Constituents of Holothuroidea, 6[◇]

Isolation and Structure of Biologically Active Glycosphingolipids from the Sea Cucumber *Cucumaria echinata*

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Five cerebrosides, **CE-1-1**, **CE-1-2**, **CE-1-3**, **CE-3-1**, and **CE-3-2**, and a ganglioside molecular species **CG-1** have been obtained from the less polar and polar fractions, respectively, of the chloroform/methanol extract of the sea cucumber *Cucumaria echinata*. The structures of these glycosphingolipids have been determined on the basis of chemical and spectroscopic evidence. The cerebrosides show lethality toward brine shrimps. On the other hand, the ganglioside **CG-1** exhibits neuritogenic activity toward the rat pheochromocytoma cell line, PC-12 cells.

A series of studies on the isolation and structure elucidation of the glycosphingolipids from sea cucumber species have been performed in our laboratory[1][2]. In continuation of the preceding studies on the sea cucumber *Pentacta australis*, the isolation and characterization of the biologically active glycosphingolipids from the sea cucumber *Cucumaria echinata* (Gumi in Japanese) has now been conducted in order to develop the novel medicinal resources from marine natural products.

Regarding the glycosphingolipid constituents of *C. echinata*, we have already described the isolation and structure determination of three sphingosine-type glucocerebrosides[1]. In this paper, we report on the isolation and characterization of a further five glucocerebrosides as well as a ganglioside molecular species from the whole bodies of *C. echinata*. The biological activities of the glycosphingolipids are also reported.

The acetone-insoluble constituents, which were obtained from the less polar fraction of the chloroform/methanol extract of the whole bodies of *C. echinata*, were separated by silica-gel column-chromatography to give two cerebroside molecular species, **CE-1** and **CE-3**. On the other hand, the water-soluble lipid fraction of the extract was subjected to reversed-phase- followed by silica-gel column-chromatography to give a ganglioside molecular species **CG-1**. Each of these compounds moved as a single spot on normal-phase (silica gel) TLC.

[◇] Part 5: See ref.[2].

Structure of the Cerebroside Molecular Species CE-1

CE-1 exhibits strong hydroxy and amide absorptions in its IR spectrum and a series of molecular ion peaks in the positive FAB mass spectrum. In the ^{13}C-NMR spectrum, the characteristic signals of a sphingosine-type cerebroside possessing fatty acid and β-glucopyranose moieties are observed (Scheme 1 and Table 1). Therefore, **CE-1** must be a molecular species of a sphingosine-type cerebroside bearing such moieties. Furthermore, **CE-1** is presumed to have mainly *normal*-type[3] fatty acids and branched-type[4] long-chain bases, since the carbon signals for the terminal methyl groups are observed at δ = 14.2 (*normal* form) and at δ = 11.5 and 19.3 (branched form) in the ^{13}C-NMR spectrum (Scheme 1 and Table 1).

The ^{1}H-NMR signals of the basic structure of **CE-1** (Table 2) are in good agreement with those of the known cerebroside molecular species **PA-0**[2], which is composed of (2*S*,3*R*,4*E*)-sphingosine, fatty acid, and β-D-glucopyranose subunits, and is a component of the related sea cucumber *Pentacta australis*. In addition, the absolute configuration of the glucose unit is verified as being of D-form by means of the Hara method[5]. These results suggest that **CE-1** has the same absolute configuration as that of **PA-0** for the basic moiety at C-2, -3, -4, and -5, and for the glucopyranose (Scheme 1).

CE-1 undergoes methanolysis upon treatment with methanolic hydrochloric acid to yield a mixture of fatty acid methyl esters (FAM) and long-chain bases (LCB), together

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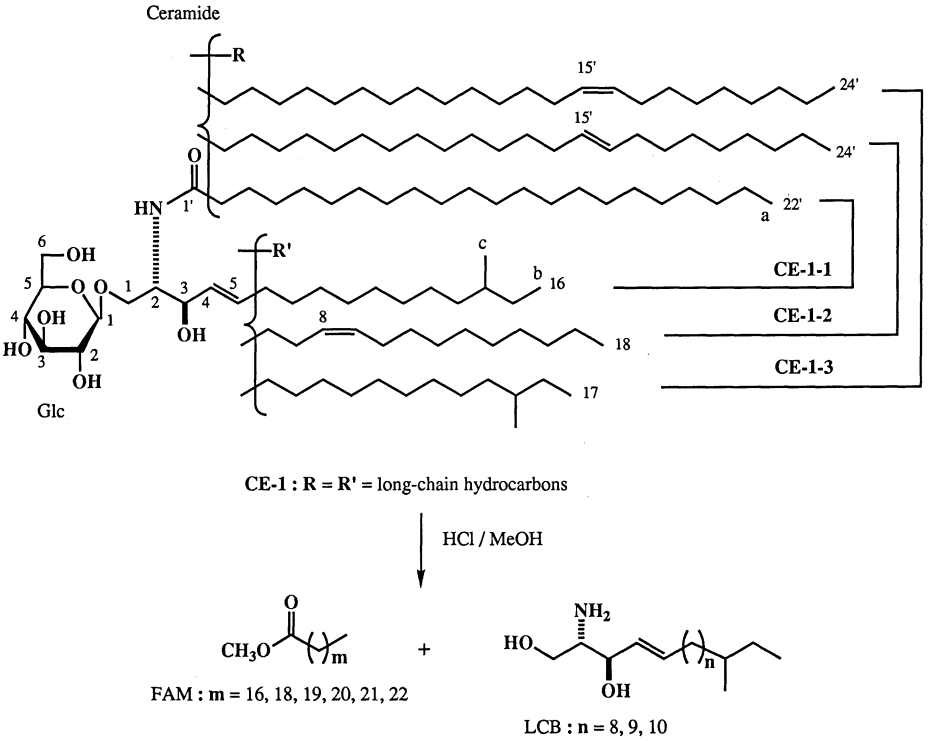
Scheme 1



with methyl D-glucopyranoside. The FAM mixture exhibits a ^{13}C-NMR signal at $\delta = 14.2$ attributable to the terminal methyl groups of *normal* fatty acid methyl esters. Analysis of the FAM mixture by GC-MS reveals the presence of six components, which are characterized as methyl octadecanoate (methyl stearate), methyl icosanoate (methyl arachidate), methyl henicosanoate, methyl docosanoate (methyl behenate), methyl tricosanoate, and methyl tetracosanoate (methyl lignocerate). Thus, the fatty acid composition of **CE-1** is characterized as shown in Scheme 1. On the other hand, the LCB components are suggested to be 2-amino-1,3-dihydroxy-14-methyl-4-hexadecene, -15-methyl-4-heptadecene, and -16-methyl-4-octadecene on the basis of GC-MS analysis of the TMS-derivatized LCB mixture (Scheme 1). Accordingly, **CE-1** is designated as a sphingosine-type cerebroside molecular species as depicted in Scheme 1, which is composed of the aforementioned fatty acids and long-chain bases.

