Constituents of Holothuroidea, 7[◇]

Isolation and Structure of Biologically Active Gangliosides from the Sea Cucumber *Holothuria pervicax*

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Three ganglioside molecular species, **HPG-8**, **HPG-3**, and **HPG-1**, have been obtained from the polar fraction of the chloroform/methanol extract of the sea cucumber *Holothuria pervicax*. The structures of these gangliosides have been determined on the basis of chemical and spectroscopic evidence. They represent new ganglioside molecular species. **HPG-8** is a sulfated monosialo-ganglioside, while **HPG-3** and **HPG-1** are disialo-gangliosides possessing 2→4-linked tandem-type disialosyl moieties. These three gangliosides qualitative neuritogenic activity toward the rat pheochromocytoma cell line, PC-12 cells.

In the course of our continuing research on biologically active glycosphingolipids from echinoderms, a series of studies on the isolation and structure elucidation of the glycosphingolipids from sea cucumber species have been performed in our laboratory[1]. In continuation of the preceding studies on the sea cucumber *Cucumaria echinata*[1c], the isolation and characterization of the biologically active glycosphingolipids from the sea cucumber *Holothuria pervicax* (Torafunamako in Japanese) has now been carried out in order to develop the novel medicinal resources from marine natural products. In this paper, we report on the isolation and characterization of three ganglioside molecular species from the whole bodies of *H. pervicax*. The biological activities of the gangliosides are also reported.

The water-soluble lipid fraction, which was obtained from the chloroform/methanol extract of the whole bodies of *H. pervicax*, was subjected to reversed-phase followed by silica-gel column chromatography to give three ganglioside molecular species, **HPG-8**, **HPG-3**, and **HPG-1**, each showing a single spot on silica gel thin-layer chromatography (TLC).

Structure of HPG-8

In its ^{13}C-NMR spectrum (Scheme 1 and Table 1), **HPG-8** exhibits the characteristic signals of a phytosphingosine-type ceramide possessing a 2-hydroxy fatty acid and a sugar moiety at C-1 [δ = 70.5 (C-1), 51.2 (C-2), 75.9 (C-3), 72.2 (C-4), 175.8 (C-1′) and 72.2 (C-2′)]. The ^{13}C-NMR spectrum of **HPG-8** also features signals due to two anomeric carbon atoms at δ = 104.7 and 101.7, one of which (δ = 101.7) is a quaternary carbon atom signal, indicating the presence of a sialic acid function. The negative FAB mass spectrum exhibits a series of quasi-molecular ion peaks $[M - H]^-$ at *m/z* = 1150−1250, and the fragment-ion peaks due to $[SO_4H]^-$ and $[SO_3]^-$ at *m/z* = 97 and 80. Therefore, **HPG-8** is suggested to be a molecular species of a sulfated phytosphingosine-type ganglioside, possessing 2-hydroxy fatty acid groups and two monosaccharide units. Furthermore, **HPG-8** is presumed to have mainly *normal*-type[2] fatty acids and branched-type[3] long-chain bases, since the carbon atom signals for the terminal methyl groups are observed at δ = 14.0 (*normal* form) and δ = 11.3 and 19.2 (*branched* form) in the ^{13}C-NMR spectrum (Scheme 1 and Table 1).

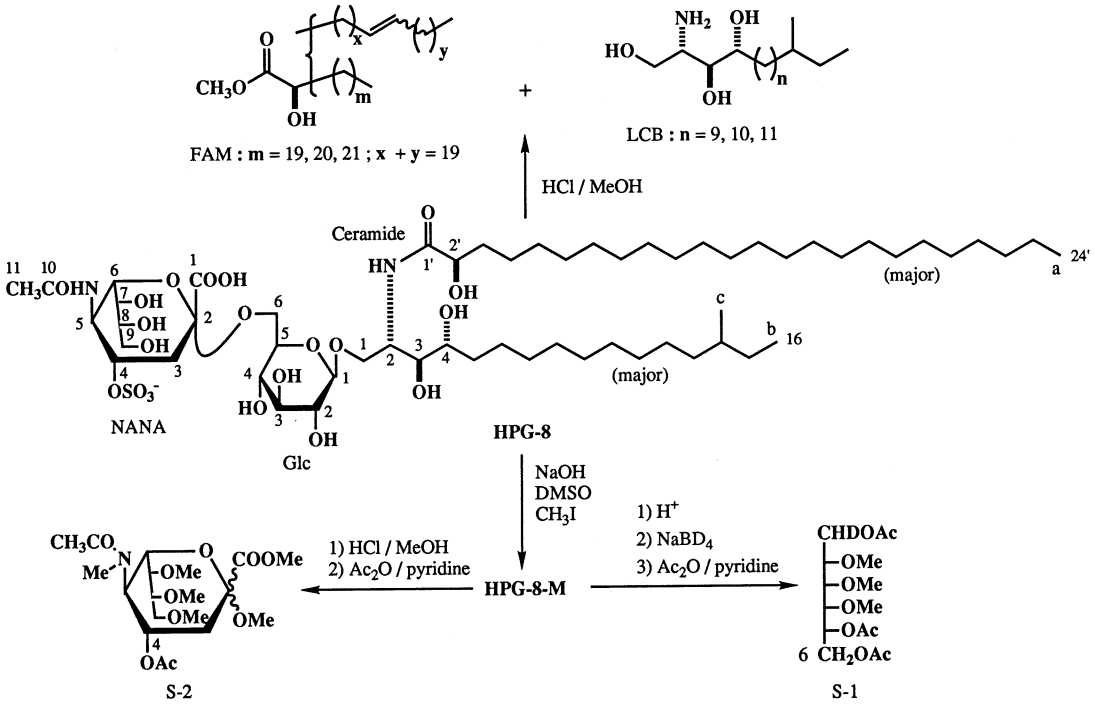
The structure of the ceramide moiety was examined first. When **HPG-8** was methanolyzed with methanolic hydrochloric acid, a mixture of fatty acid methyl esters (FAM) and long-chain bases (LCB) was obtained, together with methyl glucopyranoside. The FAM mixture was analyzed by GC MS, which revealed the presence of four components. These were characterized as methyl 2-hydroxydocosanoate, methyl 2-hydroxytricosanoate, methyl 2-hydroxytetracosanoate (major), and methyl 2-hydroxytetracosenoate. The LCB mixture was found to be composed of 2-amino-1,3,4-trihydroxy-14-methylhexadecane (major),

