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Chemoenzymatic synthesis and biological evaluation of ganglioside GM3 and lyso-GM3 as potential agents for cancer therapy

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ABSTRACT

A highly efficient chemoenzymatic method for synthesizing ganglioside GM3 and lyso-GM3 was reported here. Enzymatic extension of the chemically synthesized lactosyl sphingosine using efficient one-pot multienzyme (OPME) reaction allowed glycosylation to be carried out in aqueous solutions realizing the greening of reactions. Ganglioside GM3 was synthesized through 10 steps with a total yield of 22%. Lyso-GM3 was very useful for kinds of derivatization. The anti-proliferation activity studies demonstrated that these compounds **14-16** with sphingosine exhibited more potency than the corresponding lyso-GM3 with ceramide. All ganglioside GM3 and lyso-GM3 can effectively inhibit the migration of melanoma B16-F10 cells. These chemoenzymatically synthesized GM3 and lyso-GM3 exhibited antitumor activities, which can provide valuable sights to search new antitumor agents for cancer therapy.

1. Introduction

Gangliosides are sialic acid-containing glycosphingolipids (GSLs) that are ubiquitous components of mammalian plasma membranes and play an important role in the interaction of cells with their environment [1-3]. A typical example is GM3 ganglioside clustering at the cell surface of mouse melanoma B16, forming a “glycosignaling domain (GSD)” [4,5]. GM3, the first and simplest member in the metabolic series of a GSLs family, contains a single terminal sialic acid, lactose and ceramide (Scheme 1). Aberrant expression of GM3 has been found to be associated with glycosphingolipid storage diseases and cancer progression [6,7].

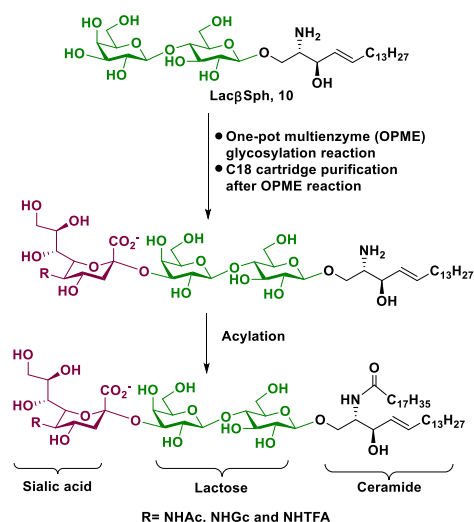
To better understand GM3 functions at molecular level and therapeutic potentials, it is of our great interest to develop an efficient synthetic route to these compounds. A general strategy for synthesizing GM3 is applying multi-step chemical synthesis of a glycosyl donor for coupling with an azido derivative of the protected glycosphingosine followed by reduction of the azido group, coupling with an fatty acid chain, and deprotection [8-10]. Especially, chemical sialylation in structure is usually more challenging and time consuming than other glycosidation reactions for the structural complexity of sialic acid including a sterically hindered tertiary anomeric center, lacking of a stereo-directing group adjacent to the anomeric position, and the

presence of an electron-withdrawing carboxyl group in sialic donors [11-15]. Recently, Komura *et al.* reported a robust method for the selective α -glycosidation of sialic acid using macrobicyclized sialic acid donors to impart stereoselectivity [16]. In spite of great progress, chemical synthesis of sialosides in good yield with complete stereoselectivity remains a challenge. Isolation of GM3 from natural sources is also difficult for their low abundance, lability, and the complication of diverse sialic acid forms, different linkages, and various internal glycans. All complex GSLs share a common lactosyl ceramide (Lac β Cer) core, which has a low solubility in water and is a poor acceptor for *in vitro* enzymatic reactions using glycosyltransferases in aqueous solutions [17]. To overcome these difficulties, efficiently chemoenzymatic methods were developed in our laboratory to obtain homogenous GM3 and lyso-GM3 (fatty acyl free ganglioside GM3). Lysogangliosides also play a role in the pathogenesis of gangliosidoses [18,19].

Herein we report the synthesis of GM3 and lyso-GM3 using highly efficient one-pot multienzyme (OPME) sialylation systems [20-23]. Sialyltransferase acceptor, lactosyl sphingosine (Lac β Sph) were chemically synthesized and used in the one-pot three-enzyme system containing a sialic acid aldolase, a CMP-sialic acid synthetase, and an α 2,3-sialyltransferase for the production of natural occurring or non-natural α 2,3-linked sialosides [24-27]. Three different sialic acid precursors, *N*-acetylmannosamine (ManNAc), *N*-glycolylmannosamine (ManNGc) and *N*-trifluoroacetylmannosamine (ManNHTFA) were used to produce

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Scheme 1. An efficient chemoenzymatic strategy for synthesizing GM3 and lyso-GM3 by enzymatic extension of lactosyl sphingosine (LacβSph, **10**) using OPME reaction with C18-cartridge purification after sialylation reaction followed by a simple acylation process.

α 2,3-linked sialosides containing *N*-acetylneuraminic acid (Neu5Ac), the most abundant sialic acid form, *N*-glycolylneuraminic acid (Neu5Gc), a common animal sialic acid form and non-natural *N*-trifluoroacetylneuraminic acid (Neu5TFA), respectively. The biological activity evaluation of GM3 and lyso-GM3 was assessed by cytotoxicity assays and wound healing tests.

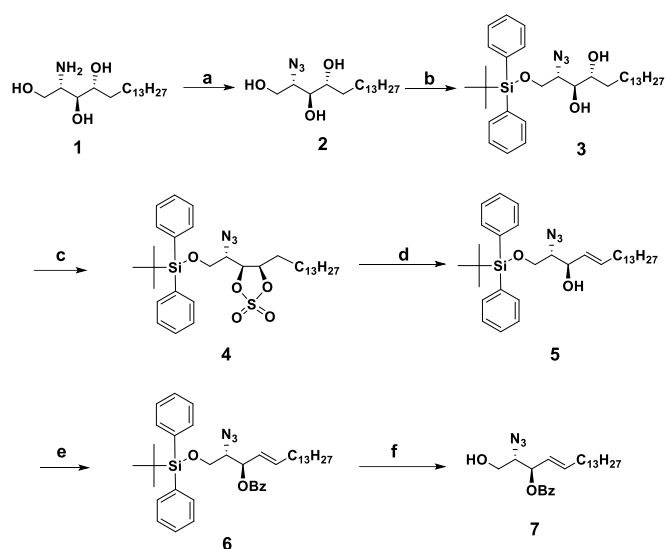
2. Results and discussion

2.1. Chemoenzymatic synthesis of GM3 and lyso-GM3

As shown in Scheme 1, we envisioned that GM3 can be readily obtained using sialyltransferase-dependent OPME system to extend the glycan chain in lactosyl sphingosine (LacβSph), a glycolipid that is readily soluble in aqueous solutions and laying on the flexible substrate specificity of the sialic acid aldolase and sialyltransferase PmST1. Meanwhile, sphingosine (lipid) component owns hydrophobic tag, allowing for facile purification using a C18 cartridge with simple green solvents. Then, acylation of lyso-GM3 with a fatty acid will form the desired gangliosides.