Table 1. ^{13}C-NMR chemical shifts (δ values) of glycosphingolipids in [D_5]py

C		CE-1	CE-1-1	CE-1-2	CE-1-3	CE-3	CE-3-1	CE-3-2	CG-1[d]	C		CE-1	CE-1-1	CE-1-2	CE-1-3	CE-3	CE-3-1	CE-3-2	CG-1[d]
ceramide										Glc									
1	(t)	70.5	70.5	70.4	70.5	70.4	70.4	70.4	70.5	1	(d)	105.8	105.8	105.8	105.8	105.5	105.5	105.5	105.1
2	(d)	55.0	55.0	55.0	55.0	51.7	51.7	51.7	51.6	2	(d)	75.2	75.2	75.2	75.2	75.1	75.1	75.1	74.7
3	(d)	72.7	72.7	72.6	72.7	75.8	75.8	75.8	75.9	3	(d)	78.5	78.5	78.5	78.5	78.4**	78.4**	78.4**	78.1
4	(d)	132.1	132.1	131.8	132.1	72.5*	72.5*	72.5*	72.5*	4	(d)	71.6	71.6	71.6	71.6	71.5	71.5	71.4	71.9
5	(d)	132.6	132.5	132.3	132.5					5	(d)	78.5	78.5	78.5	78.5	78.5**	78.5**	78.5**	77.5
7	(t)			27.5						6	(t)	62.7	62.7	62.7	62.7	62.6	62.6	62.6	73.5
8	(d)			130.2						NGNA									
9	(d)			130.2						1	(s)								174.9
10	(t)			27.5						2	(s)								101.3
1'	(s)	173.4	173.3	173.3	173.3	175.6	175.6	175.6	175.8	3	(t)								41.6
2'	(d)					72.4*	72.4*	72.4*	72.6*	4	(d)								68.5
14'	(t)			32.8*	27.5		27.4			5	(d)								54.4
15'	(d)			129.8**	130.2		132.5			6	(d)								75.4
16'	(d)			131.0**	130.2		132.5			7	(d)								69.3
17'	(t)			32.9*	27.5		27.4			8	(d)								80.4
CH_3[a]	(q)	14.2	14.2	14.2	14.2	14.2	14.2	14.2	14.2	9	(t)								61.7
CH_3[b]	(q)	11.5	11.5		11.5	11.5	11.5	11.5	11.5	10	(s)								176.0
CH_3[c]	(q)	19.3	19.3		19.3	19.3	19.3	19.3	19.4	11	(t)								62.5

*,**: Assignments may be interchanged in each vertical column. – [a] Terminal methyl group in normal type of side chain (see Scheme 1). – [b][c] Terminal methyl groups in branched type of side chain (see Scheme 1). – [d] Measured in [D_5]py/D_2O (98:2).

Table 2. ^{1}H-NMR chemical shifts (δ values) of **CE-1**, **CE-3** and **CG-1**[f]

H	CE-1[d]	CE-3[d]	CG-1[e]
ceramide			
1a	4.26 *	4.50 (q, *J* = 11, 4 Hz)	3.60 *
1b	4.76 *	4.69 (q, *J* = 11, 6 Hz)	3.84 *
2	4.80 *	5.24 (m)	4.06 (m)
3	4.81 *	4.28 *	3.41 (t, *J* = 10, 10 Hz)
4	6.02 (q, *J* = 16, 6 Hz)	4.18 *	3.33 *
5	5.91 (sext, *J* = 16, 6, 6 Hz)		
2'		4.56 (q, *J* = 7, 3 Hz)	3.84 *
CH_3[a]	0.85 *	0.85 *	0.80 *
CH_3[b]	0.85 *	0.85 *	0.80 *
CH_3[c]	0.85 *	0.85 *	0.80 *
NH	8.42 (d, *J* = 8 Hz)	8.59 (d, *J* = 9 Hz)	7.49 (d, *J* = 7 Hz)
Glc			
1	4.96 (d, *J* = 8 Hz)	4.93 (d, *J* = 8 Hz)	4.13 (d, *J* = 7 Hz)
2	4.06 (t, *J* = 8, 9 Hz)	3.98 (q, *J* = 9, 8 Hz)	2.95 (m)
3	4.23 *	4.16 *	3.15 *
4	4.21 *	4.16 *	3.15 *
5	3.94 (m)	3.84 (m)	3.17 *
6a	4.35 (q, *J* = 12, 5 Hz)	4.31 *	3.58 *
6b	4.53 (q, *J* = 12, 3 Hz)	4.46 (q, *J* = 13, 2 Hz)	3.91 *
NGNA			
3a			1.38 *
3b			2.47 *
4			3.67 (m)
5			3.58 *
6			3.84 *
7			3.83 *
8			4.33 (m)
9a			3.58 *
9b			3.91 *
11a			3.60 *
11b			3.85 *
NH			7.14 (d, *J* = 9 Hz)

*: Submerged by other signals. – [a] Terminal methyl group in *normal* type of side chain (see Schemes 1). – [b][c] Terminal methyl groups in branched type of side chain (see Schemes 1). – [d] Measured in [D_5]py. – [e] Measured in [D_6]DMSO/D_2O (98:2). – [f] Signal assingments are based on the 2D-NMR (^{1}H-^{1}H COSY) spectra.

Isolation and Structure of Cerebrosides from CE-1

CE-1 was separated by reversed-phase HPLC into twelve peaks, and three compounds could be isolated, namely **CE-1-1**, **CE-1-2**, and **CE-1-3**. **CE-1-1**, the major component of **CE-1**, was confirmed as being the cerebroside component of **CE-1**, since its ^{13}C-NMR spectrum is essentially identical to that of **CE-1** (Table 1). Upon methanolysis, **CE-1-1** yielded methyl docosanoate and 2-amino-1,3-dihydroxy-14-methyl-4-hexadecene as its fatty acid and LCB components, respectively. Therefore, **CE-1-1** must be 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*R*,4*E*)-2-(docosanoylamino)-14-methyl-4-hexadecene-1,3-diol, as shown in Scheme 1. The molecular mass of **CE-1-1** (769), obtained from its positive FAB mass spectrum, supports the proposed structure. **CE-1-1** has been found to be identical to **PA-0-5**[2], isolated from the related sea cucumber of the same family *Pentacta australis*.

