Enzymatic Synthesis of Glycosphingolipids: A Review

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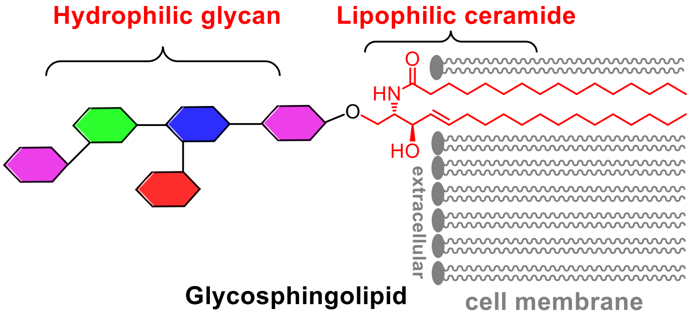

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Abstract Glycosphingolipids (GSLs) are the major vertebrate glycolipids, which contain two distinctive moieties, a glycan and a ceramide, stitched together by a *β*-glycosidic linkage. The hydrophobic lipid chains of ceramide can insert into the cell membrane to form "lipid rafts" and anchor the hydrophilic glycan onto the cell surface to generate microdomains and function as signaling molecules. GSLs mediate signal transduction, cell interaction, and many other biological activities, and are also related to many diseases. To meet the need of biological studies, chemists have developed various synthetic methodologies to access GSLs. Among them, the application of enzymes to GSL synthesis has witnessed significant advancements in the past decades. This review summarizes briefly the history and progress of enzymatic GSL synthesis.

Key words. glycosphingolipid, carbohydrate, ceramide, enzymatic synthesis, glycosyltransferase, glycosynthase

1. Introduction

Glycosphingolipid Structure—Two distinctive and biologically vital molecules, carbohydrate and lipid, combine to form a unique class of biomolecules, known as glycolipids, which are important constituents of the cell membrane across all species. There are two major subclasses of glycolipids: glycoglycerolipids, which are abundant in plants and bacteria, and glycosphingolipids (GSLs), which are predominant in higher animals.[1] While largely diverse in the structure, all GSLs have a ceramide, the hydrophobic lipid moiety, linked to hydrophilic glycans through a *β*-glycosidic bond (Figure 1). The two lipid chains of ceramide are embedded in the cell membrane and segregated into domains to anchor the glycan in proper positions to function.[2] Although quantitatively minor relative to glycerophospholipid—the main structural component of cell membranes, ceramide is critical to the membrane integrity by imparting considerable rigidity due to its presence of an amide group and long acyl chain.[3] The GSL glycan can contain up to 60 monosaccharide residues, different in type, number, linkage form and modification pattern, which extends on the cell surface into the extracellular matrix to interact with other molecules.[1b, 2b, 4]

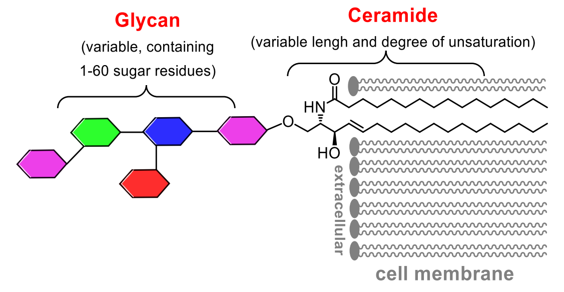


Figure 1. An illustrative structure of GSLs and the attachment of GSLs onto the cell membrane

GSL Biosynthesis—Due to the distinctive features of the glycan and ceramide moieties in GSL, GSL biosynthesis is performed in a stepwise fashion to separately furnish the ceramide and glycan in sequence (Figure 2). First, in the endoplasmic reticulum (ER), L-serine is modified with a long acyl chain using Acyl-CoA, usually palmitoyl CoA, catalyzed by serine palmitoyltransferase (SPT), followed by 3-keto reduction to provide sphinganine.[1a, 5] Then, *N*-acyltransferase catalyzes the attachment of another lipid chain, which is followed by desaturation to afford ceramide. Ceramide is transferred into Golgi apparatus, where carbohydrate residues are added sequentially by glycosyltransferases (GTs) using sugar nucleotides as glycosyl donors. The first glycosylation step forms two types of monoglycosylceramides, also called cerebrosides, with either a galactose (Gal) or a glucose (Glc) residue attached to the primary hydroxyl group of ceramide. Only less than ten percent of the cerebrosides is galactosylated, which may subject to further modification, such as sulfation.[2b] About ninety percent of ceramide is glucosylated to produce glucosylceramide (GlcCer) that is then converted into lactosylceramide (LacCer), at which point further glycosylation pattern becomes very complicated to develop the great diversity in GSL structures. It should be noted that GSLs can be catabolized and ceramides are recycled for GSL biosynthesis or directly integrated into membranes.

