Constituents of Holothuroidea. 10.[1] Isolation and Structure of a Biologically Active Ganglioside Molecular Species from the Sea Cucumber *Holothuria leucospilota*

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Three ganglioside molecular species, HLG-1 (1), HLG-2 (2), and HLG-3 (3) have been obtained from the lipid fraction of the chloroform/methanol extract of the sea cucumber *Holothuria leucospilota.* The structures of these gangliosides have been determined, on the basis of chemical and spectroscopic evidence, as 1-*O*-[(*N*-glycolyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranosyl]-ceramide (1), 1-*O*-[(*N*-glycolyl-α-D-neuraminosyl)-(2→4)-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranosyl]-ceramide (2) and 1-*O*-[α-L-fucopyranosyl-(1→11)-(*N*-glycolyl-α-D-neuraminosyl)-(2→4)-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranosyl]-ceramide (3). The ceramide moieties were composed of heterogeneous phytosphingosine, sphingosine and 2-hydroxy fatty acid units. Compounds 2 and 3 represent new ganglioside molecular species. These three ganglioside molecular species showed neuritogenic activity toward the rat pheochromocytoma cell line, PC-12 cell, in the presence of NGF (nerve growth factor).

Key words glycosphingolipid; ganglioside; sea cucumber; *Holothuria leucospilota*; neuritogenic activity

In our continuing research on biologically active glycosphingolipids (GSLs) from echinoderms, a series of studies on the isolation and structure elucidation of the GSLs from sea cucumber species have been performed in our laboratory.[2] In continuation of the preceding studies on the sea cucumber *Holothuria pervicax*,[1] the isolation and characterization of the biologically active GSLs from the sea cucumber *Holothuria leucospilota* (Nisekuronamako in Japanese) has now been carried out in order to develop the novel medicinal resources from natural marine products. In this paper, we report on the isolation and characterization of three ganglioside molecular species from the whole bodies of *H. leucospilota.* The biological activities of the gangliosides are also reported.

The polar lipid fraction, which was obtained from the chloroform/methanol extract of the whole bodies of *H. leucospilota*, was subjected to repeated silica gel column chromatography to give three ganglioside molecular species, HLG-1 (**1**), HLG-2 (**2**), and HLG-3 (**3**), each showing a single spot on silica gel thin-layer chromatography (TLC).

In its ^{13}C-NMR spectrum (Chart 1, Table 1), **1** exhibits the characteristic signals of a phytosphingosine-type ceramide, possessing a 2-hydroxy fatty acid and a sugar moiety at C-1 [δ: 70.1 (C-1), 51.0 (C-2), 75.8 (C-3), 72.0 (C-4), 175.8 (C-1′), 72.0 (C-2′)]. The ^{13}C-NMR spectrum of **1** also features signals due to two anomeric carbons at δ: 104.4 and 101.2, one of which (δ: 101.2) is a quaternary carbon signal, indicating the presence of a sialic acid residue. The negative FAB-MS exhibits a series of quasi-molecular ion peaks $[M-H]^-$ at *m/z*: 1080—1180. Therefore, **1** is suggested to be a molecular species of a phytosphingosine-type ganglioside, possessing 2-hydroxy fatty acid groups and two monosaccharide units. Furthermore, **1** is presumed to have mainly normal-type fatty acids and iso and *ante*-iso type long-chain bases, since the carbon signals for the terminal methyl groups are observed at δ: 13.9 (normal form) and δ: 22.5 (iso form), 11.3 and 19.1 (*ante*-iso form) in the ^{13}C-NMR spectrum (Chart 1, Table 1).

The structure of the ceramide moiety was examined first. When **1** 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-hydroxytetracosenoate, and methyl 2-hydroxytetracosanoate. The LCB mixture was found to be composed of 2-amino-1,3,4-trihydroxy-heptadecane, -octadecane, and -nonadecane, based on GC-MS analysis of its TMS (trimethylsilyl) derivative (Chart 1).

The relative 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 **1** are in good agreement with those of the phytosphingosine-type glucocerebroside molecular species possessing (2*S*,3*S*,4*R*, 2′*R*) configurations.[2c]

The structure of the disaccharide moiety of **1** was established as follows. The presence of glucose (Glc) was obvious from the results of the methanolysis of **1** (*vide supra*). A detailed analysis of the ^{13}C-NMR spectrum of **1** revealed the characteristic signals [δ: 173.5 (C-1), 101.2 (C-2), 42.4 (C-3), 53.4 (C-5), 63.7 (C-9), 176.2 (C-10), 61.9 (C-11)] of an *N*-glycolylneuraminic acid (NeuGc) derivative residue coupled with a β-glucopyranose derivative residue (Table 1). In the negative FAB-MS of **1**, the molecular ion and fragment ion peaks arising from cleavage of the glycosidic linkages are observed at *m/z*: 1080—1180, 800—850, and 630—700, indicating the presence of the disaccharide moiety, NeuGc→Hexose(β-glucopyranose), as shown in Fig 1.

