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Chemoenzymatic Synthesis of *N*-Acetyl Analogues of 9-*O*-Acetylated β -Series Gangliosides

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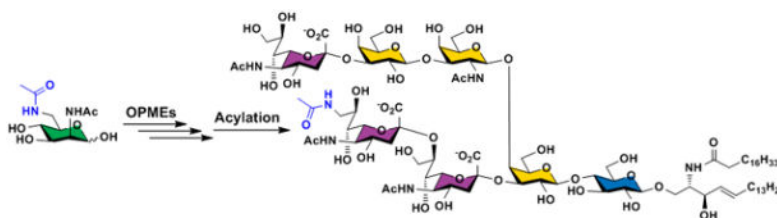
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Abstract

The stable *N*-acetyl analogues of biologically important 9-*O*-acetylated β -series gangliosides including 9NAc-GD3, 9NAc-GD2, 9NAc-GD1b, and 9NAc-GT1b were chemoenzymatically synthesized from a GM3 sphingosine. Two chemoenzymatic methods using either 6-azido-6-deoxy-*N*-acetylmannosamine (ManNAc6N₃) as a chemoenzymatic synthon or 6-acetamido-6-deoxy-*N*-acetylmannosamine (ManNAc6NAc) as an enzymatic precursor for 9-acetamido-9-deoxy-*N*-acetylneuraminic acid (Neu5Ac9NAc) were developed and compared for the synthesis of 9NAc-GD3. The latter method was found to be more efficient and was used to produce the desired 9-*N*-acetylated glycosylsphingosines. Furthermore, glycosylsphingosine acylation reaction conditions were improved to obtain target 9-*N*-acetylated gangliosides in a faster reaction with an easier purification process compared to the previous acylation conditions.

Graphical Abstract



Keywords

β -series ganglioside; chemoenzymatic synthesis; glycosphingolipid; 9-*N*-acetyl sialic acid; *O*-acetyl sialic acid; one-pot multienzyme

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tet.xxx>.

1. Introduction

Gangliosides are biologically important sialic acid (Sia)-containing glycosphingolipids. Those in the ganglio-series containing a disialylated lactosyl ceramide core with an α 2–8-linked terminal sialic acid [1] are called b-series gangliosides which include GD3, GD2, GD1b, GT1b (Figure 1), and more complex GQ1b and GQ1ba. GD3 (also called CD60a [2]) and GD2 are oncofetal markers [3] and are among the four gangliosides (two others are gangliosides GM3 and fucosyl GM1) on the list of prioritized cancer antigens [4, 5] while GD1b and GT1b are among the four most abundant gangliosides in vertebrate brains (two others are a-series gangliosides GM1a, which is also called GM1, and GD1a) [6].

N-Acetylneuraminic acid (Neu5Ac, Figure 1A) is the major sialic acid form in gangliosides [7]. The terminal α 2–8-linked Neu5Ac of the disialylated lactosyl ceramide core in the b-series gangliosides can be modified by *O*-acetylation, with 9-*O*-acetyl Neu5Ac (Neu5,9Ac₂, Figure 1A) as the most common *O*-acetylated form [7]. 9OAc-GD3, which is also called CD60b [2], was shown to be expressed selectively on the neuroepithelial cells of developing rat cerebellum [8], in perinatal rat retina [9, 10], and on human malignant melanoma cells [11, 12]. It was shown to protect cells including glioblastoma cells [13], lymphoblasts [14], and lymphocytes [15] from apoptosis including the apoptosis induced by GD3. On the contrary, it was shown to induce apoptosis in mature erythrocytes in a different report [16]. 9OAc-GD3 has also been shown to be involved in modulating cell motility such as neuronal and tumor cell migration, as well as neurite outgrowth [17, 18].

Different from GD2 which is expressed in peripheral nerve fibers in addition to its cancer cell expression, 9-*O*-acetylated GD2 (9OAc-GD2) is more selectively expressed by cancer cells such as neuroblastoma [19], glioblastoma [20], and breast cancer cells [21]. Therefore, 9OAc-GD2 is predicted to be a more selective anti-cancer immunotherapeutic target than GD2 [22]. A recent mass spectrometry-based profiling approach showed that *O*-acetylation could occur on the inner in addition to the terminal Sia in gangliosides [3].

9OAc-GD1b (also called “neurostatin”) is a potent inhibitor of astroblast and astrocytoma proliferation and also induces necrosis at nanomolar concentrations [23]. More recently, 9OAc-GD1b has been tested to treat neuronal inflammation and has shown strong anti-inflammatory effects even at nanomolar concentrations [24]. 9OAc-GD1b can be potentially used as an acceptor substrate by ST3GAL II [25] to form 9OAc-GT1b.

Despite the recognition of the important functions of *O*-acetylated gangliosides [26, 27], their specific biological roles are not clear. This is mainly due to the instability of Sia *O*-acetyl group towards pH change and/or esterase activities which presents special challenges for elucidating functions and developing applications for structurally complex *O*-acetylated b-series gangliosides.

To address the instability issue of the *O*-acetylated Sias and sialosides, we and others reported previously a chemical biology strategy by substituting the oxygen atom in the ester group of Neu5,9Ac₂ with a nitrogen atom to generate a stable analogue 9-acetamido-9-deoxy-*N*-acetylneuraminic acid (Neu5Ac9NAc) [28–32] (Figure 1A). Here we report the development of efficient approaches for synthesizing Neu5Ac9NAc-containing

b-series gangliosides including 9NAc-GD3, 9NAc-GD2, 9NAc-GD1b, and 9NAc-GT1b (Figure 1B). Two chemoenzymatic methods have been developed and compared for the synthesis of 9NAc-GD3 using either 6-azido-6-deoxy-*N*-acetylmannosamine (ManNAc6N₃) as a chemoenzymatic synthon [33] or 6-acetamido-6-deoxy-*N*-acetylmannosamine (ManNAc6NAc) as an enzymatic precursor for Neu5Ac9NAc. The latter method was found to be more efficient and was used to produce 9NAc-GD3, 9NAc-GD2, 9NAc-GD1b, and 9NAc-GT1b glycosylsphingosines. Furthermore, glycosylsphingosine acylation reaction conditions were improved to obtain target gangliosides in a faster reaction with an easier purification process compared to the previous acylation conditions [5]. The resulting 9NAc-gangliosides can serve as stable analogs of 9-*O*-acetylated b-series gangliosides enabling studies to advance the understanding of the biological roles of ganglioside *O*-acetylation.

