Tetrahedron 142 (2023) 133522

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Chemoenzymatic synthesis of *N*-acetyl analogues of 9-*O*-acetylated b-series gangliosides

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ARTICLE INFO

Article history:
Received 23 April 2023
Received in revised form
9 June 2023
Accepted 13 June 2023
Available online 15 June 2023

Keywords:
b-Series ganglioside
Chemoenzymatic synthesis
Glycosphingolipid
9-*N*-acetyl sialic acid
O-acetyl sialic acid
One-pot multienzyme

ABSTRACT

The stable *N*-acetyl analogues of biologically important 9-*O*-acetylated b-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 ($ManNAc6N_3$) 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.

1. Introduction

Gangliosides are biologically important sialic acid (Sia)-containing glycosphingolipids. Those in the ganglio-series containing a disialylated lactosyl ceramide core with an α2−8-linked terminal sialic acid [1] are called b-series gangliosides which include GD3, GD2, GD1b, GT1b (Fig. 1), and more complex GQ1b and GQ1bα. 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, Fig. 1A) is the major sialic acid form in gangliosides [7]. The terminal α2−8-linked Neu5Ac of the disialylated lactosyl ceramide core in the b-series gangliosides can be modified by *O*-acetylation, with 9-*O*-acetyl Neu5Ac ($Neu5,9Ac_2$, Fig. 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.

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https://doi.org/10.1016/j.tet.2023.133522

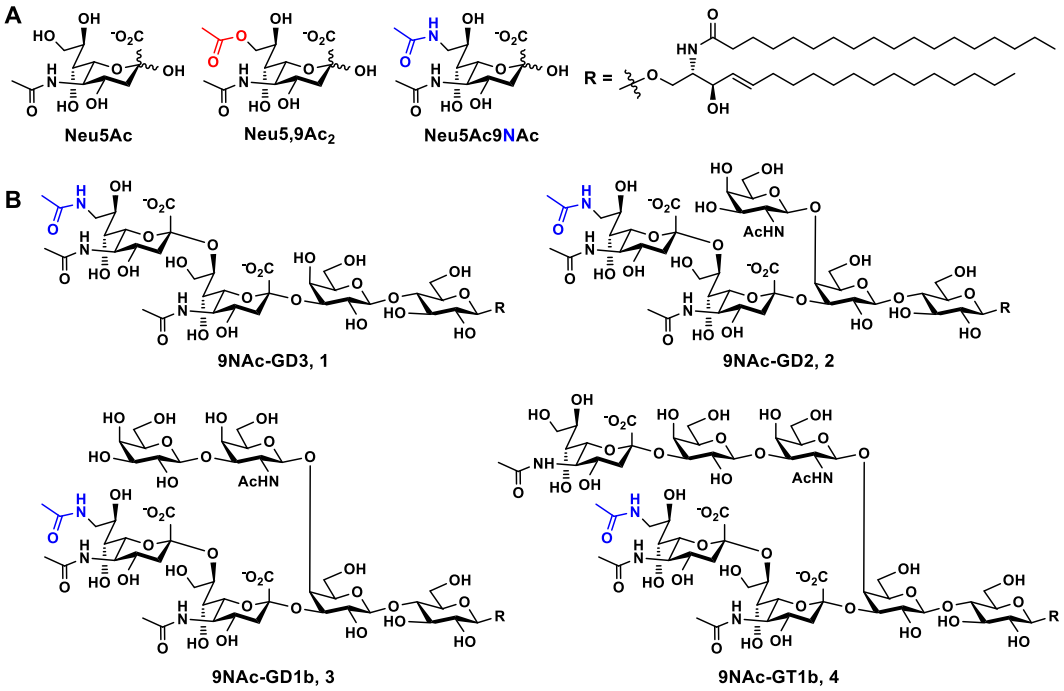


Fig. 1. Structures of **A.** *N*-Acetylneuraminic acid (Neu5Ac) as well as 9-*O*-acetyl Neu5Ac (Neu5,9Ac$_2$) and its 9-*N*-acetyl analog, 9-acetamido-9-deoxy-*N*-acetylneuraminic acid (Neu5Ac9NAc); and **B.** 9-*N*-acetylated b-series gangliosides including 9NAc-GD3 (**1**), 9NAc-GD2 (**2**), 9NAc-GD1b (**3**), and 9NAc-GT1b (**4**) synthesized in the current study.

