy.[607] Radiolabeling has also been used in combination with other labeling strategies to obtain corroborative data sets. Fluorescent and radiolabled GM1 was generated with

modifications to the oxidation/reduction approach. From GM1, the *C*-6 hydroxyl of the terminal Gal was oxidized and conjugated to AlexaFluor hydrazide.[608] The resulting hydrazone was reduced with NaB^3H_4, and the probe was used to compare the results of microscopic and biochemical techniques. Finally, labeling of GSL with mass tags has emerged as a method for imaging by mass spectrometry.[368,609]

4.2 Fluorescent glycolipids

Fluorescent labeling produces sensitive probes that are compatible with real-time monitoring at high resolution. However, the fluorochrome itself is often large and may perturb the very properties of the lipid being investigated, resulting in an undesired influence on biological function.[142,610] As a result, there has been significant effort towards probes whose overall features resemble that of natural lipids.[611,612] In systems where the biological properties under investigation relate primarily to the glycan, one may choose to label the fatty acid of the GSL.[613] However, there is growing recognition of the role of lipid-lipid interactions,[173,614] and studies of fluorescent lipids have provided a strong counterargument for preserving the integrity of the lipid chain by labeling the carbohydrate moiety.[614,615] Thoughtful selection of fluorophore location and identity can produce probes with partitioning properties analogous to native lipids.[616]

Fluorophores commonly used for labeling glycolipids include NBD, BODIPY, and tetramethylrhodium (TMR). The respective properties, advantages, and disadvantages of these fluorochromes are detailed elsewhere.[617] Polyene chromophores have been used for phospho- and sphingo-lipids[618,619] and in at least one example have been applied to GSL, such as Gb3.[620] Historically, the most common fluorophore used for lipids was NBD because of its small size and increased fluorescence intensity within a non-polar environment.[621] However, due to its polarity, NBD has been shown to act as a poor mimic of an acyl chain for interactions with natural lipid analogues within the membrane.[622,623] Cholesterol labeled with NBD did not mimic native

cholesterol activity, and the NBD label was sequestered near the lipid-water interface.[624] BODIPY has emerged as the most popular fluorophore for glycolipid labeling because it is neutral, nonpolar, and photostable; it also has a higher fluorescence yield compared to NBD. In addition, its interactions within a membrane environment are more comparable to non-polar acyl chains as determined through excimer formation.[625] The popularity of fluorescence as an analytical and imaging tool has led to the commercial availability of a variety of simple fluorescent glycolipids including cerebrosides, sphingolipids, and (to a lesser extent) gangliosides.

4.2.1 Lipid labelling

Examples of metabolic glycolipid labeling with fluorophores are limited. Fluorophores are more likely to perturb the natural activity or biosynthesis of glycolipids compared to radiolabels (see **Sec 4.1.1**) or bioorthogonal labels (see **Sec 4.4.1**). In all instances, the Sph or Cer was first fluorescently labeled and introduced to cells. Metabolic conversion of fluorescently labeled Cer to cerebrosides has been observed.[626-628] Metabolic labeling with fluorescent Sph (generated through a cross metathesis reaction between Garner's aldehyde **5** and an alkene labeled with an ω-BODIPY group) enabled the study of both acylated and non-acylated sphingolipids.[629,630]

Semisynthetic methods employing lysoglycolipids have long been used to generate glycolipids containing NBD and pyrene fluorophores. Fluorescent fatty acids have been coupled to deacylated glycosphingolipids.[631-636] Six-carbon chain NBD fatty acids are known but do not mimic the phase properties of native fatty acids incorporated into Sph mixtures.[637] Semisynthetic methods using NHS coupling have been employed to make a variety of BODIPY-labeled[638-641] and TMR-labeled glycolipids.[642-644] As an alternative to NHS activation, BODIPY-labeled fatty acids have been activated with benzotriazole-1-yloxy-tris(dimethylamino)phosphonium (BOP)[645] or as mixed anhydrides.[646]

Complex gangliosides with fluorescent labels in the acyl chain have been prepared by first modifying the amine of lyso-LacCer or GM1 with a BODIPY or TMR fatty acid through NHS activation; followed by sialyltransferase, sialidase, or galactosidase reactions to generate the desired glycan.[640,642,643,647-650] These probes have been applied in metabolic cytometry experiments.[650,651] A convergent approach employing both synthetic and chemoenzymatic methods generated TMR-labeled gangliosides with a thioglycosidic linkage between galactose and glucose. Sph was glycosylated with a thiolactoside, after which the amine was acylated with a TMR label. The resulting labeled thiolactosylsphingolipid was further modified using glycosyltransferases.[652]

The unnatural glycosphingolipid, BODIPY-maltosylceramide, was generated through a convergent approach. Azido-benzylsphingosine was glycosylated with per-*O*-acetylmaltosyl trichloroacetimidate. After deprotection, the azide was reduced, and the BODIPY-labelled fatty acid was incorporated through NHS activation.[639] A similar synthetic approach enabled the synthesis LacCer with unnatural stereochemistry to study the role of stereochemistry at *C*-2 and *C*-3 of Sph.[653,654] This strategy has also been used for the synthesis of more complex labeled GSLs such as sLe^x.[655,656] A convergent method generated ω-amino-α-GalCer. Sph was acylated with a protected ω-amino fatty acid. After glycosylation and deprotection, the terminal amine on the fatty acid was coupled to a BODIPY[247,657] or Cy-3[658] fluorophore. Starting from lyso-GM1, fatty acids with α- or ω-amines were incorporated and then labeled with a variety of fluorophores through NHS activation.[360] This method is likely to be compatible with other ganglioside targets. A modification of this approach placed an azido group at the α-methylene position on the fatty acid chain of α-GalCer, which allowed for the incorporation of a fluorophore alkyne using copper-catalyzed click chemistry.[659]

4.2.2 Glycan labeling

Fluorescent glycolipids tagged through the carbohydrate moiety may be more likely to preserve partitioning of the glycolipids into raft-like domains. Fluorescent labeling at *C*-6 of the Gal residue has been used as an alternative to lipid labeling to study the role of α-GalCer in activation of natural killer T cells. In the first synthetic strategy, α-GalCer was converted it to a 6-amino-6-deoxy-galactosyl analogue for conjugation to a dansyl fluorophore.[660] Sulfatide was prepared with a similar approach.[661] This strategy gave poor yields because glycosylceramides are generally insoluble.[660] An amended strategy achieved improved yields by labeling Gal with a dansyl group prior to glycosylation.[662]