To test our hypothesis, LacβSph was synthesized using chemical approach as previously report (Scheme 2) [28,29]. Commercially available inexpensive phytosphingosine (**1**) and lactose were chose as starting materials. According to previous reports, the azido-derivative of sphingosine was a better acceptor than other *N*-protected sphingosine derivatives for glycosylation with trichloroacetimidate glycosylation donors [30,31]. Specially, converting amino group in phytosphingosine (**1**) to an azido group by treating with triflicazide in the presence of catalytic CuSO₄ and triethylamine gave **2** with 96% yield. Then, the primary hydroxyl group in **2** was selectively protected by *tert*-butyldiphenylsilyl group (TBDPS). Next, the conversion of the 3,4-vicinal diol in **3** to its cyclic sulfate **4** was achieved in good yield (87%) by using thionyl chloride in the presence of triethylamine followed by oxidation with RuCl₃/NaIO₄.

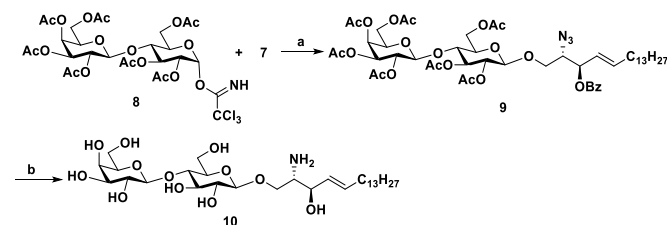
Selective opening of the cyclic sulfate by tetrabutylammonium iodide and 1,8-diazabicyclo [5.4.0] undec- by acidic hydrolysis to remove allylic *O*-sulfate group were carried out in one pot to furnish **5** in 83% yield. The conventional benzoylation of **5** produced **6** in 89% yield. The removal of the *O*-sialyl ether of **6** was carried out using HF•pyridine to produce the glycosylation acceptor **7** in 82% yield. Total yield for chemical synthesis of **7**



Scheme 2. Synthesis of sphingosine acceptor **11**. Reagents and conditions: (a) 1H-imidazole-1-sulfonyl azide, CuSO₄, K₂CO₃, MeOH, r.t., 6 h, 96%; (b) TBDPSCl, Et₃N, DMAP, CH₂Cl₂, r.t., 12 h, 96%; (c) (i) SO₂Cl₂, Et₃N, CH₂Cl₂, 0 °C, 0.5–1 h; (ii) RuCl₃·3H₂O, NaIO₄, CCl₄: CH₃CN: H₂O (1 : 1 : 1), r.t., 2 h; 87% in two steps; (d) (i) Bu₄NI, DBU, toluene, reflux, 2 h; (ii) H₂SO₄/H₂O/THF, r.t., 45 min; 83% in two steps; (e) BzCl, DMAP, Et₃N, CH₂Cl₂, r.t., 12 h, 89%; (f) HF•pyridine, THF, r.t., 12 h, 82%

from phytosphingosine **1** was 49% in six steps.

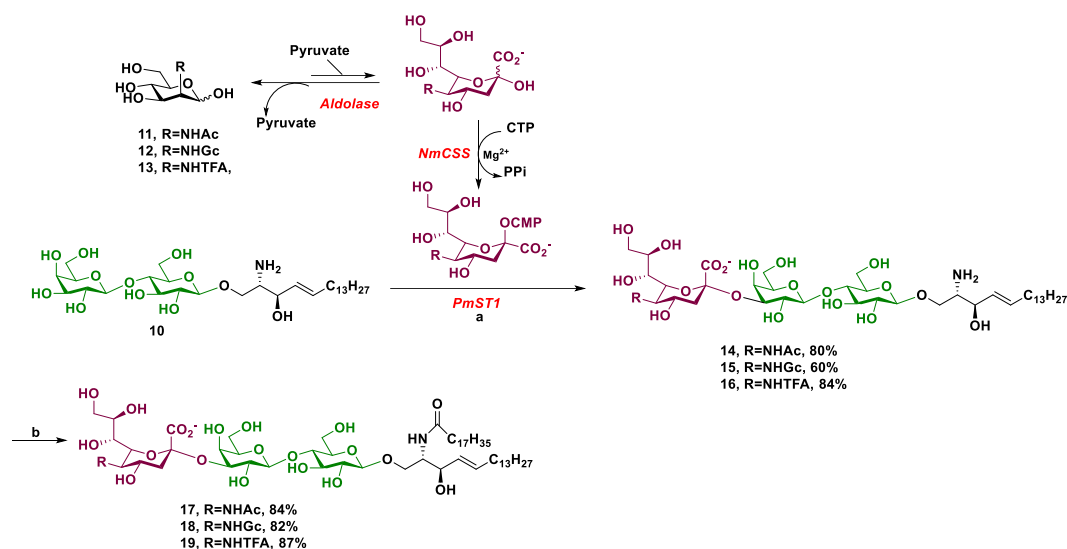
The glycosylation of acceptor **7** with per-*O*-acetyl trichloroacetimidate (**8**) in the presence of BF₃•OEt₂ in CH₂Cl₂ at -18°C produced per-*O*-acetyl lactoside (**9**) in 83% yield (Scheme 3). Then, removal of all acetyl and benzoyl protecting groups under the Zemplén conditions in a quantitative yield. Finally, the azide group was selectively reduced under the conditions of 1,3-propanedithiol and triethylamine to produce LacβSph (**10**) in an excellent yield (93%).



Scheme 3. Synthesis of lactosyl sphingosine (LacβSph, **10**). Reagents and conditions: (a) **7**, BF₃•OEt₂, CH₂Cl₂, -18 °C, 1 h, 83%; (b) (i) NaOMe, MeOH, r.t., 12 h; (ii) 1,3-propanedithiol, Et₃N, pyridine–water (1 : 1 v/v), 50 °C, 12 h, 93% in two steps.

