Biologically Active Glycosides from Asteroidea, 42.[1] Isolation and Structure of a New Biologically Active Ganglioside Molecular Species from the Starfish *Asterina pectinifera*

Ryuichi Higuchi,*[,a] Shinichi Inoue,[a] Kouji Inagaki,[a] Maki Sakai,[a] Tomofumi Miyamoto,[a] Tetsuya Komori,[a] Masanori Inagaki,[a] and Ryuichi Isobe[b]

[a] *Faculty of Pharmaceutical Sciences, Kyushu University; 3–1–1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan: and* [b] *Department of Medical Electronics Engineering, Faculty of Engineering, Towa University; 1–1–1 Chikusigaoka, Minami-ku, Fukuoka 815–0036, Japan.* Received June 6, 2005; accepted December 15, 2005

A ganglioside molecular species GP-3 (1) has been obtained from the water-soluble lipid fraction of the chloroform/methanol extract of the starfish *Asterina pectinifera*. The structure of the ganglioside has been determined on the basis of chemical and spectroscopic evidence. Compound 1 represents new ganglioside molecular species possessing two moles of sialic acids at the inner part of the sugar moiety. Partial hydrolysis by hot water and an enzymatic hydrolysis by means of endoglycoceramidase (EGCase) have proved useful for structure elucidation of the complex oligosaccharide moiety. Moreover, 1 exhibits neuritogenic activity toward the rat pheochromocytoma cell line, PC-12 cells, in the presence of nerve growth factor (NGF).

Key words glycosphingolipid; ganglioside; starfish; *Asterina pectinifera*; neuritogenic activity

As described in the previous paper,[2] three ganglioside molecular species and a homogeneous ganglioside were obtained and characterized from the water-soluble lipid fractions of the chloroform/methanol extract of the starfish *Asterina pectinifera*, and one of the ganglioside molecular species, GP-2, was found to support the survival of cultured neuronal cells. Continuing the previous studies, isolation and structure elucidation of the more polar biologically active gangliosides from the starfish *A. pectinifera* (Itomakihitode in Japanese) has been conducted with the object of searching for lead compounds of new medicines. In this paper, we report on the isolation and characterization of a ganglioside molecular species, GP-3 (**1**), obtained from the whole bodies of *A. pectinifera*. The biological activity of the ganglioside is also reported.

The water-soluble lipid fraction, which was obtained from the chloroform/methanol extract of the whole bodies of *A. pectinifera*, was subjected to reverse-phase followed by silica gel column chromatography to give a polar ganglioside molecular species, GP-3 (**1**), which showing a single spot on silica gel thin-layer chromatography (TLC).

Compound **1** shows strong hydroxy and amide absorptions in the IR spectrum. **1** reveals the characteristic signals of a phytosphingosine-type ceramide possessing a 2-hydroxy fatty acid and a sugar moiety at C-1 in its ^{13}C-NMR spectrum (Fig. 1, Table 1) [δ: 70.0 (C-1), 51.4 (C-2), 75.8 (C-3), 72.6 (C-4), 176.0 (C-1′), 72.6 (C-2′)]. The ^{13}C-NMR spectrum of **1** also reveals signals due to nine anomeric carbons at δ: 111.2, 110.7, 110.4, 104.8, 104.3, 101.3, 100.9, 96.9 and 96.6, and two of which (δ: 101.3, 100.9) are quaternary carbon signals indicating the presence of two sialic acid residues. The negative-ion FAB mass spectrum of **1** exhibits a series of quasi-molecular ion peaks $[M-H]^-$ at *m/z*: 2250—2350. Therefore, **1** is suggested to be a molecular species of phytosphingosine-type ganglioside possessing 2-hydroxy fatty acids and nine monosaccharides. In addition, the presence of one mole of glucose (Glc), two moles of arabinose (Ara) and four moles of galactose (Gal) was obvious from the results of the hydrolysis of this compound, and the existence of two moles of *N*-acetylneuraminic acids (NeuAc) was indicated by the characteristic signals in its ^{13}C-NMR spectrum (Table 1). Furthermore, **1** is presumed to have mainly normal-type[3] fatty acids and iso-type[4] long-chain bases, since the carbon atom signals for the terminal methyl groups are observed at δ: 14.1 (normal form) and 22.7 (iso form) in the ^{13}C-NMR spectrum (Fig. 1, Table 1). The detailed structure of the ceramide and oligosaccharide moieties was determined as follows.