[◇] Part 6 : See ref.[1c].

  1434−193X/98/1111−2519 $ 17.50+.50/0

-15-methylheptadecane, and -16-methyloctadecane, based on GC-MS analysis of its TMS derivative (Scheme 1).

Scheme 1

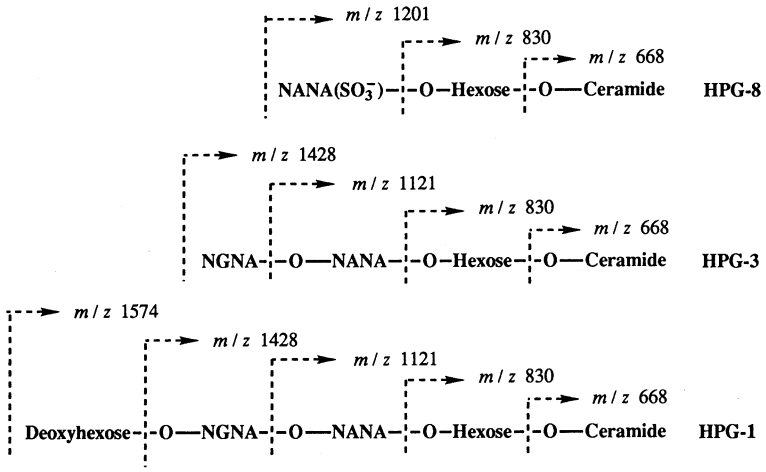


The stereochemistry of the ceramide moiety is presumed to be (2*S*,3*S*,4*R*,2′*R*), since the aforementioned ^{13}C-NMR signals ascribable to C-1, 2, 3, 4, 1′ and 2′ of **HPG-8** are in good agreement with those of phytosphingosine-type glucocerebroside molecular species possessing (2*S*,3*S*,4*R*,2′*R*) configurations[1c].

The structure of the disaccharide moiety of **HPG-8** was established as follows. The presence of glucose (Glc) was obvious from the results of the methanolysis of this species (vide supra). A detailed analysis of the ^{13}C-NMR spectrum of **HPG-8** revealed the characteristic signals [δ = 174.0 (s, C-1), 101.7 (s, C-2), 39.0 (t, C-3), 53.7 (d, C-5), 63.5 (t, C-9), 175.7 (s, C-10), 22.6 (q, C-11)] of an *N*-acetylneuraminic acid (NANA) derivative residue coupled with a β-glucopyranose derivative residue (Table 1). In the negative FAB mass spectrum of **HPG-8**, the molecular-ion and fragment-ion peaks arising from cleavage of the glycosidic linkages of the major component are observed at *m/z* = 1201, 830, and 668, indicating the presence of the disaccharide moiety NANA(SO_3^-)→hexose(β-glucopyranose), as shown in Scheme 2.

Methylation of **HPG-8** according to the Ciucanu-Kerek method[4] afforded the permethylated product **HPG-8-M**. Partially methylated alditol acetates (S-1) prepared from **HPG-8-M** were analyzed by GC MS and identified as the alditols derived from 6-linked hexopyranose. **HPG-8-M** was methanolyzed, the methanolysate was acetylated, and the acetate of partially methylated NANA (S-2) derived from

Scheme 2. Negative FAB mass fragmentation of the major component of **HPG-8**, **HPG-3**, and **HPG-1**



4-linked NANA was detected by means of GC-MS analysis. On the basis of the above evidence, the disaccharide moiety of **HPG-8** must be 4-*O*-sulfo-NANA-(2→6)-β-glucopyranose. The configuration of NANA is presumed to be α, since the characteristic carbon atom signals attributable to the NANA moiety are in good agreement with those of a known ganglioside[5] possessing α-linked NANA.

