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Changping ZHENG[a, ≠], Huanhuan QU[b, ≠, *], Wenfeng LIAO[b], Teodora BAVARO[d], Marco TERRENI[d], Matthieu SOLLOGOUB[a], Kan DING[b,*], Yongmin ZHANG[a,c,*]

[a] *Sorbonne Universités, UPMC Univ Paris 06, CNRS, UMR 8232, IPCM, 4 place Jussieu, 75005 Paris, France.*

[b] *Glycochemistry & Glycobiology Lab, Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Pudong, Shanghai 201203, China.*

[c] *Institute for Interdisciplinary Research, Jianghan University, Wuhan Economic and Technological Development Zone, Wuhan 430056, China.*

[d] *Drug Sciences Department, University of Pavia, Via le Taramelli 12, 27100 Pavia, Italy.*

* *Corresponding authors:*

Sorbonne Universités, UPMC Univ Paris 06, CNRS, UMR 8232, IPCM, 75005 Paris, France, e-mail address: yongmin.zhang@upmc.fr (Yongmin ZHANG).

Glycochemistry & Glycobiology Lab, Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Pudong Shanghai 201203, China, e-mail address: quhuanhuan@simm.ac.cn (Huanhuan QU), dingkan@simm.ac.cn (Kan DING).

[≠] *These two authors contributed equally to this work.*

Abstract

Ganglioside GM3 is implicated in a variety of physiological and pathological processes. Due to GM3 exposes on the outer surface of cell membranes, it is strongly associated with cell adhesion, motility and differentiation. Neurite outgrowth is a key process in the development of functional neuronal circuits and regeneration of the nervous system after injury. In the present study, we used enzymatic hydrolysis and chemical synthesis to obtain novel galactose containing GM3 analogues. By enzymatic hydrolysis to prepare GM3 building block, we can avoid multiple chemical procedures. Next, we employed the PC12 cells as a model to evaluate the effects of GM3 analogues on neurite outgrowth with or without NGF induction. The biological tests showed that GM3 analogues could induce neurite outgrowth, which provides the valuable sights for potential nervous system treatment after injury.

Keywords: ganglioside GM3, chemoenzymatic synthesis, PC12 cells, neurite outgrowth.

1. Introduction

Glycosphingolipids (GSLs) are ubiquitous components on mammalian cell membranes, and a variety of studies demonstrated that they are involved in many cellular functions, including cell proliferation, adhesion, motility and differentiation [1-3]. The changes of GSLs structures associated with biological functions have been a matter of research recently because structural change is the starting point for understanding biological significance, and enzymatic or genetic mechanisms [4]. Ganglioside GM3, the first and simplest member in the metabolic series of a GSLs family, contains a single terminal sialic acid, lactose and ceramide (**Figure 1**). It has been reported that ganglioside GM3 is implicated in some human serious diseases, such as cancers and diabetes [5,6].

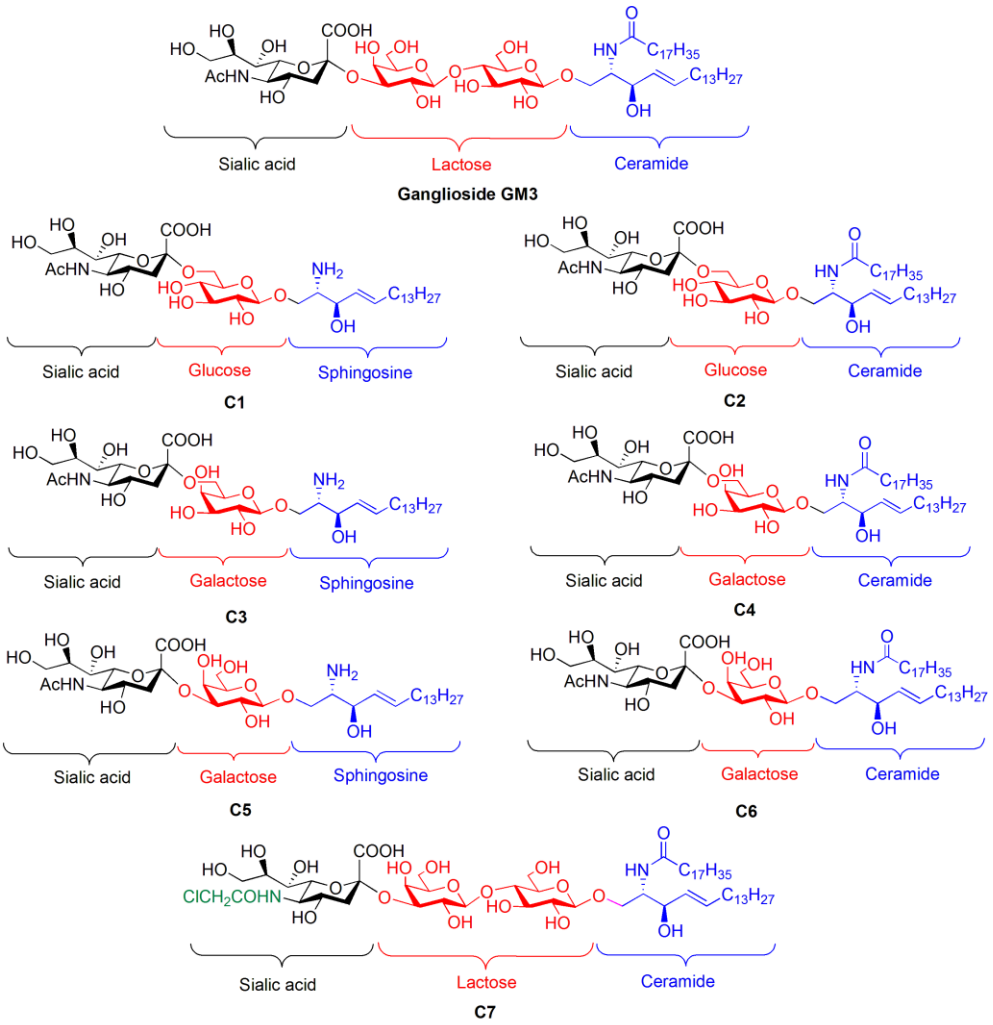


Figure 1. Chemical structure of ganglioside GM3 and its seven analogues, including compound

C1, C2, C3, C4, C5, C6 and C7, respectively.