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 Neu5,9Ac$_2$ with a nitrogen atom to generate a stable analogue 9-acetamido-9-deoxy-*N*-acetylneuraminic acid (Neu5Ac9NAc) [28–32] (Fig. 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 (Fig. 1B). Two chemoenzymatic methods have been developed and compared for the synthesis of 9NAc-GD3 using either 6-azido-6-deoxy-*N*-acetylmannosamine (ManNAc6N$_3$) 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 analogues of 9-*O*-acetylated b-series gangliosides enabling studies to advance the understanding of the biological roles of ganglioside *O*-acetylation.

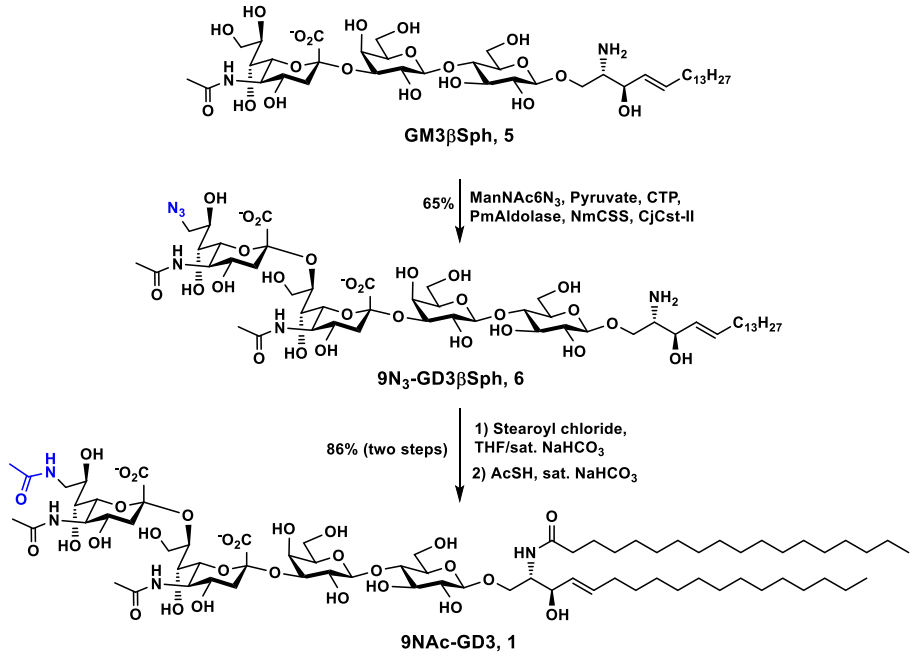
2. Results and discussion

*2.1. Synthesis of 9NAc-GD3 (**6**) using 6-azido-6-deoxy-N-acetylmannosamine (ManNAc6N$_3$) as a chemoenzymatic synthon*

Initially, we explored the synthesis of 9NAc-GD3 (**1**) from GM3 sphingosine (GM3βSph, **5**) [5] via a 9-azido-GD3 (9N$_3$-GD3, **6**) intermediate using 6-azido-6-deoxy-*N*-acetylmannosamine (ManNAc6N$_3$) [28] as a chemoenzymatic synthon. As shown in Scheme 1, 9N$_3$-GD3 glycosylsphingosine (9N$_3$-GD3βSph, **6**) was produced from ManNAc6N$_3$ and GM3βSph (**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* α2–3/8-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 α2–8-linked Neu5Ac could be added to the desired GD3βSph product to form GT3βSph as a minor product [5,37]. In contrast, we found that when ManNAc6N$_3$ was used as the donor precursor, the undesired additional sialylation did not occur to the target product 9N$_3$-GD3βSph which was obtained with a 65% yield. Acylation of the amino group in 9N$_3$-GD3βSph (**6**) using stearoyl chloride in THF/saturated aqueous NaHCO$_3$ (1:1, v/v), a condition that we developed previously [5], produced 9N$_3$-GD3 ganglioside. The 9-N$_3$ group was then converted to 9-NAc by incubating with thioacetic acid in saturated NaHCO$_3$ 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 9N$_3$-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-N$_3$ to 9-NAc on glycans which was completed in 20 h.

