Title

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Journal

Chemistry - A European Journal, 29(25)

ISSN

0947-6539

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Publication Date

2023-05-02

DOI

10.1002/chem.202300005

Peer reviewed

Published in final edited form as:

Chemistry. 2023 May 02; 29(25): e202300005. doi:10.1002/chem.202300005.

Process Engineering and Glycosyltransferase Improvement for Short Route Chemoenzymatic Total Synthesis of GM1 Gangliosides

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Abstract

Large-scale synthesis of GM1, an important ganglioside in mammalian cells especially those in the nervous system, is needed to explore its therapeutic potential. Biocatalytic production is a promising platform for such a purpose. We report herein the development of process engineering and glycosyltransferase improvement strategies to advance chemoenzymatic total synthesis of GM1. Firstly, a new short route was developed for chemical synthesis of lactosylsphingosine from the commercially available Garner's aldehyde. Secondly, two glycosyltransferases including *Campylobacter jejuni* β1–4GalNAcT (CjCgtA) and β1–3-galactosyltransferase (CjCgtB) were improved on their soluble expression in *E. coli* and enzyme stability by fusing with an N-terminal maltose binding protein (MBP). Thirdly, the process for enzymatic synthesis of GM1 sphingosines from lactosylsphingosine was engineered by developing a multistep one-pot multienzyme (MSOPME) strategy without isolating intermediate glycosphingosines and by adding a detergent, sodium cholate, to the later enzymatic glycosylation steps. Installation of a desired fatty acyl chain to GM1 glycosphingosines led to the formation of target GM1 gangliosides. The combination of glycosyltransferase improvement with chemical and enzymatic process engineering represents a significant advance in obtaining GM1 gangliosides containing different sialic acid forms by total chemoenzymatic synthesis in a short route and with high efficiency.

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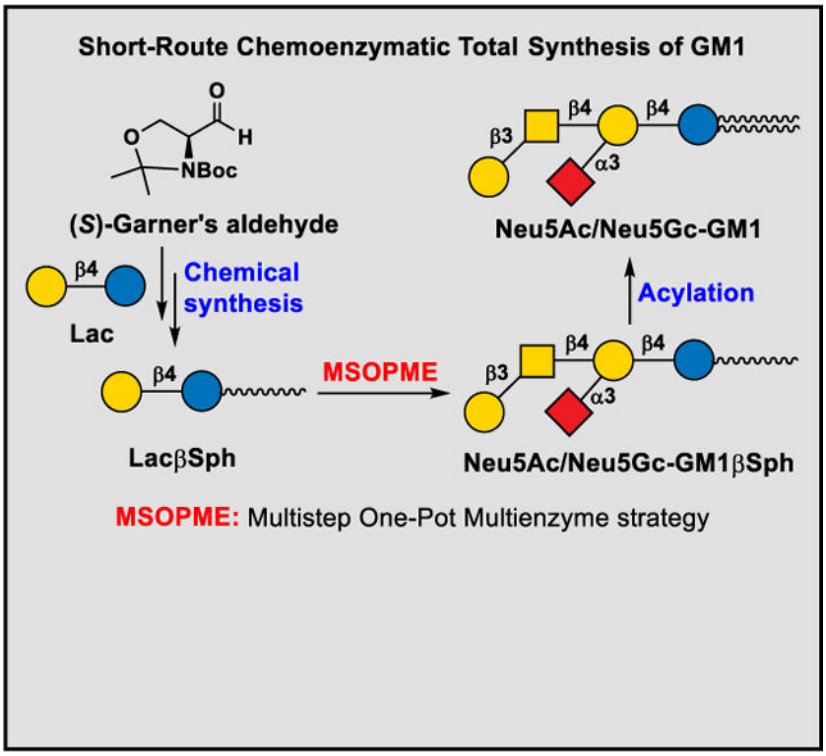

Chemoenzymatic total synthesis of GM1 gangliosides:

Combined process engineering and biocatalyst improvement strategies are developed for chemoenzymatic total synthesis of GM1 gangliosides from (*S*)-Garner's aldehyde. Two key glycosyltransferases are improved on their soluble expression in *E. coli* and enzyme stability. The multistep one-pot multienzyme (MSOPME) process with the addition of a detergent in the latter glycosylation steps have been found to be highly efficient for obtaining GM1 gangliosides containing different sialic acid forms.

Keywords

biocatalysis; chemoenzymatic synthesis; ganglioside; glycosphingolipid; GM1

Introduction

GM1a, or more commonly named as GM1, is an important member of sialic acid-containing glycosphingolipids (GSLs) called gangliosides.[1] The structure of GM1, Galβ3GalNAcβ4(Neu5Acα3)Galβ4Glcβ-ceramide (**1**, Figure 1), consists of a sialic acid-containing pentasaccharide linked via a β-glycosidic bond to a special type of lipid called ceramide.[2] In humans and other mammals, the ceramide contains a fatty acyl chain attached to the amino group of a sphingosine via an amide bond.[3] Gangliosides are presented in the outer leaflet of the plasma membrane of different cell types but are the most abundant in those of the nervous system.[4] GM1, and its more highly sialylated counterparts including GD1a, GD1b, and GT1b, constitute the four major gangliosides in human and

animal brains.[5] While GM1 in the brains of both humans and animals contains mainly the most common sialic acid form, *N*-acetylneuraminic acid (Neu5Ac),[6] GM1 containing a non-human sialic acid form, *N*-glycolylneuraminic acid (Neu5Gc) (**2**, Figure 1), has also been found in bovine brains.[7]

The important roles of GM1 and other gangliosides are well recognized.[5] Specific ganglioside-binding domains (GBDs) have been identified in a diverse array of proteins including neurotransmitter receptors, bacterial toxins, viral surface proteins, and proteins involved in various neurodegenerative diseases.[8] GM1 is a well-known cell surface receptor for pentameric cholera toxin B-subunits[9] and has been used to develop biosensors.[10] SARS-CoV-2 receptor binding domain (RBD) was also shown to bind to GM1, GM2, and GM3.[11] The therapeutic potential of exogenously admitted gangliosides in treating patients with the Rett Syndrome, Huntington's Disease (HD), and Parkinson's Disease (PD) is emerging.[8a] More specifically, the neurotrophic and neuroprotective effects of GM1 have been identified.[4a, 12] GM1 as well as GD3, GD1a, GD1b, and GT1b, but not GM3 or GQ1b, were shown to decrease inflammatory microglia responses in vitro and in vivo.[13] GM1 or GM1-containing gangliosides purified from animal brains have been used as medicines for treating peripheral neuropathies, brain and spinal cord injuries, and are being developed as potential drugs for treating HD and PD.[4a, 12, 14] GM1 oligosaccharide (OligoGM1) is also emerging as a potential candidate for treating PD.[4a, 12, 15] Furthermore, GM1 micelles[16] and GM1 sphingosine (or lysoGM1) have been used to develop drug delivery vesicles with or without poly(lactic-co-glycolic acid) (PLGA).[17] They have been shown to be able to cross the brain blood barrier (BBB).[18]

To explore their therapeutic potentials, it is crucial to obtain structurally defined GM1 gangliosides and GM1 sphingosines in sufficient amounts. Many biological studies reported were performed with GM1 purified from mammalian brains which were mixtures of GM1 molecules containing different sphingosines (e.g. d18:1, d20:1 are the most common)[19] and various fatty acyl structures including those with varied lengths and different degrees of unsaturation.[20] Using pure and structurally defined GM1 obtained by synthesis may be able to resolve some of the inconsistent or even controversial results that have been reported and will avoid the concern of applying animal brain-derived products[14b] as human therapeutics. Structurally defined gangliosides are also essential standards for analyzing ganglioside structures and components in tissue samples.[21]

Chemical synthesis of GM1 by glycosylation of ceramide was achieved from its partially protected derivative[22] or a cyclic glucosylceramide intermediate[23] using glycosyl trichloroacetimidate donors. It was also chemically synthesized from a partially protected azido-derivative of sphingosine acceptor and a thioethyl glycosyl donor.[24] Long synthetic schemes with multiple protection and deprotection steps as well as numerous glycosylation and purification processes were involved, which were time consuming and often resulted in low yields for total synthesis of GM1.

We previously reported a chemoenzymatic total synthetic strategy for the production of GM1 and other glycosphingolipids.[25] The method involves chemical synthesis of lactosylsphingosine (LacβSph) as a key intermediate. LacβSph is a water-soluble

substrate for glycosyltransferase-based one-pot multienzyme (OPME) reactions for the formation of more complex glycosylsphingosines which are readily converted to target glycosphingolipids by chemical installation of a desired fatty acyl chain. The product purification of both glycosphingosines and glycosphingolipids is facilitated by the presence of their hydrophobic tails, which can be achieved in less than 30 min using a simple C18-cartridge purification process.

Nevertheless, LacβSph was synthesized by chemical glycosylation of sphingosine glycosyl acceptors which were obtained from either phytosphingosine[25a, 25c] or a partially protected L-serine.[25b] Both methods involved an eight-step sphingosine glycosyl acceptor preparation process which was time-consuming. In addition, two of the glycosyltransferases used previously for enzymatic formation of GM1 sphingosine from LacβSph, including *Campylobacter jejuni* β1–4GalNAcT (CjCgtA) and β1–3-galactosyltransferase (CjCgtB), were not stable. Furthermore, the glycosphingosines were poorer acceptor substrates compared to the corresponding oligosaccharides for these two glycosyltransferases. Therefore, larger amounts of CjCgtA and CjCgtB and longer reaction times were needed for the production of GM1 sphingosines compared to GM1 oligosaccharides. Also, in previous efforts for glycosphingosine synthesis, a product purification process was carried out after every OPME to obtain intermediate glycosylsphingosines (such as GM3 and GM2 sphingosines) which was ideal when all intermediates were targets but the process could be simplified if a glycosphingolipid (e.g. GM1) with a long glycan chain is the desired target.

Here we report a significantly improved process for chemoenzymatic total synthesis of GM1 gangliosides containing either the most abundant Neu5Ac or the non-human Neu5Gc sialic acid form. LacβSph was chemically synthesized from a sphingosine glycosyl acceptor obtained by a four-step process, a much shorter route than the ones that we reported previously.[25] Both CjCgtA and CjCgtB were improved on their soluble expression in *E. coli* and enzyme stability. GM1 sphingosines containing either Neu5Ac (in gram-scale) or Neu5Gc were synthesized from LacβSph using a streamlined sequential multistep OPME (MSOPME) process without the need of isolating intermediate glycosphingosines. The addition of a detergent, sodium cholate, was found to improve the efficiency of the last two OPME steps for the GM1 sphingosine synthesis, leading to shorter reaction times and less amounts of CjCgtA and CjCgtB needed. These developments pave the way for large-scale production of GM1 sphingosines and GM1 gangliosides in a time-efficient manner.