Consequently, if Glc and NANA are assumed to belong to the most commonly found D-series, then **HPG-8** is the *O*-4-*O*-sulfo-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranoside of a ceramide composed of heterogeneous (2*S*,3*S*,4*R*)-phytosphingosine and (2*R*)-2-hydroxy fatty acid units. The major components of the fatty acid and LCB moieties of **HPG-8** are *n*-(2*R*)-2-hydroxytetracosanoic acid and (2*S*,3*S*,4*R*)-1,3,4-trihydroxy-2-amino-14-methylhexadecane, respectively (Scheme 1).

Structure of HPG-3

In its ^{13}C-NMR spectrum, **HPG-3** exhibits characteristic signals due to the ceramide moiety corresponding to those of **HPG-8**, together with three anomeric carbon signals at δ = 105.0, 101.8, and 100.5, two of which (δ = 101.8 and 100.5) are quaternary carbon atom signals derived from two sialic acid moieties (Table 1). In the negative FAB MS, **HPG-3** shows a series of quasi-molecular ion peaks $[M - H]^-$ at *m/z* = 1400−1500. Accordingly, **HPG-3** is suggested to be a molecular species of a ganglioside like **HPG-8**, possessing three monosaccharide units. The ceramide moiety of **HPG-3** must be the same as that of **HPG-8**, since FAM and LCB mixtures derived from **HPG-3** correspond with those obtained from **HPG-8**. Therefore, the major components of the fatty acid and long-chain base moieties of **HPG-3** should be *n*-(2*R*)-2-hydroxytetracosanoic acid and (2*S*,3*S*,4*R*)-2-amino-1,3,4-trihydroxy-14-methylhexadecane, as in the case of **HPG-8**.

The structure of the trisaccharide moiety of **HPG-3** was elucidated as outlined below. The presence of glucose (Glc) was obvious from the results of the methanolysis of this species. In addition, the absolute configuration of the glucose unit was verified as being of D-form by means of the Hara method[6]. In its ^{13}C-NMR spectrum (Table 1), **HPG-3** shows characteristic signals due to one mole each of NANA and *N*-glycolylneuraminic acid (NGNA) derivative residues, together with those of a β-glucopyranose derivative residue. These data suggest that the trisaccharide moiety of **HPG-3** is composed of one mole each of Glc, NANA, and NGNA. The negative FAB MS of **HPG-3** shows the molecular and fragment ion peaks at *m/z* = 1428, 1121, 830, and 668, corresponding to cleavage of the glycosidic linkages of the major component, thus indicating the linear trisaccharide moiety, NGNA→NANA→hexose, as shown in Scheme 2.

Partially methylated alditol acetates prepared from **HPG-3-M**, the permethylated **HPG-3**, were characterized as the alditols derived from 6-linked hexopyranose (S-1) by means of GC MS. The acetates of partially methylated NANA (S-2), derived from 4-linked NANA, and of permethylated NGNA (S-3), derived from terminal NGNA, were detected in the acetate of methanolysate prepared from **HPG-3-M**. These facts establish the structure of the trisaccharide moiety as NGNA-(2→4)-NANA-(2→6)-β-Glc(p).

The configuration of NANA is also thought to be α, as in the case of **HPG-8**, while the configuration of NGNA is suggested to be α by comparison of the appropriate carbon atom signals with those of a known ganglioside[7] possessing α-linked NGNA.

Consequently, if NGNA and NANA are assumed to belong to the D-series, then **HPG-3** is the (*N*-glycolyl-α-D-neuraminosyl)-(2→4)-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranoside of a ceramide composed of heterogeneous (2*S*,3*S*,4*R*)-phytosphingosine and (2*R*)-2-hydroxy fatty acid units. The major components of the fatty acid and LCB moieties of **HPG-3** are *n*-(2*R*)-2-hydroxytetracosanoic acid and (2*S*,3*S*,4*R*)-2-amino-1,3,4-trihydroxy-14-methylhexadecane, respectively, as in the case of **HPG-8** (Scheme 3).

