Zwitterionic Digalactosylceramides from the Earthworm, *Pheretima asiatica*

Ryuichiro TANAKA, Kazumoto MIYAHARA, and Naoki NODA*

Faculty of Pharmaceutical Sciences, Setsunan University, 45–1, Nagaotoge-cho, Hirakata, Osaka 573–01, Japan.
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Nine homogenous glycosphingolipids were isolated from the earthworm, *Pheretima asiatica* (Annelida). All of them are digalactosylceramides carrying a choline phosphate group in the outer galactose moiety. Their full structures including the position of a phosphocholine unit were determined based on chemical and spectral evidence.

Key words glycosphingolipid; digalactosylceramide; choline phosphate group; earthworm; *Pheretima asiatica*; Annelida

Our previous studies[1] demonstrated that members of the phylum Annelida contain unique monogalactosylceramides having a phosphocholine group in the sugar moiety. In search of a new series of glycosphingolipids differing in structure from known zwitterionic-type monogalactocerebrosides (MGCs), we have examined a phospholipid fraction of the land annelid, *Pheretima asiatica*, and have noted that a more polar fraction compared with that of MGCs showed, on TLC, a positive tailing band with Dittmer–Lester's reagent,[2] indicating the presence of another phosphorus-containing lipid. By application of the recycling HPLC technique, nine homogeneous compounds were isolated from the polar lipid fraction. Their chemical and spectral data revealed that all of them are digalactosylceramides having a choline phosphate group in the sugar moiety to form an amphoteric-ion structure. In this paper, we describe the isolation and structure determination of these compounds.

A glycolipid fraction[3] obtained from a polar lipid fraction of the $CHCl_3$–MeOH extract of the dried bodies of *P. asiatica* was separated on an Asahipack GS-310 column using $CHCl_3$–MeOH to give a fraction (fr. 6b′). Fraction 6b′ gave a similar tailing band to those of MGCs with Dittmer–Lester's reagent. It was subjected to HPLC with a reversed-phase column using $CHCl_3$–MeOH–H_2O to give better-separated peaks on the chromatogram (Fig. 1). Eight portions corresponding to peaks (II—IX) were collected and each of them was further separated by means of preparative HPLC to afford nine compounds (**1**—**9**) as individual molecular species.

The ^{1}H-NMR spectrum of **2** (*m/z*: 1111 $[M+H]^+$, positive ion FAB-MS) gave signals due to a choline phosphate group and the common ceramide residue, together with those ascribable to two sugar units. Furthermore, in the negative ion FAB-MS, besides the $[M-H]^-$ ion peaks at *m/z* 1109, characteristic fragment ion peaks were observed at *m/z* 1051 $[M-N(CH_3)_3]^-$, 1024 $[M-\text{choline}+H]^-$, 783 $[M-(\text{hexose and phosphocholine units})]^-$ and 620 $[M-(2\times\text{hexose}+\text{phosphocholine units})]^-$, suggesting that **2** is an amphoteric-type ceramide disaccharide in which an outer sugar moiety has a choline phosphate group (Fig. 2).

On HF degradation[4] followed by methanolysis with 7.5% methanolic HCl, **2** gave a fatty acid methyl ester and a long-chain base (LCB) together with a sugar unit. The fatty acid methyl ester produced was revealed to be methyl *n*-docasanoate by GC comparison with an authentic sample, and the sugar part was determined to be a galactopyranoside having D form according to the method of Hara *et al.*[5] On the other hand, the LCB was converted to an acetate, which was identified as D-*erythro*-(4*E*)-16-methylsphingenine triacetate by comparison of the ^{1}H-NMR and FAB-MS with those of an authentic sample obtained previously.[3]

The position of the glycosidic linkage was determined by two-dimensional shift correlation (^{1}H–^{13}C COSY) and nuclear Overhauser effect spectroscopy (NOESY). In