Results and Discussion

Synthesis of Lactosylsphingosine (LacβSph) from (*S*)-Garner's Aldehyde

To search for a more efficient route for the synthesis of LacβSph as a key intermediate for chemoenzymatic total synthesis of GM1, we identified (*S*)-1,1-dimethylethyl 4-formyl-2,2-dimethyloxazolidine-3-carboxylate [(*S*)-Garner's aldehyde, **3**][26] as a well suited starting material. It is commercially available (e.g. $142 for 1 g from Fisher Scientific) and is much more affordable than D-erythro-sphingosine (d18:1) (e.g. $181 for 25 mg from Fisher Scientific). It can also be readily prepared from L-serine.[27]

From (*S*)-Garner's aldehyde (**3**), the desired sphingosine glycosyl acceptor (**6**)[25a, 25c] was synthesized in 1.37 gram in an overall yield of 52% with a short four-step route (Scheme 1). Briefly, (*S*)-Garner's aldehyde (**3**) reacted with 1-(*E*)-pentadecenylzirconocene chloride formed from 1-pentadecyne and zirconocene chloride hydride $Cp_2Zr(H)Cl$ (Schwartz' reagent) with catalytic amount (25 mol%) of $ZnBr_2$ in tetrahydrofuran (THF)[26a, 28] to form diastereoselectively the *anti*-adduct (**4**) with the desired *R*-configuration at the newly formed chiral carbon center (C-3) and the *E*-alkene isomer. A ratio of 12:1 favoring the *anti*- versus the *syn*- product was determined by ^1H NMR which was consistent with that reported by others.[28–29] The product isomers were not separated at this step. Benzoylation of the hydroxyl group in the partially protected sphingosine intermediate **4** by benzoyl chloride (BzCl) in dichloromethane (CH_2Cl_2), and removal of both *N,O*-isopropylidene acetal and *tert*-butyloxycarbonyl (Boc) protection groups by incubating with 2 N hydrochloric acid in ethanol at 75 °C led to the formation of compound **5** with a 60% isolated yield over three steps. Its amino group was converted to an azido group by reacting with freshly prepared triflic azide in the presence of catalytic $CuSO_4$ and triethylamine to form the desired 2-azido-3-*O*-benzoyl sphingosine (**6**)[25a, 25c] in 86% yield in gram-scale.

Glycosylation of the glycosyl acceptor **6** with per-*O*-benzoyl lactosyl trichloroacetimidate glycosyl donor (**7**)[25a, 25c] in the presence of $BF_3{\cdot}OEt_2$ in CH_2Cl_2 at −20 °C produced the protected lactoside **8** in 90% yield. Removal of all benzoyl protecting groups using NaOMe/MeOH and selective reduction of the azido group by 1,3-propanedithiol and triethylamine produced the desired LacβSph **9** (1.05 gram) in an excellent 91% yield.

Glycosyltransferase Improvement and Characterization

Campylobacter jejuni β1–4GalNAcT (CjCgtA) and β1–3-galactosyltransferase (CjCgtB) were cloned and expressed in *E. coli* as N-terminal or C-terminal truncated, and C-terminal hexahistidine-tagged recombinant proteins. We previously applied Δ15CjCgtA-His_6[30] and CjCgtBΔ30-His_6[31] in chemoenzymatic synthesis of glycans,[30] glycopeptides,[31] and glycosphingolipids.[25a] With an expression level of 40 mg purified protein per liter culture, Δ15CjCgtA-His_6 was nevertheless not stable for storage at 4 °C[30] and precipitation was observed during dialysis. Therefore, cell lysate, instead of purified enzyme, was used previously for enzymatic synthesis.[30] On the other hand, CjCgtBΔ30-His_6 with an expression level of 20 mg purified protein per liter culture[31] was more stable but its expression level had room for improvement. To increase their soluble expression levels and stability,[32] an maltose-binding protein (MBP) was fused to the N-terminus of Δ15CjCgtA-His_6 and CjCgtBΔ30-His_6. The expression levels of the resulting recombinant MBP-Δ15CjCgtA-His_6 (Figure S1, ESI) and MBP-CjCgtBΔ30-His_6 (Figure S2, ESI) were improved to 85 mg L^{-1} culture and 110 mg L^{-1} culture, respectively (Figure S3, ESI). They were also stable throughout nickel-nitrilotriacetate (Ni^{2+}-NTA) column purification and dialysis processes. Furthermore, both could be lyophilized without losing enzymatic activity (Figure S4, ESI).

MBP-Δ15CjCgtA-His_6 was shown to be active in a broad pH range of pH 6.0–10.5 and optimal activity was found in the range of pH 7.5–9.5 (Figure S5A, ESI). MBP-CjCgtBΔ30-His_6 was also active in a broad pH range (pH 4.5–10.0) with optimal activity

in pH ranging from 4.5 to 5.5 (Figure S5B, ESI). Both MBP-Δ15CjCgtA-His_6 and MBP-CjCgtBΔ30-His_6 required a divalent metal cation for activity (Figure S6, ESI). Mn^{2+} was a preferred cation for both. Mg^{2+} was equally effective for MBP-Δ15CjCgtA-His_6 but was less effective for MBP-CjCgtBΔ30-His_6. Ca^{2+} was suitable for MBP-Δ15CjCgtA-His_6 but not for MBP-CjCgtBΔ30-His_6. The addition of dithiothreitol (DTT, 10 mM) deactivated MBP-Δ15CjCgtA-His_6 but improved the activity of MBP-CjCgtBΔ30-His_6 (Figure S6, ESI). Thermostability assays (Figure S7, ESI) showed that purified and dialyzed MBP-Δ15CjCgtA-His_6 and MBP-CjCgtBΔ30-His_6 samples lost most catalytic activity after incubating at 37 °C for 3 hours while about 50% activity retained after incubation at 30 °C for 3 hours and 30 °C was chosen as a more suitable reaction temperature for enzymatic synthesis purpose.

Sodium Cholate Improvement of Glycosphingosine Synthesis with MBP-Δ15CjCgtA-His_6 or MBP-CjCgtBΔ30-His_6

We observed previously[25a] that glycosphingosines were much weaker acceptor substrates than the corresponding glycans for both CjCgtA and CjCgtB.[30] The property made it prohibitive for synthesizing GM2βSph and GM1βSph in large scales using the OPME strategy. We were able to synthesize GM2βSph (120 mg) and GM1βSph (57 mg) in preparative scales and high yields were achieved with the use of large amounts of glycosyltransferases and relatively long reaction times.[25a] An anionic detergent sodium cholate,[33] and a non-ionic detergent Triton X-100[34] were shown to improve the activity of some enzymes which use glycosphingolipids as substrates. We tested the effects of these detergents in influencing the activity of MBP-Δ15CjCgtA-His_6 or MBP-CjCgtBΔ30-His_6 in using glycosphingosine acceptor substrates and the reactions were analyzed by high-resolution mass spectrometry (HRMS) (Figures S8–S9, ESI) and thin-layer chromatography (Figure S10, ESI). We found that the addition of sodium cholate in a concentration of 8–10 mM greatly enhanced the reaction yields for both enzymes (Figures S8a, S9a, and S10, ESI). The non-ionic detergent Triton X-100 (10 mM) also improved the enzyme activities in using the glycosphingosine acceptor substrates (Figures S8b and S9b, ESI) although the effect was slightly less compared to that of sodium cholate at the same molar concentration.

Multistep One-pot Multienzyme (MSOPME) Synthesis of GM1βSph from LacβSph

After producing LacβSph (**9**) via a fast chemical synthetic route, gaining access to MBP-Δ15CjCgtA-His_6 and MBP-CjCgtBΔ30-His_6 with improved properties, and obtaining a good understanding of the benefit and the optimal concentration of a detergent as well as biochemical properties of glycosyltransferases, small-scale reactions were carried out to optimize the conditions for enzymatic synthesis of GM1 sphingosine (GM1βSph) from LacβSph (**9**) using three one-pot multienzyme (OPME) reaction systems including an OPME α2–3-sialylation (OPME1), an OPME β1–4-GalNAcylation (OPME2), and an OPME β1–3-galactosylation (OPME3) processes (Scheme 2). As GM1βSph is our target molecule at this stage, it is not necessary to purify GM3βSph or GM2βSph intermediates after individual OPME reactions unlike what we reported previously for synthesizing a collection of ganglioside targets.[25a] We envisioned that a multistep OPME strategy similar to that we developed for human milk oligosaccharide (HMO) synthesis[35] would work well. Due to the non-overlapping acceptor substrate specificities of the

glycosyltransferases involved (e.g. the product of the previous OPME is the only acceptor for the glycosyltransferase in the next OPME), unlike the MSOPME processes used previously for HMO synthesis,[35] it is not necessary to deactivating the enzymes after each OPME step for the synthesis of GM1βSph here. Small-scale assays indicated that the addition of sodium cholate was detrimental to the PmST3-catalyzed sialylation of LacβSph. In addition, 10 mM of LacβSph was an appropriate concentration for the reaction, higher concentrations of LacβSph resulted in precipitation. Addition of excess donor substrates (1.3 equiv. of monosaccharides) and nucleoside triphosphates (1.5–1.6 equiv. of ATP, and CTP or UTP) in each OPME glycosylation reaction was helpful to drive the reaction to completion.

Once optimal enzymatic reaction conditions were determined from small-scale assays, a reaction for preparative-scale synthesis of Neu5Ac-containing GM1βSph was carried out and the reaction progress was monitored using HRMS. Starting from 100 mg (10 mM) of LacβSph (**9**) and Neu5Ac (1.3 eq.), GM3βSph was formed in the OPME1 α2–3-sialylation reaction containing *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS)[36] and *Pasteurella multocida* α2–3-sialyltransferase 3 (PmST3)[37] (Scheme 2). The reaction at 30 °C was shown to be completed in 20 h.