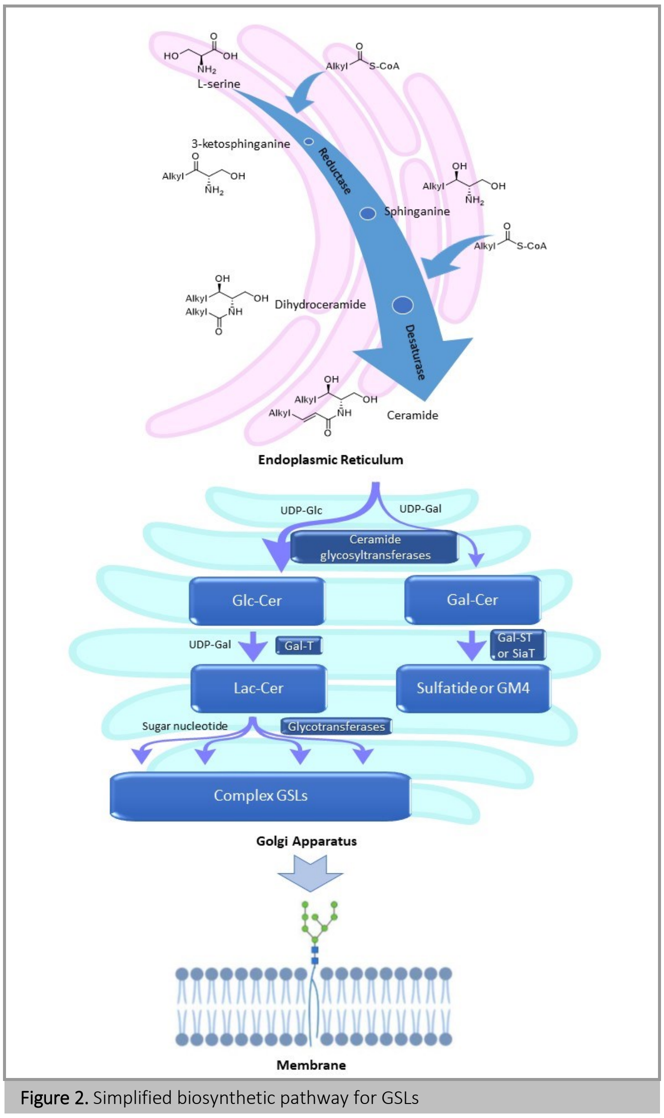


Figure 2. Simplified biosynthetic pathway for GSLs

Functions and Biological Significances—The glycan moiety of GSLs can interact with each other or with other biomolecules to mediate cell-cell interaction and other activities, thus involved in many biological events, such as cell growth, adhesion, regulation, differentiation, etc.[6] The GSL glycan structure/composition may change during oncogenic and other pathologic transformations to cause disturbed cell communication;[1b] the resulting GSLs can serve as biomarkers or therapeutic targets for related diseases. Moreover, the lipid moiety of GSLs as a key component of the cell membrane also play multiple physiological roles. Ceramide and sphingosine homeostasis, which is affected by GSL biosynthesis and degradation, is closely related to various storage disorders, such as the Gaucher and the Fabry diseases.[5] In addition, the GSL lipid moiety with varied structures dictates the formation of "lipid rafts" and GSL domains, thereby affecting the functioning of GSLs. The distinctive glycan and lipid moieties of GSLs affect the functions of each other and are both critical for the biological roles of GSLs.[2b]

Overview of GSL Synthesis—Due to their amphiphilic property and structural diversity/heterogeneity, acquiring homogeneous GSLs via isolation from natural sources is difficult, leaving total synthesis the only viable option. From a synthetic point of view, coupling glycan with lipid of distinctive properties brings nothing but trouble, thus GSL synthesis poses a great challenge. Initially, chemical synthesis was the predominant method to access these molecules and is still a powerful method in providing modified or labeled GSLs as probes for various biological studies. In fact, since the 1980's, synthetic chemists have been continuously exploring GSL total synthesis, which topic has been extensively reviewed.[7] Intuitively, convergent strategies were investigated by pioneers, represented by Schmidt,[8] Ogawa,[9] Nicolaou,[10] Hasegawa[11] and other groups,[7] based on the introduction of ceramide at the final stage of the synthesis. Recently, a "cassette strategy" featuring the coupling of GlcCer with the rest of a glycan was demonstrated to give better yields for complex GSLs.[7d] Regardless of these heroic efforts, GSL synthesis still remains a significant challenge. Among various issues, the inherit difficulties associated with chemical oligosaccharide assembly, e.g., it needs sophisticated protection and deprotection strategies to accomplish satisfactory regio- and stereoselectivity, have consistently confronted chemists.

Unlike chemical glycosylations, enzymatic glycosylations can be achieved regio- and stereo-selectively without any protection of the substrates thus to significantly improve synthetic efficiencies. Among various enzymes involved in carbohydrate syntheses and modifications, two classes of them are more widely used for GSL synthesis. One class is GTs, which transfer monosaccharides from corresponding sugar nucleotides to specific acceptors, same as the process of natural GSL glycan biosynthesis. GTs are effective and high yielding and, therefore, have found broad applications in the past decades, especially after the introduction of bacterial GTs, which are abundant, easy to purify, characterize, engineer, and tend to have a wide range of acceptors, when compared to mammalian GTs.[12] GT application to enzymatic synthesis used to be limited by the availability of sugar nucleotides—a problem that has been addressed in large by the combination of *in situ* enzymatic production of corresponding sugar nucleotides with GT-catalyzed enzymatic glycosylation in one pot.[13]

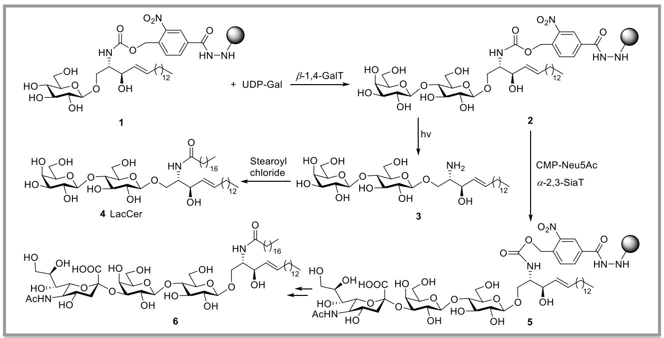
The other class of commonly utilized enzymes is glycosynthases, which invert the function of naturally hydrolytic glycosidases via mutation and directed evolution. This strategy is made possible because glycosidases cleave glycosidic bonds in a reversible way. However, recruitment of glycosidases to catalyze glycosylation reactions had been plagued by the side hydrolysis reaction until the Withers group[14] and others[15] had effectively suppressed the hydrolysis using engineered glycosidases.

Scope of Review—This review is narrowly focused on enzymatic synthesis of GSLs, although chemical synthesis of GSLs is still an active field.[7c] Enzymatic assembly of oligosaccharides, including those of GSLs, is a rather mature field and has been extensively reviewed,[15f, 16] thus it will not be revisited herein. At the center of the enzymatic GSL synthesis is how to efficiently incorporate both the hydrophobic lipid and the hydrophilic oligosaccharide moieties in one molecular entity. In addition, achieving structural diversity on both halves of GSLs to meet the growing demands from biological studies is also a hot topic. A case-by-case survey of previous accomplishments may shed light on the direction to overcome these problems.

Enzymatic synthesis of GSL can be traced back to the 1970's even before conclusive characterization of these complex molecules.[17] However, some reports[18] were focused on the characterization and evaluation of enzymatic activities rather than on production of homogeneous GSLs. Others reported the partial synthesis by cleavage or addition of carbohydrate residues with enzymes to generate other GSLs of interest.[19] Although GSLs were described as the final products in these reports, they are out of the scope of this review, which covers only reports about chemoenzymatic synthesis of complete GSLs.

2. **Glycosyltransferases for GSL Synthesis**

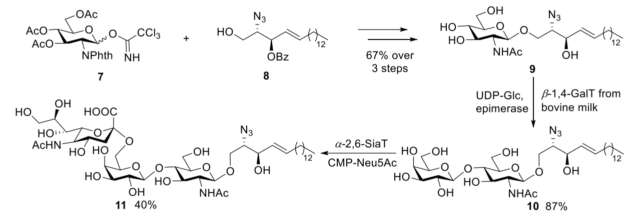
The first enzymatic synthesis of a well-defined GSL was reported by Zehavi and coworkers in 1990.[20] As discussed, hydrophobic lipids or glycolipids are not good substrates for GTs. Therefore, a glucosyl sphingosine derivative was chosen as the acceptor and a water-soluble polymer was employed as the support to improve substrate water solubility. As outlined in Scheme 1, the synthesis commenced with compound **1**, which was obtained via chemical synthesis to have glycosylated sphingosine linked to the polymer support through a light-cleavable linker, 2-nitrobenzyl urethane. A galactosyl unit was transferred onto **1** from UDP-Gal by β-1,4-galactosyltransferase (GalT) to provide compound **2**, which was cleaved from the polymer with light. Attachment of the second lipid chain to the resultant **3** using stearoyl chloride was rather straightforward to afford the final product LacCer **4** in a 20% overall yield. Glycolipids **3** and **4** was readily purified with Sep-Pak® C18 cartridges.