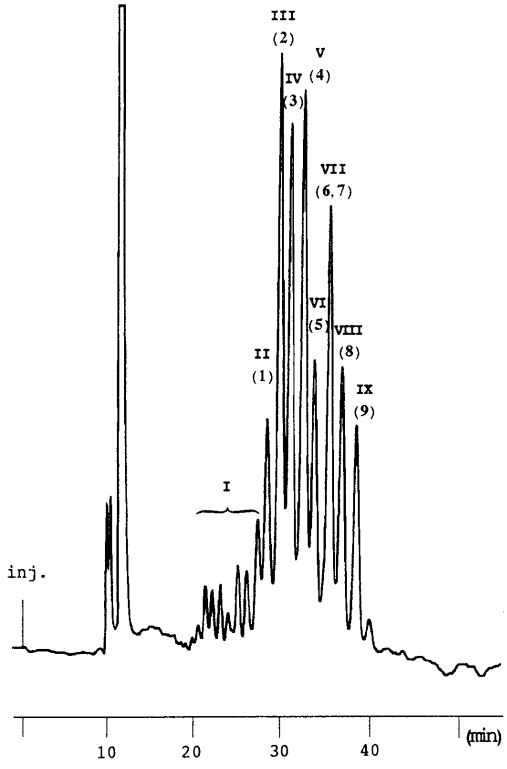

Fig. 1. HPLC Chromatogram of Fr. 6b′

Column, L-Column (10 × 250 mm) × 2; solvent, $CHCl_3$–MeOH–H_2O (9 : 20 : 2); flow rate, 2.5 ml/min; detector, RI.

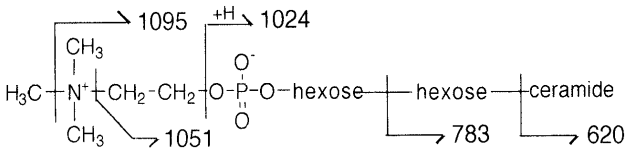

Fig. 2. Negative Ion FAB-MS of **2**

* To whom correspondence should be addressed.

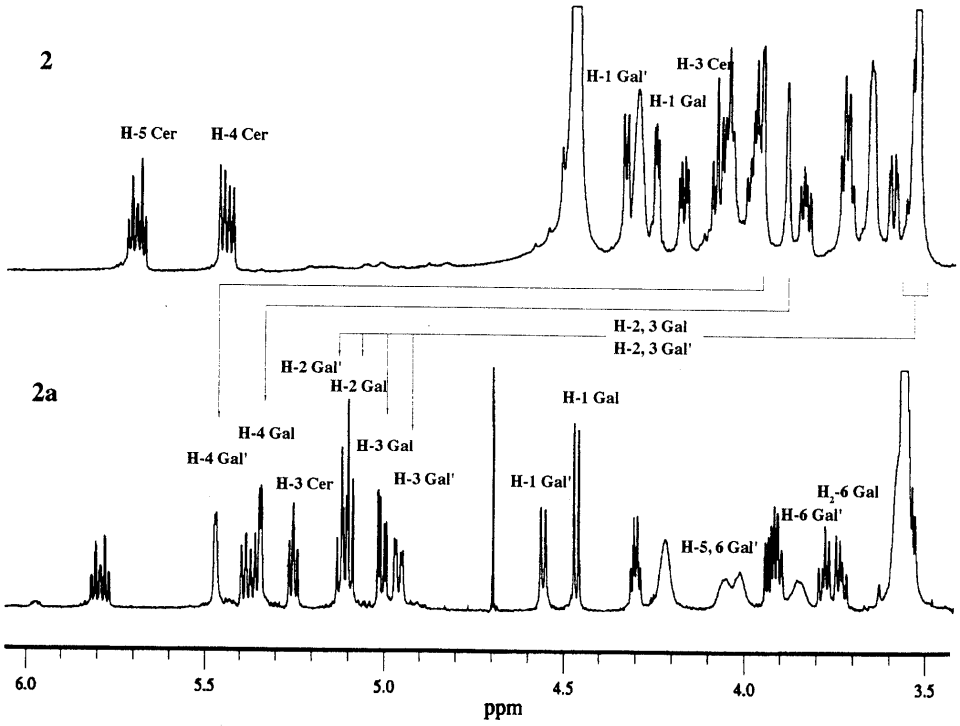

Fig. 3. ^{1}H-NMR Spectra of **2** ($CDCl_3$–CD_3OD) and **2a** ($CDCl_3$)

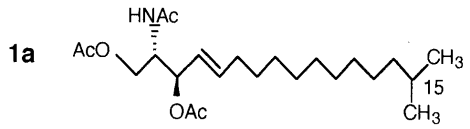

Fig. 4

the NOESY spectrum of **2**, an NOE correlation of H_2-1 of ceramide/H-1 of the inner galactose (Gal) and H_2-6 of Gal/H-1 of the outer galactose (Gal′) appeared. In addition, the carbon signals of C-5 and C-6 of Gal′ appeared as doublets owing to coupling with ^{31}P. These findings and the FAB-MS spectral data suggested that **2** has a 1→6 galactosyl linkage and that the location of the choline phosphate group is C-6 of Gal′.

