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Glycosphingolipids: From metabolism to chemoenzymatic total synthesis

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Abstract: GSLs are the major glycolipids in vertebrate mediating many key biological processes from intercellular recognition to cis regulation of signal transduction. The fast-expanding glycobiology poses a growing demand for diverse and structurally defined GSLs, and enzymatic GSL synthesis is developing rapidly in accordance. This article provides an overview of natural GSL biosynthetic pathways and surveys the bacterial enzymes applied to GSL synthesis and recent progresses in the synthetic strategy. By correlating these three areas, this article aims to define the gaps between GSL biosynthesis and chemoenzymatic synthesis and evaluate the opportunities of harnessing the natural forces to access GSLs efficiently.

1. Introduction

Glycosphingolipid (GSL) is a subclass and the major form of glycolipids in animals. GSLs are amphiphiles with a hydrophilic glycan head group and a hydrophobic lipid tail connected by a glycosidic bond. The lipid moiety, known as ceramide (Cer), contains two lipid chains that are inserted into the cell membrane to cluster GSLs in the "lipid raft" rich of cholesterol, sphingomyelin (SM), and transmembrane proteins.[1, 2] The glycan moiety extents into the extracellular medium to mediate recognition and cellular interactions. Although sharing the conserved core of Cer with β-glycosidic linkage to glycans, GSLs are structurally diverse in both moieties. Jointed by an amide bond, the sphingosine and the fatty acyl (FA) chain of Cer vary in chain length, unsaturation pattern, branching, and hydroxylation or other modifications. The glycan moiety consists of 1-60 monosaccharide residues connected by glycosidic bonds varying in regio- and stereochemistry, accounting for over 400 different glycans.[3] Accordingly, GSLs are classified based on the common core sequence of glycans into seven major "series": *ganglio*-, *globo*-, *isoglobo*-, *lacto*-, *neolacto*-, *mollu*-, and *arthro*-series. A less common way of GSL classification is based on acidity resulting from sulfation and sialyation.

1.1 Functions and biological significance

The Cer and glycan moieties of GSLs have distinct functions. The Cer moiety comprises *ca*. 5-20% of total lipids in the plasma membrane of vertebrate cells and contributes significantly to the rigidity of cell membrane due to their long and saturated lipid chains.[4] Together with cholesterol and other biomolecules, GSLs and Cers cluster into dynamic microdomains in the cell membrane, which also called lipid rafts that are believed to play a pivotal role in gathering signalling molecules, including glycans, glycosylphosphatidylinositol-anchored proteins and transmembrane proteins, for enhanced and specified signal transduction. The glycans of GSLs are the signalling motifs to mediate signal transduction in two ways, i.e., *trans* recognition and *cis* regulation. The former engages the recognition of GSL glycans by receptors on neighbouring cells, whereas the *cis* regulation means the mediation of protein activities on the same plasma membrane by GSLs. Pathological applications of GSLs have been focused on the *trans* recognition, so GSLs are used as biomarkers for diagnostic and therapeutic purposes. However, recent progress in glycobiology has helped elucidate the mechanisms of many *cis* regulated processes, enabling us to use GSLs as functional molecules in modulating signalling pathways. As a result, a new trend emerges.[5]

1.2 Scope of this review

The fast-growing glycobiology poses a synthetic challenge to provide structurally diverse but homogeneous GSLs. This synthetic challenge lies in two facets: coupling hydrophilic glycans with hydrophobic Cer and constructing glycosidic linkages in a regio- and stereo-selective manner. Since the pioneering work in the 1980's, synthetic chemists have developed various strategies addressing these two problems. However, the chemical construction of glycosidic bonds still suffers from tedious protection/deprotection procedures and lengthy routes. On the other hand, enzymatic synthesis has witnessed great progress in recent years, especially after the discovery that various bacterial glycosyltransferases (GTs) recognize common sugar nucleotides as donors and transfer sugars to a wide scope of acceptors. Despite the requirement of expensive enzymes and sugar nucleotides, enzymatic synthesis is still more efficient than chemical synthesis, especially for natural glycans. Another type of enzymes, glycosynthases developed from glycosidase bioengineering, can avoid the dependence on sugar nucleotides, thus providing an economical alternative to GTs.

Enzymatic GSL synthesis is confronted by its unique challenges, such as coupling of hydrophilic glycans and hydrophobic lipids in aqueous media and efficient ways to address the structural diversity for both moieties.[6] Solutions to these unique challenges may lie in the natural metabolic pathways of GSLs as enzymes involved in GSL biosynthesis and catabolism are highly efficient for specialized tasks. For example, a special class of glycosynthases that can directly couple sugars with Cer has been developed by engineering of glycosidases, the enzymes that break down GSLs. These findings inspired us to write this mini review to examine in detail GSL biosynthesis, the status of enzymatic GSL synthesis, and the enzymes involved in both areas in hoping to shed lights on further improvement of enzymatic synthesis of GSLs by mimicking the natural way more closely.

Since our last review on enzymatic GSL synthesis,[7] several new and important reports have emerged, such as the streamlined synthesis combining GTs and glycosynthases.[8] At the end of this review, recent progresses in enzymatic synthesis will be discussed.

2. Glycosphingolipid Biosynthesis

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GSL biosynthesis is a stepwise process that involves many enzymes and translocation of the synthetic intermediates among intracellular organelles, such as endoplasmic reticulum (ER) and Golgi apparatus. Cer is synthesized in the ER and then equilibrated to the luminal face, followed by transfer to the Golgi apparatus, where monosaccharides are sequentially attached by GTs, starting with addition of a glucose (Glc) residue. The attachment of galactose (Gal) to Cer to form GalCer is completed in ER followed by transfer to and further decoration in Golgi. GTs that connect inner sugar residues are normally specific to GSL synthesis, while the GTs transferring external sugar residues may also be involved in glycoprotein synthesis. Elongation of the glycan moiety by GTs leads to most of the structural diversity in a competing manner, depending upon the availability of GTs in various cells. As a result, GSL composition and structure may be loosely controlled by cell types. Another mechanism of GSL regulation is the streamlined assembly of glycans by multienzyme complexes without dissociation of intermediate until final product is formulated.

2.1 Ceramide biosynthesis

Three major pathways are involved in Cer biosynthesis, including *de novo* synthesis, salvage pathway, and sphingomyelinase hydrolysis pathway (Figure 1).[9] The *de novo* pathway occurs in ER, as shown in the orange panel in Figure 1. This synthetic pathway initiates with the condensation of palmitoyl-CoA and L-serine via decarboxylation of L-serine. This reaction is the rate-limiting step in Cer biosynthesis and is catalysed by serine C-palmitoyl transferase (SPT), resulting in the production of 3-ketodihydrosphingosine (3KDS).[10] SPT has three main isoforms, which are combined to form a multi-subunit structure. In humans, the main SPT isoforms, including three SPTLC and two small subunits (ssSPT a and b), contribute to the Claisen condensation reaction of L-serine and acyl-CoA.[9, 11, 12] These isoforms generate four heterostructure complexes, i.e., SPTLC1/2/ssSPTa, SPTLC1/3/ssSPTa, SPTLC1/2/ssSPTb, and SPTLC1/3/ssSPTb, in which SPTLC species are stabilized by hydrogen bonds and salt bridges with ssSPTs to regulate the enzymatic function.[13] SPT isoforms have distinct preferences for the carbon chain in Acyl-CoA. It has been observed that the SPTLC1-SPTLC2-ssSPTa complex promotes the formation of 3KDS with 16 carbons, whilst the SPTLC1-SPTLC3-ssSPTa complex is slightly more reactive with C14-CoA. On the other hand, heterodimers with ssSTPb prefer longer acyl-CoAs.[14]

The second step in *de novo* synthesis is the reduction of the ketone group in 3KDS to a hydroxyl group, catalysed by 3-ketosphinganine reductase, resulting in dihyrosphingosine (DhSph).[15] It is followed by converting DhSph into dihydroceramide (DhCer) under the influence of a 6-member family of enzymes termed Cer synthases (CerSs). This enzyme family catalyse DhSph N-acylation with a distinct preference for the FA chain ranging from C14 to C30.[16] CerS1 is the first enzyme in this class found in mammals and is highly expressed in mammalian brain cells and skeletal muscles. This enzyme specifically reacts with C18-CoA substrate to generate C18 DhCer. Meyers-Needham *et al.* have proved the presence of another CerS1 variant, spliced isoform of CerS1 called CerS1-2, which is expressed in multiple human cancer cells.[17] CerS2 is broadly expressed in organs such as liver and kidneys, using very long chain acyl-CoAs ranging from C22 to C24.[13, 16] CerS3 is widely distributed in mouse testis and skin. As the main isoform of the Cer synthase family, it is involved in almost 90% of epidermal Cer synthesis and produces Cers with long acyl chain (C26-Cers). Sassa *et al.* have shown that ultra long-chain Cers (ULC), with the FA chain of more than 26 carbons, are specifically synthesized by CerS3 and the deficiency of this enzyme in mammals is associated with defects in epidermal permeability barrier caused by reduced levels of ULC-Cer species.[18] Furthermore, it has been confirmed that the chain length is directly related to cancer development, especially breast cancer, where the levels of C24-Cers are upregulated.[18, 19] CerS4 targets acyl-CoAs with carbon chains from C18 to C22 and is mainly discovered in heart, liver, and leukocytes. CerS4 is the main component in adult epidermis, expressed in interfollicular epidermis and hair follicles. Ebel *et al.* have shown that the loss of CerS4 can lead to hair protein deficiency due to sebum composition change and hair canal blockage in mammals, which is akin to age-related hair-loss.[20, 21] CerS5 and CerS6 are expressed at lower levels and exhibit a preference for C16-CoA, and Gosejacob *et al.* have discovered that CerS5 is abundantly expressed in lung epithelial cells, and C16-Cer level is very low in the liver and muscle.[9, 13, 22] In the last step, DhCer is desaturated at d4 position of sphingosine to Cer or hydroxylated to phytoceramide.[16] There are two desaturases, DES1 and DES2. DES1 possesses only desaturase activity but DES2 has evolved into a bifunctional desaturase/hydroxylase.[23]

The second pathway of Cer synthesis, as illustrated in the green panel in Figure 1, is the sphingomyelinase pathway. It has been reported that SM can be hydrolysed to Cer by sphingomyelinases under acidic, basic, and neutral conditions at various cellular locations, including the plasma membrane, mitochondria, and the endosome/lysosome compartment.[24-26]