Structure of HPG-1

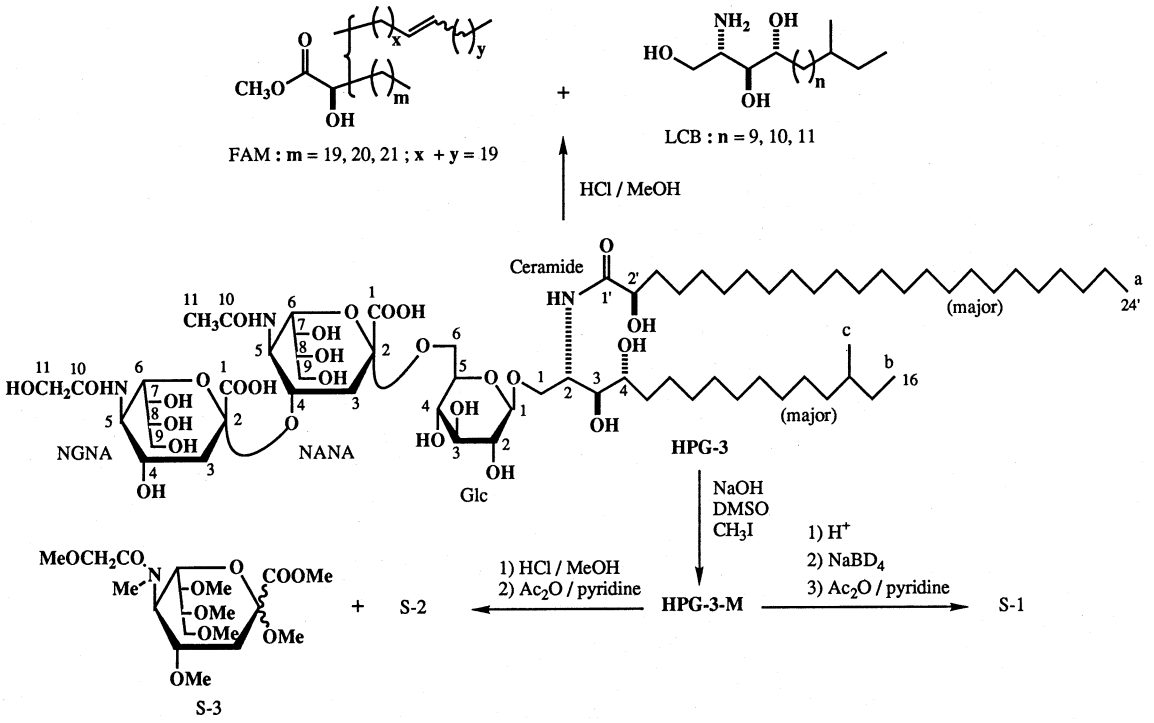
In its ^{13}C-NMR spectrum, **HPG-1** exhibits characteristic signals attributable to the ceramide moiety, which corre-

Table 1. ^{13}C-NMR chemical shifts (δ values) of the gangliosides in [D_5]pyridine/D_2O (95:5)

C		HPG-8	HPG-3	HPG-1	C		HPG-8	HPG-3	HPG-1
Ceramide					NGNA				
1	(t)	70.5	70.3	70.4	1	(s)		173.8	174.1
2	(d)	51.2	51.9	51.6	2	(s)		100.5	100.9
3	(d)	75.9	75.9	75.8	3	(t)		42.9	41.4
4	(d)	72.2	72.5*	72.3	4	(d)		69.0	68.6
1'	(s)	175.8	176.2	175.9	5	(d)		54.3	54.6
2'	(d)	72.2	72.6*	72.3	6	(d)		74.8	75.3
CH_3[a]	(q)	14.0	14.2	14.0	7	(d)		71.3	70.4
CH_3[b]	(q)	11.3	11.5	11.3	8	(d)		72.6*	77.1
CH_3[c]	(q)	19.2	19.4	19.2	9	(t)		64.1	61.3
Glc					10	(s)		176.3	175.9
1	(d)	104.7	105.0	104.6	11	(t)		62.4	62.3
2	(d)	74.8*	74.7**	74.9*	Fuc				
3	(d)	77.8	78.1	78.2	1	(d)			101.3
4	(d)	72.1	72.0	71.4	2	(d)			69.7
5	(d)	77.0	77.2	77.7	3	(d)			71.0
6	(t)	68.5	69.0	68.6	4	(d)			72.7
NANA					5	(d)			67.5
1	(s)	174.0	173.8	173.8	6	(q)			16.5
2	(s)	101.7	101.8	101.3					
3	(t)	39.0	39.3	39.1					
4	(d)	82.1	73.9	74.3					
5	(d)	53.7	53.6	53.4					
6	(d)	74.3*	74.6**	74.6*					
7	(d)	69.9	70.2	69.7					
8	(d)	71.8	73.0	73.1					
9	(t)	63.5	64.3	64.3					
10	(s)	175.7	176.3	175.9					
11	(q)	22.6	22.8	22.7					