Lactosyl sphingosine (LacβSph, **10**) was readily soluble in aqueous solutions for up to 30 mM, allowing it to be used efficiently as a starting sialyltransferase acceptor for enzymatic extension using OPME reaction. Here, two natural occurring sialic acid forms Neu5Ac and Neu5Gc and one nonnatural Neu5TFA are introduced into the structures of the target ganglioside glycans. Both Neu5Ac (in humans and animals) and Neu5Gc (in animals and small amounts in humans) are common sialic acid forms found in gangliosides [32,33]. Its unique property of resistance to sialidases makes the glycans containing Neu5TFA moiety interesting for biofunctional studies.

The synthesis of lyso-GM3 **14–16** started from *N*-acetylmannosamine (ManNAc), *N*-glycolylmannosamine (ManGc) or *N*-trifluoroacetylmannosamine (ManNHTFA) and LacβSph (**10**) by the one-pot three-enzyme approach described in our previous reports [34,35]. For example, in this enzymatic reaction, starting material ManNAc is converted by an recombinant sialic acid aldolase to *N*-acetylneuraminic acid,



Scheme 4. High-yield synthesis of ganglioside GM3 and lyso-GM3 by enzymatic extension of lactosyl sphingosine (Lac β Sph, **10**) using OPME reaction. One-pot three-enzyme synthesis of sialy Lac β Sph: Aldolase, *N. meningitidis* CMP-Sialic Acid Synthetase (NmCSS) and *Pasteurella multocida* Multifunctional sialyltransferase (PmST1). Reagents and conditions: (a) Tris-HCl buffer (100 mM, pH 8.5), 20 mM MgCl₂, 37 °C, 0.5 h.; (b) Stearic acid, EDC, HOBt, Et₃N, DMF, r.t., 12 h .

Table 1. IC₅₀ (μ M) values of compounds **14-19**.

Cells lines	IC ₅₀ (μ M)					
	Compound 14	Compound 15	Compound 16	Compound 17	Compound 18	Compound 19
HCT-116	0.05±0.01	0.04±0.03	0.04±0.01	2.95±0.33	0.30±0.03	0.30±0.01
B16-F10	<0.01	0.02±0.01	0.01±0.01	<0.01	0.03±0.01	0.26±0.07

which is activated by an *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS) before being transferred to Lac β Sph (**10**) by a multifunctional *Pasteurella multocida* sialyltransferase (PmST1) in Tris-HCl buffer (100 mM, pH 8.0) containing 20 mM Mg²⁺ at 37 °C for 0.5 h (Scheme 4).

The efficiency and the flexibility of the PmST1 in the synthesis of GM3 lyso-GM3 were tested using one-pot three-enzyme system. PmST1 had the most flexible donor substrate specificity among all bacterial SiaTs reported to date [36-39]. PmST1 also accepted CMP activated Neu5Gc (*N*-glycolylneuraminic acid) and Neu5TFA (*N*-trifluoroacetylneuraminic acid) obtained *in situ* from their corresponding precursors ManNGc (*N*-glycolylmannosamine) and ManNHTFA (*N*-trifluoroacetylmannosamine), respectively. Upon the completion of the enzymatic reaction as monitored by thin-layer chromatography (TLC) and high-resolution mass spectrometry (HRMS), the reaction mixture was diluted with the same volume of ethanol. The solution was incubated at 4 °C for 30 minutes and centrifuged to remove precipitates. The supernatant was concentrated and dissolved in water for directly loaded on a pre-conditioned C18 cartridge. The sphingosine (lipid) component of the acceptor and the product can be used as an anchor to allow facile purification of the glycosphingosines by reverse phase column chromatography. The purification process took less than 30 min in contrast to several hours using standard silica gel chromatography.

Also, we found that Lac β Sph (**10**) indeed was a good acceptor for PmST1. Using the one-pot three-enzyme system, we obtained natural Neu5Ac α 2,3Lac β Sph **14**, novel Neu5Gc α 2,3Lac β Sph **15** and Neu5TFA α 2,3Lac β Sph **16** in 80%, 60% and 84% yields, respectively. The results further confirmed the flexible acceptor specificity of PmST1. Comparing with **14** and **15**, the OPME synthesis of **16** showed more efficient, which indicated that C-5

fluoroine-substitution *N*-acetylneuraminic acid was a good substrate for PmST1, which is consistent with our previous finding [34]. It is worth mentioning that lyso-GM3 was very useful for the derivatization into a variety of analogs including fluorescent probes. For example, for the preparation of labeled gangliosides, the usual procedure is to reacylate lysogangliosides with an activated labeled fatty acid or with an activated fatty acid bearing a functional group [40,41].

Finally, the target **17-19** was readily obtained in 82~87% yield via the *N*-acylation of lyso-GM3 **14-16** with stearic acid in the presence of EDC, HOBt and Et₃N. Overall, the chemoenzymatic route provided the target **17-19** with 22%, 16% and 24% yield in ten steps, respectively.

2.2. Biological evaluation

2.2.1 Anti-proliferation activities

The effect of six compounds **14-19** on proliferation of human colon cancer HCT-116 cells and highly metastatic murine melanoma B16-F10 cells was evaluated by MTT assay [42,43]. The IC₅₀ values were shown in Table 1. Firstly, for anti-proliferation ability of **14-19** in HCT116 cell line, we found that the lyso-GM3 **14-16** with sphingosine showed much better inhibitory effect than corresponding GM3 with ceramide moieties **17-19** and the IC₅₀ values of **14-16** were 0.05±0.01, 0.04±0.03 and 0.04±0.01 μ M, respectively. For in the melanoma B16-F10 cell line, except for nonnatural ceramide **19** containing Neu5NHTFA with higher IC₅₀ value of 0.26±0.07 μ M, compounds **14-18** showed good inhibitory effect on the proliferation of B16-F10 cells. Interestingly, sphingosine **14** and its corresponding ceramide **17** showed best inhibitory effect with IC₅₀ values < 10 nM.