In the salvage pathway (black panel in Figure 1), Cers are produced by CerSs *via* direct N-acylation of sphingosine, which is generated by degradation of higher order sphingolipids such as glycosphingolipids, glucosylceramide (GlcCer) and SM, as well as deacetylation of Cer by ceramidase.[11, 25, 27] The salvage and *de novo* pathways share CerSs. In the salvage pathway, free sphingosine can be altered either to Cer by CerSs or to sphingosine-1-phosphate (S1P).[28] Moreover, three types of enzymes, acid, neutral and alkaline ceramidases, characterized by the pH level required for their optimal functions are engaged.[29] Acid ceramidase has a specific preference to hydrolyse short to medium-chain Cers ranging from C6 to C16.[28] Neutral ceramidases (NCDases) exhibit higher expressions in the kidney and liver, but are relatively less abundant in the brain, lung, and heart. Alkaline ceramidase specifically catalyses the hydrolysis of phytoceramide and react with long-chain Cers ranging from C20 to C24.[25, 29, 30]

A novel pathway to produce Cers has also been identified within the liver mitochondria. This process is mediated by an NCDase that also catalyses the reversible process of Cer deacetylation, thus acylating sphingosine. This enzyme uses the palmitate derived from palmitoyl-CoA by Acyl-CoA thioesterase to synthesize Cers.[10]

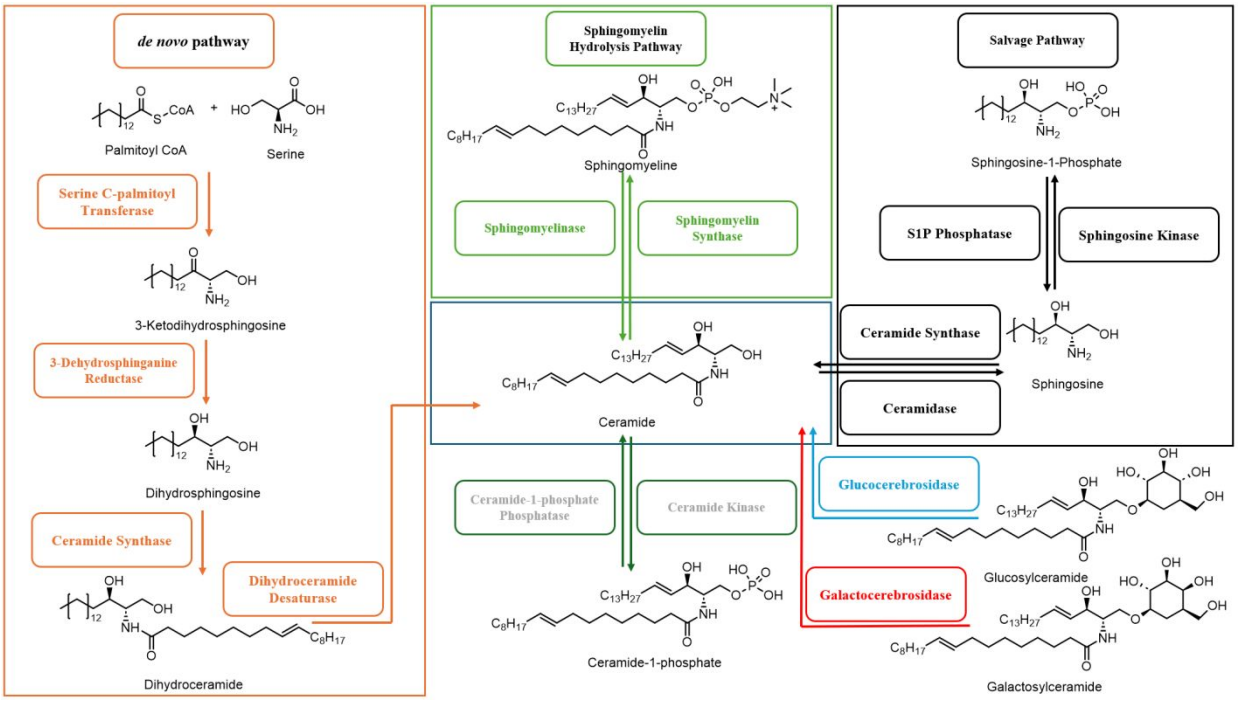


Figure 1. The major pathways involved in ceramide biosynthesis, including *de novo* synthesis (orange box) and the sphingomyelin hydrolysis (green box) and salvage (black box) pathways.

Besides being consumed in GSL synthesis, Cers may face other fates after being transferred to the Golgi apparatus. They can be added to phosphocholine to generate SM or be phosphorylated to form Cer-1-phosphate (C1P).[16] Cer kinase (CerK) is a sphingolipid metabolic enzyme found in mammals and exclusively exists in the membrane fraction of the neural secretory (synaptic) vesicles.[31] This lipid kinase regulates the intracellular level of Cers and phosphorylates Cers to generate C1P. The expression levels of CerK have been elevated in several cancers, such as breast cancer, and accordingly leads to increased levels of C1P.[31, 32] The reverse reaction of C1P to form Cer by dephosphorylation is catalysed by C1P phosphatase.[33]

2.2 GlcCer and GalCer biosynthesis

Further transformations of Cer occur in ER and the Golgi complex and are catalysed by three enzymes, that is, galactosylceramide synthase (GalCerS), GlcCer synthase (GlcCerS) and SM synthase. A majority of Cers is rapidly transported from ER to the Golgi through two distinct methods: the non-vesicular pathway, which results in the production of SM in the *trans*-Golgi compartment, and vesicular pathway, which occurs in the *cis*-Golgi apparatus to produce GlcCer.[34, 35]

2.2.1 Galactosylceramide (GalCer)

Cer galactosylation is the reaction of transferring Gal from uridine-5'-diphospho-galactose (UDP-Gal) to the hydroxyl group at the Cer C-1 carbon to generate a β-glycosidic linkage catalysed by GalCerS, also called Cer galactosyltransferase (CGT), which is expressed in selective cell types.[36] GalCer is the primary GSL component in myeline in the central nervous system and is involved in the regulation of calcium channels.[37] Additionally, GalCer has been demonstrated to present in Schwann cells and oligodendrocytes, and in spinal, cerebellar, and brainstem neurons at lower levels.[38] The biosynthesis of GalCer occurs in ER catalysed by CGT, which is oriented towards the lumen of ER. Then it is transported to the *trans*-Golgi compartment, which involves Cer transport protein (CerT), for the production of higher galactosphingolipids and sulfatides, such as 3-O-sulfogalactosylceramide.[34, 36] Sulfatides are the products of 3-O-sulfation of Gal in GalCer catalysed by cerebroside sulfotransferase (CST) with 3'-phosphoadenosine-5'-phosphosulfate. In sulfatides, the Cers are mainly composed of 4-sphingenine (d18:1) and saturated (C22:0-C24:0), unsaturated (C24:1) and hydroxylated (C22:0 h, C24:0 h) FA chains. Shorter (C16-C20) and longer (C26) FA chains are minor because CST and CGT prefer Cers with C22 and C24 FAs. Abnormal metabolism of sulfatides is linked to various diseases, such as type I diabetes, autoimmune diseases, cancer, and HIV infection.[39]

2.2.2 Glucosylceramide (GlcCer)

GlcCer is synthesized in *cis*-Golgi and then transported to the luminal side of *trans*-Golgi to form more complex GSLs. GlcCerS, also called *N*-acetylsphingosine D-glucosyltransferase or Cer glucosyltransferase (UGCG), is an essential enzyme to attach the first β-linked Glc residue to Cer to produce GlcCer, which is the primer for the biosynthesis of many other GSLs.[40, 41] The FA chains in GlcCer range from C16 to C24.

GlcCer trafficking from *cis*- to *trans*-Golgi compartments is executed through two pathways, i.e., vesicular route resulting in production of *lacto*- and *neolacto*-series GSLs and protein-aided transport leading to *globo*- and *asialo*-series GSLs.[42, 43] In the vesicular route, GlcCer is transferred to the luminal leaflet of late Golgi compartments, where complex GSLs are synthesized, started with the conversion of GlcCer into lactosylceramide (LacCer) catalysed by LacCer synthase (LCS), an enzyme found throughout the Golgi compartment.[43]

The protein-aided transport is carried out by ATP-dependent GlcCer flippases (P4-ATPase) and ATP binding cassette (ABC) transporters, which are two distinct membrane proteins transporting lipids across the cell membrane.[44] Some subfamilies of ABC transporter, including ABCB4 and ABCA12, translocate GlcCer to *trans*-Golgi

compartment, which facilitates the synthesis of *globo*-series GSLs, whereas ABCB1 flips GlcCer to the *cis*-Golgi, where *ganglio*-series GSLs are formed.[34] These enzymes function differently due to their specific expression, subcellular localization, and substrate specificity. ATP10D is one of the members of this family and selectively transports GlcCer, but not GalCer. Mutation of transporters may cause abnormal accumulation of GlcCer, which is related to the development of some diseases such as Parkinson's and Gaucher diseases.[45] GlcCer is also present in the virus envelope, facilitating virus binding and spreading to other cells. This mechanism has been proposed for the influenza virus, Sindbis virus, and SARS-CoV-2 virus.[36, 46]

2.2.3 Glycan synthesis and glycosyltransferases

GTs are enzymes that catalyse glycosidic bond formation employing sugar nucleotides as activated glycosyl donors.[47] The acceptors can be other sugars or lipids, proteins, nucleic acids, antibiotics, and other small molecules. The type of glycosidic bonds that these enzymes can generate varies from N- and O- to S- and C-linkages. By mechanisms, these enzymes can be categorized into retaining or inverting species based on the stereochemistry of their glycosylation reactions, which can be α→α (retaining) or α→β (inverting). Inverting GTs uses an S_N2-like mechanism, whereas retaining GTs is most likely to produce an oxocarbenium intermediate with the phosphate as a leaving group.[48] The GT family can be further classified based on the donor, acceptor, specificity, and amino acid sequence. The number of newly identified GTs has been growing rapidly.[49] For example, the CAZy database has documented over a million GTs (1,406,220 presently) of 135 distinct families, classified based on the amino acid sequence. Among them, GT2, GT29, GT31, GT 42, and GT52 families (Table 1) contain the GTs for GSL biosynthesis. The GT2 family also includes cellulose synthase, chitin synthase, mannosyltransferase (Man-T), glucosyltransferase (Glc-T), and galactosyltransferase (Gal-T).

Table 1. Major families of glycosyltransferases (GTs) involved in GSL glycan biosynthesis.