In the course of the studies on gangliosides from the starfish species, we found that the sialic acid–hexose linkage was cleaved by heating with water to give partial hydrolysis products.[5] When **1** was heated in water, three products, compounds **2** and **3**, and a mixture of oligosaccharides (**6**) were obtained.

Compound **2** was suggested to be a lactosyl ceramide from its behavior on TLC, and the ^{1}H- and ^{13}C-NMR spectra of **2** are in good agreement with those of the synthetic lactosyl ceramide[6] composed of (2*S*,3*S*,4*R*)-C_{16}-phytosphingosine and (2*R*)-2-hydroxytetracosanoic acid, except the signals due to side chain moiety. The above fact and the optical rotations of **2** (+13) and the synthetic lactosyl ceramide (+8) suggest that **2** has the same absolute configuration as the synthetic compound for the core structure (C-2, -3, -4, -2′ and lactose). Therefore, the absolute configuration of the ceramide part of **1** is must be 2*S*,3*S*,4*R*,2′*R* (Fig. 1).

When **2** was methanolyzed with methanolic hydrochloric acid, a mixture of fatty acid methyl ester (FAM) and long-chain bases (LCB) was obtained. The FAM mixture was analyzed by GC-MS to show the existence of three components which were characterized as methyl 2-hydroxydocosanoate (major), methyl 2-hydroxytricosanoate and methyl 2-hydroxytetracosanoate. The LCB mixture was found to be composed of iso-C_{16}-phytosphingosine, iso-C_{17}-phytosphingosine (major), iso-C_{18}-phytosphingosine, based on the GC analysis of its acetyl derivative. Accordingly, the major components of the ceramide moiety of **1** are suggested to be 2-hydroxy-

* To whom correspondence should be addressed. e-mail: rhiguchi@ phar.kyushu-u.ac.jp

Table 1. ^{13}C-NMR Spectral Data (δ Values) of **1**, **3** and **2** in C_5D_5N/D_2O (96 : 4)

C		**1**	**3**	**2**	C		**1**	**3**
Ceramide					Ara-1			
1	(t)	70.0	70.3	70.3	1	(d)	111.2[e]	111.0[h]
2	(d)	51.4	51.4	51.5	2	(d)	82.5	82.5
3	(d)	75.8	75.7	75.8	3	(d)	78.4	78.6
4	(d)	72.6	72.5	72.4	4	(d)	85.9	86.8
1′	(s)	176.0	175.9	175.5	5	(t)	62.4[d]	62.7[g]
2′	(d)	72.6	72.5	72.4	NeuAc-2			
CH_3[a]	(q)	14.1	14.4	14.3	1	(s)	173.5	
CH_3[b]	(q)	22.7	23.0	22.8	2	(s)	101.3	
Glc					3	(t)	38.5	
1	(d)	104.8[c]	105.2[f]	105.0	4	(d)	74.1	
2	(d)	74.2	74.4	74.5	5	(d)	51.3	
3	(d)	76.5	77.1	76.4	6	(d)	74.1	
4	(d)	81.1	81.5	81.6	7	(d)	69.6	
5	(d)	77.4	77.4	77.2	8	(d)	73.1	
6	(t)	62.2[d]	62.1[g]	62.0	9	(t)	64.4	
Gal-1					CH_3CO	(s)	174.0	
1	(d)	104.3[c]	104.8[f]	105.6	CH_3CO	(q)	23.2	
2	(d)	70.3	70.2	72.5	Gal-4			
3	(d)	78.3	78.4	75.1	1	(d)	96.9	
4	(d)	68.3	68.7	70.2	2	(d)	68.4	
5	(d)	76.1	76.4	76.4	3	(d)	78.4	
6	(t)	61.6[d]	61.4[g]	62.0	4	(d)	70.2	
NeuAc-1					5	(d)	72.9	
1	(s)	173.3	174.2		6	(t)	61.5[d]	
2	(s)	100.9	100.9		Ara-2			
3	(t)	38.2	39.4		1	(d)	110.7[e]	
4	(d)	73.9	74.8		2	(d)	83.2	
5	(d)	51.1	51.0		3	(d)	78.3	
6	(d)	74.1	74.4		4	(d)	86.4	
7	(d)	69.8	70.1		5	(t)	62.7[d]	
8	(d)	73.0	73.0					
9	(t)	64.3	64.4					
CH_3CO	(s)	173.9	173.3					
CH_3CO	(q)	23.1	23.2					
Gal-2								
1	(d)	96.6	96.7					
2	(d)	68.9	67.3					
3	(d)	72.2	72.9					
4	(d)	75.4	76.2					
5	(d)	70.8	70.2					
6	(t)	61.9[d]	61.5[g]					
Gal-3								
1	(d)	110.4[e]	110.9[h]					
2	(d)	82.9	82.8					
3	(d)	78.3	79.7					
4	(d)	86.0	86.8					
5	(d)	71.8	70.8					
6	(t)	65.0	62.8[g]					

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

docosanoic acid and iso-C_{17}-phytosphingosine as shown in Fig. 1.