Gangliosides can be modified at various positions on the sialic acid moiety or at *C*-6 of a Gal residue. GM1 was labeled at the glycerol chain of Neu5Ac through a semisynthetic method employing sodium periodate to generate an aldehyde at *C*-7, which was subsequently conjugated to AlexaFluor hydrazide.[161] A more recent study generated fluorescently labelled gangliosides GM1, GM2, GM3, and GD1b (**69–71**, **Figure 16**) through a convergent synthetic route using the GlcCer cassette strategy[663] (see **Sec 2.3**). The general method involved synthesis of the modified carbohydrate moiety with a protected amine at either *C*-6 of Gal or *C*-9 of Neu5Ac, followed by glycosylation of GlcCer, global deprotection, and coupling of the free amine (at *C*-6 of Gal or *C*-9 of Neu5Ac) onto a hydrophilic fluorophore through NHS activation. Many of the fluorescent ganglioside analogues exhibited lipid raft-partitioning behavior similar to that of the natural lipid.[663] A comprehensive discussion of the precedent for the synthesis of these glycan-labelled fluorescent gangliosides has recently been detailed[615] and reviewed.[38]

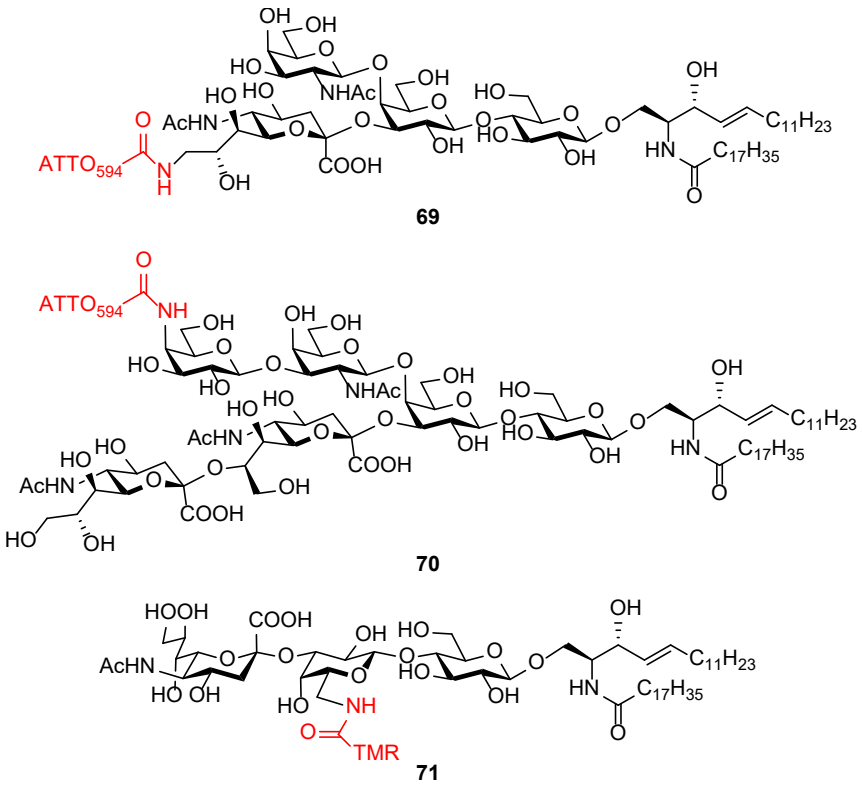


Figure 16: Select fluorescent gangliosides with fluorescent labels in the glycan, made with a convergent approach.[663] The fluorescent labels were incorporated in the final step through amine coupling at *C*-9 of Neu5Ac (**69**), *C*-6 of a terminal Gal residue (**70**), or *C*-6 of an internal Gal residue (**71**).

Less conventional methods have been used to generate fluorescently labeled unnatural glycolipids. A pyrene group was used to label *N*-(9-*cis*-octadecenoyl)-β-D-glucosamine through a dehydration reaction between the sugar hydroxyl groups and 1-pyreneboronic acid.[664] A synthetic glycolipid was formed from a trisaccharide containing a bioorthogonal tag, which was designed as a multifunctional spacer. The trisaccharide crosslinked a cholesterol moiety at its reducing end to a dansyl group at *C*-4 of the non-reducing end glucose and was used to investigate lipid raft domains.[665]

4.3 FRET-based glycolipid probes

Fluorescent glycolipids can be used in Förster resonance energy transfer (FRET) experiments, which may provide improved sensitivity or be used to monitor chemical reactions and molecular associations. FRET is a non-radiative energy transfer between donor and acceptor chromophores. Intermolecular glycolipid-based FRET systems have been used extensively to measure intermolecular distances, and therefore molecular interactions within membranes,[666] while intramolecular glycolipid-based FRET systems have been used for monitoring enzymatic processing of glycolipids.

4.3.1 Intermolecular FRET assays

One of the earliest glycolipid-based FRET systems used 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH propinoic acid) as the FRET donor, and NBD-aminohexanoic acid as the FRET acceptor to study glycolipid distribution in a membrane. The fluorophores were incorporated into the fatty acid tail using semisynthetic methods. Lyso lipids of GM1, GalCer, sulfatide, and Ga1 were re-acylated with DPH propinoic acid or NBD-aminohexanoic acid after NHS activation.[667] Another early glycolipid-based FRET system used anthrylvinyl (AV) as the FRET donor- and perylenoyl as the FRET acceptor. These probes were introduced as lipid-specific probes in 1985 by Bergelson et al.[611] The semisynthetic labeling strategy employing NHS activation of fluorescent fatty acids was later expanded to galactocerebrosides and a variety of gangliosides.[613] AV-labeled GalCer (AV-GalCer) and perylenoyl-triglyceride probes were used in a FRET assay to monitor the activity of the glycolipid transfer protein. Both probes were inserted into a donor vesicle, and the glycolipid transfer protein transferred AV-GalCer from the donor vesicle to an empty acceptor vesicle which led to an increase in anthrylvinyl emission and a decrease in perylenoyl emission.[668] This FRET system is still popular for studying glycolipid transfer proteins and glycolipid mixing in membranes.[513,669-

