Constituents of Holothuroidea, 18.[1] Isolation and Structure of Biologically Active Disialo- and Trisialo-Gangliosides from the Sea Cucumber *Cucumaria echinata*

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Three new disialo- and trisialo-gangliosides, CEG-6 (6), CEG-8 (8), and CEG-9 (9), were obtained, together with one known ganglioside, HLG-3 (7), from the lipid fraction of the chloroform/methanol extract of the sea cucumber *Cucumaria echinata*. The structures of the new gangliosides were determined on the basis of chemical and spectroscopic evidence to be 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 (6) and 1-*O*-[(*N*-glycolyl-D-neuraminosyl)-(2→11)-(*N*-glycolyl-D-neuraminosyl)-(2→4)-(*N*-acetyl-D-neuraminosyl)-(2→6)-D-glucopyranosyl]-ceramide (8, 9). The ceramide moieties of each compound were composed of an homogeneous sphingosine or phytosphingosine base and heterogeneous 2-hydroxy or nonhydroxylated fatty acid units. These gangliosides showed neuritogenic activity toward the rat pheochromocytoma cell line PC-12 in the presence of nerve growth factor.

Key words glycosphingolipid; ganglioside; sea cucumber; *Cucumaria echinata*; neuritogenic activity

In the course of our continuing research on biologically active glycosphingolipids (GSLs) from echinoderms, a series of studies on the isolation and structural elucidation of the GSLs from sea cucumber species have been performed in our laboratory.[2—14] In the study of the GSLs of the sea cucumber *Cucumaria echinata* (*gumi* in Japanese), we reported the isolation and structure of glucocerebrosides[2,4] and monosialogangliosides.[1] In a continuation of the preceding studies,[1] the further isolation and characterization of the more polar biologically active gangliosides from the sea cucumber *C. echinata* were carried out to develop novel medicinal resources from natural marine products. In this paper, we report the isolation and characterization of three new disialo- and trisialo-gangliosides from the whole bodies of *C. echinata*. 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 *C. echinata*, was subjected to repeated silica gel column chromatography to yield four compounds (**6**—**9**), each showing a single spot on TLC.

In its ^{13}C-NMR spectrum (Fig. 1, Table 1), compound **6** exhibits the characteristic signals of a sphingosine-type ceramide, with a nonhydroxylated fatty acid and a sugar moiety at C-1 [δ: 70.6 (C-1), 54.1 (C-2), 72.5 (C-3), 131.0 (C-4), 132.3 (C-5), and 175.9 (C-1′)]. The ^{13}C-NMR spectrum of **6** also features signals due to four anomeric carbons at δ 104.8, 101.2, 101.0, and 100.5, two of which (δ 101.2, 101.0) are quaternary carbon signals, indicating the presence of two sialic acid residues. Therefore **6** is suggested to be a sphingosine-type ganglioside, with nonhydroxylated fatty acid groups and four monosaccharide units. Furthermore, **6** is presumed to have normal-type fatty acids and *ante*-iso-type long-chain bases (LCBs), since the carbon signals for the terminal methyl groups are observed at δ 14.1 (normal form) and δ 11.5 and 19.2 (*ante*-iso form) in the ^{13}C-NMR spectrum (Fig. 1, Table 1).

The structure of the ceramide moiety was examined first. When **6** was methanolyzed with methanolic hydrochloric acid, a mixture of fatty acid methyl esters (FAMs) and an LCB was obtained, together with methyl glucoside and fucoside. The FAM mixture was analyzed using GC-MS, which revealed the presence of 10 components (see Experimental), and the major one was characterized as methyl docosanoate. The LCB was found to be 2-amino-1,3-dihydroxy-4-heptadecene based on GC-MS analysis of its TMS derivative. The stereochemistry of the ceramide moiety of **6** is presumed to be (2*S*,3*R*,4*E*) the same as that of the precursor compound, glucocerebroside CE-1,[4] coexisting in the same organism.

The structure of the tetrasaccharide moiety of **6** was established as follows. The presence of glucose (Glc) and fucose (Fuc) was obvious from the results of the methanolysis of **6** (*vide supra*). A detailed analysis of the ^{13}C-NMR spectrum of **6** revealed the characteristic signals [δ 172.3 and 172.4 (C-1), 101.2 and 101.0 (C-2), 42.0 and 41.0 (C-3), 53.8 and 53.7 (C-5), 63.9 and 62.7 (C-9), 176.5 and 175.5 (C-10), 22.8 and 66.8 (C-11)] of an *N*-acetylneuraminic acid (NeuAc) and an *N*-glycolylneuraminic acid (NeuGc) residue (Table 1). In the negative FAB-MS of **6**, the molecular ion and fragment ion peaks arising from cleavage of the glycosidic linkages of the major component are observed at *m*/*z* 1512, 1366, 1059, 768, and 606, indicating the presence of a tetrasaccharide moiety, deoxyhexose→NeuGc→NeuAc→hexose, as shown in Fig. 2.