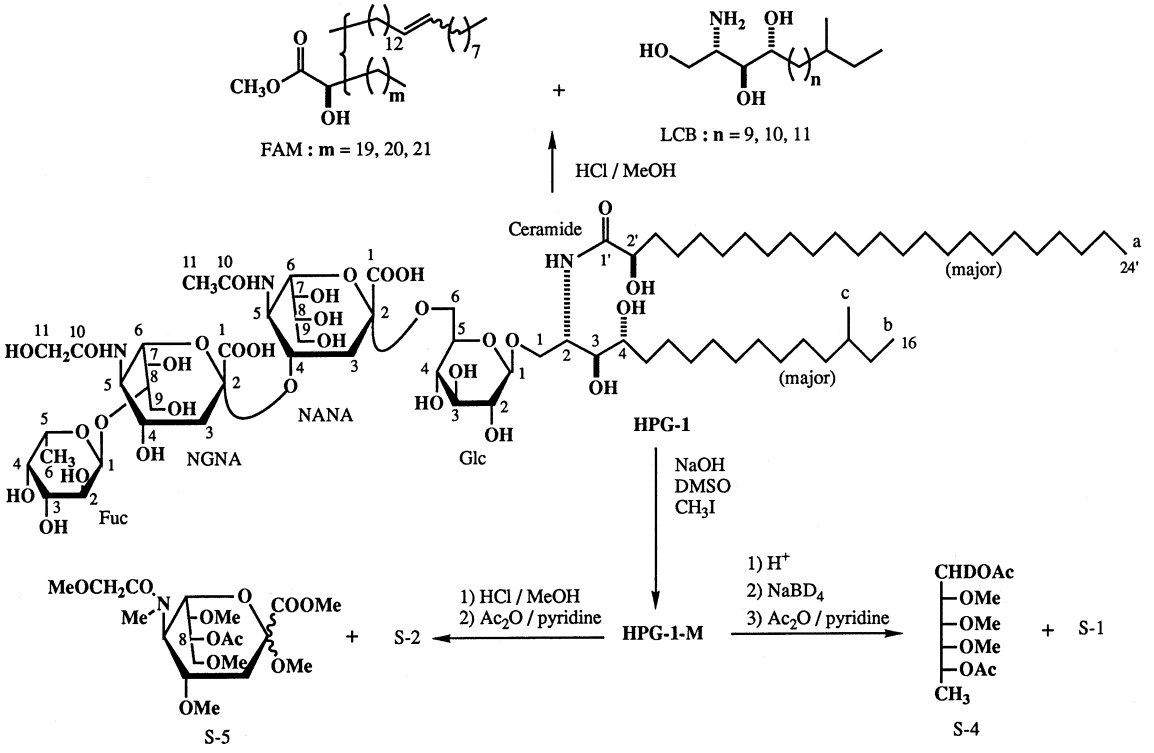
*, ** Assignments may be interchanged in each vertical column. − [a] Terminal methyl group in the *normal*-type of side chain (see Scheme 1). − [b][c] Terminal methyl groups in the *branched*-type of side chain (see Scheme 1).

Scheme 3

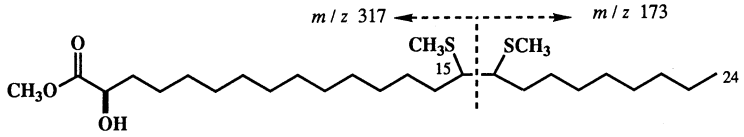


spond to those of **HPG-3** (Table 1). The ^{13}C-NMR spectrum of **HPG-1** also features signals due to four anomeric carbon atoms at δ = 104.6, 101.3, 101.3, and 100.9, two of which (δ = 101.3 and 100.9) are quaternary carbon atom signals, indicating the presence of two sialic acid residues. The negative FAB MS exhibits a series of quasi-molecular ion peaks $[M - H]^-$ at *m/z* = 1500−1600. Therefore, **HPG-1** is suggested to be a molecular species of a ganglioside like **HPG-3**, having four monosaccharide units. Since **HPG-1** gave the same FAM and LCB mixtures as those derived from **HPG-8**, the major components of the fatty acid and long-chain base moieties of **HPG-1** must be *n*-(2*R*)-2-

Scheme 4



Scheme 5. Mass fragmentation of the DMDS derivative of methyl 2-hydroxytetracosenoate



hydroxytetracosanoic acid and (2*S*,3*S*,4*R*)-2-amino-1,3,4-trihydroxy-14-methylhexadecane, as for **HPG-8** and **HPG-3**. The position of the double bond in the *n*-(2*R*)-2-hydroxytetracosenoic acid, a fatty acid component of **HPG-1**, was determined to be as shown in Scheme 4, by means of mass-spectral fragmentation of its dimethyl disulfide (DMDS) derivative[8] (Scheme 5).

That methanolysis and acidic hydrolysis of **HPG-1** gave the same results as for **HPG-3**, together with the signals due to sugar moiety in the ^{13}C-NMR spectrum of **HPG-1** (Table 1), suggests that the sialosyl tetrasaccharide moiety of **HPG-1** is composed of one mole each of β-D-glucopyanose, α-L-fucopyranose, α-NANA, and α-NGNA. In its negative FAB MS, **HPG-1** shows molecular (*m/z* = 1574) and fragment ion peaks (*m/z* = 1428, 1121, 830, and 668) arising from cleavage of the glycosidic linkages of the major component, which are indicative of the linear tetrasaccharide moiety, deoxyhexose→NGNA→NANA→hexose, as shown in Scheme 2.

GC-MS analysis of the partially methylated alditol acetates of the neutral sugars and of the acetates of partially methylated sialic acids, which were synthesized from **HPG-1-M**, the permethylated **HPG-1**, indicated the presence of terminal 6-deoxyhexopyranose (S-4), 6-linked hexopyranose (S-1), 4-linked NANA (S-2), and 8-linked NGNA (S-5) in the sugar moiety. On the basis of the above evidence, the sialosyl tetrasaccharide moiety of **HPG-1** must be α-L-Fuc(p)-(1→8)-α-NGNA-(2→4)-α-NANA-(2→6)-β-Glc(p).