Scheme 1. Zehavi's polymer-supported enzymatic synthesis of LacCer **3** and natural GM3 **6**

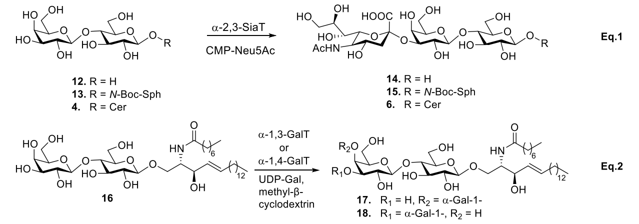
In 1998, the same group utilized compound **2** to prepare GM3 (Scheme 1).[21] First, **2** was converted into **5** in a 63% yield upon sialylation using α-2,3-sialyltransferase (SiaT). In this reaction, calf intestinal alkaline phosphatase was utilized to decompose byproduct CMP to prevent its inhibition on SiaT. This work had demonstrated the great potential of enzymes for stereoselective sialylation, one of the most difficult reactions in carbohydrate synthesis,[22] in good yields without protection and deprotection steps. Following a procedure similar to that used for **4**, compound **5** was cleaved from the polymer support and then *N*-acylated to provide GM3 **6**. A GM3 analogue with glycan linked to the 3-hydroxyl group in sphingosine was also synthesized by the same procedure.

Due to the lack of enzymes for lipid and glycan coupling, earlier enzymatic GSL syntheses relied on chemical methods to link the first sugar unit to sphingosine to access primers required for GT-catalyzed glycosylation, as demonstrated by Flitsch's synthesis of a GM3 analogue **11** (Scheme 2).[23] First, glucosamine was coupled with 2-azidosphingosine to afford **9** as the glycosyl acceptor to react with UDP-Gal, which was produced *in situ* via epimerization of UDP-Glc, in the presence of a β-1,4-GalT from bovine milk. The resulting lactosylsphingosine (LacSph) **10** was then treated with α-2,6-SiaT from porcine liver to provide GM3 analogue **11**.



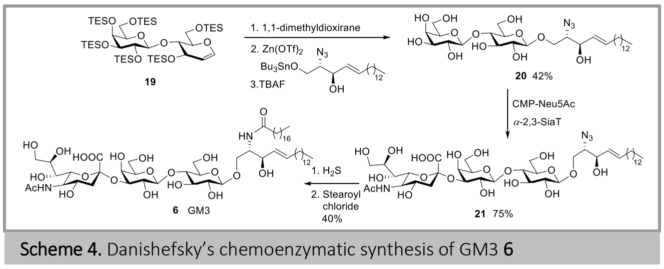
Scheme 2. Flitsch's enzymatic synthesis of a GM3 analogue **11**

Early-stage installation of the acyl group on ceramide of lactosyl substrate causes solubility issues to result in low efficacy in the enzymatic glycosylation. Wong group[24] cloned the bacterial α-2,3-SiaT from *Neisseria gonorrheae* and systematically compared the relative catalytic rate of this enzyme using lactosyl acceptors with varied lipid moiety (scheme 4, Eq. 1). Measured by isotope assay, it was found that LacSph and LacCer could be converted into the corresponding GM3 analogs in the relative rates of 0.43 and 0.28 as compared to lactose. In a report by Palcic group,[25] the activities of two galactosyl transferases, *N. meningitidis* α-1,4-GalT and bovine α-1,3-GalT were evaluated. As shown in Scheme 4, lactosyl acceptor **16** with a short C8 lipid chain was converted into Gb3 and iGb3 derivatives **17** and **18**. With the addition of methyl-*β*-cyclodextrin to address the solubility issue, the relative activities of enzymes for acceptor **16** were measured to be 70% and 5% compared to 8-methoxycarbonyloctyl lactose acceptor. Due to the significant decrease in enzyme activity, sphingosine or smaller lipid groups have been ubiquitously adopted in the initial acceptors for the enzymatic glycosylation towards the synthesis of GSLs.



Scheme 3 Enzymatic synthesis of GSLs with LacCer as the acceptor

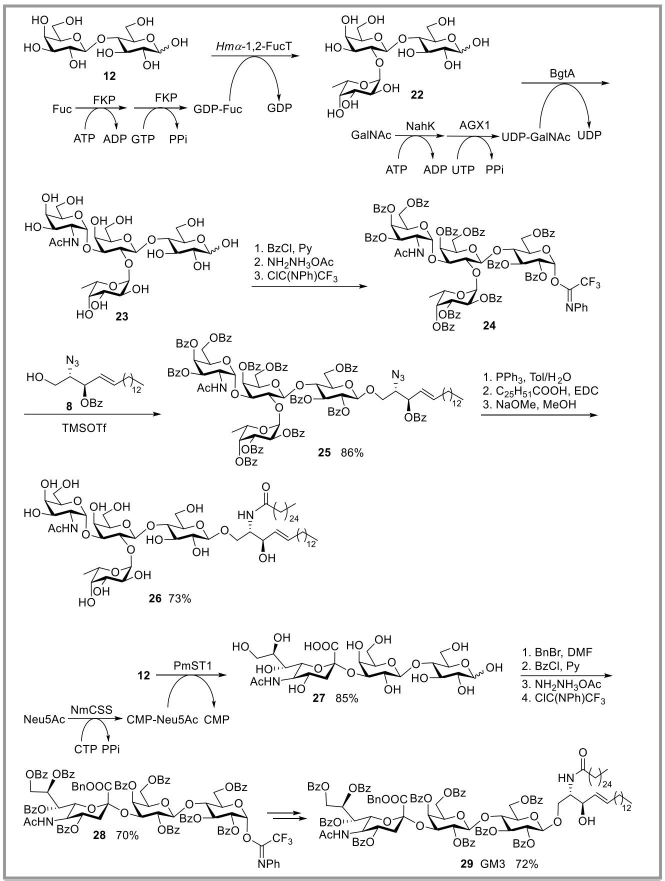
With LacSph derivative **20** as acceptor for enzymatic sialylation, Danishefsky group[26] achieved the chemoenzymatic synthesis of natural GM3 **6** by a similar method (Scheme 4). Chemical synthesis of lactosyl azidosphingosine followed by α-2,3-SiaT-catalyzed sialylation afforded **21** in a 75% yield. This *lyso*-GM3 analogue containing an azido group was deprotected and then acylated with stearoyl chloride to provide GM3 in a 40% yield for two steps.