2.2.2 Anti-migration activities

In order to further investigate the effect of the compounds **14-19** on the migration ability of B16-F10 cells, an *in vitro* wound healing experiment was performed [44,45]. According to the bar

graph, the inhibitory effects of all compounds on cell migration showed significantly different comparing with control group within 48 h as shown in Figure 1. Cell migration rate showed negatively correlated with compounds concentration. Comparing

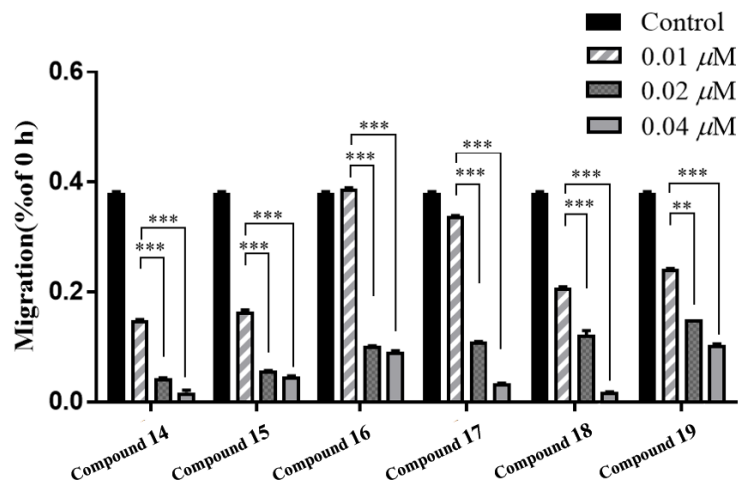
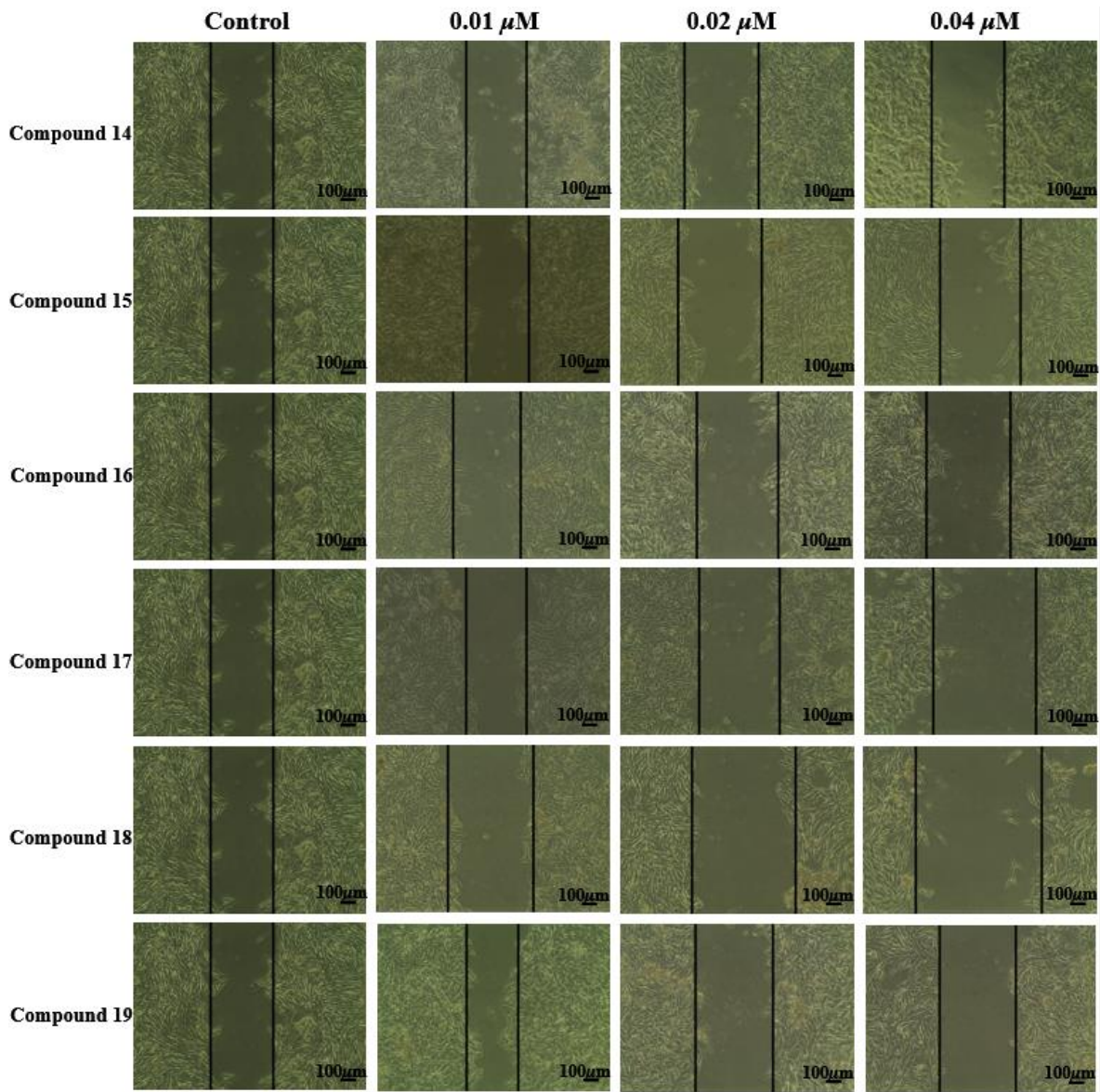


Figure 1. Compounds inhibited highly metastatic murine melanoma B16-F10 cell migration after 48 h of exposure. Inhibition in migration of cancer cells was observed upon treatment with compounds. Values were calculated as a percentage of the 0 h (wound width difference of 0 h and exposure time point/wound width at 0 h %). Comparison between groups, ** $p < 0.01$, *** $p < 0.001$

the effect of compounds on the cell migration rate at low concentration of 0.01 μM , natural compounds **14** and **15** with sphingosine showed much better inhibitory effect than corresponding ganglioside **17** and **18** with ceramide. However, nonnatural **16** showed lower inhibitory migration ability than its corresponding **19** with ceramide. GM3(Neu5Ac) **17** and GM3(Neu5Gc) **18** showed similar anti-migration activities. Furthermore, at high concentration (0.04 μM) of the intermediate **16** and end product **19**, B16-F10 cells showed highest migration comparing with other groups, which indicated that the fluorine substitution at C5 of sialic acid may reduce the recognition of GM3 on the cell surface. Interestingly, lyso-GM3 **14** showed excellent inhibitory effect on cell migration under different compound concentration. This is our preliminary result and more compounds need to be developed using this efficient chemoenzymatic synthesis route to study structure-activity relationships based on this structure.

3. Conclusions

In summary, we have described a simple and efficient strategy for chemoenzymatic synthesis of biologically important ganglioside GM3 and lyso-GM3 containing natural and non-natural sialic acids using bacterial sialyltransferase-catalyzed sialylation of Lac β Sph acceptor. Similar strategies also can be explored for the synthesis of other gangliosides. In addition, lyso-GM3 was very useful for kinds of derivatization. The activities of GM3 and lyso-GM3 against cancer cell proliferation and migration were also studied. These results demonstrated that GM3 and lyso-GM3 exhibited anti-proliferation and anti-migration effects on cancer cells, which can provide valuable entry into glycosphingolipid for therapeutic development.