2. Results and discussion

2.1. Synthesis of 9NAc-GD3 (6) using 6-azido-6-deoxy-*N*-acetylmannosamine (ManNAc6N₃) as a chemoenzymatic synthon

Initially, we explored the synthesis of 9NAc-GD3 (**1**) from GM3 sphingosine (GM3 β Sph, **5**) [5] via a 9-azido-GD3 (9N₃-GD3, **6**) intermediate using 6-azido-6-deoxy-*N*-acetylmannosamine (ManNAc6N₃) [28] as a chemoenzymatic synthon. As shown in Scheme 1, 9N₃-GD3 glycosylsphingosine (9N₃-GD3 β Sph, **6**) was produced from ManNAc6N₃ and GM3 β Sph (**5**) in the presence of sodium pyruvate and cytidine 5'-triphosphate (CTP) in an efficient one-pot multienzyme (OPME) sialylation system containing *Pasteurella multocida* sialic acid aldolase (PmAldolase) [34], *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS) [35], and *Campylobacter jejuni* α 2-3/8-sialyltransferase (CjCst-II) [36]. We showed previously, when unmodified Neu5Ac or *N*-acetylmannosamine (ManNAc) was used as the donor precursor for CjCstII-catalyzed sialylation, an additional α 2-8-linked Neu5Ac could be added to the desired GD3 β Sph product to form GT3 β Sph as a minor product [5, 37]. In contrast, we found that when ManNAc6N₃ was used as the donor precursor, the undesired additional sialylation did not occur to the target product 9N₃-GD3 β Sph which was obtained with a 65% yield. Acylation of the amino group in 9N₃-GD3 β Sph (**6**) using stearoyl chloride in THF/saturated aqueous NaHCO₃ (1:1, v/v), a condition that we developed previously [5], produced 9N₃-GD3 ganglioside. The 9-N₃ group was then converted to 9-NAc by incubating with thioacetic acid in saturated NaHCO₃ aqueous solution [33] to produce 9NAc-GD3 (**1**) with an overall yield of 86% in two steps. It was found that the reaction converting the azido group in 9N₃-GD3 (**6**) to an acetamido group in 9NAc-GD3 (**1**) was very slow and took three days or longer to complete, which was much longer than the reaction for converting 9-N₃ to 9-NAc on glycans which was completed in 20 h.

2.2. Synthesis of 9NAc-GD3 β Sph (7) using 6-acetamido-6-deoxy-*N*-acetylmannosamine (ManNAc6NAc) as a 9-acetamido-9-deoxy-*N*-acetylneuraminic acid (Neu5Ac9NAc) precursor

Due to the relatively low efficiency in converting the azido group in 9N₃-GD3 to an acetamido group in 9NAc-GD3, we explored the strategy of using ManNAc6NAc [28] as a precursor for enzymatic synthesis of 9NAc-GD3 β Sph (**7**) directly from GM3 β Sph

(5). We previously showed that ManNAc6NAc was a suitable precursor for the synthesis of α 2–3- and α 2–6-linked Neu5Ac9NAc-terminated sialosides using one-pot three-enzyme (OP3E) systems containing a sialic acid aldolase, NmCSS, and a sialyltransferase such as PmST1_M144D and Pd2,6ST, respectively [31]. When ManNAc6NAc was tested in small-scale reactions for the synthesis of 9NAc-GD3 β Sph (7) directly from GM3 β Sph (5) using an OPME sialylation system containing PmAldolase, NmCSS, and CjCst-II [36], the reaction went quite slowly but it was able to be pushed to give satisfied yields by adding additional amounts of CjCst-II and using longer reaction times. Once this was confirmed, preparative-scale synthesis was carried out. As shown in Scheme 2, 9NAc-GD3 β Sph (7) was synthesized from GM3 β Sph (5) as the acceptor substrate and ManNAc6NAc as the donor precursor using the OPME sialylation system containing PmAldolase, NmCSS, and CjCst-II (Figure S1A, ESI). Similar to the case for synthesizing 9N₃-GD3 β Sph (6), no additional sialylation of the desired product 9NAc-GD3 β Sph (7) was observed. The sialylated product was readily purified by passing the concentrated reaction mixture through a C18 cartridge and eluting with a mixed solvent gradient of CH₃CN in water. 9NAc-GD3 β Sph (7) (130 mg, 63%) was eluted first with 40% acetonitrile in water (v/v) and the unreacted GM3 β Sph (5) was eluted with 60% acetonitrile in water (v/v).

2.3. Synthesis of 9NAc-GD2 β Sph (8) from 9NAc-GD3 β Sph (7) using a one-pot multienzyme (OPME) GalNAc activation and transfer system

With 9NAc-GD3 β Sph (7) in hand, the synthesis of 9NAc-GD2 β Sph (8) was accomplished using an OPME β 1–4-GalNAc activation and glycosylation reaction containing *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-15CjCgtA-His₆ which was a recombinant *Campylobacter jejuni* β 1–4-*N*-acetylgalactosaminyltransferase designed with improved *E. coli* expression and increased stability [41]. As shown in Scheme 2, incubating 9NAc-GD3 β Sph (7), *N*-acetylgalactosamine (GalNAc), adenosine 5'-triphosphate (ATP), and uridine 5'-triphosphate (UTP) in the reaction mixture containing an anionic detergent sodium cholate [41] at 30 °C produced 9NAc-GD2 β Sph (8) smoothly (Figure S1B, ESI). Sodium cholate was shown to increase the accessibility of ganglioside substrates by different enzymes, most likely by dispersing gangliosides molecules to decrease steric hindrance [42–44]. Indeed, similar to what we observed previously [41], the presence of sodium cholate greatly improved the enzyme properties and the OPME reaction was completed in 16 h. Pure 9NAc-GD2 β Sph (8) (56 mg, 97% yield) was readily obtained by removing sodium cholate using a silica gel column chromatography followed by passing the concentrated product-containing fractions through a C18 cartridge and eluting with 30% CH₃CN in water.

2.4. Synthesis of 9NAc-GD1b β Sph (9) from 9NAc-GD3 β Sph (7) using a multistep one-pot multienzyme (MSOPME) strategy

From 9NAc-GD3 β Sph (7), a recently developed multistep one-pot multienzyme (MSOPME) strategy was used to directly access 9NAc-GD1b β Sph (9). In the first step, 9NAc-GD2 β Sph (8) was formed from 9NAc-GD3 β Sph (7) as described above in section 2.3. When the reaction went to completion, the reaction mixture was used directly in the second step

without purification to produce 9NAc-GD1b β Sph (**9**) using an OPME β 1–3-galactosylation system by adding galactose (Gal), ATP, UTP, and four enzymes including *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK) [45], *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP) [46], PmPpA, and MBP-CjCgtB 30-His₆ which was a recombinant *Campylobacter jejuni* β 1–3-galactosyltransferase designed with improved *E. coli* expression and increased stability [41]. The second OPME glycosylation was completed at 30 °C in 20 h (Figure S1C, ESI). The desired pure 9NAc-GD1b β Sph (**9**) (123 mg) was obtained in 96% yield after removing sodium cholate using a silica gel column chromatography followed by purification using a C18 cartridge.