Methylation of **1**, according to Ciucanu–Kerek method,[3] afforded the permethylated product **4**. Partially methylated alditol acetate (S-1), prepared from **4**, was analyzed by GC-MS and identified as the alditol derived from 6-linked hexopyranose. On the other hand, **4** was methanolyzed, the methanolysate was acetylated, and the permethylated NeuGc (S-2) derived from the terminal NeuGc was detected by means of GC-MS analysis. On the basis of the above evi-

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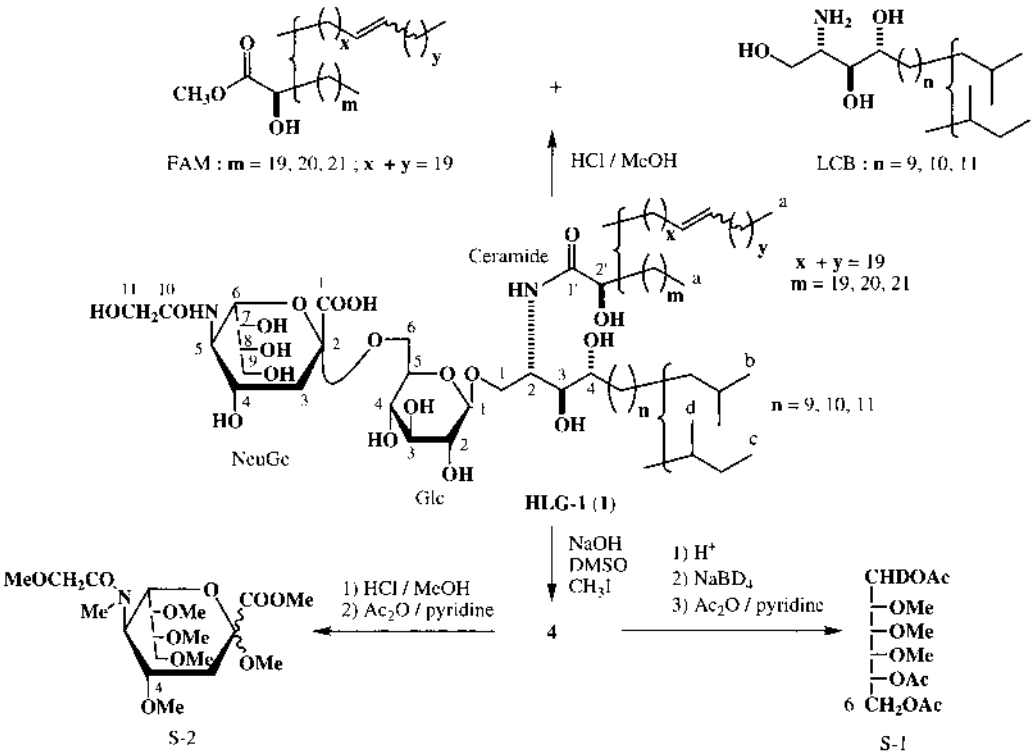

