Constituents of Holothuroidea, 8[+]

Structure of Neuritogenic Active Ganglioside from the Sea Cucumber *Stichopus japonicus*

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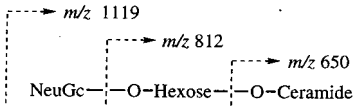
A ganglioside molecular species SJG-1 has been obtained from the *n*-hexane soluble lipid fraction of the chloroform/methanol extract of the sea cucumber *Stichopus japonicus*. On the basis of chemical and spectroscopic evidence, the structure of SJG-1 has been determined. SJG-1 possesses an *N*-glycolylneuraminic acid (sialic acid), nonhydroxy fatty acids and phytosphingosine-type long-chain bases as major ceramide components. The ganglioside SJG-1 exhibits neuritogenic activity toward the rat pheochromocytoma cell line PC12 cells in the presence of NGF.

In our continuing research on biologically-active glycosphingolipids from echinoderms, a series of studies on the isolation, structure elucidation and biological activities of the glycosphingolipids from the sea cucumber species have been performed in our laboratory.[1] In continuation of the preceding studies on the sea cucumber *Holothuria pervicax*,[1d] the isolation and characterization of the biologically active glycosphingolipids from the sea cucumber *Stichopus japonicus* (Manamako in Japanese) has been conducted to develop novel medicinal resources from marine natural products. In this paper, we report the isolation and the characterization of a ganglioside molecular species **SJG-1** 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 reversed-phase followed by normal-phase column chromatography to give a ganglioside molecular species **SJG-1**, which appeared as a single spot on normal-phase TLC.

Structure of SJG-1

SJG-1 showed strong hydroxy and amide absorptions in the IR spectrum. The ^{13}C NMR spectrum of **SJG-1** (Table 1) exhibited the characteristic signals of a phytosphingosine-type ceramide possessing a nonhydroxy fatty acid and two monosaccharide moieties. The ^{13}C NMR spectrum also revealed signals due to two anomeric carbon atoms at δ = 101.4 and 104.1, one of which was a quaternary carbon signal (δ = 101.4) indicating the existence of a sialic acid. The negative FAB mass spectrum exhibited seven quasi-molecular ion peaks due to differences in alkyl chain of about 14 mass units $[M - H]^-$ at *m/z* = 1077−1161, and the fragment ion peaks arising from cleavage of the glycosidic linkages of the major component (*m/z* = 1119) were observed at *m/z* = 812 and 650, indicating the presence of the disaccharide moiety NeuGc−hexose, as shown in Scheme 1.



Scheme 1. Negative FAB mass fragmentation of the major component of **SJG-1**

Furthermore, **SJG-1** was presumed to have *normal*-type fatty acids and *normal*- or *iso*- and *anteiso*-type long-chain bases (LCB) at the terminus, since the carbon signals for the terminal methyl groups were observed at δ = 14.3 (*normal* form), δ = 22.8 (*iso* form) and 11.6, 19.4 (*anteiso* form) in the ^{13}C NMR (Table 1 and Scheme 2). The main type of LCB was the *iso* form [$CH_2CH_2(CH_3)_2$].

The structure of the ceramide moiety was elucidated first. When **SJG-1** was methanolyzed with 5% HCl/MeOH, a mixture of fatty acid methyl esters (FAM) and long chain bases (LCB) was obtained together with methyl glucopyranoside (Scheme 2). A GC-MS analysis of the FAM mixture showed the existence of five components, which were characterized as methyl octadecanoate (methyl stearate) (FAM-1), methyl eicosanoate (FAM-2), methyl docosanoate (FAM-3), methyl tricosanoate (FAM-4) and methyl 2-hydroxytricosanoate (FAM-5). The major FAM was methyl octadecanoate (FAM-1). On the other hand, the long-chain base (LCB) mixture was found to be composed of 2-amino-1,3,4-trihydroxy-15-methylhexadecane (major), 2-amino-1,3,4-trihydroxy-16-methylheptadecane, 2-amino-1,3,4-trihydroxy-17-methyloctadecane on the basis of GC-MS analysis of the TMS-derived LCB mixture. The stereochemistry of the ceramide moiety is presumed to be (2*S**,3*S**,4*R**), since the aforementioned ^{13}C NMR signals attributable to C-1, 2, 3, 4 of **SJG-1** were in good agreement with those