Sialic acids are nine-carbon monosaccharides also involved in a wide range of biological phenomena. Being at the terminus of natural cell-surface glycoconjugates, sialic acids are ideally positioned to mediate carbohydrate-protein interactions in cell-cell recognition. Sialic acids also play important masking roles to prevent biological recognition. In addition, sialic acids act as receptors for some toxins, bacteria, and viruses [7]. In spite of extensive efforts and notable progress, the chemical synthesis of sialosides in high yield with complete stereoselectivity remains a significant challenge [8-10]. The presence of a destabilizing electron-withdrawing carboxylic group along with a tertiary anomeric center and the lack of a participating auxiliary often drive glycosylation reactions toward competitive elimination reactions resulting in poor stereoselectivity (β-anomer) and in the formation of a 2,3-dehydro derivative [11].

Neurite outgrowth is a key process in the development of functional neuronal circuits and regeneration of the nervous system after injury [12]. The addition of neurotrophic factors such as nerve growth factor (NGF) to rat PC12 pheochromocytoma cells leads to cell cycle arrest and differentiate into neuron-like cells with elongated neurites [13]. The phenotypic changes associated with NGF-induced differentiation include the biosynthesis of neurotransmitters, the acquisition of electrical excitability and the growth of axon-like extensions named neuritis through a process known as neuritogenesis [14].

In the present study, we firstly used enzymatic hydrolysis and chemical procedures to prepare GM3 analogues, and novel GM3 analogues C3 and C4 (**Figure 1**) were efficiently synthesized. Then along with a series of known analogues C1, C2, C5, C6 and C7 (**Figure 1**) [15,16], we employed the PC12 cells as a model to study the effects of these analogues on neurite outgrowth.

2. Results and discussion

2.1. Synthesis of GM3 analogues

For synthesizing GM3 analogues C3 and C4, three key building blocks were needed (**Figure 2**). Firstly, for the sialic acid block, it was activated as the sialyl xanthates form based on literature [17]. Then the galactose block G1 was prepared by enzymatic hydrolysis using the published method [18,19]. Finally, the 3-*O*-benzoyl-azidosphingosine block was synthesized from commercial D-(+)-Galactose as previous report with some modifications [20,21].

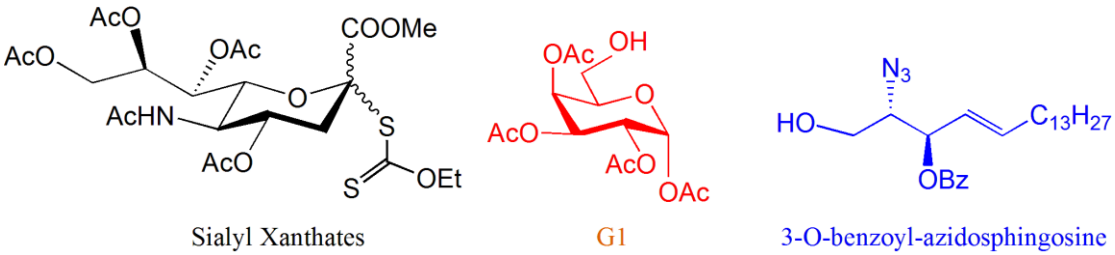
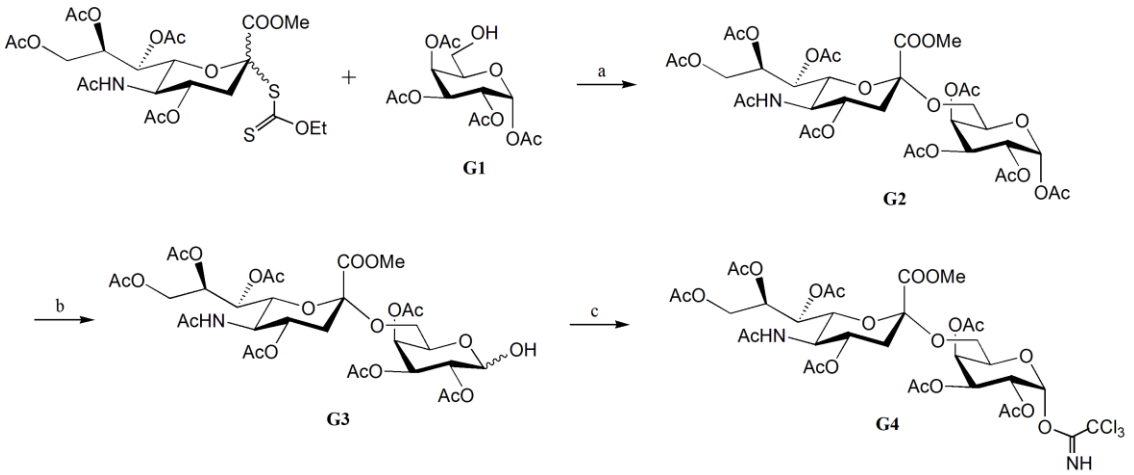


Figure 2. Three key building blocks for synthesizing analogues C3 and C4: sialyl xanthate block, galactose residue G1 block and 3-*O*-benzoyl-azidosphingosine block.

In fact, the study of complex molecules such as glycoconjugated products is some time limited by the complex procedure required for the preparation of oligosaccharides by the classical orthogonal synthetic strategy. In particular, the preparation of different sugar building blocks having only one free hydroxyl group in the position desired for glycosylation is the most time consuming task. In this study, the enzymatic hydrolysis is firstly introduced to prepare GM3 building block G1. The enzyme is specific for removing C-6 position acetyl group from all acetylated galactose. By enzymatic hydrolysis, we can avoid multiple chemical procedures.

Further the key step was α-sialylation between sialyl xanthates and free 6-OH of all acetylated galactose residue G1. In our previous studies, we tried to optimize the α-sialylation reaction between acceptors and the sialic acid donors. The reaction in the presence of benzenesulfenyl chloride (PhSCl) and silver trifluoromethanesulfonate (AgOTf) as promoters in a 2:1 mixture of CH_3CN/CH_2Cl_2 at low temperature afforded α sialoside in significantly higher yield [16]. So for the sialylation of galactose residue with free 6-OH, we directly selected this Martichonok and Whitesides method. As shown in **Scheme 1**, the α-sialylation reaction was carried out at -68 ℃ with PhSCl and AgOTf as promoters in the mixture of CH_2Cl_2:CH_3CN (1:2)

