Constituents of Holothuroidea, 13.[1] Structure of Neuritogenic Active Ganglioside Molecular Species from the Sea Cucumber *Stichopus chloronotus*

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Three ganglioside molecular species, SCG-1, SCG-2, and SCG-3, were obtained from the lipid fraction of the chloroform–methanol extract of the sea cucumber *Stichopus chloronotus*. On the basis of chemical and spectroscopic evidence, the structures of these gangliosides have been determined to be 1-*O*-[(*N*-glycolyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranosyl]-ceramide (SCG-1), 1-*O*-[8-*O*-sulfo(major)-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranosyl]-ceramide (SCG-2), and 1-*O*-[α-L-fucopyranosyl-(1→11)-(*N*-glycolyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranosyl]-ceramide (SCG-3). The ceramide moieties were composed of heterogeneous long-chain base and fatty acid units. SCG-3 is the first type of ganglioside containing a fucopyranose in the sialosyl trisaccharide moiety. Moreover, these three gangliosides exhibited neuritogenic activity toward the rat pheochromocytoma PC12 cells in the presence of nerve growth factor.

Key words glycosphingolipid; ganglioside; sea cucumber; *Stichopus chloronotus*; neuritogenic activity

In our continuing research on biologically active glycosphingolipids from echinoderms, a series of studies on the isolation, structure elucidation, and evaluation of biological activities of glycosphingolipids from the sea cucumber species have been performed in our laboratory.[2—7] Continuing the previous studies, our search for the biologically active ganglioside from the sea cucumber *Stichopus chloronotus* (*shikakunamako* in Japanese) has led to the isolation of three ganglioside molecular species designated **SCG-1**, **SCG-2**, and **SCG-3**. In this paper, we report on the isolation and characterization of these three gangliosides from the body walls of *S. chloronotus*. The biological activities of the gangliosides are also reported.

The lipid fraction, obtained from the chloroform–methanol extract of the body walls of *S. chloronotus*, was subjected to reverse-phase followed by normal-phase column chromatography to give the three ganglioside molecular species **SCG-1**, **SCG-2**, and **SCG-3**, which appeared as a single spot on normal-phase TLC.

The IR spectrum of **SCG-1** showed prominent peaks due to hydroxy and amide groups. Its ^{13}C-NMR spectrum exhibited the characteristic signals of a sphingosine-type ceramide,[2,3] possessing a 2-hydroxy fatty acid and a sugar moiety at C-1 [δ: 70.2 (C-1), 54.1 (C-2), 72.3 (C-3), 131.4 (C-4), 132.9 (C-5), 175.7 (C-1′), and 72.2 (C-2′)]. The spectrum also revealed signals attributed to two anomeric carbons at δ: 101.1 and 105.2, one of which (δ: 101.1) was a quaternary carbon signal, indicating the existence of one sialic acid function. Negative-ion FAB-MS exhibited a quasimolecular ion peak $[M-H]^-$ at *m/z*: 1100—1160. Therefore **SCG-1** was suggested to be a molecular species of a sphingosine-type ganglioside, with 2-hydroxy fatty acids and two monosaccharide units. Furthermore, **SCG-1** was presumed to have mainly *normal*-type fatty acids and *iso*-type long-chain bases (LCBs) at the terminus, since the carbon signals for the terminal methyl groups were observed at δ: 14.2 (*normal* form), and δ: 22.8 (*iso* form) in ^{13}C-NMR (Table 1, Chart 1).

The structure of the ceramide moiety was elucidated first. When **SCG-1** was methanolyzed with 10% HCl–MeOH, a mixture of fatty acid methyl esters (FAM) and LCBs was obtained together with methyl glycoside. GC-MS analysis of the FAM mixture showed the existence of seven components, which were characterized as methyl docosanoate (FAM-1), methyl tricosanoate (FAM-2), methyl 2-hydroxydocosanoate (FAM-3), methyl tetracosenoate (FAM-4), methyl 2-hydroxytricosanoate (FAM-5), methyl 2-hydroxytetracosenoate (FAM-6), and methyl 2-hydroxytetracosanoate (FAM-7). The major FAM was methyl 2-hydroxytetracosenoate (FAM-6). The locations and geometries of the double bonds in the methyl tetracosenoate (FAM-4) and methyl 2-hydroxytetracosenoate (FAM-6) were determined as follows. The mass spectra of the bismethylthio derivatives of FAM,[8,9] which were obtained by methanolysis of **SCG-1**, showed remarkable fragment-ion peaks due to cleavage of the bond between the carbons bearing a methylthio group, as shown in Chart 2. These data indicated that the double bonds in FAM-4 and FAM-6 are located between C-15′ and C-16′, respectively, as shown in Chart 2. Furthermore, the chemical shifts of the C-14′ and C-17′ methylene carbons (δ: 27.6), which were assigned based on ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond connectivity (HMBC) spectra, suggested a 15′-*Z*-geometry, as shown in Table 1. It is known that the geometry of the double bond in a long-chain alkene can be determined on the basis of the ^{13}C-NMR chemical shift of the methylene carbon adjacent to the olefin carbon, which is observed at $\delta\approx27$ in (*Z*) isomers and at $\delta\approx32$ in (*E*) isomers.[10,11] On the other hand, the LCB mixture was found to be composed of 2-amino-1,3-dihydroxy-4-heptadecene (major) and 2-amino-1,3,4-trihydroxyheptadecane on the basis of GC-MS analysis of the TMS-derived LCB mixture.