Without purification, the reaction mixture was directly used for OPME2 β1–4-GalNAcylation reaction[30] by adding GalNAc, ATP, UTP, sodium cholate (8 mM final concentration), and four enzymes including *Bifidobacterium longum* strain ATCC55813 *N*-acetylhexosamine-1-kinase (BLNahK),[38] *Pasteurella multocida N*-acetylglucosamine uridylyltransferase (PmGlmU),[39] *Pasteurella multocida* inorganic pyrophosphatase (PmPpA),[40] and MBP-Δ15CjCgtA-His_6. The reaction mixture was incubated at 30 °C to generate GM2βSph. The presence of sodium cholate and the improvement of enzyme properties decreased the reaction time and the amount of MBP-Δ15CjCgtA-His_6 needed (compared to previous OPME synthesis of GM2βSph[25a]) to a level similar to GM2 glycan synthesis.[30] The OPME2 reaction was completed in 20 h.

Again without purification, the resulting reaction mixture was applied for OPME3 β1–3-galactosylation reaction in the third step by adding Gal, ATP, UTP, and four enzymes including *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK),[41] *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP),[42] PmPpA, and MBP-CjCgtBΔ30-His_6. As sodium cholate was added in the previous step, no additional detergent was needed in this step. The formation of GM1βSph at 30 °C was completed in 16 h. Both the reaction time and the amount of MBP-CjCgtBΔ30-His_6 were decreased (compared to previous OPME synthesis of GM1βSph[25a]) due to the presence of sodium cholate and the improvement of enzyme properties.

It is worthy to note that even though thermostability assays (Figure S7, ESI) showed that incubation of the purified and dialyzed MBP-Δ15CjCgtA-His_6 and MBP-CjCgtBΔ30-His_6 samples at 30 °C lost about 50% of their activity after 3 hours and deactivated them completely after 15 h, the product formation of GM2βSph or GM1βSph during individual OPME reaction steps increased over time. The observed higher enzyme stability in the reactions could be contributed by the presence of substrates and other components.[43]

After stopping the reaction by incubating the reaction mixture in a boiling water bath for 5 min and removing precipitates by centrifugation, the supernatant was concentrated and the GM1βSph product was purified by passing the residue through a C18 cartridge and eluting with a mixed solvent gradient of CH_3CN in water. We found that using this process, GM1βSph could be separated efficiently from other components except for sodium cholate in the reaction mixture. The removal of sodium cholate from GM1βSph was achieved by silica gel column chromatography, in which sodium cholate was eluted out first using $CHCl_3$:MeOH = 5:2 (by volume) and then GM1βSph was eluted using $CHCl_3$:MeOH:H_2O = 5:4:1 (by volume).

Once the optimized synthetic procedures and purification processes were established, gram-scale synthesis of GM1βSph (1.88 g) from LacβSph (1.00 g) was carried out similarly and an excellent yield (90%) was achieved.

Multistep One-pot Multienzyme (MSOPME) Synthesis of Neu5Gc-containing GM1βSph (Neu5Gc-GM1βSph)

The optimized procedures for synthesis and purification were also applied for the production of Neu5Gc-GM1βSph. As shown in Scheme 3, Neu5Gc-containing GM3 sphingosine (Neu5Gc-GM3βSph) was readily synthesized from LacβSph as the acceptor substrate and *N*-glycolylmannosamine (ManNGc)[36] as the Neu5Gc precursor using a three-enzyme OPME α2–3-sialylation system (OPME4) containing *Pasteurella multocida* sialic acid aldolase (PmNanA),[44] NmCSS, and PmST3. Without purification, the reaction mixture was applied to the next step to produce Neu5Gc-GM2βSph via OPME2 with sodium cholate (10 mM). When the formation of Neu5Gc-GM2βSph was completed, the reaction mixture was directly applied to the next step without purification to produce Neu5Gc-GM1βSph via OPME3. The desired Neu5Gc-GM1βSph was obtained in 91% yield after purification using a C18 cartridge followed by a silica gel column chromatography purification processes.

One-step OPME Synthesis of GM1βSph from GM3βSph

We also examined the approach of synthesizing GM1βSph from GM3βSph in one-pot in a single step by adding all reagents and enzymes needed at the beginning. This approach worked well and the formation of GM1βSph from GM3βSph was completed in two days (Scheme 4). Employing the same C18 cartridge and silica gel column purification processes described above, pure GM1βSph product was obtained in 95% yield.

An attempt to produce GM1βSph directly from LacβSph in one-step by adding all reagents and enzymes at once produced GM1βSph with only a moderate yield. It was found that the presence of sodium cholate slowed the sialylation process. Therefore, the multistep OMPE (MSOPME) approach is better than the one-step approach for producing GM1βSph starting from LacβSph.

Synthesis of GM1 Gangliosides via Acylation of GM1 Sphingosines

The production of target GM1 gangliosides (d18:1–18:0) was completed by installing the stearoyl chain to the amino group in GM1 sphingosines using stearoyl chloride in a mixed solvent of THF/aq. $NaHCO_3$ similar to what we reported previously[25a, 25b] (Scheme 5).

The acylation reaction progress was monitored by HRMS and reached completion in less than 4 h. The reaction mixture was purified using a C18 cartridge then a silica gel column to obtain the desired gangliosides GM1 (97%) and Neu5Gc-GM1 (98%), respectively.

Conclusions

In conclusion, a rapid route for chemical synthesis of lactosylsphingosine (LacβSph) from a commercially available starting material (*S*)-Garner's aldehyde has been developed. Two glycosyltransferases, CjCgtA and CjCgtB, have been improved on their soluble expression in *E. coli* and enzyme stability. A multistep one-pot multienzyme (MSOPME) strategy has been successfully developed for enzymatic synthesis of GM1 sphingosines from lactosylsphingosine without the purification of intermediate glycosphingosines. The addition of a detergent (sodium cholate) has been found to drastically improve the glycosylation efficiency of glycosphingosines by CjCgtA and CjCgtB. The combined process engineering and glycosyltransferase improvement strategies allow rapid access to GM1 gangliosides containing different sialic acid forms. They can be applied to the synthesis of other glycosphingolipids, glycoconjugates, and glycans.

Experimental Section

Materials and methods:

All chemicals were obtained from commercial suppliers and used without further purification. ^{1}H NMR (600 Hz) and ^{13}C NMR (150 MHz) spectra were recorded on a Bruker Avance-600 Spectrometer. High-resolution electrospray ionization (ESI) mass spectra were recorded using a Thermo Scientific Q Exactive HF Orbitrap Mass Spectrometer at the Mass Spectrometry Facilities in the University of California, Davis. Thin-layer chromatography (TLC, Sorbent Technologies) was performed on silica gel plates using anisaldehyde sugar stain for detection. Purified enzymes without lyophilization were used for syntheses.

Protein expression and purification:

Recombinant enzymes were expressed and purified as described previously for PmAldolase,[44] PmST3,[45] SpGalK,[41] BLUSP,[42] BLNahK,[38] PmGlmU,[39] NmCSS,[36] PmPpA.[40] Briefly, *E. coli* BL21 (DE3) cells harboring the recombinant plasmid containing the target gene were cultured in Luria-Bertani (LB) broth (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} NaCl) containing ampicillin (0.1 mg mL^{-1}) with rapid shaking (220 rpm) at 37 °C for overnight. Then the overnight culture (5 mL) was transferred into 1 L of LB broth containing ampicillin (0.1 mg mL^{-1}) and incubated at 37 °C. When the $OD_{600\ nm}$ of the cell culture reached 0.6−0.8, isopropyl-1-thio-β-D-galactopyranoside (IPTG, 0.1 mM) was added to induce the expression of the recombinant enzyme. The culture was then incubated at 20 °C with shaking (220 rpm) for 20 h. Cells were collected by centrifugation at 4392 × g for 30 min at 4 °C. The cell pellet was re-suspended in lysis buffer (100 mM Tris-HCl buffer, pH 7.5, containing 0.1% Triton X-100) and the cells were lysed using a homogenizer (EmulsiFlex-C3). Cell lysate was obtained by centrifugation at 9016 × g for 1 h at 4 °C. The supernatant was filtered using a 0.45 μm syringe filter and loaded to a

nickel-nitrilotriacetic acid (Ni^{2+}-NTA) affinity column pre-equilibrated with a binding buffer (50 mM Tris-HCl buffer, pH 7.5, 5 mM imidazole, 0.5 M NaCl). The column was washed with 10 column volumes of a binding buffer and 10 column volumes of a washing buffer (50 mM Tris-HCl buffer, pH 7.5, 10 mM imidazole, 0.5 M NaCl) and eluted using 10 column volumes of an elution buffer (50 mM Tris-HCl buffer, pH 7.5, 200 mM imidazole, 0.5 M NaCl). Fractions containing the target protein were combined and dialyzed against a dialysis buffer (20 mM Tris-HCl buffer, pH 7.5, 10% glycerol). The samples were then stored at −20 °C.

Plasmid construction for MBP-Δ15CjCgtA-His_6:

To construct the plasmid for expressing MBP-Δ15CjCgtA-His_6, the Δ15CjCgtA-His_6 gene[30] in a pET22b(+) vector plasmid was subcloned into pMAL-c2X vector. The primers used were: Forward, 5'-GACCGAATTC GTGCTGGACAACGAGCAC-3' (EcoRI restriction site is underlined); Reverse, 5'-CAGCAAGCTTTCAGTGGTGGTGGTGGTG-3' (HindIII restriction site is underlined). The polymerase chain reaction (PCR) for amplifying the target gene was performed in a 50 μL reaction mixture containing the plasmid DNA (10 ng), forward and reverse primer (0.2 μM each), 1 × Phusion HF buffer, dNTP mixture (0.2 mM each), and 1 U (0.5 μL) of Phusion® High-Fidelity DNA Polymerase. The reaction mixture was subjected to 30 cycles of amplification at an annealing temperature of 55 °C. The resulting PCR product was purified and double digested with EcoRI and HindIII restriction enzyme. The digested and purified PCR product was inserted by ligating with the pMAL-c2X vector predigested with the same restriction enzymes and transformed into *E. coli* DH5α Z-competent cells. Selected clones were grown for plasmid minipreps and the gene sequence was confirmed by customer sequencing by Genewiz.

Plasmid construction for MBP-CjCgtBΔ30-His_6:

To construct the plasmid for expressing MBP-CjCgtBΔ30-His_6, the CjCgtBΔ30-His_6 gene[31] in a pET22b(+) vector plasmid was subcloned into pMAL-c2X vector. The primers used were: Forward, 5'- GACCGAATTCTTCAAAATTTCTATCATCCTGCCG 3' (EcoRI restriction site is underlined); Reverse, 5'-CAGCAAGCTTTTAGTGGTGGTGATGATGATGCTTAATTTTGTAGATCTGAATATAC-3' (HindIII restriction site is underlined). The PCR for amplifying the target gene was performed similarly to that described above except that 52 °C was used as the annealing temperature. The subcloning process and gene sequence confirmation were the same as described above.