Methylation of **6**, following the Ciucanu–Kerek method,[15] afforded the permethylated product **6-Me**. Partially methylated alditol acetates (S-1, S-2) prepared from **6-Me** were analyzed using GC-MS and identified as the alditols derived from 6-linked hexopyranose and terminal 6-deoxy-hexopyranose, respectively. On the other hand, **6-Me** was methanolyzed, the methanolysate was acetylated, and the acetates of partially methylated NeuAc (S-3) and NeuGc (S-4) derived from 4-linked NeuAc and 11-linked NeuGc, respectively, were detected by means of GC-MS analysis. On the basis of the above evidence, the tetrasaccharide moiety of **6** must be Fuc-(1→11)-NeuGc-(2→4)-NeuAc-(2→6)-Glc. The configurations of Fuc, NeuGc, NeuAc, and Glc are believed to be α, α, α, and β, respectively, on the basis of their

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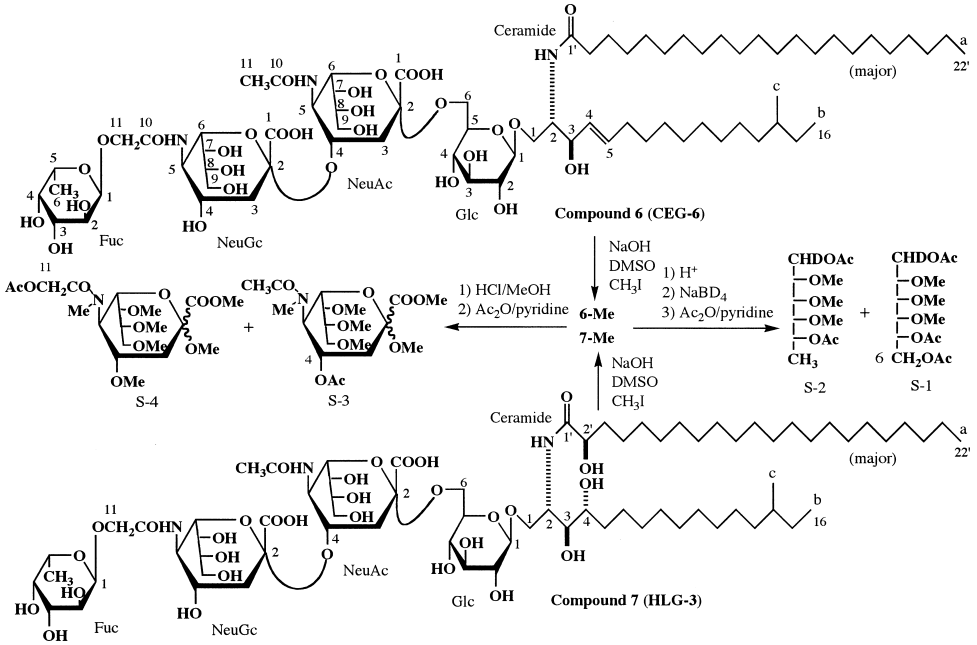

Fig. 1. Structure of Compounds **6** and **7**

Table 1. ^{13}C-NMR Spectral Data (δ Values) of Compounds **6** and **7** in C_5D_5N–D_2O (9 : 1)

C		**6**	**7**	C		**6**	**7**
Ceramide				NeuGc			
1	(t)	70.6	70.0	1	(s)	172.4[d]	173.1[g]
2	(d)	54.1	51.4	2	(s)	101.0[e]	101.1[h]
3	(d)	72.5	75.8	3	(t)	41.0	41.4
4	(d)	131.0	72.5[f]	4	(d)	66.8	66.8
5	(d)	132.3		5	(d)	53.7	53.6
1′	(s)	175.9	175.6	6	(d)	76.2	76.6
2′	(d)		72.4[f]	7	(d)	67.4	67.5
CH_3[a]	(q)	14.1	14.1	8	(d)	72.5	72.5
CH_3[b]	(q)	11.5	11.5	9	(t)	62.7	63.1
CH_3[c]	(q)	19.2	19.2	10	(s)	175.5	175.8
Glc				11	(t)	66.8	67.5
1	(d)	104.8	104.8	Fuc			
2	(d)	74.4	74.2	1	(d)	100.5	100.4
3	(d)	76.8	76.5	2	(d)	70.0	69.9
4	(d)	70.4	70.2	3	(d)	72.0	71.8
5	(d)	75.7	76.1	4	(d)	72.5	72.5
6	(t)	67.5	67.7	5	(d)	68.5	68.8
NeuAc				6	(q)	16.5	16.5
1	(s)	172.3[d]	173.3[g]				
2	(s)	101.2[e]	101.3[h]				
3	(t)	42.0	41.5				
4	(d)	74.0	74.2				
5	(d)	53.8	54.0				
6	(d)	76.0	76.0				
7	(d)	68.6	68.5				
8	(d)	72.5	72.5				
9	(t)	63.9	64.1				
10	(s)	176.5	176.3				
11	(q)	22.8	22.7				

a—*c*) Terminal methyl groups in the normal and *ante*-iso type of side chain (see Fig. 1). *d*—*h*) Assignments may be interchanged in each vertical column.