More polar partial hydrolysis product **3** is a hexasaccharide of ceramide possessing Glc, NeuAc, Ara, Gal in the ratio of 1 : 1 : 1 : 3, based on the results of its methanolysis, and its negative-ion FAB mass and ^{13}C-NMR spectra (Table 1), which giving a series of quasi-molecular ion peaks $[M-H]^-$ at *m*/*z*: 1700—1750 and characteristics carbon signals ascribable to NeuAc. Furthermore, the detailed analysis of negative-ion FAB-MS of **3** shows the molecular and fragment ion peaks at *m*/*z*: 1711, 1549, 1417, 1255, 964, 802 and 640 corresponding to cleavage of the glycosidic linkages of the major component, thus indicating the linear or branched hexasaccharide moiety, (Hexose, Pentose)→Hexose→NeuAc→Hexose→Hexose, as shown in Fig. 2.

Methylation of **3** according to the Hakomori method[7] afforded the permethylated product **4**. Partially methylated alditol acetates (S-1—S-5) prepared from **4** were analyzed by GC-MS and identified as the alditols derived from terminal hexofuranose (S-1), terminal pentofuranose (S-2), 3,4-linked hexopyranose (S-3), 3-linked hexopyranose (S-4) and 4-linked hexopyranose (S-5). On the other hand, **4** was methanolyzed, the methanolysate was acetylated, and the acetate of partially methylated NeuAc (S-6) derived from 4-linked NeuAc was detected by means of GC-MS.

On the basis of the above evidence and the existence of lactosyl ceramide moiety (**2**), the hexasaccharide moiety of **3** must be Gal(*f*)-(1→3 or 1→4)-[Ara(*f*)-(1→4 or 1→3)]-