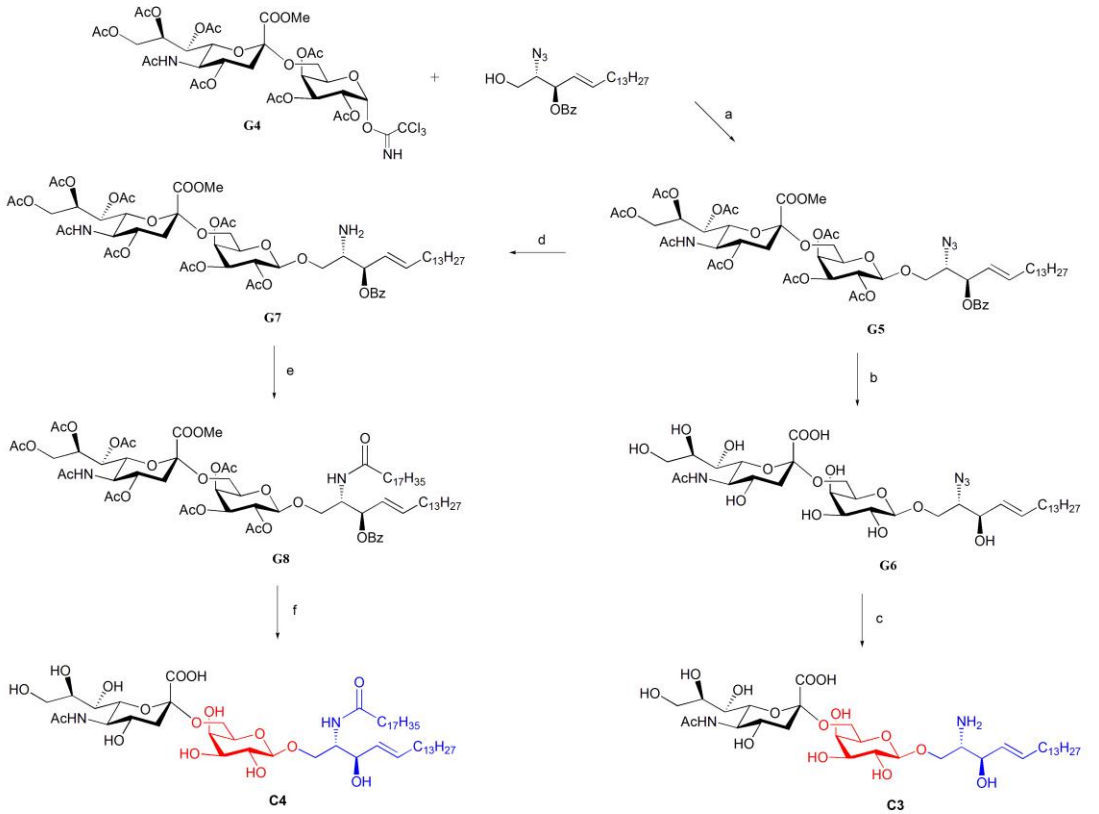
for 3 h. PhSCl was freshly prepared each time according to the previous method [17]. At last, the sialylation product G2 was absolute α isomer in the yield of 70%. In the ^{1}H NMR, for H-3eq of sialic acid residue, the chemical shift was smaller for α-glycosides than for β-glycosides. Next, for the α-sialosides G2, the acetyl group at the anomeric center was selectively removed by $PhCH_2NH_2$ in the dry THF at RT for 24 h to provide the hemiacetal G3 as α and β mixture in **Scheme 1**. This crude product G3 was not separated and could be directly used for the next step. This hemiacetal was then treated with trichloroacetonitrile in the presence of 1,8-diazabicyclo [5,4,0] undec-7-ene (DBU) to give the trichloroacetimidate G4 in 70% yield for two steps in **Scheme 1**. The ^{1}H NMR spectrum showed that G4 is the α-trichloroacetimidate on the basis of the H-1' and H-2' coupling constant ($J_{1',2'}$ = 3.7 Hz) of the galactose residue.



Scheme 1. Reagents and conditions: (a) CH_3CN, CH_2Cl_2, molecular sevies, r.t., 1 h, AgOTf, DTBP, -68 °C, PhSCl, 3 h, 70%; (b) $PhCH_2NH_2$, THF, r.t., 24 h; (c) CCl_3CN, DBU, CH_2Cl_2, -5 °C, 5 h, 70% (two steps from G2).

Conjugation of trichloroacetimidate G4 with 3-*O*-benzoyl-azidosphingosine was performed using $BF_3{\cdot}Et_2O$ as a promoter to provide the desired glycolipid G5 in 65% yield, as shown in **Scheme 2**. The β configuration of the newly introduced glycosidic linkage was confirmed from the ^{1}H NMR spectrum ($J_{1',2'}$ = 8.0 Hz). We adopted the 3-*O*-benzoyl-azidosphingosine block to conjugate with sugar block, not directly using ceramide block. Then all the acetyl groups of compound G5 were removed by NaOMe/MeOH. After adding several drops of water, compound G6 was obtained. Then the azide in the G6 was reduced with propanedithiol/triethylamine to afford the

new analogue C3 in 85% yield. On the other hand, the azide group of G5 was reduced by triphenylphosphine in a mixture of toluene and water at 50 ℃ for 12 h to give an amino free derivative G7. Again, G7 was not separated and directly reacted with stearic acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in dry CH_2Cl_2 to give the glycosyl ceramide G8 in 60% yield for two steps from G5. The acetyl groups of compound G8 were subsequently removed by NaOMe/MeOH. After adding several drops of water, ganglioside C4 was obtained with the yield of 95%.



Scheme 2. Reagents and conditions: (a) BF_3-Et_2O, CH_2Cl_2, molecular sevies, -15 ℃, 3 h, 65%; (b) NaOMe, MeOH, r.t., 14 h; H_2O, 0 ℃, 2 h; (c) $HS(CH_2)_3SH$, Et_3N, MeOH, r.t., 4 days, 85% (two steps from G5); (d) PPh_3, toluene, H_2O, 50 ℃, 12 h; (e) Stearic acid, EDC, CH_2Cl_2, r.t., 24 h, 60% (two steps from G5); (f) NaOMe, MeOH, r.t., 14 h; H_2O, 0 ℃, 2 h, 95%.

2.2. Biological evaluation of GM3 analogues

In this study, we used two models to investigate the effects of the compounds on neurite outgrowth. Firstly, we employed no NGF induced model, and in this model, NGF was used as a positive control. Generally, the cells maintained in low-serum medium were treated with NGF (25 ng/ml), C1, C2, C3, C4, C5, C6 and C7 (10 μM). After 72 h, the morphology of the cells was photographed under an inverted

microscope and the representative micrographs were exhibited in **Figure 3A**, and the number of neurite bearing cells was counted and shown in **Figure 3B**. Interestingly, neuritogenesis was augmented robustly by compound C1 comparing to the blank group (**Figure 3A-c**), the percentage of neurite-bearing cells reached 26%, even higher than that of the NGF group at the tested concentration (**Figure 3B**). The compound C3 (**Figure 3A-e**), C5 (**Figure 3A-g**) and C7 (**Figure 3A-i**) could significantly promote neurite outgrowth, while the percentage of neurite-bearing cells was 9%, 6% and 8%, respectively (**Figure 3B**). While the compound C2 (**Figure 3A-d**), C4 (**Figure 3A-f**) and C6 (**Figure 3A-h**) had no obvious effects on neuritogenesis, as the proportion of neurite-bearing cells was closed to the blank group (**Figure 3B**).