Scheme 4. Danishefsky's chemoenzymatic synthesis of GM3 **6**

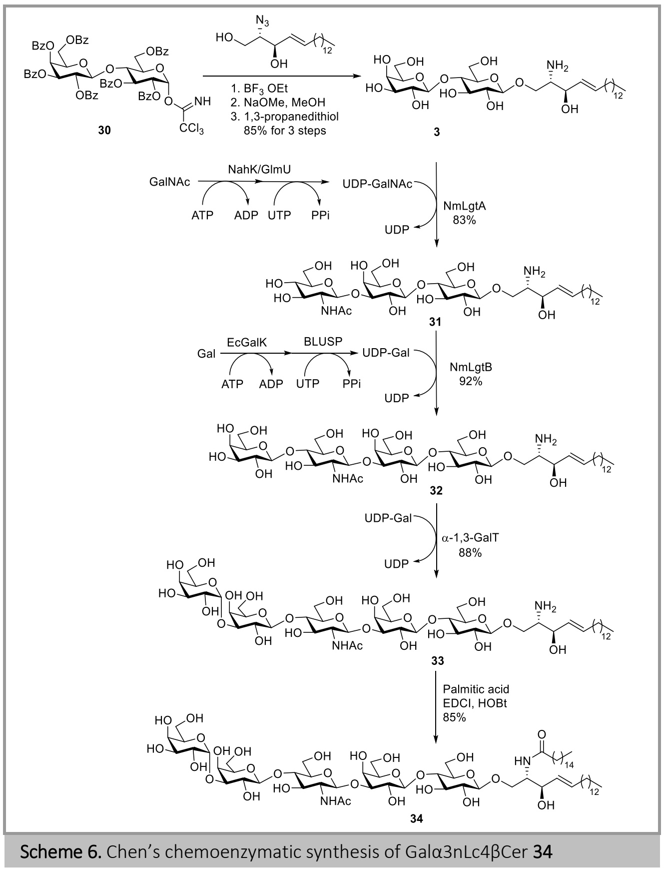
The GT pool has been significantly enriched after the discovery that bacterial GTs can be used for oligosaccharide synthesis and are advantageous over mammalian GTs in several aspects, such as abundancy, stability, and substrate scope. Along with the progressive engineering of bacterial GTs, many GSL free glycans have been synthesized, represented by works from Blixt group[27] on ganglioside, Wong group[28] on globo series, Boons group[29] on automated platform, and Samain group[30] on *in vivo* synthesis using engineered *E-coli*. As a result, most GSL glycans are now within the reach, especially by the one-pot multienzyme (OPME) systems,[31] reinvigorated by Chen group.[13c]

In 2016, Chen group reported a synthesis of the tetrasaccharide moiety **23** of a blood group H-antigen with the OPME strategy.[32] As outlined in Scheme 5, lactose was converted into trisaccharide **22** upon treatment with fucose (Fuc), ATP, GTP, bifunctional enzyme L-fucokinase/GDP-Fuc pyrophosphorylase (FKP), and *Helicobacter mustelae* *α*-1,2-fucosyltranferase (Hmα1,2FT). During this reaction, Fuc was first converted into GDP-Fuc by FKP and then transferred onto the 2'-*O*-position of lactose by Hmα1,2FT. Similarly, introduction of a galactosamine (GalNAc) residue to **22** by OPME synthesis employing *Homo sapiens* UDP-GalNAc pyrophosphorylase (AGX1) and an α-1,3-GalNAcT (BgtA) provided free oligosaccharide **23**. Eventually, **23** was converted into GSL **26** upon a series of chemical transformations. First, **23** was fully protected with benzoyl groups to generate the glycosyl donor *N*-trifluoroacetimidate **24** in a 78% yield. Thereafter, the sphingosine motif was coupled with the glycan through chemical glycosylation of **8** with **24** to afford **25**. Then, the azido group in **25** was reduced to expose the amino group for the attachment of the aliphatic chain under standard *N*-acylation condition to fulfill the ceramide moiety. Finally, global deprotection of the product provided GSL antigen **26**. GM3 **29** was also synthesized by the same strategy (Scheme 5).



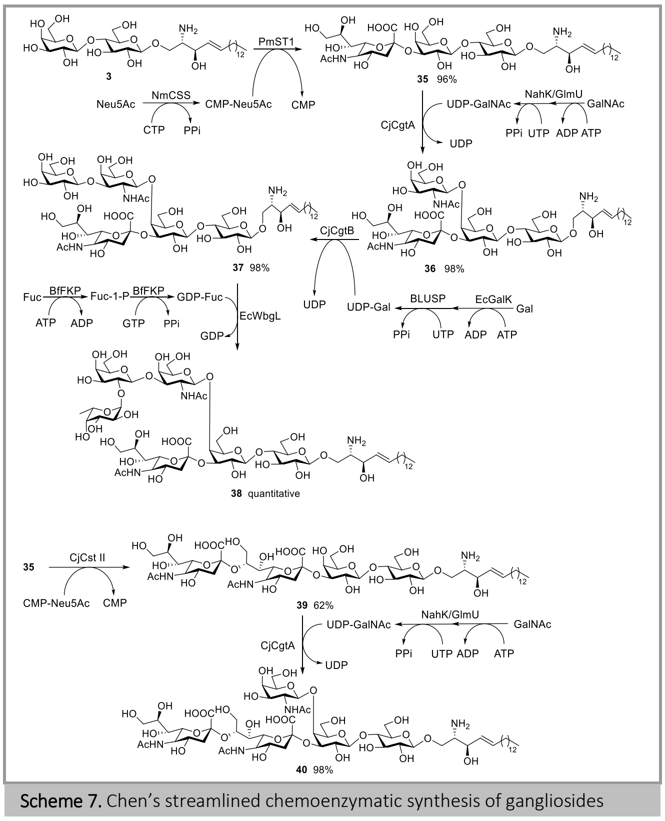
Scheme 5. Chen's chemoenzymatic synthesis of GSLs **26** and **29** by OPME glycosylations

A year later, Chen and coworkers[33] reported the chemoenzymatic synthesis of another GSL, Galα3nLc4βCer **34**, which is responsible for the immune rejection of pig to human xenotransplantation. In this synthesis, the sphingosine moiety was introduced onto the glycan reducing end at an early stage, as depicted in Scheme 6, with **3** as the initial acceptor of enzymatic glycosylations. Glycosylation of a sphingosine derivative with benzoyl group-protected disaccharyl trichloroacetimidate **30** followed by global deprotection of the product in two steps gave LacSph **3** in an 85% yield. Protecting the amino group in sphingosine as an azide was necessary for the effective glycosylation. Enzymatic glycosylation of the lactose primer in **3** by the OPME strategy using bacterial enzymes *Bifidobacterium longum* *N*-acetylhexosamine-1-kinase (BLNahK),[34] *Pasteurella multocida* *N*-acetylglucosamine uridylyltransferase (PmGlmU),[35] *P. multocida* inorganic pyrophosphatase (PmPpA),[36] and *N. meningitidis* *β*-1,3-*N*-acetylglucosaminyltransferase (NmLgtA)[37] gave rise to **31** in an 83% yield. Similarly, **31** was converted into **32** in the presence of *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK),[38] *B. longum* UDP-sugar pyrophosphorylase (BLUSP),[39] PmPpA, and *N. meningitidis* *β*-1,4-GalT (NmLgtB).[37] The last OPME glycosylation was achieved with a recombinant bovine *α*-1,3-Gal (B*α*-1,3-GalT)[40] to afford **33**, which was treated with palmitic acid and EDCI to provide the synthetic target **34** in a 57% overall yield from **3**.



