Structure of a New Neuritogenic-Active Ganglioside from the Sea Cucumber *Stichopus japonicus*[‡]

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A new ganglioside molecular species **SJG-2** has been obtained from the *n*-hexane-soluble lipid fraction of the chloroform/methanol extract of the sea cucumber *Stichopus japonicus*. On the basis of chemical and spectroscopic evidence, the structure of **SJG-2** has been determined, as NeuAcα2→4(NeuAcα2→3)Galβ1→8NeuAcα2→3GalNAcβ1→3Galβ1→4Glcβ1→1Cer. The new ganglioside **SJG-2**, possessing a unique carbohydrate moiety, is the first ganglioside containing either a branched sugar chain moiety or an *N*-acetylgalactosamine residue to be isolated from a sea cucumber. Partial hydrolysis with hot water and dilute acetic acid has proved useful for the structure elucidation of the complex oligosaccharide moieties. Moreover, the ganglioside **SJG-2** exhibits neuritogenic activity toward the rat pheochromocytoma cell line PC12 cells in the presence of NGF.

Introduction

In our continuing research on biologically active glycosphingolipids from echinoderms, we have performed a series of studies on the isolation, structure elucidation, and evaluation of biological activities of the glycosphingolipids from the sea cucumber species.[1] In the preceding study, we reported the structure and the neuritogenic activity of a new ganglioside molecular species **SJG-1** from the sea cucumber *Stichopus japonicus* ("Manamako" in Japanese).[1e] Our continuing search for the biologically active gangliosides from *S. japonicus* has led to the further isolation of a new polar ganglioside molecular species designated **SJG-2**. In this paper, we report on the isolation and characterization of **SJG-2** from the body walls of *S. japonicus*. The *n*-hexane-soluble lipid fraction, obtained from the chloroform/methanol extract of the body walls of *S. japonicus*, was subjected to reverse-phase, followed by normal-phase, column chromatography to give a ganglioside molecular species **SJG-2**, which appeared as a single spot on normal-phase TLC.

Results and Discussion

Structure of SJG-2

The IR spectrum of **SJG-2** shows absorptions belonging to hydroxyl and amido groups. The ^{13}C NMR spectrum of **SJG-2** (Table 1) exhibits the characteristic signals of a phytosphingosine-type ceramide possessing an unsubstituted fatty acid and a sugar moiety at C-1 [$\delta = 70.3$ (C-1), 51.1 (C-2), 75.5 (C-3), 72.2 (C-4), 174.4 (C-1′), 36.0 (C-2′) ppm]. The spectrum also reveals signals attributed to seven anomeric carbon atoms at $\delta = 100.4$, 101.0, 101.5, 103.6, 103.7, 103.8 and 105.0 ppm, three of which ($\delta = 100.4$, 101.0, 101.5 ppm) are signals of quaternary carbon atoms, indicating the existence of three sialic acid units. The negative-ion FABMS spectrum exhibits a series of quasi-molecular ion peaks $[M - H]^-$ at $m/z = 2100$ to 2200. Therefore, **SJG-2** was suggested to be a molecular species of ganglioside comprising the above-mentioned ceramide and trisialoheptaose units. Furthermore, **SJG-2** was presumed to have *normal*-type fatty acids and *iso*- and *anteiso*-type long-chain bases (LCB) at the terminus, since the signals of the carbon nuclei of the terminal methyl groups are observed at $\delta = 14.0$ (*normal* form), 22.8 (*iso* form), and 11.5 and 19.3 ppm (*anteiso* form) in its ^{13}C NMR spectrum (Table 1 and Scheme 1).