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

Position		δ [ppm]			δ [ppm]
Ceramide			NeuGc		
C-1	(t)	70.6	C-1′′′	(s)	174.5
C-2	(d)	53.7	C-2′′′	(s)	101.4
C-3	(d)	76.0	C-3′′′	(t)	42.6
C-4	(d)	72.5	C-4′′′	(d)	66.0
C-5	(t)	32.9	C-5′′′	(d)	53.7
C-1′	(s)	176.5	C-6′′′	(d)	76.0
C-2′	(t)	36.9	C-7′′′	(d)	68.6
CH_3[a]	(q)	14.3	C-8′′′	(d)	77.4
CH_3[b]	(q)	22.8	C-9′′′	(t)	63.9
CH_3[c]	(q)	11.6	C-10′′′	(s)	176.5
CH_3[d]	(q)	19.4	C-11′′′	(t)	62.2
Glc					
C-1′′	(d)	104.1			
C-2′′	(d)	74.4			
C-3′′	(d)	76.8			
C-4′′	(d)	70.0			
C-5′′	(d)	76.0			
C-6′′	(t)	70.8			

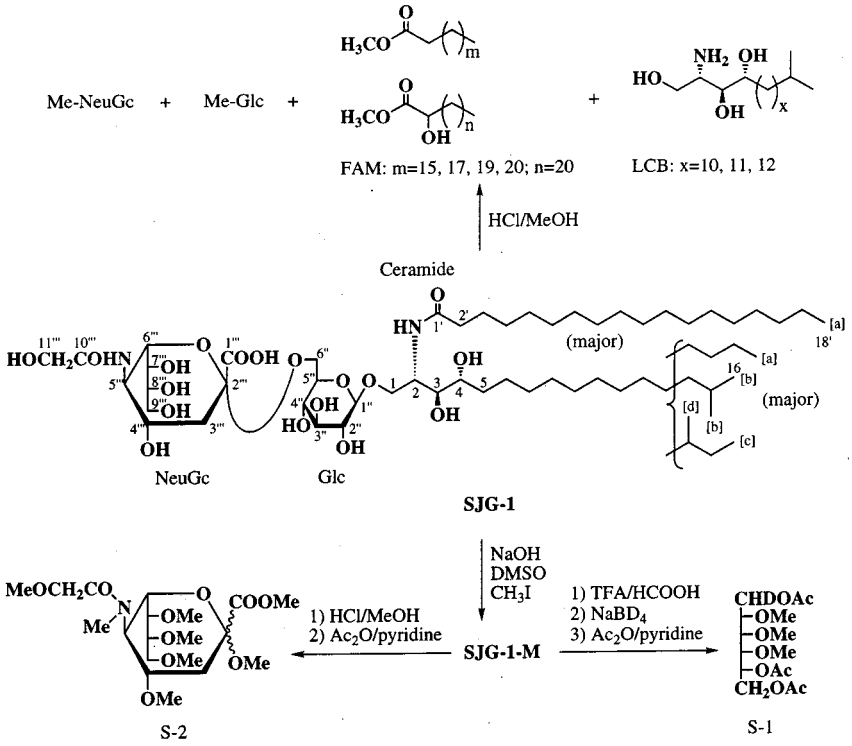
[a] Terminal methyl group in the *normal*-type side chain (see Scheme 2). – [b–d] Terminal methyl group in the *branched*-type side chain.

of the known phytosphingosine-type cerebroside molecular species possessing (2*S**,3*S**,4*R**) configurations.[1]

The structure of the disaccharide moiety of **SJG-1** was established as follows. The presence of glucose (Glc) was obvious from the GC analysis of the TMS derivative of methyl glycoside, which was obtained by methanolysis of **SJG-1**. A detailed analysis of the ^{13}C-NMR spectrum of **SJG-1** revealed signals characteristic of an *N*-glycolylneuraminic acid (NeuGc), together with those of a β-glucopyranose residue (Table 1).