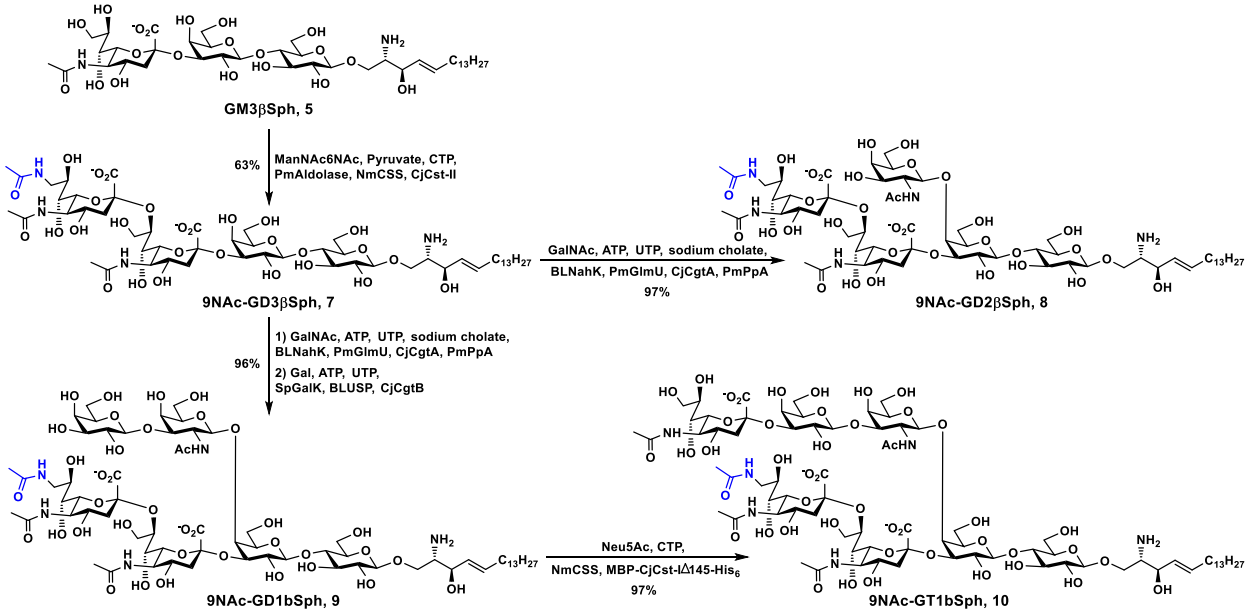
2.2. Synthesis of 9NAc-GD3βSph (7) using 6-acetamido-6-deoxy-N-acetylmannosamine (ManNAc6NAc) as a 9-acetamido-9-deoxy-N-acetylneuraminic acid (Neu5Ac9NAc) precursor

Due to the relatively low efficiency in converting the azido group in 9N$_3$-GD3 to an acetamido group in 9NAc-GD3, we



Scheme 1. Synthesis of 9NAc-GD3 (**1**) from GM3βSph (**5**) via a $9N_3$-GD3 (**6**) intermediate using ManNAc6N_3 as a chemoenzymatic synthon in a one-pot multienzyme (OPME) sialylation system following by chemical acylation and N_3 to NHAc conversion.

explored the strategy of using ManNAc6NAc [28] as a precursor for enzymatic synthesis of 9NAc-GD3βSph (**7**) directly from GM3βSph (**5**). We previously showed that ManNAc6NAc was a suitable precursor for the synthesis of α2−3- and α2−6-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-GD3βSph (**7**) directly from GM3βSph (**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-GD3βSph (**7**) was synthesized from GM3βSph (**5**) as the acceptor substrate and ManNAc6NAc as the donor precursor using the OPME sialylation system containing PmAldolase, NmCSS, and CjCst-II (Fig. S1A, ESI). Similar to the case for synthesizing $9N_3$-GD3βSph (**6**), no addition sialylation of the desired product 9NAc-GD3βSph (**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 CH_3CN



Scheme 2. Chemoenzymatic synthesis of 9NAc-GD3βSph (**7**), 9NAc-GD2βSph (**8**), 9NAc-GD1bβSph (**9**), and 9NAc-GT1bβSph (**10**) using ManNAc6NAc as a Neu5Ac9NAc precursor.

in water. 9NAc-GD3βSph (**7**) (130 mg, 63%) was eluted first with 40% acetonitrile in water (v/v) and the unreacted GM3βSph (**5**) was eluted with 60% acetonitrile in water (v/v).

2.3. Synthesis of 9NAc-GD2βSph (8) from 9NAc-GD3βSph (7) using a one-pot multienzyme (OPME) GalNAc activation and transfer system

With 9NAc-GD3βSph (**7**) in hand, the synthesis of 9NAc-GD2βSph (**8**) was accomplished using an OPME β1−4-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-Δ15CjCgtA-His_6 which was a recombinant *Campylobacter jejuni* β1−4-*N*−acetylgalactosaminyltransferase designed with improved *E. coli* expression and increased stability [41]. As shown in Scheme 2, incubating 9NAc-GD3Sph (**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-GD2βSph (**8**) smoothly (Fig. 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-GD2βSph (**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% CH_3CN in water.