The ^{1}H-NMR spectrum of a heptaacetate (**2a**) derived from **2** showed, when compared with that of **2**, remarkable downfield shifts of 2, 3 and 4-H of Gal and Gal′, in addition to the broad signals of H_2-6 of Gal′ coupled with ^{31}P (Fig. 3); the above suggestion was thus confirmed. Based on the coupling constants (each of H-1, $J=7.3$ Hz) and the chemical shifts (δ 104.2 and 104.5) of the anomeric centers, the mode of glycosidic linkages of the two galactose units is concluded to be β.[6]

From the information described above, compound **2** was concluded to be *N*-docosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-(4*E*)-16-methylheptadecasphingenine.

The ^{1}H- and ^{13}C-NMR spectra of compounds **1** and **3**—**9** were almost identical to that of **2**, showing doublets of C-5 and C-6 of sugar carbon signals as well as the presence of a choline phosphate and two sugar units, indicating that they are analogs of **2** consisting of a Galβ1–6Galβ1–Cer core with a different ceramide residue. The HF degradation of each compound followed by methanolysis in the same manner as described for **2** gave uniformly a methyl galactopyranoside and a fatty acid methyl ester. On direct comparison by GC with authentic samples,[3] the fatty acids were identified as *n*-docosanoate from **1**, **3**, and **4**, *n*-tricosanoate from **5** and **6**, and *n*-tetracosanoate from **7**, **8**, and **9**. Further, the LCB parts formed were converted into their acetates, which were identified as known LCB triacetates having D-*erythro*-(4*E*) configuration, namely, octadecasphingenine from **3** and **8**, 16-methylheptadecasphingenine from **5** and **7**, and 17-methyloctadecasphingenine from **4**, **6**, and **9**, by comparison with authentic samples.[1,3] The unknown LCB triacetate (**1a**) produced from **1** gave a quite similar ^{1}H-NMR spectrum to those of iso-type sphingenines, and in the FAB-MS, exhibited the $[M+Na]^+$ ion peak at 434 which was 14 mass units less than that of 16-methylheptadecasphingenine triacetate. From these observations, together with the optical rotation ($[\alpha]_D$ −14.3°), **1a** was concluded to be 15-methylhexadecasphingenine triacetate (Fig. 4), and hence the constitution of all the ceramide parts of compounds **1** and **3**—**9** were definitively established. The remaining structure of the sugar moiety was determined from the NMR spectral data. The detailed assignment of the proton and carbon signals arising from each substance revealed that all of them have the same carbohydrate linkage, Galβ1–6Galβ1–Cer, and that the phosphocholine group is attached to the C-6 position of Gal′.

On the basis of the results obtained above, the full structures of the nine compounds (**1**—**9**) are as shown in Fig. 5.

In conclusion, nine zwitterionic-type glycosphingolipids were obtained in pure form. All of them have a Galβ1–6Galβ1–Cer glycosidic linkage carrying a choline phosphate group attached to the C-6 position of the outer