Methylation of **SJG-1** according to the Ciucanu-Kerek method[2] afforded the permethylated product **SJG-1-M**. Partially methylated alditol acetate (S-1), prepared from **SJG-1-M,** was analyzed by GC-MS and identified as the alditol derived from 6-linked hexopyranose. **SJG-1-M** was subsequently methanolyzed and the methanolysate was acetylated. Only the permethylated NeuGc (S-2), derived from the terminal NeuGc, was detected as sialic acid derivative by means of GC-MS, giving characteristic fragment ion peaks (*m*/*z* = 89, 159, 284, 328, 348 and 378).

The configuration of Glc was considered to be β on the basis of its anomeric carbon signal (δ = 104.1) in the ^{13}C NMR spectrum of **SJG-1**. The configuration of NeuGc was presumed to be α, since the carbon signals assigned to the NeuGc moiety in **SJG-1** were in good agreement with those of the known gangliosides[1d] possessing the α-linked NeuGc. Moreover, the α configuration of NeuGc was supported by the proton chemical shifts of 3′′′-H.[3] On the basis of the above evidence, the disaccharide moiety of **SJG-1** must be NeuGcα-(2→6)-Glcβ. The absolute configuration of the glucose moiety of **SJG-1** was determined as the D-form by means of Hara′s method.[4] The absolute configuration of NeuGc was not investigated. Therefore, **SJG-1** is the *O*-(*N*-glycolyl-α-neuraminosyl)-(2→6)-β-D-glucopyranoside of a ceramide composed of heterogeneous (2*S**,3*S**,4*R**)-phytosphingosine and fatty acid units. The major components of the fatty acid and long-chain base moieties of **SJG-1** are *n*-octadecanoic acid (stearic acid)



Scheme 2

and (2*S**,3*S**,4*R**)-2-amino-1,3,4-trihydroxy-15-methylhexadecane.

Neuritogenic Activity

The effect of the isolated ganglioside molecular species on the neuritogenesis of the rat pheochromocytoma cell line (PC12 cells) has been investigated. The results showed that **SJG-1** displays neuritogenic activity in the presence of NGF (nerve growth factor) relative to H_2O (control) at a concentration above 10 μg.mL^{-1}. The effect was the same as those of the ganglioside from the sea cucumber *Holothuria pervicax*.[1d]

Although gangliosides having a 2→6 linked *N*-glycolylneuraminosyl glucose moiety have been obtained from the sea cucumber,[5][6] sea urchin[7] and brittle star,[8] **SJG-1** differs from these gangliosides in the structure of its ceramide part. The isolation and characterization of the neuritogenically-active ganglioside from *S. japonicus*, which is important as a crude drug, is attracting considerable attention, and is worthy of note

Experimental Section

General: IR spectra: Jasco IR-700 infrared spectrophotometer. − ^{1}H- 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-120A (xenon atom beam); matrix: HMPA/TEG (negative ion mode); carrier: Xe. − GC-MS: Shimadzu QP-1000; EI mode (ionizing potential of 70 eV, separator and ion-source temperature of 250°C); column: TC-1701 (0.53 mm × 15m, GL Sciences); carrier: He. − GC: Shimadzu GC-14B (FID mode); column: fused-silica capillary column DB-17 (0.317 mm × 30 m, J. and W. Scientific); carrier: N_2.

Separation of SJG-1: 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 further extracted three times with MeOH (3 L), and then the MeOH layer was concentrated in vacuo to give a residue. The residue was dissolved in acetone. The acetone-insoluble part (46.0 g) was purified by chromatography on Cosmosil 140 C_{18}-PREP (reversed phase, eluent: 60%, 100% MeOH). The crude ganglioside fraction (32.2 g), (100% MeOH eluant), was further subjected to chromatography on silica gel (solvent: $CHCl_3$/MeOH/H_2O, 7:3:0.5 to 6:4:1) to afford **SJG-1** (11.5 mg,). − R_f = 0.71) [silica gel TLC, solvent: $CHCl_3$/MeOH/H_2O, 6:4:1].