Chart 1

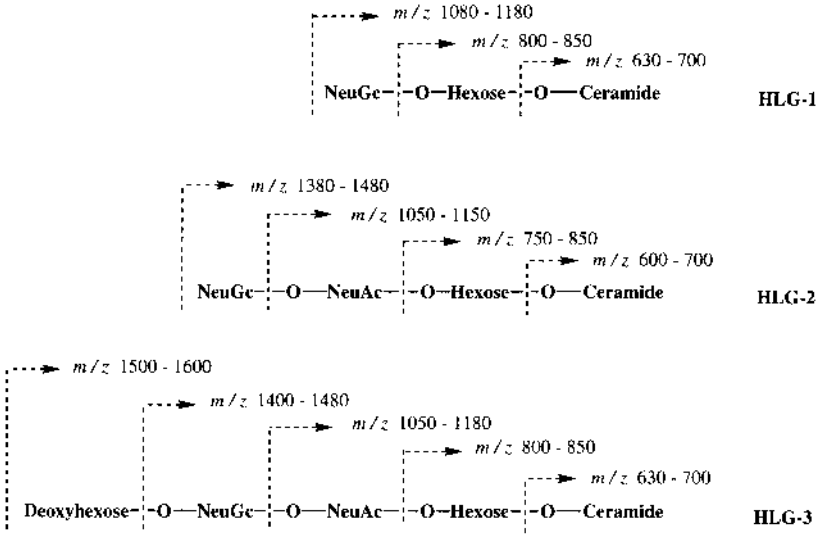

Fig. 1. The Negative Ion FAB-MS Fragmentation of HLG-1, HLG-2, and HLG-3

dence, the disaccharide moiety of **1** must be NeuGc-(2→6)-β-glucopyranose. The configuration of NeuGc is believed to be α on the basis of its anomeric carbon signal (δ: 101.2)[4] in the ^{13}C-NMR spectrum of **1**. In addition, the absolute configuration of the glucose unit was verified as being of D-form by means of the Hara method.[5]

Consequently, if NeuGc is assumed to belong to the most commonly found D-series, then **1** is the (*N*-glycolyl-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, as shown in Chart 1.

Compound **2** exhibits the characteristic signals due to the sphingosine-type ceramide, possessing a 2-hydroxy fatty acid and a sugar moiety at C-1 [δ: 70.2 (C-1), 54.3 (C-2), 72.5 (C-3), 131.6 (C-4), 133.0 (C-5), 175.8 (C-1′), 72.5 (C-2′)] in its ^{13}C-NMR spectrum (Chart 2, Table 1). The ^{13}C-NMR spectrum of **2** also shows three anomeric carbon signals at δ: 105.3, 101.0, and 99.7, two of which (δ: 101.0, 99.7) are quaternary carbon signals derived from two sialic acid moieties (Table 1). In the negative FAB-MS, **2** shows a series of quasi-molecular ion peaks $[M-H]^-$ at *m/z*: 1380—1480. Accordingly, **2** is suggested to be a molecular species of a sphingosine-type ganglioside, possessing 2-hydroxy fatty acid groups and three monosaccharide units. The terminal methyl groups of the ceramide moiety of **2** must be the same as that of **1** from their carbon atom signals (Table 1).

Table 1. ^{13}C-NMR Spectral Data (δ values) of the Gangliosides in C_5D_5N–D_2O (98 : 2)

C		HLG-1	HLG-2	HLG-3	C		HLG-1	HLG-2	HLG-3
Ceramide					NeuGc				
1	(t)	70.1	70.2	70.3	1	(s)	173.5	173.8	174.0
2	(d)	51.0	54.3	50.9	2	(s)	101.2	99.7[f]	100.5
3	(d)	75.8	72.5	75.5[e]	3	(t)	42.4	43.1	42.0
4	(d)	72.0	131.6	72.0	4	(d)	68.4	68.7	68.5
5	(d)		133.0		5	(d)	53.4	53.9	53.0
1′	(s)	175.8[e]	175.8[e]	176.0	6	(d)	73.8	74.8	74.0
2′	(d)	72.0	72.5	72.6	7	(d)	69.7	71.2	69.5
CH_3[a]	(q)	13.9	14.3	14.3	8	(d)	72.4	72.5	72.0
CH_3[b]	(q)	22.5	22.8	22.8	9	(t)	63.7	64.9	63.0
CH_3[c]	(q)	11.3	11.6	11.6	10	(s)	176.2[e]	176.2[e]	174.0
CH_3[d]	(q)	19.1	19.4	19.4	11	(t)	61.9	62.6	64.0
Glc					Fuc				
1	(d)	104.4	105.3	105.3	1	(d)			101.9
2	(d)	74.2	74.8	74.8	2	(d)			69.5
3	(d)	76.9	77.7	77.4	3	(d)			70.8
4	(d)	70.4	72.5	70.7	4	(d)			72.4
5	(d)	75.8	76.4	76.3	5	(d)			67.5
6	(t)	70.1	70.6	70.0	6	(q)			17.1
NeuAc									
1	(s)		173.8	174.0					
2	(s)		101.0[f]	100.5					
3	(t)		39.3	39.0					
4	(d)		74.3	71.8					
5	(d)		51.8	52.7					
6	(d)		74.8	75.3[e]					
7	(d)		70.6	70.4					
8	(d)		73.0	72.0					
9	(t)		64.0	62.0					
10	(s)		176.2[e]	176.0					
11	(q)		22.8	22.5					

a—*d*): Terminal methyl groups in the normal, iso, and *ante*-iso type of side chain (see Chart 1). *e*, *f*): Assignments may be interchanged in each vertical column.