Furthermore, the relative stereochemistry of the ceramide moiety was presumed to be (2*S*, 3*R*, 4*E*, 2′*R*), since the aforementioned ^{13}C-NMR signals attributable to C-1, 2, 3, 4, 5, and 2′ of **SCG-1** were in good agreement with those of the known sphingosine-type glucocerebroside molecular species[2] with (2*S*, 3*R*, 4*E*, 2′*R*) configurations obtained from another sea cucumber.

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

Position		SCG-1	SCG-2	SCG-3	Position		SCG-1	SCG-2	SCG-3
Ceramide					NeuGc				
1	(t)	70.2	70.1	70.4	1	(s)	173.3		173.0
2	(d)	54.1	54.1	54.5	2	(s)	101.1		101.3
3	(d)	72.3	72.7	72.3	3	(t)	42.8		42.4
4	(d)	131.4	131.4	131.4	4	(d)	68.5		67.6
5	(d)	132.9	132.9	133.0	5	(d)	54.1		54.5
1′	(s)	175.7[e)]	175.8[e)]	175.7[e)]	6	(d)	74.5		74.6
2′	(d)	72.2	72.3	72.3	7	(d)	68.5		69.6
14′	(t)	27.6		28.1	8	(d)	77.5		77.2
15′	(d)	130.1		130.1	9	(t)	64.1		64.1
16′	(d)	130.1		130.1	10	(s)	176.3[e)]		175.9[e)]
17′	(t)	27.6		28.1	11	(t)	62.3		67.6
CH_3[a)]	(q)	14.2	14.1	14.1	NeuAc				
CH_3[b)]	(q)	22.8	22.8	22.8	1	(s)		173.4	
CH_3[c)]	(q)			11.4	2	(s)		100.8	
CH_3[d)]	(q)			19.3	3	(t)		41.6	
Glc					4	(d)		68.1	
1	(d)	105.2	105.2	105.2	5	(d)		54.1	
2	(d)	74.6	74.6	74.6	6	(d)		74.6	
3	(d)	77.5	77.4	77.2	7	(d)		69.5	
4	(d)	70.8	71.1	71.0	8	(d)		80.6	
5	(d)	76.3	76.1	76.2	9	(t)		62.2	
6	(t)	68.5	67.6	67.7	10	(s)		175.8[e)]	
					11	(q)		22.7	
					Fuc				
					1	(d)			100.6
					2	(d)			69.7
					3	(d)			71.0
					4	(d)			72.6
					5	(d)			66.8
					6	(q)			16.8

a—*d*) Terminal methyl groups in the normal, iso, and *ante*-iso type of side chain (see Charts 1, 4, and 5), *e*) Assignments may be interchanged in each vertical column.

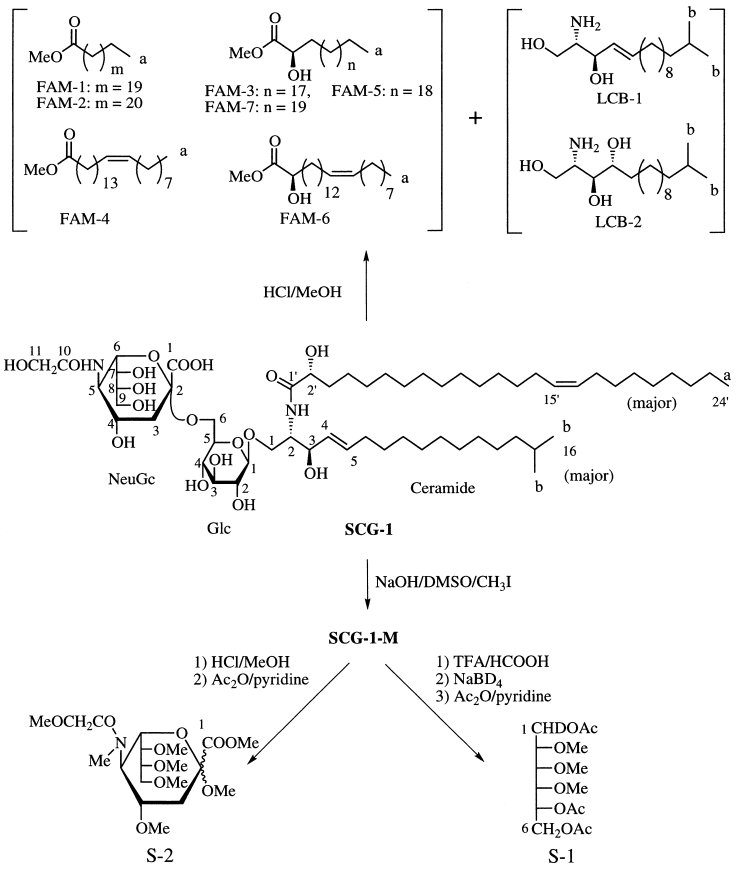

Chart 1

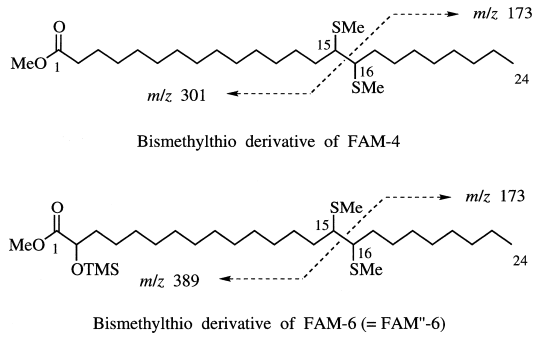

Chart 2. Mass Fragmentation of Bismethylthio Derivaties of FAM-4 and FAM-6 from **SCG-1**