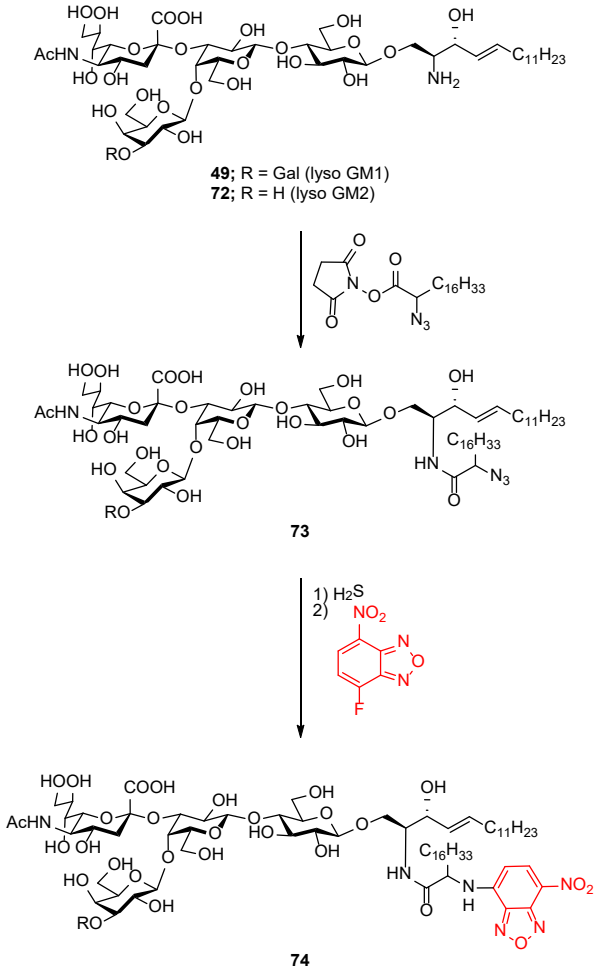
[673] A 3,3'-dihexadecyloxacarbocyanine perchlorate (Dio-C_{16}) acceptor has been used in place of the perylenoyl group acceptor.[674-677] Recently, the assay has been implemented with BODIPY-GalCer in place of AV-GalCer[678] and adapted to a microfluidics platform.[679]

A similar FRET assay was developed to monitor GM2-activator protein (GM2-AP) activity. GM1 and GM2, both with NBD-labelled acyl chains, were incorporated into donor vesicles containing another lipid to act as a FRET acceptor (rhodamine-dioleoyl phosphoglycerol ethanolamine). The transfer of the labeled gangliosides by GM2-AP from the donor vesicle to an empty acceptor vesicle was detected as an increase in NBD fluorescence emission. The NBD labels were incorporated into the α-methylene position of the fatty acid tail (**74**, **Scheme 6**) by acylation of lyso-GM1 **49** or lyso-GM2 **72** with 2-azido-octadecanoic acid, reduction of the azide, and treatment with NBD-fluoride. Reduction of the azide with H_2S left the double bond of the sphingosine intact.[680]

FRET has also been used to detect the binding of proteins specific for GSL. The fatty acid of GM1 was labelled with one of two BODIPY fluorophores (a FRET donor or a FRET acceptor) using a semisynthetic strategy. In the absence of cholera toxin, labeled GM1 was dispersed homogeneously in a phospholipid bilayer. Upon addition of cholera toxin, the FRET donor- and acceptor-labelled GM1 were brought into proximity, causing an increase in the FRET signal which could be observed at the membrane,[681,682] or using a flow cytometry assay.[683] A modification of this assay has used pyrene-labelled phospholipids as the FRET donor within a phospholipid bilayer. An application of GM1 labelled with BODIPY or 4-((4-(dimethylamino)phenyl)azo)benzoic acid (DABCY) as the FRET acceptor was used to detect GM1 clustering upon addition of cholera toxin based on self-quenching.[684] More recently, GM1 labeled with BODIPY was mixed with vesicles made up of polymerized 10,12-pentacosadiynoic acid (PDA). Insertion of the probe into the lipid vesicle caused fluorescence quenching, but in the

presence of cholera toxin, the ganglioside was blocked from inserting into the vesicle, increasing the fluorescence signal.[685]

Scheme 6. Synthesis of NBD-GM1 and NBD-GM2 FRET donors to probe GM2-AP activity by Schwarzmann et al., 2005.[680]



BODIPY-labelled GSLs, prepared using semisynthetic strategies, have also been used to study lipid aggregation. Lyso-sulfatide was re-acylated with BODIPY-pentanoic acid after NHS activation.[686] Gangliosides GM1 and GD1a were de-acetylated at *N*-5 of Neu5Ac and re-acetylated with a mixed anhydride of BODIPY-pentanoic acid. Notably, GD1a, a disialo-ganglioside, was labelled with two BODIPY groups. Using these probes, evidence of BODIPY dimers and monomer-to-dimer resonance energy transitions were detected.[646,686] The same synthetic

methodology was used to generate two different BODIPY-labelled GM1 probes as a FRET pair that was used to study the intrinsic self-aggregation properties of GM1.[687,688]

4.3.2 Intramolecular FRET assays

An intramolecular FRET assay has been used to detect SiaT activity. Neu5Ac was labeled with a naphthalene group at *C*-9 and was used to synthesize the modified CMP-Neu5Ac. Lac was labeled with a dansyl group through a propylamine linker on its aglycone. Sialyltransferase activity brought the two fluorophores into proximity, as detected by FRET.[689] The assay was also adapted to monitor FucT activity.[690]

Despite the precedent of using FRET to study many lipid-modifying enzymes,[550,691] intramolecular FRET assays have only recently been implemented to study glycolipid catabolism, most likely due to the synthetic challenges presented by such targets. Intramolecular FRET assays based on lipid probes have been used to study enzyme activity at the membrane including phospholipases[692,693] and ceramidases.[694] Ganglioside degradation has been studied with GM3 FRET probe **75** (**Figure 17**), made through a chemoenzymatic approach in which a novel EGCase II glycosynthase was used for glycosylation. The FRET donor was a coumarin group at *C*-9 of Neu5Ac, while a BODIPY acceptor was incorporated into the fatty acid.[394] The probe was able to insert into cellular membranes and was used to detect SCDase, EGCase, and NEU activity.