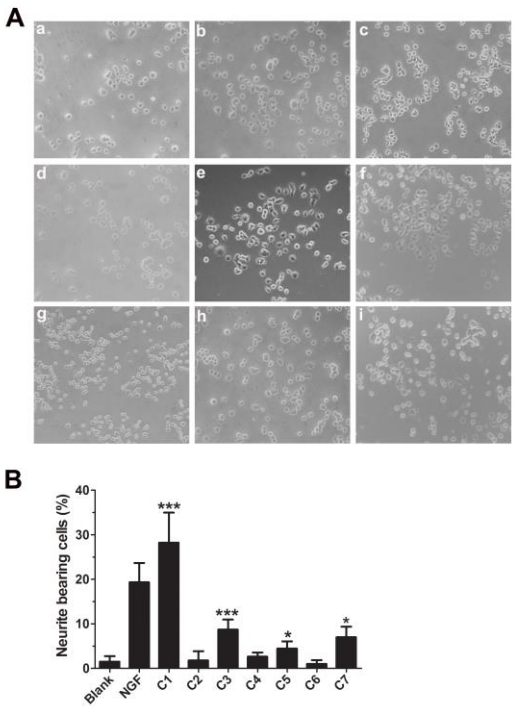


Figure 3. Compound C1, C3, C5 and C7 could induce the neuritogenesis of PC12 cells. (A): PC12 cells were seeded in a 12-well plate, incubated overnight and then were maintained in a low-serum medium (DMEM supplemented with 1.0% horse serum and 0.5% FBS) for 24 h, followed by the treatment with the compound C1 (c), C2 (d), C3 (e), C4 (f), C5 (g), C6 (h), C7 (i) at the concentration of 10 μM or 25 ng/ml NGF (j) or without (a) for an additional 72 h. The cells were photographed by a phase contrast microscope, and the representative micrographs of each group were exhibited. (B): Quantification of PC12 cells bearing one neurite at least twice the length of the cell body diameter in each group were shown. Results are presented as means ± S.D., scale bar, 5 μm, and experiments were repeated at least three times.***, $p<0.001$; *, $p<0.05$.

The NGF-induced PC12 cell model was a common tool to study neuritogenesis, we then investigated the effects of these compounds on neurite outgrowth in the NGF-induced model. In this model, NGF (at the concentration of 25 ng/ml) was added into the cell culture medium along with the compounds. The results showed that the proportion of neurite-bearing cells in C1 is more than 30% (**Figure 4A-b** and **Figure 4B**), and the C5 (**Figure 4A-f** and **Figure 4B**) also could significantly accelerate neuritogenesis comparing to NGF group. It seemed that C2 (**Figure 4A-c**), C3 (**Figure 4A-d**), C4 (**Figure 4A-e**), C6 (**Figure 4A-g**) and C7 (**Figure 4A-h**) had no obvious effects on neurite outgrowth comparing to NGF group. These data indicated that GM3 analogues C1 and C5 potently induce neurite outgrowth in PC12 cells both in NGF free or NGF-induced systems.

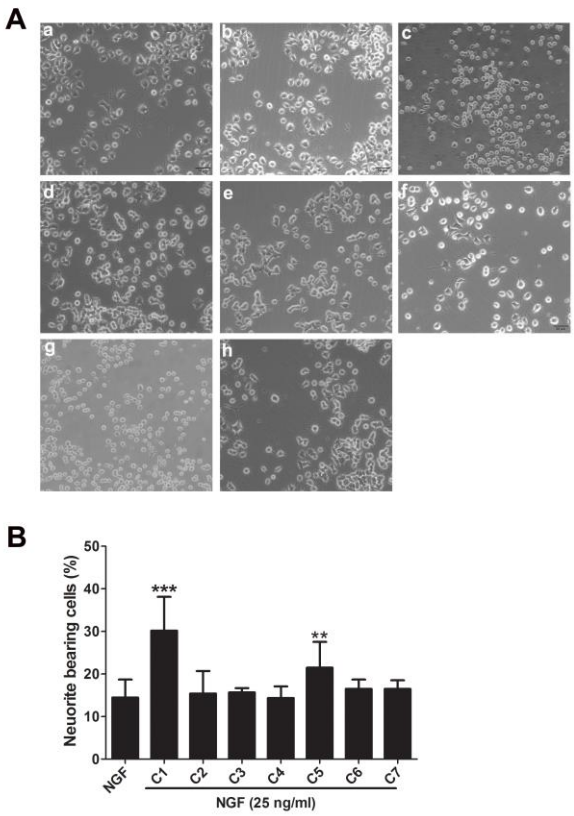


Figure 4. Compound C1 and C5 could promote NGF-induced PC12 cells neuritogenesis. (A): The representative micrographys of neuritigenesis of PC12 cells in each group were presented. In this assay, PC12 cells were plated in a 12-well plate, 12 h later, the culture medium was changed by the low-serum medium and the cells were maintained for further 24 h, followed by the treatment of the compound C1 (b), C2 (c), C3 (d), C4 (e), C5 (f), C6 (g) and C7 (h) accompanied by NGF for an additional 72 h. The morphology of the cells were photographed by the inverted microscope. (B): The proportion of PC12 cells bearing one neurite at least twice the length of the cell body

diameter in each group were showed. Results are presented as means ± S.D., scale bar, 5 μm, and experiments were repeated at least three times.***, $p<0.001$; *, $p<0.05$.

Several literatures had emphasized the important role of GM3 and glycolipids in neuritogenesis [22,23]. For example, in mouse neuroblastoma Neuro2a cells, studies found that GM3 enriched microdomains were involved in signal transduction during neurite formation [24]. PC12 cell line has been widely used as a model system for studying neuronal differentiation and neurosecretion [13,25]. In the present study, we firstly employed the PC12 cells as a model to evaluate the effects of the GM3 analogues on neuritogenesis. Our studies demonstrated that some GM3 analogues could induce neurite outgrowth. From the preliminary studies, it seems that GM3 analogues with free amino group NH_2 at the sphingosine are more prone to induce neurite outgrowth, despite of different sugar composition or linkages.

It has been reported that nervous system injuries affect many people every year, and due to its high incidence, nerve regeneration or repair, is becoming a rapidly growing field dedicated to the discovery of new ways to recover nerve functionality after injury [26,27]. Neurite outgrowth is a key process in the development of functional neuronal circuits and regeneration of the nervous system after injury. The ability of GM3 analogues to induce neurite outgrowth can provide a valuable sight for developing potential therapeutic agents to recover nervous functionality after injury.