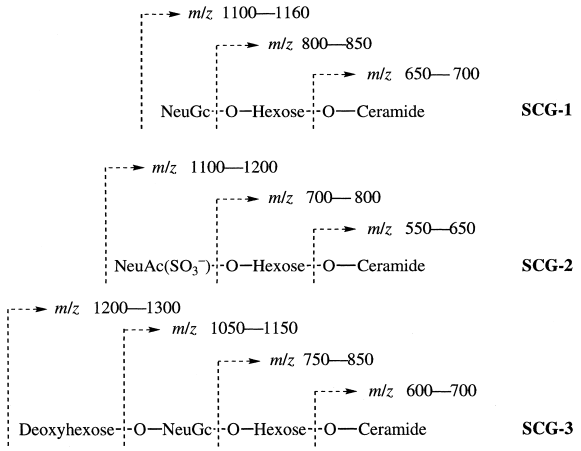

Chart 3. Negative-Ion FAB-MS Fragmentation of **SCG-1**, **SCG-2**, and **SCG-3**

The structure of the disaccharide moiety of **SCG-1** was established as follows. Negative-ion FAB-MS exhibited quasimolecular ion peaks $[M-H]^-$ at *m/z*: 1100—1160, and the fragment ion peaks arising from cleavage of glycosidic linkages of the major component were observed at *m/z*: 800—850, and 650—700, corresponding to cleavage of the glycosidic linkages of **SCG-1**, thus indicating the disaccharide moiety, *N*-glycolylneuramic acid (NeuGc)→hexose→ceramide, as shown in Chart 3.

The GC-MS analysis of the TMS derivatives of the methyl glycoside mixture, which was obtained by methanolysis of **SCG-1**, showed the existence of one mole of glucose (Glc). Methylation of **SCG-1** according to Ciucanu-Kerek's method[12] afforded the permethylated product **SCG-1-M**. Partially methylated alditol acetates prepared from **SCG-1-M** were characterized as the alditols (S-1) derived from 6-linked hexopyranose by means of GC-MS (*m/z*: 118, 162, 189, 233). On the other hand, **SCG-1-M** was methanolyzed and the methanolysate was acetylated, and then the permethylated NeuGc (S-2) derived from the terminal NeuGc was analyzed by GC-MS. On the basis of the above evidence, the disaccharide moiety of **SCG-1** must be NeuGc (2→6)–Glc.

The configuration of Glc was assigned to be β, while that of NeuGc was α on the basis of their anomeric carbon signals (δ: 105.2, 101.1) in the ^{13}C-NMR spectrum of **SCG-1**.[5,13] In addition, the absolute configuration of the Glc unit was verified to be the D-form using the Hara *et al.* method.[14] Consequently, if NeuGc, which is commonly found in natural sources, is assumed to be of the D-series, then **SCG-1** is the (*N*-glycolyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranoside of a ceramide composed of heterogeneous (2*S*,3*R*,4*E*)-sphingosine and (2*R*)-2-hydroxy fatty acid units. The major components of the fatty acid and LCB moieties were (*R*)-2-hydroxy-15-tetracosenoic acid and (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-15-methyl-4-hexadecene, as shown in Chart 1.

In its ^{13}C-NMR spectrum, **SCG-2** exhibited characteristic signals due to the ceramide moiety corresponding to those of **SCG-1**, together with two anomeric carbon signals at δ: 105.2 and 100.8, one of which (δ: 100.8) was a quaternary carbon atom signal ascribable to the sialic acid moiety (Table 1). In negative-ion FAB-MS, **SCG-2** showed a series of quasimolecular ion peaks $[M-H]^-$ at *m/z*: 1110—1200, and fragment-ion peaks due to $[SO_4H]^-$ and $[SO_3]^-$ at *m/z*: 97 and 80. Accordingly, **SCG-2** was suggested to be a molecular species of a sulfated ganglioside with two monosaccharide units. The structure of the ceramide moiety of **SCG-2** was elucidated in the same manner as in the case of **SCG-1**. As a result, it has become apparent that the components of fatty acid are six constituents, which were characterized as methyl octadecanoate (FAM′-1), methyl 2-hydroxyoctadecanoate (FAM′-2), methyl icosanoate (FAM′-3), methyl henicosanoate (FAM′-4), methyl docosanoate (FAM′-5), and methyl 2-hydroxydocosanoate (FAM′-6). The major FAM was methyl 2-hydroxyoctadecanoate (FAM′-2). On the other hand, the component of the LCB was one constituent of 2-amino-1,3-dihydroxy-4-heptadecene, as shown in Chart 4.