GT families	Enzymes
GT2	α-GlcNAc-PP-lipid β-1,3-Gal-T
	β1,3-GalNAc-T
	GalNAc-T
	β1,3-Gal-T
	α1,3-Fuc-T
	α1,4-Fuc-T
	α1,6-Fuc-T
	β1,4-Gal-T
GT52	β-galactoside α-2,6-ST
	β-galactoside α-2,3-ST
	N-acetyllactoside α-2,3-ST
GT29	β1,3-galactan β1,6-Gal0T
	β1,6-galactan β1,6-Gal0T
	α2,8-polysialyltransferase (α2,8-PST)
	β-galactoside α2,6-ST
	α-*N*-acetylgalactoside α2,6-ST
	β-galactoside α2,3-ST
	N-acetyllactoside α2,3-ST
	α-*N*-acetylneuraminyl-2,3-β-galactosyl-1,3-*N*-acetylgalactoside α2,6-ST
	α-*N*-acetylneuraminate α2,8-ST
	lactosylceramide α2,3-ST
GT31	β1,3-GalNAcT
	β1,3-GalT
	β1,3-GlcNAcT/Lc3 synthase
GT42	α-*N*-acetylgalactoside α2,6-ST
	β-galactoside α2,3-ST
	N-acetyllactoside α2,3-ST
	α-*N*-acetylneuraminate α2,8-ST

GTs involved in the initial steps during GSL biosynthesis can recognize hydrophobic accepters with Cer and, thus, are relatively specific. GTs that attach external residues, such as sialic acid and fucose residues, are shared with the synthetic pathways of other glycoconjugates like glycoproteins. As a result of this sharing mechanism and a large pool of GTs identified, it is hard to specify all GTs specific to GSL synthesis. In this section, only some of the GTs are identified as specific to GSL synthesis if verified in literature reports.

2.2.4 Galactosyltransferase (Gal-T)

Gal-Ts are located in the Golgi apparatus to catalyse the transfer of a Gal residue from UDP-Gal to acceptor to generate α1,3-, α1,4-, β1,3-, and β1,4-Gal linkages. Seven members of the β1,4-Gal-T family have been identified to date, including β1,4-Gal-T I, II, III, IV, V, VI, and VII (Table 2). *In vitro* studies have shown that these enzymes recognize specific acceptors, including Glc, *N*-acetylglucosamine (GlcNAc), and xylose (Xyl). β1,4-Gal-T I mediates the transfer of Gal from UDP-Gal to acceptors containing a GlcNAc residue. It has been confirmed that β1,4-Gal-T I and II are active for lactose synthesis in the presence of α-lactalbumin, in contrast to β1,4-Gal-T III and V that are inactive for α-lactalbumin.[50] Recent studies show that β1,4-Gal-T I also processes Glc, deoxy-Glc, GalNAc, GlcNAc, and arabinose (Ara) donors, albeit at lower rates (0.3-5%) compared to the transfer of Gal.[51] This enzyme has two isoforms on the *trans*-Golgi surface to facilitate the synthesis of disaccharides, oligosaccharides, and polysaccharides.[52] β1,4-Gal-T II and V, like β1,4-Gal-T I, catalyse the formation of a Galβ1,4-GlcNAc motif, but the activity of β1,4-Gal-T V is not limited to the formation of β1,4-glycosidic bonds between Gal and GalNAc. It has a preference to generate Galβ1,4-GlcNAcβ1,6-GlcNAcβ1,2-Man on the branching mannose (Man) and, thus, is essential for oligosaccharide branching.[53] However, it exhibits no preference to asialo-agalacto-transferrin or lactotriosylceramide (Lc_3Cer).[50] β1,4-Gal-T III is usually found in the reproductive organs, such as testis, ovary and placenta, and it catalyses efficient synthesis of *N*-acetyllactosamine (LacNAc). β1,4-Gal-T IV is highly expressed in placenta, and it is homologous to β1,4-Gal-T III to catalyse LacNAc synthesis with GlcNAcβ1,3-Galβ1,4-GlcCer as the substrate.[54] β1,4-Gal-T IV has shown a high preference in transferring Gal to βGlcNAc in Lc_3Cer and nLc_5Cer. In contrast to β1,4-Gal-T I to III, β1,4-Gal-T IV shows no activity to asialo-agalacto-fetuin, asialo-agalacto-

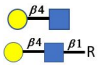
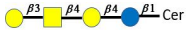
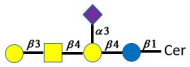
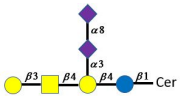
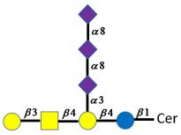
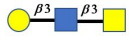
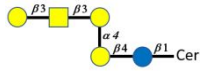
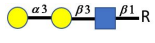
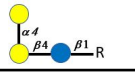
transferrin, and ovalbumin.[50, 55] β1,4-Gal-T VII is different from other β1,4-Gal-Ts as it transfers Gal to Xyl residue to generate a Galβ1-4Xylβ1-R motif in proteoglycans.[56]

β1,4-Gal-T V is highly expressed in brain, whereas the expression of β1,4-Gal-T VI in brain follows a restricted pattern.[55] The expression levels of β1,4-Gal-T II and III are limited. β1,4-Gal-T V has been shown to play a role in human colorectal cancer, where it can be utilized as a diagnostic marker due to its activity in glycosylating GlcCer and the GlcNAc β1,6-Man epitope present on tumour cells.[57]

The β1,3-Gal-T family consists of seven members that are involved in transferring Gal to GlcNAc to form Galβ1-3Hex(NAc)-α/β linkages in various GSLs, such as gangliosides.[55] Four isoforms of β1,3-Gal-T (I, II, III, and V) are involved in the synthesis of GSL type I chain structures (Galβ1,3-GlcNAcβ1-R) by transferring Gal to the GlcNAc O-3-position. β1,3-Gal-T V possesses higher catalytic activity on various glycolipids compared to β1,3-Gal-T I and II. The main function of β1,3-Gal-T IV is to transfer a Gal residue to GM2 and Gg3 gangliosides to form Galβ1-3GalNAcβ1-4Galβ1-R in other gangliosides, including GA1, GM1 and GD1. β1,3-Gal-T I and II use nLc4Cer as an acceptor, while β1,3-Gal-T V generates the Galβ1-3Gb4 structure.[55, 58] In addition, β1,3-Gal-T VI is involved in glycosaminoglycan synthesis to form the Galβ1,3-Galβ motif by transferring Gal to a Gal residue.[59]

α1,3-Gal-T resides in the Golgi membrane and transfers a Gal residue to Lac or LacNAc.[60] The resulting epitope was first identified in rabbit red blood cells that contain a range of glycolipids with various lengths of Galα1,3-Galβ1,4-GlcNAc-R structure. This moiety is proved to exist on bovine neutral glycolipids and gangliosides.[61]

Table 2. Major Gal-Ts involved in GSL glycan biosynthesis.

Linkages	Enzymes	Acceptors	Products	Ref
β1,4-	β1,4-Gal-T I (B4GALT1)	GlcNAc		62, 63
		Glc		
	β1,4-Gal-T II (B4GALT2)	GlcNAc		62, 64
		Glc		
	β1,4-Gal-T III (B4GALT3)	GlcNAc		62
	β1,4-Gal-T IV (B4GALT4)	GlcNAc		62
	β1,4-Gal-T V (B4GALT5)	GlcNAc		62
	β1,4-Gal-T VI (B4GALT6)	GlcNAc		65
	β1,4-Gal-T VII (B4GALT7)	Xyl		65
β1,3-	β1,3-Gal-T I (B3GALT1)	GlcNAc-based		55
	β1,3-Gal-T II (B3GALT2)	GlcNAc-based		55
	β1,3-Gal-T III (B3GALT3)	GlcNAc-based		55
	β1,3-Gal-T IV (B3GALT4) GM1 Synthase	GA2		66
		GM2		
		GD2		
		GT2		
	β1,3-Gal-T V	O-linked GlcNAcβ1-3GalNAc		55
		Gb4		
		GlcNAc		
	β1,3-Gal-T VI	Galβ1,4-Xyl		59
α1,3-	α1,3-Gal-T	Galβ1,4-GlcNAc-R		61, 67
		Galβ1,3-GlcNAc-R		
α1,4-	α1,4-Gal-T	LacCer		68

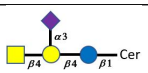
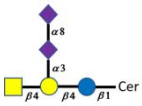
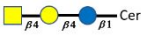
2.2.5 N-Acetylgalactosaminyltransferase (GalNAc-T)

The β1,4-GalNAc-T family contains six enzymes, which are also called GM2/GD2 synthase. These enzymes transfer a GalNAc unit to various acceptors, as listed in Table 3,[54, 65, 69, 70] and they play a crucial role in converting GM3, GD3, and LacCer into GM2, GD2, and asialo-GM2, respectively. Two of the enzymes, β1,4Gal-NAc-T III and IV, exhibit a specificity for acceptors and transfer GalNAc to the terminal GlcNAc residue.[71] β1,4-GalNAc-T II can transfer GalNAc to NeuAc2-3Galβ1,4-Glc(NAc),[72] and it can also transfer GalNAc to the Gal residue in Sd[a] antigen to form Siaα2,3(GalNAcβ1,4)-Galβ1,4-GlcNAc.[73]

The β1,3-GalNAc-T family enzymes transfer a GalNAc residue to form GalNAcβ1,3-linkage. This type of enzyme is involved in the synthesis of the major blood group P antigen in humans with the structure of GalNAcβ1,3-Galα1,4-Galβ1,4-GlcCer (Gb_4Cer).[74]

Table 3. Major GalNAc-Ts involved in GSL glycan biosynthesis.