3. Conclusion

Taken together, two new GM3 analogues were synthesized by enzymatic hydrolysis and chemical procedures from simple and commercially available substrates and reagents. By chemoenzymatic synthesis, we can avoid multiple procedures. Further, we firstly employed PC12 cells model to demonstrate that some GM3 analogues could induce neurite outgrowth with or without NGF induction, which provides the valuable sights for potential nervous system treatment after injury.

4. Experimental section

4.1. General methods

All chemicals were purchased as reagent grade and used without further purification. All reactions were carried out under N_2 atmosphere and anhydrous conditions with freshly distilled solvents, unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of silica gel 60 F254 (Merck) and detection by staining with sulfuric acid. Solvents were evaporated under reduced pressure and below 40 °C (water bath). Flash column chromatography was performed on silica gel 60 (230-400 mesh, Merck). ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz with Bruker AVANCE DRX 400 spectrometer. The chemical shifts were referenced to the solvent peak, 7.26 ppm (^{1}H) and 77.16 ppm (^{13}C) for $CDCl_3$, 3.31 ppm (^{1}H) and 49.00 ppm (^{13}C) for CD_3OD, 2.50 ppm (^{1}H) and 39.52 ppm (^{13}C) for DMSO, at 25 °C, and coupling constants were given in Hz. High-resolution mass spectra (HRMS) were recorded with a Bruker micrOTOF spectrometer in electrospray ionization (ESI) mode, using Tuning-Mix as reference. Optical rotations were measured at 589 nm (Na line) at 20 °C with a Perkine Elmer Model 343 digital polarimeter, using a 10 cm, 1 mL cell.

4.2. O - (Methyl 5 - acetamido - 4,7,8,9 - tetra - O - acetyl - 3,5 - dideoxy - D - glycero - α - D - galacto - 2 - nonulopyranosylonate) - (2 → 6) - 1,2,3,4 - tetra - O - acetyl - α - D - galactopyranoside (G2)

A mixture of sialyl xanthates (1.03g, 1.73 mmol) and compound G1 (400 mg, 1.15 mmol) with 4Å powdered molecular sieves (2.2 g) were dissolved in the dry CH_3CN (24 mL) and CH_2Cl_2 (12 mL) stirring at RT for 1 h. Further, AgOTf (488 mg, 1.90 mmol) and DTBP (0.45 mL, 2.00 mmol) were added, and the mixture was cooled to -68 ^{o}C and kept protected from light. Then PhSCl (0.23 mL, 2.00 mmol) in dry CH_2Cl_2 (1.2 mL) was added by running the solution down the cold wall of the reaction flask. Finally, the mixture was stirred for 3 h at -68 ^{o}C. After that, the mixture was diluted with a suspension of silica gel (6 g) in EtOAc (36 mL), filtered through celite, washed with saturated aqueous $NaHCO_3$ and water, dried with $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed (Cy-EtOAc 1:3) to give the compound G2 as a white foam (659.4 mg, 70%). R_f = 0.29

(Cy-EtOAc 1:3, twice). $[\alpha]_D^{20} = -11.9$ (*c* 1.0 in $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$): δ 6.29 (d, *J*=3.7 Hz, 1H, H-1'), 5.42 (t, *J*=9.8 Hz, 1H, H-3'), 5.32–5.27 (m, 3H, H-7, H-8, NH), 5.19 (d, *J*=9.3 Hz, 1H, H-5'), 5.06 (dd, *J*=10.2, 3.6 Hz, 1H, H-2'), 4.88–4.81 (m, 1H, H-4), 4.25 (dd, *J*=11.3, 3.2 Hz, 1H, Ha-9), 4.07–4.00 (m, 4H, H-4', H-5, Ha-6', H-6), 3.93 (dd, *J*=11.2, 3.3 Hz, 1H, Hb-9), 3.78 (s, 3H, COOCH_3), 3.36 (dd, *J*=11.4, 1.9 Hz, 1H, Hb-6'), 2.61 (dd, *J*=13.4, 4.7 Hz, 1H, H-3eq), 2.15 (s, 3H, OAc), 2.14 (s, 6H, 2 × OAc), 2.07 (s, 3H, OAc), 2.02 (s, 9H, 3 × OAc), 1.98 (s, 3H, OAc), 1.92 (t, 1H, *J*=9.8 Hz, H-3ax), 1.86 (s, 3H, NAc). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.49 (C=O), 172.40 (C=O), 171.89 (C=O), 171.80 (C=O), 171.67 (C=O), 171.61 (C=O), 171.52 (C=O), 171.48 (C=O), 170.96 (C=O), 169.18 (C=O), 100.09 (C-2), 90.82 (C-1'), 73.46, 71.06, 70.56, 69.32, 69.18, 68.95, 68.54, 68.15 (C-6, C-4', C-3', C-2', C-4, C-8, C-5', C-7), 63.93 (C-9), 63.53 (C-6'), 53.51 (COOCH_3), 50.01 (C-5), 38.91 (C-3), 22.73 (CH_3, NAc), 21.22 (CH_3, OAc), 20.91 (CH_3, OAc), 20.80 (CH_3, OAc), 20.73 (CH_3, OAc), 20.70 (CH_3, OAc), 20.68 (CH_3, OAc), 20.61 (CH_3, OAc), 20.43 (CH_3, OAc). ESI-HRMS (*m*/*z*) calcd for $C_{34}H_{47}NO_{22}Na$ $[M+Na]^+$: 844.2487, found: 844.2469.

4.3. O - (Methyl 5 - acetamido - 4,7,8,9 - tetra - O - acetyl - 3,5 - dideoxy - D - glycero - α - D - galacto - 2 - nonulopyranosylonate) - (2 → 6) - 2,3,4 - tri - O - acetyl - α - D-galactopyranosyl trichloroacetimidate (G4)