The structure of the disaccharide moiety of **SCG-2** was elucidated as outlined below. The presence of glucose was obvious from the results of the methanolysis of this species. In its ^{13}C-NMR spectrum (Table 1), **SCG-2** showed characteristic signals due to an α-linked *N*-acetylneuraminic acid derivative residue together with those of a β-linked glucopyranose derivative residue. These data suggested that the disaccharide moiety of **SCG-2** was composed of one mole each of β-glucose and α-*N*-acetylneuraminic acid (NeuAc). Negative-ion FAB-MS of **SCG-2** showed molecular and fragment ion peaks at *m/z*: 1100—1200, 700—800, and 550—650, corresponding to cleavage of the glycosidic linkages of the major component, thus indicating a linear disaccharide moiety, NeuAc(SO_3^-)→Glc→ceramide, as shown in Chart 3.

Partially methylated alditol acetate prepared from **SCG-2-M**, the permethylated **SCG-2**, was characterized as the alditol derived from 6-linked hexopyranose (S-1) by GC-MS analysis. The acetates of partially methylated NeuAc (S-3 and S-4), derived from 8-linked NeuAc and 4-linked NeuAc were detected in the acetate of methanolysate prepared from **SCG-2-M**, as shown in Chart 4. The major NeuAc derivative was S-3 from 8-linked NeuAc. These findings established the structure of the disaccharide moiety of the major component to be 8-*O*-sulfo-α-NeuAc(2→6)β-Glc.

The absolute configuration of Glc was also determined to be the D-form. Consequently, if NeuAc is assumed to belong to the D-series, then **SCG-2** is the *O*-8-*O*-sulfo(major)-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranoside of ceramide composed of heterogeneous (2*S*,3*R*,4*E*)-sphingosine and (2*R*)-hydroxy fatty acid units. The major components of the fatty acid and LCB moieties were (*R*)-2-hydroxyoctadecanoic acid and (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-15-methyl-4-hexadecene, respectively, as shown in Chart 4.

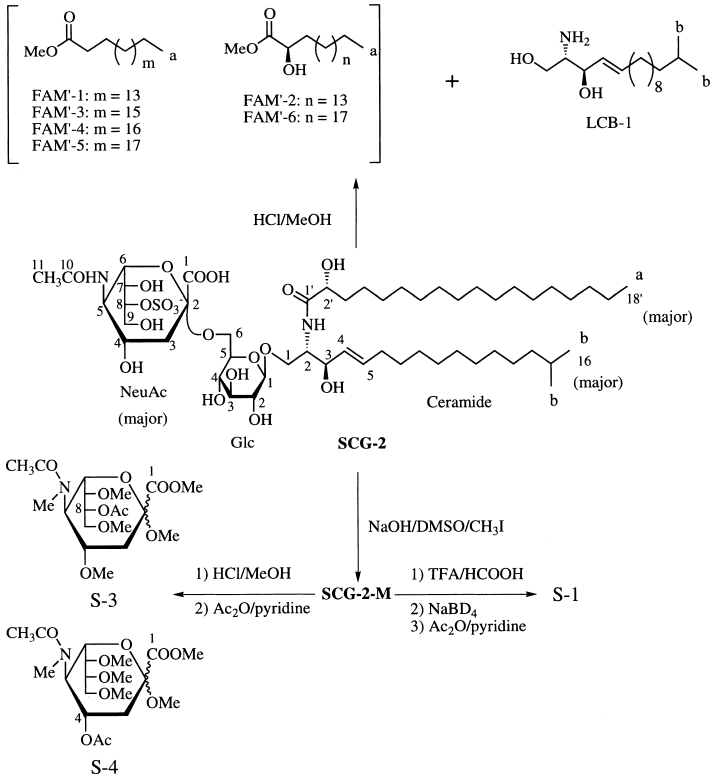

Chart 4

In its ^{13}C-NMR spectrum, **SCG-3** exhibited characteristic signals due to the ceramide moiety corresponding to those of **SCG-1** and **SCG-2** together with signals ascribable to the methyl groups of the *ante*-iso form at δ: 11.4 and 19.3. The ^{13}C-NMR spectrum of **SCG-3** also showed conspicuous signals due to three anomeric carbon atoms at δ: 105.2 101.3, and 100.6, one of which (δ: 101.3) was a quaternary carbon atom signal ascribable to the sialic acid moiety (Table 1). Negative-ion FAB-MS exhibited a series of quasimolecular ion peaks $[M-H]^-$ at *m/z*: 1200—1300. Therefore **SCG-3** was suggested to be a molecular species of a ganglioside like **SCG-1** and **SCG-2**, with three monosaccharide units. **SCG-3** gave the FAM and LCB mixtures upon methanolysis. The components of fatty acid were six constituents, which were characterized as methyl hexadecanoate (FAM″-1), methyl henicosanoate (FAM″-2), methyl tricosanoate (FAM″-3), methyl 2-hydroxydocosanoate (FAM″-4), methyl 2-hydroxytricosanoate (FAM″-5), and methyl 2-hydroxytetracosenoate (FAM″-6). The major FAM was methyl 2-hydroxydocosanoate (FAM″-4), as shown in Chart 5. The position of the double bond in the methyl 2-hydroxytetracosenoate (FAM″-6) was determined to be as shown in Chart 2 by GC-MS analysis of its bismethylthio derivative as performed for FAM-6. On the other hand, the LCB component was 2-amino-1,3-dihydroxy-4-heptadecene.