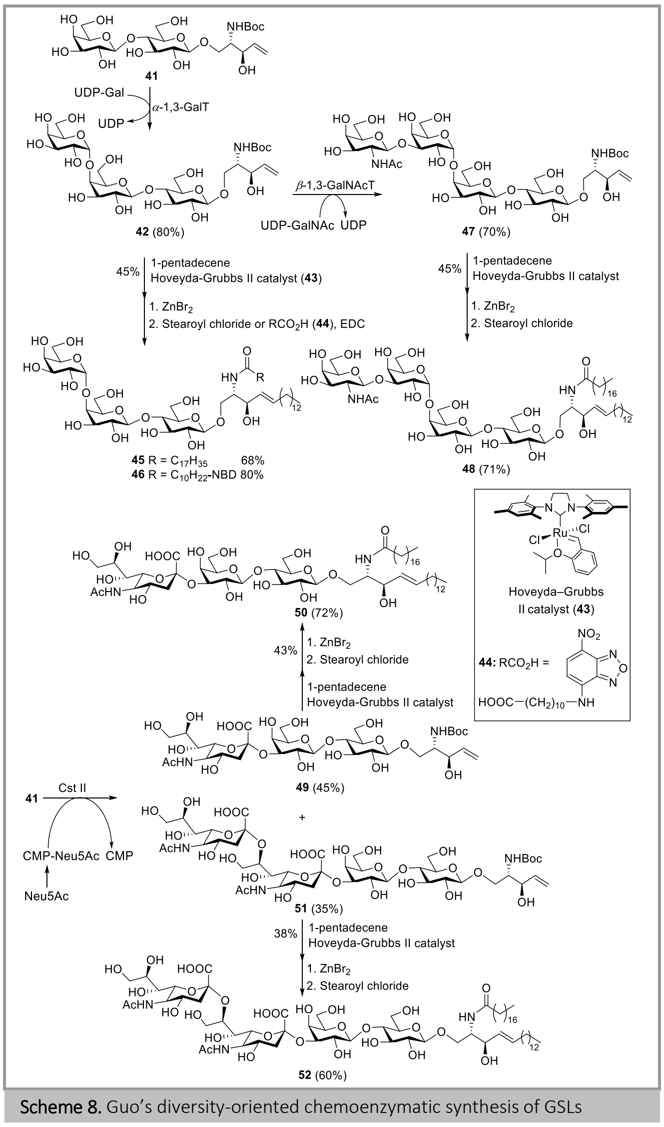
Scheme 6. Chen's chemoenzymatic synthesis of Galα3nLc4βCer **34**

In 2018, Chen group[41] exploited further applications of GTs, especially SiaTs, in streamlined chemoenzymatic synthesis of a series of gangliosides. As outlined in Scheme 7, the synthesis started from compound **3**, which was prepared chemically as described above. The first OPME glycosylation introduced a sialic acid (Neu5Ac) residue to the 3'-*O*-position of lactose selectively by the one-pot protocol to give *lyso*-GM3 **35** in an excellent yield (96%). *N. meningitidis* CMP-sialic acid synthase (NmCSS)[42] was employed to generate CMP-Neu5Ac *in situ*, and then *P. multocida* α-2,3-SiaT 3 (PmST3),[43] which could accept glycolipids as substrates, transferred sialic acid onto **3**. Compound **35** was used to synthesize both **36** and **39**. Installing a GalNAc residue at the lactose 4'-*O*-position in **35** utilizing *Campylobacter jejuni β*-1,4-GalNAcT (CjCgtA)[31] by the OPME method gave **36** in a 98% yield after Sep-Pak® cartridge purification. On the other hand, 8''-*O*-sialyation of **35** using *C. jejuni* α-2,3/8-SiaT (CjCst II)[44] generated *lyso*-GD3 **39** in a 62% yield, along with byproducts containing additional sialic acid residues. The enzymes involved in the conversion of **35** to **36** were also utilized to transform **39** into *lyso*-GD2 **40** in a similar yield at the cost of a larger amount of CjCgtA to compensate for the lower efficiency of this reaction. Fucosyl *lyso*-GM1 **38** was synthesized from **36** after two OPME reactions. The first one was to attach a Gal residue by *C. jejuni β*-1,3-GalT (CjCgtB),[45] and the second one installed a Fuc residue by *Escherichia coli α*-1,2-FucT (EcWbgL)[46] with *in situ* generation of the corresponding nucleotides. After enzymatic glycosylations, all the *lyso*-gangliosides were subjected to chemical *N*-acylation under previously described conditions to install the second lipid chain of ceramide. Several members of the ganglioside family, including GM3, GM2, GM1 and the challenging GD3 and GD2, were synthesized in this manner in high overall yields.



Scheme 7. Chen's streamlined chemoenzymatic synthesis of gangliosides

In addition to the synthetic challenges caused by the structural complexity of GSLs, the demand for structurally diverse GSLs is also well acknowledged. The target-oriented synthetic methods are usually adapted for preparing specific GSLs of interest. A synthetic method for efficient and rapid access to various GSLs, including different lipid forms critical for the formation of GSL microdomains in cell membranes, is highly desired. Accordingly, our group have explored a diversity-oriented chemoenzymatic strategy for the synthesis of GSLs, which could reach structural complexity and diversity simultaneously.[47] As shown in Scheme 8, all of the syntheses started from **41**, the core structure shared by most GSLs. It is hydrophilic enough to allow for enzymatic glycan elongation in aqueous media to accomplish various GSL glycans. In the meantime, the sphingosine head group in **41** is a superb primer for on-site construction of various lipids through chemical transformations, e.g., aqueous cross metathesis[48] and *N*-acylation. This simple core was readily prepared in large scales to satisfy the demand for divergent synthesis of multiple targets. As the proof of concept, stepwise enzymatic elongation of the glycan in **41** using various GTs, as delineated in Scheme 8, afforded oligosaccharides **42**, **47**, **49**, and **51**. These products were subjected to the same sequence of transformations, that is, cross metathesis with alkenes in the presence of Hoveyda-Grubbs II catalyst, removal of the *N*-Boc protecting group and then chemoselective *N*-acylation, to eventually afford a series of natural GSLs **45**, **48**, **50**, and **52**. When different alkenes and fatty acids were utilized for cross metathesis and *N*-acylation, GSL derivatives carrying different lipids and functional groups, such as **46**, were obtained alone with the natural GSLs. Compared to conventional chemoenzymatic synthetic methods, this strategy to conduct chemical lipidation at the final stage allows for dual diversification of both glycans and lipids so that to have a broader application scope.