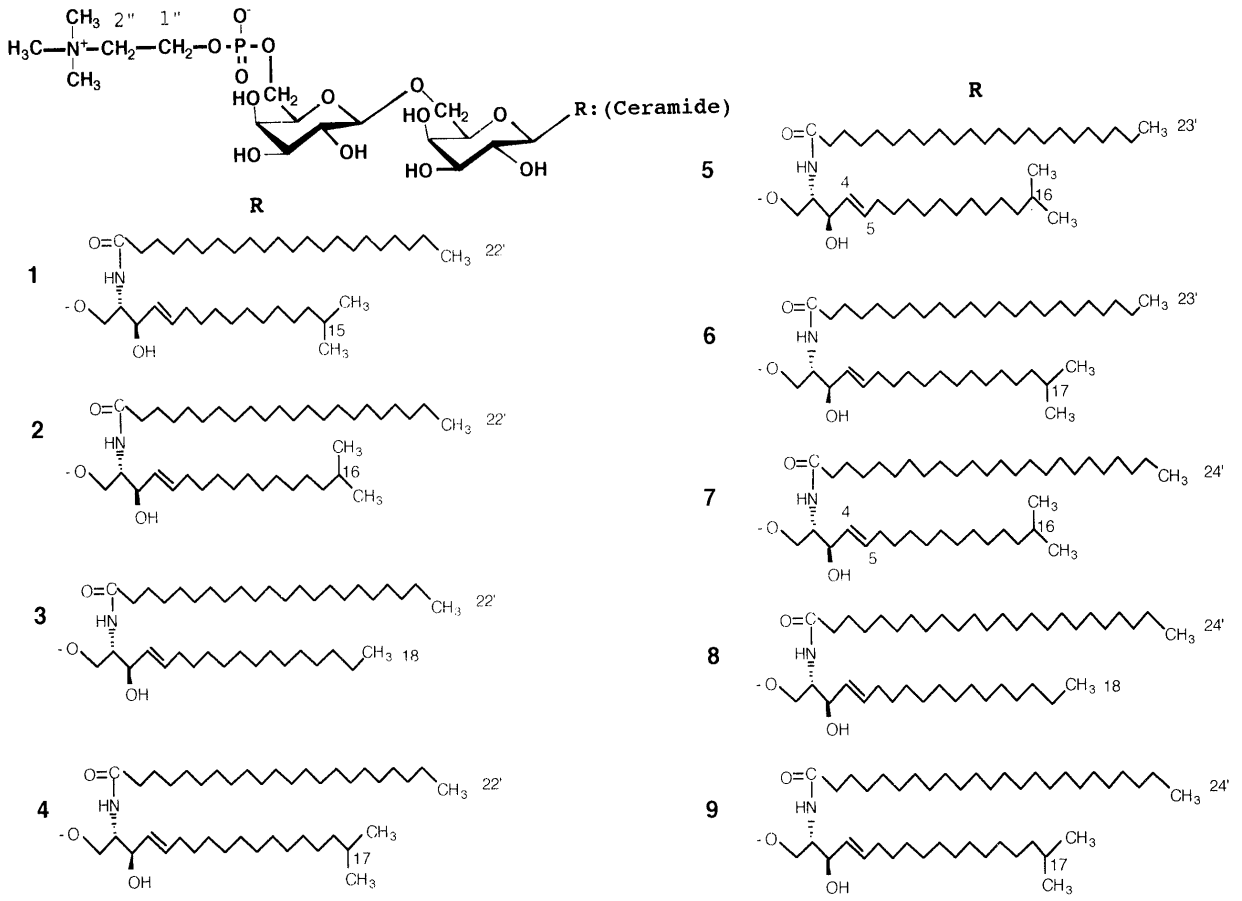

Fig. 5

galactose moiety. The fatty acid and LCB compositions of their ceramide units were very similar to those of MGCs characterized previously.[3] Although to date the function and role of these compounds remain unsolved, they may play physiologically important roles in the phylum organism, perhaps a source of choline in the nervous system and as intermediates in the transport of sodium and potassium ions. These compounds are expected to have unique biological activities.

Experimental

Details of the instruments and chromatographic conditions used throughout this work were the same as described in the previous papers.[1,3] The specific rotations were taken at 24—26 °C. The NMR spectra were recorded on a JEOL JNM GSX 400 instrument at 400 MHz (^{1}H) and 100 MHz (^{13}C) at a probe temperature of 35 °C. The NOESY spectrum was obtained using a mixing time of 120 ms.

Isolation of Glycosphingolipids The MeOH extractives (75 g) of the dried body walls (1 kg) of the commercial crude drug "Jiryu" (*P. asiatica*) (purchased from Tochimoto Tenkaido) were treated with $CHCl_3$–MeOH–H_2O (1 : 2 : 1, 900 ml), and the lower phase was concentrated to give a brown extractive (27 g). This was subjected to silica-gel and Cosmosil 75C_{18}-OPN column chromatographies with various solvent systems as described in the previous paper[3] to give fractions 5 (4 g) and 6 (2 g). Fraction 6 (2.0 g) (positive to Dittmer–Lester's reagent)[2] was applied to an Asahipack GS-320 column (7.6 mm × 50 cm, Asahi Chemical Industry Co., Ltd.) and eluted with MeOH to provide two fractions (crude glycosphingolipid fractions), fr. 6a (1.5 g) and fr. 6b (0.4 g). The latter was subjected to HPLC with an Asahipack GS-310 column (7.6 mm × 50 cm, Asahi Chemical Industry Co., Ltd.) by using $CHCl_3$–MeOH (3 : 7) as the eluent, to separate fr. 6b′ (240 mg). Fraction 6b′ was fractionated by HPLC (L-column, Chemicals Inspection & Testing Institute; 5 μm, 1 cm × 25 cm × 2; solvent, $CHCl_3$–MeOH–H_2O, 9 : 20 : 2) to give frs. I—IX. Each of frs. II—IX obtained was further separated by conventional HPLC (solvent, $CHCl_3$–MeOH–H_2O, 9 : 20 : 2) to give **1** (16 mg) from fr. II, **2** (30 mg) from fr. III, **3** (20 mg) from fr. IV, **4** (10 mg) from fr. V, **8** (16 mg) from fr. VIII, and **9** (12 mg) from fr. IX. Recycling HPLC was conducted with a Waters recycling valve (solvents; $CHCl_3$–MeOH–H_2O, 9 : 20 : 2) to give **5** (8 mg) from fr. VI (4 cycles), and **6** (8 mg) and **7** (11 mg) from fr. VII (8 cycles).