The ^{13}C-NMR spectrum of **CE-1-2** features signals characteristic of *normal* sphingosine-type cerebrosides bearing a *normal* fatty acid and a β-D-glucopyranose unit (Table 1 and Scheme 1). Furthermore, **CE-1-2** is suggested to contain two olefinic groups in the fatty acid and LCB side chains, since four olefinic carbon signals (δ = 130.2, 130.2, 129.8, and 131.0) are observed in addition to the carbon signals due to the double bond at C-4/C-5 (Table 1). In fact, **CE-1-2** gives methyl tetracosenoate and 2-amino-1,3-dihydroxyoctadecadiene as fatty acid and LCB components upon methanolysis. The location and geometries of the double bonds in the side chains of **CE-1-2** were determined as follows.

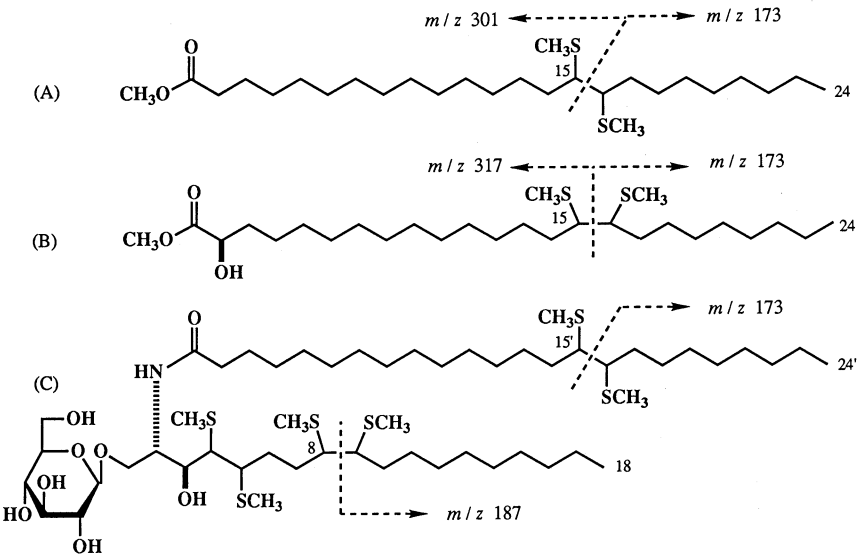
The mass spectra of the dimethyl disulfide (DMDS) derivatives[6] of FAM from **CE-1-2** [(A) in Scheme 2] and **CE-1-2** [(C) in Scheme 2] show remarkable fragment-ion peaks at *m/z* = 173 for A and *m/z* = 173 and 187 for C due to cleavage of the bond between the carbons bearing a methylthio group (Scheme 2). These data indicate that the double bonds in the fatty acid and LCB residues of **CE-1-2** are located at C-15′ and C-8, respectively, as shown in Scheme 1.

Furthermore, it is known[7] 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 olefinic carbon, which is observed at δ ≈ 27 in (*Z*) isomers and at δ ≈ 32 in (*E*) isomers. The proton signals at δ = 5.49 and 5.47 were assigned to the olefinic groups at C-8 and C-15′, respectively, by means of a ^{1}H-^{1}H COSY spectrum of **CE-1-2**. When an HMBC spectrum of **CE-1-2** was measured, significant correlations were observed between the signal of the olefinic protons at δ = 5.49 and that of the methylene carbon atoms at δ = 27.5, and between the signal at δ = 5.47 and those at δ = 32.8, 32.9, as shown in Scheme 3. Accordingly, these methylene carbon atoms must be the carbon atoms adjacent to the double bonds and are thus assigned to C-7 and C-10 (δ = 27.5), and to C-14′ and C-17′ (δ = 32.8, 32.9). Thus, the olefinic groups in the LCB and fatty acid side chains of **CE-1-2** are characterized as being of *cis* (*Z*) and *trans* (*E*) geometries, respectively (Scheme 1).

Taking the above facts and the molecular mass of **CE-1-2** (807) into account, the structure of **CE-1-2** is proposed to be 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*R*,4*E*,8*Z*)-2-[(15*E*)-15-tetracosenoylamino]-4,8-octadecadiene-1,3-diol, as shown in Scheme 1.

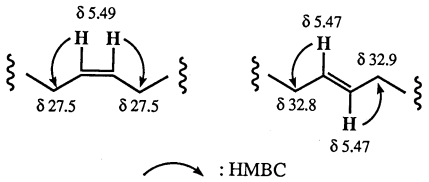
The ^{13}C-NMR spectrum of **CE-1-3** is essentially identical to that of **CE-1** (Table 1). This proves that this compound is also a cerebroside component of **CE-1**. When **CE-1-3** was methanolyzed, methyl tetracosenoate (FAM), and 2-amino-1,3-dihydroxy-15-methyl-4-heptadecene (LCB) were detected. The position and stereochemistry of the double bond in the fatty acid part was determined in the same manner as described for **CE-1-2**. Thus, from the mass fragmentation (*m/z* = 173) of the DMDS derivative of the FAM and the δ value (27.5) of the allylic carbon atoms obtained from the HMBC spectrum of **CE-1-3**, the presence of a *cis* (*Z*) double bond at C-15′ is indicated. On the basis of the above data and the molecular mass of **CE-1-3** (809), the structure of **CE-1-3** is determined to be 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*R*,4*E*)-2-[(15*Z*)-15-tetracosenoylamino]-15-methyl-4-heptadecene-1,3-diol (Scheme 1).