SJG-1: Amorphous powder, m.p. 159−160°C. − IR (KBr): $\tilde{\nu}$ = 3375 cm^{-1} (OH), 1720, 1646, 1548 cm^{-1} (amide). − Negative FAB-MS; *m/z*: 1077, 1091, 1105, 1119, 1133, 1147, 1161 [M − H]$^-$ series, 812, 650 (fragment ions of major component, see Scheme 1). − ^{1}H NMR (C_5D_5N): δ = 4.55 (dd, 1 H, *J* = 4.3 Hz, *J* = 10.6 Hz, 1-H^a), 4.75 (dd, 1 H, *J* = 6.6 Hz, *J* = 10.6 Hz, H-1^b), 5.31 (m, 1 H, 2-H), 4.08 (m, 1 H, 3-H), 4.22 (m, 1 H, 4-H), 4.61 (m, 1 H, 2′-H), 0.87 (m, 9 H, terminal Me), 4.70 (d, 1 H, *J* = 7.9 Hz, 1′′-H), 3.93 (m, 1 H, 2′′-H), 4.14 (m, 1 H, 3′′-H), 4.09 (m, 1 H, 4′′-H), 4.08 (m, 1 H, 5′′-H), 3.72 (m, 1 H, 6′′-H^a), 4.08 (m, 1 H, 6′′-H^b), 3.57 (m, 1 H, 3′′′-H^{eq}), 2.35 (m, 1 H, 3′′′-H^{ax}), 4.57 (m, 1 H, 5′′′-H), 4.42 (m, 2 H, 11′′′-H). − ^{13}C NMR: See Table 1.

Methanolysis of SJG-1: SJG-1 (0.7 mg) was heated with 5% HCl in MeOH (0.5 mL) at 70°C for 18 h. The reaction mixture was then 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 glycoside.

GC-MS Analysis of FAM from SJG-1: The FAM mixture derived from **SJG-1** was subjected to GC-MS [column temp.: 180−250°C (rate of temp. increase 4°C·min^{-1})]. The results were as follows: **Methyl Octadecanoate (FAM-1):** t_R [min] = 7.1. − *m/z*: 298 [M$^+$], 255 [M − 43]$^+$. − **Methyl Eicosanoate (FAM-2):** t_R = 9.0. − *m/z* = 326 [M$^+$], 283 [M − 43]$^+$. − **Methyl Docosanoate (FAM-3):** t_R = 10.8. − *m/z* = 354 [M$^+$], 311 [M − 43]$^+$. − **Methyl Tricosanoate (FAM-4):** t_R = 11.7. − *m/z* = 368 [M$^+$], 325 [M − 43]$^+$. − **Methyl 2-Hydroxytricosanoate (FAM-5):** t_R = 12.6. − *m/z* = 384 [M$^+$], 325 [M − 59]$^+$; FAM-1/FAM-2/FAM-3/FAM-4/FAM-5 ≈ 3:1:1:1:2.

GC-MS Analysis of TMS Ethers of LCB from SJG-1: The mixture of LCB and methyl glycoside derived from **SJG-1** was heated with 1-(trimethylsilyl)imidazole/pyridine (1:1) for 10 min at 60°C and then the reaction mixture [trimethylsilyl (TMS) ethers] was analyzed by GC-MS [column temp. 180−250°C (rate of temp. increase 4°C·min^{-1})]. The results were as follows: **2-Amino-1,3,4-trihydroxy-15-methylhexadecane (major):** t_R [min] = 8.6. − *m/z* = 326 [M − 193]$^+$, 285 [M − 234]$^+$, 132. − **2-Amino-1,3,4-trihydroxy-16-methylheptadecane:** t_R = 10.2. − *m/z* = 340 [M − 193]$^+$, 299 [M − 234]$^+$, 132. − **2-Amino-1,3,4-trihydroxy-17-methyloctadecane:** t_R = 11.6. − *m/z* = 354 [M − 193]$^+$, 313 [M − 234]$^+$, 132.

GC Analysis of TMS Ethers of Methyl Glycoside from SJG-1: The mixture of trimethylsilyl ethers of LCB and methyl glycoside was analyzed by GC [column temp.: 100−250°C (rate of temp. increase 5°C·min^{-1})]: t_R [min] = 18.0 (methyl-2,3,4,6-tetra-*O*-(trimethylsilyl)-glucose from **SJG-1**), t_R [min] = 17.9 (standard methyl-2,3,4,6-tetra-*O*-(trimethylsilyl)-glucose).