N-Docosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-*β*-D-galactopyranosyl-(1→6)-*β*-D-galactopyranosyl]-(4*E*)-15-methylhexadecasphingenine (**1**): Powder, mp 175—178 °C. $[\alpha]_D$ +2.5° (*c* = 1.6, $CHCl_3$–MeOH, 1 : 1). Positive ion FAB-MS *m/z*: 1097 $(M+H)^+$. Negative ion FAB-MS *m/z* (%): 1095 (35) $(M-H)^-$, 1081 (27), 1037 (20), 1010 (30), 769(10), 606 (10), 297 (100).

N-Docosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-*β*-D-galactopyranosyl-(1→6)-*β*-D-galactopyranosyl]-(4*E*)-16-methylheptadecasphingenine (**2**): Powder, mp 173—176 °C. $[\alpha]_D$ +2.4° (*c* = 1.5, $CHCl_3$–MeOH, 1 : 1). Positive ion FAB-MS *m/z*: 1111 $(M+H)^+$. Negative ion FAB-MS *m/z* (%): 1109 (64) $(M-H)^-$, 1095 (36), 1051 (30), 1024 (46), 783 (16), 620 (12), 297 (100).

N-Docosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-*β*-D-galactopyranosyl-(1→6)-*β*-D-galactopyranosyl]-(4*E*)-octadecasphingenine (**3**): Powder, mp 174—178 °C. $[\alpha]_D$ +2.9° (*c* = 1.8, $CHCl_3$–MeOH, 1 : 1). Positive ion FAB-MS *m/z*: 1111 $(M+H)^+$. Negative ion FAB-MS *m/z* (%): 1109 (42) $(M-H)^-$, 1095 (28), 1051 (21), 1024 (32), 782 (16), 620 (14), 297 (100).

N-Docosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-*β*-D-galactopyranosyl-(1→6)-*β*-D-galactopyranosyl]-(4*E*)-17-octadecasphingenine (**4**): Powder, mp 173—176 °C. $[\alpha]_D$ +2.8° (*c* = 1.0, $CHCl_3$–MeOH, 1 : 1). Positive ion FAB-MS *m/z*: 1125 $(M+H)^+$. Negative ion FAB-MS *m/z* (%): 1123 (40) $(M-H)^-$, 1109 (22), 1065 (14), 1038 (30), 796 (10), 634 (10), 297 (100).

N-Tricosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-*β*-D-galactopyranosyl-(1→6)-*β*-D-galactopyranosyl]-(4*E*)-16-methylheptadecasphingenine (**5**): Powder, mp 175—178 °C. $[\alpha]_D$ +3.0° (*c* = 0.8, $CHCl_3$–MeOH, 1 : 1). Positive ion FAB-MS *m/z*: 1125 $(M+H)^+$. Negative ion FAB-MS *m/z* (%): 1123 (42) $(M-H)^-$, 1109 (21), 1065 (14), 1038 (30), 796 (12), 634 (10), 297 (100).

N-Tricosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-(4*E*)-17-methyloctadecasphingenine (**6**): Powder, mp 173—176 °C. $[\alpha]_D$ +2.8° (c=0.8, $CHCl_3$–MeOH, 1 : 1). Positive ion FAB-MS m/z: 1139 $(M+H)^+$. Negative ion FAB-MS m/z (%): 1137 (50) $(M-H)^-$, 1123 (22), 1079 (16), 1052 (32), 810 (12), 648 (20), 446 (100).