Scheme 2



Mass fragmentation of DMDS derivatives of **CE-1-2** FAM (A), **CE-3-1** FAM (B) and **CE-1-2** (C)

Scheme 3

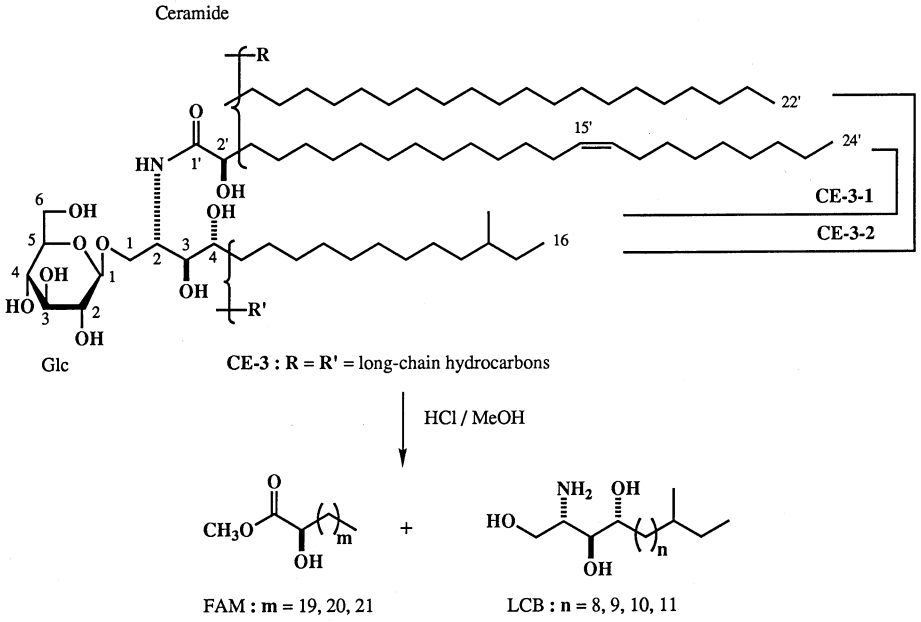


Structure of the Cerebroside Molecular Species CE-3

In the IR and positive FAB mass spectra of **CE-3**, strong hydroxy and amide absorptions and a series of molecular ion peaks are observed. The ^{1}H- and ^{13}C-NMR spectra of **CE-3** are in good agreement with those of the known glucocerebroside molecular species **PA-2**[2], which was obtained from the related sea cucumber *Pentacta australis* and is composed of (2*S*,3*S*,4*R*)-phytosphingosines with branching methyl groups, *normal*-(2*R*)-2-hydroxy fatty acids, and β-D-glucopyranose (Scheme 4, Tables 1 and 2).

Methanolysis of **CE-3** with methanolic hydrochloric acid yielded a mixture of FAM and LCB, together with methyl glucopyranoside. The FAM mixture, exhibiting a ^{13}C-NMR signal at δ = 14.2 diagnostic of *normal* side chains, was analyzed by GC-MS. This showed the presence of three

Scheme 4



components, which were characterized as methyl 2-hydroxydocosanoate, methyl 2-hydroxytricosanoate, and methyl 2-hydroxytetracosanoate. The LCB mixture was found to be composed of 2-amino-1,3,4-trihydroxy-13-methylpentadecane, -14-methylhexadecane, -15-methylheptadecane, and -16-methyloctadecane, on the basis of a GC-MS analysis of its TMS derivative (Scheme 4).

Since the absolute configuration of the glucose residue of **CE-3** was determined as being of the D series, as in **CE-1**, **CE-3** is designated as a (2*S*,3*S*,4*R*,2′*R*)-phytosphingosine-type cerebroside molecular species, composed of the aforementioned fatty acids and LCBs, as depicted in Scheme 4.

Isolation and Structure of Cerebrosides from CE-3

Like **CE-1**, **CE-3** was also separated by reversed-phase HPLC into six peaks, and two compounds, **CE-3-1** and **CE-3-2**, could be recovered. These showed molecular masses of 829 (**CE-3-1**) and 803 (**CE-3-2**), and their ^{13}C-NMR spectra were found to be essentially identical to that of **CE-3** (Table 1). This proves that these two compounds are cerebroside components of **CE-3**.

When **CE-3-1** and **CE-3-2** were methanolyzed, methyl 2-hydroxytetracosenoate and methyl 2-hydroxydocosanoate, respectively, were detected as fatty acid components. On the other hand, 2-amino-1,3,4-trihydroxy-14-methylhexadecane was found as a common LCB component. In the same manner as for **CE-1-2**, the position (C-15′) and geometry (*Z*) of the double bond in the fatty acid chain of **CE-3-1** was determined on the basis of the mass fragmentation (*m*/*z* = 173) of the DMDS derivative of the FAM from **CE-3-1** (Scheme 2) and from the δ value (27.4) of the allylic carbon atoms (C-14′ and C-17′). Therefore, the structures of **CE-3-1** and **CE-3-2** are proposed to be 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*S*,4*R*)-2-[(2*R*,15*Z*)-2-hydroxy-15-tetracosenoylamino]-14-methylhexadecane-1,3,4-triol and -(2*S*,3*S*,4*R*)-2-[(2*R*)-2-hydroxydocosanoylamino]-14-methylhexadecane-1,3,4-triol, as shown in Scheme 4.

Structure of the Ganglioside Molecular Species CG-1

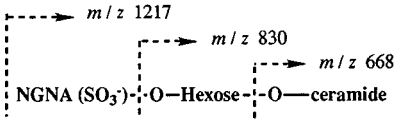
In its ^{13}C-NMR spectrum, **CG-1** exhibits the characteristic signals of a phytosphingosine-type ceramide possessing a 2-hydroxy fatty acid and a sugar moiety at C-1 (Table 1) [δ = 70.5 (C-1), 51.6 (C-2), 75.9 (C-3), 72.5 (C-4), 175.8 (C-1′), and 72.6 (C-2′)]. The ^{13}C-NMR spectrum of **CG-1** also features signals due to two anomeric carbons at δ = 105.1 and 101.3, one of which (δ = 101.3) is a quaternary carbon signal, indicating the presence of a sialic acid residue. The negative FAB mass spectrum exhibits a series of quasi-molecular ion peaks $[M - H]^-$ at *m*/*z* = 1245, 1231, 1217, 1203, 1189, and 1175, and a fragment-ion peak due to $[SO_4H]^-$ at *m*/*z* = 97. Therefore, **CG-1** is suggested to be a molecular species of a sulfated phytosphingosine-type ganglioside, possessing 2-hydroxy fatty acids and two monosaccharides. Furthermore, **CG-1** is presumed to have mainly *normal*-type fatty acids and branched-type LCBs, like the co-existing cerebroside molecular species **CE-3**, since the carbon signals for the terminal methyl groups are observed at δ = 14.2 (*normal* form), and at δ = 11.5 and 19.4 (branched form) (Table 1).