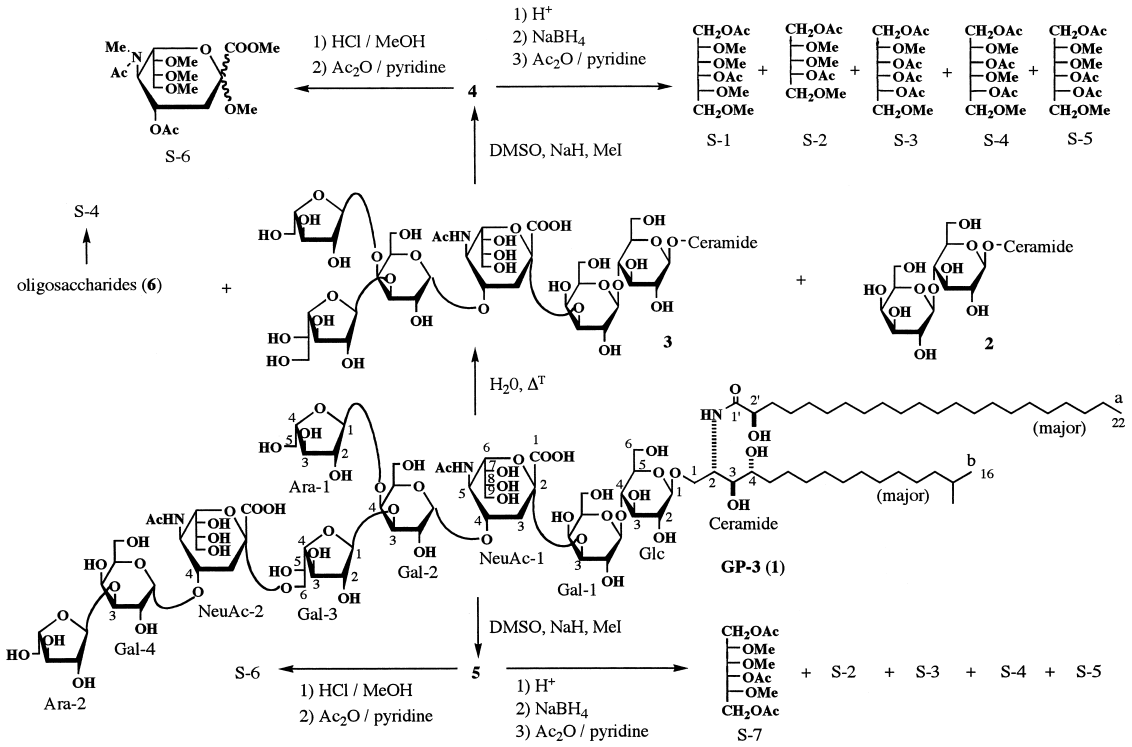

Fig. 1. Structure of GP-3 (**1**)

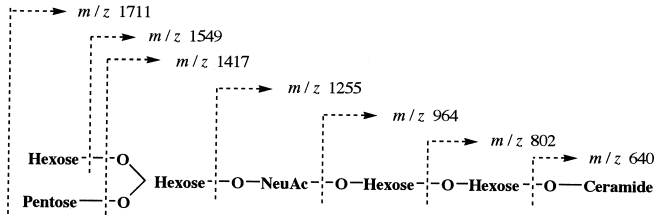

Fig. 2. Negative-Ion FAB Mass Fragmentation of the Major Component of **3**

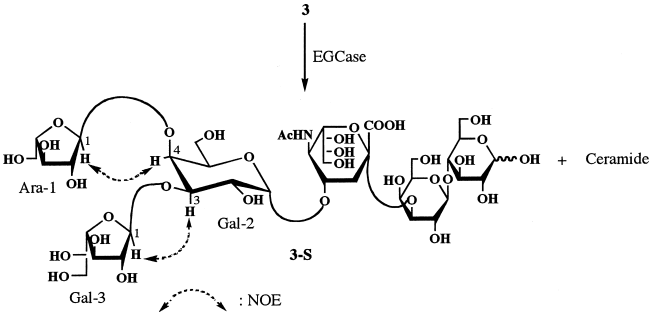

Fig. 3. NOE Correlations of **3-S**

Gal(*p*)-(1→4)-NeuAc-(2→3)-Gal(*p*)-(1→4)-Glc(*p*) as shown in Fig. 1. The sites of linkage of Gal(*f*) (Gal-3) and Ara(*f*) (Ara-1) to Gal-2 were determined with the aid of the 2D-NMR experiments as follows. The enzymatic hydrolysis of **3** by means of endoglycoceramidase (EGCase)[8] afforded the intact oligosaccharide (**3-S**) and ceramide moieties as shown in Fig. 3. When NOE Correlation Spectroscopy (NOESY) of **3-S** is measured, clear NOE correlations are observed between 1-H (anomeric H) of Gal-3 (δ_H: 5.19) and 3-H of Gal-2 (δ_H: 3.86), 1-H of Ara-1 (δ_H: 5.43) and 4-H of Gal-2 (δ_H: 4.21) (see Fig. 3), those signals were assigned by ^1H–^1H Correlation Spectroscopy (COSY) of **3-S**. Therefore, Gal-3 and Ara-1 combined with the 3- and 4-hydroxy groups of Gal-2, respectively, and the correct structure of **3** must be described in Fig. 1.

By taking the structure of the partial hydrolysis product **3** and the carbohydrate components of **1** (*vide supra*) into account, the sugar moiety of **1** is such a nonasaccharide as one mole each of NeuAc, Gal and Ara are combined to the hexasaccharide of **3**. When the partially methylated alditol acetates and the acetate of partially methylated NeuAc, which were prepared from the permethylated **1** (**5**), were analyzed by GC-MS, alditols derived from 6-linked hexofuranose (S-7), terminal pentofuranose (S-2), 3,4-linked hexopyranose (S-3), 3-linked hexopyranose (S-4), 4-linked hexopyranose (S-5), and the partially methylated NeuAc (S-6) originated from 4-linked NeuAc were detected. Therefore, a linear trisaccharide Ara(*f*)-(1→3 or 1→4)-Gal(*p*)-(1→4)-NeuAc is thought to be attached to C_6-OH of Gal(*f*) (Gal-3 in Fig. 1). Furthermore, the site of linkage of Ara(*f*) (Ara-2) to Gal(*p*) (Gal-4) in the trisaccharide moiety was determined as follows. Since the alditol (S-4) derived from 3-linked hexopyranose was obtained from the permethylate of the mixture of oligosaccharides (**6**), i.e. mixture of Ara-2→Gal-4→NeuAc-2, Gal-3→[Ara-1→]Gal-2→NeuAc-1, and Ara-2→Gal-4→NeuAc-2→Gal-3→[Ara-1→]Gal-2→NeuAc-1, terminal Ara (Ara-2) must be combined to C_3-OH of Gal-4.