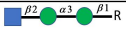
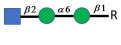
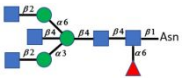
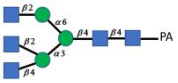
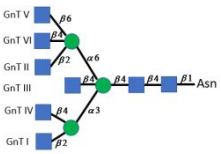
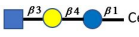
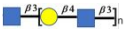
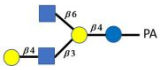
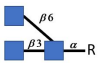
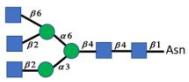
Linkages	Enzymes	Acceptors	Products	ref

β1,4-	β1,4-GalNAc-T	GM3		65, 70
		GD3		
		LacCer		
β1,3-	β1,3-GalNAc-T	Gb_3Cer		74

2.2.6 N-Acetylglucosaminyltransferase (GlcNAc-T)

GlcNAc-T is another family of GTs (Table 4) that are responsible for transferring GlcNAc from UDP-GlcNAc to various acceptors, including carbohydrates and other molecules, to form an α or β linkage. β1,2-*N*-GlcNAc-T I (GnT I) catalyses the transfer of a GlcNAc residue to the Manα1,3 branch in the trimannoside core of N-glycans to generate a β1,2-linkage during N-glycan biosynthesis. β1,2-*N*-GlcNAc-T II (GnT II) is an enzyme that is active on the Manα1,6 arm of the N-glycan core. The product of GnT I (GlcNAcβ1-2Manα1-3Manβ-R) is the substrate of other enzymes, such as GnT II, III, IV, α3/6-mannosidase and α1,6-Fuc-T, for further glycosylation reactions.[75] In contrast to GnT I and II, GnT III can generate β1,4-glycosidic linkages between GlcNAc and Man, which leads to the attachment of a bisecting GlcNAc between the two main arms in the core structure of N-glycans.[76] This bisecting GlcNAc can mediate the formation of β1,6-GlcNAc branches. Another enzyme in this family is β1,4-GlcNAc-T IV (GnT IV), which is involved in the synthesis of multi-branched N-glycans. This enzyme catalyses the formation of β1,4-linkage between GlcNAc and Manα1,3 on the GlcNAcβ1-2Manα1,3 arm. Thus, this activity relies on the presence of a GlcNAcβ1-2Manα1,3 epitope in the acceptor, which is formed by GnT I, and it enhances when the number of branches with terminal GlcNAc increases. GnT IV possesses two isoforms (GnT IVa and GnT IVb), which have similar substrate preferences. It has been reported that GnT IVa is more involved in the glycan biosynthesis compared to GnT IVb.[77] β1,6-GlcNAc-T V (GnT V) transfers GlcNAc to the Manα1,6 residue in the core structure of N-glycans.[78] β1,4-GlcNAc-T VI (GnT VI) is the sixth member of this family, which transfers GlcNAc to the Man1,6 to form a GlcNAcβ1,4-Manα1,6 linkage. It is mainly involved in the generation of pentaantennary arms with a GlcNAc residue in a bisecting position. The preferred substrate of GnT VI need to contain a specific sequence, which is GlcNAcβ1-2(GlcNAcβ1-6)Manα1-R.[79]

Table 4. Major *N*-acetylglucosaminyltransferases (GlcNAc-Ts) involved in GSL and N-glycan biosynthesis.

Linkages	Enzymes	Acceptors	Product s	Ref
β1,2-	β1,2-*N*-GlcNAc-T I (GnT I)	Manα1,3-		75
	β1,2-*N*-GlcNAc-T II (GnT II)	Manα1,6-		75
β1,4-	β1,4-*N*-GlcNAc-T III (GnT III)	Manβ1,4-		76
	β1,4-*N*-GlcNAc-T IV (GnT IV)	Manα1,3-		77, 78
	β1,4-GlcNAc-T VI (GnT VI)	Manα1,6-		79
β1,3-	β1,3-*N*-GlcNAc-T II	LacCer		80, 81
		Lc_4Cer		
		Poly-LacNAc		
	β1,3-*N*-GlcNAc-T (*Fringe*)	*O*-fucose		82
β1,6-	I β1-6GlcNAc-T (I GnT)	Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA		83
	C4 β1-6GlcNAc-T (C4GnT)	GlcNAcβ1-3GlcNAcα		84
	core 2 β1-6GlcNAc-T (C2GnT)	Galβ1-3GlcNAc		85
	β1,6-GlcNAc-T V (GnT V)	Manα1,6-		86

In glycan biosynthesis, the β1,3-linkage can be generated by β1,3-*N*-GlcNAc-T. β1,3-*N*-GlcNAc-T II transfers GlcNAc to Gal to form β1,3-linkage, contributing to the synthesis and elongation of poly-LacNAc chains. Stults *et al*., have studied the catalytic activity of this enzyme in the synthesis of type 2 GSLs. It is reported that β1,3-*N*-GlcNAc can transfer GlcNAc to LacCer to produce lactotriaosylceramide (Lc_3Cer). However, the elongation process is accompanied by β1,4-Gal-T that converts Lc_3Cer to nLc_4Cer by connecting Gal to GlcNAc through β1,4- linkage. The sequential actions of β1,3-*N*-GlcNAc and β1,4-Gal-T lead to LacNAc and poly-LacNAc structures. [87-89]

The identification of a gene named *Fringe* leads to the recognition of a Fuc-specific β1,3-*N*-GlcNAc-T that mediates cell signal transmission in mammals and flies. It was first identified during the studies on *Drosophila* wing development and is involved in transferring GlcNAc to *O*-Fuc on Notch, a signalling receptor, and forming GlcNAcβ1-3Fuc structure.[82] It can be further extended to a tetrasaccharide structure (Neu5Acα2-3/6Galβ1-4GlcNAcβ1-3Fuc).[54]

The I and i blood group antigens are composed of LacNAc units linked to each other linearly via β1,3-bonds with branches. The conversion of linear poly-LacNAc to branched structures is achieved by β1,6-N-GlcNAc-T.[83] Four different β1-6GlcNAc linkages have been identified, supporting the presence of four β1,6-GlcNAc-Ts involved in the formation of these bonds. Generally, these enzymes transfer GlcNAc to various Gal residues in the acceptor. The first enzyme is named as β1-6GlcNAc-T V (GnT V) and forms GlcNAcβ1-2(GlcNAcβ1-6)Man structure. Core 2 β1-6GlcNAc-T (C2GnT) transfers GlcNAc to the core Gal and generate Galβ1-3(GlcNAcβ1-6)GlcNAc. Core 4 β1-6GlcNAc (C4GnT) is involved in generating GlcNAcβ1-3(GlcNAcβ1-6)GlcNAc, whereas I β1-6GlcNAc-T (IGnT) mediates the synthesis of GlcNAcβ1-3(GlcNAcβ1-6)Gal structure.[84] C2GnT converts core 1 structures into core 2 structures to produce different ligands such as sialyl Le^x.[85] The β1,6-branching activity of GnT V and C2GnT is essential in controlling the production of poly-LacNAc, which plays a key role in biological functions such as cell-cell adhesion.[84] The activity of C2GnT and C4GnT have been recognized on the Galβ1-3GalNAcα1-R substrate, where R can be a hydroxyl group or protein and a hydrophobic aglycon.[84]

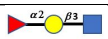
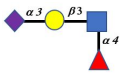
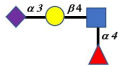
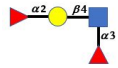
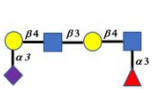
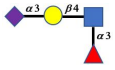
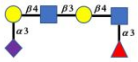
2.2.7 Fucosyltransferase (Fuc-T)

Fuc-Ts (Table 5) are type II membrane-associated enzymes that can transfer the Fuc unit from guanosine-5'-diphospho-fucose (GDP-Fuc) to glycan, glycolipid, and glycoprotein. Fucosyl glycans are involved in many biological processes, e.g., tissue development, fertilization, blood grouping, cell differentiation, inflammation or metastasis.[90, 91] The classification of Fuc-Ts depends on the type of linkages that they catalysed, which include α1,2-, α1,3-, α1,3/4, α1,6- and O-linkages.[90] Five isoforms have been identified, including Fuc-T III, IV, V, VI, and VII, for α1,3-Fuc-T, and each of these subtypes can attach Fuc to a GlcNAc residue in acceptors by an α1,3-linkage. Among these isoforms, α1,3-Fuc-T III possesses the broadest acceptor specificity. α1,2- and α1,3/4-Fuc-Ts are involved in the last step during the biosynthesis of H Lewis blood antigens, Lewis-related carbohydrate antigens, and difucosyl Lewis antigens, such as Le^x, Le^y, Le^a, Le^b, sialyl-Le^x and sialyl-Le^a antigens, through terminal and subterminal fucosylations.[90] α1,3/4-Fuc-Ts catalyses the transfer of a Fuc residue to the GlcNAc residue by α1,3- and α1,4-linkages.[54]

2.2.8 Sialyltransferase (ST)

Mammalian STs (Table 6) reside in the Golgi apparatus using cytidine-5'-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) as a donor to transfer sialic acid (Neu5Ac) residues to various acceptors. STs are primarily categorized by the glycosidic linkages that they generate, including α2,3-, α2,6- and α2,8-linkages, as well as the primary target monosaccharide residues in their acceptors like Gal, GalNAc, GlcNAc or another Neu5Ac unit. The ST family contains at least 20 members, and they are type II transmembrane glycoproteins that reside in the *trans*-Golgi compartment.[92] They are divided to 4 subfamilies, ST3Gal I-VI, ST6GalNAc I-VI, ST6GalI/II, and ST8Sia I-VI, based on the types of glycosidic bond that they form.[93] ST3Gal, ST6Gal, and ST8Sia form α2,3-, α2,6-, and α2,8-Sia linkages, respectively. Investigations show that ST3Gal and ST6Gal are associated with inflammation and metastasis, and cancer cell hypersialylation can lead to resistance to chemo or targeted therapy in some cancers, e.g., breast and ovarian cancers.[94, 95] The ST8Sia family is composed of six members, which are classified into three variations, including mono-STs (ST8Sia I, V, and VI), oligo-ST (ST8Sia III), and poly-STs (ST8Sia II and IV). These enzymes can catalyse glycoconjugate sialylation by adding Neu5Ac to the O-8-position of another Neu5Ac residue.[93]

Table 5. Major Fuc-Ts involved in GSL biosynthesis.