The structure of the trisaccharide moiety of **SCG-3** was elucidated in the same manner as in the case of **SCG-1** and **SCG-2**. The presence of β-glucopyranose, α-fucopyranose (Fuc), and α-*N*-glycolylneuraminic acid was obvious from the results of methanolysis and acidic hydrolysis and from the ^{13}C-NMR signals due to the sugar moiety of **SCG-3** (Table 1). The absolute configurations of Glc and Fuc were also determined to be the D- and L-form, respectively, as previously. Its negative-ion FAB-MS showed molecular and fragment ion peaks at *m/z*: 1200—1300, 1050—1150, 750—850, and 600—700, corresponding to cleavage of the glycosidic linkages of **SCG-3**, thus indicating the linear trisaccharide moiety deoxyhexose→NeuGc→hexose→ceramide, as shown in Chart 3.

GC-MS analysis of the partially methylated alditol acetates of the neutral sugars and of the acetate of partially methylated sialic acid, which were derived from **SCG-3-M** prepared from **SCG-3** by permethylation, indicated the presence of terminal 6-deoxyhexopyranose (S-5) [*m/z*: 118, 131, 162, 175], 6-linked hexopyranose (S-1) [*m/z*: 118, 162, 189, 233], and 11-linked NeuGc (S-6) in the sugar moiety, as shown in Chart 5. On the basis of the above evidence, the sialosyl trisaccharide moiety of **SCG-3** must be α-L-Fuc-(1→11)-α-NeuGc-(2→6)-β-D-Glc.

Accordingly, if NeuGc is assumed to belong to the D-series, **SCG-3** must be α-L-fucopyranosyl-(1→11)-(*N*-glycolyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranoside of a ceramide composed of the heterogeneous (2*S*,3*R*,4*E*)-sphingosine and (2*R*)-2-hydroxy fatty acid units. The major components of the fatty acid and LCB moieties were (*R*)-2-hydroxydocosanoic acid and (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-14(and 15)-methyl-4-hexadecene, as shown in Chart 5.

The effects of the isolated ganglioside molecular species on the neuritogenesis of the rat pheochromocytoma cell line (PC12 cells) have been investigated. The results showed that the three ganglioside molecular species **SCG-1**, **SCG-2**, and **SCG-3** displayed neuritogenic activity in the presence of nerve growth factor. The proportions of the neurite-bearing cells of **SCG-1**, **SCG-2**, and **SCG-3** at a concentration of

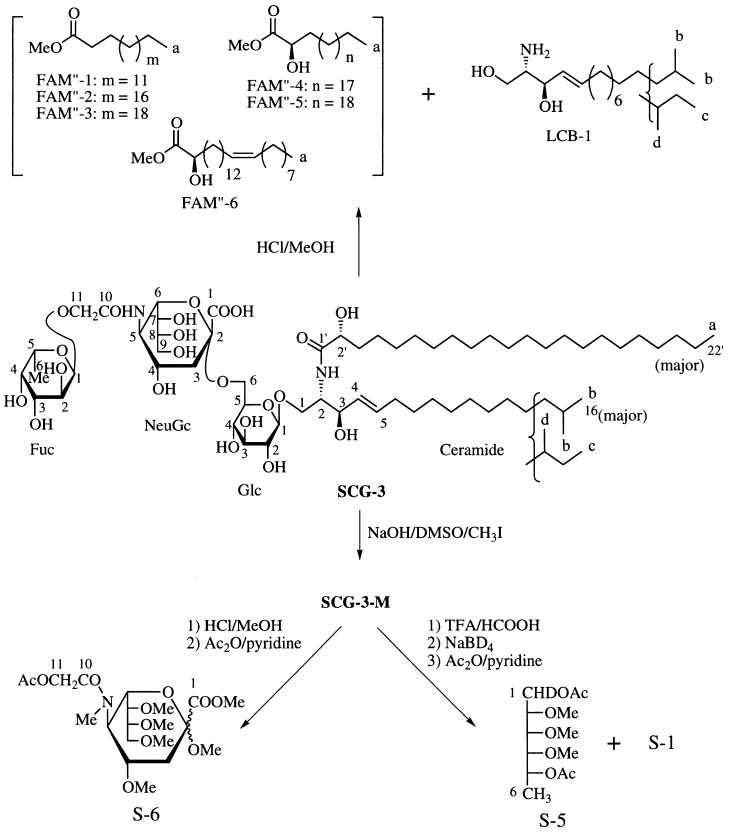

Chart 5

3.3 μg/ml, were 34.1%, 24.4%, and 24.5%, respectively. These effects were compared with that of the mammalian ganglioside **GM1** (22.1% at a concentration of 3.3 μg/ml).

Although gangliosides with the same sugar moiety as that of **SCG-1** have been obtained from *Cucumaria japonica*,[15] *Holothuria atra*,[16] *Telenota ananas*,[16] *Stichopus japonicus*,[6] *Holothuria leucospilota*,[7] *Ophiura sarsi*,[17] *Ophiocoma echinata*,[18] *Ophiomastix annulosa*,[18] *Ophiocoma scolopendrina*,[19] and *Anthocidaris crassispina*,[20] **SCG-1** differs from them in the structure of the ceramide moiety. Gangliosides with the same sugar moiety as that of the major component of **SCG-2** have also been obtained from *O. sarsi*,[17] *O. scolopendrina*,[19] and *Hemicentrotus pulcherrimus*,[21] although the major component of **SCG-2** is different from them in the ceramide structure. On the other hand, to the best of our knowledge, **SCG-3** is a new ganglioside with a unique sialosyl trisaccharide moiety. The isolation and characterization of such neuritogenically active gangliosides are attracting considerable attention with regard to the manufacture of new medicines from marine natural products.