The structure of the ceramide moiety was elucidated first. When **SJG-2** was methanolyzed with 5% HCl−MeOH, a mixture of fatty acid methyl esters (FAM) and long-chain bases (LCB) was obtained together with methyl glycoside. GC-MS analysis of the FAM mixture showed the existence of four components, which were characterized as methyl hexadecanoate (methyl palmitate, FAM-1), methyl octadecanoate (methyl stearate, FAM-2), methyl eicosanoate (FAM-3), and methyl docosanoate (FAM-4). The major FAM was methyl octadecanoate (FAM-2). On the other hand, the LCB mixture was found to be composed of 2-

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[‡] Constituents of Holothuroidea, 11. Part 10: Ref.[1g]

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Table 1. ^{13}C NMR (125 MHz) chemical shifts (δ values) of the ganglioside **SJG-2** in C_5D_5N/D_2O (95:5)

Position	δ [ppm]	Position	δ [ppm]	Position	δ [ppm]
Ceramide		GalNAc		NeuAc-2,3	
C-1 (t)	70.3	C-1 (d)	105.0	C-1 (s)	172.3[e]
C-2 (d)	51.1	C-2 (d)	54.2	C-1 (s)	172.6[e]
C-3 (d)	75.5	C-3 (d)	76.2	C-2 (s)	101.0
C-4 (d)	72.2	C-4 (d)	68.5	C-2 (s)	101.5
C-5 (t)	32.9	C-5 (d)	77.4	C-3 (t)	42.1
C-1′ (s)	174.4	C-6 (t)	62.2	C-3 (t)	42.1
C-2′ (t)	36.0	C-7 (s)	170.8	C-4 (d)	69.0
CH_3[a] (q)	14.0	C-8 (q)	25.5	C-4 (d)	69.2
CH_3[b] (q)	22.8			C-5 (d)	52.4
CH_3[c] (q)	11.5	NeuAc-1		C-5 (d)	52.5
CH_3[d] (q)	19.3	C-1 (s)	173.6[e]	C-6 (d)	75.3
		C-2 (s)	100.4	C-6 (d)	75.5
Glc		C-3 (t)	42.0	C-7 (d)	69.9
C-1 (d)	103.6	C-4 (d)	68.4	C-7 (d)	70.0
C-2 (d)	74.1	C-5 (d)	52.1	C-8 (d)	72.1
C-3 (d)	75.3	C-6 (d)	75.2	C-8 (d)	72.3
C-4 (d)	80.7	C-7 (d)	70.0	C-9 (t)	63.7
C-5 (d)	76.4	C-8 (d)	78.2	C-9 (t)	63.8
C-6 (t)	62.0	C-9 (t)	64.0	C-10 (s)	172.0[e]
		C-10 (s)	172.5[e]	C-10 (s)	172.2[e]
Gal-1		C-11 (q)	22.1	C-11 (q)	22.1
C-1 (d)	103.7			C-11 (q)	22.2
C-2 (d)	69.8	Gal-2			
C-3 (d)	84.8	C-1 (d)	103.8		
C-4 (d)	69.5	C-2 (d)	69.5		
C-5 (d)	74.8	C-3 (d)	79.8		
C-6 (t)	61.0	C-4 (d)	71.4		
		C-5 (d)	73.7		
		C-6 (t)	60.8		

[a] Terminal methyl group in the *normal*-type side chain (see Scheme 1). [b] Terminal methyl group in the *branched*-type side chain (see Scheme 1). [c] Terminal methyl group in the *branched*-type side chain (see Scheme 1). [d] Terminal methyl group in the *branched*-type side chain (see Scheme 1). [e] Assignments may be interchanged.

amino-1,3,4-trihydroxy-14(and 15)-methyl-hexadecane, 2-amino-1,3,4-trihydroxy-15(and 16)-methyl-heptadecane (major), and 2-amino-1,3,4-trihydroxy-16(and, 17)-methyl-octadecane, on the basis of GC-MS analysis of the TMS-derived LCB mixture.

The relative stereochemistry of the ceramide moiety was presumed to be (2*S**,3*S**,4*R**), since the aforementioned ^{13}C NMR spectroscopic signals attributable to C-1, 2, 3, and 4 of **SJG-2** are in good agreement with those of the known phytosphingosine-type glucocerebroside molecular species possessing such configurations.[1c]

The structure of the heptasaccharide moiety of **SJG-2** was established as follows. The negative-ion FAB mass spectrum exhibits a quasi-molecular ion peak $[M - H]^-$ at *m*/*z* = 2130, and fragment ion peaks arising from cleavage of the glycosidic linkages of the major component are observed at *m*/*z* = 1839, 1548, 1386, 1095, 892, 730, and 568, indicating the heptasaccharide moiety as shown in Scheme 2.