Accordingly, if NGNA and NANA are assumed to belong to the D-series, the major component of **HPG-1** must be 1-*O*-[α-L-fucopyranosyl-(1→8)-(*N*-glycolyl-α-D-neuraminosyl)-(2→4)-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranosyl]-(2*S*,3*S*,4*R*)-2-[(2*R*)-2-hydroxytetracosanoylamino]-14-methylhexadecane-1,3,4-triol, as shown in Scheme 4.

Neuritogenic Activity

The effects of the above, newly isolated ganglioside molecular species on the neuritogenesis of the rat pheochromocytoma cell line (PC-12 cells) have been investigated. The results show that the three ganglioside molecular species, **HPG-8**, **HPG-3**, and **HPG-1**, display neuritogenic activity compared with H_2O (control) at a concentration of above 10 μg/ml.

From the sea cucumbers *Cucumaria japonica*[9], *Holothuria atra*[10], *Telenota ananas*[10], and *Cucumaria echinata*[1c], four kinds of ganglioside molecular species have been obtained and characterized. However, the ganglioside molecular species isolated in this study, **HPG-8**, **HPG-3**, and **HPG-1** are, to the best of our knowledge, new gangliosides. Most notably, a sulfated ganglioside possessing a 4-*O*-sulfated sialic acid residue, i.e. as in **HPG-8**, has been isolated for the first time. Furthermore, it is noteworthy that the gangliosides having 2→4-linked tandem-type disialoyl moieties, **HPG-3** and **HPG-1**, are akin to those isolated from the starfish *Lethasterias fuska*[11]. 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.

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Experimental Section

IR spectra: Jasco IR-700 infrared spectrophotometer. − ^{1}H- and ^{13}C-NMR spectra: Jeol GX-270 spectrometer (270 MHz and 67.8 MHz). − FAB mass-spectra: Jeol DX-300 (xenon atom beam); matrix: HMPA/TEG (negative ion mode). − GC MS: Shimadzu QP-1000; EI mode (ionizing potential of 70 eV, separator and ion-source temperature of 250°C); column: CBP10-W12-100 (0.53 mm × 12 m, Shimadzu); 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 **HPG-8**, **HPG-3** *and* **HPG-1**: Whole bodies of the sea cucumber *Holothuria pervicax* (126 kg), which were collected at Tsuyazaki, Fukuoka Prefecture, Japan, in 1994, were chopped and extracted four times with $CHCl_3$/MeOH (1:2, 54 l). The combined extracts were concentrated in vacuo to give an aqueous solution (36 l), which was extracted three times with AcOEt/*n*BuOH (2:1, 12 l). The aqueous phase was further extracted with *n*BuOH (12 l, 3 times), and then concentrated in vacuo to give a residue. This residue was extracted with $CHCl_3$/MeOH (1:1, 22 l), and the extract was concentrated in vacuo to give a crude water-soluble lipid fraction (1059 g). The polar lipid fraction was chromatographed on Chromatorex ODS DM1020T (reversed phase, eluent: 60%, 80% and 100% MeOH) to give three fractions. The crude ganglioside fraction (13 g), the 100% MeOH eluate, was further chromatographed on silica gel (solvent $CHCl_3$/MeOH/H_2O, 7:3:0.5 to 5:5:1) to afford **HPG-8** (5 mg, R_f = 0.57), **HPG-3** (7 mg, R_f = 0.46), and **HPG-1** (30 mg, R_f = 0.42) [silica gel TLC, solvent $CHCl_3$/MeOH/H_2O (6:4:1)].

HPG-8: Amorphous powder, m.p. 200−205°C. − Negative FAB MS: *m/z* = 1150−1250 $[M - H]^-$ series, 830, 668 (fragment ions

of major component, see Scheme 2), 97 $[SO_4H]^-$, 80 $[SO_3]^-$. – ^{13}C NMR: See Table 1.

Methanolysis of **HPG-8**: **HPG-8** (0.3 mg) was heated with 5% HCl in MeOH (0.4 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 **HPG-8**: The FAM mixture derived from **HPG-8** was subjected to GC MS [column temp.: 180−250°C (rate of temp. increase 4°C/min)]. The results were as follows: methyl 2-hydroxydocosanoate, t_R [min] = 7.5, *m/z* = 370 $[M^+]$, 311 $[M - 59]^+$; methyl 2-hydroxytricosanoate, t_R = 9.0, *m/z* = 384 $[M^+]$, 325 $[M - 59]^+$; methyl 2-hydroxytetracosenoate, t_R = 9.9, *m/z* = 396 $[M^+]$, 337 $[M - 59]^+$; methyl 2-hydroxytetracosanoate (major), t_R = 10.6, *m/z* = 398 $[M^+]$, 339 $[M - 59]^+$.