Glucocerebrosidase activity in live mammalian cells has been detected with FRET probe **76**. The key intermediate in the synthesis was a bifunctionalized Glc with a pendant alkyne at *C*-6 and an aminoalkyl aglycone. From that intermediate, only four synthetic steps were required to obtain the final probe containing BODIPY 576/589 at *C*-6 of Glc and the FRET acceptor (Black Hole Quencher 2) in the aglycone.[695,696] The same group also developed a quenched bis-acetal-based substrate (BABS) probe **77** to monitor exo-glycosidase activity. The BABS tethered the fluorophore and the FRET acceptor/quencher close together; however, upon cleavage of the

glycosidic bond, the hemiacetal spontaneously broke down, freeing the fluorophore from the quencher. Although this probe was used to monitor O-GlcNAcase activity (an enzyme that releases GlcNAc from serine and threonine), the study demonstrated that BABS-based quenched FRET probes can be used to monitor glycosidase activity.[697]

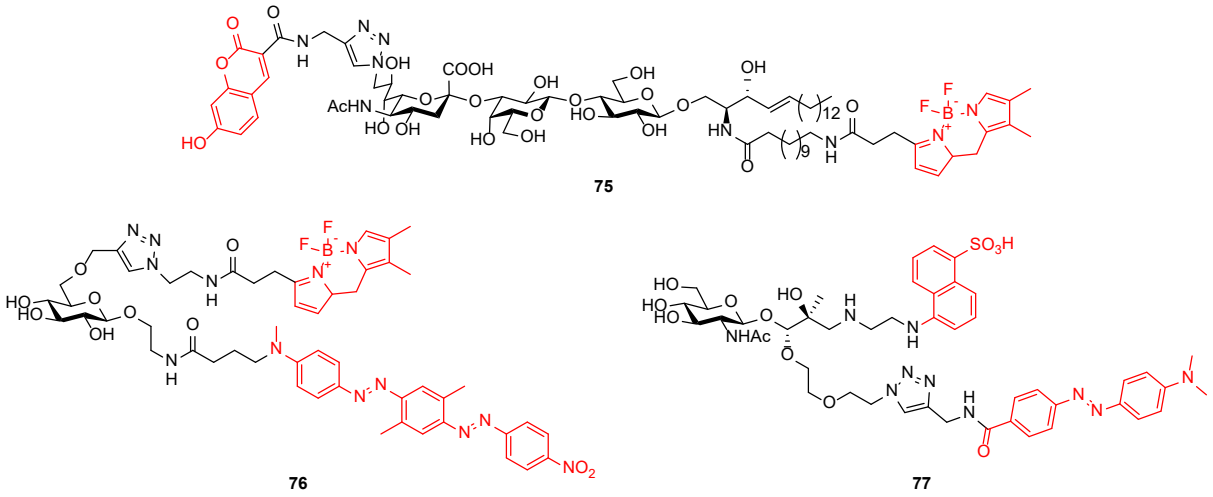


Figure 17. Structure of intramolecular FRET probes to study ganglioside-degrading enzymes (**75**), glucocerebrosidase (**76**), and exo-glycosidase enzymes(**77**).

4.4 Bioorthogonal-tagged glycolipids

Biomolecular probes containing functional groups which undergo selective reactions, or bioorthogonal groups, have become popular tools for elucidating cellular function of specific biomolecules. The specific chemistry of bioorthogonal functional groups should minimize non-specific labeling to allow for detection in a complex cellular environment. In the context of carbohydrate probes, the most popular chemistries are azide-alkyne cycloaddition[698] and the Staudinger ligation.[699,700] Bioorthogonal chemical reactivity has been reviewed extensively elsewhere.[701-705]

4.4.1 Tagging the glycan

Metabolic labeling with bioorthogonal probes offers a solution to some of the problems associated with metabolic labeling using radioactive or fluorescent tags. Small bioorthogonal groups are generally more readily incorporated into biosynthetic pathways, and sensitive fluorescence detection can be used to observe the probe after the labelling reaction. The bioorthogonal metabolic labeling of carbohydrates is immensely popular; however, the majority of studies employing this approach have tended to focus on glycoproteins, despite potential incorporation of the probe into both glycoproteins and glycolipids.[706] *N*-acetylmannose (ManNAc) derivatives, which are incorporated as Neu5Ac residues, are the most common monosaccharides used for metabolic oligosaccharide engineering, and their use has been reviewed.[707-709] Providing ManNAc derivatives to cells with bioorthogonal or photoactivatable groups (see **Sec 4.5**) on their *N*-acyl groups has become an invaluable tool for understanding sialic acid biology. Bertozzi and coworkers have summarized the biosynthesis of Neu5Ac from ManNAc.[710] While *C*-9 azido Neu5Ac has been used as a metabolic label,[711] the Neu5Ac biosynthetic pathway does not tolerate modifications to *C*-6 of ManNAc; therefore, methods to target this position require production of a modified Sia, rather than its ManNAc precursor. Sia modified both at the *N*-5 acyl side chain and *C*-9 has been used in metabolic labeling experiments and has the potential to expand the applications of bioorthogonal metabolic probes.[708] Chemoenzymatic glycan labeling using glycosyltransferases to introduce labeled monosaccharides onto specific acceptors presents an alternative to metabolic oligosaccharide engineering.[712-714]

The most common bioorthogonal functional group introduced into carbohydrates is the azide because of its small size, metabolic stability, and requisite bioorthogonal reactivity.[715] Alkynes have been proposed as a less toxic and more metabolically efficient alternative.[716-718] The popular copper-catalyzed azide-alkyne cycloaddition was considered to be incompatible with

living systems because of toxicity from the catalyst. Strategies to mitigate copper toxicity are a partial solution to this problem.[719,720] To avoid the use of copper in live-cell or live-organism imaging, the Staudinger ligation[706,721,722] and strain-promoted (3+2) cycloaddition[715,723,724] have been developed. ManNAc labeled with perfluoroaryl azide is compatible with the Neu5Ac biosynthetic machinery and accelerates the Staudinger ligation.[725]

An alternative to bioorthogonal metabolic labeling is bioorthogonal *in situ* labeling. This strategy takes advantage of intrinsic reactivity in natural carbohydrates to selectively label the residue of interest. Because of the lack of chemical handles present in carbohydrates, these methods are limited to selective methods such as periodate oxidation and aniline catalyzed oxime ligation (PAL, selective for terminal sialic acids). An analogous approach with Gal-6-ox and aniline catalyzed oxime ligation is known as GAL (selective for terminal Gal residues). Both methods selectively introduce aldehydes into the target carbohydrate residue, followed by the selective addition of a tag through aniline-catalyzed oxime ligation.[363,726] PAL and GAL are complementary[727] and have recently been used together to probe for NEU activity on the cell surface.[728] The *in situ* labeling of carbohydrates has been used primarily for studies of glycoproteins; however, these methods should be applicable to glycolipid targets.