GC-MS analysis of the TMS derivatives of the methyl glycoside mixture, which was obtained by methanolysis of **SJG-2**, showed the existence of one mole equivalent each of glucose (Glc) and *N*-acetylgalactosamine (GalNAc) and two mole equivalents of galactose (Gal). Methylation of **SJG-2**, according to the Ciucanu−Kerek method,[2] afforded the permethylated product **SJG-2-M**. Partially methylated alditol acetates prepared from **SJG-2-M** were characterized as the alditols (S-1, S-2, S-3, and S-4) derived from 3-linked hexopyranose, 4-linked hexopyranose, 3,4-linked hexopyranose, and 3-linked *N*-acetylhexosamine, respectively, by means of GC-MS. On the other hand, **SJG-2-M** was methanolyzed and the methanolysate was acetylated, and then the partially methylated sialic acid derivatives were analyzed by GC-MS. The existence of one mole quivalent of 8-linked NeuAc (S-6) and two mole equivalents of terminal NeuAc (S-5) was detected. Furthermore, **SJG-2** was partially hydrolyzed by heating with 5% AcOH (aq.), then with H_2O, to confirm the structure of the oligosaccharide moiety. Asialo products **SJG-2−1**, **SJG-2−2**, and **SJG-2−3**, were obtained and analyzed by positive-ion FAB mass spectrometry and TLC, which confirmed the structures to be cerebroside, ceramide dihexoside and ceramide trihexoside, respectively, as shown in Scheme 3.

On the basis of the above evidence, the heptasaccharide moiety of **SJG-2** must be NeuAc2→3(NeuAc2→4)-Gal1→8NeuAc2→3GalNAc1→3Gal1→4Glc.

The configurations of Glc, Gal, and GalNAc were believed to be β,[3] while those of NeuAc were α,[4] on the basis of the signals of their anomeric carbon atoms [δ = 103.6, 103.7, 103.8, 105.0, 100.4, 101.0, 101.5 ppm] in the ^{13}C NMR spectrum of **SJG-2**.

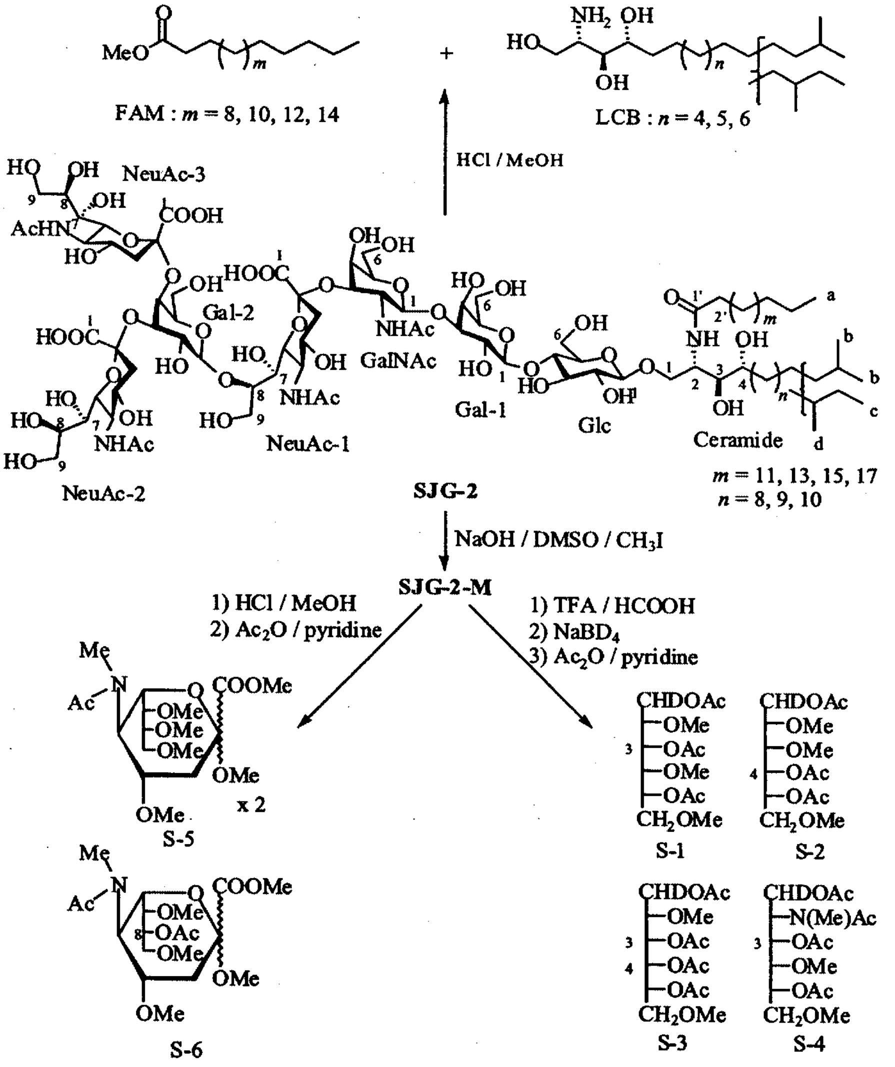
Consequently, if those monosaccharides are assumed to belong to the most commonly found D-series, then **SJG-2** is the (*N*-acetyl-α-D-neuraminosyl)-(2→3)-[(*N*-acetyl-α-D-neuraminosyl)-(2→4)]-(β-D-galactopyranosyl)-(1→8)-(*N*-acetyl-α-D-neuraminosyl)-(2→3)-(β-D-*N*-acetylgalactopyranosaminyl)-(1→3)-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside of a ceramide composed of heterogeneous (2*S*,3*S*,4*R*)-phytosphingosine and fatty acid units, as shown in Scheme 1.