2.5. Synthesis of 9NAc-GT1b β Sph (**10**) from 9NAc-GD1b β Sph (**9**) using an OPME sialylation system

To synthesize 9NAc-GT1b β Sph (**10**) from 9NAc-GD1b β Sph (**9**), a suitable α 2–3-sialyltransferase was needed. *Pasteurella multocida* α 2–3-sialyltransferases PmST1 [47] and PmST3 [48] had no or very low activity on sialylating the terminal Gal in 9NAc-GD1b β Sph (**9**). We previously cloned a recombinant human ST3GAL II (MBP- 27hST3GAL II-His₆) which was successfully expressed in *E. coli*, purified, and used in the synthesis of GT1b glycan from GD1b glycan [49]. Its low expression level and less efficiency in using glycosylsphingosines as acceptors compared to the corresponding glycans, however, limited its application in the synthesis of 9NAc-GT1b β Sph (**10**). To our delight, we found that MBP-CjCst-I 145-His₆, a recombinant *Campylobacter jejuni* α 2–3-sialyltransferase that we cloned recently and used in sialylating glycoprotein N-glycans [50], showed very good activity towards sialylating GD1b sphingosine and glycan (data not shown). Compared to the recombinant MBP- 27hST3GAL II-His₆ expressed in *E. coli* which has an expression level of less than 1 mg (~9 mU) per liter LB culture [49], MBP-CjCst-I 145-His₆ has a much higher expression level (60 mg purified enzyme from per liter LB culture) [50]. Preparative-scale synthesis of 9NAc-GT1b β Sph (**10**) was achieved efficiently from 9NAc-GD1b β Sph (**9**) and Neu5Ac in the presence of CTP using an OPME sialylation reaction containing NmCSS and MBP-CjCst-I 145-His₆. The reaction was completed in 15 h (Figure S1D, ESI) and pure 9NAc-GT1b β Sph (**10**) (58 mg, 97%) was obtained after purification via a C18 cartridge by eluting with 20% of CH₃CN in water.

2.6. Improved acylation conditions for converting glycosylsphingosines to glycosphingolipids

With 9-*N*-acetylated GD3 β Sph, GD2b β Sph, GD1b β Sph, and GT1b β Sph (**7–10**) in hand, the final step for the formation of target 9-*N*-acetylated GD3, GD2b, GD1b, and GT1b (**1–4**) was acylation. The acylation conditions that we developed previously using THF/saturated aqueous NaHCO₃ [5] led to high yields (97–100%). Nevertheless, a large amount of the salt was introduced to the reaction mixture which formed a two-phase mixture. The reaction conditions had room for improvement, especially for large-scale reactions. Using GM3 β Sph (**5**) as a model substrate, several acylation conditions were compared, including THF/saturated NaHCO₃ (1:1 by volume) [5], THF/50 mM carbonate buffer (pH 9.2) (1:1 by volume), THF/100 mM CAPS buffer (pH 9.5) (1:1 by volume), and THF/1% Na₂CO₃ (1:1 by volume). The reaction mixtures were analyzed by thin-layer chromatography (Figure

S2, ESI). Among these conditions, THF/1% Na₂CO₃ (1:1 by volume) was identified as the optimal condition, with which the acylation rate was the fastest and the reaction for the formation of GM3 was completed in one hour. Furthermore, the reaction mixture was a homogeneous solution without any undissolved residues. Using the improved acylation reaction conditions (stearoyl chloride in THF/1% Na₂CO₃), the production of target gangliosides 9NAc-GD3 (**1**), 9NAc-GD2 (**2**), 9NAc-GD1b (**3**), and 9NAc-GT1b (**4**) (d18:1–18:0) was achieved by installing a stearoyl chain to the amino group in the corresponding glycosylsphingosines. The acylation reaction progress was monitored by HRMS and additional amounts of stearoyl chloride were added during the acylation reaction when needed. The reactions for the production of more complex glycosphingolipids such as 9NAc-GD1b (**3**) and 9NAc-GT1b (**4**) took longer time (3–4 h) to complete compared to less complex glycosphingolipids such as 9NAc-GD3 (**1**), 9NAc-GD2 (**2**) (reactions were completed in 2 h). The desired products were purified using C18 cartridges.

3. Conclusions

In conclusion, we have developed and identified an efficient chemoenzymatic approach for synthesizing 9-*N*-acetylated b-series gangliosides 9NAc-GD3 (**1**), 9NAc-GD2 (**2**), 9NAc-GD1b (**3**), and 9NAc-GT1b (**4**) from GM3 sphingosine (GM3βSph, **5**) with ManNAc6NAc as the donor precursor for Neu5Ac9NAc. The 9-*N*-acetylated b-series gangliosides are stable analogues of the corresponding labile 9-*O*-acetylated counterparts which play important biological and pathological roles. The chemoenzymatic strategy reported here can be generally applied to access *N*-acetyl analogues of other gangliosides or glycans containing *O*-acetyl sialic acids.

4. Experimental section

4.1. Materials and general methods

D-Galactose (Gal) was purchased from Fisher Scientific (Hampton, New Hampshire, USA). D-GalNAc was from Carbosynth US. Neu5Ac was bought from Ningbo Hongxiang Bio-chem Co., Ltd. (Ningbo, China). UTP, CTP, and ATP were from Hangzhou Meiya Pharmacy (Hangzhou, China). Recombinant enzymes *Pasteurella multocida* sialic acid aldolase (PmAldolase) [34], *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS) [35], *Campylobacter jejuni* multifunctional α2–3/8-sialyltransferase (CjCst-II) [36], *Bifidobacterium longum* strain ATCC55813 *N*-acetylhexosamine-1-kinase (BLNahK) [38], *Pasteurella multocida* *N*-acetylglucosamine uridylyltransferase (PmGlmU) [39], *Pasteurella multocida* inorganic pyrophosphatase (PmPpA) [40], *Campylobacter jejuni* β1–4-*N*-acetylgalactosaminyltransferase MBP-15CjCgtA-His₆ [41], *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK) [45], *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP) [46], *Campylobacter jejuni* β1–3-galactosyltransferase MBP-CjCgtB-30-His₆ [41], and *Campylobacter jejuni* α2–3-sialyltransferase MBP-CjCst-I-145-His₆ [50] were expressed and purified as described previously.

4.2. OPME synthesis of 9N₃-GD3βSph (**5**): (5-Acetamido-9-azido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→8)-(5-acetamido-3,5-dideoxy-D-

glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 3)- β -D-galactopyranosyl-(1 \rightarrow 4)- β -D-glucopyranosyl-(1 \rightarrow 1)-(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol

A reaction mixture in Tris-HCl buffer (100 mM, pH 8.5) in a total volume of 5 mL containing GM3 β Sph (**5**) (30 mg, 0.032 mmol), ManNAc6N₃ (16 mg, 0.065 mmol), sodium pyruvate (25 mg, 0.227 mmol), CTP (37 mg, 0.070 mmol), MgCl₂ (20 mM), PmAldolase (1.5 mg), NmCSS (0.8 mg), and CjCst-II (2 mg) was incubated in an incubator shaker at 30 °C with agitation at 100 rpm. The product formation was monitored by HRMS. After 24 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 via a preconditioned Discovery DSC-18 SPE cartridge (bed wt. 10 g). After the sample was loaded, the C18 cartridge was washed with water (30 mL), and the 9N₃-GD3 β Sph was eluted with 40% acetonitrile in water (v/v). The unreacted GM3 β Sph was eluted with 60% acetonitrile in water (v/v). The fractions containing the pure product were collected, concentrated, and lyophilized to produce the desired pure 9N₃-GD3 β Sph (**6**) (27 mg, 65% yield). ¹H NMR (600 MHz, CD₃OD) δ 5.75 (dt, *J* = 14.1, 6.9 Hz, 1H), 5.49 (dd, *J* = 15.4, 7.6 Hz, 1H), 4.48 (d, *J* = 7.9 Hz, 1H), 4.34 (d, *J* = 7.8 Hz, 1H), 4.19–3.33 (m, 40H), 3.04–2.64 (m, 3H), 2.09 (q, *J* = 7.4 Hz, 2H), 2.03 (d, *J* = 2.2 Hz, 8H), 1.73 (q, *J* = 11.9, 11.1 Hz, 2H), 1.30 (d, *J* = 14.8 Hz, 31H), 0.90 (t, *J* = 7.0 Hz, 3H). ¹³C NMR (150 MHz, CD₃OD) δ 174.16, 173.57, 173.29, 134.95, 134.30, 129.52, 103.35, 102.64, 101.58, 99.98, 79.26, 75.42, 75.31, 75.05, 74.57, 73.72, 73.35, 73.27, 73.11, 72.43, 70.77, 70.48, 69.62, 69.46, 68.10, 67.94, 67.10, 63.00, 61.88, 61.41, 54.79, 53.44, 52.73, 52.49, 41.51, 41.16, 32.06, 31.67, 29.39, 29.36, 29.28, 29.24, 29.07, 29.04, 28.99, 28.93, 22.34, 21.64, 21.26, 13.07. HRMS (ESI-Orbitrap) *m/z*: [M-H]⁻ calculated for C₅₂H₈₉N₆O₂₇ 1229.5781; found 1229.5776.

4.3. Conversion of 9N₃-GD3 β Sph (6**) to 9NAc-GD3 (**1**)**

To a solution of 9N₃-GD3 β Sph (**6**) (15 mg) in sat. NaHCO₃-THF (2 mL, 2:1), stearoyl chloride (1.5 eq) in 0.5 mL of THF was added. The resulting mixture was stirred vigorously at room temperature for 2 h. An additional amount of stearoyl chloride (1.0 eq) in THF was added and the reaction was stirred for another 1.5 h. The product was purified via a preconditioned Discovery DSC-18 SPE cartridge (bed wt. 10 g). After the sample was loaded, the C18 cartridge was washed with water (30 mL), and the ganglioside product was eluted using a solution of 60% acetonitrile in water. The fractions contained pure target compounds were collected, concentrated, and lyophilized. The obtained 9N₃-GD3 was dissolved in sodium bicarbonate saturated solution in water (3 mL), thioacetic acid (10 equiv.) was added, and the reaction was stirred at 70 °C for 20 h. An additional amount of thioacetic acid (5 equiv.) was added every 10 h. After three days, HRMS analysis indicated the completion of the reaction. The solvent was concentrated and purified via a preconditioned Discovery DSC-18 SPE cartridge (bed wt. 10 g) to produce the desired 9NAc-GD3 (**1**) (15 mg, 86%).

4.4. OPME synthesis of 9NAc-GD3 β Sph (7**): (5,9-Diacetamido-3,5,9-trideoxy-D-glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 8)-(5-acetamido-3,5-dideoxy-D-**

glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 3)- β -D-galactopyranosyl-(1 \rightarrow 4)- β -D-glucopyranosyl-(1 \rightarrow 1)-(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol

A reaction mixture in Tris-HCl buffer (100 mM, pH 8.5) in a total volume of 16 mL containing GM3 β Sph (**5**) (150 mg, 0.16 mmol), ManNAc6NAc (84 mg, 0.32 mmol), sodium pyruvate (211 mg, 1.92 mmol), CTP (202 mg, 0.38 mmol), MgCl₂ (20 mM), PmAldolase (3.2 mg), NmCSS (1.6 mg), and CjCst-II (4 mg) was incubated in an incubator shaker at 30 °C with agitation at 100 rpm. The product formation was monitored by HRMS. After 20 h, an additional amount of CjCstII (4 mg) was added. After three days, 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 via a preconditioned Discovery DSC-18 SPE cartridge (bed wt. 10 g). After the sample was loaded, the C18 cartridge was washed with water (30 mL), and 9NAc-GD3 β Sph (**7**) was eluted with 40% acetonitrile in water (v/v). Unreacted GM3 β Sph (**5**) was eluted with 60% acetonitrile in water (v/v). The fractions containing the pure product were collected, concentrated, and lyophilized to produce the desired pure 9NAc-GD3 β Sph (**7**) (130 mg, 63% yield). ¹H NMR (800 MHz, CD₃OD) δ 5.81 (dt, J = 14.4, 6.4 Hz, 1H), 5.50 (dd, J = 15.2, 6.4 Hz, 1H), 4.50 (d, J = 8.0 Hz, 1H), 4.35 (d, J = 8.0 Hz, 1H), 4.24–3.12 (m, 29H), 2.93 (dd, J = 12.8, 4.8 Hz, 1H), 2.73 (dd, J = 12.8, 4.8 Hz, 1H), 2.10 (q, J = 7.2 Hz, 2H), 2.04 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.73–1.70 (m, 2H), 1.47–1.15 (m, 24H), 0.90 (t, J = 7.2 Hz, 3H). ¹³C NMR (200 MHz, CD₃OD) δ 173.98, 173.63, 173.29, 173.15, 172.27, 134.64, 128.34, 103.44, 102.59, 101.06, 100.09, 79.22, 77.01, 75.45, 75.09, 74.64, 74.10, 73.21, 73.03, 71.51, 70.58, 69.68, 69.42, 68.90, 68.25, 68.08, 67.28, 61.99, 61.41, 60.39, 55.07, 52.80, 52.50, 42.69, 41.40, 40.90, 32.04, 31.68, 29.41, 29.37, 29.33, 29.26, 29.08, 29.07, 29.02, 28.93, 22.34, 21.67, 21.32, 21.29, 21.27, 13.08, 13.07. HRMS (ESI-Orbitrap) m/z : [M-H]⁻ calculated for C₅₄H₉₃N₄O₂₈ 1245.5982; found 1245.5957.