Methanolysis of **2** afforded a mixture of FAM, LCB and methyl glucopyranoside. The FAM mixture was analyzed by GC-MS and four components corresponding with those obtained from **1** were detected. On the other hand, the LCB component was found to be 2-amino-1,3-dihydroxy-4-heptadecene by means of GC-MS analysis of its TMS derivative (Chart 2). Furthermore, the relative stereochemistry of the ceramide moiety is presumed to be (2*S*, 3*R*, 4*E*, 2′*R*) by comparison of the ^{13}C-NMR signals due to C-1, 2, 3, 4, 5 and 2′ of **2** and a sphingosine-type glucocerebroside molecular species possessing (2*S*, 3*R*, 4*E*, 2′*R*) configurations.[2a]

The structure of the trisaccharide moiety of **2** was elucidated as outlined below. The presence of glucose (Glc) was obvious from the results of the methanolysis of this species and the absolute configuration (D-form) of the glucose unit was verified as before. In its ^{13}C-NMR spectrum (Table 1), **2** shows characteristic signals due to one mole each of NeuGc and *N*-acetylneuraminic acid (NeuAc) derivative residues, together with those of a β-glucopyranose derivative residue. These data suggest that the trisaccharide moiety of **2** is composed of one mole each of Glc, NeuGc, and NeuAc. The negative FAB-MS of **2** shows the molecular and fragment ion peaks at *m*/*z*: 1380—1480, 1050—1150, 750—850, and 600—700, corresponding to cleavage of the glycosidic linkages of **2**, thus indicating the linear trisaccharide moiety, NeuGc→NeuAc→Hexose, as shown in Fig. 1.

Partially methylated alditol acetate prepared from **5**, the permethylated **2**, was characterized as the alditol derived from 6-linked hexopyranose (S-1) by means of GC-MS. The acetate of partially methylated NeuAc (S-3) derived from 4-linked NeuAc and of permethylated NeuGc (S-2), derived from terminal NeuGc, were detected in the acetate of methanolysate prepared from **5**. These facts establish the structure of the trisaccharide moiety as NeuGc-(2→4)-NeuAc-(2→6)- β-D-Glc (p).

The configurations of the sialic acids (NeuGc, NeuAc) are also thought to be α, as in the case of **1**, based on their anomeric carbon signals (δ: 99.7, 101.0) in the ^{13}C-NMR spectrum of **2**.

Consequently, if NeuGc and NeuAc are assumed to belong to the D-series, then **2** is the (*N*-glycolyl-α-D-neuraminosyl)-(2→4)-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranoside of a ceramide composed of (2*S*,3*R*,4*E*)-C_{17}-sphingosine and heterogeneous (2*R*)-2-hydroxy fatty acid units as shown in Chart 2.

In its ^{13}C-NMR spectrum, **3** exhibits characteristic signals attributable to the ceramide moiety, which correspond to those of **1** (Table 1). The ^{13}C-NMR spectrum of **3** also features signals due to four anomeric carbon atoms at δ: 105.3, 101.9, 100.5, and 100.5, two of which (δ: 100.5) 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, **3** is suggested to be a molecular species of ganglioside, like **1**, having four monosaccharide units. Since **3** gave the same FAM and LCB mixture as those derived from **1**, the components of the fatty acid and long-chain base moieties of **3** must be (2*R*)-2-hydroxy fatty acids and (2*S*,3*S*,4*R*)-