Neuritogenic Activity of the Ganglioside SJG-2

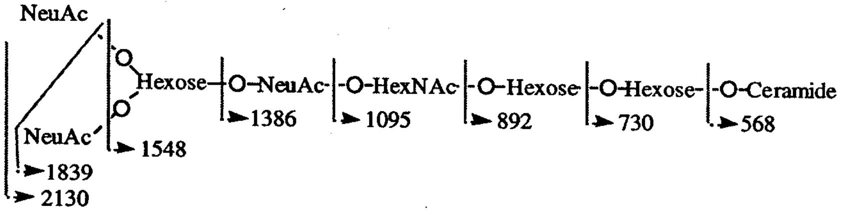
We investigated the effect of the isolated ganglioside molecular species on the neuritogenesis of the rat pheochromocytoma cell line (PC12 cells). The results showed that **SJG-2** displayed neuritogenic activity in the presence of NGF (Nerve Growth Factor) and that the ganglioside alone did not cause the neurite formation. The proportion of neurite-bearing cells of **SJG-2** (64.8 ± 7.6%) was larger than that of **SJG-1**[1e] (NeuGcα2→6Glcβ1→1Cer; 35.4 ± 4.0%) when compared with the control (NGF, 5 ng/mL: 20.6 ± 2.2%). Furthermore, the effect of **SJG-2** was more considerable than that of the mammalian ganglioside [5] **GM1** (47.0 ± 2.5%).

Conclusion

To the best of our knowledge, **SJG-2**, possessing a unique sugar moiety, is a new ganglioside, and is the first