4. Experimental

4.1 Materials and methods

Chemicals and materials were obtained from commercial sources and were used as received without additional purification unless otherwise noted. Air and moisture sensitive reactions were carried out under a stream of argon. Thin layer chromatography (TLC) was performed on silica gel HF₂₅₄ plates with detection by staining with 5% H₂SO₄ in ethanol or a UV detector. Flash column chromatography was performed on silica gel 300~400 mesh. All the new compounds were fully characterized by ¹H and ¹³C NMR, as well as HRMS. ¹H and ¹³C NMR spectra were recorded at a Bruker Avance 400 MHz instrument. The chemical shifts were referenced to the solvent peak, 7.26 ppm (¹H) and 77.16 ppm (¹³C) for CDCl₃, 3.31 ppm (¹H) and 49.00 ppm (¹³C) for CD₃OD at 25 °C, and coupling constants (*J*) were given in Hz. High resolution ESI (*m/z*) were recorded on a hybrid IT-TOF mass spectrometer (Shimadzu LCMS-IT-TOF, Kyoto, Japan).

4.2 Synthesis

4.2.1 (2S,3R,4E)-2-Azido-3-O-benzoyloxy-1-O-tertbutyldiphenylsilyloxy-octadec-4-ene (6)

Compound **5** was synthesized from commercially available phytosphingosine (**1**) as reported previously [28]. For the synthesis of compound **6**, to a solution of compound **5** (2.7 g, 4.8 mmol) in dry CH₂Cl₂ (15 mL), Et₃N (4.0 mL, 28.8 mmol) and 4-dimethyl amino pyridine (DMAP) (59 mg, 0.5 mmol) were added and the mixture was stirring at 0 °C. Benzoyl chloride (1.1 mL,

9.7 mmol) was then added to the stirring reaction mixture drop-wisely. The reaction was allowed to warm up to r.t. and stirred for overnight, until TLC analysis (hexane:ethyl acetate = 20:1 by volume) showing total consumption of the starting material. The reaction was then diluted with CH₂Cl₂, washed with 1 M HCl, saturated NaHCO₃ solution and brine, then was dried with Na₂SO₄. After filtration, the solvent was removed under reduced pressure and the product was purified by silica gel chromatography using hexane: EtOAc = 9:1 (by volume) as an eluent to produce compound **6** (2.85 g, 89%) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 8.02 (d, 2H), 7.69–7.64 (m, 4H), 7.58 (t, *J* = 7.2 Hz, 1H), 7.46–7.40 (m, 6H), 7.33 (t, *J* = 7.2, 2H), 5.95–5.84 (m, 1H), 5.69 (dd, *J* = 7.8, 4.8 Hz, 1H), 5.52 (dd, *J* = 15.4, 7.8 Hz, 1H), 3.85–3.82 (m, 1H), 3.76 (d, *J* = 5.8 Hz, 2H), 2.03 (dd, *J* = 14.0, 7.0 Hz, 2H), 1.49–1.19 (m, 22H), 1.09 (br s, 9H), 0.89 (t, *J* = 6.6 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 165.15, 138.46, 135.53, 134.42, 134.39, 133.05, 132.82, 132.68, 130.22, 130.06, 129.84, 129.80, 129.71, 128.37, 127.87, 127.79, 127.74, 123.22, 74.30, 65.75, 63.34, 32.32, 31.91, 29.64, 29.55, 29.40, 29.34, 29.10, 28.69, 26.67, 25.96, 22.68, 19.09, 14.10.

4.2.2 (2S,3R,4E)-2-Azido-3-O-benzoyloxy-octadec-4-ene-1-ol (7)

To a solution of compound **6** (2.85 g, 4.3 mmol) in dry THF (40 mL) in a plastic flask, 65–70% HF·pyridine solution (1.4 mL) was added drop-wisely to the stirred mixture at 0 °C. The reaction mixture was stirred at r.t. for about 6 h until complete consumption of compound **7** as judged by TLC analysis (hexane:ethyl acetate = 9:1 by volume). The reaction mixture was then quenched using solid NaHCO₃. EtOAc (40 mL) and H₂O (40 mL) were then added. The aqueous phase was extracted twice with EtOAc. The combined organic phase was washed with a saturated aqueous solution of NaHCO₃ and dried over Na₂SO₄. After filtration, the solvent was removed under reduced pressure and the product was purified by silica gel chromatography using hexane:EtOAc=6:1 (by volume) as an eluent to produce compound **7** (1.52 g, 82%) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 8.06 (d, *J* = 7.9 Hz, 2H), 7.58 (t, *J* = 7.4 Hz, 1H), 7.46 (t, *J* = 7.7 Hz, 2H), 5.96 (dt, *J* = 13.8, 6.7 Hz, 1H), 5.67–5.53 (m, 2H), 3.87–3.71 (m, 2H), 3.64 (d, *J* = 8.3 Hz, 1H), 2.08 (dd, *J* = 14.4, 7.0 Hz, 2H), 1.51–1.11 (m, 23H), 0.98–0.79 (m, 3H). ¹³C NMR δ 165.48, 138.74, 133.27, 129.74, 129.68, 128.42, 123.14, 77.32, 77.00, 76.68, 74.64, 66.15, 61.88, 32.31, 31.86, 29.62, 29.61, 25.59, 29.51, 29.35, 29.29, 29.08, 28.62, 22.63, 14.06. HRMS (ESI) *m/z* calcd for C₂₅H₃₉N₃O₃Na, [M+Na]⁺: 452.2884; found: 452.2863.

4.2.3 O-(2,3,4,6-Tetra-O-acetyl- β -D-galactopyranosyl)-(1 \rightarrow 4)-(2,3,6-tri-O-acetyl- β -D-glucopyranosyl)-(1 \rightarrow 1)-(2S, 3R, 4E)-2-azido-3-O-benzoyloxy-octadec-4-ene (9)

To a solution of peracetylated lactosyl trichloroacetimidate **8** (1.0 g, 1.3 mmol) and acceptor **7** (418 mg, 1.0 mmol) in 35 mL of dry CH₂Cl₂, powdered molecular sieves (4Å, 1.0 g) were added. The mixture was stirred under argon at r.t. for 30 min. The reaction mixture was cooled down to -18 °C and BF₃·OEt₂ (170 μL , 1.3 mmol) was added. The reaction mixture was then stirred at -18 °C until TLC analysis (hexane: ethyl acetate = 4:1 by volume) showed fully conversion of the acceptor. The reaction was quenched with Et₃N, and the solid was filtered off. The filtrate was concentrated under vacuum, and the residue was purified by silica gel chromatography using hexane:EtOAc = 2:1 (by volume) as an eluent to produce compound **9** (1.12 g, 83%) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 8.01 (d, *J* = 7.4 Hz, 2H), 7.54 (t, *J* = 7.4 Hz, 1H), 7.42 (t, *J* = 7.6 Hz, 2H), 5.88 (dd, *J* = 14.4, 7.2 Hz, 1H), 5.57–5.31 (m, 2H), 5.31 (d, *J* = 3.0 Hz,