N-Tetracosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-β-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-(4*E*)-16-methylheptadecasphingenine (**7**): Powder, mp 173—176 °C. $[\alpha]_D$ +2.8° (c=1.1, $CHCl_3$–MeOH, 1 : 1). Positive ion FAB-MS m/z: 1139 $(M+H)^+$.

Table 1. ^{1}H-NMR Spectral Data of **1**—**9** (400 MHz)

No.		**2**	**1**	**3**	**4**	**5**
Cer	1	3.60 (dd, 7.0, 10.0)	3.61 (dd, 7.0, 10.0)	3.62 (dd, 7.0, 10.0)	3.61 (dd, 7.0, 10.0)	3.60 (dd, 7.0, 10.0)
		4.17 (dd, 4.0, 10.0)	4.16 (dd, 4.0, 10.0)	4.16 (dd, 4.0, 10.0)	4.17 (dd, 4.0, 10.0)	4.16 (dd, 4.0, 10.0)
	2	3.96 (m)	3.98 (m)	3.96 (m)	3.98 (m)	3.96 (m)
	3	4.08 (t, 7.0)	4.07 (t, 7.0)	4.08 (t, 7.0)	4.08 (t, 7.0)	4.09 (t, 7.0)
	4	5.44 (dd, 7.0, 15.0)	5.45 (dd, 7.0, 15.0)	5.44 (dd, 7.0, 15.0)	5.45 (dd, 7.0, 15.0)	5.45 (dd, 7.0, 15.0)
	5	5.70 (ddd, 7.0, 8.0, 15.0)	5.70 (ddd, 7.0, 8.0, 15.0)	5.69 (ddd, 7.0, 8.0, 15.0)	5.69 (ddd, 7.0, 8.0, 15.0)	5.69 (ddd, 7.0, 8.0, 15.0)
	6	2.22 (2H, m)	2.03 (2H, m)	2.13 (2H, m)	2.03 (2H, m)	2.02 (2H, m)
–CH–$(CH_3)_2$		1.54[a)]	1.53[a)]	—	1.53[a)]	1.54[a)]
	–CH_3	0.87 (6H, d, 7.0)	0.87 (6H, d, 7.0)	0.86 (3H, t, 7.0)	0.87 (6H, d, 7.0)	0.87 (6H, d, 7.0)
FA	2′	2.28 (2H, br t, 7.0)	2.18 (2H, br t, 7.0)	2.19 (2H, br t, 7.0)	2.18 (2H, br t, 7.0)	2.17 (2H, br t, 7.0)
	–CH_3	0.88 (3H, t, 7.0)	0.89 (3H, t, 7.0)	0.88 (3H, t, 7.0)	0.89 (3H, t, 7.0)	0.89 (3H, t, 7.0)
Gal	1	4.24 (d, 7.3)	4.25 (d, 7.3)	4.24 (d, 7.3)	4.25 (d, 7.3)	4.25 (d, 7.3)
	2	3.52	3.52	3.51	3.52	3.51
	3	3.52	3.52	3.51	3.52	3.51
	4	3.88 (dd, 1.0, 3.0)	3.88 (dd, 1.0, 3.0)	3.88 (dd, 1.0, 3.0)	3.88 (dd, 1.0, 3.0)	3.88 (dd, 1.0, 3.0)
	5	3.84 (td, 1.0, 6.0)	3.84 (td, 1.0, 6.0)	3.84 (td, 1.0, 6.0)	3.85 (td, 1.0, 6.0)	3.86 (td, 1.0, 6.0)
	6	3.73 (m)	3.73 (m)	3.72 (m)	3.73 (m)	3.71 (m)
		3.98 (m)	3.99 (m)	3.97 (m)	3.98 (m)	3.97 (m)
Gal′	1	4.34 (d, 7.3)	4.32 (d, 7.3)	4.32 (d, 7.3)	4.33 (d, 7.3)	4.33 (d, 7.3)
	2	3.52	3.52	3.51	3.52	3.51
	3	3.52	3.52	3.51	3.52	3.51
	4	3.95 (dd, 1.0, 3.0)	3.94 (dd, 1.0, 3.0)	3.94 (dd, 1.0, 3.0)	3.94 (dd, 1.0, 3.0)	3.96 (dd, 1.0, 3.0)
	5	4.04	4.04	4.04	4.04	4.04
	6	3.71 (m)	3.72 (m)	3.71 (m)	3.72 (m)	3.71 (m)
		4.04	4.02	4.04	4.04	4.04
Cho	1″	4.30 (2H, m)	4.30 (2H, m)	4.29 (2H, m)	4.29 (2H, m)	4.29 (2H, m)
	2″	3.65 (2H, m)	3.65 (2H, m)	3.63 (2H, m)	3.64 (2H, m)	3.63 (2H, m)
	–CH_3	3.23 (9H, s)	3.23 (9H, s)	3.23 (9H, s)	3.23 (9H, s)	3.22 (9H, s)