4.5. OPME synthesis of 9NAc-GD2 β Sph

(8): 2-Acetamido-2-deoxy- β -D-galactopyranosyl-(1 \rightarrow 4)-(5,9-diacetamido-3,5,9-trideoxy-D-glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 8)-(5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 3)- β -D-galactopyranosyl-(1 \rightarrow 4)- β -D-glucopyranosyl-(1 \rightarrow 1)-(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol

9NAc-GD3 β Sph (**7**) (50 mg, 0.039 mmol), GalNAc (13 mg, 0.059 mmol), ATP (36 mg, 0.066 mmol), and UTP (35 mg, 0.066 mmol) were incubated in 5 mL of Tris-HCl buffer (100 mM, pH 7.5) containing BLNahK (1.2 mg), PmGlmU (1.3 mg), MBP-15CjCgtA-His₆ (2 mg), PmPpA (1.0 mg), MgCl₂ (20 mM), and sodium cholate (10 mM). The reaction was carried out by incubating the solution in an incubator shaker at 30 °C for 16 h with agitation at 100 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 was purified by a 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 to elute the product. The fractions containing the product were collected and concentrated. The residue was dissolved in ddH₂O (1 mL) and loaded to a preconditioned Discovery DSC-18 SPE cartridge (bed wt. 10 g). After washed with water

(30 mL), a mixed solvent of acetonitrile in water (30%) was used as an eluent to obtain pure 9NAc-GD2 β Sph (**8**) (56 mg, 97 %) as a white powder. ^1H NMR (600 MHz, CD_3OD) δ 5.88 (dt, J = 14.4, 6.4 Hz, 1H), 5.50 (dd, J = 15.2, 6.4 Hz, 1H), 4.47 (d, J = 8.0 Hz, 1H), 4.36 (d, J = 8.0 Hz, 2H), 4.27–3.25 (m, 35H), 2.86 (dd, J = 12.8, 4.8 Hz, 1H), 2.75 (dd, J = 12.8, 4.8 Hz, 1H), 2.10 (q, J = 7.2 Hz, 2H), 2.05 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.80–1.74 (m, 2H), 1.51–1.20 (m, 24H), 0.90 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, CD_3OD) δ 173.97, 173.50, 173.43, 173.22, 172.86, 172.26, 135.13, 126.94, 103.57, 102.97, 102.43, 101.06, 100.52, 79.05, 77.07, 76.68, 75.20, 74.91, 74.60, 74.39, 74.03, 73.17, 73.05, 72.63, 70.66, 69.55, 69.47, 68.56, 68.35, 68.22, 65.78, 61.96, 61.37, 61.23, 60.38, 60.08, 59.76, 55.39, 53.23, 52.87, 52.62, 42.76, 40.78, 40.06, 31.98, 31.66, 29.38, 29.34, 29.23, 29.05, 29.02, 28.81, 22.32, 22.25, 21.75, 21.30, 21.29, 13.04. HRMS (ESI-Orbitrap) m/z : $[\text{M}-2\text{H}]^{2-}$ calculated for $\text{C}_{62}\text{H}_{107}\text{N}_5\text{O}_{33}$ 723.8351; found 723.8365.

4.6. MSOPME synthesis of 9NAc-GD1 β Sph

(9): β -D-Galactopyranosyl-(1 \rightarrow 3)-(2-acetamido-2-deoxy- β -D-galactopyranosyl)-(1 \rightarrow 4)-(5,9-diacetamido-3,5,9-trideoxy-D-glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 8)-(5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 3)- β -D-galactopyranosyl-(1 \rightarrow 4)- β -D-glucopyranosyl-(1 \rightarrow 1)-(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol

A reaction mixture in Tris-HCl buffer (100 mM, pH 7.5) in a total volume of 8 mL containing 9NAc-GD3 β Sph (**7**) (100 mg, 0.077 mM), GalNAc (26 mg, 0.116 mmol), ATP (156 mg, 0.132 mmol), UTP (70 mg, 0.132 mmol), BLNahK (2.0 mg), PmGlmU (2.0 mg), MBP-15CjCgtA-His₆ (3.0 mg), PmPpA (1.5 mg), MgCl_2 (20 mM), and sodium cholate (10 mM) was incubated at 30 °C with agitation at 100 rpm. The formation of the product 9NAc-GD2 β Sph (**8**) was monitored by HRMS and 9NAc-GD3 β Sph (**7**) was completely consumed after 16 h. In the same reaction container without workup or purification, galactose (21 mg, 0.116 mmol), ATP (73 mg, 0.132 mmol), and UTP (70 mg, 0.132 mmol), SpGalK (1.5 mg), BLUSP (2.0 mg), MBP-CjCgtB-30-His₆ (3.0 mg), and PmPpA (1.0 mg) were added. The reaction mixture with a total volume of 11 mL was incubated at 30 °C for 20 h with agitation at 100 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 a 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 to elute the product. The fractions containing the product were collected and concentrated. The residue was dissolved in ddH₂O (1 mL) and loaded to a preconditioned Discovery DSC-18 SPE cartridge (bed wt. 10 g). After washed with water (30 mL), a mixed solvent of acetonitrile in water (30%) was used as an eluent to obtain pure 9NAc-GD1 β Sph (**9**) (123 mg, 96 %) as a white powder. ^1H NMR (600 MHz, CD_3OD) δ 5.88 (dt, J = 14.4, 6.4 Hz, 1H), 5.50 (dd, J = 15.2, 6.4 Hz, 1H), 4.46–4.34 (m, 4H), 4.27–3.23 (m, 41H), 2.86 (dd, J = 12.8, 4.8 Hz, 1H), 2.71 (dd, J = 12.8, 4.8 Hz, 1H), 2.11 (q, J = 7.2 Hz, 2H), 2.03 (s, 3H), 2.01 (s, 6H), 1.99 (s, 3H), 1.87–1.71 (m, 2H), 1.49–1.22 (m, 24H), 0.90 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, CD_3OD) δ 174.00, 173.47, 173.08, 172.29, 135.16, 126.94, 105.09, 103.66, 102.98, 102.38, 100.92, 81.00, 79.10, 76.80, 75.19, 74.74, 74.61, 74.39, 73.98, 73.15, 71.13, 70.67, 69.48,

68.92, 68.31, 68.20, 65.76, 61.96, 61.26, 61.14, 60.35, 60.09, 59.74, 55.35, 52.91, 52.66, 51.56, 42.74, 40.47, 39.76, 32.00, 31.95, 31.67, 29.39, 29.36, 29.30, 29.25, 29.19, 29.07, 29.03, 28.87, 28.81, 22.44, 22.33, 21.76, 21.32, 13.06. HRMS (ESI-Orbitrap) m/z : $[M-2H]^{2-}$ calculated for $C_{68}H_{117}N_5O_{38}$ 804.8616; found 804.8634.

4.7. OPME synthesis of 9NAc-

GT1b β Sph (10): (5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 3)- β -D-Galactopyranosyl-(1 \rightarrow 3)-(2-acetamido-2-deoxy- β -D-galactopyranosyl)-(1 \rightarrow 4)-(5,9-diacetamido-3,5,9-trideoxy-D-glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 8)-(5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 3)- β -D-galactopyranosyl-(1 \rightarrow 4)- β -D-glucopyranosyl-(1 \rightarrow 1)-(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol

9NAc-GD1b β Sph (**9**) (50 mg, 0.030 mmol), Neu5Ac (14 mg, 0.045 mmol), and CTP (27 mg, 0.051 mmol) were incubated at 37 °C in a Tris-HCl buffer (6 mL, 100 mM, pH 8.5) containing MgCl₂ (20 mM), NmCSS (0.8 mg), and MBP-CjCst-I 145-His₆ (2 mg). The reaction mixture was incubated in an incubator shaker at 30 °C for 15 h with agitation at 100 rpm. The product formation was monitored by HRMS. Upon completion, 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 via a preconditioned Discovery DSC-18 SPE cartridge (bed wt. 10 g). After the sample was loaded, the C18 cartridge was washed with water (30 mL), a mixed solvent of acetonitrile in water (20%) was then used as an eluent to obtain pure 9NAc-GT1b β Sph (**10**) (58 mg, 98 %) as a white powder. ¹H NMR (600 MHz, CD₃OD) δ 5.86 (dt, J = 14.4, 6.4 Hz, 1H), 5.50 (dd, J = 15.2, 6.4 Hz, 1H), 4.51 (d, J = 8.0 Hz, 1H), 4.46 (d, J = 8.0 Hz, 1H), 4.35 (d, J = 8.0 Hz, 1H), 4.28 (s, 1H), 4.24–3.21 (m, 48H), 2.94–2.66 (m, 3H), 2.11 (q, J = 7.2 Hz, 2H), 2.04 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.79–1.71 (m, 3H), 1.49–1.22 (m, 24H), 0.90 (t, J = 7.2 Hz, 3H). ¹³C NMR (150 MHz, CD₃OD) δ 174.05, 173.48, 173.42, 173.02, 172.26, 134.90, 127.41, 104.58, 103.51, 102.93, 102.52, 101.02, 100.41, 99.68, 76.78, 76.13, 75.21, 74.60, 74.33, 73.83, 73.49, 73.17, 71.51, 70.54, 70.10, 69.65, 69.31, 68.52, 68.30, 68.07, 67.96, 67.54, 66.57, 62.95, 62.06, 61.30, 60.63, 60.34, 55.22, 52.93, 52.55, 51.36, 42.62, 40.89, 32.04, 31.68, 29.41, 29.37, 29.33, 29.27, 29.08, 29.06, 28.88, 22.59, 22.34, 21.79, 21.41, 21.36, 13.08. HRMS (ESI-Orbitrap) m/z : $[M-2H]^{2-}$ calculated for $C_{79}H_{134}N_6O_{46}$ 950.4093; found 950.4108.

4.8. Optimizing the acylation reaction conditions for the formation of gangliosides from sphingosines using GM3 β Sph as a model substrate

Acylation reaction assays were performed at room temperature for 1 h in a 3 mL of solution containing GM3Sph (2 mg) and stearoyl chloride (1.5 equiv.). Solution used were: THF/sat NaHCO₃; THF/50 mM carbonate buffer (pH 9.2); THF/100 mM CAPS buffer (pH9.5); and THF/1% Na₂CO₃. The reactions were analyzed by thin-layer chromatography. Developing solvent used was *i*-PrOH:H₂O:NH₄OH = 10:2:1.

4.9. General procedures for converting glycosylsphingosines (7–10) to gangliosides (1–4)

To a solution of a glycosylsphingosine selected from 7–10 (15–20 mg) in THF/1% Na₂CO₃ (3 mL, 1:1), stearoyl chloride (1.5 eq) in 0.1 mL of THF was added. The resulting mixture was stirred vigorously at room temperature for 1.5 h. The solution was then concentrated and dissolved in ddH₂O (2 mL). The product formation was monitored by HRMS. An additional amount of stearoyl chloride (0.5 eq) in THF was added. The reaction was completed in 2 h for 9NAc-GD3βSph (7) and 9NAc-GD2βSph (8), and in 4 h for 9NAc-GD1bβSph (9) and 9NAc-GT1bβSph (10). The product was purified via a preconditioned Discovery DSC-18 SPE cartridge (bed wt. 10 g). After the sample was loaded, the C18 cartridge was washed with water (30 mL), and the ganglioside product was eluted using a solution of 40–80% acetonitrile in water. The product was further purified by a silica gel column chromatography using chloroform:methanol = 5:2 (by volume) then chloroform:methanol:water = 5:4:1 (by volume) as an eluant.

4.10. Compounds

4.10.1. 9NAc-GD3 (1)—The product 9NAc-GD3 (1) was eluted from the C18 cartridge using 50% CH₃CN in water. The pure product (24 mg, 99% yield) was a white powder. ¹H NMR (800 MHz, CD₃OD) δ 5.68 (dt, *J* = 15.2, 6.4 Hz, 1H), 5.44 (dd, *J* = 15.2, 8.0 Hz, 1H), 4.49 (d, *J* = 8.0 Hz, 1H), 4.31 (d, *J* = 8.0 Hz, 1H), 4.24–3.28 (m, 23H), 3.21 (dd, *J* = 12.6, 8.0 Hz, 1H), 2.92 (dd, *J* = 12.0, 4.0 Hz, 1H), 2.73 (dd, *J* = 12.0, 4.0 Hz, 1H), 2.16 (t, *J* = 7.2 Hz, 2H), 2.02 (s, 3H, CH₃), 2.01 (s, 3H, CH₃), 1.99 (s, 3H, CH₃), 1.72–1.67 (m, 2H), 1.62–1.55 (m, 2H), 1.41–1.21 (m, 50H), 0.90 (t, *J* = 7.2 Hz, 6H). ¹³C NMR (200 MHz, CD₃OD) δ 174.53, 173.95, 173.61, 173.22, 172.30, 133.65, 130.00, 103.45, 103.04, 100.81, 100.08, 79.35, 76.99, 75.44, 75.37, 75.00, 74.75, 74.13, 73.44, 72.98, 71.55, 70.66, 69.55, 69.45, 68.54, 68.31, 68.26, 67.20, 62.10, 61.41, 60.33, 53.29, 52.80, 52.49, 42.76, 41.46, 40.84, 37.96, 35.98, 32.08, 31.71, 31.69, 29.49, 29.46, 29.44, 29.43, 29.41, 29.39, 29.38, 29.35, 29.32, 29.28, 29.25, 29.12, 29.09, 29.07, 29.04, 26.45, 25.78, 22.83, 22.36, 22.35, 22.33, 21.60, 21.19, 13.07, 13.06, 13.03. HRMS (ESI-Orbitrap) *m/z*: [M-2H]²⁻ calculated for C₇₂H₁₂₈N₄O₂₉ 755.4259; found 755.4267.

4.10.2. 9NAc-GD2 (2)—The product 9NAc-GD2 (2) was eluted from the C18 cartridge using 40% CH₃CN in water. The pure product (22 mg, 97% yield) was a white powder. ¹H NMR (600 MHz, CD₃OD) δ 5.69 (dt, *J* = 14.4, 6.4 Hz, 1H), 5.44 (dd, *J* = 15.2, 6.4 Hz, 1H), 4.43 (d, *J* = 8.0 Hz, 1H), 4.30 (d, *J* = 8.0 Hz, 1H), 4.19–3.25 (m, 36H), 2.85 (dd, *J* = 12.8, 4.8 Hz, 1H), 2.71 (dd, *J* = 12.8, 4.8 Hz, 1H), 2.19–2.04 (m, 4H), 2.04 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H), 1.99 (s, 3H), 1.83–1.68 (m, 2H), 1.61–1.20 (m, 52H), 0.90 (t, *J* = 7.2 Hz, 6H). ¹³C NMR (150 MHz, CD₃OD) δ 174.52, 173.94, 173.40, 172.34, 133.62, 130.01, 103.64, 103.09, 102.91, 79.45, 78.06, 76.71, 75.06, 74.90, 74.69, 74.34, 73.46, 73.12, 72.60, 71.54, 70.59, 69.47, 68.54, 68.20, 62.14, 61.19, 60.29, 53.30, 53.23, 52.63, 48.17, 42.73, 39.79, 35.98, 32.08, 31.70, 31.68, 29.49, 29.45, 29.43, 29.41, 29.37, 29.31, 29.24, 29.18, 29.11, 29.08, 29.04, 25.78, 22.36, 22.34, 22.17, 21.66, 21.22, 13.06. HRMS (ESI-Orbitrap) *m/z*: [M-2H]²⁻ calculated for C₈₀H₁₄₁N₅O₃₄ 856.9656; found 856.9665.