Scheme 8. Guo's diversity-oriented chemoenzymatic synthesis of GSLs

Accredited to the characterization, cloning, and broad application of bacterial GTs, enzymatic assembly of complex GSL glycans has made great progresses in the past decades. However, streamlined synthesis of whole GSLs by their natural synthetic pathways is still beyond our reach because some of the key enzymes involved in ceramide glycosylation, e.g., ceramide glucosyltransferase (GlcT) (UGCG)[49] or galactosyltransferase (UGCGal) (Figure 2), are still not available; thus, glycan-ceramide coupling still relies on chemical methods. In this regard, glycosynthases derived from glycosidases have brought about some hopes.

3. Glycosynthases for GSL Synthesis

Two types of glycosidases can catalyze the cleavage of glycosidic linkages. The exoglycosidase cuts off terminal sugar units of a glycan while endoglycosidase cuts internal glycosidic bonds. As they are reversible reactions, a glycosidase can be utilized, theoretically, to create glycosidic linkages (Figure 3) for the synthesis of oligosaccharides, but the efficiencies or yields of such glycosylation reactions are typically low. To push the equilibrium of a reaction towards the synthetic direction, strategies such as using more reactive glycosyl donors, utilizing a large excess for one of the substrates, manipulating the pH value of reaction media and adding an organic solvent, have been commonly adopted, but the outcome has been moderate. Only after the application of action mechanism-based enzyme evolution, can glycosidases devoid of hydrolytic activities be discovered and applied to oligosaccharide synthesis.[50] Accordingly, once the action mechanism of a glycosidase is elucidated, mutations can be performed on its catalytic site to significantly or completely inhibit its hydrolytic function, thereby converting it into a glycosylation enzyme, called glycosynthase.[51] As shown in Figure 3, a glutamic acid residue in a glycosidase is believed to mediate hydrolysis. When it is changed to a nonnucleophilic amino acid bearing the similar property in protein folding, the resultant enzyme mutant will not possess hydrolytic activity but may retain the activity to promote glycosidic bond formation. As a result, the engineered enzyme can be used for transglycosylation reactions with appropriate glycosyl donors. For GSL synthesis, endoglycoceramidase (EGCase), an enzyme that cleaves the ceramide-glycan linkage,[52] has attracted much attention.

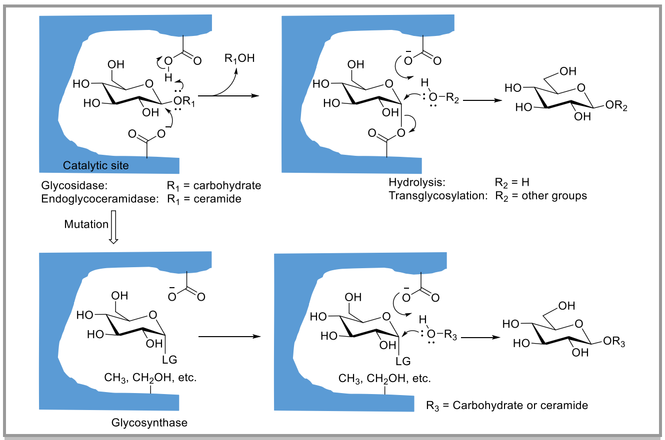
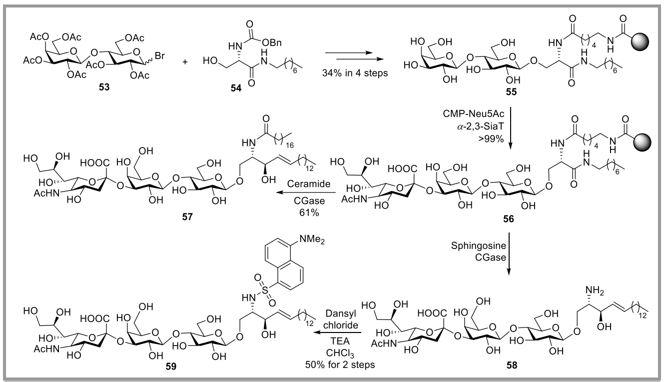


Figure 3. Mechanisms for glycosydase and glycosynthase to catalyze glycoside hydrolysis and transglycosylation reactions

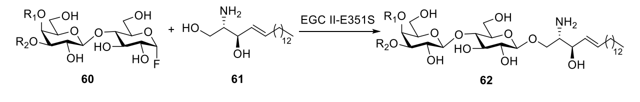
In 1997, Nishimura et al.[53] reported the synthesis of GM3 by directly transferring GM3 glycan from a water-soluble polymer to ceramide using leech ceramide glycanase (CGase, an earlier name for EGCase) (Scheme 9).[54] In this synthesis, lactose was attached to a water-soluble polymer support by a ceramide mimicking linker to generate compound **55**. Sialylation of **55** catalyzed by α-2,3-SiaT provided polymer-supported GM3 analogue **56** in a quantitative yield. Successively, the glycan in **56** was transferred to ceramide by a CGase-catalyzed reversible reaction. A large excess of ceramide was used to push the reaction equilibrium to the desired direction to get **57** in a 61% yield. Besides ceramide, the transfer of GM3 glycan to sphingosine under the influence of CGase was also achieved later by the same group.[55] The resultant *lyso*-GM3 **58** was subsequently labeled with a fluorescent tag at the sphingosine amino group to afford compound **59**. Although an extra swap step was needed to introduce the ceramide moiety, CGase's ability to accept ceramide and sterically hindered **56** as substrates had opened the door for the application of ceramide glycanase to GSL synthesis.



Scheme 9. Nishimura's chemoenzymatic synthesis of GM3 using CGase

In 2001, Ito and coworkers applied EGCase from jellyfish *Cyanea nozakii* to the semi-total synthesis of a number of GSLs and their analogues.[56] For example, they used EGCase to condense lactose with ceramide to obtain alkylated LacCer. They also used EGCase to transfer the glycans of crude gangliosides, such as GM1, GD1b, and GT1b, to alcohols in varied chain lengths under acidic conditions and to a fluorescent-tagged ceramide in the presence of acetone to get GSL analogs. Later, the same group identified the first EGCase that hydrolyzes gala series GSLs, R-Galβ1-6Galβ1-1'Cer, and performed similar transglycosylation studies.[57] This new EGCase, known as endogalactosylceramidase (EGALC), was also used to synthesize fluorescent-tagged GSLs. Although only qualitative results were described in this report, its significance is apparent as it had set up the stage for further development of glycosynthases.