GC-MS Analysis of TMS Ethers of LCB from **HPG-8**: The mixture of LCB and methyl glycoside derived from **HPG-8** 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)]. The results were as follows: 2-amino-1,3,4-trihydroxy-14-methylhexadecane (major), t_R [min] = 4.5, *m/z* = 326 $[M - 193]^+$, 285 $[M - 234]^+$, 132; 2-amino-1,3,4-trihydroxy-15-methylheptadecane, t_R = 5.7, *m/z* = 340 $[M - 193]^+$, 299 $[M - 234]^+$, 132; 2-amino-1,3,4-trihydroxy-16-methyloctadecane, t_R = 7.0, *m/z* = 354 $[M - 193]^+$, 313 $[M - 234]^+$, 132.

GC Analysis of TMS Ethers of Methyl Glycoside from **HPG-8**: 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)]: t_R [min] = 17.9 and 18.1 (methyl α- and β-glucopyranoside).

Methylation of **HPG-8** (Ciucanu-Kerek Method)[4]: NaOH/DMSO solution, which was prepared from powdered NaOH (40 mg) and DMSO (1 ml), and MeI (0.2 ml) were added to **HPG-8** (2.6 mg), and the mixture was stirred for 30 min The reaction mixture was then diluted with H_2O (15 ml), and extracted with $CHCl_3$ (10 ml × 3). The combined $CHCl_3$ phases were washed with H_2O, and the solvent was evaporated in vacuo to give permethylated **HPG-8**, denoted **HPG-8-M** (2.5 mg).

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetate from **HPG-8-M**: **HPG-8-M** (1.1 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 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/C_5H_5N (1:1, 0.3 ml) at 70°C for 2 h. After dilution with H_2O, the mixture was extracted with $CHCl_3$ (0.2 ml × 3). 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)]. The results were as follows: S-1, t_R [min] = 7.1, *m/z* = 118, 162, 189, 233 [1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol (derived from 6-linked hexopyranose)].

Preparation and GC-MS Analysis of Acetate of Partially Methylated Sialic Acid from **HPG-8-M**: **HPG-8-M** (0.6 mg) was heated with 5% HCl in MeOH (0.5 ml) at 75°C for 6 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/C_5H_5N (1:1, 0.2 ml) at 70°C for 2 h. The resulting mixture was diluted with H_2O and extracted with $CHCl_3$ (0.2 ml × 3), 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)]: S-2, t_R = 16.9 min, *m/z* = 157, 346, 376 [methyl *N*-acetyl-4-*O*-acetyl-*N*-methyl-2,7,8,9-tetra-*O*-methylneuraminate (derived from 4-linked NANA)].

HPG-3: Amorphous powder, m.p. > 300°C. – Negative FAB MS: *m/z* = 1400−1500 $[M - H]^-$ series, 1121, 830, 668 (fragment ions of major component, see Scheme 2). – ^{13}C NMR: See Table 1.

Methanolysis of **HPG-3**: In the same manner as described for **HPG-8**, **HPG-3** was methanolyzed and the reaction mixture was worked up to give a FAM mixture and a residue composed of long-chain bases (LCB) and methyl glycoside.

GC-MS Analysis of FAM from **HPG-3**: The FAM mixture from **HPG-3** was subjected to GC MS under the same conditions as described for the FAM mixture obtained from **HPG-8**. Methyl 2-hydroxydocosanoate, methyl 2-hydroxytricosanoate, methyl 2-hydroxytetracosenoate, and methyl 2-hydroxytetracosanoate (major) were detected.

GC-MS and GC Analyses of TMS Ethers of LCB and Methyl Glycoside from **HPG-3**: The residue (mixture of LCB and methyl glycoside) from **HPG-3** was trimethylsilylated and the reaction mixture was analyzed by GC MS and GC in the same manner as described for **HPG-8**. LCB (GC MS): 2-Amino-1,3,4-trihydroxy-14-methylhexadecane (major), 2-amino-1,3,4-trihydroxy-15-methylheptadecane, 2-amino-1,3,4-trihydroxy-16-methyloctadecane. – Methyl glycoside (GC): Methyl α- and β-glucopyranoside were detected.

Determination of Absolute Configuration of the Glucose Moiety of **HPG-3** (Hara Method[6]): **HPG-3** (0.4 mg) was heated with 4 N H_2SO_4 (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-cysteine methyl ester hydrochloride (0.3 mg) and pyridine (0.3 ml) at 60°C for 1 h. Then, 0.1 ml of 1-(trimethylsilyl)imidazole was added and the mixture was heated at 60°C for a 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)]; t_R = 13.3 min (derivative of D-glucose, 13.3 min; L-glucose, 14.0 min).

Preparation of **HPG-3-M** *and Partially Methylated Alditol Acetates from* **HPG-3-M**: **HPG-3** (1.2 mg) was methylated according to the Ciucanu-Kerek method and the reaction mixture was worked up in the same manner as described for **HPG-8**, thereby yielding **HPG-3-M** (1.3 mg). **HPG-3-M** (0.4 mg) was hydrolyzed, reduced, and then acetylated, and the partially methylated alditol acetate was analyzed by GC MS in the same manner as described for **HPG-8-M**, whereupon S-1, derived from 6-linked hexopyranose, was detected.