4.10.3. 9NAc-GD1b (3)—The product 9NAc-GD1b (3) was eluted from the C18 cartridge using 40% CH₃CN in water. The pure product (22 mg, 97% yield) was a white

powder. ^1H NMR (600 MHz, CD_3OD) δ 5.68 (dt, J = 14.4, 6.4 Hz, 1H), 5.44 (dd, J = 15.2, 6.4 Hz, 1H), 4.43 (d, J = 8.0 Hz, 2H), 4.29 (d, J = 8.0 Hz, 1H), 4.19–3.26 (m, 42H), 2.85 (dd, J = 12.8, 4.8 Hz, 1H), 2.67 (dd, J = 12.8, 4.8 Hz, 1H), 2.19–2.14 (m, 4H), 2.03 (s, 3H), 2.01 (s, 6H), 2.00 (s, 3H), 1.84–1.72 (m, 2H), 1.62–1.23 (m, 52H), 0.90 (t, J = 7.2 Hz, 6H). ^{13}C NMR (150 MHz, CD_3OD) δ 174.52, 173.95, 173.47, 172.31, 133.63, 130.00, 105.02, 103.73, 103.08, 102.93, 79.48, 78.06, 75.26, 75.06, 74.68, 74.59, 74.31, 73.82, 73.46, 73.17, 71.54, 71.13, 69.47, 68.97, 68.54, 68.20, 62.14, 61.25, 60.26, 53.30, 52.67, 51.56, 48.17, 42.69, 35.98, 32.08, 31.73, 31.70, 31.68, 29.48, 29.45, 29.42, 29.41, 29.37, 29.31, 29.24, 29.11, 29.08, 29.03, 25.78, 22.35, 22.34, 21.67, 21.31, 21.24, 13.07, 13.06. HRMS (ESI-Orbitrap) m/z : $[\text{M}-2\text{H}]^{2-}$ calculated for $\text{C}_{86}\text{H}_{151}\text{N}_5\text{O}_{39}$ 937.9920; found 937.9934.

4.10.4. 9NAc-GT1b (4)—The product 9NAc-GT1b (4) was eluted from the C18 cartridge using 40% CH_3CN in water. The pure product (16 mg, 95% yield) was a white powder. ^1H NMR (600 MHz, CD_3OD) δ 5.69 (dt, J = 14.4, 6.4 Hz, 1H), 5.45 (dd, J = 15.2, 6.4 Hz, 1H), 4.60–4.28 (m, 4H), 4.20–3.26 (m, 49H), 2.91–2.70 (m, 3H), 2.19–2.02 (m, 4H), 2.02 (s, 9H), 2.01 (s, 3H), 1.99 (s, 3H), 1.85–1.72 (m, 3H), 1.62–1.22 (m, 52H), 0.90 (t, J = 7.2 Hz, 6H). ^{13}C NMR (150 MHz, CD_3OD) δ 174.56, 174.11, 174.01, 173.43, 172.50, 133.71, 129.97, 104.30, 103.66, 103.09, 99.78, 79.38, 76.18, 75.22, 75.04, 74.65, 74.57, 74.28, 73.44, 73.22, 71.53, 70.32, 69.46, 68.58, 68.00, 63.16, 62.30, 61.56, 61.31, 60.23, 53.28, 52.64, 52.52, 51.19, 48.46, 48.18, 42.56, 40.71, 35.99, 32.08, 31.70, 31.67, 29.48, 29.45, 29.42, 29.40, 29.37, 29.36, 29.30, 29.28, 29.25, 29.10, 29.08, 29.03, 25.79, 22.35, 22.34, 21.65, 21.37, 21.34, 21.26, 13.08. HRMS (ESI-Orbitrap) m/z : $[\text{M}-2\text{H}]^{2-}$ calculated for $\text{C}_{97}\text{H}_{168}\text{N}_6\text{O}_{47}$ 1083.5397; found 1083.5401.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Xi Chen is collaborating with Integrated Micro-Chromatography System (IMCS) on a current National Institutes of Health (NIH) grant (grant number: R44GM139441) focusing on developing chemoenzymatic strategies, enzymes, and kits for accessible and affordable gangliosides. IMCS played no role in the design, execution, interpretation, or publication of this study.

Data availability

No data was used for the research described in the article.