1H), 5.16 (t, $J = 9.0$ Hz, 1H), 5.07 (dd, $J = 10.4, 8.0$ Hz, 1H), 4.98-4.83 (m, 2H), 4.56-4.39 (m, 3H), 4.05 (dq, $J = 12.2, 4.8$ Hz, 3H), 3.94-3.73 (m, 4H), 3.63-3.45 (m, 2H), 2.11-1.87 (m, 21H), 1.42-1.11 (m, 24H), 0.93-0.75 (m, 3H). ^{13}C NMR (101MHz, CDCl_3) δ 170.29, 170.23, 170.09, 169.99, 169.76, 169.49, 169.06, 165.03, 138.97, 133.18, 129.89, 129.70, 128.42, 122.64, 101.09, 100.28, 76.11, 74.60, 72.82, 72.66, 71.46, 70.93, 70.67, 69.07, 68.27, 66.62, 63.44, 61.86, 60.81, 32.33, 31.87, 29.60, 29.53, 29.34, 29.30, 29.09, 28.68, 22.63, 20.74, 20.64, 20.58, 14.08. HRMS (ESI) m/z calcd for $\text{C}_{51}\text{H}_{73}\text{N}_3\text{O}_{20}\text{Na}$, $[\text{M}+\text{Na}]^+$: 1070.4685; found: 1070.4701.

4.2.4 *O*-(β -D-galactopyranosyl)-(1 \rightarrow 4)-(β -D-glucopyranosyl)-(1 \rightarrow 1)-(2S, 3R, 4E)-2-aminooctadec-4-ene-1,3-diol (10)

To a solution of **9** (1.12 g, 1.1 mmol) in dry MeOH (20 mL), NaOMe (96 mg) was added. After being stirred at r.t. for 10 h, the reaction mixture was neutralized with Dowex 50W (H^+), filtered and concentrated under reduced pressure. This intermediate was used in the next step without further purification. To the dry intermediate (620 mg, 1.0 mmol) in pyridine-water (1:1 v/v, 6 mL), 1,3-propanedithiol (0.94 mL, 9.4 mmol) and Et_3N (1 mL) were added and the mixture was stirred at 50 °C for 12 h. The reaction mixture was concentrated and purified by silica gel chromatography using EtOAc:MeOH= 1:1 (by volume) as an eluent to afford **10** (554 mg, 93%) as a white amorphous powder. ^1H NMR (400 MHz, CD_3OD) δ 5.84 – 5.73 (m, 1H), 5.50 (dd, $J = 15.4, 7.6$ Hz, 1H), 4.37 (d, $J = 7.6$ Hz, 1H), 4.32 (d, $J = 8.0$ Hz, 1H), 4.01 (t, $J = 6.8$ Hz, 1H), 3.95 – 3.89 (m, 1H), 3.88 – 3.82 (m, 3H), 3.78 (dt, $J = 10.2, 3.8$ Hz, 2H), 3.71 (dd, $J = 11.4, 4.6$ Hz, 1H), 3.63 – 3.48 (m, 5H), 3.46 – 3.40 (m, 1H), 3.32 (dd, $J = 18.7, 11.5$ Hz, 2H), 2.95 (td, $J = 6.8, 3.6$ Hz, 1H), 2.10 (q, $J = 6.8$ Hz, 2H), 1.49 – 1.21 (m, 24H), 0.91 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 134.34, 129.26, 103.72, 102.80, 79.21, 75.69, 75.11, 74.88, 73.42, 73.29, 72.98, 71.15, 69.95, 68.90, 61.11, 60.46, 54.85, 32.06, 31.69, 29.41, 29.38, 29.26, 29.09, 29.00, 28.99, 22.35, 13.09. HRMS (ESI) m/z calcd for $\text{C}_{30}\text{H}_{57}\text{NO}_{12}\text{Na}$, $[\text{M}+\text{Na}]^+$: 646.3778; found: 646.3762.

General procedures for one-pot three-enzyme synthesis of sphingolipid 14-16

A acceptor **10** (30 mg, 0.05 mmol), ManNAc, ManGc or ManNHTFA (2.0 equiv.) and CTP (2.0 equiv.) were dissolved in water in a 50 mL centrifuge tube containing Tris-HCl buffer (100 mM, pH 8.5) and MgCl_2 (20 mM). NmCSS (0.1 mg) and PmST1 (0.1 mg) were added, and water was added to bring the total volume of the reaction mixture to 10 mL. The reaction mixture was incubated at 37 °C for 30 min with agitation at 140 rpm in an isotherm incubator. The product formation was monitored by TLC (EtOAc: MeOH: H_2O : AcOH = 4: 2: 1: 0.5, v/v/v/v) and stained with *p*-anisaldehyde sugar stain. The reaction was stopped by adding 10 mL of ice-cold EtOH followed by incubation at 4 °C for 30 min. The mixture was centrifuged (12 000 rpm) at 4 °C for 30 min. The precipitates were removed by centrifugation and the supernatant was concentrated. The residue was dissolved in 2~3 mL of water at 40~45 °C and the solution was directly loaded to a preconditioned RediSep[®]Rf C18 cartridge (5.5 g media) through a 10 mL plastic syringe. The product was eluted from the C18 cartridge with 30% acetonitrile and 0.01% TFA in water (v/v). The elute solvent was collected in 1–1.5 mL fractions.