The structure of the ceramide moiety was elucidated first. When **CG-1** was methanolyzed with methanolic hydrochloric acid, a mixture of FAM, which were characterized as methyl 2-hydroxyoctadecanoate, methyl 2-hydroxydocosanoate, methyl 2-hydroxytricosanoate, and methyl 2-hydroxytetracosanoate (major), was obtained, together with methyl glucopyranoside. Taking into account the mass of the ceramide part (669) of the major component of **CG-1**, obtained from its negative FAB mass spectrum (Scheme 6), and the major fatty acid component from **CG-1** (2-hydroxytetracosanoate), we regard the major LCB component of **CG-1** as being a 14-methyl-hexadecane derivative.

The stereochemistry of the ceramide moiety is presumed to be 2*S*,3*S*,4*R*,2′*R*, since the aforementioned ^{13}C-NMR signals attributable to C-1, -2, -3, -4, -1′, and -2′ of **CG-1** are in good agreement with those of the cerebroside molecular species **CE-3** (Table 1) and those of synthetic (2*S*,3*S*,4*R*)-1-*O*-[*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl]-2-[(2*R*)-2-hydroxytetracosanoylamino]-1,3,4-hexadecanetriol[8].

The structure of the disaccharide moiety of **CG-1** was characterized as follows. Firstly, the presence of glucose (Glc) was obvious from the results of the methanolysis of **CG-1** (vide supra). A detailed analysis of the ^{13}C-NMR spectrum of **CG-1** revealed the characteristic signals [δ = 174.9 (s) (C-1), 101.3 (s) (C-2), 41.6 (t) (C-3), 54.4 (d) (C-5), 61.7 (t) (C-9), 176.0 (s) (C-10), 62.5 (t) (C-11)] of an *N*-glycolylneuraminic acid (NGNA) derivative residue, together with those of a β-glucopyranose derivative residue (Table 1). In the negative FAB mass spectrum of **CG-1**, the molecular-ion and fragment-ion peaks resulting from cleavage of the glycosidic linkages of the major component were observed at *m*/*z* = 1217, 830, and 668, indicating the presence of the disaccharide moiety, NGNA(SO_3^-) → hexose(β-glucopyranose), as shown in Scheme 5.

Scheme 5

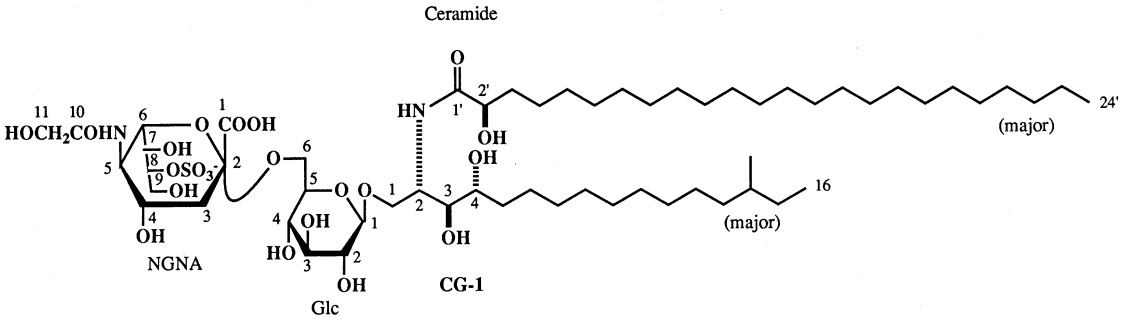


Negative FAB-mass fragmentation of the major component of **CG-1**

When the ^{13}C-NMR spectra of **CG-1** and **CE-3** (ceramide β-D-glucopyranoside) are compared, the carbon signal of C-6 of the Glc residue in **CG-1** is seen to be shifted downfield (δ = 62.6 → 73.5) upon glycosylation[9], which indicates that the NGNA residue is located at C-6 of the Glc residue. Furthermore, the downfield signals for C-8 (δ = 80.4) of the NGNA unit resulting from esterification[10], as well as for 8-H (δ = 4.33), in the ^{13}C- and ^{1}H-NMR spectra of **CG-1** (Tables 1 and 2), indicate the presence of the sulfate group at C-8 of the NGNA unit.

On the basis of the above evidence, the disaccharide moiety of **CG-1** must be 8-*O*-sulfo-NGNA-(2→6)-β-glucopyranose. The β-configuration of Glc is supported by the ^{1}H-

Scheme 6



NMR signal of its anomeric proton [δ = 4.13 (d, J = 7 Hz)]. The configuration of the NGNA is presumed to be α, since the characteristic carbon signals attributable to the NGNA moiety are in good agreement with those of a known ganglioside[11], possessing α-linked NGNA.

Consequently, if Glc and NGNA are assumed to belong to the most commonly found D-series, then **CG-1** is the *O*-8-*O*-sulfo-(*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. The major components of the fatty acid and LCB moieties of **CG-1** are *n*-(2*R*)-2-hydroxytetracosanoic acid and (2*S*,3*S*,4*R*)-2-amino-1,3,4-trihydroxy-14-methylhexadecane, respectively (Scheme 6).

Biological Activities

The newly isolated cerebrosides, **CE-1-1**, **CE-1-2**, **CE-1-3**, **CE-3-1**, and **CE-3-2**, have been tested for their lethality towards brine shrimps at a 30-ppm concentration according to the brine shrimp lethality bioassay[12]. They exhibit lethal rates of 27, 11, 31, 19, and 22%, respectively. On the other hand, the ganglioside molecular species **CG-1** is found to exhibit neuritogenic activity toward the rat pheochromocytoma cell line, PC-12 cells. Details of the latter biological activity will be reported elsewhere.

In conclusion, **CE-1-2**, **CE-1-3**, **CE-3-1**, and **CE-3-2** are, to the best of our knowledge, new cerebrosides. **CE-1-1** has been found to be identical to **PA-0-5**[2], isolated from the related sea cucumber *Pentacta australis* of the same family. On the other hand, from the sea cucumber *Cucumaria japonica*, three kinds of glucocerebroside molecular species have been obtained and characterized[13]. Regarding the gangliosides derived from sea cucumbers, our study shows similarities to the results of those on *C. japonica*[14], *Holothuria atra* and *Telenota ananas*[15]. However, as far as we are aware, this is the first time that a sulfated ganglioside, **CG-1**, has been isolated from a sea cucumber and characterized as such. Although a sulfated ganglioside possessing the same sugar moiety as that of **CG-1** has been obtained from the eggs of the sea urchin *Anthocidaris crassispina*[16], **CG-1** differs from this ganglioside in the structure of its ceramide part. The isolation and characterization of the biologically active glycosphingolipids is attracting considerable attention with regard to the manufacture of new medicines from marine natural products.