anomeric carbon signals (δ 100.5, 101.0,[16] 101.2,[16] 104.8) in the ^{13}C-NMR spectrum of **6**. In addition, the absolute configurations of the glucose and fucose units were verified as the D- and L-form using the Hara method.[17]

Consequently, if NeuAc and NeuGc are assumed to belong to the most commonly found D-series, then compound **6** is the 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 (2*S*,3*R*,4*E*)-C_{17}-sphingosine and heterogeneous nonhydroxylated fatty acids (with docosanoic acid as the major component), as shown in Fig. 1.

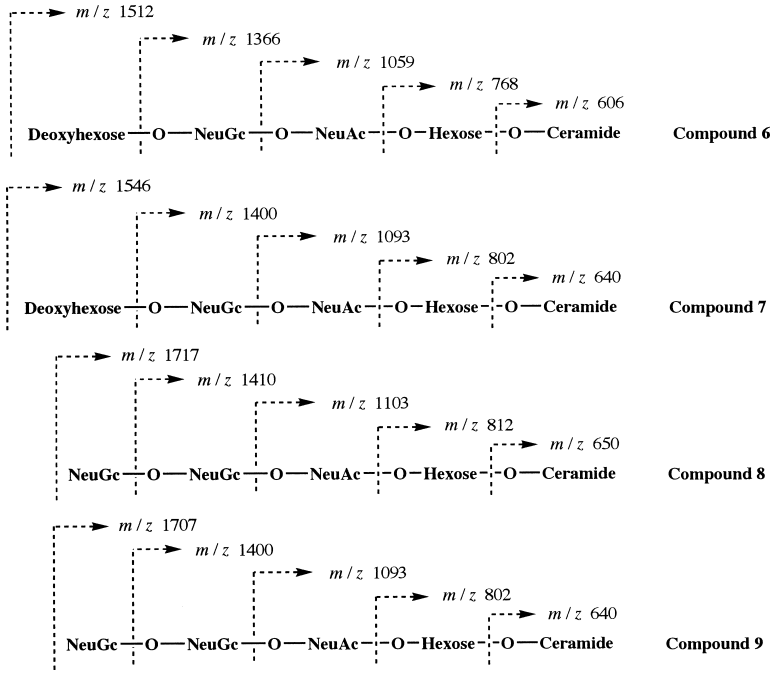

Fig. 2. Negative FAB Mass Fragmentation of the Major Component of Compounds **6**—**9**

Compound **7** exhibits characteristic signals due to a phytosphingosine-type ceramide, with a 2-hydroxy fatty acid and a sugar moiety at C-1 [δ 70.0 (C-1), 51.4 (C-2), 75.8 (C-3), 72.5 (C-4), 175.6 (C-1′), 72.4 (C-2′)] in its ^{13}C-NMR spectrum (Fig. 1, Table 1). The ^{13}C-NMR spectrum of **7** also shows four anomeric carbon signals at δ 104.8, 101.3, 101.1, and 100.4, two of which (δ 101.3, 101.1) are quaternary carbon signals derived from two sialic acid moieties (Table 1). Accordingly, **7** is suggested to be a phytosphingosine-type ganglioside, with 2-hydroxy fatty acid groups and four monosaccharide units. The terminal methyl groups of the ceramide moiety of **7** must be the same as that of **6** based on their carbon atom signals (Table 1).

Methanolysis of **7** afforded a mixture of FAM, LCB, and methyl glycosides identical to Glc and Fuc. The FAM mixture was analyzed using GC-MS and eight components were detected (see Experimental). The major component was methyl 2-hydroxydocosanoate. On the other hand, the LCB component was found to be 2-amino-1,3,4-trihydroxy-heptadecane by means of GC-MS analysis of its TMS derivative. Furthermore, the stereochemistry of the ceramide moiety of **7** is presumed to be (2*S*,3*S*,4*R*,2′*R*) because the ceramide part of glucocerebroside CE-3[4] coexisting in the same organism has the same absolute configuration.

The structure of the oligosaccharide moiety of **7** was indicated to be the same tetrasaccharide as that of **6** by comparison of the signals due to sugar moieties in their ^{13}C-NMR spectra (Table 1) and the fragmentations in their negative FAB-MS (Fig. 2). Furthermore, chemical degradation of **7-Me**, permethylated **7**, providing S-1, S-2, S-3, and S-4, verified the above suggestion (Fig. 1). Since the absolute configurations (D-, L-form) of the Glc and Fuc units were confirmed, if NeuAc and NeuGc are assumed to belong to the D-series, then **7** is the 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 (2*S*,3*S*,4*R*)-C_{17}-phytosphingosine and heterogeneous (2*R*)-2-hydroxy fatty acids (with docosanoic acid as the major component), as shown in Fig. 1.