Experimental

Melting points were determined on a micromelting point apparatus (Yanako MP-3) without correction. IR spectra were obtained using a Jasco FT/IR1140 infrared spectrophotometer. NMR spectra were recorded on a Varian Unity-500 spectrometer (500 MHz). Negative-ion FAB-MS spectra were acquired with a JEOL SX/SX-102A mass spectrometer (xenon atom beam; matrix, HMPA-TEG). GC-MS were recorded with a Shimadzu QP-5000 [EI mode; ionizing potential, 70 eV; separator and ion-source temperature 250 °C; column, TC-1701 (0.53 mm×15 m, GL Sciences); carrier gas, He]. GC was performed on a Shimadzu GC-14B [FID mode; column, Fused Silica Capillary Column DB-17 (0.317 mm×30 m, J&W Scientific); carrier, N_2].

Separation of SCG-1, SCG-2, and SCG-3 Body walls of the sea cucumber *S. chloronotus* (187.8 kg), which were collected at Cape Zanpa, Okinawa Prefecture, Japan, in 1999, were chopped and extracted three times with $CHCl_3$–MeOH (1 : 2, 270.0 l). The combined extracts were concentrated *in vacuo* to give an aqueous solution (109.0 l), which was extracted three times with *n*-hexane (40.0 l). The *n*-hexane layer was concentrated *in vacuo* to give a residue (173.0 g). The residue was dissolved in acetone. The acetone-insoluble part (98.0 g), the polar lipid fraction, was chromatographed on silica gel (solvent $CHCl_3$–MeOH–H_2O, 95 : 5 : 0→40 : 60 : 10) to give 10 fractions (fractions 1—10). Successive column chromatography of fraction 10 with silica gel (solvent $CHCl_3$–MeOH–H_2O=70 : 30 : 5→50 : 50 : 10), ion-exchange column (DEAE-Sephadex A-25, $CHCl_3$–MeOH–H_2O=30 : 60 : 8→$CHCl_3$–MeOH–0.5 N NH_4OAc=30 : 60 : 8), and gel filtration (Sephadex LH-20, $CHCl_3$–MeOH–H_2O=50 : 50 : 10) afforded **SCG-1** (196.5 mg) (*Rf*=0.47), **SCG-2** (12.5 mg) (*Rf*=0.40), and **SCG-3** (30.6 mg) (*Rf*=0.36) [silica gel TLC, solvent $CHCl_3$–MeH–H_2O=60 : 40 : 10].

SCG-1: Amorphous powder, mp 187—192 °C. IR (KBr) cm^{-1}: 3390 (OH), 1640, 1540 (amide). Negative-ion FAB-MS *m/z*: 1100—1160 $[M-H]^-$ series (see Chart 3). ^{13}C-NMR: See Table 1.

SCG-2: Amorphous powder, mp 160—163 °C. IR (KBr) cm^{-1}: 3410 (OH), 1650, 1560 (amide), 1230 (sulfate). Negative-ion FAB-MS *m/z*: 1100—1200 $[M-H]^-$ series (see Chart 3). ^{13}C-NMR: See Table 1.

SCG-3: Amorphous powder, mp 214—216 °C. IR (KBr) cm^{-1}: 3380 (OH), 1650, 1550 (amide). Negative-ion FAB-MS *m/z*: 1200—1300 $[M-H]^-$ series (see Chart 3). ^{13}C-NMR: See Table 1.

Methanolysis of SCG-1 **SCG-1** (1.0 mg) was heated with 10% 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 evaporated *in vacuo* to yield a mixture of FAM. The MeOH layer was neutralized with Ag_2CO_3, centrifuged, and the supernatant was concentrated *in vacuo* to give a mixture of LCB and methyl glycoside.

GC-MS Analysis of FAM from SCG-1 A FAM mixture from **SCG-1** was subjected to GC-MS [column temp.: 150—250 °C (rate of temp. increase: 2 °C/min)]. The results were as follows: methyl docosanoate, t_R[min]=24.4, *m/z*: 354 (M^+), 311 $(M-43)^+$; methyl tricosanoate, t_R[min]=26.7, *m/z*: 368 (M^+), 325 $(M-43)^+$; methyl 2-hydroxydo-

cosanoate, t_R[min]=29.2, *m/z*: 370 (M^+), 311 ($M-59)^+$; methyl tetracosenoate, t_R[min]=29.3, *m/z*: 380 (M^+), 348 ($M-32)^+$; methyl 2-hydroxytricosanoate, t_R[min]=32.5, *m/z*: 384 (M^+), 325 ($M-59)^+$; methyl 2-hydroxytetracosenoate, t_R[min]=36.5, *m/z*: 396 (M^+), 337 ($M-59)^+$; methyl 2-hydroxytetracosanoate, t_R[min]=37.0, *m/z*: 398 (M^+), 339 ($M-59)^+$.

GC-MS Analysis of TMS Ethers of LCB from SCG-1 The mixture of LCB and methyl glycoside from **SCG-1** was heated with 1-(trimethylsilyl) imidazole–pyridine (50 : 50, 40 ml) 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: 5 °C/min)]. The results were as follows: 2-amino-1,3-di-*O*-trimethylsilyl-4-heptadecene, t_R[min]=13.5, *m/z*: 326 ($M-103)^+$, 324 ($M-105)^+$, 297 ($M-132)^+$, 132; 2-amino-1,3,4-tri-*O*-trimethylsilyl-heptadecane, t_R[min]=15.8, *m/z*: 326 ($M-193)^+$, 285 ($M-234)^+$, 132.