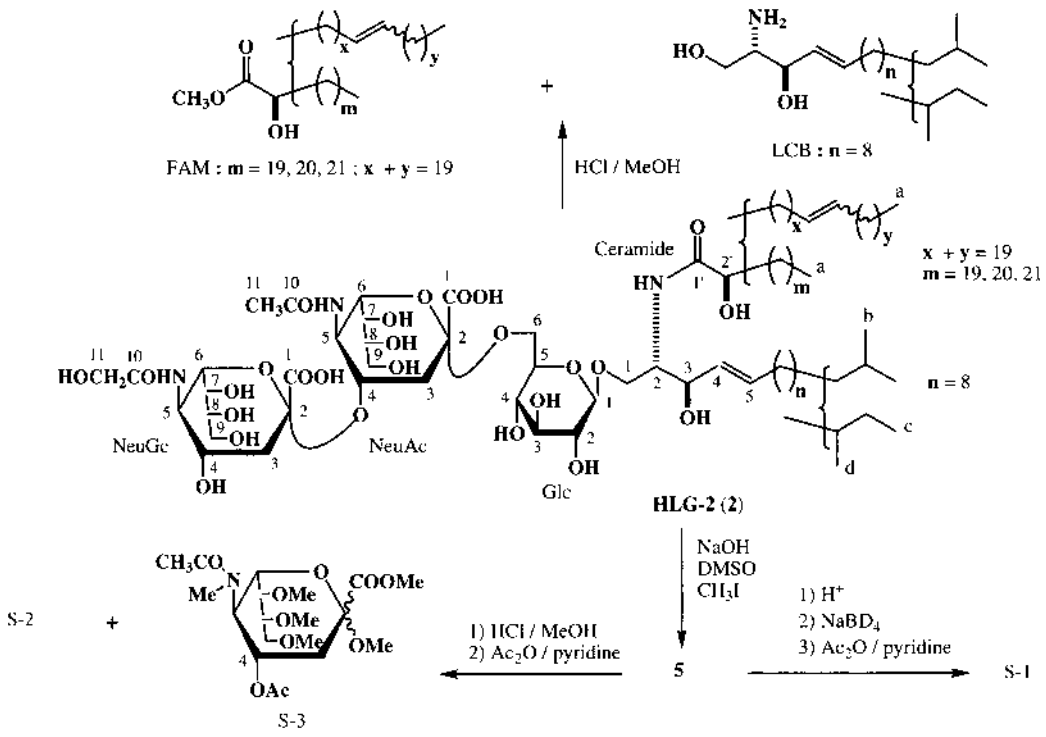

Chart 2

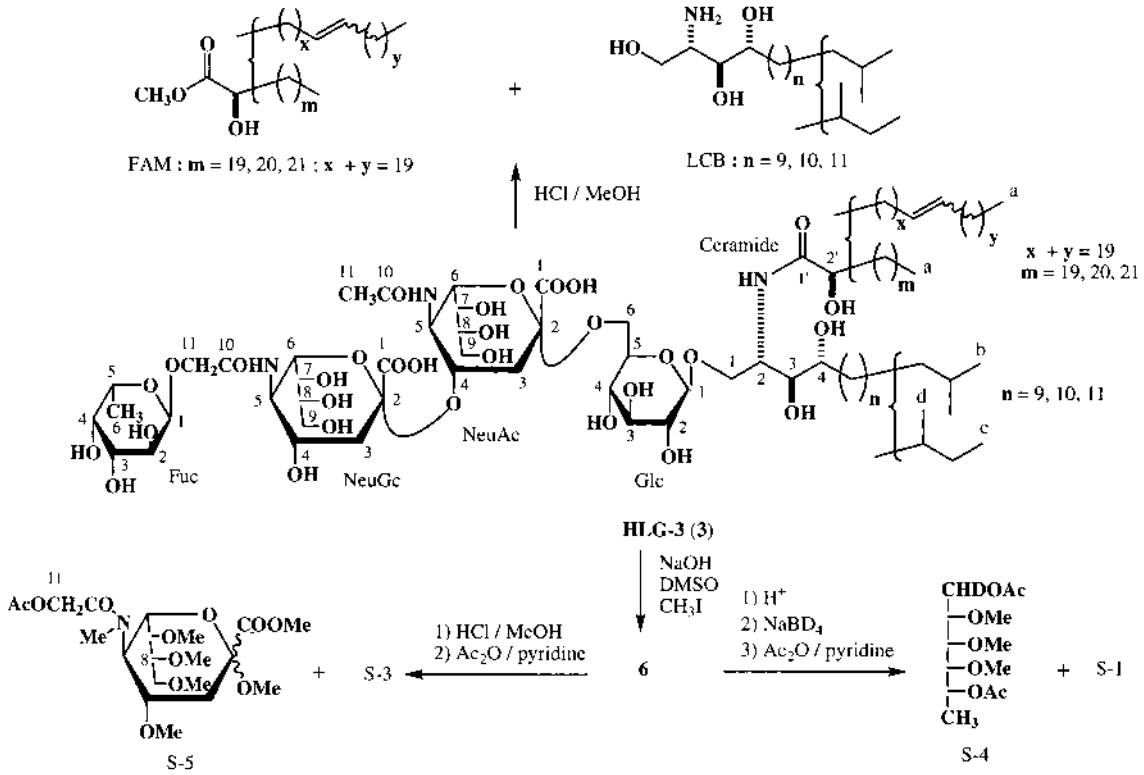

Chart 3

phytosphingosines as for **1**.