Since the ^{13}C-NMR spectra of compounds **8** and **9** were not available due to a lack of samples, their structures were determined mainly through chemical means.

When compound **8** was methanolyzed, a mixture of FAM, LCB, and the methyl glycoside Glc was obtained. The major component of the FAM mixture was methyl 2-hydroxytetracosanoate and the LCB was 2-amino-1,3-dihydroxy-4-heptadecene, as determined by means of GC-MS analysis (see Experimental).

The presence of the tetrasaccharide moiety NeuGc→NeuGc→NeuAc→hexose was suggested on the basis of the molecular ion and fragment ion peaks at *m*/*z* 1717, 1410, 1103, 812, and 650 arising from cleavage of the glycosidic linkages of the major component in the negative FAB-MS of **8**, as shown in Fig. 2. Furthermore, chemical degradation of permethylated **8**, **8-Me**, yielded S-1, S-3, S-4, and the permethylated NeuGc (S-5) derived from the teminal NeuGc, as shown in Fig. 3. Therefore the tetrasaccharide moiety of **8** must be NeuGc-(2→11)-NeuGc-(2→4)-NeuAc-(2→6)-Glc.

On the basis of the above findings and the absolute configurations (D-form) of Glc, NeuGc, and NeuAc (assumed), compound **8** is (*N*-glycolyl-D-neuraminosyl)-(2→11)-(*N*-glycolyl-D-neuraminosyl)-(2→4)-(*N*-acetyl-D-neuraminosyl)-(2→6)-D-glucopyranoside of a ceramide, composed of C_{17}-sphingosine and heterogeneous 2-hydroxy fatty acids (with tetracosanoic acid as the major component), as shown in Fig. 3.

Meanwhile, compound **9** afforded methyl 2-hydroxydocosanoate as the major component of the FAM mixture and 2-amino-1,3,4-trihydroxy-heptadecane as the LCB on methanolysis. By taking the negative FAB mass fragmentation of the major component of **9**, as shown in Fig. 2, and the products obtained by the chemical degradation of **9-Me**, per-

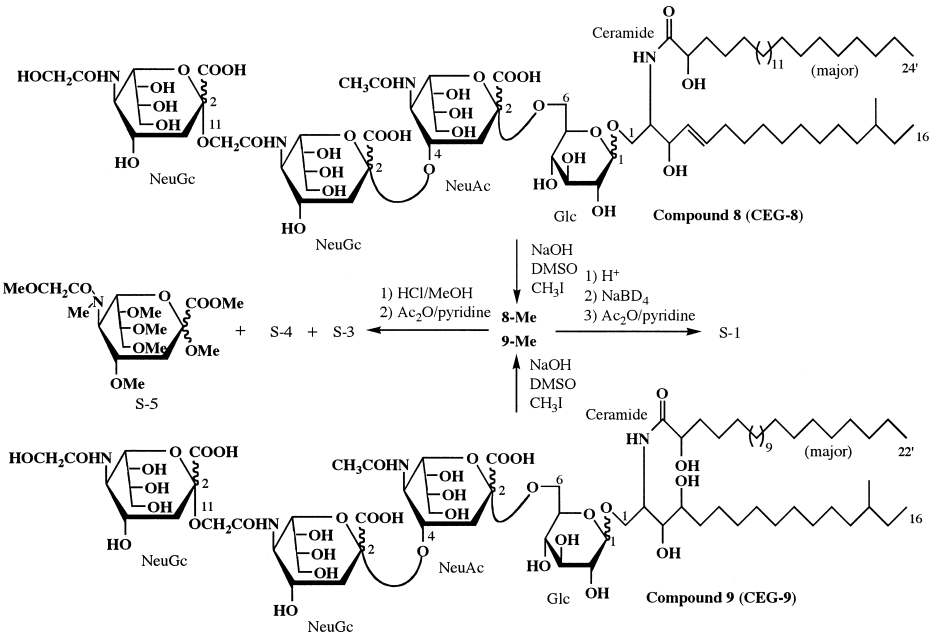

Fig. 3. Structure of Compounds **8** and **9**

methylated **9**, as revealed in Fig. 3, into account, **9** must have the same tetrasaccharide as that of **8**. Therefore compound **9** is the (*N*-glycolyl-D-neuraminosyl)-(2→11)-(*N*-glycolyl-D-neuraminosyl)-(2→4)-(*N*-acetyl-D-neuraminosyl)-(2→6)-D-glucopyranoside of a ceramide, composed of C_{17}-phytosphingosine and heterogeneous 2-hydroxy fatty acids (with docosanoic acid as the major component), as shown in Fig. 3. Compounds **8** and **9** are presumed to have the *ante*-iso-type LCB as do other gangliosides coexisting in the same organism.