No.		**6**	**7**	**8**	**9**
Cer	1	3.61 (dd, 7.0, 10.0)	3.61 (dd, 7.0, 10.0)	3.61 (dd, 7.0, 10.0)	3.62 (dd, 7.0, 10.0)
		4.17 (dd, 4.0, 10.0)	4.16 (dd, 4.0, 10.0)	4.17 (dd, 4.0, 10.0)	4.17 (dd, 4.0, 10.0)
	2	3.96 (m)	3.97 (m)	3.97 (m)	3.98 (m)
	3	4.08 (t, 7.0)	4.08 (t, 7.0)	4.08 (t, 7.0)	4.08 (t, 7.0)
	4	5.46 (dd, 7.0, 15.0)	5.45 (dd, 7.0, 15.0)	5.45 (dd, 7.0, 15.0)	5.45 (dd, 7.0, 15.0)
	5	5.71 (ddd, 7.0, 8.0, 15.0)	5.70 (ddd, 7.0, 8.0, 15.0)	5.69 (ddd, 7.0, 8.0, 15.0)	5.70 (ddd, 7.0, 8.0, 15.0)
	6	2.03 (2H, m)	2.03 (2H, m)	2.03 (2H, m)	2.04 (2H, m)
–CH–$(CH_3)_2$		1.54[a)]	1.53[a)]	—	1.54[a)]
	–CH_3	0.87 (6H, d, 7.0)	0.87 (6H, d, 7.0)	0.88 (2H, t, 7.0)	0.87 (6H, d, 7.0)
FA	2′	2.16 (2H, br t, 7.0)	2.17 (2H, br t, 7.0)	2.18 (2H, br t, 7.0)	2.17 (2H, br t, 7.0)
	–CH_3	0.88 (3H, t, 7.0)	0.88 (3H, t, 7.0)	0.88 (3H, t, 7.0)	0.89 (3H, t, 7.0)
Gal	1	4.24 (d, 7.3)	4.24 (d, 7.3)	4.24 (d, 7.3)	4.25 (d, 7.3)
	2	3.52	3.52	3.51	3.52
	3	3.52	3.52	3.51	3.52
	4	3.88 (dd, 1.0, 3.0)	3.88 (dd, 1.0, 3.0)	3.88 (dd, 1.0, 3.0)	3.88 (dd, 1.0, 3.0)
	5	3.84 (td, 1.0, 6.0)	3.84 (td, 1.0, 6.0)	3.84 (td, 1.0, 6.0)	3.84 (td, 1.0, 6.0)
	6	3.72 (m)	3.72 (m)	3.73 (m)	3.72 (m)
		3.97 (m)	3.97 (m)	3.98 (m)	3.98 (m)
Gal′	1	4.33 (d, 7.3)	4.33 (d, 73)	4.33 (d, 7.3)	4.33 (d, 7.3)
	2	3.52	3.52	3.51	3.52
	3	3.52	3.52	3.51	3.52
	4	3.94 (dd, 1.0, 3.0)	3.94 (dd, 1.0, 3.0)	3.98 (dd, 1.0, 3.0)	3.93 (dd, 1.0, 3.0)
	5	4.04	4.04	4.04	4.03
	6	3.72 (m)	3.72 (m)	3.72 (m)	3.71 (m)
		4.04	4.04	4.04	4.03
Cho	1″	4.29 (2H, m)	4.29 (2H, m)	4.29 (2H, m)	4.30 (2H, m)
	2″	3.64 (2H, m)	3.64 (2H, m)	3.64 (2H, m)	3.64 (2H, m)
	–CH_3	3.22 (9H, s)	3.22 (9H, s)	3.23 (9H, s)	3.26 (9H, s)

Coupling constants (J) in Hz are given in parentheses. Cer, ceramide unit; FA, fatty acid unit; Gal, galactose unit; Cho, phosphocholine unit. *a*) The signal was observed as a septet (J=7.0 Hz).