The methanolysis and acidic hydrolysis of **3**, indicating the existence of D-Glc and L-fucose (Fuc), together with the signals due to sugar moiety in the ^{13}C-NMR spectrum of **3** (Table 1), suggest that the sialosyl tetrasaccharide moiety of **3** is composed of one mole each of β-D-glucopyranose, α-L-fucopyranose, α-NeuAc, and α-NeuGc. In its negative FAB-MS, **3** shows molecular (*m*/*z*: 1500—1600) and fragment ion peaks (*m*/*z*: 1400—1480, 1050—1180, 800—850, 630—700) arising from cleavage of the glycosidic linkages of **3**, which are indicative of the linear tetrasaccharide moiety, deoxyhexose→NeuGc→NeuAc→Hexose, as shown in Fig. 1.

The 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 **6**, the permethylated **3**, indicated the presence of terminal 6-deoxyhexopyranose (S-4), 6-linked hexopyranose (S-1), 4-linked NeuAc (S-3), and 11-linked NeuGc (S-5) in the sugar moiety. On the basis of the above evidence, the sialosyl tetrasaccharide moiety of **3** must be α-L-Fuc (p)-(1→11)-α-NeuGc-(2→4)-α-NeuAc-(2→6)-β-D-Glc (p).

Accordingly, if NeuGc and NeuAc are assumed to belong to the D-series, **3** must be α-L-fucopyranosyl-(1→11)-(*N*-glycolyl-α-D-neuraminosyl)-(2→4)-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranoside of a ceramide composed of the same fatty acid and long-chain base units as **1**.

The effects of the 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, **1**, **2**, and **3**, display neuritogenic activity, compared with H_2O (control), at a concentration of 10 μM in the presence of NGF (5 ng/ml).

From the sea cucumber *Cucumaria japonica*,[6] *Holothuria atra*,[7] *Telenota ananas*,[7] *Cucumaria echinata*,[2c] *Holothuria pervicax*,[1,2d] and *Stichopus japonicus*,[2e] nine kinds of ganglioside molecular species have been obtained and characterized. However, the ganglioside molecular species isolated in this study, HLG-2 and HLG-3, are, to the best of our knowledge, new gangliosides. Although a ganglioside possessing the same sugar and core of ceramide moieties as that of HLG-1 has been obtained from the eggs of the sea urchin *Anthocidaris crassispina*,[8] HLG-1 slightly differs from the ganglioside in the structure of its fatty acyl and LCB components. The isolation and characterization of such neuritogenically active gangliosides is attracting considerable attention with regard to the manufacturing of new medicines from natural marine products.

Experimental

Melting points were determined on a micro melting point apparatus (Yanako MP-3) without correction. NMR spectra were recorded on a Varian Unity-500 spectrometer (500 MHz). Negative-ion FAB-MS spectra were acquired with a JEOL SX-102 mass spectrometer (xenon atom beam; matrix, HMPA-TEG). GC-MS were taken with a Shimadzu QP-1000 [EI mode; ionizing potential, 70 eV; separator and ion-source temperature 250 °C; column, CBP10-W12-100 (0.53 mm×12 m, Shimadzu); carrier gas, He]. GC were run on a Shimadzu GC-14B [FID mode; column, Fused Silica Capillary Column DB-17 (0.317 mm×30 m, J & W Scientific); carrier, N2].

Separation of HLG-1 (1), HLG-2 (2) and HLG-3 (3) Whole bodies of the sea cucumber *Holothuria leucospilota* (170 kg), which was collected at Ushibuka, Kumamoto Prefecture, Japan, in 1997, were chopped and extracted three times with CHC_3–MeOH (1 : 2, 55 l). The combined extracts were concentrated *in vacuo* to give an aqueous solution (120 l), which was extracted three times with *n*-hexane (55 l). The *n*-hexane phase was concentrated *in vacuo* to give a residue (352 g). The residue was dissolved in acetone. The acetone-insoluble part (146 g), the polar lipid fraction, was chromatographed on silica gel (solvent $CHCl_3$–MeOH–H_2O, 8 : 2 : 0 to 5 : 5 : 1) to give seven fractions. Successive column chromatography of fraction 2 (silica gel, solvent $CHCl_3$–MeOH–H_2O, 7 : 3 : 0.2 to 6 : 4 : 0.2) afforded HLG-1 (**1**) (22 mg) (*Rf*=0.53) and HLG-2 (**2**) (13 mg) (*Rf*=0.42). On the other hand, fraction 6 was further chromatographed on silica gel (solvent $CHCl_3$–MeOH–3.5 N NH_3 aq., 65 : 35 : 5 to 6 : 4 : 1) to afford HLG-3 (**3**) (25 mg) (*Rf*=0.36) [silica gel TLC, solvent $CHCl_3$–MeOH–H_2O (6 : 4 : 1)].