Linkages	Enzymes	Acceptors	Products	Ref
α1,2-	α1,2-Fuc-T I	Galβ1-3GlcNAc		90
	α1,2-Fuc-T II	Galβ1-4GlcNAc (LacNAc)		90
	H type α1,2-Fuc-T	Galβ1-3GlcNAc (H type I)		90
		Galβ1-4GlcNAc (H type II)		
α1,3/4-	α1,3/4-Fuc-T III (Lewis α1-3/4-Fuc-T)	Galβ1-3GlcNAc		96
		Galβ1-4GlcNAc		97
		Galβ1-4Glc		97
		Fucα1-2Galβ1-4Glc		97
		NeuAcα2-3Galβ1-3GlcNAc (Sialyl-type-I LacNAc)		96
		NeuAcα2-3Galβ1-4GlcNAc (Sialyl-type-II LacNAc)		96
		Fucα1-2Galβ1-4GlcNAc		96
		Fucα1-2Galβ1-3GlcNAc (H-type-I LacNAc)		97
		NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc		96
	α1,3-Fuc-T IV	Galβ1-4GlcNAc		96
		NeuAcα2-3Galβ1-4GlcNAc		
		NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc		
	α1,3-Fuc-T V	Galβ1-4GlcNAc		96
		Galβ1-3GlcNAc		96
		Galβ1-4Glc		97

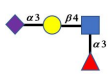
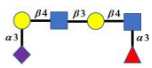
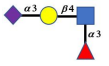
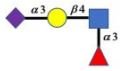
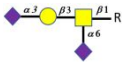
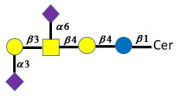
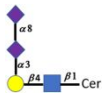
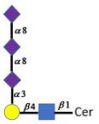
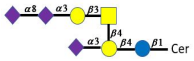
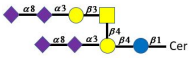
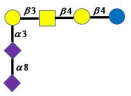
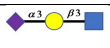
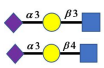
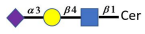
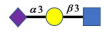
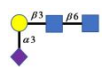
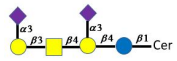
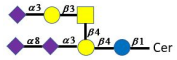
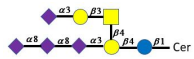
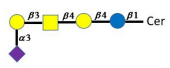
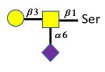
		NeuAcα2-3Galβ1-4GlcNAc		
		Fucα1-2Galβ1-4GlcNAc		
		NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc		
	α1,3-Fuc-T VI	Galβ1-4GlcNAc		96
		NeuAcα2-3Galβ1-4GlcNAc		
		Fucα1-2Galβ1-4GlcNAc		
	α1,3-Fuc-T VII	NeuAcα2-3Galβ1-4GlcNAc		96
	ST6GalNAc III, IV, V, VI	Neu5Acα2-3Galβ1-3GalNAc-R		99
	ST6GalNAc V	GM1b		99
	ST6Gal I ST6Gal II	Galβ1,4-GlcNAc-R		100
α2,8-	ST8Sia I	Neu5Acα1,3-Galβ1,4-GlcNAcβ1Cer		101, 102
	ST8Sia V	Neu5Acα2,8-Neu5Acα1,3-Galβ1,4-GlcNAcβ1Cer		
	ST8Sia I, III, V	GD1a		68
		GT1b		
		GQ1c		
		GD3		
	ST8Sia I, V	GM1b		

Table 6. Major STs involved in GSL biosynthesis.

Linkages	Enzymes	Acceptors	Products	Ref
α2,3-	ST3Gal I, II	Galβ1,3-GlcNAc		68, 92, 98
	ST3Gal IV, V, VI	Galβ1,3/4-GlcNAc		99
	ST3Gal V	Galβ1,4-GlcCer		99
	ST3Gal III	Galβ1,3-GlcNAc		99
		Galβ1,3-GlcNAcβ1,6-GalNAc		
	ST3Gal II, III	GM1a		99
		GD1b		
		GT1c		
		GA1		
α2,6-	ST6GalNAc I, II	Galβ1,3-GalNAc		68

3. Enzymes for GSL Synthesis

Compared to labour-intense, lengthy chemical synthesis, enzymatic glycosylation has evident advantages in terms of efficiency and regio- and stereo-selectivity. After the incorporation of bacterial enzymes in glycosylations, which can be readily expressed in large scales and have demonstrated broad substrate scopes, enzymatic glycosylation has become very popular in complex carbohydrate synthesis. It has been widely adopted to assemble GSL glycans. Recent development in the one-pot multiple enzyme (OPME) protocol, which combines glycosylation with *in-situ* generation of sugar nucleotides, has further improved the synthetic efficiency.[103] A major challenge in enzymatic syntheses of GSLs is the coupling of glycan with Cer that is hydrophobic. Incorporating lipids to glycan accepters also decreases the enzyme activity of GTs. An innovative engineering strategy that reverses the function of glycosidase, giving rise to a new class of enzymes known as glycosynthase, enables the coupling of lipid chains with glycosyl fluorides at a late stage. These enzymes will also be summarized in this section and their advantages and disadvantages will be briefly discussed.

3.1 Bacterial Sialyltransferases (STs)

STs are the key enzymes for the attachment of sialic acids. The most common sialyl donor used by STs is CMP-Neu5Ac. These enzymes can generate different sialyl bonds including α2,3-, α2,6-, α2,8- and α2,9-linkages. In the CAZy database, bacterial STs are classified into GT-4, GT-38, GT-42, GT-52, GT-80, and GT-97 families.[104] There are reviews about bacterial STs identified thus far, which are classified

based on the linkages they catalyse in reaction with hexoses/hexosamines. For instance, STs present in GT-42 and GT-52 families generate the α2,3- linkage, whereas the GT-80 family can form diverse linkages including α2,3-, α2,6- and the combination of α2,3-/α2,6-linkages.[105]

Bacteria *Escherichia (E.) coli, Campylobacter (C.) jejuni*, *Neisseria (N.) meningitidis*, *Streptococcus (S.) agalactiae*, and *Fusobacterium* (*F*.) *nucleatum* express various STs for synthesizing sialoglycans.[106] CMP-Neu5Ac is the main donor substrate to produce sialic acid-containing GSLs by STs, and it is generated by CMP-Neu5Ac synthetase. The bacterial CMP-Neu5Ac synthetase used in synthesis is usually from *N. meningitidis* and *E. coli*.[107]

A comparison of the bacterial and mammalian enzymes reveals that bacterial STs are more stable and possess broader acceptor substrate specificity. For instance, mammalian ST6Gal I forms an α2,6-linkage between Neu5Ac and the Gal residue in Galβ1-4GlcNAc, but exhibits low activity towards Gal in lactose (Galβ1-4Glc), *N*-acetyllactosamine (Galβ1-3GlcNAc), and LacdiNAc (GalNAcβ1-4GlcNAc), and no activity towards Galβ1,3-GalNAc. In contrast, a *photobacterium damselae*-originated bacterial ST, Pd2,6ST belonging to the GT-80 family, shows more flexibility to various substrates and thus has been widely used to synthesize sialo-GSLs. Specifically, Pd2,6ST can use fucosyllactose and sialyllactose as acceptors to provide fucosyl-2,6-sialyllactose and 2,6-disialyllactose, respectively.[108]

N. meningitidis α2,8-polyST, belonging to the GT-38 family, can use CMP-Neu5Ac to generate mammalian polysialic acids, which are then translocated to the surface of cells.[109] *Haemophilus (H.) parasuis* has another enzyme of this family, which functions as α2,3-ST to transfer Neu5Ac in α2,3-linked form to the termini of lipooligosaccharides.[110] Enzymes of the GT-42 and 52 families are involved in the attachment of sialic acids to lipopolysaccharides. *N. Haemophilus* and *Pasteurella* contain enzymes of the GT-52 family. A multifunctional ST (Cst II) is found in *C. jejuni*, belonging to the GT-42 family and with both α2,3- and α2,8-ST activities. This enzyme transfers Neu5Ac to Gal in lactose and sialyllactose.[111] α2,8- and α2,9-Linkages of Neu5Ac residues have been identified in the capsular polysaccharides of *N. meningitidis*. The STs responsible for these linkages belong to the GT-97 family.[112]

STs have also been identified in marine bacteria. A recombinant ST from marine *Photobacterium* sp. JT-ISH-224 shows unique substrate specificity. It transfers Neu5Ac to Glc in lactose, which contrasts with mammalian α2,3-ST that transfers Neu5Ac to Gal or GalNAc. An α2,6-ST from *P. leiognathi* JT-SHIZ-119 can catalyse the formation of α2,6-sialyl linkages to Gal.[113]

The majority of sialidases, known as hydrolases, cleave the glycosidic bond of terminal α2,3/6/8-linked Neu5Ac, whereas *trans*-sialidases transfer the released Neu5Ac to other glycoconjugates.[114] Sialidases can also create sialyl linkages in a regio- and stereo-specific manner using sugar donors that are less expensive than those used by STs. Therefore, sialidases are useful for chemoenzymatic synthesis of sialyloligosaccharides.[115] Sialidases derived from *Vibrio (V.) cholerae Clostridium perfringens, Salmonella* (*S.) typhimurium* and Newcastle disease virus generate α2,6-sialyl linkages selectively. Some of these organisms, like *S. typhimurium* and Newcastle disease virus, express STs that exhibit distinct specificity for α2,3-sialylation.[115]

3.2 Bacterial Galactosyltransferases (Gal-Ts)

Gal-Ts derived from various bacteria can utilize diverse acceptors to generate α1,3-, α1,6-, β1,3-, and β1,4-linked Gal epitopes. Bacterial Gal-Ts used in GSL synthesis have been reviewed previously.[62] β1,4-Linked Gal is one of the most abundant epitopes in natural glycans, such as Galβ1,4-GlcNAc in LacNAc and Galβ1,4-Glc in lactose, which can be enzymatically generated using two bacterial β1,4-Gal-Ts, *N. meningitidis* NmLgtB and *Helicobacter* (*H.) pylori* Hp1,4-Gal-T. Both enzymes are flexible and can transfer a Gal unit to *para*-nitrophenyl-β-D-mannopyranoside to give Galβ1,4-Manβ*p*NP.[116]

A disaccharide epitope, Galβ1,3-GalNAc, has recently been identified on the membrane of *E. coli*, confirming the presence of β1,3-Gal-T in this organism, which mediates the synthesis of oligosaccharides with a repeating Galβ1,3-GalNAcα structure.[117] *E. coli* also expresses α1,3-Gal-T, which generates Galα1,3-GlcNAc-R structure. Another enzyme that has been found in *E. coli* is α1,4-Gal-T. It mediates the transfer of Gal to terminal Neu5Ac of a glycolipid acceptor.[118] In *S. agalactiae*, the biosynthesis of a polysaccharide having the main chain consisting of Galβ1-4Glcβ with a trisaccharide Neu5Acα2-3Galβ1-4GlcNAcβ1-3Gal as side chains involves β1,4- and β1,3-Gal-Ts, which link β-Gal to the GlcNAc O-4- and O-3-positions, respectively.[119]

Some bacterial Gal-Ts have novel functions that are different from mammalian Gal-Ts. It has been found that an α1,4-Gal-T from *E. coli* can transfer Gal from UDP-Gal to the terminal Neu5Ac of Neu5Acα2-3Galβ1-3GalNAcα-diphosphate-lipid, which leads to the formation of Galα1-4Neu5Acα2-3Galβ1-3GalNAcα-diphosphate-lipid. Neu5Acα2-3Galβ1-3GalNAcα-PP-PhU turns out to be the only effective acceptor for this enzyme.[118] The synthesis of the polysaccharide core structure in *S. typhimurium* is performed by a Gal-T that forms an α1,6-linkage of Gal to the Glc residue of the core.[120]