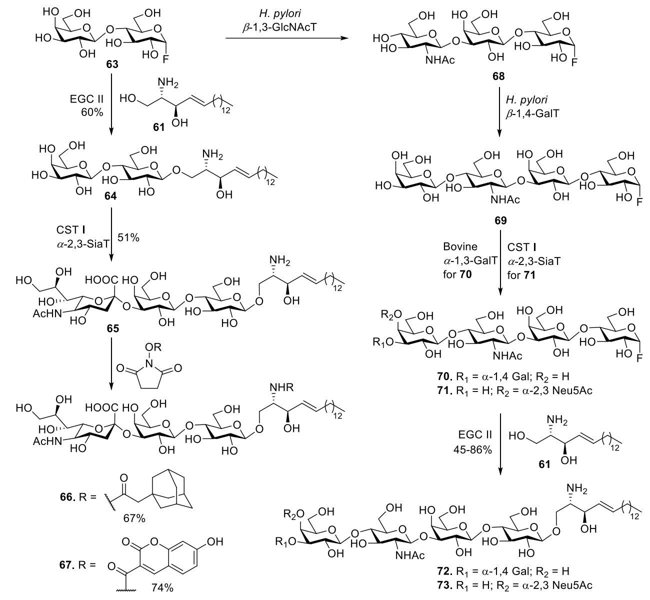
Upon elucidation of the amino acid sequence in the catalytic site of endoglycoceramidase II (EGC II) from *Rhodococcus*,[58] Withers group turned it into a glycosynthase E351S by replacing Glu351 with Serine.[59] The condensation reactions of glycosyl fluorides, which were chemically synthesized as donors, with sphingosine derivatives (acceptors) afforded excellent yields as determined by thin-layer chromatography (Scheme 10). Besides sphingosine, several of its analogues, including *N*-octanoyl sphingosine, were also accepted by this glycosynthase, although ceramide failed to be glycosylated under similar conditions. Because the reactivity towards ceramide was lost during mutagenesis, the final step of GSL synthesis was achieved by chemical acylation of *lyso*-GSLs **62** with *N*-palmitoyl succinimide.



Donor strcuture	Yield (%)
$R_1 = R_2 = H$	97
R_1 = α-Gal-1,4; R_2 = H	96
R_1 = β-Gal-1,3-α-GalNAc-1,4; R_2 = α-Neu5Ac-2,3	95
R_1 = H; R_2 = α-Neu5Ac-2,3	95
R_1O = (4*R*)-4-hydroxyl; R_2 = H	94

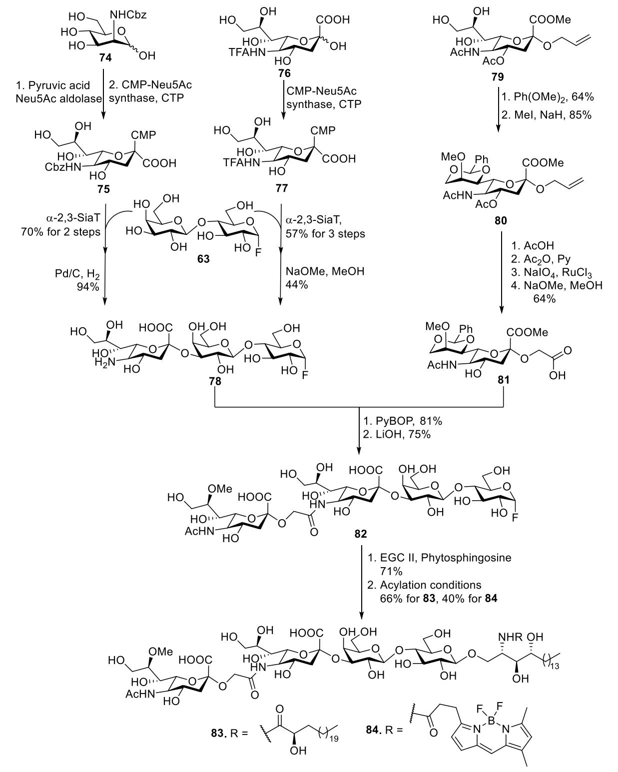
Scheme 10. Withers' enzymatic synthesis of GSLs using EGC II mutant

Following this breakthrough, Withers group performed a series of studies targeting at further improvement of the enzyme and expanding/demonstrating its scope in GSL synthesis. To improve its activity, E351S EGC II enzyme was further modified through directed evolution.[60] The resultant third-generation enzyme D314Y exhibited improved activity towards phytosphingosine, which is a sphingosine analogue with the double bond hydroxylated, by 10,000-fold in k_{cat}/k_m as compared to EGC II. In 2011, GTs and EGC II were incorporated into GSL synthesis in two ways (Scheme 11).[61] In one synthetic route (Scheme 11, left), lactosyl fluoride **63** was coupled with sphingosine under the influence of EGC II to provide LacSph **64** in a 60% yield. Thereafter, the product was subjected to CST I-catalyzed 3'-*O*-sialyation and then *N*-acylation using activated esters to produce GM3 derivatives **66** and **67** carrying an adamantyl group and a fluorescent tag, respectively. In the other synthetic route (Scheme 11, right), lactosyl fluoride **62** was subjected to a series of GT-catalyzed glycosylations to get pentasaccharide donors **70** and **71**, which were finally coupled with sphingosine in the presence of EGC II to afford *lyso*-GSLs **72** and **73**. The yields of these EGC II-catalyzed coupling reactions were 45-86%, which were similar to that of the reaction between **63** and sphingosine. These results suggested that the size of the oligosaccharide donor might not have a major impact on EGC II-catalyzed reactions.



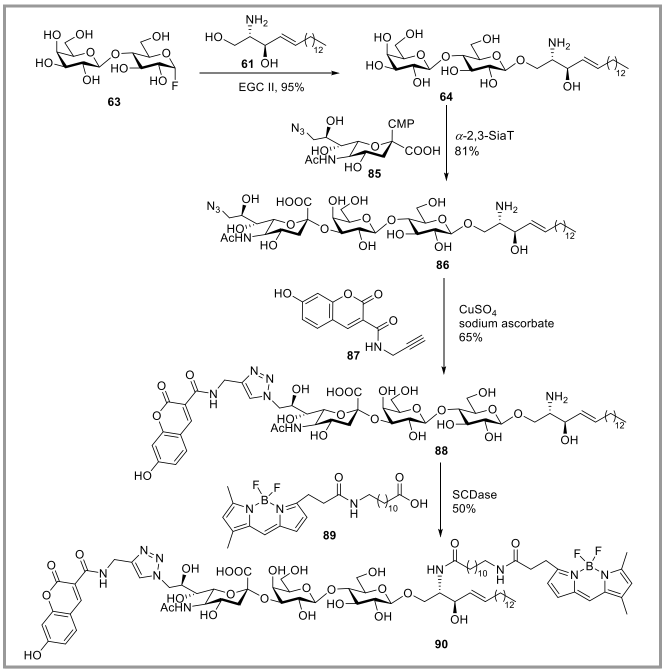
Scheme 11. Withers' synthesis of GSLs by combining GTs with EGC II