HLG-1 (**1**): Amorphous powder, mp 182—185 °C. Negative-ion FAB-MS *m/z*: 1080—1180 $[M-H]^-$ series (see Fig. 1). ^{13}C-NMR: See Table 1.

HLG-2 (**2**): Amorphous powder, mp 186—188 °C. Negative-ion FAB-MS *m/z*: 1380—1480 $[M-H]^-$ series (see Fig. 1). ^{13}C-NMR: See Table 1.

HLG-3 (**3**): Amorphous powder, mp 186—188 °C. Negative-ion FAB-MS *m/z*: 1500—1600 $[M-H]^-$ series (see Fig. 1). ^{13}C-NMR: See Table 1.

Methanolysis of 1 Compound **1** (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 FAM. The MeOH layer was neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated *in vacuo* to give a mixture of LCB and methyl glycoside.

GC-MS Analysis of FAM from 1 A FAM mixture from **1** 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, t_R=10.6, *m/z*: 398 (M^+), 339 $(M-59)^+$.

GC-MS Analysis of TMS Ethers of LCB from 1 The mixture of LCB and methyl glycoside from **1** was heated with 1-(trimethylsilyl) imidazole–pyridine (1 : 1) for 10 min at 60 °C and the reaction mixture (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-trihydroxyheptadecane, t_R [min]=4.5, *m/z*: 326 $(M-193)^+$, 285 $(M-234)^+$, 132; 2-amino-1,3,4-trihydroxyoctadecane, t_R=5.7, *m/z*: 340 $(M-193)^+$, 299 $(M-234)^+$, 132; 2-amino-1,3,4-trihydroxynonadecane, t_R=7.0, *m/z*: 354 $(M-193)^+$, 313 $(M-234)^+$, 132.

GC Analysis of TMS Ethers of Methyl Glycoside from 1 The mixture of TMS 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).

Determination of Absolute Configuration of Glucose Moiety of 1 (Hara Method[5]) **1** (0.5 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 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).

Methylation of 1 (Ciucanu–Kerek Method)[3] NaOH–dimethylsulfoxide (DMSO) solution, which was prepared from powdered NaOH (40 mg) and DMSO (1 ml), and MeI (0.2 ml) were added to **1** (2 mg), and the mixture was stirred for 30 min. The reaction mixture was then diluted with H_2O (15 ml), extracted with $CHCl_3$ (10 ml×3), and the $CHCl_3$ phases were washed with H_2O, and the solvent was evaporated *in vacuo* to give permethylated **1**, denoted **4** (1.9 mg).

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetate from 4 Compound **4** (0.7 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 4 Compound **4** (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=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 NeuGc)].

Methanolysis of 2 In the same manner as described for **1**, **2** was

methanolyzed and the reaction mixture was worked up to give a mixture of FAM and a residue composed of LCB and methyl glycoside.

GC-MS Analysis of FAM from 2 A FAM mixture from **2** was subjected to GC-MS under the same conditions as described for the FAM mixture obtained from **1**. Methyl 2-hydroxydocosanoate, methyl 2-hydroxytricosanoate, methyl 2-hydroxytetracosenoate, and methyl 2-hydroxytetracosanoate were detected.

GC-MS and GC Analyses of TMS Ethers of LCB and Methyl Glycoside from 2 The residue (mixture of LCB and methyl glycoside) from **2** was trimethylsilylated and the reaction mixture was analyzed by GC-MS and GC in the same manner as described for **1**. LCB (GC-MS): 2-Amino-1,3-dihydroxy-4-heptadecene, t_R [min]=3.1, *m*/*z*: 326 $(M-103)^+$, 297 $(M-132)^+$, 236 $(M-193)^+$, 132. Methyl glycoside (GC): methyl α- and β-glucopyranoside were detected.