The introduction of a bioorthogonal group on glycolipids through metabolic or *in situ* labelling are synthetically inexpensive. However, they are analytically costly as these labels may be incorporated broadly into multiple species. Convergent or semisynthetic methods for the synthesis of glycolipids with bioorthogonal groups incorporated into the carbohydrate moiety, thus, have advantages for restricting the label to a single target. GlcCer was tagged with an azido group at *C*-6 of the Glc residue.[729] Peracetylated *C*-6 azido Glc was converted to a trichloroacetimidate donor prior to glycosylation with benzoyl-protected Cer in the presence of boron trifluoride diethyl etherate. Azido-LacCer has been generated by a similar convergent approach.[729]

4.4.2 Tagging the lipid

There are few examples of biorthogonal tags as applied to the lipid portion of GSLs; however, there are several strategies which have been applied on other classes of lipids and could likely be translated to GSLs. The semisynthesis of lipids containing azido- or alkynyl-fatty acids can be accomplished through acylation of lyso-lipids with the appropriately modified fatty acids. Incorporation of a bioorthogonal tag into the lipid moiety of glycolipids is likely to make the bioorthogonal group less accessible for subsequent reaction. Consequently, the tag used to detect the bioorthogonal probe must be membrane permeable, or else the lipid may need to be extracted from the membrane. Reports of glycolipids with bioorthogonal labels in the lipid moiety tend to have poor detection, with the most plausible explanation being that the membrane is shielding reactivity of the bioorthogonal group.[729,730]

Several strategies for the generation of fatty acids with bioorthogonal tags are known, and acylation of lyso-glycolipids with these fatty acids could be used to generate labeled GSLs. Fatty acids with an ω-azide have been generated through the conjugation of diphenylphosphorylazide to the methyl ester of a hydroxy fatty acid in the presence of diisopropylazodicarboxylate and PPh_3. The α-azide was obtained by converting the isobutyl 3-hydroxy fatty acid to the isobutyl 3-mesyl fatty acid. After hydrolysis of the isobutyl ester, reaction with NaN_3 generated the final product.[731] An azide was introduced to the alkene of oleate by converting the olefin to the 1,2 epoxide and obtaining the azide through treatment with NaN_3.[732] Ceramides were labeled with an azido group at either the α or ω position of the fatty acid through a convergent method. An α- or ω-bromocarboxylic acid was stirred with NaN_3 in dimethylformamide to generate the azide-modified acid. Carbodiimide coupling was used to form the azide-modified Cer.[730] These azide-modified ceramides could be glycosylated to generate azide-labeled glycolipids. Azide-labeled Sph has been used for the metabolic labeling of sphingolipids, including glycolipids, in vitro.[733]

Alkynes may perturb the hydrophobic properties of the lipid moiety less than the more polar azido group. Fatty acids with a pendant alkyne have been generated by converting propargyl alcohol to a trichloroacetimidate and stirring it with a fatty acid ester containing an alcohol at the α or ω position.[731] Alkyne oleate was made from ω-bromononanol and 9-decynol, followed by oxidation and coupling by Wittig reaction.[734] Saturated ω-alkyne fatty acids were generated through the oxidation of ω-hydroxy fatty acid esters, followed by conversion of the aldehyde to an alkyne with the Bestmann-Ohira reagent.[734] This method has been applied to the synthesis of alkyne-containing glycolipids.[735] Oleate can be converted to a saturated ω-alkyne fatty acid through bromination/debromination, followed by an acetylene zipper reaction.[736,737] Alkyne-terminated fatty acids have been generated using a convergent method from 1-bromo alkyl chains.[738]

4.5 Photoresponsive glycolipids

Photosensitive functional groups have been introduced into glycolipids to generate labeled glycolipid probes. These labels undergo a chemical change in response to irradiation with UV light. The most common class of photoresponsive glycolipid probes generated are photocrosslinking glycolipids that can be covalently cross-linked to other biomolecules upon irradiation with UV light, which provides information on interactions of these glycolipids with other biomolecules.[739,740] This strategy can help to identify proteins that are in proximity to the glycolipid of interest within the cellular environment. Photocrosslinking groups typically incorporated into glycolipids include nitrophenyl azides, diazirines, and benzophenones. Most photocrosslinking glycolipid probes also contain a secondary label (e.g. radionuclide, fluorochrome, or bioorthogonal tag) for detection of the crosslinked product. The location of the photocrosslinking group within the probe may vary depending on the nature of the interactions being investigated.

4.5.1 Glycan photocrosslinkers

Metabolic labeling is a common method for generating glycolipids with a photocrosslinking group contained in the glycan. Like metabolic labeling of glycolipids with bioorthogonal tags, the most extensive work in this field has been done with metabolic labeling of Sia (**Figure 18**). A ManNAc precursor with a pendant *N*-2 acyl group containing a tag (**78**) will be incorporated biosynthetically into Sia with an *N*-5 tag (**79**). Examples of this strategy using ManNAc derivatives containing an *N*-2 diazirine[741] or aryl azide[709] were incorporated into cell surface glycoconjugates. Modification of the *C*-9 position of Sia with an aryl azide required the use of cells lacking Sia biosynthetic machinery.[742] Photocrosslinking of *N*-5 diazirine-modified Sia has been used to detect ganglioside–protein interactions.[743,744] A bifunctional Sia probe containing an azide at *C*-9 and diazirine at the *C*-5 was provided to cells, and was confirmed to incorporate into glycoproteins, while glycolipids were likely also labeled.[708]