MBP-Δ15CjCgtA-His_6 and MBP-CjCgtBΔ30-His_6 expression and purification:

Escherichia coli BL21(DE3) cells were transformed with the desired plasmid and grown on an LB agar plate containing ampicillin (0.1 mg mL^{-1}). A single colony was inoculated in LB broth supplemented with 0.1 mg mL^{-1} ampicillin. The protein expression and purification procedures were similar to that described above for other enzymes. The enzymes were dialyzed against a buffer containing Tris-HCl (50 mM, pH 7.5) and NaCl (250 mM). The dialyzed samples were either lyophilized or added with 10% of glycerol, and then stored at −20 °C.

Enzyme activity assays:

The enzymatic assays were carried out in duplicate at 37 °C for 10 min in a reaction mixture (10 μL) containing the donor substrate (1.5 mM, UDP-GalNAc for MBP-Δ15CjCgtA-His_6 and UDP-Gal for MBP-CjCgtBΔ30-His_6), an acceptor substrate (1 mM, GM3βNHCbz for MBP-Δ15CjCgtA-His_6 and GM2βNHCbz for MBP-CjCgtBΔ30-His_6), Tris-HCl buffer (100 mM, pH 7.5), a metal cation (10 mM, $MgCl_2$ for MBP-Δ15CjCgtA-His_6 and $MnCl_2$ for MBP-CjCgtBΔ30-His_6), and the enzyme (0.32 μM for MBP-Δ15CjCgtA-His_6 and 4 μM for MBP-CjCgtBΔ30-His_6). The reactions were stopped by adding 10 μL of ice-cold methanol followed by incubation of the mixture on ice for 20 min and centrifugation at 16200 g for 5 min. The supernatant (about 20 μL) was transferred into another tube containing ddH_2O (40 μL) and the resulting mixture was analyzed by LC-MS (SHIMADZU LCMS-2020 system with electrospray ionization) for confirming the product and UHPLC (monitored at 215 nm on an Agilent Infinity 1290 II HPLC system equipped with 1260 Infinity II Diode Array Detector WR) for reaction yield determination. The column used for the UHPLC analysis was Dionex™ CarboPac™ PA-100 (1.8 μm particle, 4 × 250 mm, Thermo scientific, CA) for both glycosyltransferases. A gradient flow (100% water to 70% water/30% 1 M NaCl in 16 min) was used for analyzing the reactions catalyzed by MBP-Δ15CjCgtA-His_6 and a different gradient flow (100% water to 75% water /15 % 1 M NaCl in 16 min) was used for analyzing MBP-CjCgtBΔ30-His_6-catalyzed reactions. The flow rate was 0.75 mL min^{-1}.

pH Profile assays:

Enzymatic assays were performed in a buffer (100 mM) with a pH in the range of 3.0–10.0. Buffers used were: citric acid-sodium citrate, pH 4.0–5.5; PBS, pH 6.0–7.0; Tris-HCl, pH 7.5–8.5; and glycine-NaOH, pH 9.0–11.0. The MBP-Δ15CjCgtA-His_6 reactions were performed in the presence of $MgCl_2$ (10 mM) and the MBP-Δ15CjCgtA-His_6 reactions were performed in the presence of $MnCl_2$ (10 mM). Other conditions were the same as described above for the enzyme activity assays.

Effects of divalent metal cations, ethylenediaminetetraacetic acid (EDTA), and dithiothreitol (DTT):

The effect of various metal ions (10 mM), the chelating reagent EDTA (10 mM), and the reducing reagent DTT (10 mM) on the enzyme activity of MBP-Δ15CjCgtA-His_6 and MBP-CjCgtBΔ30-His_6 were examined at pH 7.5 in a Tris-HCl buffer (100 mM). Reactions without adding metal ions, DTT, nor EDTA were used as controls. Other conditions were the same as described above for the enzyme activity assays.

Thermostability studies:

Thermostability studies of MBP-Δ15CjCgtA-His_6 (in the presence of 10 mM $MgCl_2$) and MBP-CjCgtBΔ30-His_6 (in the presence of 10 mM $MnCl_2$) were performed by incubating the enzyme in a Tris-HCl buffer (100 mM, pH 7.5) at different temperatures for different durations (1 h, 3 h, 15 h, and 24 h) in the reaction buffer. The substrates were then added and the reaction mixtures were incubated at 37 °C for 10 min followed by reaction quenching and sample analyses. Other conditions were the same as described above for the enzyme activity assays.

Enzyme activity comparison before and after lyophilization:

Enzymatic assays were carried out in duplicates at 37 °C for 15 min in a reaction mixture (10 μL) containing the donor substrate (1.5 mM), an acceptor substrate (1 mM, GM3βNHCbz for MBP-Δ15CjCgtA-His_6 and GM2βNHCbz for MBP-CjCgtBΔ30-His_6), Tris-HCl buffer (100 mM, pH 7.5), $MgCl_2$ (10 mM) for MBP-Δ15CjCgtA-His_6 or $MnCl_2$ (10 mM) for MBP-CjCgtBΔ30-His_6, and 0.32 μM (0.025 mg mL^{-1}) of MBP-Δ15CjCgtA-His_6 or 4 μM (0.3 mg mL^{-1}) of MBP-CjCgtBΔ30-His_6. To prepare lyophilized samples, purified enzyme was dialyzed in Tris-HCl (50 mM, pH 7.5) and NaCl (250 mM). The concertation was determined by measuring $OD_{280\ nm}$ and calculation using the molecular absorption coefficient of the enzyme. The sample was then lyophilized, and the weight of the dried powder was obtained using an analytical balance. The value of mg protein per mg power was determined. The lyophilized enzyme samples were stored at −20 °C. For activity comparison, ddH_2O was used to dissolve the lyophilized enzyme before the assays. The enzyme solution with the same enzyme concentration without lyophilization was used for comparison.

Chemical synthesis of lactosylsphingosine (LacβSph, 9)

(2*S*,3*R*,*E*)-2-Amino-3-*O*-benzoyloxy-octadec-4-ene-1-ol (**5**): 1-Pentadecyne (20 mL, 76.1 mmol) in anhydrous THF (105 mL) was added into a suspension of $Cp_2Zr(H)Cl$ (19.63 g, 76.1 mmol) in anhydrous THF (75 mL) with stirring under argon at 0 °C by incubation in an ice-water bath. The mixture was stirred at room temperature for 1 h and then cooled to 0 °C by incubating in an ice-water bath. To the resulting yellow solution, Garner's aldehyde **3** (8.72 g, 38.0 mmol) in THF (105 mL) was added followed by the addition of $ZnBr_2$ (4.28 g, 19.0 mmol, dried under vacuum overnight before use). The mixture was stirred at room temperature for 24 h, then diluted with EtOAc (200 mL) and aq. potassium sodium tartrate (200 mL), and stirred for 15 min. The resulting suspension was filtered through a pad of Celite and washed thoroughly with EtOAc. The combined filtrate was washed with H_2O and then brine (saturated solution of NaCl in water). The combined organic layers were dried over Na_2SO_4 and filtered. The filtrate was concentrated and passed through a silica gel column chromatography using EtOAc:hexane = 1:7 to 1:5 (by volume) as an eluant to produce the crude product **4** (15.38 grams) containing a small amount of *syn* isomer.

To a solution of the crude product obtained above (13.6 g, 30.9 mmol) in anhydrous CH_2Cl_2 (150 mL) at 0 °C, triethylamine (30 mL, 216.5 mmol), 4-dimethyl amino pyridine (DMAP) (378 mg, 3.1 mmol) and BzCl (7.19 mL, 61.9 mmol) were added. The reaction mixture was stirred at room temperature for 24 h. After the starting material was completely consumed as monitored by TLC analysis, the reaction mixture was washed with 1 N HCl, sat. $NaHCO_3$, and then brine. The combined organic layers were collected and dried over Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure and the residue was purified by passing through a silica gel column using toluene:EtOAc = 40:1 (by volume) as an eluant. The fractions were collected and concentrated to obtain the benzoylated intermediate (16.8 g) as a colorless oil.

The intermediate obtained above (5.0 g) was dissolved in EtOH (16 mL), and aqueous 2 N hydrochloric acid (4 mL) was added. The mixture was stirred at 75 °C for 5 h. $CHCl_3$/

MeOH (100 mL, 7:1, by volume) and water (100 mL) were added into the reaction mixture, the layers were separated and the aqueous phase was extracted the $CHCl_3$/MeOH (7:1, 4 × 80 mL). The combined organic phase was evaporated and the residue was purified by silica gel column chromatography using CH_2Cl_2:MeOH = 30:1 to 20:1 then 10:1 (by volume) as an eluant to obtain compound **5** (2.42 g, 60% yield over three steps) as a white solid. ^{1}H NMR (600 MHz, MeOD) δ 8.00 (d, *J* = 8.4 Hz, 2H), 7.53 (m, *J* = 7.8 Hz, 1H), 7.40 (t, *J* = 7.8 Hz, 2H), 5.93 (dt, *J* = 14.4, 6.6 Hz, 1H), 5.69–5.67 (m, 1H), 5.47 (dd, *J* = 15.6, 7.2 Hz, 1H), 3.90 (dd, *J* = 12.0, 4.2 Hz, 1H), 3.76 (dd, *J* = 12.0, 7.8 Hz, 1H), 3.55–3.52 (m, 1H), 2.05–2.00 (m, 2H), 1.34–1.12 (m, 25H), 0.80 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, MeOD) δ 165.58, 139.41, 133.56, 129.75, 129.25, 128.48, 122.01, 121.99, 72.49, 58.20, 55.71, 32.28, 31.84, 29.61, 29.59, 29.56, 29.50, 29.35, 29.27, 29.14, 28.57, 22.59, 13.90. HRMS (ESI-Orbitrap) m/z: $[M+Na]^+$ calculated for $C_{25}H_{41}NO_3Na$ 426.2984, found 426.2967.