The solution of compound G2 (610 mg, 0.74 mmol) in 3 mL of dry THF was added $PhCH_2NH_2$ (122 µL, 1.12 mmol). The mixture was stirred at RT for 24 h. The solvent was evaporated and the residue was taken into CH_2Cl_2 and washed progressively with 1.0 M HCl, saturated aqueous $NaHCO_3$ and water. The organic layer was dried over $MgSO_4$ and concentrated to give the crude intermediate G3, R_f = 0.43 (EtOAc). The crude intermediate was directly engaged in the following step without further purification. Then the intermediate G3 was dissolved in the dry CH_2Cl_2 (19.6 mL), and 2 mL of trichloroacetonitrile was added to the solution. After that, 192 µL of DBU was added dropwise at -5 ^oC, and the mixture was stirred at -5 ^oC for 5 h. After concentration, the residue was purified by flash column chromatography (Cy-EtOAc 1:4) to yield compound G4 as pale yellow foam (514.1 mg, 70% for two steps from G2). R_f = 0.48 (EtOAc). $[\alpha]_D^{20} = +7.8$ (*c* 1.0 in $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.74 (s, 1H, NH), 6.40 (d, *J*=3.7 Hz, 1H, H-1'), 5.42 (t, *J*=9.8 Hz, 1H, H-3'), 5.32–5.27 (m, 3H, H-7, H-8, H-5'), 5.06 (dd, *J*=10.2, 3.6 Hz, 1H, H-2'), 4.88–4.81 (m, 1H, H-4), 4.25 (dd, *J*=11.3, 3.2 Hz, 1H, Ha-9), 4.07–4.00 (m, 4H, H-4', H-5, Ha-6', H-6), 3.93 (dd, *J*=11.2, 3.3 Hz, 1H, Hb-9), 3.78 (s, 3H, COO*CH*$_3$), 3.36 (dd, *J*=11.4, 1.9 Hz, 1H, Hb-6'), 2.61 (dd, *J*=13.4, 4.7 Hz, 1H, H-3eq), 2.16 (s, 3H, OAc), 2.14 (s, 6H, 2 × OAc), 2.07 (s, 3H, OAc), 2.02 (s, 6H, 2 × OAc), 1.98 (s, 3H, OAc), 1.92 (t, 1H, *J*=9.8 Hz, H-3ax), 1.86 (s, 3H, NAc). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.40 (C=O), 171.89 (C=O), 171.80 (C=O), 171.67(C=O), 171.61 (C=O), 171.52 (C=O), 171.48 (C=O), 170.96 (C=O), 169.18 (C=O), 161.17 (C=NH), 100.09 (C-2), 92.50 (C-1'), 90.00 (CCl_3) 73.46, 71.06, 70.56, 69.32, 69.18, 68.95, 68.54, 68.15 (C-6, C-4', C-3', C-2', C-4, C-8, C-5', C-7), 63.93 (C-9), 63.53 (C-6'), 53.51 (COO*CH*$_3$), 50.01 (C-5), 38.91 (C-3), 22.73 (CH_3, NAc), 20.91 (CH_3, OAc), 20.80 (CH_3, OAc), 20.73 (CH_3, OAc), 20.70 (CH_3, OAc), 20.68 (CH_3, OAc), 20.61 (CH_3, OAc), 20.43 (CH_3, OAc). ESI-HRMS (*m/z*) calcd for $C_{35}H_{48}N_2Cl_3O_{21}Na$ $[M+Na]^+$: 960.1713, found: 960.1701.

4.4. O- (Methyl 5 - acetamido - 4,7,8,9 - tetra - O - acetyl - 3,5 - dideoxy - D - glycero - α - D - galacto - 2 - nonulopyranosylonate) - (2 → 6) - (2,3,4 - tri - O - acetyl - β - D - galactopyranosyl) - (1 → 1) - (2S, 3R, 4E) - 2 - azido - 3 - O - benzoyl - 4 - octadecene - l,3 - diol (G5)

The compound G4 (114.6 mg, 0.124 mmol) and 3-*O*-benzoyl-azidosphingosine (90.2 mg, 0.21 mmol) in 6 mL of dry CH_2Cl_2 was stirred with 4Å powdered molecular sieves (500 mg) at RT for 1 h. The mixture was then cooled to -15 ^{o}C, and BF_3•Et_2O (78 µL, 0.62 mmol) was added dropwise, stirred for 3 h at -15 ^{o}C, and then filtered through Celite. The filtrate was washed with saturated aqueous $NaHCO_3$ and water, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was applied to a flash chromatography eluted with Cy-EtOAc 1:5 to give the product G5 (96.0 mg, 65%) as an amorphous solid. R_f = 0.58 (EtOAc). $[\alpha]_D^{20}$ = −14.5 (*c* 1.0 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02–7.99 (m, 2H, Ar-H), 7.58–7.54 (m, 1H, Ar-H), 7.46–7.42 (m, 2H, Ar-H), 5.85 (dd, *J*=14.2, 7.3 Hz, 1H, H-5''), 5.59–5.48 (m, 2H, H-3'', H-4''), 5.36–5.23 (m, 5H, H-4, H-7, H-8, NH, H-3'), 5.12 (d, *J*=9.59, 1H, H-2'), 4.92–4.84 (m, 1H, H-4'), 4.73 (d, *J*=8.0 Hz, 1H, H-1'), 4.50 (m, 1H, Hb-9), 4.27 (dd, *J*=12.4, 2.7 Hz, 1H, Ha-9), 4.08–3.99 (m, 3H, Ha-6', H-2'', H-5), 3.94–3.87 (m, 2H, H-5', Hb-6'), 3.79 (m, 1H, Ha-1''), 3.76 (s, 3H, COO*CH*$_3$), 3.65 (dd, *J*=10.8, 4.3 Hz, 1H, Hb-1''), 3.40 (d, *J*=9.1 Hz, 1H, H6), 2.59 (dd, *J*=12.7, 4.6 Hz, 1H, H-3eq),

2.20 (m, 2H, H_2-6''), 2.13 (s, 3H, OAc), 2.12 (d, *J*=2.2 Hz, 6H, 2 × OAc), 2.05 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.94 (t, *J*=11.2 Hz, 1H, H-3ax), 1.86 (s, 3H, NAc), 1.36 (m, 2H, H_2-7''), 1.24 (s, 20H, 10 × CH_2), 0.86 (t, *J*=6.8 Hz, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 172.40 (C=O), 171.89 (C=O), 171.80 (C=O), 171.67(C=O), 171.61 (C=O), 171.52 (C=O), 171.48 (C=O), 170.96 (C=O), 169.18 (C=O), 166.51 (PhC=O), 138.24 (C-5''), 133.53 (C, CH aromatic), 130.48 (C aromatic), 130.15 (2C, CH aromatic), 128.90 (2C, CH aromatic), 125.08 (C-4''), 100.09 (C-1'), 97.53 (C-2), 75.05 (C-3''), 73.46 (C-3'), 71.06 (C-4'), 70.56 (C-6), 69.81 (C-2'), 69.32 (C-4), 69.18 (C-5'), 68.95 (C-1''), 68.54 (C-8), 68.15 (C-7), 64.05 (C-2''), 63.93 (C-6'), 63.53 (C-9), 53.51 (COO*CH*$_3$), 50.01 (C-5), 38.94 (C-3), 32.38 (C-6''), 30.17, 30.16, 30.12, 29.82 (11 × CH_2), 22.73 (CH_3, NAc), 20.91 (CH_3, OAc), 20.80 (CH_3, OAc), 20.73 (CH_3, OAc), 20.70 (CH_3, OAc), 20.68 (CH_3, OAc), 20.61 (CH_3, OAc), 20.44 (CH_3, OAc), 14.57 (CH_3). ESI-HRMS (*m*/*z*) calcd for $C_{57}H_{82}N_4O_{23}Na$ $[M+Na]^+$: 1213.5268, found: 1213.5286.