Determination of Absolute Configuration of Glucose Moiety of 2 Compound **2** (0.5 mg) was subjected to acid hydrolysis and the sugar fraction was treated in the same manner as described for **1**, thereby affording the TMS ethers of the methyl thiazolidine-4(*R*)-carboxylate derivatives. The derivative was analyzed by GC under the same conditions as before, and D-glucose was detected.

Preparation of 5 and Partially Methylated Alditol Acetates from 5 Compound **2** (1.1 mg) was methylated according to the Ciucanu–Kerek method and the reaction mixture was worked up in the same manner as described for **1**, thereby yielding permethylated **2**, denoted **5** (1.2 mg). Compound **5** (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 **4**, whereupon S-1, derived from 6-linked hexopyranose, was detected.

Preparation and GC-MS Analysis of Acetates of Partially Methylated Sialic Acids from 5 Compound **5** (0.5 mg) was methanolyzed and then acetylated in the same manner as described for **4**. The acetates were subjected to GC-MS under the same conditions as mentioned above, and S-2 (derived from terminal NeuGc) and S-3, 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 NeuAc)], were detected.

Analyses of FAM, LCB and Methyl Glycosides from 3 Experiments were conducted in the same manner as in the case of **1**, leading to a mixture of FAM and a residue composed of LCB and methyl glycosides derived from the **3**. The FAM mixture was subjected to GC-MS under the same conditions as described for **1**, and methyl 2-hydroxydocosanoate, -tricosanoate, -tetracosenoate, and -tetracosanoate 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 **1**. The results were as follows: LCB (GC-MS): 2-Amino-1,3,4-trihydroxyheptadecane, -octadecane, -nonadecane. Methyl glycosides (GC): methyl α- and β-fucopyranoside, t_R [min]=12.6 and 13.0, and methyl α- and β-glucopyranoside were detected.

Determination of Absolute Configuration of the Fucose and Glucose Moieties of 3 In the same manner as described for **1**, **3** (1.0 mg) was subjected to acid hydrolysis and the sugar fraction was treated. The sugar derivatives were analyzed by GC in the same condition 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 6 and Partially Methylated Alditol Acetates from 6 The partially methylated alditol acetates were obtained from **6** (prepared from **3** as above) and analyzed by GC-MS in the same way as for those from **4**. 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 6 The acetates were prepared from **6** and subjected to GC-MS in the same manner as described for **4**. S-3 (derived from 4-linked NeuAc) and S-5, t_R=19.9 min, *m*/*z*: 187, 201, 376, 406 [methyl *N*-glycolyl-11-*O*-acetyl-*N*-methyl-2,4,7,8,9-penta-*O*-methylneuraminate (derived from 11-linked NeuGc)], were detected.

Biological Assay Neuritogenic activity of **1**, **2**, and **3** on PC-12 cells was observed according to the method previously reported.[2e] Cells treated with 10 μM of each of three gangliosides with NGF (5 ng/ml) showed neurite outgrowth compared with those treated with H_2O (control).

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References

1) Part 9: Yamada K., Harada Y., Miyamoto T., Isobe R., Higuchi R., *Chem. Pharm. Bull*., **48**, 157—159 (2000).
2) *a*) Higuchi R., Inagaki M., Togawa K., Miyamoto T., Komori T., *Justus Liebigs Ann. Chem*., **1994**, 79—81; *b*) *Idem, ibid*., **1994**, 653—658; *c*) Yamada K., Hara E., Miyamoto T., Higuchi R., Isobe R., Honda S., *Eur. J. Org. Chem*., **1998**, 371—378; *d*) Yamada K., Harada Y., Nagaregawa Y., Miyamoto T., Isobe R., Higuchi R., *ibid*., **1998**, 2519—2525; *e*) Kaneko M., Kisa F., Yamada K., Miyamoto T., Higuchi R., *ibid*., **1999**, 3171—3174.P
3) Ciucanu I., Kerek F., *Carbohydr. Res*., **131**, 209—217 (1984).
4) Higuchi R., Mori T., Sugata T., Yamada K., Miyamoto T., *Eur. J. Org. Chem*., **1999**, 3175—3178.
5) Hara S., Okabe H., Mihashi K., *Chem. Pharm. Bull*., **35**, 501—506 (1987).
6) Chekareva N. V., Smirnova G. P., Kochetkov N. K., *Bioorg. Khim*., **17**, 398—402 (1991).
7) Smirnova G. P., *Bioorg. Khim*., **22**, 134—139 (1996).
8) Kubo H., Irie A., Inagaki F., Hoshi M., *J. Biochem*., **108**, 185—192 (1990).