Accordingly, the structure of the nonasaccharide of **1** is suggested to be Ara(*f*)-(1→3)-Gal(*p*)-(1→4)-NeuAc-(2→6)-Gal(*f*)-(1→3)-[Ara(*f*)-(1→4)]-Gal(*p*)-(1→4)-NeuAc-(2→3)-Gal(*p*)-(1→4)-Glc(*p*). The configurations of Glc, Gal-1 and Gal-3 are considered to be β, on the other hand, NeuAc-1, NeuAc-2, Gal-2, Gal-4, Ara-1 and Ara-2 are α on

the basis of their anomeric carbon signals (δ: 104.8, 104.3, 110.4, 100.9, 101.3, 96.6, 96.9, 111.2, 110.7) in the ^{13}C-NMR spectrum of **1**.

Consequently, if Glc, Gal, NeuAc and Ara are presumed to belong to the most commonly found D and L series, GP-3 (**1**) is 1-*O*-α-L-arabinofuranosyl-(1→3)-α-D-galactopyranosyl-(1→4)-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-galactofuranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-D-galactopyranosyl-(1→4)-(*N*-acetyl-α-D-neuraminosyl)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside of ceramide composed of heterogeneous (2*S*,3*S*,4*R*)-phytosphingosine (*iso*-C_{17}-phytosphingosine as major component) and (2*R*)-2-hydroxy fatty acid units (docosanoic acid as major component) (Fig. 1).

From the starfish *A. pectinifera*, several kinds of ganglioside molecular species have been obtained by Sugita,[9] Smirnova[10] and Kochetkov[11] *et al*. However, GP-3 (**1**) is, to the author's knowledge, new ganglioside molecular species. Furthermore, **1** is the second ganglioside possessing two sialic acid residue at the inner part of the sugar moiety next to the disialo-ganglioside from *A. pectinifera*[11] as starfish ganglioside.

The effect of **1** on the neuritogenesis of the rat pheochromocytoma cell line (PC-12 cells) have been investigated. The results showed that **1** displayed neuritogenic activity in the presence of nerve growth factor (NGF). The proportion of the cells with neurite longer than the diameter of the cell body of **1** at a concentration of 10 μM was 38.2% when compared with the control (NGF, 5 ng/ml: 20.6%). The effect of **1** was lower than that of the mammalian ganglioside GM_1 (47.0%).

Experimental

Optical rotations were measured with a Jasco DIP-370 digital polarimeter at 27 °C. IR spectra were obtained on a Jasco IR-700 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 Jeol DX-300 mass spectrometer (xenon atom beam; matrix, HMPA/TEG). GC-MS was taken with a Shimadzu QP-1000 [EI mode; ionizing potential of 70 eV; separator and ion-source temperature 250 °C; column, 2% OV-17 or 2% OV-1 on Chromosorb W (1.1 m×2.6 mm); carrier gas, He]. GC was run on a Shimadzu GC-14B [FID mode; column, Fused Silica Capillary Column DB-17 (0.32 mm×30 m, J & W Scientific); carrier gas, N_2].

Separation of GP-3 (1) Whole bodies of the starfish *Asterina pectinifera* (30 kg) were chopped and extracted with $CHCl_3$/MeOH (1 : 2) (64 l). The $CHCl_3$/MeOH solutions were concentrated *in vacuo* to give a residue of 2.41 kg. This residue was dissolved in H_2O (3 l) and the solution was extracted with AcOEt/*n*-BuOH (2 : 1, 5 l, 3 times) for separation of less polar lipids. The aqueous layer was further extracted with *n*-BuOH (2 l, 4 times) to remove saponins, and the aqueous layer was concentrated *in vacuo* to give a residue (0.96 kg). This residue was extracted with $CHCl_3$/MeOH (1 : 1, 0.5 l, 3 times), and the extract was concentrated *in vacuo* to give a crude water-soluble lipid fraction (186 g). The polar lipid fraction was chromatographed over Cosmosil 140C_{18}-OPN (reverse-phase) (solvent 20%, 40%, 60%, 80%, 100% MeOH) to give five fractions. The crude polar ganglioside fraction (7.04 g), the 80% MeOH eluate, was chromatographed on silica gel [solvent $CHCl_3$/MeOH/14% NH_3 (13 : 9 : 2)] to afford **1** (250 mg) (*Rf*=0.10) [solvent of TLC (silica gel), *n*-BuOH/*i*-PrOH/H_2O (7 : 3 : 6) (upper layer)].