In 2012, Withers group reported a total synthesis of LLG-3 **83** and its fluorescent-tagged analogue **84** (Scheme 12).[62] This unique GSL has a sialic acid residue linked to the hydroxyl group of 5-*N*-glycolyl residue in Neu5Gc and a ceramide analogue containing phytosphingosine and a longer *N*-acyl chain. First, two sialic acid nucleotides **75** and **77** containing different 5-*N*-protecting groups were enzymatically created *in situ*. CMP-Neu5Cbz **75** was formed by aldolase-catalyzed conversion of *N*-Cbz mannosamine into Neu5Cbz followed by a reaction with CTP and CMP-Neu5Ac synthase, while CMP-Neu5TFA **77** was generated directly from Neu5TFA. Both **75** and **77** were reacted with lactosyl fluoride **63** in the presence of α-2,3-SiaT to afford trisaccharide in moderate yields. The protecting group on the amino group was removed to generate **78** for introduction of the terminal sialic acid residue. Synthesis of terminal sialic acid **81** having a 2-hydroxylacetic acid motif at its anomeric position was achieved with allylic sialoside **80** as the key intermediate, as the allylic group could be readily converted into carboxylic acid upon oxidation with sodium periodate to get **81**. The coupling reaction between **78** and **81** was accomplished in the presence of benzotriazolyloxy-1-tripyrollidinophosphonium hexafluorophosphate (PyBOP) to afford an 81% yield. After removal of the protecting group on the carboxylic group under basic condition, the resultant tetrasaccharide **82** was coupled with phytosphingosine by the third generation EGC II-D314Y enzyme to afford *lyso*-LLG-3 in a 71% yield, which was then *N*-acylated under conventional conditions to produce **83** and **84**.



Scheme 12. Withers' chemoenzymatic synthesis of LLG-3 and its analogue

Recently, Withers group[63] combined EGC II glycosynthase with *N*-deacylase (SCDase), an enzyme that catalyzes the hydrolysis of ceramide reversibly as reported by Ito et al,[64] for the synthesis of a GM3 derivative carrying 7-hydroxycoumarin and BODIPY **90** (Scheme 13). Compound **90** was used as a fluorescence resonance energy transfer (FRET) probe for *in vivo* detection and quantification of enzymes involved in ganglioside degradation. Following the established protocols described above, LacSph was prepared and subjected to α-2,3-SiaT-catalysed sialyation using 9-azido Neu5Ac to produce GM3 analogue **86** in an 81% yield. Conjugation of 7-hydroxycoumarin derivative **87** carrying a terminal alkyne with the azido group in **86** by click reaction provided **88**, which was subjected to SCDase-catalysed *N*-acylation with **89** to afford FRET probe **90** as the final product. Aliphatic acid **89** with a BODIPY functionality was proved to be a good substrate for the enzyme.



Scheme 13. Withers' chemoenzymatic synthesis of a GM3 derivative as a FRET probe

SCDase has also been used for the preparation of functionalized GSLs via semi-total synthesis. For example, radio-labeled GSLs, including Gb4 and gangliosides GM3 and GM1a, were obtained through remodeling of GSLs using a SCDase derived from marine bacteria, *Shewanella alga*, to catalyze GSL *N*-deacylation and then, after purification, reacylation with ^{14}C labeled stearic acid. These reactions gave moderate yields (54.4-71.6%). This enzyme was systematically studied by Han and co-workers[65] and applied to GSL lipid remodeling under optimized conditions. Several other SCDases were also cloned and studied independently by different groups.[66]

The discovery of SCDases complimented GTs and EGCases and completed the enzyme collection for GSL synthesis. Combining with GTs and EGCases that generate *lyso*-GSL, SCDases provide the opportunity for streamlined enzymatic synthesis. However, the substrate scope of enzymes can not compete with chemical methods, which are well-established to install lipids with various functional groups.

4. Enzymatic synthesis of ceramide

As discussed, ceramide biosynthesis involves multiple enzymes in the ER and replication of this process is challenging. Besides these difficulties, development of the enzymatic system is also less appealing compared to that of glycan synthesis, since large scale chemical syntheses of ceramides and/or sphingosines have been well documented.

Nevertheless, the first *in vitro* enzymatic synthesis of sphingosine was reported by Brady et al. in 1957,[67] using an enzyme system isolated from mammal tissue. Although, in this work, sphingosine production was investigated by radioactive labeling, the specific enzymes involved in the process were a mixture and thus were not fully characterized. In a recent report by Börgel et al.,[68] triacetylated sphingosine was produced in up to 890 mg/kg yield by fermentation using genetically engineered yeast *P. ciferrii*. However, incorporation of these enzymes or this process in the synthesis of GSLs has not been reported.

5. Conclusion

In the past decades, researchers have explored various methods for GSL synthesis. Regardless of these efforts, GSL synthesis still remains a great challenge, whereas the demand for structurally diverse and defined GSLs from various biological studies is ever growing. In addition to the inherent difficulties associated with chemical glycosylation, and the difficulty to couple glycan and ceramide that have distinct properties are among the key hurdles to hinder efficient GSL synthesis. To overcome these problems, enzymatic reactions have found wide applications.

GTs have been commonly adopted for the assembly of the glycan moieties of GSLs. With improved access to various GTs, especially with the introduction of bacterial GTs, the majority of GSL glycans can be achieved by enzymatic synthesis, and its efficiency is generally much higher than that of traditional chemical synthesis. Although totally enzymatic synthesis of GSLs by the natural biosynthetic pathway is currently not a viable option yet, the combination of enzymatic glycosylation and chemical assembly of the ceramide moiety has been demonstrated as being a powerful strategy for obtaining various natural GSLs and their derivatives. In addition, EGCases and their engineered mutants have given encouraging results about the direct coupling of glycans with lipids, especially sphingosines. Thus, a combination of GTs to assemble glycans and EGCases to attach sphingosine to glycans posts great promises for the development of streamlined totally enzymatic GSL synthesis. However, EGCases and GTs are not perfect match spontaneously to reach optimal efficiencies in GSL synthesis. GTs are often more effective in the absence of a large lipid moiety, while the donors accepted by EGCases, usually glycosyl fluorides, often require chemical synthesis. Nonetheless, looking forward, an optimized combination of EGCases and GTs seems to be a promising direction.

Furthermore, the expression and use of UDP-Glc ceramide UGCG,[49] the natural enzyme that catalyzes ceramide and glycan coupling, and its mutants represent another promising direction to achieve totally enzymatic GSL synthesis. To further improve synthetic efficiency, solid-phase or polymer-supported synthesis[69] and other automated systems[70] should be considered in the future as well.

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Biosketches

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