4.5. O - (5 - Acetamido - 3,5 - dideoxy - D - glycero - α - D - galacto - 2 - nonulopyranosylonic acid) - (2 → 6) - (β - D - galactopyranosyl) - (1 → 1) - (2S, 3R, 4E) - 2 - amino - 4 - octadecene - l,3 - diol (C3)

The compound G5 (40.0 mg, 0.034 mmol) in 6.7 mL of NaOMe/MeOH (0.04 M) was stirred at RT for 14 h. Then a few drops of water were added at 0 ^{o}C. After stirring at RT for 2 h, the mixture was neutralized by Amberlite IR 120/H^+ ion exchange resin. After filtration and concentration, the residue was dried in vacuo to afford crude intermediate G6, R_f = 0.39 (EtOAc-*i*PrOH-H_2O 4:2:1). The crude intermediate G6 in the anhydrous MeOH (1.8 mL) was added propane-1,3-dithiol (0.18 mL) and triethylamine (0.18 mL), and the mixture was stirred at RT for 4 days. A white precipitate was formed. After filtration and washing with MeOH, the filtrate was concentrated under reduced pressure. The residue obtained was flash-chromatographed, eluting with $CHCl_3$-MeOH 3:1 to yield C3 (21.5 mg, 85% for two steps from G5) as a white amorphous solid. R_f = 0.33 (EtOAc-*i*PrOH-H_2O 4:2:1). $[\alpha]_D^{20}$ = −2.1 (*c* 1.0 in $CHCl_3$-MeOH 1:1). ^{1}H NMR (400 MHz, DMSO-d_6): *δ* 8.48 (s, 1H, NH), 5.74–5.67 (m, 1H, H-5''), 5.49–5.43 (m, 1H, H-4''), 4.45 (d, *J*=7.4 Hz, 2H, H-3'', H-1'), 4.12 (d, *J*=9.1 Hz, 1H, Ha-1''), 3.90 (dt, *J*=10.5, 9.1 Hz, 3H, Ha-9, Hb-1'', Hb-9), 3.70 (d, *J*=9.2 Hz, 3H, Ha-6', H-4, H-4'), 3.58–3.45 (m, 3H, H-3', H-5, Hb-6'), 3.35 (d, *J*=8.6 Hz, 2H, H-8, H-5'), 3.30–3.21 (m, 2H, H-6, H-2''), 3.10 (dd, *J*=16.4, 8.5 Hz, 2H, H-2', H-7), 2.73 (d, *J*=7.4, 3.8 Hz, 1H, H-3eq), 2.06–2.01 (m, 2H,

H_2-6’’), 2.00 (s, 3H, NAc), 1.61–1.56 (m, 3H, H-3ax, H_2-7’’), 1.29 (s, 20H, 10 × CH_2), 0.90 (t, *J*=6.8 Hz, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.18 (C=O), 168.65 (C=O), 134.18 (C-5’’), 128.44 (C-4’’), 100.09 (C-1’), 97.53 (C-2), 75.05 (C-3’’), 73.46 (C-3’), 71.06 (C-4’), 70.56 (C-6), 69.81 (C-2’), 69.32 (C-4), 69.18 (C-5’), 68.95 (C-1’’), 68.54 (C-8), 68.15 (C-7), 64.36 (C-2’’), 63.93 (C-6’), 63.53 (C-9), 50.97 (C-5), 40.12 (C-3), 30.17, 30.16, 30.12, 29.82 (CH_2), 22.71 (CH_3, NAc), 15.47 (CH_3). ESI-HRMS (*m*/*z*) calcd for $C_{35}H_{63}N_2O_{15}$ $[M-H]^+$: 751.4307, found: 751.4321.

4.6. O - (Methyl 5 - acetamido - 4,7,8,9 - tetra - O - acetyl - 3,5 - dideoxy - D - glycero - α - D - galacto - 2 - nonulopyranosylonate) - (2 → 6) - (2,3,4 - tri - O - acetyl - β - D - galactopyranosyl) - (1 → 1) - (2S, 3R, 4E) - 2 - octadecanamido - 3 - O - benzoyl - 4 - octadecene - l,3 - diol (G8)

To a solution of compound G5 (40 mg, 0.034 mmol) in 3.9 mL of toluene and 0.16 mL of water was added 22.0 mg of triphenylphosphine. The mixture was stirred at 50 ^{o}C for 12 h. After concentration, the crude residue G7, R_f = 0.26 (EtOAc), was directly used for the next step. The intermediate G7, stearic acid (32.9 mg, 0.12 mmol), EDC•HCl (34.2 mg, 0.18 mmol) in 5.4 mL of CH_2Cl_2 were stirred at RT for 24 h. Then the mixture was washed with water, dried over $MgSO_4$ and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography (Cy-EtOAc 1:3) to afford compound G8 (37.2 mg, 60% for two steps from G5) as an amorphous solid. R_f = 0.33 (Cy-EtOAc 1:3). $[\alpha]_D^{20}$ = −17.5 (*c* 1.0 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02–7.99 (m, 2H, Ar-H), 7.58–7.54 (m, H, Ar-H), 7.45–7.42 (m, 2H, Ar-H), 5.93–5.83 (m, 2H, NH, H-5’’), 5.59–5.48 (m, 2H, H-3’’, H-4’’), 5.34–5.23 (m, 4H, H-8, H-7, H-3’, NH), 5.12 (d, *J*=9.3 Hz, H-5’), 4.94–4.83 (m, 1H, H-2’), 4.73 (d, *J*=9.5 Hz, 1H, H-1’), 4.54–4.47 (m, 1H, H-4), 4.26 (dd, *J*=12.4, 2.6 Hz, 1H, Ha-9), 4.07–4.01 (m, 3H, Hb-9, H-5, H-6), 3.94–3.89 (m, 3H, H-2’’, Ha-6’, Hb-6’), 3.81 (dd, *J*=10.5, 3.1 Hz, , Ha-1’’), 3.77 (s, 3H, COO*CH*$_3$), 3.66 (dd, *J*=5.7, 2.5 Hz, 1H, H-4’), 3.40 (d, *J*=8.9 Hz, 1H, Hb-1’’), 2.59 (dd, *J*=12.8, 4.6 Hz, 1H, H-3eq), 2.25–2.18 (m, 2H, CO*CH*$_2$), 2.13 (s, 3H, OAc), 2.12 (d, *J*=1.5 Hz, 6H, 2 × OAc), 2.05 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.94 (t, *J*=7.4 Hz, 1H, H-3ax), 1.86 (s, 3H, NAc), 1.64–1.59 (m, 2H, H_2-6’’), 1.42 (m, 2H, H_2-7’’), 1.24 (s, 50H, 25 × CH_2), 0.87 (t, *J*=6.9 Hz, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.49 (C=O), 172.40 (C=O), 171.89 (C=O), 171.80 (C=O), 171.67 (C=O), 171.61 (C=O), 171.52 (C=O), 171.48 (C=O), 170.96 (C=O),