The effects of the isolated gangliosides (**6**—**9**) on the neuritogenesis of a rat pheochromocytoma cell line (PC-12 cells) were investigated. They displayed neuritogenic activity in the presence of nerve growth factor (NGF). The proportion of cells with neurites longer than the diameter of the cell body of compounds **6**—**9** at a concentration of 10 μM was 43.0%, 42.0%, 40.2%, and 35.1%, respectively, compared with the control (NGF 5 ng/ml, 7.5%). Interestingly, the effects of **6**, **7**, and **8** were greater than that of the mammalian ganglioside GM_1 (35.6%).

Compounds **8** and **9** are, to the best of our knowledge, the first gangliosides with the linear trisialoyl moiety NeuGc→NeuGc→NeuAc from echinoderms. Although a ganglioside, **HLG-3**, with the same sugar moiety as that of **6** and **7** has been obtained from the sea cucumber *Holothuria leucospilota*,[8] **6** differs in the basic structure of its ceramide moiety. However, compound **7** is regarded as the same compound as **HLG-3** although its fatty acyl and LCB moieties are slightly different from those of **HLG-3**. Three new disialo- and trisialo-gangliosides (**6**, **8**, **9**) obtained in this study were designated CEG-6, -8, and -9, respectively. The isolation and characterization of such neuritogenically active gangliosides are attracting considerable attention with regard to the development of new medicines from natural marine products.

Experimental

IR spectra were obtained on a Jasco FT/IR-410 infrared spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Jeol GX-270 spectrometer (270, 67.8 MHz) or a Varian Unity-500 spectrometer (500, 125 MHz). Negative-ion FAB-MS spectra were acquired with a Jeol JMS-SX-102 mass spectrometer (xenon atom beam; matrix, triethanolamine). GC and GC-MS were recorded with a Shimadzu QP-5050A [EI mode; ionizing potential, 120 eV; column, TC-1701 (0.25 mm×30 m, GL Science Inc.); carrier gas, He].

Separation of Compounds 6—9 The whole bodies of the sea cucumber *C. echinata* (126 kg), which was collected from the Sea of Genkai, Japan, in 1997, were chopped and extracted with CHC_3–MeOH [1 : 4 (541), 1 : 2 (541)×2]. The combined extracts were concentrated *in vacuo* to give an aqueous solution (120 l), which was extracted three times with *n*-hexane (80 l). The *n*-hexane phase was concentrated *in vacuo* to give a residue (3.7 kg). The residue was dissolved in acetone. The acetone-insoluble part (2.0 kg), which was the polar lipid fraction, was chromatographed on silica gel (solvent $CHCl_3$–MeOH–H_2O, 95 : 15 : 0 to 4 : 6 : 2) to give nine fractions. Fraction 8 of the nine fractions was further chromatographed on silica gel (solvent $CHCl_3$–MeOH–H_2O, 7 : 3 : 0.5 to 5 : 5 : 1) to afford seven fractions. Fraction 6 of the seven fractions was chromatographed successively on silica gel (solvent $CHCl_3$–MeOH–AcOEt–H_2O, 3 : 4 : 3 : 0.6 to $CHCl_3$–MeOH–H_2O, 5 : 5 : 1) to give compound **6** (12.2 mg) (*Rf*=0.13) and compound **7** (10.5 mg) (*Rf*=0.12) [silica gel TLC, solvent $CHCl_3$–MeOH–2% $CaCl_2$ aq. (6 : 4 : 0.8)] together with previously reported compounds **4** and **5**.[1] On the other hand, the aqueous phase was concentrated *in vacuo* to give a residue. The residue was extracted with $CHCl_3$–MeOH (1 : 1) and the extract was concentrated *in vacuo* to give a $CHCl_3$–MeOH-soluble fraction (587 g). The fraction was chromatographed on silica gel (solvent $CHCl_3$–MeOH–H_2O, 7 : 3 : 0.5 to 4 : 6 : 2) to give seven fractions. Successive column chromatography of fraction 7 (silica gel, solvent $CHCl_3$–MeOH–H_2O, 65 : 35 : 7) afforded compound **8** (3.5 mg) (*Rf*=0.07) and compound **9** (3.0 mg) (*Rf*=0.05) [silica gel TLC, solvent $CHCl_3$–MeOH–2% $CaCl_2$ aq. (6 : 4 : 0.8)].

Compound **6** (CEG-6): Amorphous powder. IR (KBr) cm^{-1}: 3378 (OH), 1640, 1544 (amide). Negative-ion FAB-MS: see Fig. 2. ^{1}H-NMR (C_5D_5N : D_2O, 9 : 1) δ: 2.02 (3H, s, $COCH_3$), 1.42 (3H, d, *J*=4.0 Hz, CH_3 of Fuc), 0.85 (9H, m, terminal methyl groups). ^{13}C-NMR: see Table 1.