GP-3 (**1**): Amorphous powder. IR (KBr) cm^{-1}: 3400 (OH), 1645, 1545 (amide). Negative-ion FAB-MS *m/z*: 2250—2350 $[M-H]^-$ series, 1417, 1255, 964, 802, 640 (fragment ions of major component) (see Fig. 2). ^{1}H-NMR (C_5D_5N/D_2O 96 : 4) δ: 2.32 (s, 3H, CH_3CO), 2.19 (s, 3H, CH_3CO), 0.9 (m, 9H, $3CH_3$). ^{13}C-NMR: see Table 1.

Analysis of Sugar Components of 1 Compound **1** (2 mg) was heated with 2 N HCl at 100 °C for 4 h in a small-volume sealed vial, and the mixture was evaporated *in vacuo*. The residue was dissolved in H_2O (5 ml), and 28% NH_3 (2 drops), and $NaBH_4$ (40 mg) was added. After standing at room temperature for 5 h, the reaction mixture was acidified with AcOH to pH 3.5 and concentrated *in vacuo*. H_3BO_3 contained 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, and the mixture was concentrated *in vacuo*. The residue was extracted with $CHCl_3$ (1 ml), and the $CHCl_3$ solution was washed with H_2O, dried (Na_2SO_4) and the solvent evaporated to give alditol acetates. These acetates were subjected to GC (column temperature 210 °C). The results were as follows: t_R [min]=10.0 (2 mol), (1,2,3,4,5-tetra-*O*-acetyl arabitol); t_R=22.8 (4 mol), (1,2,3,4,5,6-hexa-*O*-acetyl galactitol); t_R=23.5 (1 mol), (1,2,3,4,5,6-hexa-*O*-acetyl glucitol).

Partial Hydrolysis of 1 Compound **1** was heated with H_2O at 90 °C for 7 h in a sealed vial. The reaction mixture was extracted with AcOEt/*n*-BuOH (2 : 1), the organic layer was concentrated *in vacuo*, and the residue was chromatographed on silica gel with $CHCl_3$/MeOH/H_2O (7 : 3 : 0.3) as solvent to give **2**. The aqueous layer was freeze-dried, followed by separated with chromatographed on silica gel (solvent $CHCl_3$/MeOH/14% NH_3, 13 : 9 : 2 to 6 : 4 : 1) to afford **3** and **6**.

Compound **2**: Amorphous powder, $[\alpha]_D$ +13.0° [*c*=0.2, $CHCl_3$/MeOH (1 : 1)]. **2** was identified as synthetic lactosyl ceramide,[6] $[\alpha]_D$ +8.0°, except for the side chain of the ceramide moiety [^{1}H- and ^{13}C-NMR (Table 1)].

Compound **3**: Amorphous powder. Negative-ion FAB-MS *m/z*: 1700—1750 $[M-H]^-$ series, 1711, 1549, 1417, 1255, 964, 802, 640 (molecular and fragment ions of major component) (see Fig. 2). ^{1}H-NMR (C_5D_5N/D_2O, 96:4) δ: 2.23 (s, 3H, CH_3CO), 0.88 (m, 9H, $3CH_3$). ^{13}C-NMR: see Table 1.

Compound **6**: A mixture of oligosaccharides.

Methanolysis of 2 Compound **2** (2 mg) was heated with 5% HCl in MeOH (1 ml) at 70 °C for 4 h in a small-volume sealed vial. The reaction mixture was extracted with *n*-hexane and the hexane layer was concentrated to give a mixture of fatty acid methyl esters (FAM). The MeOH layer was neutralized with Ag_2CO_3, filtered, and evaporated *in vacuo*. The residue was heated with Ac_2O/C_5H_5N (1 : 1) (1 ml) at 70 °C for 2 h, and the mixture was concentrated *in vacuo*. The residue was extracted with $CHCl_3$, and the $CHCl_3$ solution was washed with H_2O, dried (Na_2SO_4) and the solvent was evaporated to give the mixture of acetate of long-chain bases (LCB).