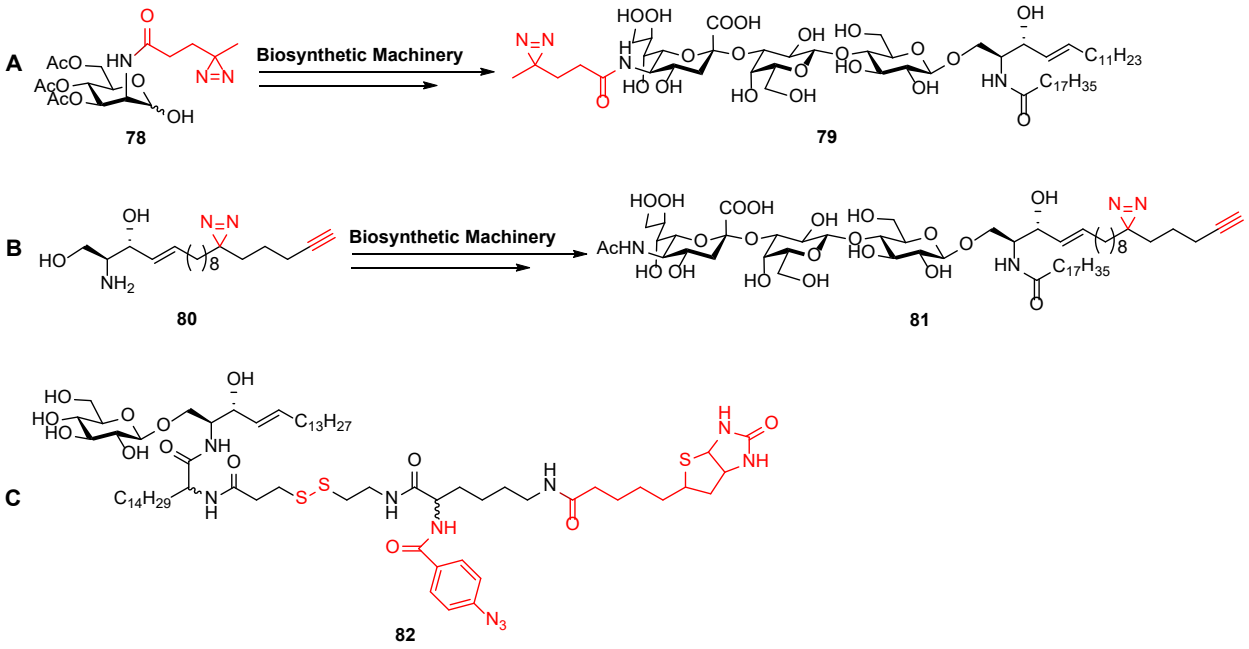


Figure 18. Photoactivatable glycolipids made by A) metabolic labeling of the glycan moiety, B) metabolic labeling of the Sph chain, and C) semisynthesis through removal of the fatty acid and addition of a modified fatty acid.

4.5.2 Lipid photocrosslinkers

Glycolipids labeled with photocrosslinking groups and radiolabels in their lipid chain have been used to study ganglioside-protein interactions. These bifunctional probes were first used by Sonnino et al. in 1989. The Neu5Ac *N*-acetyl group and the fatty acid chain of GM1 were removed using base hydrolysis. Fatty acid acylation was accomplished with an Fmoc-protected ω-amino fatty acid acid through NHS activation. After re-acetylation of the Sia moiety with ^{3}H acetic anhydride, the Fmoc group was removed and the free amine was coupled with a nitrophenylazide.[745] The method has been reviewed elsewhere[746,747] and has been used to identify several glycolipid-protein interactions.[739,740] For asialo glycolipids, the *C*-3 position of Cer was labeled with tritium using an oxidation/reduction strategy.[740] Alternatively, ^{125}I can be incorporated into the phenylazide group.[748,749] As such, the method can be applied to free sphingolipids.[605] Glycolipid-like probes were used to obtain information on the depth of proteins and phospholipids in membrane structures. Glucosamine was acylated at *N*-2 with a fatty acid containing a nitrophenyl azide. The photocrosslinking group was located either at the α-position, or near the terminus, of the fatty acid chain.[750-752] Diazirines and benzophenones have been used as photoactivatable groups in the lipid portion of glycolipids. Bifunctional Sph-containing diazirine and tritium labels were incorporated into GSLs in a metabolic labeling experiment.[753] A bifunctional Sph with a diazirine photocrosslinker and an alkyne tag was incorporated into the GSL metabolic pathway (e.g. **81**).[754] On the other hand, a bifunctional fatty acid containing diazirine and alkyne labels was reported to be incompatible with the metabolic labeling of GSLs.[162] Bifunctional fatty acids were, however, incorporated into glycolipids synthetically.[755] A semisynthetic method incorporating an amine into the α position of the fatty acid chain was used to generate a trifunctional GlcCer probe **82** containing an aryl azide, a biotin tag, and a cleavable disulfide bond.[756] Benzophenone was incorporated into the sphingosine moiety of a glycolipid

using a convergent approach. Following glycosylation of galactose onto a truncated Sph containing a terminal alkene, a cross-metathesis reaction generated the labeled product.[757]

Aryl azide, benzophenone, and diazirine crosslinkers have been incorporated into the lipid moiety of glycolipids through a convergent synthetic approach starting from Boc protected lysine. Comparative studies concluded that the aryl azides had the highest crosslinking yield; however, diazirine groups had the lowest proportion of nonspecific crosslinking.[758,759] A similar study comparing diazirine labeled glycolipids to trifluoromethylphenyldiazirines found the diazirine had reduced crosslinking yields, but was more specific.[760]

4.5.3 Caged glycolipids

Lipids featuring an unnatural protecting group which can be released upon irradiation with UV light are commonly referred to as "caged lipids." This term can be misleading because the lipid is not trapped; rather, a photocleavable group is typically attached to the lipid in a position that renders it biologically inactive. The active lipid is released upon irradiation with a suitable wavelength of light. Caged lipids are attractive in their capability for rapid and specific control over the concentration of a single lipid species, making them useful for investigating the role of lipids in signaling.[761] To date, there are few examples of caged glycolipids or GSL probes, and relevant examples are provided below.

The photocleavable *N*-4-carboxymethyl-2-nitrobenzyloxycarbonyl was originally used as a protecting group for the chemoenzymatic synthesis of GSLs. Lyso-LacCer was acylated with 1-methyl-3-(4-carboxymethyl-2-nitrobenzyloxycarbonyl)-imidazolium chloride, which was prepared by stirring methyl-4-hydroxyl-3-methoxybenzoate, 1-methylimidazole, and phosgene in toluene. The protecting group could be removed upon irradiation with light.[762] This concept was expanded from sphingolipid protecting group chemistry to caged sphingolipids nearly a decade later.[763] *N*-2-nitrobenzyl caged lipids have also been prepared.[762] An *N*-2-nitrobenzyl caged

galactosylsphingosine and glucosylsphingosine were prepared by reaction of nitrobenzyl bromide with the corresponding sphingosine.[763] A similar strategy was used for the chemoenzymatic synthesis of *N*-2-nitrobenzyloxycarbonyl-caged lyso-GM3. *N*-2-nitrobenzyloxycarbonyl-caged Sph was glycosylated with per-*O*-acetylated lactosyl bromide. After deprotection to afford caged lyso-LacCer, a SiaT reaction generated caged lyso-GM3.[764] The compatibility of *N*-2-nitrobenzyloxycarbonyl-caged Sph with both chemical and enzymatic transformations indicate this approach may be feasible for the synthesis of other lyso-gangliosides.