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Experimental Section

Melting points: micro melting point apparatus (Yanaco MP-3), uncorrected values. − Optical rotations: Jasco DIP-370 digital polarimeter at 25°C. − IR spectra: Jasco IR-700 infrared spectrophotometer. − ^{1}H- and ^{13}C-NMR spectra: Jeol GX-270 spectrometer (270 MHz and 67.8 MHz), Varian Unity-500 spectrometer (500 MHz and 125 MHz), Varian Unity-600 spectrometer (600 MHz and 150 MHz). − FAB mass spectra: Jeol DX-300 (xenon atom beam); matrix, HMPA/TEG (negative-ion mode) and *m*-nitrobenzyl alcohol (positive-ion mode). − High-resolution FAB mass spectra (HR FAB MS): Jeol SX/SX102A tandem mass spectrometer. − GC-MS: Shimadzu QP-1000; EI mode (ionizing potential of 70 eV, separator and ion-source temperature of 250°C); carrier gas He (30 ml/min). − GC: Shimadzu GC-14B (FID mode); column, fused-silica capillary column DB-17 (0.317 mm × 30 m, J. and W. Scientific); carrier gas, N_2 (1.3 ml/min). − HPLC: Jasco PU-980; RI detector; column, Wakosil 5C18 (C-18, 5 μ, Wako).

Separation of **CE-1**, **CE-3**, *and* **CG-1**: Whole bodies of the sea cucumber *Cucumaria echinata* (49 kg), which was collected from the Sea of Genkai in 1995, were chopped and extracted four times with $CHCl_3$/MeOH (1:2, 41 l). The combined extracts were concentrated in vacuo to give an aqueous solution (32 l), which was extracted with three portions of AcOEt/*n*BuOH (2:1, 11 l). The organic layer was concentrated in vacuo, and the residue was washed with cold acetone (21 l) to give an acetone-insoluble fraction (1.15 kg). A portion of the acetone-insoluble material (59 g) was chromatographed on Sephadex LH-20 (solvent $CHCl_3$/MeOH, 1:1) to give three fractions, Fr.1, Fr.2 and Fr.3. Fr.2 (16.1 g) was further separated by silica-gel column-chromatography (solvent $CHCl_3$/MeOH, 9:1 to 1:1) to afford **CE-1** (649 mg) and **CE-3** (336 mg). **CE-1** and **CE-3** each showed a single spot on silica-gel TLC ($CHCl_3$/MeOH, 85:15); R_f = 0.68 (**CE-1**), 0.54 (**CE-3**).

The aqueous solution was further extracted with *n*BuOH (3 × 5 l), and the aqueous layer was concentrated in vacuo to give a residue. This residue was extracted with $CHCl_3$/MeOH (1:1, 5.5 l), and the extract was concentrated in vacuo to give a crude water-soluble lipid fraction (558 g). The polar lipid fraction was chromatographed on Cosmosil 140C_{18}-OPN (reversed-phase) (solvent, 60%, 80%, and 100% MeOH) to give three fractions. The crude ganglioside fraction (1.7 g), i.e. the 100% MeOH eluate, was further chromatographed on silica gel (solvent $CHCl_3$/MeOH/H_2O, 7:3:0.5 to

6:4:1) to afford **CG-1** (12 mg); R_f = 0.61, silica-gel TLC, solvent $CHCl_3$/MeOH/H_2O (6:4:1).

CE-1: Amorphous powder, m.p. 136−139°C. − IR (KBr): $\tilde{\nu}$ = 3420 cm^{-1} (OH), 1645, 1540 (amide). − Positive FAB MS; *m/z*: 725−825 $[M + H]^+$ series. − ^{13}C NMR: Table 1. − ^1H NMR: Table 2.

Methanolysis of **CE-1**: **CE-1** (2 mg) was heated with 5% HCl in MeOH (1 ml) at 70°C for 6 h. The reaction mixture was then extracted with *n*-hexane, and the extract was concentrated in vacuo to yield a mixture of fatty acid methyl esters (FAM). − ^{13}C NMR ([D_5]pyridine): δ = 14.2 ($[CH_2]_nCH_3$). − The MeOH layer was neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated in vacuo to give a mixture of long-chain bases (LCB) and methyl glycoside.

GC-MS Analysis of FAM from **CE-1**: An FAM mixture from **CE-1** was subjected to GC-MS [column, 2% OV-17; column temp. 180−250°C (rate of temp. increase 4°C/min)]. The results were as follows: methyl octadecanoate, t_R [min] (ratio of peak areas) = 4.3 (2.6), *m/z*: 298 $[M^+]$, 255 $[M - 43]^+$; methyl icosanoate, t_R = 7.5 (1), *m/z*: 326 $[M^+]$, 283 $[M - 43]^+$; methyl henicosanoate, t_R = 9.2 (1.2), *m/z*: 340 $[M^+]$, 297 $[M - 43]^+$; methyl docosanoate, t_R = 10.9 (4.6), *m/z*: 354 $[M^+]$, 311 $[M - 43]^+$; methyl tricosanoate, t_R = 13.1 (6.2), *m/z*: 368 $[M^+]$, 325 $[M - 43]^+$; methyl tetracosanoate, t_R = 15.3 (7.6), *m/z*: 382 $[M^+]$, 339 $[M - 43]^+$.

GC-MS Analysis of TMS Ethers of LCB from **CE-1**: The mixture of LCB and methyl glycoside from **CE-1** was heated with 1-(trimethylsilyl)imidazole/pyridine (1:1) for 10 min at 70°C and then the reaction mixture [trimethylsilyl (TMS) ethers] was analyzed by GC-MS [column, 2% OV-17; column temp. 180−250°C (rate of temp. increase 4°C/min)]. The results were as follows: 2-amino-1,3-dihydroxy-14-methyl-4-hexadecene, t_R [min] (ratio of peak areas) = 7.9 (1), *m/z*: 326 $[M - 103]^+$, 297 $[M - 132]^+$, 236 $[M - 193]^+$, 132; 2-amino-1,3-dihydroxy-15-methyl-4-heptadecene, t_R = 8.5 (7.9), *m/z*: 340 $[M - 103]^+$, 311 $[M - 132]^+$, 250 $[M - 193]^+$, 132; 2-amino-1,3-dihydroxy-16-methyl-4-octadecene, t_R = 10.2 (3.8), *m/z*: 354 $[M - 103]^+$, 325 $[M - 132]^+$, 264 $[M - 193]^+$, 132.