(2*S*,3*R*,*E*)-2-Azido-3-*O*-benzoyloxy-octadec-4-ene-1-ol (**6**): *Preparation of TfN_3*: To a solution of NaN_3 (1.45 g, 22.3 mmol) in water (4 mL), CH_2Cl_2 (4 mL) was added. The reaction mixture was cooled down to 0 °C in an ice-water bath. To the vigorously stirred mixture, Tf_2O (1.85 mL, 11.2 mmol) was added drop-wisely. After the mixture was stirred at 0 °C for 2 h, sat. $NaHCO_3$ (2 mL) was added. The mixture was stirred at r.t. for 5 min. The aqueous phase was separated and extracted with CH_2Cl_2 (3 mL) twice. The combined solution of the organic layers containing TfN_3 was used immediately in the next step.

The freshly prepared solution of TfN_3 (1.5 eq., 5.6 mmol in 5 mL CH_2Cl_2) was added to a mixture of compound **5** (1.5 g, 3.7 mmol), Et_3N (0.78 mL, 5.6 mmol), and $CuSO_4$ (10 mg). MeOH (1 mL) was then added dropwisely and the resulting solution was stirred at room temperature for overnight. The volume of the reaction mixture was reduced and the resulting residue was purified by silica gel column chromatography using hexane:EtOAc = 4:1 (by volume) as an eluant to obtain compound **6** (1.37 g, 86%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.07–8.05 (m, 2H), 7.60–7.57 (m, 1H), 7.45 (t, *J* = 7.8 Hz, 2H), 5.96 (dt, *J* = 13.8, 6.6 Hz, 1H), 5.64–5.58 (m, 2H), 3.82–3.79 (m, 1H), 3.76 (dd, *J* = 10.8, 4.2 Hz, 1H), 3.64 (dd, *J* = 11.4, 7.2 Hz, 1H), 2.11–2.04 (m, 3H), 1.41–1.36 (m, 2H), 1.31–1.20 (m, 22H), 0.88 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 165.48, 138.78, 133.32, 129.80, 129.77, 128.48, 123.30, 74.60, 66.22, 62.00, 32.37, 31.92, 29.68, 29.66, 29.65, 29.63, 29.57, 29.41, 29.35, 29.13, 28.69, 22.68, 14.10. HRMS (ESI-Orbitrap) m/z: $[M+Na]^+$ calculated for $C_{25}H_{39}N_3O_3Na$ 452.2889, found 452.2875.

O-(2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-benzoyl-α-D-glucopyranosyl)-(1→1)-(2*S*,3*R*,*E*)-2-azido-3-*O*-benzoyloxy-octadec-4-ene (**8**): Perbenzoylated lactosyl trichloroacetimidate (**7**, 3.48 g, 2.87 mmol)[46] and glycosyl acceptor **6** (0.82 g, 1.9 mmol) were dissolved in anhydrous CH_2Cl_2 (80 mL) containing powdered molecular sieves (4 Å, 2.0 g). The mixture was stirred under argon at r.t. for 30 min and then cooled down to −20 °C. $BF_3{\cdot}OEt_2$ (0.77 mL, 6.3 mmol) was added and the reaction mixture was stirred at −20 °C for 30 min. The temperature of the reaction was then slowly increased to 0 °C and the mixture was continuously stirred at 0 °C (by incubating the reaction container in an ice-water bath) until TLC analysis (hexane:EtOAc = 3:1 by volume and detected with *p*-anisaldehyde sugar stain) showed

the complete consumption of the acceptor (3 h). The reaction was quenched with Et_3N, and the mixture was filtered over Celite and concentrated. The residue was purified by silica gel column chromatography using toluene:EtOAc = 20:1 (by volume) as an eluant to obtain compound **8** (2.55 g, 90%) as a white foam. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.04–7.16 (m, 40H), 5.81 (t, *J* = 9.6 Hz, 1H), 5.75–5.65 (m, 3H), 5.51–5.47 (m, 2H), 5.45–5.35 (m, 2H), 4.89 (d, *J* = 7.8 Hz, 1H), 4.74 (d, *J* = 7.8 Hz, 1H), 4.58 (d, *J* = 12.0 Hz, 1H), 4.48 (dd, *J* = 12.0, 4.8 Hz, 1H), 4.29 (t, *J* = 9.6 Hz,1H), 3.92–3.84 (m, 4H), 3.77–3.69 (m, 2H), 3.55 (dd, *J* = 10.2, 6.0 Hz, 1H), 1.89 (q, *J* = 7.2 Hz, 2H), 1.37–1.05 (m, 22H), 0.88 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 165.80, 165.56, 165.40, 165.22, 165.02, 164.93, 164.81, 138.95, 133.54, 133.42, 133.38, 133.34, 133.26, 133.22, 133.20, 133.05, 130.00, 129.93, 129.87, 129.76, 129.71, 129.66, 129.58, 129.54, 129.44, 129.41, 129.29, 128.97, 128.87, 128.68, 128.64, 128.58, 128.55, 128.51, 128.38, 128.36, 128.28, 128.25, 122.41, 101.03, 100.83, 75.87, 74.78, 73.12, 72.87, 71.78, 71.65, 71.43, 69.89, 68.28, 67.54, 63.42, 62.27, 61.07, 32.28, 31.94, 29.71, 29.68, 29.65, 29.59, 29.37, 29.14, 28.60, 22.71, 14.15. HRMS (ESI-Orbitrap) m/z: $[M+Na]^+$ calculated for $C_{86}H_{87}N_3O_{20}Na$ 1504.5781, found 1504.5782.

O-(β-D-Galactopyranosyl)-(1→4)-(β-D-glucopyranosyl)-(1→1)-(2*S*, 3*R*, *E*)-2-aminooctadec-4-ene-1,3-diol (LacβSph, **9**): To a solution of **8** (2.50 g, 1.7 mmol) in dry MeOH (30 mL), NaOMe (250 mg) was added. After being stirred at r.t. for 14 h, the reaction mixture was neutralized using Dowex 50W (H^+), filtered and concentrated under a reduced pressure. The intermediate was used in the next step without further purification. To the dry intermediate in pyridine-water = 1:1 (by volume, 30 mL), 1,3-propanedithiol (1.83 mL, 16.8 mmol) and Et_3N (0.43 mL) were added, and the mixture was stirred at 50 °C for 36 h. The reaction mixture was concentrated and purified by silica gel column chromatography using chloroform:methanol:water = 5:4:0.5 (by volume) as an eluant to obtain LacβSph (**9**, 1.05 g, 91%) as a white powder. ^{1}H NMR (600 MHz, MeOD) δ 5.72 (dt, *J* = 14.4, 6.6 Hz, 1H), 5.41 (dd, *J* = 15.6, 7.2 Hz, 1H), 4.28 (d, *J* = 7.8 Hz, 1H), 4.24 (d, *J* = 7.8 Hz, 1H), 4.00 (t, *J* = 6.6 Hz, 1H), 3.84 (dd, *J* = 12.0, 2.4 Hz, 1H), 3.83–3.69 (m, 5H), 3.62 (dd, *J* = 11.4, 4.8 Hz, 1H), 3.52–3.44 (m, 4H), 3.41 (dd, *J* = 10.2, 3.6 Hz, 1H), 3.37–3.34 (m, 1H), 3.21 (t, *J* = 8.4 Hz, 1H), 2.98 (ddd, *J* = 7.8, 6.0, 3.6 Hz, 1H), 2.04–1.94 (m, 2H), 1.42–1.08 (m, 20H), 0.83 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, MeOD) δ 134.61, 128.61, 103.71, 102.68, 79.10, 75.71, 75.14, 74.89, 73.42, 73.25, 72.07, 71.13, 68.89, 68.84, 61.10, 60.37, 54.96, 32.01, 31.67, 29.39, 29.36, 29.23, 29.07, 28.97, 28.91, 22.33, 13.03. HRMS (ESI-Orbitrap) m/z: $[M+H]^+$ calculated for $C_{30}H_{58}NO_{12}$ 624.3959, found: 624.3950.

Effects of detergents on MBP-Δ15CjCgtA-His$_6$-catalyzed OPME formation of GM2βSph:

Assays were carried out at 30 °C for 12 h, each with a total volume of 10 μL in Tris-HCl buffer (100 mM, pH 7.5) containing GM3βSph (10 mM), GalNAc (15 mM), ATP (15 mM), UTP (15 mM), $MgCl_2$ (20 mM), BLNahK (2 μg), PmGlmU (2 μg), MBP-Δ15CjCgtA-His$_6$ (0.5 μg), PmPpA (1 μg), and various concentrations of sodium cholate (0, 1, 3, 5, 8, 10, 15 mM) or Triton X-100 (2, 5, 10, 15 mM). Reactions were quenched by adding 10 μL of pre-chilled ethanol and the mixtures were incubated at 0 °C for 30 min, centrifuged, and the supernatant was analyzed by HRMS.

MSOPME preparative-scale synthesis of GM1βSph from LacβSph:

A reaction mixture containing LacβSph (75 mg, 0.12 mmol), Neu5Ac (48 mg, 0.16 mmol), and CTP (101 mg, 0.19 mmol), $MgCl_2$ (20 mM), NmCSS (1.0 mg), and PmST3 (2.3 mg) in a Tris-HCl buffer (12 mL, 100 mM, pH 8.5) was incubated at 30 °C with agitation at 100 rpm. The product formation was monitored by mass spectrometry. After 20 h, HRMS indicated that the LacβSph was almost consumed. GalNAc (35 mg, 0.16 mmol), ATP (100 mg, 0.18 mmol), and UTP (100 mg, 0.18 mmol) were then added, and the pH of the reaction was adjusted to 7.5 by adding NaOH (4 M). After adding BLNahK (0.6 mg), PmGlmU (0.8 mg), MBP-Δ15CjCgtA-His_6 (1.5 mg), PmPpA (0.6 mg), and 0.12 mL of sodium cholate (1 M in water), the reaction mixture (14 mL) was incubated at 30 °C with agitation at 100 rpm. The product formation was monitored by HRMS and GM3βSph was completely consumed after 20 h. In the same reaction container without workup or purification, galactose (25 mg, 0.16 mmol), ATP (100 mg, 0.18 mmol), and UTP (100 g, 0.18 mmol), SpGalK (0.8 mg), BLUSP (0.8 mg), MBP-CjCgtBΔ30-His_6 (1.5 mg), and PmPpA (0.6 mg) were added. The reaction mixture (16 mL) was incubated at 30 °C for 16 h with agitation at 180 rpm. The product formation was monitored by HRMS. After the reaction was completed, the reaction mixture was incubated in a boiling water bath for 5 min and then centrifuged to remove precipitates. The supernatant was concentrated, and the residue obtained was purified by passing through a ODS-SM column (50 μM, 120 Å, Yamazen) using a CombiFlash® Rf 200i system. The fractions containing the product were collected and concentrated. The residue was purified by silica gel column chromatography. A mixed solvent chloroform:methanol = 5:2 (by volume) was used to remove sodium cholate and chloroform:methanol:water = 5:4:1 (by volume) was used as an eluent to obtain pure GM1βSph (141 mg, 91%) as a white powder. ^{1}H NMR (600 MHz, MeOD) δ 5.81 (dt, J = 15.0, 7.2 Hz, 1H), 5.45 (dd, J = 15.0, 7.2 Hz, 1H), 4.86 (d, J = 9.0 Hz, 1H), 4.41 (d, J = 7.8 Hz, 1H), 4.37 (d, J = 7.8 Hz, 1H), 4.31 (d, J = 7.8 Hz, 1H), 4.25–4.08 (m, 3H), 4.01–3.24 (m, 32H), 2.69 (dd, J = 12.6, 5.4 Hz, 1H), 2.06 (q, J = 7.2 Hz, 2H), 1.98 (s, 3H), 1.96 (s, 3H), 1.86 (t, J = 12.0 Hz, 1H), 1.45–1.20 (m, 22H), 0.86 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, MeOD) δ 174.26, 173.81, 173.41, 135.05, 127.34, 105.22, 103.52, 102.73, 102.44, 102.03, 81.62, 79.73, 78.06, 77.60, 75.19, 75.11, 74.98, 74.76, 74.52, 74.29, 73.70, 73.22, 73.13, 71.99, 71.13, 70.10, 69.62, 69.08, 68.85, 68.34, 68.27, 66.48, 64.02, 61.60, 61.02, 60.40, 55.23, 52.41, 51.44, 51.35, 37.24, 31.98, 31.66, 29.38, 29.34, 29.26, 29.22, 29.05, 28.99, 28.82, 22.41, 22.32, 21.23, 13.04. HRMS (ESI-Orbitrap) m/z: $[M-H]^-$ calculated for $C_{55}H_{96}N_3O_{30}$ 1278.6084, found 1278.6070.

MSOPME gram-scale synthesis of GM1βSph from LacβSph:

LacβSph (1.00 g, 1.6 mmol), Neu5Ac (0.64 g, 2.1 mmol), and CTP (1.45 g, 2.6 mmol) were incubated at 30 °C in a Tris-HCl buffer (150 mL, 100 mM, pH 8.5) containing $MgCl_2$ (20 mM), NmCSS (12 mg), and PmST3 (33 mg). The reaction was incubated in an incubator shaker at 30 °C with agitation at 100 rpm. The product formation was monitored by mass spectrometry. After 15 h, an additional amount of PmST3 (10 mg) was added. After 2 days, the TLC and HRMS indicated that the LacβSph was consumed almost completely. GalNAc (462 mg, 2.1 mmol), ATP (1.33 g, 2.4 mmol), and UTP (1.32 g, 2.4 mmol) were added, and the pH of the mixture was adjusted to 7.5 by adding NaOH (4 M). BLNahK (8 mg), PmGlmU (10 mg), MBP-Δ15CjCgtA-His_6 (16 mg), PmPpA (8 mg), and 1.5 mL of sodium

cholate (1 M in water) were then added and the reaction mixture (170 mL) was incubated at 30 °C with agitation at 100 rpm. The product formation was monitored by TLC and HRMS. When GM3βSph was completely consumed (30 h), in the same reaction container without workup or purification, galactose (375 mg, 2.1 mmol), ATP (1.33 g, 2.4 mmol), and UTP (1.32 g, 2.4 mmol), SpGalK (10 mg), BLUSP (10 mg), MBP-CjCgtBΔ30-His_6 (12 mg), and PmPpA (8 mg) were added. The reaction mixture (185 mL) was incubated at 30 °C with agitation at 180 rpm for overnight. The product formation was monitored by HRMS. After the reaction was completed (18 h), the reaction mixture was incubated in a boiling water bath for 5 min and then centrifuged to remove precipitates. The supernatant was concentrated, and the residue was purified by passing through a ODS-SM column (50 μM, 120 Å, Yamazen) using a CombiFlash® Rf 200i system. The fractions containing the product were collected and concentrated. The residue was further purified by silica gel column chromatography. A mixed solvent chloroform:methanol = 5:2 (by volume) was used to remove sodium cholate and then chloroform:methanol:water = 5:4:1 (by volume) was used as an eluant to produce pure Neu5Ac-containing GM1βSph (1.88 g, 90%) as a white powder.

MSOPME enzymatic synthesis of Neu5Gc-GM1βSph from LacβSph:

A reaction mixture containing LacβSph (100 mg, 0.16 mM), ManNGc (57 mg, 0.24 mM), sodium pyruvate (176 mg, 1.60 mM), and CTP (180 mg, 0.32 mM) $MgCl_2$ (20 mM), PmAldolase (3.0 mg), NmCSS (2.0 mg), and PmST3 (3.0 mg) in a Tris-HCl buffer (16 mL, 100 mM, pH 8.5) was incubated at 30 °C with agitation at 100 rpm. The product formation (Neu5Gc-GM3βSph) was monitored by mass spectrometry. After 24 h, HRMS indicated that the LacβSph was almost consumed. GalNAc (53 mg, 0.24 mmol), ATP (156 mg, 0.27 mmol), and UTP (150 mg, 0.27 mmol) were then added and the pH of the reaction was adjusted to 7.5 by adding NaOH (4 M). After adding BLNahK (1.5 mg), PmGlmU (2.0 mg), MBP-Δ15CjCgtA-His_6 (4.0 mg), PmPpA (1.0 mg), and 0.16 mL of sodium cholate (1 M in water), the reaction mixture (19 mL) was incubated at 30 °C with agitation at 100 rpm. The product formation (Neu5Gc-GM2βSph) was monitored by HRMS and Neu5Gc-GM3βSph was completely consumed after 12 h. In the same reaction container without workup or purification, galactose (44 mg, 0.24 mmol), ATP (156 mg, 0.27 mmol), and UTP (150 mg, 0.27 mmol), SpGalK (1.5 mg), BLUSP (1.5 mg), MBP-CjCgtBΔ30-His_6 (4.0 mg), and PmPpA (1.0 mg) were added. The reaction mixture (21 mL) was incubated at 30 °C for 12 h with agitation at 180 rpm. The product formation was monitored by HRMS. After the reaction is completed, the reaction mixture was incubated in a boiling water bath for 5 min and then centrifuged to remove precipitates. The supernatant was concentrated, and the residue obtained was purified by passing through a ODS-SM column (50 μM, 120 Å, Yamazen) using a CombiFlash® Rf 200i system. The fractions containing the product were collected and concentrated. The residue was purified by silica gel column chromatography. A mixed solvent chloroform:methanol = 5:2 (by volume) was used to remove sodium cholate and chloroform:methanol:water = 5:4:1 (by volume) was used as an eluent to obtain pure Neu5Gc-GM1βSph (193 mg, 91.4%) as a white powder. ^{1}H NMR (600 MHz, MeOD) δ 5.81 (dt, *J* = 15.0, 7.2 Hz, 1H), 5.45 (dd, *J* = 15.0, 7.2 Hz, 1H), 4.87 (d, *J* = 8.7 Hz, 1H), 4.42 (d, *J* = 7.8 Hz, 1H), 4.38 (d, *J* = 7.8 Hz, 1H), 4.31 (d, *J* = 7.8 Hz, 1H), 4.23 (t, *J* = 6.0 Hz, 1H), 4.16–4.07 (m, 2H), 4.07–3.32 (m, 34H), 2.71 (dd, *J* = 12.6, 5.4 Hz, 1H), 2.06

(q, *J* = 7.2 Hz, 2H), 1.96 (s, 3H), 1.88 (t, *J* = 12.0 Hz, 1H), 1.56–1.02 (m, 22H), 0.86 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, MeOD) δ 176.01, 173.87, 173.85, 135.68, 127.12, 105.04, 103.24, 102.69, 102.38, 102.00, 81.23, 79.38, 78.00, 77.47, 75.07, 74.81, 74.65, 74.46, 74.22, 73.30, 73.03, 72.98, 72.17, 71.08, 70.39, 69.81, 68.84, 68.79, 68.24, 68.15, 66.82, 63.69, 61.49, 61.15, 61.03, 60.38, 60.20, 55.09, 51.97, 51.27, 37.14, 31.93, 31.58, 29.27, 29.26, 29.24, 29.13, 28.95, 28.92, 28.72, 22.52, 22.27, 13.11. HRMS (ESI-Orbitrap) m/z: $[M-H]^-$ calculated for $C_{55}H_{96}N_3O_{31}$ 1294.6033, found 1294.6051.

One-pot preparative-scale enzymatic synthesis of GM1βSph from GM3βSph:

GM3βSph[25b] (57 mg, 0.061 mmol), GalNAc (17.5 mg, 0.079 mmol), Gal (15 mg, 0.079 mmol), ATP (100 mg, 0.18 mmol), and UTP (100 mg, 0.18 mmol) were dissolved in water in a 50 mL centrifuge tube containing Tris-HCl buffer (100 mM, pH 7.5) and $MgCl_2$ (20 mM). The pH of the mixture was adjusted to 7.5 by adding NaOH (4 M). BLNahK (0.8 mg), PmGlmU (0.8 mg), SpGalK (0.8 mg), BLUSP (0.8 mg), MBP-Δ15CjCgtA-His_6 (1.2 mg), MBP-CjCgtBΔ30-His_6 (1.0 mg), PmPpA (0.5 mg), and 0.05 mL of sodium cholate (1 M in water) were then added and water was added to bring the final volume to 5 mL, resulting in a solution containing 12 mM GM3βSph. The reaction mixture was incubated at 30 °C in an incubator shaker with agitation at 180 rpm. The reaction was monitored by TLC assays and HRMS analyses. After the reaction was completed (48 h), the reaction mixture was incubated in a boiling water bath for 5 min, cooled down, and centrifuged. The supernatant was concentrated and purified similar to that described above to obtain pure GM1βSph (75 mg, 95%) as a white powder.