Compound **7** (HLG-3): Amorphous powder. IR (KBr) cm^{-1}: 3401 (OH), 1637, 1540 (amide). Negative-ion FAB-MS: see Fig. 2. ^{1}H-NMR (C_5D_5N : D_2O, 9 : 1) δ: 2.02 (3H, s, $COCH_3$), 1.42 (3H, d, *J*=4.0 Hz, CH_3 of Fuc), 0.85 (9H, m, terminal methyl groups). ^{13}C-NMR: see Table 1.

Compound **8** (CEG-8): Amorphous powder. IR (KBr) cm^{-1}: 3410 (OH), 1650, 1547 (amide). Negative-ion FAB-MS: see Fig. 2. ^{1}H-NMR (C_5D_5N : D_2O, 5 : 1) δ: 2.01 (3H, s, $COCH_3$), 0.86 (9H, m, terminal methyl groups).

Compound **9** (CEG-9): Amorphous powder. IR (KBr) cm^{-1}: 3392 (OH), 1643, 1550 (amide). Negative-ion FAB-MS: see Fig. 2. ^{1}H-NMR ($C_5D_5N : D_2O$, 5 : 1) δ: 2.00 (3H, s, $COCH_3$), 0.85 (9H, m, terminal methyl groups).

Methanolysis of 6 Compound **6** (0.5 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 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. FAM: ^{1}H-NMR ($CDCl_3$) δ: 3.67 (3H, s, $COOCH_3$), 0.88 (3H, t, J=6.8 Hz, terminal CH_3).

GC-MS Analysis of FAM from 6 A FAM mixture from **6** was subjected to GC-MS [column temperature: 150—300 °C (rate of temperature increase 5 °C/min)]. The results were as follows: methyl hexadecanoate, t_R [min] (ratio of peak areas)=8.3 (5), *m/z*: 270 (M^+), 227 $(M-43)^+$; methyl heptadecanoate, t_R=9.7 (3), *m/z*: 284 (M^+), 241 $(M-43)^+$; methyl octadecanoate, t_R=11.9 (16), *m/z*: 298 (M^+), 255 $(M-43)^+$; methyl icosanoate, t_R=15.2 (9), *m/z*: 326 (M^+), 283 $(M-43)^+$; methyl heneicosanoate, t_R=16.6 (6), *m/z*: 340 (M^+), 297 $(M-43)^+$; methyl docosanoate, t_R=18.7 (32), *m/z*: 354 (M^+), 311 $(M-43)^+$; methyl tricosenoate, t_R=20.6 (2), *m/z*: 366 (M^+), 334 $(M-32)^+$; methyl tricosanoate, t_R=21.0 (10), *m/z*: 368 (M^+), 325 $(M-43)^+$; methyl tetracosenoate, t_R=23.4 (7), *m/z*: 380 (M^+), 348 $(M-32)^+$; methyl tetracosanoate, t_R=23.7 (10), *m/z*: 382 (M^+), 339 $(M-43)^+$.

GC-MS Analysis of TMS Ethers of LCB from 6 The mixture of LCB and methyl glycoside from **6** was heated with 1-(trimethylsilyl)imidazole–pyridine (1 : 1) for 15 min at 70 °C and the reaction mixture (TMS ethers) was analyzed using GC-MS [column temperature: 150—300 °C (rate of temperature increase 5 °C/min)]. The results were as follows: 2-amino-1,3-dihydroxy-4-heptadecene, t_R [min]=13.8, *m/z*: 326 $(M-103)^+$, 297 $(M-132)^+$, 132.

GC Analysis of TMS Ethers of Methyl Glycosides from 6 The mixture of TMS ethers of the LCB and methyl glycosides from **6** was analyzed using GC-MS [column temperature: 100—300 °C (rate of temperature increase 5 °C/min)]: t_R [min]=15.6, 16.5, and 17.3 (methyl Fuc); 17.9 and 18.1 (methyl Glc).

Determination of Absolute Configuration of Fuc and Glc Moieties of 6 (Hara Method) Compound **6** (0.5 mg) was heated with 4 N H_2SO_4 (0.5 ml) at 100 °C for 24 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 (1.0 mg) and pyridine (0.05 ml) at 70 °C for 1 h. Then, 0.05 ml of 1-(trimethylsilyl)imidazole was added and the mixture was heated at 70 °C for a further 15 min to yield trimethylsilyl ether of the methyl (4*R*)-thiazolidine-4-carboxylate derivatives. The derivatives were analyzed using GC-MS [column temperature: 180—300 °C (rate of temperature increase 2.5 °C/min)]; t_R [min]=19.2 and 24.0 (derivative of D-Fuc, 20.5; L-Fuc, 19.2; D-Glc, 24.0; L-Glc, 24.8).