GC Analysis of TMS Ethers of Methyl Glycoside from **CE-1**: The mixture of trimethylsilyl ethers of the LCB and methyl glycoside was analyzed by GC [column temp. 100−250°C (rate of temp. increase 5°C/min)]: t_R [min] = 18.5 and 18.7 (methyl β- and α-glucopyranoside).

Determination of Absolute Configuration of the Glucose Moiety of **CE-1** *(Hara Method*[5]*):* **CE-1** (1.2 mg) was heated with 10% H_2SO_4 (0.2 ml) at 70°C for 14 h. The reaction mixture was then extracted with *n*-hexane, and the acidic aqueous layer was neutralized with $Ba(OH)_2$, centrifuged, and the supernatant was concentrated. The residue (sugar fraction) was heated with L-cysteine methyl ester hydrochloride (0.25 mg) and pyridine (0.3 ml) at 70°C for 1 h. Then, 0.1 ml of 1-(trimethylsilyl)imidazole was added and the mixture was heated at 60°C for a further 0.5 h to yield the trimethylsilyl ether derivative of the methyl thiazolidine-4(*R*)-carboxylate. The derivative was analyzed by GC [column temp.: 200−250°C (rate of temp. increase 5°C/min)]; t_R = 12.4 min (derivative of D-glucose, 12.4 min; L-glucose, 13.0 min).

Isolation of Cerebrosides **CE-1-1**, **CE-1-2**, *and* **CE-1-3**: HPLC of **CE-1** (solvent MeOH, flow rate 3.0 ml/min) showed twelve peaks. Using these conditions, 648 mg of **CE-1** was separated by HPLC to give three compounds: **CE-1-1** (30 mg, t_R = 40 min), **CE-1-2** (8 mg, t_R = 47 min), **CE-1-3** (4 mg, t_R = 55 min).

CE-1-1: Amorphous powder, m.p. 135−136°C, $[\alpha]_D$ = −5.2 (*c* = 1.42 in 1-PrOH). − Positive FAB MS ; *m/z*: 770 $[M + H]^+$, 590 $[(M + H) - 180]^+$. − ^{13}C NMR: Table 1. − Methanolysis of **CE-1-1** in the same manner as described for **CE-1** afforded methyl docosanoate and 2-amino-1,3-dihydroxy-14-methyl-4-hexadecene (vide supra). − **CE-1-1** was found to be identical to **PA-0-5** {ref.[2]: m.p. 138−140°C, $[\alpha]_D$ = −7.6 (*c* = 0.89 in 1-PrOH)} by comparison of TLC and ^{13}C-NMR data[2].

CE-1-2: Amorphous powder, m.p. 136−137°C, $[\alpha]_D$ = −1.9 (*c* = 0.33 in 1-PrOH). − Positive FAB MS; *m/z*: 808 $[M + H]^+$, 628 $[(M + H) - 180]^+$. − ^1H NMR ([D_5]pyridine): δ = 5.49 [m, 2 H, CH=CH (8-H and 9-H)], 5.47 [m, 2 H, CH=CH (15′-H and 16′-H)]. − ^{13}C NMR: Table 1. − HMBC spectrum ([D_5]pyridine): Correlations were observed between the signals at δ = 5.49 (olefinic protons) and δ = 27.5 (methylene carbon atoms), and between those at δ = 5.47 (olefinic protons) and δ = 32.8, 32.9 (methylene carbon atoms). − $C_{48}H_{90}O_8N$; $[M + H]^+$: calcd. 808.6666; found 808.6649 (HR positive FAB MS). − **CE-1-2** was methanolyzed and the products (FAM and LCB) were treated and analyzed by GC-MS in the same way as described for **CE-1**. The results were as follows: methyl tetracosenoate, t_R = 15.8 min, *m/z*: 380 $[M^+]$, 337 $[M - 43]^+$; 2-amino-1,3-dihydroxy-octadecadiene, t_R = 9.5 min, *m/z*: 338 $[M - 103]^+$, 309 $[M - 132]^+$, 248 $[M - 193]^+$.

DMDS Derivatives of **CE-1-2** *FAM, and* **CE-1-2**: **CE-1-2** FAM (methyl tetracosenoate, 0.7 mg) was dissolved in carbon disulfide (0.2 ml) and dimethyl disulfide (DMDS, 0.2 ml) and iodine (1 mg) were added to the solution. The resulting mixture was kept at 60°C for 40 h in a small-volume sealed vial. The reaction was subsequently quenched with aqueous $Na_2S_2O_3$ (5%), and the mixture was extracted with *n*-hexane (0.3 ml). The extract was concentrated and the residue (**CE-1-2** FAM DMDS derivative) was analyzed by GC-MS (column, 2% OV-1; column temp. 250°C): t_R = 9.6 min; *m/z*: 474 $[M^+]$, 301, 173. In the same manner, **CE-1-2** (1.5 mg) was derivatized and the crude reaction mixture was purified by silica-gel column-chromatography (solvent $CHCl_3$/MeOH/H_2O, 10:1:0.05) to give the **CE-1-2** DMDS derivative. − Positive FAB MS: *m/z*: 187, 173.

CE-1-3: Amorphous powder, m.p. 128−129°C, $[\alpha]_D$ = −0.4 (*c* = 0.15 in 1-PrOH). − Positive FAB MS; *m/z*: 810 $[M + H]^+$, 630 $[(M + H) - 180]^+$. − ^1H NMR ([D_5]pyridine): δ = 5.50 [m, 2 H, CH=CH (15′-H and 16′-H)]. − ^{13}C NMR: Table 1. − HMBC spectrum ([D_5]pyridine): A correlation was observed between the signals at δ = 5.50 (olefinic protons) and δ = 27.5 (methylene carbon atoms). − $C_{48}H_{92}O_8N$; $[M + H]^+$: calcd. 810.6823; found 810.6794 (HR positive FAB MS). − **CE-1-3** was methanolyzed in the same way as described for **CE-1** to yield methyl tetracosenoate and 2-amino-1,3-dihydroxy-15-methyl-4-heptadecene as FAM and LCB components, respectively. − In the same manner as in the case of **CE-1-2**, **CE-1-3** FAM (methyl tetracosenoate) was converted to its DMDS derivative, which was examined by GC-MS: t_R = 9.6 min; *m/z*: 474 $[M^+]$, 301, 173.