Synthesis of GM1 gangliosides by acylation

GM1: To a solution of GM1βSph (90 mg, 0.071 mmol) in sat. $NaHCO_3$-THF (4.5 mL, 2:1), stearoyl chloride (32 mg, 0.105 mmol, 1.5 eq) in 1.5 mL THF was added. The resulting mixture was stirred vigorously at room temperature for 2 h. An additional 0.5 eq of stearoyl chloride was added and the mixture was stirred for another 2 h and concentrated. The sample was loaded to a pre-conditioned (by washing the cartridge with three column volumes of MeOH and then three column volumes of deionized water) C18 cartridge (bed weight 10 g) and eluted with a solution of 50–80% acetonitrile in water. The fractions containing the final product were collected, combined, and concentrated. The residue was further purified by silica gel column chromatography using chloroform:methanol:water = 5:4:0.5 (by volume) as an eluant to obtain pure GM1 (105 mg, 97%) as a white powder. ^{1}H NMR (600 MHz, MeOD) δ 5.60 (dt, *J* = 14.4, 7.8 Hz, 1H), 5.36 (dd, *J* = 15.0, 7.8 Hz, 1H), 4.83 (d, *J* = 9.0 Hz, 1H), 4.37 (d, J = 7.8 Hz, 1H), 4.33 (d, *J* = 7.8 Hz, 1H), 4.22 (d, *J* = 7.8 Hz, 1H), 4.12–4.05 (m, 3H), 3.99 (t, *J* = 8.4 Hz, 1H), 3.95–3.26 (m, 30H), 3.20 (t, *J* = 8.4 Hz, 1H), 2.65 (dd, *J* = 12.6, 4.8 Hz, 1H), 2.09 (t, *J* = 7.8 Hz, 2H), 1.96–1.92 (m, 2H), 1.93 (s, 3H), 1.91 (s, 3H), 1.83 (t, *J* = 12.0 Hz, 1H), 1.55–1.06 (m, 52H), 0.82 (t, *J* = 6.6 Hz, 6H). ^{13}C NMR (150 MHz, MeOD) δ 174.53, 174.23, 173.83, 173.41, 133.64, 129.99, 105.24, 103.55, 103.04, 102.71, 102.08, 81.63, 79.88, 77.62, 75.08, 75.03, 74.94, 74.71, 74.51, 74.21, 73.70, 73.44, 73.18, 71.98, 71.56, 71.10, 69.64, 69.05, 68.83, 68.51, 68.34, 68.29, 63.99, 61.59, 60.99, 60.39, 60.32, 53.30, 52.38, 51.34, 37.16, 35.97, 32.08, 31.71, 31.69, 29.49, 29.46, 29.43, 29.42, 29.38, 29.31, 29.25, 29.12, 29.09, 29.08, 29.04, 25.78, 22.38, 22.37, 22.35, 21.19, 13.08,

13.07. HRMS (ESI-Orbitrap) m/z: $[M-H]^-$ calculated for $C_{73}H_{130}N_3O_{31}$ 1544.8694, found 1544.8661.

Neu5Gc-GM1: To a solution of Neu5Gc-GM1βSph (80 mg, 0.061 mmol) in sat. $NaHCO_3$-THF (3 mL, 2:1 by volume), stearoyl chloride (28 mg, 0.92 mmol, 1.5 eq) in 1 mL THF was added. The resulting mixture was stirred vigorously at room temperature for 2 h. An additional 0.5 eq of stearoyl chloride was added and the mixture was stirred for another 2 h and concentrated. The sample was loaded to a pre-conditioned C18 cartridge (bed weight 10 g) and eluted with a solution of 50–80% acetonitrile in water. The fractions containing the final product were collected, combined, and concentrated. The residue was further purified by silica gel column chromatography using chloroform:methanol:water = 5:4:0.5 (by volume) as an eluant to obtain Neu5Gc-GM1 (94 mg, 98%) as a white powder. ^{1}H NMR (600 MHz, MeOD) δ 5.66 (dt, *J* = 15.0, 7.2 Hz, 1H), 5.42 (dd, *J* = 15.0, 7.2 Hz, 1H), 4.90 (d, *J* = 8.4 Hz, 1H), 4.42 (d, *J* = 8.4 Hz, 1H), 4.39 (d, *J* = 8.4 Hz, 1H), 4.27 (d, *J* = 7.8 Hz, 1H), 4.18–4.13 (m, 3H), 4.06–3.62 (m, 22H), 3.56–3.35 (m, 11H), 3.25 (t, *J* = 7.8, 1H), 2.72 (dd, *J* = 12.6, 5.4 Hz, 1H), 2.16–1.99 (m, 4H), 1.97 (s, 3H), 1.90 (t, *J* = 12.0 Hz, 1H), 1.60–1.22 (m, 52H), 0.87 (t, *J* = 6.6 Hz, 6H). ^{13}C NMR (150 MHz, MeOD) δ 176.00, 174.55, 173.87, 173.41, 133.61, 129.97, 105.20, 103.56, 103.07, 102.71, 102.08, 81.60, 79.86, 77.59, 75.10, 75.06, 74.99, 74.72, 74.53, 74.21, 73.45, 73.21, 72.06, 71.59, 71.12, 69.65, 69.05, 68.86, 68.56, 68.35, 68.11, 64.02, 61.59, 61.19, 61.02, 60.43, 60.34, 53.35, 52.09, 51.33, 37.94, 37.24, 35.98, 32.06, 31.69, 31.67, 31.66, 29.49, 29.46, 29.43, 29.41, 29.39, 29.37, 29.34, 29.33, 29.29, 29.26, 29.22, 29.09, 29.07, 29.05, 29.02, 26.43, 25.76, 22.37, 22.34, 22.33, 22.31, 13.05, 13.04. HRMS (ESI-Orbitrap) m/z: $[M-H]^-$ calculated for $C_{73}H_{130}N_3O_{32}$ 1560.8643, found 1560.8624.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Research reported in this publication was partially supported by the United States (U.S.) National Institutes of Health (NIH) Common Fund Glycoscience Program under the award number U01GM120419 and an NIH SBIR grant under the award number R44GM139441. The Thermo Scientific Q Exactive HF Orbitrap Mass Spectrometer was purchased with a U.S. NIH Shared Instrumentation Grant under the award number S10OD025271.

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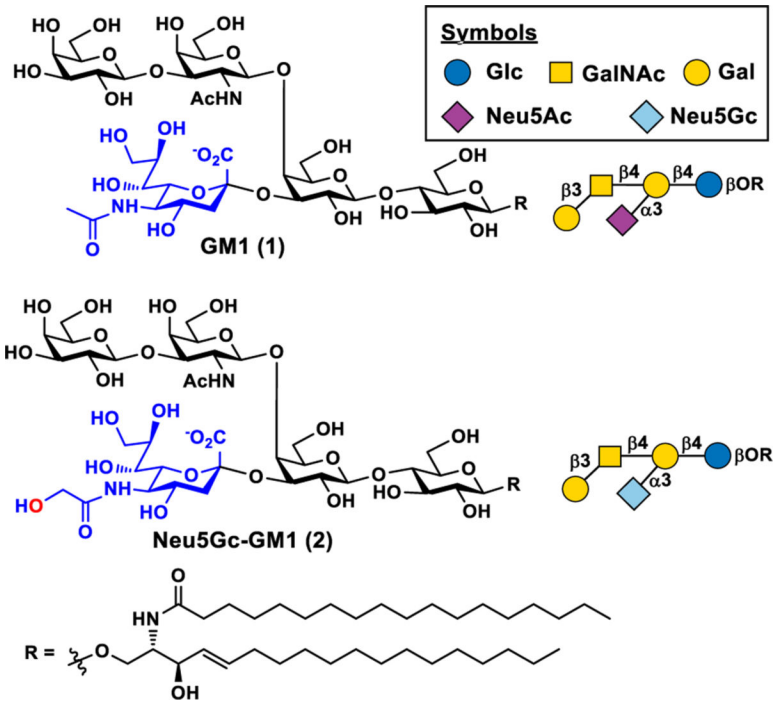
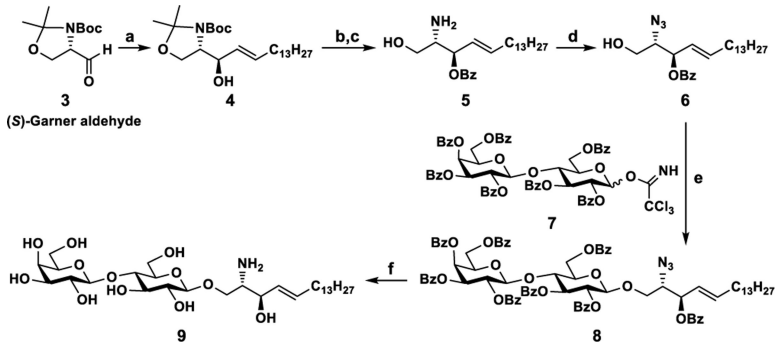