Scheme 1



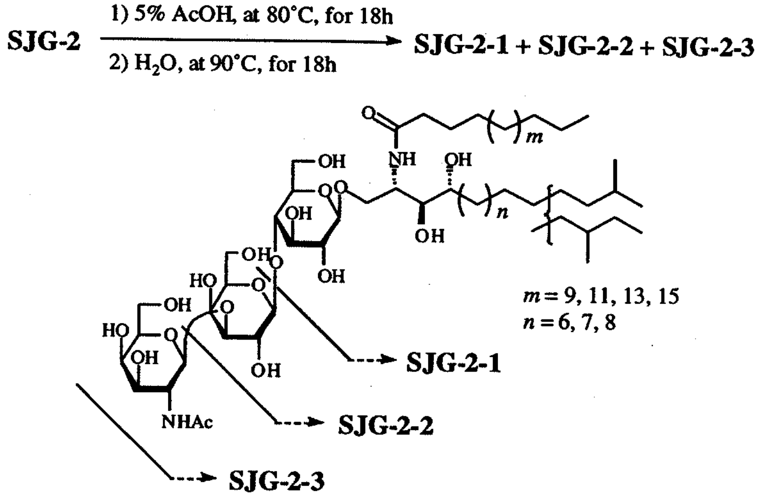
Scheme 2

type of ganglioside containing either the branched sugar chain moiety or the *N*-acetylgalactosamine residue found in sea cucumber gangliosides. **SJG-2** shows potent neuritogenic activity toward PC12 cells in the presence of NGF. The isolation and characterization of such neuritogenically active gangliosides is attracting considerable attention with regard to the manufacture of new medicines from marine natural products.

Experimental Section

General Remarks: IR spectra: Jasco FT/IR-140 infrared spectrophotometer. ^1H and ^{13}C NMR spectra: Jeol GX-270 spectrometer (270 MHz and 67.8 MHz), Varian Unity-500 spectrometer (500 MHz and 125 MHz). FAB mass spectra: Jeol SX/SX-102A (xenon atom beam); matrix: *m*-nitrobenzyl alcohol (positive-ion mode), HMPA/TEG (negative-ion mode); GC-MS: Shimadzu QP-5000; EI mode (ionizing potential of 70 eV, separator and ion-source temperature of 250 °C); column: TC-1701 (0.53 mm × 15 m, GL Sciences); carrier He.

Separation of SJG-2: The body walls of the sea cucumber *Stichopus japonicus* (30.3 kg) were chopped and extracted three times with $CHCl_3$/MeOH (1:2, 18 L). The combined extracts were concentrated in vacuo to give an aqueous solution (9 L), which was extracted three times with *n*-hexane (3 L). The *n*-hexane phase was then extracted three times with MeOH (3 L), and then the MeOH



Scheme 3

layer was concentrated in vacuo to give a residue, which was dissolved in acetone. The acetone insoluble part (61.5 g) was chromatographed on Cosmosil 140C18-PREP (reverse phase, eluent: 60 to 100% MeOH. The crude ganglioside fraction (46.2 g), which eluted with 100% MeOH, was chromatographed further on Cosmosil 140C18-PREP (reversed phase, eluent: 60% to 100% MeOH) to give four fractions (fractions 1 − 4). The second fraction (30.4 g) was chromatographed on silica gel (solvent: $CHCl_3/MeOH/H_2O$, 70:30:0 to 30:70:10) to give six fractions (fractions 5−10). Fraction 9 (5.2 g) was chromatographed on silica gel ($CHCl_3/MeOH/H_2O$, 70:30:0 to 30:70:10) to give six fractions (fractions 11−16). Successive column chromatography of fraction 14 (3.2 g) with an ion-exchange column (DEAE-TOYOPEARL 650S, $CHCl_3/MeOH/H_2O$, 30:60:8, to $CHCl_3$/MeOH/0.8 N aq. NaOAc, 30:60:8) and gel filtration (Sephadex LH-20, $CHCl_3/MeOH/H_2O$, 50:50:10) afforded **SJG-2** [13.1 mg; R_f = 0.28 (silica gel TLC, solvent: $CHCl_3/MeOH/H_2O$, 60:40:10)].

SJG-2: Amorphous powder. IR (KBr): $\tilde{\nu}$ = 3388 (OH), 1634 (amide) cm^{-1}. Negative-ion FABMS: *m/z* (%) = 2130 (0.6) $[M - H]^-$, 1839 (0.1), 1548 (0.2), 1386 (0.1), 1095 (0.2), 892 (0.2), 730 (0.7), 568 (1.6) (fragment ions of major component, see Scheme 2). ^{13}C NMR: See Table 1.

Methanolysis of SJG-2: SJG-2 (0.7 mg) was heated with 5% HCl in MeOH (0.5 mL) at 70 °C for 18 h. The reaction mixture was extracted with *n*-hexane, and the extract was concentrated in vacuo to yield a mixture of fatty acid methyl esters (FAM). The MeOH layer was neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated in vacuo to give a mixture of long-chain bases (LCB) and methyl glycosides.