CE-3: Amorphous powder, m.p. 122−124°C. − IR (KBr): $\tilde{\nu}$ = 3410 cm^{-1} (OH), 1630, 1540 (amide). − Positive FAB MS ; *m/z*: 800−900 $[M + Na]^+$ series. − ^{13}C NMR: Table 1. − ^1H NMR: Table 2. − The absolute configuration of glucose residue was determined to be D in the same way as described for **CE-1**.

Methanolysis of **CE-3**: In the same manner as described for **CE-1**, **CE-3** was methanolyzed and the reaction mixture was worked-up to give an FAM mixture and a residue composed of long-chain bases (LCB) and methyl glycoside. − FAM mixture: ^{13}C NMR ([D_5]pyridine), δ = 14.2 [$(CH_2)_nCH_3$].

GC-MS Analysis of FAM from **CE-3**: The FAM mixture from **CE-3** was subjected to GC-MS using the same conditions as de-

scribed for the FAM mixture obtained from **CE-1**. The results were as follows: methyl 2-hydroxydocosanoate, t_R [min] (ratio of peak areas) = 15.3 (1.2), *m/z*: 370 [M^+], 311 [$M - 59]^+$; methyl 2-hydroxytricosanoate, t_R = 17.0 (1), *m/z*: 384 [M^+], 325 [$M - 59]^+$; methyl 2-hydroxytetracosanoate, t_R = 19.3 (1), *m/z*: = 398 [M^+], 339 [$M - 59]^+$.

GC-MS and GC Analyses of TMS Ethers of LCB and Methyl Glycoside from **CE-3**: The residue (a mixture of LCB and methyl glycoside) from **CE-3** was trimethylsilylated and the reaction mixture was analyzed by GC-MS and GC in the same way as described for **CE-1**. The results were as follows. LCB (GC-MS): 2-amino-1,3,4-trihydroxy-13-methylpentadecane, t_R [min] (ratio of peak areas) = 8.7 (1), *m/z*: 312 [$M - 193]^+$, 271 [$M - 234]^+$, 132; 2-amino-1,3,4-trihydroxy-14-methylhexadecane, t_R = 9.8 (9.7), *m/z*: 326 [$M - 193]^+$, 285 [$M - 234]^+$, 132; 2-amino-1,3,4-trihydroxy-15-methylheptadecane, t_R = 11.3 (2), *m/z*: 340 [$M - 193]^+$, 299 [$M - 234]^+$, 132; 2-amino-1,3,4-trihydroxy-16-methyloctadecane, t_R = 13.2 (3.1), *m/z*: 354 [$M - 193]^+$, 313 [$M - 234]^+$, 132. Methyl glycoside (GC): methyl β- and α-glucopyranoside were detected.

Isolation of Cerebrosides **CE-3-1** *and* **CE-3-2**: HPLC of **CE-3** (under the same conditions as used for **CE-1**) revealed six peaks. 328 mg of **CE-3** was separated by HPLC under these conditions to give two compounds: **CE-3-1** (4 mg, t_R = 29 min), **CE-3-2** (18 mg, t_R = 34 min).

CE-3-1: Amorphous powder, m.p. 138−140°C, $[\alpha]_D$ = +5.8 (*c* = 0.13 in 1-PrOH). − Positive FAB MS ; *m/z*: 852 [M + Na]$^+$, 668 [(M + H) − 162]$^+$. − ^{1}H NMR ([D$_5$]pyridine): δ = 5.49, 5.51 [each m, 1 H, CH=CH (15′-H and 16′-H)]. − ^{13}C NMR: Table 1. − HMBC spectrum ([D$_5$]pyridine): Correlation was observed between the signals at δ = 5.49, 5.51 (olefinic protons) and δ = 27.4 (methylene carbon atoms). − In the same manner as described for **CE-1**, **CE-3-1** was methanolyzed, to yield methyl 2-hydroxytetracosenoate [GC-MS (column, CBP10-W12-100): t_R = 10.2 min; *m/z*: 396 (M^+), 337 ($M - 59)^+$] and 2-amino-1,3,4-trihydroxy-14-methylhexadecane as FAM and LCB components, respectively. − **CE-3-1** FAM (methyl 2-hydroxytetracosenoate) was converted to its DMDS derivative and examined by GC-MS in the same way as described for **CE-1-2**: t_R = 14.2 min; *m/z*: 490 [M^+], 317, 173.

CE-3-2: Amorphous powder, m.p. 147−149°C, $[\alpha]_D$ = +10.2 (*c* = 0.63 in 1-PrOH). − Positive FAB MS; *m/z*: 826 [M + Na]$^+$, 642 [(M + H) − 162]$^+$. − ^{13}C NMR: Table 1. − Methanolysis of **CE-3-2**, in the same manner as described for **CE-1**, afforded methyl 2-hydroxydocosanoate and 2-amino-1,3,4-trihydroxy-14-methylhexadecane.

CG-1: Amorphous powder, m.p. 129−130°C. − Negative FAB MS: *m/z* 1245, 1231, 1217, 1203, 1189, 1175 [M − H]$^-$, 830, 668 (fragment ions of major component, see Scheme 6), 97 [$SO_4H]^-$, 80 [$SO_3]^-$. − ^{13}C NMR: Table 1. − ^{1}H NMR: Table 2.

Methanolysis of **CG-1**: Conducted in the same manner as described for **CE-1** to give an FAM mixture and a residue composed of long-chain bases (LCB) and methyl glycoside. The FAM mixture was subjected to GC-MS [column, CBP10-W12-100; column temp. 180−250°C (rate of temp. increase 8°C/min)]. The results were as follows: methyl 2-hydroxyoctadecanoate, t_R [min] (ratio of peak areas) = 3.9 (1), *m/z*: 314 [M^+], 255 [$M - 59]^+$; methyl 2-hydroxydocosanoate, t_R = 7.1 (2.2); methyl 2-hydroxytricosanoate, t_R = 7.9 (4.2); methyl 2-hydroxytetracosanoate, t_R = 8.8 (5.2). − Methyl glucopyranoside was detected in the mixture of LCB and methyl glycoside by TLC. The LCB components could not be identified because of the small amount of sample available.

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[3] *normal* means straight chain (−$CH_2CH_2CH_2CH_3$).
[4] Branched chain possessing a methyl group on the third carbon atom from the terminal methyl group [−CH_2CH-(CH_3)CH_2CH_3].
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