GC Analysis of TMS Ethers of Methyl Glycoside from SCG-1 The mixture of TMS ethers of LCB and methyl glycoside was analyzed by GC [column temp.: 150—200 °C (rate of temp. increase: 5 °C/min)]; t_R[min]= 15.5 and 16.0 (methyl α- and β-glucopyranoside).

Determination of the Position of the Double Bond in Unsaturated Fatty Acid FAM (0.3 mg) obtained by methanolysis of **SCG-1** was dissolved in carbon disulfide (0.2 ml), and dimethyl disulfide (DMDS, 0.2 ml) and iodine (1.0 mg) were added. The reaction mixture was stored at 70 °C for 40 h in a small-volume sealed vial. The reaction was then quenched with 5% $Na_2S_2O_3$ (0.4 ml), and the mixture was extracted with *n*-hexane (0.5 ml×3) . The extract was concentrated *in vacuo* and the residue was heated with 1-(trimethylsilyl) imidazole–pyridine (50 : 50, 40 μl) for 10 min at 60 °C. The reaction mixture (TMS ethers) was analyzed by GC-MS [column temp.: 250 °C]. The results were as follows: methyl-15,16-S-methyl tetracosanoate, t_R[min]=72.1, *m/z*: 301, 173; methyl-2-*O*-trimethylsilyl-15,16-*S*-methyl tetracosanoate, t_R[min]=111.2, *m/z*: 389, 173.

Methylation of SCG-1 (Ciucanu–Kerek Method[12]) NaOH–dimethylsulfoxide (DMSO) solution (1.0 ml), which was prepared from powdered NaOH (40.0 mg) and DMSO (5.0 ml), and MeI (0.2 ml) were added to **SCG-1** (1.1 mg), and the mixture was stirred for 30 min. The reaction mixture was then diluted with H_2O (15.0 ml), extracted with $CHCl_3$ (10.0 ml×3), the $CHCl_3$ phases were washed with H_2O, and the solvent was evaporated *in vacuo* to give permethylated **SCG-1**, denoted **SCG-1-M** (1.9 mg).

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetate from SCG-1-M **SCG-1-M** (0.5 mg) was heated with 90% HCOOH–10% CF_3COOH (50 : 50) (1.0 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 (5.0 ml), and 28% NH_3 (2 drops) and $NaBD_4$ (10.0 mg) were added. After allowing the mixture to stand at room temperature 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 (50 : 50, 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.: 150—250 °C (rate of temp. increase: 5 °C/min)]. The results were as follows: S-1, t_R[min]=15.2, *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 SCG-1-M **SCG-1-M** (0.7 mg) was heated with 10% HCl in MeOH (0.5 ml) at 75 °C for 18 h in a small-volume sealed vial. The reaction mixture was then neutralized with Ag_2CO_3, centrifuged, and the supernatant was concentrated *in vacuo.* The residue (methanolysate) was heated with Ac_2O–C_5H_5N (50 : 50, 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.: 200—250 °C (rate of temp. increase: 5 °C/min)]; S-2, t_R[min]=23.1, *m/z*: 89, 159, 201, 284, 328, 348, 378, 406; [methyl *N*-glycolyl-*N*-methyl-2,4,7,8,9,11-hexa-*O*-methylneuraminate (derived from terminal NeuGc)].

Determination of the Absolute Configuration of the Glucose Moiety of SCG-1 (Hara method[14]) **SCG-1** (1.0 mg) was heated with 2 N H_2SO_4 (0.5 ml) at 70 °C for 18 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 *in vacuo.* The residue (sugar fraction) was heated with L-cysteine methyl ester hydrochloride (2.0 mg) and pyridine (0.1 ml) at 70 °C for 1 h. Then 0.1 ml of 1-(trimethylsilyl) imidazole was added and the mixture was heated at 70 °C for a further 1 h to yield trimethylsilyl ether of the methyl (4*R*)-thiazolidine-4-carboxylate derivative. The derivative was analyzed by GC [column temp.: 180—250 °C (rate of temp. increase: 2.5 °C/min)]; t_R[min]=23.8 (derivative of D-glucose, 23.8 min; derivative of L-glucose, 24.5 min).

Methanolysis of SCG-2 In the same manner as described for **SCG-1**, **SCG-2** (1.0 mg) was methanolyzed and the reaction mixture was worked up to give a mixture of FAM and residue composed of LCB and methyl glycoside.

GC-MS Analysis of FAM from SCG-2 A FAM mixture from **SCG-2** was subjected to GC-MS under the same conditions as described above for the FAM mixture obtained from **SCG-1**. Methyl octadecanoate, methyl 2-hydroxyoctadecanoate, methyl icosanoate, methyl henicosanoate, methyl docosanoate, and methyl 2-hydroxydocosanoate were detected.

GC-MS Analysis of TMS Ethers of LCB from SCG-2 The mixture of LCB and methyl glycoside from **SCG-2** was trimethylsilylated, the reaction mixture was analyzed by GC-MS in the same manner as for **SCG-1**, and 2-amino-1,3-di-*O*-trimethylsilyl-4-heptadecene was detected.