GC-MS Analysis of FAM from SJG-2: The FAM mixture derived from **SJG-2** was subjected to GC-MS (column temp.: 150 − 250 °C; rate of temp. increase: 5 °C/min). The results were as follows: methyl hexadecanoate (FAM-1), t_R [min] = 12.9, *m/z* = 270 $[M]^+$, 227 $[M - 43]^+$; methyl octadecanoate (FAM-2), t_R = 16.6, *m/z* = 298 $[M]^+$, 255 $[M - 43]^+$; methyl eicosanoate (FAM-3), t_R = 20.1, *m/z* = 326 $[M]^+$, 283 $[M - 43]^+$; methyl docosanoate (FAM-4), t_R = 23.5, *m/z* = 354 $[M]^+$, 311 $[M - 43]^+$; FAM-1:FAM-2:FAM-3:FAM-4 ≈ 3:10:2:1.

GC-MS Analysis of TMS Ethers of LCB from SJG-2: The mixture of LCB and methyl glycoside derived from **SJG-2** was heated with 1-(trimethylsilyl)imidazole/pyridine (50:50, 0.2 mL) for 10 min at 60 °C and then the reaction mixture of TMS ethers was analyzed by GC-MS (column temp.: 150 − 250 °C; rate of temp. increase: 2.5 °C/min). The results were as follows: 2-amino-1,3,4-tri-*O*-trimethylsilyl-14(and, 15)-methyl-hexadecane, t_R [min] = 16.4, *m/z* = 326 $[M - 193]^+$, 285 $[M - 234]^+$; 2-amino-1,3,4-tri-*O*-trimethylsilyl-15(and, 16)-methyl-heptadecane (major), t_R = 18.1, *m/z* = 340 $[M - 193]^+$, 299 $[M - 234]^+$; 2-amino-1,3,4-tri-*O*-trimethylsilyl-16(and, 17)-octadecane, t_R = 19.9, *m/z* = 354 $[M - 193]^+$, 313 $[M - 234]^+$.

GC-MS Analysis of TMS Ethers of Methyl Glycoside from SJG-2: The mixture of TMS ethers of LCB and methyl glycosides was analyzed by GC-MS (column temp.: 150 − 250 °C; rate of temp. increase: 2.5 °C/min): t_R [min] = 13.8 (methyl-2,3,4,6-tetra-*O*-trimethylsilyl-galactose), t_R [min] = 15.5 (methyl-2,3,4,6-tetra-*O*-trimethylsilyl-glucose), t_R [min] = 27.6 (methyl-2-*N*-acetyl-3,4,6-tri-*O*-trimethylsilyl-galactosamine).

Methylation of SJG-2: (Ciucanu−Kerek Method[2]) An NaOH/DMSO solution, which was prepared from powdered NaOH (40.0 mg) and DMSO (1.0 mL), and MeI (0.2 mL) were added to **SJG-2** (1.1 mg), and the mixture was stirred for 30 min. The reaction mixture was then diluted with H_2O (15 mL) and extracted with $CHCl_3$ (3 × 10 mL). The combined $CHCl_3$ phases were washed with H_2O and then the solvent was evaporated in vacuo to give permethylated **SJG-2** (denoted **SJG-2-M**, 1.9 mg).

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetates from SJG-2-M: SJG-2-M (0.6 mg) was heated with 90% HCOOH/10% CF_3COOH, 50:50 (1 mL) at 70 °C for 18 h in a small-volume sealed vial, and then the solvents were evaporated in vacuo. The residue was dissolved in H_2O (5 mL), and 2% aq. NH_3 (2 drops) and $NaBD_4$ (10.0 mg) were added. After standing at room temperature for 7 h, the mixture was acidified with AcOH to pH = 3.5 and concentrated in vacuo. H_3BO_3 present in the residue was removed by distillation with MeOH (three times). The residue was heated with Ac_2O/pyridine, 50:50 (0.3 mL) at 70 °C for 2 h. After dilution with H_2O (0.6 mL), the mixture was extracted with $CHCl_3$ (3 × 0.2 mL). The combined $CHCl_3$ extracts were washed with H_2O and then the solvent was evaporated to give partially methylated alditol acetates. The acetates were subjected to GC-MS (column temp.: 150−250 °C; rate of temp. increase: 5 °C/min). The results were as follows: S-1, t_R [min] = 14.9 (1 mol), *m/z* = 45, 118, 161, 234 [1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol (derived from