2.4. Synthesis of 9NAc-GD1bβSph (9) from 9NAc-GD3βSph (7) using a multistep one-pot multienzyme (MSOPME) strategy

From 9NAc-GD3βSph (**7**), a recently developed multistep one-pot multienzume (MSOPME) strategy was used to directly access 9NAc-GD1bβSph (**9**). In the first step, 9NAc-GD2βSph (**8**) was formed from 9NAc-GD3βSph (**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-GD1bβSph (**9**) using an OPME β1−3-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-CjCgtBΔ30-His_6 which was a recombinant *Campylobacter jejuni* β1−3-galactosyltransferase designed with improved *E. coli* expression and increased stability [41]. The second OPME glycosylation was completed at 30 °C in 20 h (Fig. S1C, ESI). The desired pure 9NAc-GD1bβSph (**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-GT1bβSph (10) from 9NAc-GD1bβSph (9) using an OPME sialylation system

To synthesize 9NAc-GT1bβSph (**10**) from 9NAc-GD1bβSph (**9**), a suitable α2−3-sialyltransferase was needed. *Pasteurella multocida* α2−3-sialyltransferases PmST1 [47] and PmST3 [48] had no or very low activity on sialylating the terminal Gal in 9NAc-GD1bβSph (**9**). We previously cloned a recombinant human ST3GAL II (MBP-Δ27hST3GAL II-His_6) 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-GT1bβSph (**10**). To our delight, we found that MBP-CjCst-IΔ145-His_6, a recombinant *Campylobacter jejuni* α2−3-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-Δ27hST3GAL II-His_6 expressed in *E. coli* which has an expression level of less than 1 mg (~9 mU) per liter LB culture [49], MBP-CjCst-IΔ145-His_6 has a much higher expression level (60 mg purified enzyme from per liter LB culture) [50]. Preparative-scale synthesis of 9NAc-GT1bβSph (**10**) was achieved efficiently from 9NAc-GD1bβSph (**9**) and Neu5Ac in the presence of CTP using an OPME sialylation reaction containing NmCSS and MBP-CjCst-IΔ145-His_6. The reaction was completed in 15 h (Fig. S1D, ESI) and pure 9NAc-GT1bβSph (**10**) (58 mg, 97%) was obtained after purification via a C18 cartridge by eluting with 20% of CH_3CN in water.

2.6. Improved acylation conditions for converting glycosylsphingosines to glycosphingolipids

With 9-*N*-acetylated GD3βSph, GD2bβSph, GD1bβSph, and GT1bβSph (**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 $NaHCO_3$ [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 GM3βSph (**5**) as a model substrate, several acylation conditions were compared, including THF/saturated $NaHCO_3$ (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% Na_2CO_3 (1:1 by volume). The reaction mixtures were analyzed by thin-layer chromatography (Fig. S2, ESI). Among these conditions, THF/1% Na_2CO_3 (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 1 h. Furthermore, the reaction mixture was a homogeneous solution without any undissolved residues. Using the improved acylation reaction conditions (stearoyl chloride in THF/1% Na_2CO_3), 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-Δ15CjCgtA-His_6 [41], *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK) [45], *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP) [46], *Campylobacter jejuni* β1−3-galactosyltransferase MBP-CjCgtBΔ30-His_6 [41], and *Campylobacter jejuni* α2−3-sialyltransferase MBP-CjCst-IΔ145-His_6 [50] were expressed and purified as described previously.

4.2. OPME synthesis of 9N_3-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 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 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 GM3βSph (**5**) (30 mg, 0.032 mmol), ManNAc6N_3 (16 mg, 0.065 mmol), sodium pyruvate (25 mg, 0.227 mmol), CTP (37 mg, 0.070 mmol), $MgCl_2$ (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 9N_3-GD3βSph was eluted with 40% acetonitrile in water (v/v). The unreacted GM3βSph 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 9N_3-GD3βSph (**6**) (27 mg, 65% yield). ^{1}H NMR (600 MHz, CD_3OD) 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). ^{13}C NMR (150 MHz, CD_3OD) 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 $C_{52}H_{89}N_6O_{27}$ 1229.5781; found 1229.5776.