GC Analysis of TMS Ethers of Methyl Glycoside from SCG-2 The mixture of TMS ethers of LCB and methyl glycoside was analyzed by GC. As methylglycosides, methyl α- and β-glucopyranoside were detected.

Methylation of SCG-2 **SCG-2** (1.1 mg) was methylated and the mixture was worked up in the same manner as described for **SCG-1** to give **SCG-2-M** as the permethylated **SCG-2** (1.2 mg).

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetate from SCG-2-M **SCG-2-M** (0.4 mg) was hydrolyzed, reduced, and then acetylated. The partially methylated alditol acetate was analyzed by GC-MS in the same manner as described previously, and S-1 was detected.

Preparation and GC-MS Analysis of Acetate of Partially Methylated Sialic Acid from SCG-2-M **SCG-2-M** (0.4 mg) was methanolyzed and acetylated in the same manner as in the case of **SCG-1-M**. Then the acetate was subjected to GC-MS as described previously, and S-3 (t_R[min]=23.8, *m/z*: 129, 201, 254, 318, 326, 376, 404; [methyl *N*-acetyl-8-*O*-acetyl-*N*-methyl-2,4,7,9-tetra-*O*-methylneuraminate (derived from 8-linked NeuGc)] and S-4 (t_R[min]=24.4, *m/z*: 89, 157, 228, 254, 298, 346, 376, 404; [methyl *N*-acetyl-4-*O*-acetyl-*N*-methyl-2,7,8,9-tetra-*O*-methylneuraminate (derived from 4-linked NeuGc)] were detected. S-3 : S-4=*ca.* 6 : 4.

Determination of the Absolute Configuration of the Glucose Moiety of SCG-2 **SCG-2** (1.0 mg) was subjected to acid hydrolysis to give the sugar fraction in the same manner as in the case of **SCG-1**. Then its fraction was converted to trimethylsilyl ether of the methyl (4*R*)-thiazolidine-4-carboxylate. The derivative was determined to be D-glucose by GC under the same conditions as previously described.

Analysis of FAM, LCB, and Methyl Glycoside from SCG-3 In the same manner as described for **SCG-1** and **SCG-2**, a FAM mixture and a residue composed of LCB and methyl glycosides were obtained from **SCG-3** (1.2 mg). The FAM mixture was subjected to GC-MS under the same conditions as described above for the FAM mixture obtained from **SCG-1**, and methyl hexadecanoate, methyl henicosanoate, methyl tricosanoate, methyl 2-hydroxydocosanoate, methyl 2-hydroxytricosanoate, and methyl 2-hydroxytetracosenoate were detected. The mixture of LCB and methyl glycosides was trimethylsilylated and analyzed by GC-MS as described previously. As an LCB, 2-amino-1,3-di-*O*-trimethylsilyl-4-heptadecene was detected. As methylglycosides, methyl α- and β-fucopyranoside, t_R[min]=10.9 and 11.7, and α- and β-glucopyranoside t_R[min]=15.5 and 16.0 were detected.

Determination of the Position of the Double Bond in Unsaturated Fatty Acid In the same manner as described for **SCG-1**, the bismethylthio derivertive of FAM was prepared from FAM (0.3 mg) and analyzed by EIMS. The results were as follows: methyl-2-*O*-trimethylsilyl-15,16-S-methyl tetracosanoate, *m/z*: 389, 173.

Methylation of SCG-3 **SCG-3** (1.1 mg) was methylated by the method of Ciucanu–Kerek[12] and the mixture was worked up in the same manner as described previously to give **SCG-3-M** as the permethylated **SCG-3** (1.5 mg).

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetate from SCG-3-M **SCG-3-M** (0.5 mg) was hydrolyzed, reduced, and then acetylated. The partially methylated alditol acetate was analyzed by GC-MS in the same manner as described previously. S-5, t_R[min]=10.7, *m/z*: 118, 131, 162, 175; [1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methylhexitol (derived from terminal 6-deoxyhexopyranose)] and S-1 were detected.

Preparation and GC-MS Analysis of Acetate of Partially Methylated Sialic Acid from SCG-3-M The acetate of partially methylated sialic acid was prepared from **SCG-3-M** (0.5 mg) and analyzed by GC-MS in the same manner as in the case of **SCG-1-M**. S-6, t_R[min]=30.9, *m/z*: 89, 187, 201, 312, 356, 376, 406; [methyl *N*-glycolyl-11-*O*-acetyl-*N*-methyl-2,4,7,8,9-penta-*O*-methylneuraminate (derived from 11-linked NeuGc)] was detected.

Determination of the Absolute Configuration of the Glucose and Fu-

cose Moieties of SCG-3 In the same manner as described previously, **SCG-3** (1.0 mg) was subjected to acid hydrolysis and the sugar fraction was treated. The sugar derivatives were analyzed by GC under the same conditions as previously, and L-fucose, t_R[min]=26.1 (derivative of D-fucose, 25.0 min; derivative of L-fucose, 26.1 min), and D-glucose were detected.

Biological Assay 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 culture, the culture medium was replaced with serum-free DMEM/Ham's F12 (50 : 50) medium supplemented with N-2 Supplement (GIBCO). The gangliosides **SCG-1**, **SCG-2**, **SCG-3**, and **GM1** (3.3 μg/ml) were added to the medium with or without NGF (5 ng/ml), and the cells were further cultured at 37 °C. After 4 d, the morphological changes in the cells were observed under a light microscope.

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