169.18 (C=O), 166.49 (PhC=O), 138.24 (C-5''), 133.53 (C, CH aromatic), 130.48 (C aromatic), 130.15 (2C, CH aromatic), 128.90 (2C, CH aromatic), 125.08 (C-4''), 100.09 (C-1'), 97.53 (C-2), 75.05 (C-3''), 73.46 (C-3'), 71.06 (C-4'), 70.56 (C-6), 69.81 (C-2'), 69.32 (C-4), 69.18 (C-5'), 68.95 (C-1''), 68.54 (C-8), 68.15 (C-7), 64.36 (C-2''), 63.93 (C-6'), 63.53 (C-9), 53.51 (COO*CH*$_3$), 50.01 (C-5), 38.94 (C-3), 31.38, 30.17, 30.16, 30.12, 29.82 (CH_2), 22.73 (CH_3, NAc), 20.91 (CH_3, OAc), 20.80 (CH_3, OAc), 20.73 (CH_3, OAc), 20.70 (CH_3, OAc), 20.68 (CH_3, OAc), 20.61 (CH_3, OAc), 20.44 (CH_3, OAc), 14.57 (2 × CH_3). ESI-HRMS (*m*/*z*) calcd for $C_{75}H_{118}N_2O_{24}Na$ $[M+Na]^+$: 1453.7972, found: 1453.7988.

4.7. O - (5 - Acetamido - 3,5 - dideoxy - D - glycero - α - D - galacto - 2 - nonulopyranosylonic acid) - (2 → 6) - (β - D - galactopyranosyl) - (1 → 1) - (2S, 3R, 4E) - 2 - octadecanamido - 4 - octadecene - l,3 - diol (C4)

A solution of compound G8 (30 mg, 0.021 mmol) in 4.1 mL of NaOMe/MeOH (0.04 M) was stirred at RT for 14 h. A few drops of water were added at 0 ^oC. Then the mixture was stirred at RT for 2 h, and further neutralized by Amberlite IR 120/H^+ ion exchange resin. After filtration and concentration, the residue obtained was flash-chromatographed, eluting with $CHCl_3$-MeOH 3:1 to yield C4 (20.2 mg, 95%) as a white amorphous solid. R_f = 0.36 (EtOAc-*i*PrOH-H_2O 4:2:1). $[\alpha]_D^{20}$ = +6.1 (*c* 1.0 in $CHCl_3$-MeOH 1:1). ^1H NMR (400 MHz, CD_3OD): *δ* 5.74–5.67 (m, 1H, H-5''), 5.49–5.43 (m, 1H, H-4''), 4.41 (d, *J*=9.8 Hz, 1H, H-1'), 4.09–4.00 (m, 2H, Ha-1'', H-3''), 3.99–3.95 (m, 1H, H-3'), 3.87–3.80 (m, 5H, H-4, H-5, H-4', Ha-6', Hb-1''), 3.70–3.51 (m, 9H, H-6, H-7, H-8, Ha-9, Hb-9, H-2', H-5', Hb-6', H2''), 3.35 (s, 1H, NH), 2.71 (dd, *J*=12.1, 4.9 Hz, 1H, H-3eq), 2.18 (t, *J*=6.8 Hz, 2H, *CH*$_2$C(O)), 2.06–2.01 (m, 2H, H_2-6''), 2.00 (s, 3H, NAc), 1.79 (t, *J*=12.1 Hz, 1H, H-3ax), 1.58 (m, 2H, *CH*$_2$$CH_2$C(O)), 1.29 (s, 50H, 25 × CH_2), 0.90 (t, *J*=7.4 Hz, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, CD_3OD): *δ* 174.16 (C=O), 169.18 (C=O), 168.65 (C=O), 134.18 (C-5''), 128.44 (C-4''), 100.09 (C-1'), 97.53 (C-2), 75.05 (C-3''), 73.46 (C-3'), 71.06 (C-4'), 70.56 (C-6), 69.81 (C-2'), 69.32 (C-4), 69.18 (C-5'), 68.95 (C-1''), 68.54 (C-8), 68.15 (C-7), 64.36 (C-2''), 63.93 (C-6'), 63.53 (C-9), 50.97 (C-5), 40.12 (C-3), 30.17, 30.16, 30.12, 29.82 (CH_2), 22.71 (CH_3, NAc), 15.47 (2 × CH_3). ESI-HRMS (*m*/*z*) calcd for $C_{53}H_{97}N_2O_{16}$ $[M-H]^+$: 1017.6916, found: 1017.6934.

4.8. Biological evaluation

4.8.1. Cell culture

PC12 cells were obtained from the ATCC (Manassas, VA, USA). The cells were maintained in DMEM (Dulbecco's modified Eagle's medium; HyClone, USA), supplemented with 10% (v/v) heat-inactivated horse serum (Gibco, USA) and 5% (v/v) FBS (Gibco, USA). The cells were cultured in a humidified incubator at 37 ^{o}C with 5% CO_2 atmosphere.

4.8.2. Neurite outgrowth assay

The experiments were conducted as the previous study [28]. Briefly, PC12 cells were seeded into a 12-well plate pre-coated with 50 ng/ml poly-L-lysine (Sigma–Aldrich, USA) at a density of 2×10^4 cells/well. The cells were allowed to attach for 24 h, then treated with DMEM containing 1% horse serum and 0.5% FBS for 24 h. In the non-NGF-induced experiments, PC12 cells were treated by the indicated compounds at the concentration of 10 μM or treated by NGF (25 ng/ml) alone, subsequently cultured for another 72 h. In the NGF-induced assay, NGF (25 ng/ml) was added into the culture medium accompanied with the compounds, then maintained for further 72 h. PC12 cell morphology was observed by the inverted microscope (Olympus, Japan) and determined by counting the proportion of cells containing at least one neurite twice as long as the cell body diameter, at least over 200 cells per sample were scored (Image J software, NIH, USA).

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Supporting information

Supplementary data of NMR spectra associated with this article can be found in the online version.

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