Analysis of FAM and LCB Acetate from 2 The FAM mixture was subjected to GC-MS [column, 2% OV-1; column temp. 200—230 °C (rate of temperature increase 3 °C/min)]. The results were as follows: methyl 2-hydroxydocosanoate, t_R [min] (ratio of peak areas)=7.9 (1.14); methyl 2-hydroxytricosanoate, t_R=9.6 (0.88); methyl 2-hydroxytetracosanoate, t_R=11.5 (0.85). The acetate of LCB mixture was analyzed by GC [column temperature 200—270 °C (rate of temperature increase 5 °C/min)]. The results were as follows: 1,3,4-tri-*O*-acetyl-*iso*-C_{16}-phytosphingosine, t_R [min] (ratio of peak area)=20.6 (0.20); 1,3,4-tri-*O*-acetyl-*iso*-C_{17}-phytosphingosine, t_R=22.1 (0.88); 1,3,4-tri-*O*-acetyl-*iso*-C_{18}-phytosphingosine, t_R=25.4 (0.64).

Analysis of Sugar Components of 3 Compound **3** (1.9 mg) was heated with 5% HCl in MeOH (1 ml) at 70 °C for 4 h in a small-volume sealed vial. The reaction mixture was washed with *n*-hexane to remove FAM, and the MeOH layer was evaporated *in vacuo*. The residue was heated with 1-(trimethylsilyl) imidazole/pyridine (1 : 1, 0.2 ml) for 7 min at 70 °C and the reaction mixture (trimethylsilyl ethers of methyl glycosides) was analyzed by GC [column temperature 100—250 °C (rate of temperature increase 5 °C/min)] with the following results: t_R [min]=14.1 and 14.5 (1 mol) (methyl arabinoside), 19.5 and 20.1 (3 mol) (methyl galactoside), 20.7 and 20.8 (1 mol) (methyl glucoside).

Methylation of 3 (Hakomori Method) Compound **3** (5.2 mg) was treated with NaH (40 mg) and MeI (1 ml) in DMSO (1 ml) according to the Hakomori method.[7] The reaction mixture was diluted with H_2O, extracted with $CHCl_3$, and the $CHCl_3$ layer was washed with H_2O, dried (Na_2SO_4), and the solvent was evaporated *in vacuo*. The residue was chromatographed on silica gel [solvent, *n*-hexane/acetone (2 : 1)] to give **4** (2.3 mg).

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetates from 4 Compound **4** (1 mg) was heated with 90% HCOOH/10% CF_3COOH (1 : 1) (1 ml) at 100 °C for 4 h in a small-volume sealed vial, and the mixture was evaporated *in vacuo*. The residue was dissolved in H_2O (1 ml), and 28% NH_3 (2 drops), and $NaBH_4$ (20 mg) was added. After standing at room temperature for 5 h, the reaction mixture was acidified with AcOH to pH 3.5 and concentrated *in vacuo*. H_3BO_3 contained 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, and the mixture was concentrated *in vacuo*. The residue was extracted with $CHCl_3$ (1 ml), and the $CHCl_3$ solution was washed with H_2O, dried (Na_2SO_4) and the solvent evaporated to give partially methylated alditol acetates. These acetates were subjected to GC-MS [column, 2% OV-17; column temperature 170—

230 °C (rate of temperature increase 3 °C/min)]. The results were as follows: S-1, t_R [min]=9.8, *m/z*: 101, 117, 205, 277 [1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methylhexitol (derived from terminal hexofuranose)]; S-2, t_R=4.8, *m/z*: 101, 117, 129, 161 [1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylpentitol (derived from terminal pentofuranose)]; S-3, t_R=16.3, *m/z*: 87, 117, 129 [1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylhexitol (derived from 3,4-linked hexopyranose)]; S-4, t_R=14.0, *m/z*=101, 117, 129, 161, 201, 233 [1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol (derived from 3-linked hexopyranose)]; S-5, t_R=14.0, *m/z*: 101, 117, 129, 173, 233 [1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol (derived from 4-linked hexopyranose)].

Preparation and GC-MS Analysis of Acetate of Partially Methylated NeuAc from 4 Compound **4** (1.0 mg) was heated with 5% HCl in MeOH at 70 °C for 4 h in a small-volume sealed vial. The reaction mixture was concentrated *in vacuo*, and the residue (methanolysate) was heated with Ac_2O/C_5H_5N (1 : 1) (1 ml) at 70 °C for 2 h, then the mixture was concentrated *in vacuo*. The residue was subjected to GC-MS (column, 2% OV-17; column temperature 230 °C) : S-6, t_R=8.0 min, *m/z*: 157, 302, 346, 376, 390, 404 [methyl *N*-acetyl-4-*O*-acetyl-*N*-methyl-2,7,8,9-tetra-*O*-methylneuraminate (from 4-linked NeuAc)].