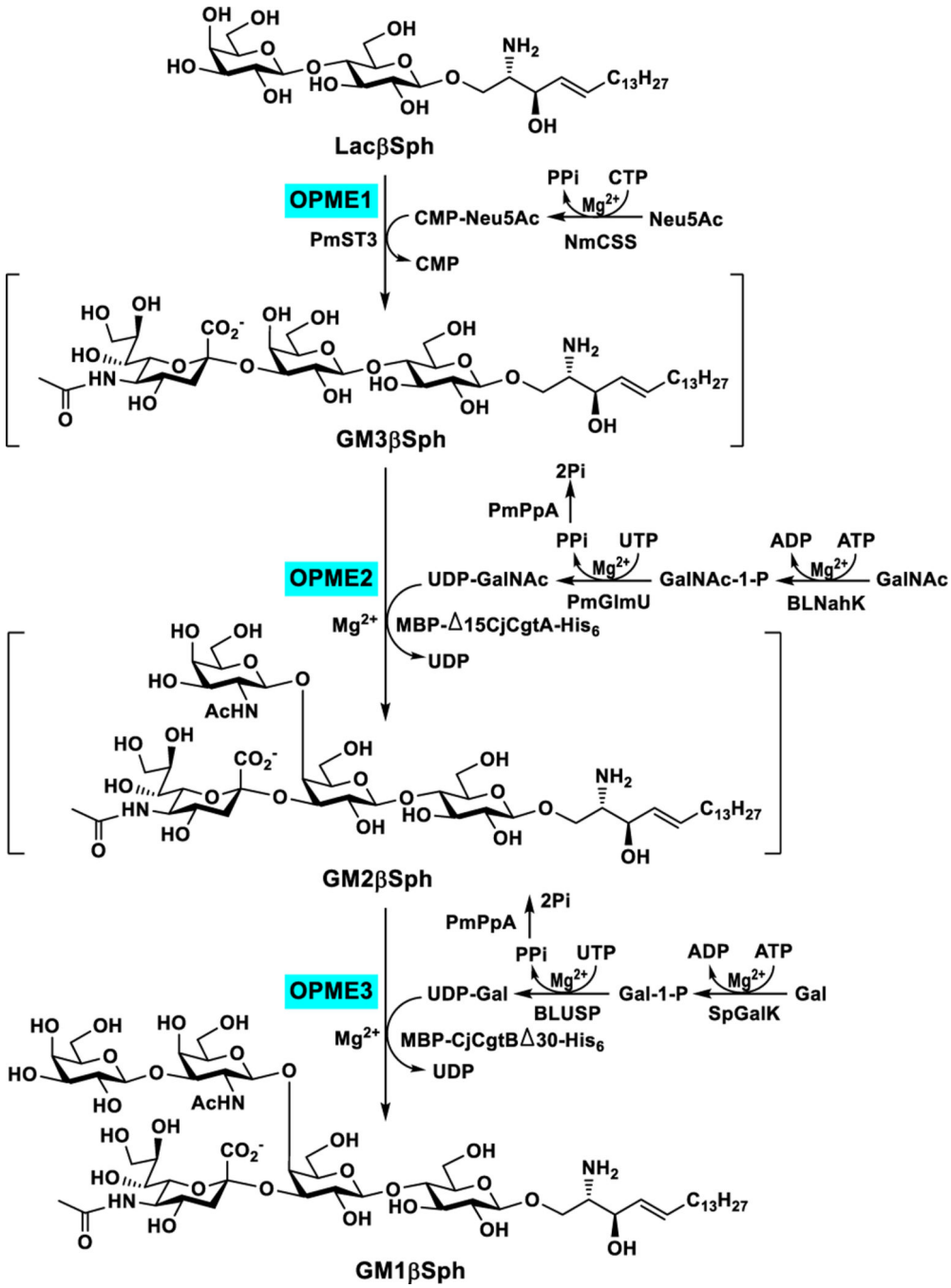
Figure 1.

Structures of GM1 gangliosides containing Neu5Ac (**1**) or Neu5Gc (**2**) with as a representative (d18:1–18:0) ceramide (R) structure.



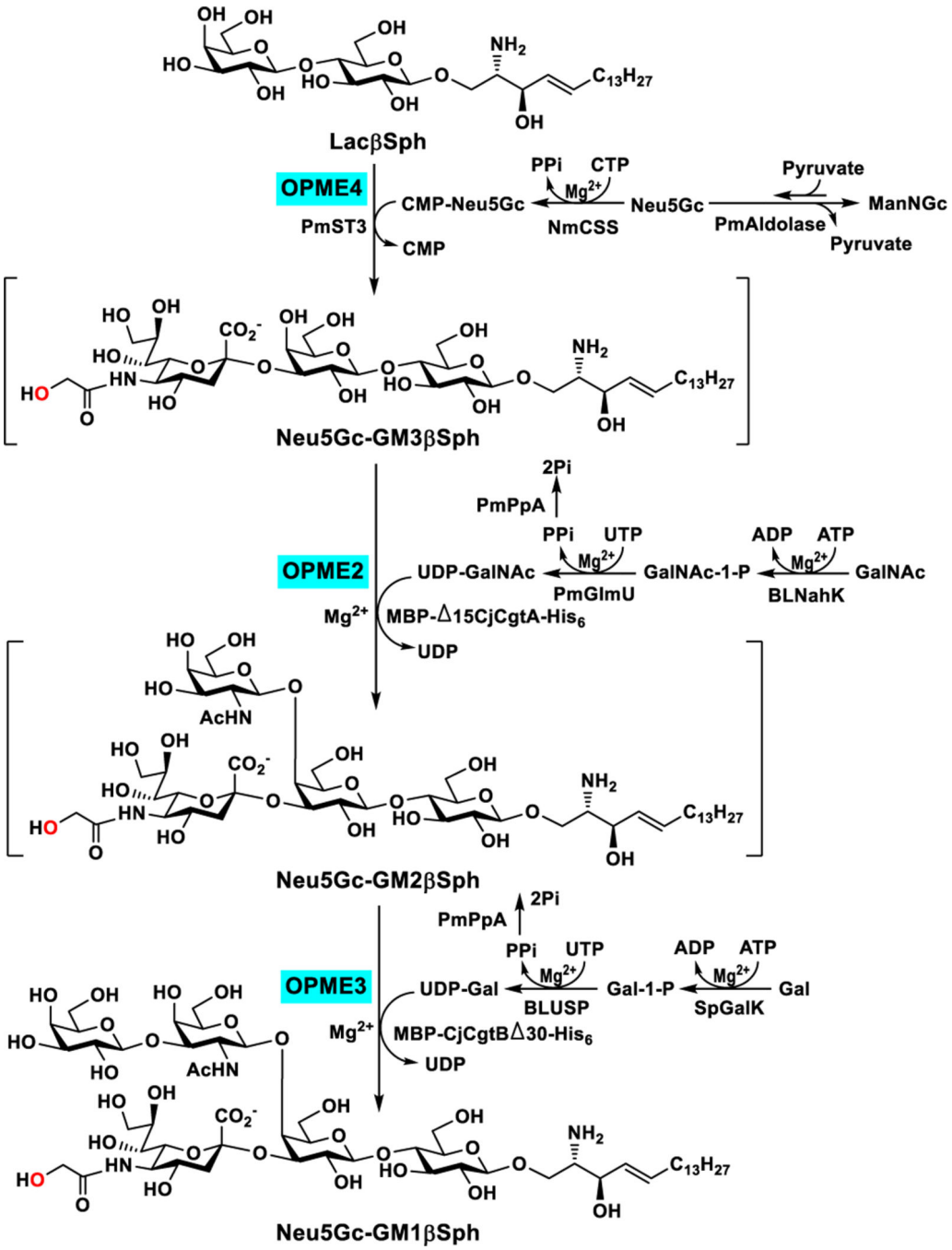
Scheme 1.

Synthesis of lactosylsphingosine (LacβSph) from (*S*)-Garner's aldehyde. *Reagents and conditions*: (a) 1-pentadecyne, $Cp_2Zr(H)Cl$, 25 mol% $ZnBr_2$, THF, r.t., 24 h; (b) BzCl, DMAP, Et_3N, CH_2Cl_2, 0 °C to r.t., 24 h; (c) 2 N HCl/EtOH, 75 °C, 5 h, 60% over three steps; (d) TfN_3, $CuSO_4$, Et_3N, MeOH, CH_2Cl_2, H_2O, r.t., overnight, 86%; (e) $BF_3{\cdot}OEt_2$, CH_2Cl_2, −20 °C to 0 °C, 3 h, 90%; (f) (i) NaOMe, MeOH, r.t., 14 h; (ii) 1,3-propanedithiol, Et_3N, pyridine-water = 1:1 (by volume), 50 °C, 36 h, 91% over two steps.



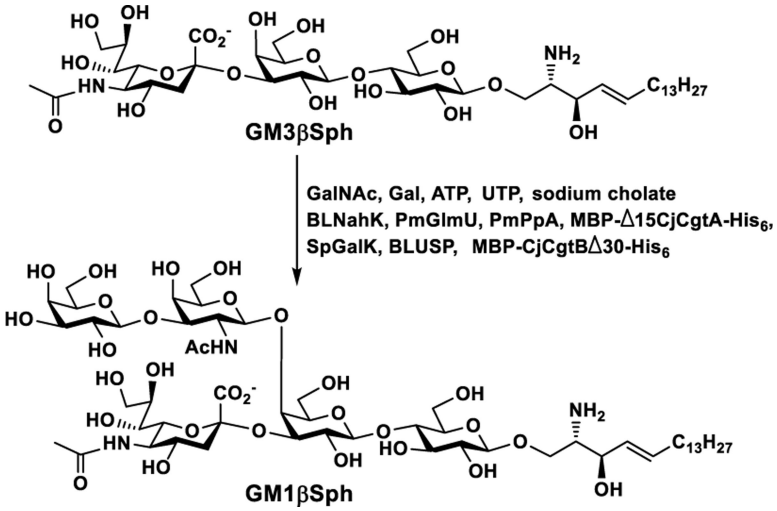
Scheme 2.

Multistep one-pot multienzyme (MSOPME) synthesis of GM1βSph from LacβSph.



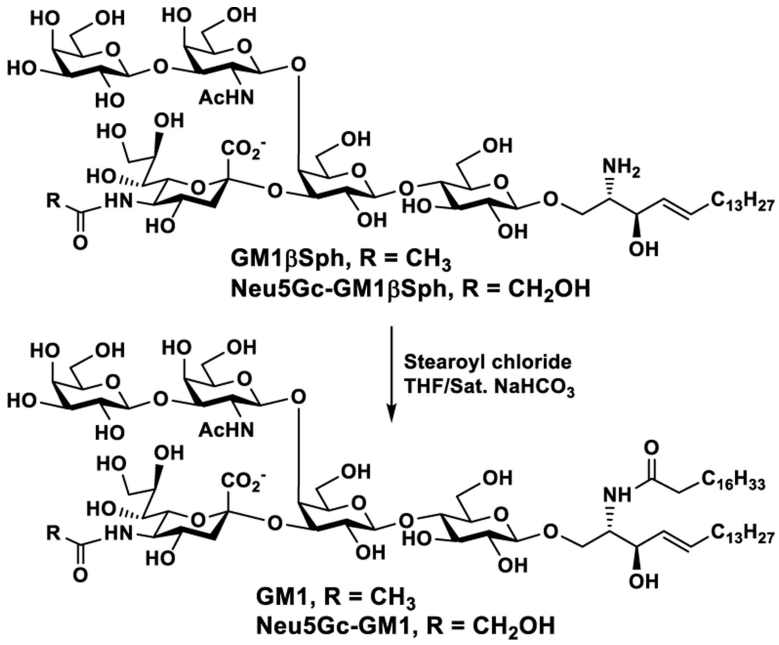
Scheme 3.

Multistep one-pot multienzyme (MSOPME) synthesis of Neu5Gc-GM1βSph from LacβSph and ManNGc.



Scheme 4.

One-step OPME synthesis of GM1βSph from GM3βSph.



Scheme 5.

Synthesis of GM1 gangliosides (d18:1–18:0) containing Neu5Ac or Neu5Gc from the corresponding GM1 sphingosines.