Preparation and GC-MS Analysis of Acetates of Partially Methylated Sialic Acids from **HPG-3-M**: **HPG-3-M** (0.5 mg) was methanolyzed and then acetylated in the same manner as described for **HPG-8-M**. The acetates were subjected to GC MS under the same conditions as mentioned above, and S-2 (derived from 4-linked NANA) and S-3, t_R = 7.2 min, *m/z* = 159, 348, 378 [methyl *N*-

glycolyl-*N*-methyl-2,4,7,8,9,11-hexa-*O*-methylneuraminate (derived from terminal NGNA)], were detected.

HPG-1: Amorphous powder, m.p. 261−270°C. − IR (KBr): $\tilde{\nu}$ = 3400 cm^{-1} (OH), 1630, 1530 (amide). − Negative FAB MS: *m/z* = 1500−1600 $[M - H]^-$ series, 1428, 1121, 830, 668 (fragment ions of major component, see Scheme 2). − ^{13}C NMR: See Table 1.

Analyses of FAM, LCB, and Methyl Glycosides from **HPG-1**: Experiments were conducted in the same manner as in the case of **HPG-8**, leading to a FAM mixture and a residue composed of long-chain bases (LCB) and methyl glycosides derived from the **HPG-1**. The FAM mixture was subjected to GC MS under the same conditions as described for **HPG-8**, and methyl 2-hydroxydocosanoate, -tricosanoate, -tetracosenoate, and -tetracosanoate (major) were detected. The mixture of LCB and methyl glycosides was trimethylsilylated and analyzed by GC MS and GC in the same way as in the case of **HPG-8**. The results were as follows: LCB (GC MS): 2-Amino-1,3,4-trihydroxy-14-methylhexadecane (major), -15-methylheptadecane, -16-methyloctadecane. − Methyl glycosides (GC): Methyl α- and β-fucopyranoside, t_R [min] = 12.6 and 13.0, and methyl α- and β-glucopyranoside were detected.

DMDS Derivative of Methyl 2-Hydroxytetracosenoate: **HPG-1** FAM (0.5 mg) was dissolved in carbon disulfide (0.2 ml), and dimethyl disulfide (DMDS, 0.2 ml) and iodine (1 mg) were added. The resulting mixture was kept at 60°C for 40 h in a small-volume sealed vial. The reaction was then quenched with aqueous $Na_2S_2O_3$ (5%, 0.4 ml), and the mixture was extracted with *n*-hexane (0.2 ml × 3). The extract was concentrated and the residue was analyzed by GC MS (column temp. 250°C): t_R = 6.7 min; *m/z* = 317, 173 (DMDS derivative of methyl 2-hydroxy-15-tetracosenoate).

Determination of Absolute Configuration of the Fucose and Glucose Moieties of **HPG-1**: **HPG-1** (0.7 mg) was subjected to acid hydrolysis and the sugar fraction was treated in the same manner as described for **HPG-3**, thereby affording the trimethylsilyl ethers of the methyl thiazolidine-4(*R*)-carboxylate derivatives. The derivative was analyzed by GC under the same conditions as before, and L-fucose, t_R = 12.3 min (derivative of D-fucose, 11.4 min; L-fucose, 12.3 min), and D-glucose were detected.

Preparation of **HPG-1-M** *and Partially Methylated Alditol Acetates from* **HPG-1-M**: The partially methylated alditol acetates were obtained from **HPG-1-M** (prepared from **HPG-1** as above) and analyzed by GC MS in the same way as for those from **HPG-8-M**. S-1 (derived from 6-linked hexopyranose) and S-4, t_R [min] = 3.4, *m/z* = 118, 162, 175, 131 [1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methylhexitol (derived from terminal 6-deoxyhexopyranose)], were detected.

Preparation and GC-MS Analysis of Acetates of Partially Methylated Sialic Acids from **HPG-1-M**: The acetates were prepared from **HPG-1-M** and subjected to GC MS in the same manner as described for **HPG-8-M**. S-2 (derived from 4-linked NANA) and S-5, t_R = 17.2 min, *m/z* = 159, 348, 356, 406 [methyl *N*-glycolyl-8-O-acetyl-*N*-methyl-2,4,7,9,11-penta-*O*-methylneuraminate (derived from 8-linked NGNA)], were detected.

Observation of Neuritogenic Activity on PC-12 Cells: PC-12 cells (Riken Cell Bank) were cultured, at a density of 1×10^5 cells in 1 ml medium, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and 10% horse serum 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 growth medium was replaced by serum-free DMEM/Ham's F12 (1:1) medium supplemented with N-2 Supplement (GIBCO). Each ganglioside, **HPG-8**, **HPG-3**, and **HPG-1**, was added to the medium at densities of 100, 10, 1, and 0.1 μg/ml, and the cells were further cultured at 37°C. After 2 d, the morphological changes in the cells were observed with a microscope. Cells treated with above 10 μg/ml of each of three gangliosides showed neurite outgrowth compared with those treated with H_2O (control).

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[2] *normal* means straight chain (...$CH_2CH_2CH_2CH_3$).

[3] *branched* means chain possessing a methyl group on the third carbon atom from the terminal methyl group [...$CH_2CH(CH_3)CH_2CH_3$].

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