4.3. Conversion of 9N_3-GD3βSph (**6**) to 9NAc-GD3 (**1**)

To a solution of 9N_3-GD3βSph (**6**) (15 mg) in sat. $NaHCO_3$-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 9N_3-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-GD3βSph (**7**): (5,9-Diacetamido-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 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 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 GM3βSph (**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), $MgCl_2$ (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-GD3βSph (**7**) was eluted with 40% acetonitrile in water (v/v). Unreacted GM3βSph (**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-GD3βSph (**7**) (130 mg, 63% yield). ^{1}H NMR (800 MHz, CD_3OD) 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). ^{13}C NMR (200 MHz, CD_3OD) 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 $C_{54}H_{93}N_4O_{28}$ 1245.5982; found 1245.5957.

*4.5. OPME synthesis of 9NAc-GD2βSph (**8**): 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1 → 4)-(5,9-diacetamido-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 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 1)-(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol*

9NAc-GD3βSph (**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-Δ15CjCgtA-His$_6$ (2 mg), PmPpA (1.0 mg), $MgCl_2$ (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 ddH_2O (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-GD2βSph (**8**) (56 mg, 97%) as a white powder. ^{1}H 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*: $[M - 2H]^{2-}$ calculated for $C_{62}H_{107}N_5O_{33}$ 723.8351; found 723.8365.

*4.6. MSOPME synthesis of 9NAc-GD1bβSph (**9**): β-D-Galactopyranosyl-(1 → 3)-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1 → 4)-(5,9-diacetamido-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 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 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-GD3βSph (**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-Δ15CjCgtA-His$_6$ (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-GD2βSph (**8**) was monitored by HRMS and 9NAc-GD3βSph (**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-CjCgtBΔ30-His$_6$ (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 ddH_2O (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-GD1bβSph (**9**) (123 mg, 96%) as a white powder. ^{1}H 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-GT1bβSph (**10**): (5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2 → 3)-β-D-Galactopyranosyl-(1 → 3)-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1 → 4)-(5,9-diacetamido-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 → 3)-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 1)-(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol*

9NAc-GD1bβSph (**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 $MgCl_2$ (20 mM), NmCSS (0.8 mg), and MBP-CjCst-IΔ145-His$_6$ (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-GT1bβSph (**10**) (58 mg, 98%) as a white powder. ^{1}H NMR (600 MHz, CD_3OD) 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). ^{13}C NMR (150 MHz, CD_3OD) 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 GM3βSph 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 $NaHCO_3$; THF/50 mM carbonate buffer (pH 9.2); THF/100 mM CAPS buffer (pH9.5); and THF/1% Na_2CO_3. The reactions were analyzed by thin-layer chromatography. Developing solvent used was *i*-PrOH:H_2O:NH_4OH = 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% Na_2CO_3 (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 ddH_2O (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% CH_3CN in water. The pure product (24 mg, 99% yield) was a white powder. ^{1}H NMR (800 MHz, CD_3OD) 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, CH_3), 2.01 (s, 3H, CH_3), 1.99 (s, 3H, CH_3), 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). ^{13}C NMR (200 MHz, CD_3OD) 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 $C_{72}H_{128}N_4O_{29}$ 755.4259; found 755.4267.

4.10.2. 9NAc-GD2 (**2**)

The product 9NAc-GD2 (**2**) was eluted from the C18 cartridge using 40% CH_3CN in water. The pure product (22 mg, 97% yield) was a white powder. ^{1}H NMR (600 MHz, CD_3OD) 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). ^{13}C NMR (150 MHz, CD_3OD) 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 $C_{80}H_{141}N_5O_{34}$ 856.9656; found 856.9665.

4.10.3. 9NAc-GD1b (**3**)

The product 9NAc-GD1b (**3**) was eluted from the C18 cartridge using 40% CH_3CN in water. The pure product (22 mg, 97% yield) was a white powder. ^{1}H 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*: [M − 2H]$^{2-}$ calculated for $C_{86}H_{151}N_5O_{39}$ 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. ^{1}H 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*: [M − 2H]$^{2-}$ calculated for $C_{97}H_{168}N_6O_{47}$ 1083.5397; found 1083.5401.

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.

Acknowledgements

The authors would like to acknowledge the collaboration of Lee-Ping Wang at the University of California Davis on studies of *N*-acetyl analogues of sialosides. Research reported in this publication was partially supported by the United States (U.S.) National Institutes of Health (NIH) awards R01AI130684, R44GM139441, R01GM032373, and NIH Common Fund Glycoscience Program award U01GM120419. The Thermo Scientific Q Exactive HF

Orbitrap Mass Spectrometer was purchased with a U.S. NIH Shared Instrumentation Grant under the award number S10OD025271. The Bruker Avance-800 NMR spectrometer was funded by the U.S. National Science Foundation grant number DBI-0722538.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tet.2023.133522.

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