3.3 Bacterial Fucosyltransferases (Fuc-Ts)

Fuc-Ts are classified into five families, α1,2-, α1,3-, α1,4-, α1,6-Fuc-T and *O*-Fuc-T, based on their preference to form specific Fuc linkages. A Fuc-T derived from *Dictyostelium discoideum*, which is very similar to the mammalian Fuc-T, can transfer Fuc to Galβ1-3GlcNAcβ-*para*-nitrobenzene to form an α1,2-linkage.[121] Another α1,2-Fuc-T from *H. pylori* has been used to synthesize the Lewis Y antigen. This enzyme exhibits specific substrate selectivity, i.e., preferring Lewis X [Galβ1-4(Fucα1-3)GlcNAcβ-R] to LacNAc (Galβ1,4-GlcNAcβ-R), which differs from mammalian α1,2-Fuc-Ts. This means that this enzyme enables a new way to synthesize Lewis Y via Lewis X.[122, 123]

In the CAZy database, α1,3- and α1,4-Fuc-Ts belong to family 10. *H. pylori* is an organism that produces A, B, and H blood group and Lewis X structures. Lewis X [Galβ1,4(Fucα1,3)-GlcNAc] contains a Fuc α1,3-linked to GlcNAc and is synthesized by the corporative action of α1,3-Fuc-T and β1,4-GT.[124, 125] α1,4-Fuc-T is another enzyme produced by *H. pylori* and transfers Fuc from GDP-Fuc to substrates with a βGal1-3βGlcNAc epitope. Some other organisms that have been found to produce α1,3/4Fuc-Ts include *V. cholerae, S. enterica serovar Typhi, Yersinia pestis,* and *Mesorhizobium loti*.[126] α1,6-Fuc-T belongs to GT23 family and transfers Fuc to GlcNAc to generate an α1,6-linkage. It has been identified in *Azorhizobium caulinodans* and found to catalyse the fucosylation of a lipo-chitin oligosaccharide.[127] It exhibits distinct acceptor specificities. A unique di-α1,6-fucosyl chitooligosaccharide was observed by combining mammalian and bacterial α1,6-Fuc-Ts, which represents a new structural motif.[128]

3.4 Bacterial *N*-Acetylhexosaminyltransferases (HexNAc-Ts)

Engineered enzymes can be useful tool to prepare complex glycans. According to the CAZy database, *N*-acetylhexosamine-transferring GTs belong to over 26 families out of 135. B3GlcNAcTGlNA derived from *H. pylori* (β3GlcNAcT) has a broad acceptor specificity and is

used to synthesize poly-LacNAc by extending the Galβ1-4GlcNAc sequence. This enzyme can selectively attach GlcNAc to Galβ1-3GlcNAc by β1,3-linkage[129, 130] and has been used to produce complex carbohydrates like Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc.[130] Some organisms, such as *Pasteurella* (*P.) multocida,* can produce bifunctional enzymes like hyaluronan synthase (PmHAS), which contains both a β1,4-GlcNAc-T domain and a β1,3-glucuronyltransferase (β1,3-GlcA-T) domain that are functional for elongating hyaluronan by transferring GlcNAc and GlcA, respectively.[131] Some bacteria express enzymes that can supply unique glycans. For example, in *P. multocida*, an α1,3-GalNAc linkage is present in the core structure of its lipopolysaccharide.[132]

3.5 Glycosynthases

Glycosidases are enzymes that inherently degrade glycosidic bonds but also catalyse the reverse reaction by means of cheap substrates and thus can be used as alternatives of GTs for large-scale production of carbohydrates. However, these hydrolytic enzymes are not very efficient to construct glycosidic linkages. To tackle the problem, they are usually engineered to supress the hydrolytic activity and enhance the glycosylation activity, hopefully to achieve the desired efficiency. The engineered glycosidases are termed glycosynthases that can be used to create glycosidic bonds in the presence of activated glycosyl donors through translycosylation.[133, 134] Glycosynthases can catalyse regio-/stereospecific reactions but oftentimes have a broad acceptor substrate scope. Another advantage of employing glycosynthases for carbohydrate synthesis is their donor substrates are much easier and cheaper to access and more stable than donors used by GT. The most common donor for glycosynthase is glycosyl fluoride, which usually reacts with acceptor by an inverting mechanism. Less commonly, the double inversion is also observed mainly with glycosynthases derived from aryl β-glycosidases. As a result, glycosynthases are useful tools for chemoenzymatic synthesis of carbohydrates. There are already several excellent reviews about glycosynthases in the literature.[69, 133] In this section, we will focus on glycosynthases that are active for specific glycosyl residue and can generate various glycosidic linkages in a regio- and stereo-specific manner.

The first successful glycosynthase was developed through mutation of a β-glycosidase (Glu358Ala) from *Agrobacterium sp*. (Abg), which utilizes glycosyl fluorides as donors to generate glycosidic linkages to various acceptors.[133] The first α-glycosynthase was the mutant of an α-glucosidase derived from *Schizosac-charomyces pombe*. Overall, β-glycosynthases are more extensively investigated and used than α-glycosynthases that are more limited and only observed in family 29, 31, and 95 glycoside hydrolases.[135]

Two α-L-fucosidases, 1,2-α- and 1,3/4-α-L-fucosidases that belong to hydrolase family 29, have been identified in *Bifidobacterium bifidum*. These enzymes have been employed to develop L-fucosylsynthases. A 1,2-α-L-fucosynthase can transfer Fuc from fucosyl fluoride donor to lactose to afford 2′-fucosyllactose (Fucα1-2Galβ1-4Glc). A 1,3/4-α-L-fucosynthase has the same substrate specificity, which can attach Fuc to the GlcNAc unit in Gal1–3/4GlcNAc(Glc) by α-1,3/4-linkage.[136] These synthases are used to synthesize fucosylated glycans, such as blood group antigens with Fuc residues, as appropriate alternatives for GT-based chemoenzymatic synthesis.

An α-galactosynthase (Asp327Gly) was developed from *Thermotoga maritima* (TmGalA) and has been identified as the third member of α-glycosynthases along with α-glucosynthase and α-fucosynthase. In order to confirm it catalytic activity, β-galactosyl azide (β-Gal-N_3) was used as the donor substrate to produce α-galactooligosaccharides.[135] β-Galactosidase is another enzyme that is active in hydrolysing β1,3- and 1,4-galactosyl linkages. β-Galactosidases from *Aspergillus oryzae* (Ao-β-gal), *Penicillium sp.* (Psp-b-gal), and *Trichoderma reesei* (Tr-b-gal), all belonging to family 35 glycoside hydrolases, have been used to produce various glycans. A novel subtype of β-glycosynthase has been derived from Ao-β-gal that has bifunctional activities, namely, β-galactosynthase and β-mannosynthase. This enzyme can convert free monosaccharide into Galβ1-6Gal and Manβ1-6Man.[137] Mutation of endo-β-*N*-acetylglucosaminidase (ENGase) for the development of useful glycosynthases represents another highly successful example, and the resultant enzymes have been widely applied to synthesizing complex N-glycoproteins and other glycoconjugates.[138-140]

An important breakthrough in chemoenzymatic GSL synthesis is the development of glycosynthases that can directly couple sphingosine with glycan, [141-143] which is not achieved yet using GTs involved in GSL biosynthesis. After elucidating the amino acid sequence in the active site of endoglycoceramidase (EGC) II from *Rhodococcus*, Withers *et al.* developed a glycosynthase by replacing Glc351 with Serine, which led to EGC II E351S[144, 145] that was further optimized through mutation and directed evolution.[146]

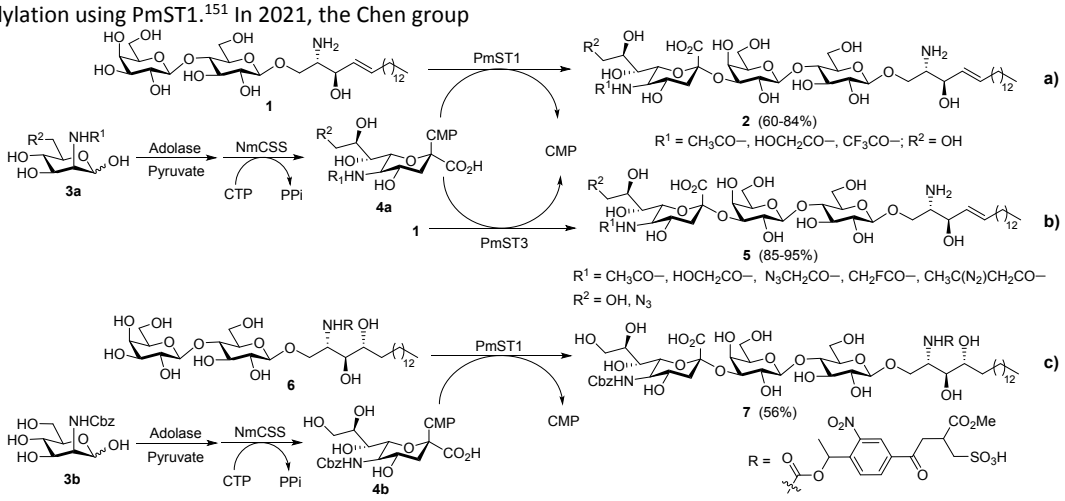
In the assembly of glycans, glycosynthases are more economic than GTs by avoiding using expensive sugar nucleotides and have broader substrate scopes. However, glycosynthases often afford lower yields and regioselectivity than GTs, thus they are alternatives rather than replacements for GTs. As mentioned, bacterial GTs exhibit a broader substrate scope than mammalian GTs, which is double-edged, as the broader substrate scope may also mean less selectivity to affect yield and product purification. Realizing this problem, researchers start to seek superior GTs for carbohydrate synthesis by taking advantage of the exceptional specificity of mammalian GTs and their compatibility with hydrophobic substrates.[117, 147] As a result, we should anticipate more applications of mammalian GTs to GSL synthesis in future.