Enzymatic Hydrolysis of 3 with EGCase The mixture contained 3.7 mg of **3** and the 200 mU of EGCase (TAKARA) in 20 mM sodium acetate buffer (pH 5.14) with 0.4% Triton X-100 (500 μl) was incubated at 37 °C for 24 h. The reaction mixture was centrifuged with 1500 rpm for 5 min, and the supernatant was chromatographed on reverse-phase (C8) (solvent, MeOH to H_2O) to afford oligosaccharide and ceramide fractions. The former was purified by Sephadex LH-20 (Pharmacia Fine Chemical) column chromatography (solvent, H_2O) to give **3-S** (0.8 mg).

Preparation of 5, and Analysis of Partially Methylated Alditol Acetates and NeuAc from 5 Compound **1** (19.8 mg) was methylated according to the Hakomori method and worked up in the same manner as described for **3** to give **5** (13.4 mg). Compound **5** (1.5 mg) was hydrolyzed, reduced and then acetylated, and the partially methylated alditol acetates were analyzed by GC-MS in the same manner as described for **4**, whereupon S-7, t_R [min]=13.4, *m/z*: 117, 159, 201, 233 [1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*-methylhexitol (derived from 6-linked hexofuranose)] was detected together with S-2, S-3, S-4, and S-5. Meanwhile, **5** (1.2 mg) was methanolyzed and then acetylated, and the acetate was subjected to GC-MS in the same way as described for **4** and S-6 was detected.

Methylation of 6 and Analysis of Partially Methylated Alditol Acetates from Permethylated 6 The mixture of oligosaccharides **6** (7.2 mg) was methylated according to the Hakomori method and worked up in the same manner as described for **3**, thereby yielding permethylated **6** (2.1 mg). The partially methylated alditol acetates prepared from the permethylated compound were analyzed by GC-MS in the same condition as for **4**, whereon S-4, derived from 3-linked hexopyranose, was detected together with S-1, S-2, S-3, and S-7.

Biological Assay Neuritogenic activity of **1** in PC-12 cells was observed according to the method previously reported.[12)]

Acknowledgments We thank Mr. Y. Tanaka and Ms. Y. Soeda of the Faculty of Pharmaceutical Sciences, Kyushu University, for the NMR measurements. This work was supported in part by a Grant-in-Aid for Scientific Research Nos. 08680632, 09470486, and 13024260 (Priority Area A) from the Ministry of Education, Culture, Science, Sports and Technology, Japan, and a grant (No. 16510163) from the Japan Society for the Promotion of Science, which are gratefully acknowledged.

References and Notes

1) Part 41: Maruta T., Saito T., Inagaki M., Shibata O., Higuchi R., *Chem. Pharm. Bull.*, **53**, 1255—1258 (2005).
2) Higuchi R., Inagaki K., Natori T., Komori T., Kawajiri S., *Liebigs Ann. Chem.*, **1991**, 1—10 (1991).
3) Normal means straight chain ($-CH_2CH_2CH_2CH_3$).
4) Iso means branched chain possessing a methyl group on the second carbon from the terminal methyl group [$-CH_2CH(CH_3)_2$].
5) Kawano Y., Higuchi R., Komori T., *Liebigs Ann. Chem.*, **1990**, 43—50 (1990).
6) Sugiyama S., Honda M., Higuchi R., Komori T., *Liebigs Ann. Chem.*, **1991**, 349—356 (1991).
7) Hakomori S., *J. Biochem.* (Tokyo), **55**, 205—208 (1964).
8) Ito M., Yamagata T., *J. Biol. Chem.*, **261**, 14278—14282 (1986).
9) Sugita M., *J. Biochem.* (Tokyo), **86**, 765—772 (1979).
10) Smirnova G. P., Kochetkov N. K., *Biochim. Biophys. Acta*, **618**, 486—495 (1980).
11) Kochetkov N. K., Smirnova G. P., *Biochim. Biophys. Acta*, **759**, 192—198 (1983).
12) Kaneko M., Kisa F., Yamada K., Miyamoto T., Higuchi R., *Eur. J. Org. Chem.*, **2003**, 1004—1008 (2003).