Methylation of 6 (Ciucanu–Kerek Method) NaOH-dimethylsulfoxide (DMSO) solution, which was prepared from powdered NaOH (80 mg) and DMSO (2 ml), and MeI (0.2 ml) were added to **6** (1 mg), and the mixture was stirred for 30 min. The reaction mixture was then diluted with water (20 ml), extracted with Et_2O (10 ml×3), the Et_2O phases were washed with water, and the solvent was evaporated *in vacuo* to give permethylated **6**, denoted **6-Me**.

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetates from 6-Me Compound **6-Me** (0.5 mg) was heated with 90% HCOOH–10% CF_3COOH (1 : 1) (0.5 ml) at 100 °C for 18 h in a small-volume sealed vial, and then the solvents were evaporated *in vacuo*. The residue was alkalified with 7% NH_3 aqueous solution and $NaBD_4$ (10 mg) was added. After allowing the mixture to stand at room temperature for 7 h, it was acidified with AcOH 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, 0.5 ml) at 70 °C for 2 h. The reaction mixture was evaporated *in vacuo* with toluene to give partially methylated alditol acetates. The acetates were subjected to GC-MS [column temperature 150—300 °C (rate of temperature increase 5 °C/min)]. The results were as follows: S-1, t_R [min]=14.9, *m/z*: 118, 162, 189, 233 [1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol (derived from 6-linked hexopyranose)]; S-2, t_R=9.2, *m/z*: 118, 131, 162, 175 [1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methylhexitol (derived from terminal 6-deoxyhexopyranose)].

Preparation and GC-MS Analysis of Acetates of Partially Methylated Sialic Acid from 6-Me Compound **6-Me** (0.5 mg) was heated with 10% HCl in MeOH (0.5 ml) at 70 °C for 18 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, 0.5 ml) at 70 °C for 2 h. The resulting mixture was evaporated *in vacuo* with toluene and the residue was subjected to GC-MS [column temperature 200—300 °C (rate of temperature increase 5 °C /min)]: S-3, t_R [min]=22,5, *m/z*: 157, 346, 376, 404 [methyl *N*-acetyl-4-*O*-acetyl-*N*-methyl-2,7,8,9-tetra-*O*-methylneuraminate (derived from 4-linked NeuAc)] and S-4, t_R=31.3, *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)].

Methanolysis of 7 In the same manner as described for **6**, compound **7** was methanolyzed and the reaction mixture was worked up to give a mixture of FAM and a residue composed of LCB and methyl glycoside. FAM: ^{1}H-NMR ($CDCl_3$) δ: 3.67 (3H, s, $COOCH_3$), 0.88 (3H, t, J=6.6 Hz, terminal CH_3).

GC-MS Analysis of FAM from 7 A FAM mixture from **7** was subjected to GC-MS under the same conditions as for **6**. The results were as follows: methyl 2-hydroxyoctadecanoate, t_R [min] (ratio of peak areas)=14.9 (13), *m/z*: 314 (M^+), 255 $(M-59)^+$; methyl 2-hydroxyicosanoate, t_R=18.2 (17), *m/z*: 342 (M^+), 283 $(M-59)^+$; methyl 2-hydroxydocosanoate, t_R=22.8 (28), *m/z*: 370 (M^+), 311 $(M-59)^+$; methyl 2-hydroxytricosanoate, t_R=26.0 (9), *m/z*: 384 (M^+), 325 $(M-59)^+$; methyl 2-hydroxytetracosenoate, t_R=29.7 (8), *m/z*: 396 (M^+), 337 $(M-59)^+$; methyl 2-hydroxytetracosanoate, t_R=30.2 (17), *m/z*: 398 (M^+), 339 $(M-59)^+$; methyl octadecanoate, t_R=11.9 (5); methyl docosanoate, t_R=18.7 (3).

GC-MS Analysis of TMS Ethers of LCB from 7 The mixture of LCB and methyl glycoside from **7** was treated in the same way as **6** and the reaction mixture (TMS ethers) was analyzed using GC-MS under the same conditions as for **6**. The results were: 2-amino-1,3,4-trihydroxy-heptadecane, t_R [min] (ratio of peak areas)=16.2, *m/z*: 326 $(M-193)^+$, 285 $(M-234)^+$, 132.

GC Analysis of TMS Ethers of Methyl Glycosides from 7 The mixture of TMS ethers of LCB and methyl glycosides from **7** was analyzed using GC-MS in the same manner as before, and methyl Fuc and Glc (methyl glycosides) were detected.

Determination of the Absolute Configuration of Fuc and Glc Moieties of 7 Compound **7** was subjected to acid hydrolysis and the sugar fraction was treated in the same manner as described for **6**, thereby affording the trimethylsilyl ether of the methyl (4*R*)-thiazolidine-4-carboxylate derivatives. The derivatives were analyzed using GC-MS under the same conditions as above, and L-Fuc and D-Glc were detected.