4. Recent Progress in Enzymatic GSL Synthesis

The rapid evolution of glycoscience has been continuously improving our understanding of GSLs, whilst this also poses a growing demand for structurally defined GSLs. In response, new or improved methods for GSL synthesis are being developed constantly. The advantage of chemical synthesis is demonstrated by customized diversification or structural modification of GSLs to meet specific requests by biological studies. Strategies like late-stage attachment of lipid chains[148, 149] and diversity-oriented synthesis[150] have been developed to tackle various challenges. This area has been reviewed recently by Ando *et al.*[6] and thus is not elaborated in this article. Enzymatic synthesis provides a greener alternative to chemical synthesis with apparent advantages. Pioneered by the Chen group, the OPME strategy has exhibited great promises and been adopted for the synthesis of many GSLs. In the meantime, new enzymes are continuously added into the toolbox of enzymes. By combining chemical modifications with protocols using various enzymes, streamlined chemoenzymatic syntheses can reach the optimal result in terms of both synthetic efficacy and structural diversification to satisfy the demand from various biological studies. In this section, we will summarize the recent progress (past 4 years) in enzymatic and chemoenzymatic syntheses of GSLs.

Sialylation is one of the most challenging reactions in carbohydrate synthesis, thus enzymatic sialylation is often favoured over

chemical sialylation. In a study by Li *et al*., chemically prepared LacβSph **1** was subjected to the OPME protocol to install Neu5Ac and its derivatives in 60-84% yields. As shown in Scheme 1a, mannosamine with various N-acyl modifications were proved compatible with enzymes involved in its conversion to corresponding CMP-sialic acid donor by aldolase and NmCSS as well as subsequent sialylation using PmST1.[151] In 2021, the Chen group employed another ST, PmST3, for the sialylation of LacβSph by the same OPME protocol (Scheme 1b). A range of Neu5Ac derivatives with modified C-2- and C-6-positions were linked to LacβSph in excellent yields (85-95%),[152] showing the broad application scope of this strategy. In both cases, the FA chain was chemically attached to **2** and **5** in the last step to furnish GM3 analogues.

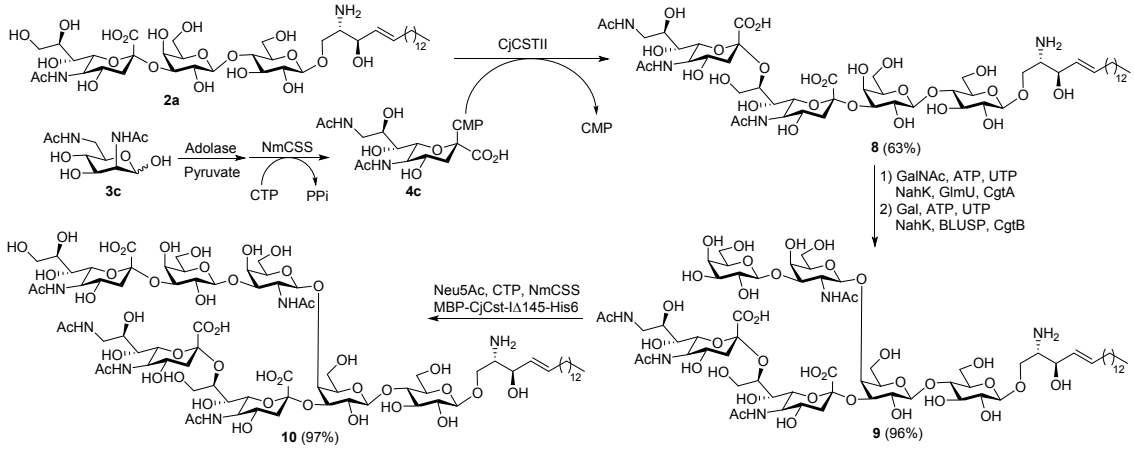


Scheme 1. Chemoenzymatic synthesis of GM3 derivatives involving late-stage enzymatic sialylation.

In the synthesis of an analog of ganglioside LLG-5, Cho *et al.* achieved the enzymatic sialylation of LacCer **6** with a functionalized acyl chain (Scheme 1c).[153] The mannosamine derivative **3b** with a Cbz group on the amino group was utilized as the substrate but the sialylation with PmST1 gave a low yield (56%), presumably caused by the Cbz group on sialic acid, the bulky acyl group on Cer, and PmST1-mediated sialyl hydrolysis. Subsequently, CST1 was used for sialylation to afford **7** in an improved yield (80%), which was further modified by chemical approaches to furnish the targeted LLG-5 analogue.

To avoid the hydrolysis of sialylation product caused by PmST1, Yang *et al*. expressed in *E. Coli* a recombinant human ST, hST3GAL II.[154] The transmembrane domain of this complex enzyme was truncated and replaced with maltose-binding protein (MBP) on the N-terminus and a His_6-Tag was attached to the C-terminus. This enzyme was applied to the OPME protocol for synthesizing a series of ganglioside glycans with a 3-azidopropyl group as a linker at the reducing end.

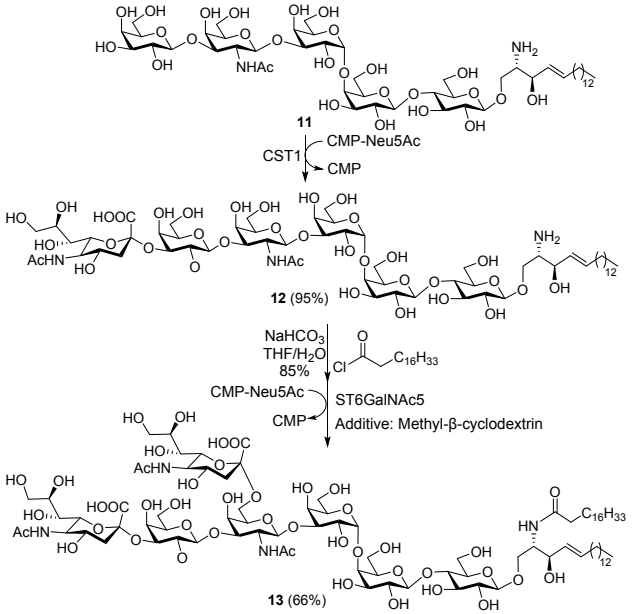
In the synthesis of b-series gangliosides, the α2,8-linkage between the two Neu5Ac residues can be achieved by Just-II. This ST has also shown good tolerance to modifications on the C-9-position of sialic acid. For instance, GM3βSph **2a** was successfully converted into GD3βSph **8** in a 63% yield (Scheme 2).[155] Due to its wide substrate scope for acceptors, additional sialylation can occur to **8** to impact yields and complicate product purification. Further elongation of the glycan in **8** by adding GalNAc using CjCgtA and Gal using CjCgtB generated the GD1βSph analogue **9** in a high yield (96%). Finally, sialylation of the 3-OH group in terminal Gal unit with MBP-CjCst-IΔ145-His_6, a recently developed α2,3-ST,[156] afforded 9NAc-GT1βSph **10** as the final product in a 97% yield. The CjCst-II enzyme was also used to furnish the α2,8-linkage in the synthesis of GD2 analogue with the 3-azido propyl group at the glycan reducing end.[157]



Scheme 2. Chemoenzymatic synthesis of GT1βSph involving multiple enzymatic sialylation steps.

CST1 can also perform sialylation of the 3-OH group of Gal in *Globo*-series glycans. In a recent study by Liu *et al*., chemically synthesized Gb5βSph **11** was treated with CST1 and CMP-Neu5Ac to give SSEA-4 analogue **12** (Scheme 3).[158] Targeting at the disialylated GSL Gb5,

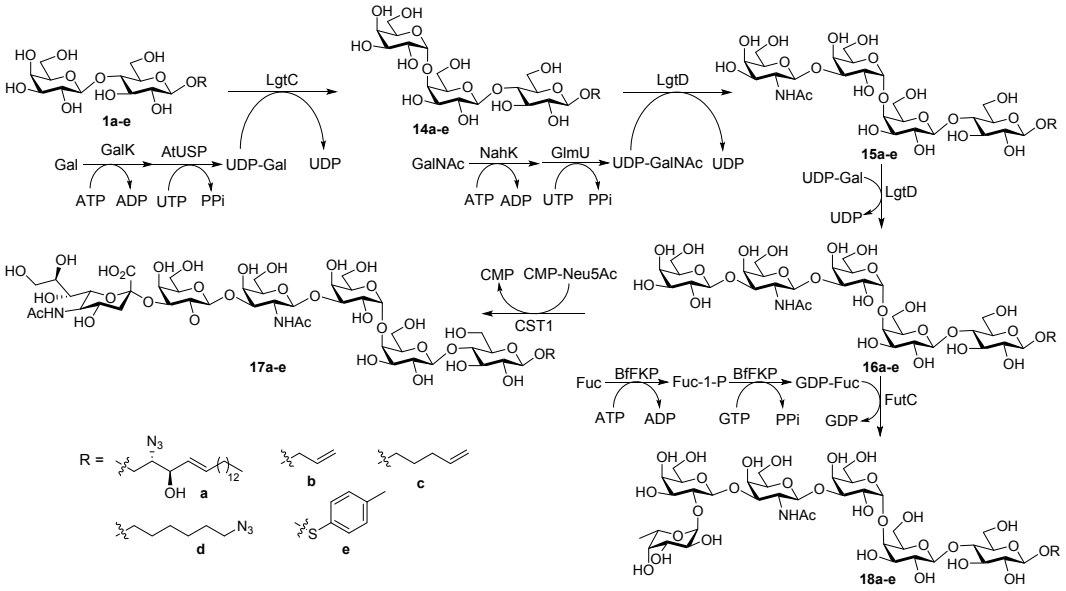
the authors tried to attach the second Neu5Ac residue to the 6-OH of GalNAc in **12** using a mammalian α2,6-ST ST6GalNAc5, but unsuccessful. It was reasoned that mammalian STs prefer Cer over sphingosine. Thus, **12** was chemical N-acylated, which was followed by sialylation using ST6GalNAc5 to afford the desired product **13** in a 66% yield. It should be noted that methyl-β-cyclodextrin was added to improve the solubility of the fully lipidated intermediate in aqueous media, while no additive was added during the sialylation of **12**. Nevertheless, it is interesting that, quite opposite to bacterial GTs, mammalian enzyme ST6GalNAc5 prefers the more hydrophobic accepter with Cer.