4.2.5 *O*-(5-Acetamido-3,5-dideoxy-D-glycero-a-D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 3)-(β -D-galactopyranosyl) - (1 \rightarrow 4)-(β -D-glucopyranosyl)-(1 \rightarrow 1)-(2S, 3R, 4E)-2-aminooctadec-4-ene-1,3-diol (14)

35 mg, 80%. ^1H NMR (400 MHz, CD_3OD) δ 5.91 – 5.82 (m, 1H), 5.49 (dd, $J = 15.4, 6.8$ Hz, 1H), 4.42 (d, $J = 7.8$ Hz, 1H), 4.36 (d, $J = 7.8$ Hz, 1H), 4.29 (t, $J = 5.3$ Hz, 1H), 4.05 (dd, $J = 9.7, 2.9$ Hz, 1H), 3.98 – 3.54 (m, 22H), 3.48 (d, $J = 7.9$ Hz, 3H), 3.41 – 3.33 (m, 3H), 2.91 – 2.84 (m, 1H), 2.11 (dd, $J = 13.5, 6.4$ Hz, 2H), 2.01 (s, 3H), 1.73 (dd, $J = 14.3, 7.8$ Hz, 1H), 1.49 – 1.22 (m, 31H), 0.90 (t, $J = 6.2$ Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 175.37, 174.82, 136.37, 128.23, 104.84, 103.58, 100.88, 80.30, 77.42, 76.81, 76.32, 75.92, 74.69, 74.25, 72.80, 70.87, 70.57, 69.87, 69.02, 68.86, 67.26, 66.43, 64.38, 62.50, 61.39, 56.46, 53.76, 41.73, 33.18, 32.85, 31.48, 30.59, 30.55, 30.44, 30.26, 30.21, 29.99, 23.52, 22.48, 13.85. HRMS (ESI) m/z calcd for $\text{C}_{41}\text{H}_{74}\text{N}_2\text{O}_{20}\text{Na}$, $[\text{M}+\text{Na}]^+$: 937.4727; found: 937.4730.

4.2.6 *O*-(5-Glycolylacetamido-3,5-dideoxy-D-glycero-a-D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 3)-(β -D-galactopyranosyl) - (1 \rightarrow 4)-(β -D-glucopyranosyl)-(1 \rightarrow 1)-(2S, 3R, 4E)-2-aminooctadec-4-ene-1,3-diol (15)

27 mg, 60%. ^1H NMR (400 MHz, CD_3OD) δ 5.75 (s, 1H), 5.40 (d, $J = 13.8$ Hz, 1H), 4.33 (d, $J = 7.4$ Hz, 1H), 4.29 – 4.15 (m, 2H), 3.96 (s, 3H), 3.88 – 3.33 (m, 19H), 3.23 (t, $J = 7.4$ Hz, 3H), 2.78 (d, $J = 12.0$ Hz, 1H), 2.01 (d, $J = 6.6$ Hz, 2H), 1.70 – 1.55 (m, 3H), 1.35 – 1.10 (m, 32H), 0.81 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 177.16, 174.80, 136.35, 128.35, 104.88, 103.63, 100.88, 80.37, 77.48, 76.92, 76.37, 75.99, 74.51, 74.29, 72.87, 71.03, 70.60, 69.88, 68.84, 68.78, 64.50, 62.55, 62.41, 56.49, 53.42, 49.65, 41.92, 33.19, 32.87, 30.60, 30.57, 30.45, 30.41, 30.28, 30.21, 30.01, 23.54, 14.26. HRMS (ESI) m/z calcd for $\text{C}_{41}\text{H}_{74}\text{N}_2\text{O}_{21}\text{Na}$, $[\text{M}+\text{Na}]^+$: 953.4676; found: 953.4656.

4.2.7 *O*-(5-Trifluoroacetamido-3,5-dideoxy-D-glycero-a-D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 3)-(β -D-galactopyranosyl) - (1 \rightarrow 4)-(β -D-glucopyranosyl)-(1 \rightarrow 1)-(2S, 3R, 4E)-2-aminooctadec-4-ene-1,3-diol (16)

39 mg, 84%. ^1H NMR (400 MHz, CD_3OD) ^1H NMR (400 MHz, MeOD) δ 5.83 (dd, $J = 15.1, 7.5$ Hz, 1H), 5.49 (dd, $J = 14.8, 6.2$ Hz, 1H), 4.42 (d, $J = 8.0$ Hz, 1H), 4.35 (d, $J = 7.8$ Hz, 1H), 4.31–4.24 (m, 1H), 4.05 (dd, $J = 9.6, 3.0$ Hz, 1H), 3.98–3.72 (m, 11H), 3.70 – 3.52 (m, 9H), 3.46 (d, $J = 7.9$ Hz, 2H), 3.35 (s, 3H), 2.85 (dd, $J = 12.5, 4.6$ Hz, 1H), 2.14 – 1.99 (m, 2H), 1.78 – 1.70 (m, 1H), 1.47 – 1.23 (m, 28H), 0.90 (t, $J = 6.8$ Hz, 5H). ^{13}C NMR (101 MHz, CD_3OD) δ 174.77, 159.92, 159.55, 136.34, 128.39, 104.81, 103.63, 100.84, 80.35, 77.37, 76.84, 76.35, 75.97, 74.28, 73.57, 73.31, 71.09, 70.61, 69.81, 69.14, 68.61, 67.49, 64.36, 62.55, 61.48, 56.44, 53.80, 41.92, 33.18, 32.86, 30.58, 30.55, 30.43, 30.26, 30.20, 30.00, 23.52, 14.25. ^{19}F NMR (376 MHz, CD_3OD) δ -76.92. HRMS (ESI) m/z calcd for $\text{C}_{41}\text{H}_{71}\text{N}_2\text{O}_{20}\text{F}_3\text{Na}$, $[\text{M}+\text{Na}]^+$: 991.4444; found: 991.4398.

General procedures for synthesis of ceramide 17-19

To a solution of sphingolipid **14** (35 mg), **15** (20 mg) or **16** (18 mg) in dry DMF (1 mL), HOBt (1.2 equiv.), EDC·HCl (1.2 equiv.), stearic acid (1.2 equiv.), and Et_3N (1.2 equiv.) were added to the solution. The reaction mixture was stirred under N_2 atmosphere at room temperature for 12 h. The reaction progress was monitored using TLC. After completion, the solution was concentrated under reduced pressure and passed through Sephadex[™] LH 20 (200×18 mm) using CH_3OH as eluant to produce pure product.