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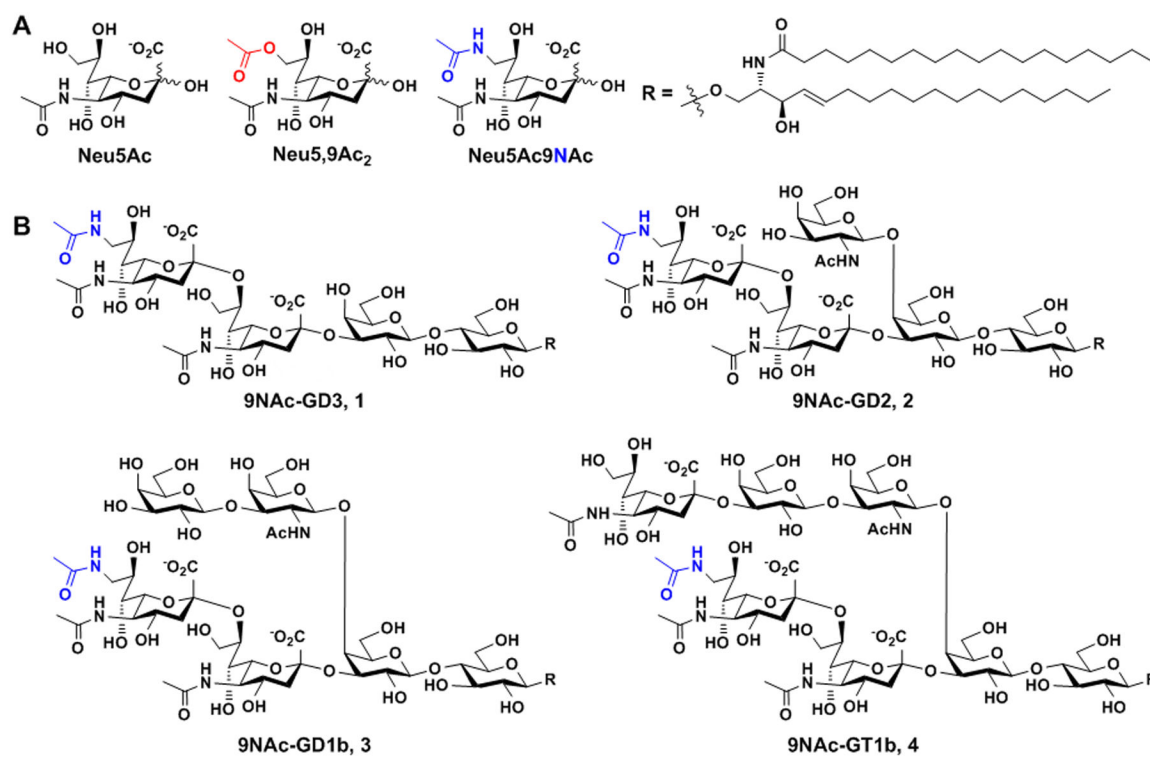
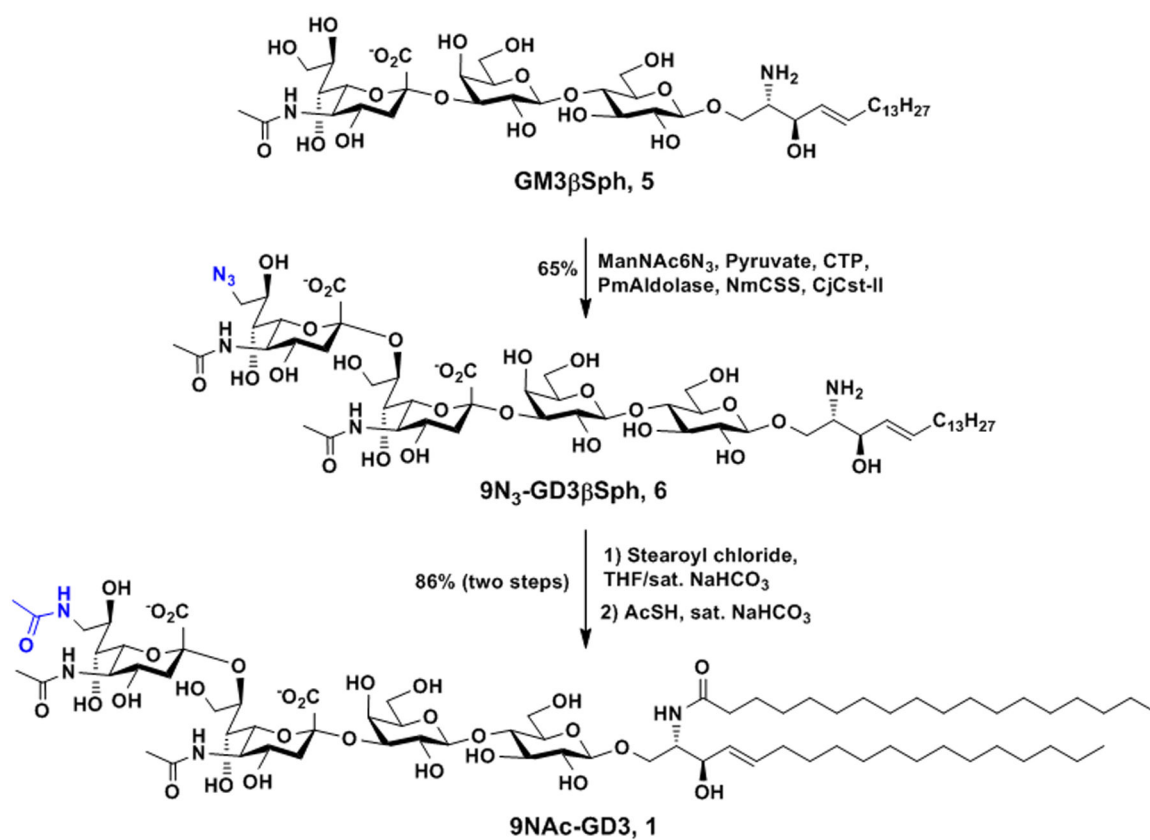
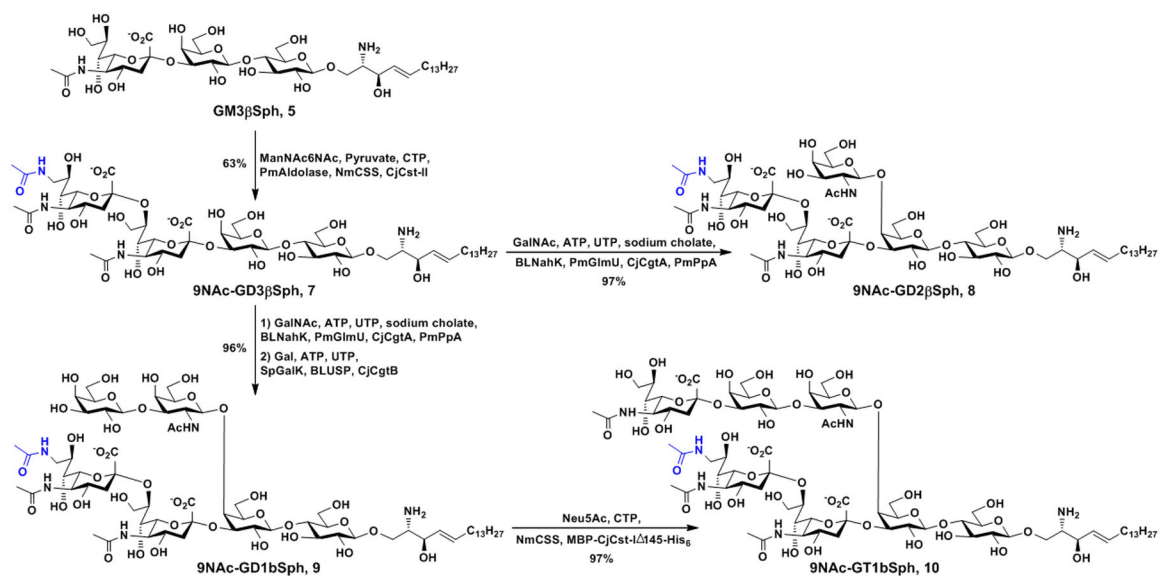


Fig. 1. Structures of **A.** *N*-Acetylneuraminic acid (Neu5Ac) as well as 9-*O*-acetyl Neu5Ac (Neu5,9Ac₂) and its 9-*N*-acetyl analog, 9-acetamido-9-deoxy-*N*-acetylneuraminic acid (Neu5Ac9NAc); and **B.** 9-*N*-acetylated b-series gangliosides including 9NAc-GD3 (1), 9NAc-GD2 (2), 9NAc-GD1b (3), and 9NAc-GT1b (4) synthesized in the current study.

**Scheme 1.**

Synthesis of 9NAc-GD3 (1) from GM3βSph (5) via a 9N₃-GD3 (6) intermediate using ManNAc6N₃ as a chemoenzymatic synthon in a one-pot multienzyme (OPME) sialylation system following by chemical acylation and N₃ to NHAc conversion.

**Scheme 2.**

Chemoenzymatic synthesis of 9NAc-GD3βSph (7), 9NAc-GD2βSph (8), 9NAc-GD1βSph (9), and 9NAc-GT1βSph (10) using ManNAc6NAc as a Neu5Ac9NAc precursor.