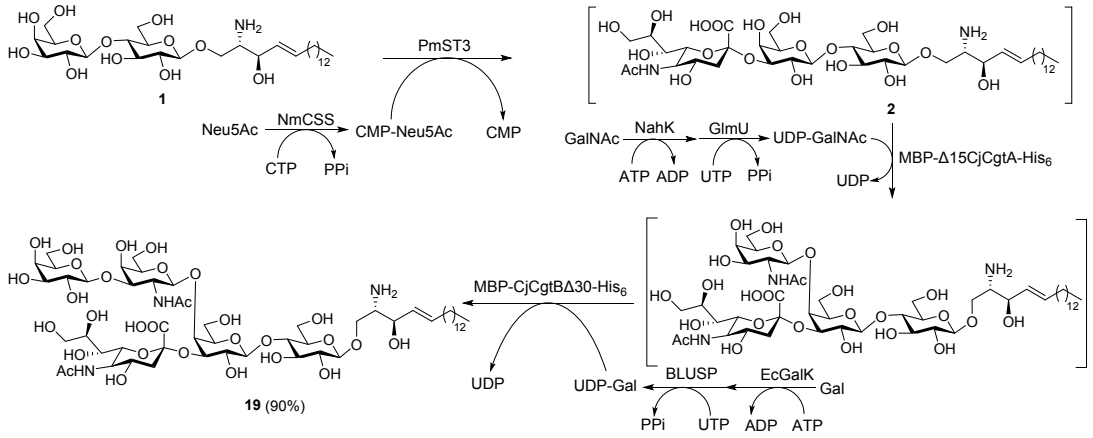
Scheme 3. Chemoenzymatic synthesis of DSGb5.

Chiang *et al*. lately studied enzymatic synthesis of two *Globo*-series GSLs bearing different terminal sugars from the same intermediate (Scheme 4).[159] This synthesis followed the general protocol described above, with minor modifications in choosing the enzymes, UDP-sugar pyrophosphorylase (AtUSP), for UDP-Gal synthesis. The authors first probed the effect of lipid chain on the first glycosylation catalysed by LgtC. It was found that galactosylation of LacβSph **1a** only gave a 47% yield, while galactosylation of lactose with a pentenyl chain **1c** gave the desired product in a 99% yield. Clearly, the bacterial enzyme is less effective for glycosyl acceptors with long hydrophobic lipid chains, especially in the early stage of the synthesis when the glycan moiety is relatively small. As the glycan moiety elongates, the influence of the long lipid chain is significantly mitigated. As demonstrated in the conversion of trisaccharide **14** to tetrasaccharide **15**, GalNAcylation of **14a** and **14c** gave 83% and 98% yields, respectively. A relatively poor yield (42%) was observed during the attachment of a Gal unit to GalNAc in **15** when bifunctional enzyme LgtD was employed to introduce both Gal and GalNAc. The second step usually suffers from low yields due to the excessive galactosylation to form hexasaccharide because the newly installed Gal residue in **16** is also an excellent acceptor for further glycosylation in the presence of LgtD. Thereafter, compound **16** was converted into the SSEA-4 derivatives **17** and Globo H derivatives **18** catalysed by CST1 and FutC to attach a Neu5Ac and Fuc residue to the Gal O-3- or O-2-position, respectively. The initial attempt to attach Neu5Ac using PmST1 was problematic, and computational docking studies indicate that **16** is not a good substrate for sialylation at the 3-OH of Gal as it is not properly positioned for interaction with the enzyme PmST1. An alternative enzyme CST1 was found to be more effective to provide SSEA-4 derivatives **17** in a 94% yield.

In line with the trend to improve the efficiencies of OPME protocols, the Chen group explored GSL synthesis by performing multiple OPME glycosylation steps consecutively without intermediate purification (Scheme 5). Starting from LacβSph **1a,** Neu5Ac/Neu5Gc, GalNAc, and Gal residues were sequentially installed by PmST3, MBP-Δ15CjCgtA-His_6, and MBP-CjCgtBΔ30-His_6, respectively, to afford GM1βSph **26** in a remarkably overall yield (90%). In this study, two new GTs derived from CgtA and CgtB, which were engineered to fuse MBP at their N-termini, were expressed in *E. Coli* and used for this synthesis. These GTs exhibited improved solubility and stability to suit the multistep OPME protocol. Addition of physiological detergent sodium cholate could also improve the efficiency of the enzymes.[160]



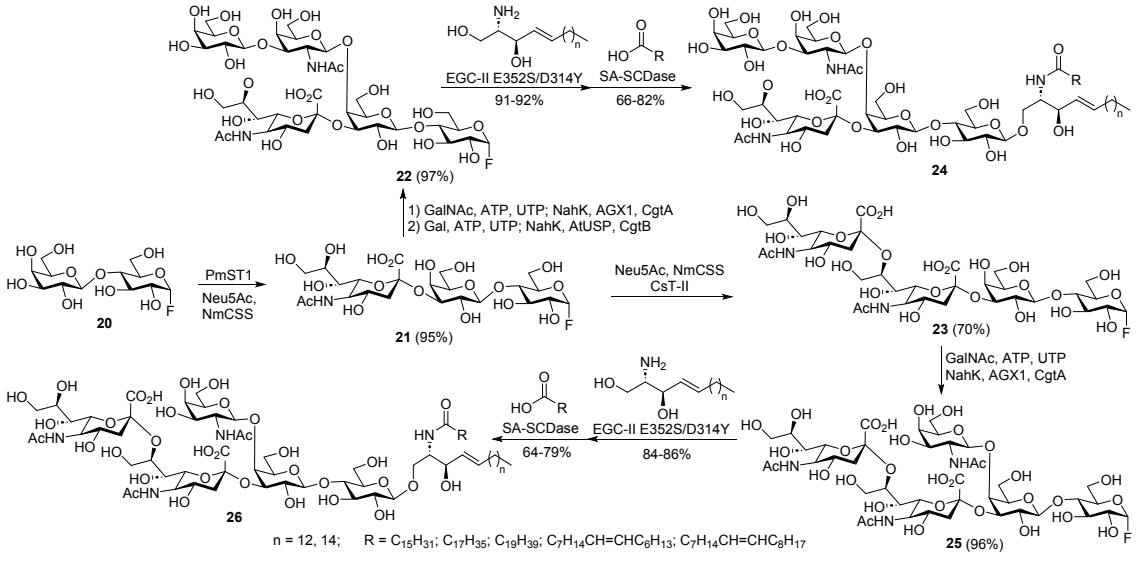
Scheme 4. Divergent chemoenzymatic synthesis of SSEA-4 and Globo H derivatives bearing different lipid tails.



Scheme 5. One-pot chemoenzymatic synthesis of GM1βSph by multiple OPME protocols using recombinant enzymes.

Structural diversity of GSLs occurs more frequently to the glycan–the extracellular epitope for recognition, thus the glycan has drawn more attention than the lipid. From the synthetic point of view, glycans are more difficult than lipids to assemble. Naturally, enzymatic synthesis of GSLs has been mainly focused on glycosylation of substrates with preinstalled Cer or sphingosine. Compared to this strategy, synthetic designs with late-stage installation of Cer/sphingosine have obvious advantages, such as facilitating enzymatic glycosylation by increasing hydrophilicity of acceptors and allowing structural diversification of the lipid chains. However, due to the difficulty to couple hydrophobic lipids to hydrophilic glycans in aqueous media, this approach is rarely practiced. On the other hand, the Cer moiety of GSLs has drawn more attention currently, due to its great impact on the various properties of plasma membrane, especially the lipid raft, interaction with other molecules in the cell membrane, and involvement in transmembrane signalling, as well as its correlation with many diseases.

Sphingolipid Cer N-deacylase (SCDase), which can delete the FA chain from Cer of GSLs and reversely add the FA chain back to sphingosine of lyso-GSLs, should be suitable for the enzymatic synthetic strategy to build Cer at the final stage.[161] SCDase, in combination with EGC and GTs, has been utilized to perform streamlined, totally enzymatic GSL synthesis. Yang *et al*. call this strategy modular chemoenzymatic cascade assembly (MOCECA).[8] As depicted in Scheme 6, the glycans are efficiently assembled starting from lactosyl fluoride **20**, which is easy to prepare in large scales and highly soluble in aqueous buffers, thus its sialyation by the OPME protocol was very efficient to afford **21** in a 95% yield. Consecutive glycosylations to install other sugar units also followed the OPME protocol, and all products and intermediates involved were soluble in aqueous media. Thus, the reactions were smooth and efficient to provide complex glycans **22** and **23** in excellent overall yields. The resulting glycosyl fluorides **22** and **23**, as well as their derivatives, served as glycosyl donors for the assembly of Cer using SCDase and EGC. The reactions between these glycosyl fluorides and sphingosine catalysed by EGC-II to form lyso-GSLs, as well as subsequent N-acylation of sphingosine using SA-SCD and fatty acids, were effective to produce the target GSLs, including complex GM1 **24** and GD2 **26**, in large quantity and excellent overall yields. As a result, this work not only establishes an practical method to access GSLs but also provides a series of GSLs with different glycan and lipid structures, which have been employed to study related biology.[8, 161] This is an excellent example to demonstrate the power of enzymes in complex glycoconjugate synthesis.



Scheme 6. Streamlined, fully enzymatic synthesis of various forms of gangliosides GM1 and GD2.

5. Conclusion

Considerable progress has been made in enzymatic syntheses of GSLs in recent years. In particular, the efficient OPME protocol has been widely adopted in the synthesis of many GSLs, especially gangliosides with Neu5Ac residues that are notoriously hard to install by chemical glycosylation. To further improve the efficiency, multistep OPME and MOCECA synthetic strategies have been explored, which aim to take advantages of the constantly enriched toolbox of enzymes, including a diversity of GTs as well as enzymes involved in the metabolism and remodelling of Cer and sphingosine in GSLs. The new strategies have enabled the streamlined and even completely enzymatic assembly of complex GSLs. In the meantime, mammalian GTs, which are more relevant and specific to human GSLs but rarely used in synthesis, are now expressed in bacteria in large scales and find applications in GSL synthesis. The challenges remaining in this field are how to improve the compatibility of current enzymes to hydrophobic acceptors, their capability to differentiate Gal from GalNAc and to minimize excessive sialylation in the synthesis of di/multisialo-gangliosides. Hopefully, the introduction of mammalian enzymes involved in GSL biosynthesis to the field will help tackle these problems. Accordingly, the enzymes involved in the biosynthetic pathways of GSLs as well as other related glycans, in addition to enzymes currently utilized in chemoenzymatic GSL synthesis, and their functions are summarized in this review. We hope that their comparisons will shed lights on finding solutions to current challenges. Overall, the seemingly improved accessibility to various GSLs through chemical and enzymatic syntheses has boosted related biological studies to result in a better understanding of the functional roles and mechanisms of GSLs and associated biological processes.

Conflicts of interest

There are no conflicts to declare.

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Data availability statement:

The data underlying this paper are available in the cited publications.