3-linked Gal)]; S-2, t_R [min] = 15.1 (1 mol), *m*/*z* = 45, 118, 233 [1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol (derived from 4-linked Glc)]; S-3, t_R [min] = 16.3 (1 mol), *m*/*z* = 118, 129, 143, 185, 305 [1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylhexitol (derived from 3,4-linked Gal)]; S-4, t_R [min] = 27.6 (1 mol), *m*/*z* = 116, 129, 158, 161, 170, 230, 274 [1,3,5-tri-*O*-acetyl-4,6-di-*O*-methyl-2-*N*-methylacetamide-2-deoxyhexitol (derived from 3-linked GalNAc)].

Preparation and GC-MS Analysis of the Permethylated Sialic Acid Derivative from SJG-2-M: SJG-2-M (0.7 mg) was heated with 10% HCl in MeOH (0.5 mL) at 70 °C for 15 h in a small-volume sealed vial. The reaction mixture was then neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated in vacuo. The residue (methanolysate) was heated with Ac_2O/pyridine (50:50, 0.2 mL) at 70 °C for 2 h. The resulting mixture was diluted with H_2O (0.3 mL), extracted with $CHCl_3$ (3 × 0.2 mL), the combined $CHCl_3$ extracts were washed with H_2O, and then the solvent was evaporated in vacuo. The residue was subjected to GC-MS (column temp.: 200−250 °C; rate of temp. increase: 2.5 °C/min): S-5, t_R [min] = 19.7 (2 mol), *m*/*z* = 89, 129, 201, 318, 348, 392 [methyl-*N*-acetyl-*N*-methyl-2,4,7,8,9-penta-*O*-methylneuraminate (derived from terminal NeuAc)]; S-6, t_R [min] = 22.8 (1 mol), *m*/*z* = 129, 201, 254, 318, 326, 376, 420 [methyl-*N*-acetyl-8-*O*-acetyl-*N*-methyl-2,4,7,9-tetra-*O*-methylneuraminate (derived from 8-linked NeuAc)].

Partial Hydrolysis of SJG-2: SJG-2 (1.0 mg) was heated with 5% AcOH in H_2O (1.0 mL) at 80 °C for 18 h in a small-volume sealed vial, and then the solvents were evaporated in vacuo. The residue was dissolved in H_2O (1.0 mL) and heated at 90 °C for 18 h. The solvents were evaporated in vacuo. The residue was purified by Preparative TLC (solvent: $CHCl_3$/MeOH/H_2O, 60:40:10) to give three compounds designated **SJG-2−1** (R_f = 0.9; authentic cerebroside, R_f = 0.9), **SJG-2−2** (R_f = 0.8; authentic ceramide dihexoside, R_f = 0.8), and **SJG-2−3** (R_f = 0.6; authentic ceramide trihexoside, R_f = 0.6). **SJG-2−1**, positive-ion FABMS: *m*/*z* = 750, 778 $[M + Na]^+$; **SJG-2−2**, positive ion FABMS: *m*/*z* = 912, 940 $[M + Na]^+$; **SJG-2−3**, positive ion FABMS: *m*/*z* = 1115, 1143 $[M + Na]^+$.

Observation of Neuritogenic Activity on PC12 Cells: PC12 cells (Riken Cell Bank) were cultured, at a density of 1×10^4 cells/mL, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 5% horse serum, and 2% penicillin−streptomycin, in collagen-coated 96-well plates (IWAKI) under a humidified atmosphere of 5% CO_2 in air at 37 °C. After 24 h, the culture medium was replaced by a medium of serum-free DMEM/Ham's F12 (50:50) supplemented with N-2 Supplement (GIBCO). The ganglioside **SJG-2**, **SJG-1**, or **GM1** (10 μM) was added with or without NGF (5 ng/mL) to the medium, and the cells were cultured further at 37 °C. After 4 days, the morphological changes in the cells were observed with a light microscope.

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