Preparation and GC-MS Analyses of Partially Methylated Alditol Acetates and Acetates of Partially Methylated Sialic Acid from 7-Me Compound **7** was methylated according to the Ciucanu–Kerek method and the reaction mixture was worked up in the same manner as described for **6**, thereby yielding permethylated **7**, denoted **7-Me**. Compound **7-Me** was hydrolyzed, reduced, and then acetylated, and the partially methylated alditol acetates were analyzed using GC-MS in the same manner as described for **6-Me**, whereupon S-1 and S-2, derived from 6-linked hexopyranose and terminal 6-deoxyhexopyranose, respectively, were detected. On the other hand, **7-Me** was methanolyzed and then acetylated as above, and the acetates were subjected to GC-MS under the same conditions as described above, and S-3 (derived from 4-linked NeuAc) and S-4 (derived from 11-linked NeuGc) were detected.

Methanolysis of 8 In the same manner as described for **6**, compound **8** was methanolyzed, and the reaction mixture was worked up to give a mixture of FAM and a residue composed of LCB and methyl glycoside. FAM: ^{1}H-NMR ($CDCl_3$) δ: 3.67 (3H, s, $COOCH_3$), 0.87 (3H, t, J=6.7 Hz, terminal CH_3).

GC-MS Analysis of FAM from 8 A FAM mixture from **8** was subjected to GC-MS under the same conditions as for **6**. The results were: methyl 2-hydroxyoctadecanoate, t_R [min] (ratio of peak areas)=14.6 (12); methyl 2-hydroxyicosanoate, t_R=18.0 (14); methyl 2-hydroxydocosenoate, t_R=22.2 (5), *m/z*: 368 (M^+), 309 $(M-59)^+$; methyl 2-hydroxydocosanoate, t_R=22.7 (21); methyl 2-hydroxytricosanoate, t_R=25.9 (8); methyl 2-hydroxytetracosanoate, t_R=30.3 (25); methyl hexadecanoate, t_R=8.4 (3); methyl octadecanoate, t_R=11.5 (8); methyl docosanoate, t_R=18.3 (5).

GC-MS Analyses of TMS Ethers of LCB and Methyl Glycoside from 8 The residue (a mixture of LCB and methyl glycoside) from **8** was trimethylsilylated and the reaction mixture was analyzed using GC-MS in the same manner as above. 2-Amino-1,3-dihydroxy-4-heptadecene (LCB) and methyl Glc (methyl glycoside) were detected.

Preparation and GC-MS Analyses of Partially Methylated Alditol Acetate and Acetates of Partially Methylated Sialic Acid from 9-Me The

partially methylated alditol acetate prepared from **8-Me**, permethylated **8**, was analyzed using GC-MS in the same manner as described above, and S-1 was detected. On the other hand, the acetates of partially methylated sialic acid from **8-Me** were analyzed as above, and S-3, S-4, and S-5, t_R [min]=26.1, *m*/*z*: 159, 348, 378, 406 [methyl *N*-glycolyl-*N*-methyl-2,4,7,8,9,11-hexa-*O*-methylneuraminate (derived from terminal NeuGc)] were detected.

Methanolysis of 9 In the same manner as described above, compound **9** was methanolyzed and the reaction mixture was worked up to give a mixture of FAM and a residue composed of LCB and methyl glycoside. FAM: ^{1}H-NMR ($CDCl_3$) δ: 3.67 (3H, s, $COOCH_3$), 0.88 (3H, t, J=6.8 Hz, terminal CH_3).

Analyses of FAM, LCB, and Methyl Glycoside from 9 Experiments were conducted as before, leading to a mixture of FAM and a residue composed of LCB and methyl glycoside derived from compound **9**. The FAM mixture was subjected to GC-MS under the same conditions as described above, and methyl 2-hydroxyoctadecanoate (ratio of peak areas, 13), methyl 2-hydroxyicosanoate (15), methyl 2-hydroxydocosenoate (7), methyl 2-hydroxydocosanoate (26), methyl 2-hydroxytricosanoate (9), methyl 2-hydroxytetracosanoate (18), and methyl octadecanoate (12) were detected. The mixture of LCB and methyl glycoside was trimethylsilylated and analyzed using GC-MS as above, and 2-amino-1,3,4-trihydroxy-heptadecane (LCB) and methyl Glc (methyl glycoside) were detected.

Preparation and GC-MS Analyses of Partially Methylated Alditol Acetate and Acetates of Partially Methylated Sialic Acid from 9-Me The partially methylated alditol acetate prepared from **9-Me**, permethylated **9**, was analyzed using GC-MS in the same manner as described above, and S-1 was detected. On the other hand, methanolysis followed by acetylation of **9-Me** under the same conditions as above afforded S-3, S-4, and S-5.

Biological Assay The neuritogenic activity of compounds **6**, **7**, **8**, and **9** in PC-12 cells was observed according to a method previously reported.[10)]

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