4.2.8 *O*-(5-Acetamido-3,5-dideoxy-D-glycero-a-D-galacto-2-nonulopyranosylonic acid)-(2 \rightarrow 3)-(β -D-galactopyranosyl)-

(1→4)-(β-D-glucopyranosyl)-(1→1)-(2S, 3R, 4E)-2-octadecanamido-4-octadecene-1,3-diol (17)

39 mg, 84%. ¹H NMR (400 MHz, CD₃OD) δ 5.68 (dd, *J* = 14.4, 7.4 Hz, 1H), 5.46 (dd, *J* = 12.5, 4.8 Hz, 1H), 4.42 (t, *J* = 6.4 Hz, 1H), 4.30 (d, *J* = 6.8 Hz, 2H), 4.21 – 4.14 (m, 2H), 4.11 – 4.02 (m, 3H), 3.99 – 3.82 (m, 8H), 3.78 – 3.39 (m, 18H), 2.86 (d, *J* = 8.6 Hz, 1H), 2.17 (t, *J* = 7.6 Hz, 2H), 2.03 (d, *J* = 4.5 Hz, 2H), 2.01 (s, 3H), 1.74 (d, *J* = 7.1 Hz, 1H), 1.59 (s, 2H), 1.37 – 1.26 (m, 46H), 0.90 (t, *J* = 7.2 Hz, 6H). ¹³C NMR (101 MHz, CD₃OD) δ 173.69, 173.24, 172.69, 130.09, 127.62, 102.84, 102.21, 98.81, 78.59, 75.41, 74.81, 74.18, 73.94, 72.67, 72.55, 70.69, 69.18, 68.58, 67.85, 67.62, 67.11, 66.71, 64.41, 62.38, 60.48, 59.52, 52.44, 51.68, 35.14, 31.23, 30.84, 29.46, 28.64, 28.60, 28.58, 28.57, 28.53, 28.46, 28.39, 28.27, 28.24, 28.19, 24.93, 21.50, 20.35, 18.01, 12.24, 11.83.

4.2.9 O-(5-Glycolylacetamido-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-octadecanamido-4-octadecene-1,3-diol (18)

21 mg, 82%. ¹H NMR (400 MHz, CD₃OD) δ 5.78–5.71 (m, 1H), 5.49 (dd, *J* = 15.6, 4.2 Hz, 1H), 4.36 (d, *J* = 7.4 Hz, 1H), 4.29 (t, *J* = 6.6 Hz, 3H), 4.04 (s, 2H), 3.92–3.77 (m, 12H), 3.59–3.47 (m, 15H), 3.44 – 3.38 (m, 2H), 2.75 (d, *J* = 6.6 Hz, 1H), 2.09 (d, *J* = 7.4 Hz, 2H), 1.74–1.70 (m, 4H), 1.43–1.25 (m, 50H), 0.90 (t, *J* = 6.6 Hz, 6H). ¹³C NMR (101 MHz, CD₃OD) δ 177.09, 175.80, 174.70, 135.69, 130.99, 120.91, 104.91, 103.96, 100.74, 80.29, 79.27, 76.90, 76.33, 76.08, 74.62, 74.48, 72.35, 70.95, 70.67, 70.65, 70.09, 66.48, 62.30, 61.59, 61.55, 56.08, 49.65, 43.29, 33.23, 32.88, 31.52, 30.94, 30.63, 30.61, 30.59, 30.56, 30.44, 30.40, 30.31, 30.28, 30.24, 30.21, 30.17, 30.13, 30.09, 30.06, 30.03, 30.00, 30.00, 29.98, 23.54, 20.06, 14.24, 13.83. HRMS (ESI) *m/z* calcd for C₅₉H₁₀₇N₂O₂₂⁻, [M-H]⁻: 1195.7321; found: 1195.7369.

4.2.10 O-(5-Trifluoroacetamido-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-octadecanamido-4-octadecene-1,3-diol (19)

20 mg, 87%. ¹H NMR (400 MHz, CD₃OD) δ 5.75 – 5.63 (m, 1H), 5.48 – 5.41 (m, 1H), 4.42 (d, *J* = 7.8 Hz, 1H), 4.29 (t, *J* = 7.3 Hz, 2H), 4.09 – 4.01 (m, 2H), 3.98 – 3.74 (m, 11H), 3.68 – 3.37 (m, 10H), 2.84 (d, *J* = 7.6 Hz, 1H), 2.17 (t, *J* = 7.6 Hz, 2H), 2.02 (d, *J* = 7.0 Hz, 2H), 1.80 – 1.67 (m, 1H), 1.57 (br s, 2H), 1.34 – 1.25 (m, 54H), 0.89 (d, *J* = 5.4 Hz, 6H). ¹³C NMR (101 MHz, CD₃OD) δ 175.75, 174.83, 169.11, 134.82, 129.67, 104.88, 104.30, 100.85, 80.70, 77.36, 76.83, 76.26, 76.04, 74.63, 73.59, 73.26, 72.77, 70.67, 69.89, 69.71, 69.25, 68.59, 66.45, 64.38, 62.55, 61.68, 54.53, 53.81, 41.99, 37.18, 33.27, 32.88, 31.52, 30.68, 30.65, 30.62, 30.61, 30.57, 30.51, 30.43, 30.31, 30.28, 30.26, 30.23, 26.97, 23.54, 20.05, 14.25, 13.84. ¹⁹F NMR (376 MHz, CD₃OD) δ -76.93. HRMS (ESI) *m/z* calcd for C₅₉H₁₀₄F₃N₂O₂₁⁻, [M-H]⁻: 1233.7089; found: 1233.7065.

4.3 Cytotoxicity assay

The cytotoxicity of compounds was measured toward human colon cancer HCT-116 cells and murine melanoma B16-F10 cells with the help of MTT assay. Briefly, the cells (5×10⁴ cells/mL) were seeded into 96-well plates, and then treated with compounds with different concentration gradients at 0.01, 0.1, 1, 10, 100 μM or vehicle DMSO for 48 h. At the end of the

incubation period, 20 μL (5 mg/mL) of MTT solution was added into each well of the 96-well plate. Following 4 h of incubation at 37 °C and 5% CO₂, absorbance was measured at wavelengths of 492 nm and 630 nm. Calculate the IC₅₀ value of the compound by the difference in absorbance. The results are presented as means ± SD from the three independent experiments.

4.4 Wound healing test

Wound healing test was used to evaluate the effect of compounds on tumor cell motility by melanoma B16-F10 cell line. The cells (5×10⁴ cells/mL) were cultured into 12-well plates and grown in DMEM (high glucose) medium containing 10% FBS to nearly confluent cell monolayer. A wound healing culture-insert (IBIDI, two wells for self-insertion) was used to draw a “wound” in the cell monolayer of each well. The monolayer was then washed twice with PBS buffer in pH 7.4 to remove debris or detached cells, and compounds were added at different concentrations in fresh medium without FBS, DMEM (high glucose) medium containing DMSO was added to the control well as the solvent control, and subsequently cultured for 48 h. The wound healing of the scratched cells was photographed under a OLYMPUS CORPORATION microscope (TOKYO, JAPAN) after removed the culture-inserts of each well. The effect of compounds on tumor cell motility was expressed as migration % of 0 h (wound width at exposure time point/wound width at 0 h). The experiments were performed in triplicate.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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

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