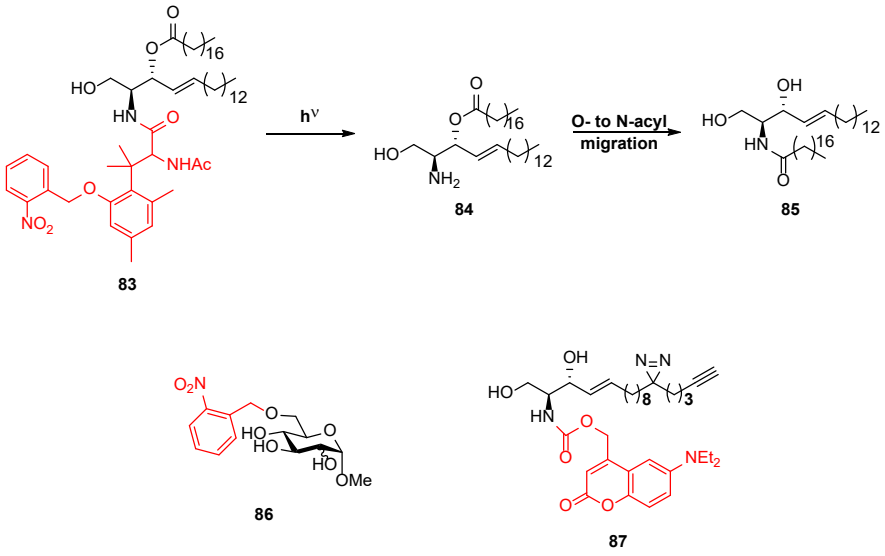


Figure 19. Structures of caged lipids

Approaches to caged phospholipids and sphingolipids may be transferable for the synthesis of caged glycolipids. An elegant caged Cer probe was developed in which release of the photocleavable group induced *O*- to *N*-acyl migration of the fatty acid to generate Cer (**83**-**85**, **Figure 19**). Starting from protected Sph, *N*-acylation with the photocleavable group was followed by *O*-acylation of the *C*-3 secondary alcohol with a fatty acid.[765] Although this method has not been applied to the synthesis of caged glycolipids, the primary hydroxyl group of the Cer moiety was not involved and could likely tolerate a glycan. Photocaged glycan residues have been generated using *o*-nitrobenzyl groups incorporated into *C*-6 of methyl glucoside and methyl

mannoside from their 4,6-*O*-*o*-benzylidene acetals through reductive ring opening (**86**).[766] A trifunctional sphingosine probe was made containing a photocleavable caging group, a bioorthogonal alkyne, and a photocrosslinking diazirine group (**87**).[767]

4.5.4 Photoswitchable glycolipids

Photoswitchable glycolipids are those that change conformation upon irradiation with UV light. Azobenzene derivatives are well known for their photoswitchable properties that have been adapted for applications in biomolecules[768] and photosensitive materials.[769] Upon irradiation with UV light, azobenzene switches from a *trans* conformation to a *cis* conformation. Other properties of azobenzene and its applications have been reviewed elsewhere.[768] Azobenzene-Glc derivatives have been constructed and used to form monolayers.[770] A similar approach was used for the synthesis of a variety of glycolipids with Glc, Gal, Man, xylose, and Lac sugar units.[771] A photoswitchable Cer was developed to convert ordered domains into disordered domains upon irradiation with UV light, a feature useful to probe lipid function within a membrane environment. Sph was converted in one step to the photoswitchable Cer through amide coupling with an azobenzene-containing fatty acid.[772] Maltose-based azobenzene-containing glycolipids were designed as photosenstitive supramolecular gelators.[773] A supramolecular amphiphile with a Gal headgroup and a distal azobenzene group has demonstrated photoswitchable properties.[774]

4.6 Glycolipid microarrays

Carbohydrate microarrays provide a high-throughput method to screen carbohydrate-protein interactions using small amounts of analyte and protein.[775] Glycolipid microarrays that mimic properties of the lipid membrane can also provide insight into the role of lipid clustering in glycolipid-protein or glycolipid-pathogen interactions. The methods for carbohydrate immobilization onto microarrays fall into two categories: covalent and non-covalent methods.

Methods for glycan microarray construction have been reviewed extensively,[310,775-778] so keeping with the scope of this review, we will only discuss methods that pertain to the construction of glycolipid microarrays.

4.6.1 Non-covalent attachment

The non-covalent immobilization of carbohydrates on glycolipid microarrays permits lateral mobility of the glycolipids and can mimic clustering that occurs within membranes. Clustering may lead to increased avidity for weak carbohydrate-protein interactions. An early glycolipid array immobilized GSLs through adsorption on a 96-well polystyrene microtiter plate.[779] An array of synthetic glycolipids with an aglycone consisting of alkyl chains ranging in length from 3-11 carbon atoms was also made by this method.[780] This strategy has been used by other groups, but requires greater quantities of glycolipid than microarrays.[781,782]

An early method for construction of carbohydrate microarrays converted carbohydrates to neoglycolipids followed by adsorption onto nitrocellulose coated glass slides.[783-787] Microarrays with glycans from a variety of sources, from synthetic oligosaccharides to whole organs, have been constructed.[310] These neoglycolipids are typically generated through reductive amination of reducing sugars.[783] An alternative which preserves the reducing-end residue is oxime ligation with an aminooxy-linker. Neoglycolipid arrays produced in this way were reported to be more sensitive than an equivalent array generated by reductive amination.[320] An alternative to nitrocellulose membranes is polyvinyl difluoride (PVDF) membranes.[788] Drawing inspiration from membrane protein microarrays,[789] glycolipid microarrays have been constructed on γ-aminopropylsilane-coated slides. This surface retained glycolipids yet allowed for their long-range lateral mobility. Gangliosides GM1 and GT1b were incorporated into vesicles and printed on γ-aminopropylsilane-coated slides.[790] A glycolipid microarray has been made by non-covalent immobilization of

glycolipids on a hydrazide-functionalized dendrimer film. The method is proposed to have reduced autofluorescence as compared to nitrocellulose and PVDF membranes.[791]