Methylation of SJG-1 (Ciucanu-Kerek′s method[2])**:** NaOH/DMSO solution, which was prepared from powdered NaOH (40 mg) and DMSO (1 mL), and MeI (0.2 mL) were added to **SJG-1** (1.2 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 $CHCl_3$ phase was washed with H_2O, and the solvent was evaporated in vacuo to give permethylated **SJG-1**, denoted **SJG-1-M** (1.8 mg).

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetate from SJG-1-M: SJG-1-M (0.6 mg) was heated with 90% HCOOH/10% CF_3COOH (1:1, 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 28% NH_3 (2 drops) and $NaBD_4$ (10.0 mg) were added. After allowing the mixture to stand at room temp. for 7 h, it 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 (1:1, 0.3 mL) at 70°C for 2 h. After dilution with H_2O, the mixture was extracted with $CHCl_3$ (3 × 0.2 mL). The combined $CHCl_3$ extracts were washed with H_2O, and the solvent was evaporated to give partially methylated alditol acetate. The acetate was subjected to GC-MS [column temp.: 170−230°C (rate of temp. increase 3°C·min^{-1})]. The results were as follows: **S-1:** t_R [min] = 4.8 − *m/z* = 118, 162, 189 [1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol (derived from 6-linked hexopyranose)].

Preparation and GC-MS Analysis of Permethylated Sialic Acid Derivative from SJG-1-M: SJG-1-M (0.7 mg) was heated with 5% HCl in MeOH (0.5 mL) at 70°C for 22 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 (1:1, 0.2 mL) at 70°C for 2 h. The resulting mixture was diluted with H_2O and extracted with $CHCl_3$ (3 × 0.2 mL), the combined $CHCl_3$ extracts were washed with H_2O, and the solvent was evaporated in vacuo. The residue was subjected to GC-MS [column temp.: 180−250°C (rate of temp. increase 4°C·min^{-1})]. **S-2:** t_R [min] = 13.1. − *m/z* = 89, 159, 284, 328, 348, 378 [methyl *N*-glycolyl-*N*-methyl-(2,4,7,8,9,11-hexa-*O*-methyl)neuraminate (derived from terminal NeuGc)].

Determination of Absolute Configuration of the Glucose Moiety of SJG-1 (Hara′s method[4])**: SJG-1** (0.7 mg) was heated with H_2SO_4 (4 N, 0.3 mL) at 100°C for 8 h. The reaction mixture was then extracted with *n*-hexane, and the acidic aqueous phase was neutralized with $Ba(OH)_2$, centrifuged, and the clear supernatant solution was concentrated. The residue (sugar fraction) was heated with L-cystein methyl ester hydrochloride (0.3 mg) and pyridine (0.3 mL) at 60°C for 1 h. Then, 1-(trimethylsilyl)imidazole (0.1 mL) was added and the mixture was heated at 60°C for further 0.5 h to yield the trimethylsilyl ether of the methyl (4*R*)-thiazolidine-4-carboxylate derivative. The derivative was analyzed by GC [column temp.: 200−250°C (rate of temp. increase 2.5°C·min^{-1})]; t_R [min] = 11.9 (derivative of D-glucose, 11.9 min; L-glucose, 12.6 min).

Observation of Neuritogenic Activity on PC12 Cells: PC12 cells (Riken Cell Bank) were cultured, at density of 1 × 10^5 cells·mL^{-1}, 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 of culture, the culture medium was replaced by serum-free DMEM/Ham′s F12 (1:1) medium supplemented with N-2 Supplement (GIBCO). The ganglioside **SJG-1** was added with or without NGF (0.1 μM) to the medium at densities of 100, 10, 1 and 0.1 μg.mL^{-1}, and the cells were further cultured at 37°C. After 4 d, the morphological changes in the cells were observed with a light microscope. Cells treated with above 10 μg·mL^{-1} of the ganglioside **SJG-1** with NGF showed neurite outgrowth compared with those treated with H_2O (control).

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