Glycolipids form heterogeneous microdomains in membranes.[171-173] Combinatorial glycolipid microarrays (where multiple glycolipids are printed on each spot) enable study of the effects of glycolipid clusters and heterodimers on protein recognition. A combinatorial ganglioside microarray was made by spotting mixtures of gangliosides on a PVDF membrane.[152,792] A variety of lectins and toxins were tested against the microarray; notably, individual gangliosides had different interactions as compared to combinations of gangliosides.[152] These data highlight the complexity of glycolipid membrane interactions,[793] and the necessity for analytical technologies to study such complex interactions. Combinatorial glycolipid microarrays have also been printed with neutral glycolipids,[794] and mixtures of gangliosides and neutral glycolipids.[795-797] As many as 11 gangliosides, 8 neutral glycolipids, and their 162 heterodimeric complexes have been assayed on a single combinatorial glycolipid microarray.[797]

4.6.2 Covalent attachment

Although covalent immobilization of glycolipids on microarrays does not permit lateral diffusion, it enables more control over intermolecular distance and may afford improved stability.[798] Early covalent carbohydrate microarrays immobilized maleimide-linked carbohydrates onto thiol-coated glass slides. The carbohydrate moiety was tethered to the maleimide through alkyl chains of varying length.[799] Another early method coupled the reducing end sugar of GM1, asialo-GM1, and GM3 to 4-(2-aminoethyl)aniline through reductive amination. The resulting pendant amine was coupled to an NHS-activated glass surface.[800] The same protocol was used more recently to make a microarray containing GM1-related carbohydrates to probe GM1 interactions with cholera toxin.[798] An alternative covalent approach has used azide-modified surfaces to link phosphine-containing liposomes via Staudinger ligation. Liposomes doped with phosphine-lipids were loaded

with gangliosides, such as GM1 and GM3, prior to immobilization on the azide-coated surface.[801] The unreacted phosphine reagents provided potential for further synthetic modification.[802] This approach provides a combination of covalent attachment while presenting gangliosides in a fluid bilayer. Alternative membrane-mimetic platforms include micelles,[803] nanodiscs,[129] and picodiscs.[128]

5 Conclusions and outlook

There has been a definite resurgence in interest for development of modified glycolipids as research tools over the last decade. This is perhaps most attributable to the maturation of synthetic methods which have put complex GSL targets within reach. We hope to see this field expand and begin to address fundamental questions about the association of GSLs with themselves and other molecular species within the membrane environment. It is notable that many of the methods discussed to generate probes in this review were first applied to phospho- or sphingo-lipids before applications in glycolipids. As more groups delve into the synthesis of modified glycolipids, it is likely that we will expand our understanding of the molecular features central to their function. There remains a need for diverse strategies that provide selective modifications in the glycan moiety of GSL. While Gal-6-Ox, periodate oxidation, and glycosyltransferase strategies are well known, there remains a need to expand available tools to address alternative sites common to these oligosaccharides. Furthermore, new analytical methods that can explore the specific interactions of glycolipids may benefit from new probe-based strategies. GSLs serve unique roles within the cell membrane and contain extensive molecular complexity as compared to other membrane lipids. The continued development of new probes to investigate these systems will be essential to revealing the details of protein-lipid and lipid-lipid interactions which GSL take part in and, by extension, the diverse biological processes in which they participate.

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Abbreviations

AV	anthrylvinyl
BABS	bis-acetal-based substrate
Boc	*tert*-butyloxylcarbonyl
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene
BOP	Benzotriazole-1-yloxy-tris(dimethylamino)phosphonium
Cav	Caveolin
CAZy	Carbohydrate Active enZYmes Database
Cer	Ceramide
Chol	Cholesterol
CM	Cross-metathesis
CMC	Critical micelle concentration
CMP	Cytidine monophosphate
CSS	CMP-sialic acid synthetase
CTP	Cytidine triphosphate
Cy-3	Cyanine 3 dye
DABCY	4-((4-(dimethylamino)phenyl)azo)benzoic acid
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
Dio-C16	3,3'-dihexadecyloxacarbocyanine perchlorate
DPH	3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenyl
DSC	Differential scanning calorimetry
EGFR	Endothelial growth factor receptor
ESR	Electron spin resonance
Fluor	Fluorophore
Fmoc	Fluorenylmethyloxycarbonyl
FRET	Förster resonance energy transfer

Fuc	Fucose
FucT	Fucosyltransferase
GAL	Galactose-6-oxidase oxidation and aniline catalyzed oxime ligation
Gal	Galactose
GalCer	Galactosylceramide
GalNAc	*N*-acetylgalactosamine
GalNAcT	*N*-acetylgalactosaminyltransferase
GalT	Galactosyltransferase
GGLs	Glycoglycerolipids
Glc	Glucose
GlcCer	Glucosylceramide
GlcNAc	*N*-acetylglucosamine
GM2-AP	GM2-activator protein
GPI	Glycophosphatidylinostitol
GSL	Glycosphingolipid
GUV	Giant unilamellar vesicle
HP-TLC	High performance thin layer chromatography
HWE	Horner-Wadsworth-Emmons
Kdn	Keto-deoxyneuraminic acid
Lac	Lactose
LacCer	Lactosyl ceramide
LacNAc	*N*-acetyllactosamine
LIPID MAPS	Lipid Metabolites and Pathways Strategy
LC-MS	Liquid chromatography and mass spectrometry
Le^a	$Lewis^a$

Le^x	Lewis^x
LNT	lacto-N-tetraose
LNnT	lacto-N-neotetraose
MAM	Mitochondria-associated membranes
ManNAc	*N*-acetylmannosamine
NBD	7-nitrobenz-2-oxa-1,3-diazole
Neu4,5Ac$_2$	4-*O*-acetyl *N*-acetylneuraminic acid
Neu5Ac	*N*-acetylneuraminic acid
Neu5Ac8OMe	8-*O*-methyl *N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
NHS	*N*-hydroxysuccinimide
NTP	Nucleotide triphosphate
OPME	One-pot multienzyme
PAL	Periodate oxidation and aniline catalyzed oxime ligation
PDA	10,12-pentacosadiynoic acid
PVDF	Polyvinyl difluoride
Sia	Sialic acid
SiaT	Sialyltransferase
sLe^x	Sialyl Lewis^x
Sph	Sphingosine
TBDPS	*Tert*-butyldiphenylsilyl
TBS	*tert*-butyldimethylsilyl
TMR	Tetramethylrhodamine
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
UDP	Uridine diphosphate
UV	Ultraviolet

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