Table 2. ^{13}C-NMR Spectral Data of **1**—**9** (100 MHz)

No.		**2**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Cer	1	69.7	69.7	69.7	69.7	69.6	69.7	69.7	69.8	69.8
	2	53.3	54.0	54.0	54.1	53.9	54.0	54.1	54.2	54.2
	3	72.2	72.3	72.3	72.3	72.2	72.3	72.2	72.4	72.4
	4	130.0	130.2	130.2	130.2	129.9	130.1	130.1	130.4	130.4
	5	134.7	134.7	134.7	134.7	134.7	134.7	134.7	134.8	134.8
	6	32.8	32.9	32.9	32.9	32.8	32.9	33.0	33.0	32.8
	$-CH_3$	22.8	23.2	14.2	23.1	23.0	22.8	22.8	14.3	23.2
FA	1′	175.0	175.2	175.1	175.2	175.0	175.0	175.0	175.0	175.3
	$-CH_3$	14.2	14.3	14.2	14.2	14.2	14.2	14.2	14.3	14.3
Gal	1	104.5	104.6	104.6	104.6	104.4	104.5	104.5	104.7	104.7
	2	71.7	71.9	71.8	71.9	71.8	71.8	71.8	72.0	72.0
	3	73.8	74.0	73.9	74.0	73.8	73.9	73.9	74.1	74.1
	4	68.9	69.1	69.0	69.1	68.9	69.0	69.0	69.2	69.2
	5	74.3	74.6	74.5	74.5	74.3	74.4	74.4	74.7	74.6
	6	68.6	68.7	68.7	68.7	68.6	68.7	68.7	68.8	68.8
Gal′	1	104.2	104.4	104.4	104.4	104.2	104.3	104.3	104.5	104.5
	2	71.9	72.0	72.0	72.0	71.9	72.0	72.0	72.2	72.2
	3	73.8	74.0	73.9	74.0	73.8	73.9	73.9	74.1	74.1
	4	68.9	69.1	69.0	69.1	68.9	69.0	68.9	69.2	69.2
	5	74.5[a]	74.7[a]	74.6[a]	74.7[a]	74.5[a]	74.6[a]	74.6[a]	74.8[a]	74.8[a]
	6	65.1[a]	65.2[a]	65.1[a]	65.2[a]	65.1[a]	65.1[a]	65.1[a]	65.3[a]	65.3[a]
Cho	1″	67.0[a]	67.1[a]	67.0[a]	67.1[a]	67.0[a]	67.1[a]	67.1[a]	67.2[a]	67.2[a]
	2″	59.7[a]	59.8[a]	59.7[a]	59.8[a]	59.6[a]	59.7[a]	59.7[a]	59.9[a]	59.9[a]
	$-CH_3$	54.6	54.6	54.6	54.6	54.5	54.6	54.6	54.6	54.6

a) The signals marked each appeared as a doublet, J=5—8 Hz, by coupling with ^{31}P.

Negative ion FAB-MS *m/z* (%): 1137 (44) $(M-H)^-$, 1123 (34), 1079 (24), 1052 (36), 810 (16), 648 (20), 297 (100).

N-Tetracosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-*β*-D-galactopyranosyl-(1→6)-*β*-D-galactopyranosyl]-(4*E*)-octadecasphingenine (**8**): Powder, mp 174—178 °C. $[\alpha]_D$ +2.4° (c=1.6, $CHCl_3$–MeOH, 1 : 1). Positive ion FAB-MS *m/z*: 1139 $(M+H)^+$. Negative ion FAB-MS *m/z* (%): 1137 (45) $(M-H)^-$, 1123 (32), 1079 (24), 1052 (35), 810 (20), 648 (22), 297 (100).

N-Tetracosanoyl-1-*O*-[6-*O*-(2-trimethylammonioethoxy)phosphinate-*β*-D-galactopyranosyl-(1→6)-*β*-D-galactopyranosyl]-(4*E*)-17-methyloctadecasphingenine (**9**): Powder, mp 175—178 °C. $[\alpha]_D$ +2.5° (c=1.2, $CHCl_3$–MeOH, 1 : 1). Positive ion FAB-MS *m/z*: 1153 $(M+H)^+$. Negative ion FAB-MS *m/z* (%): 1151 (98) $(M-H)^-$, 1137 (54), 1093 (40), 1066 (64), 824 (20), 662 (22), 297 (100). ^{1}H-NMR and ^{13}C-NMR spectral data of **1**—**9** ($CDCl_3$–CD_3OD, 1 : 1) δ: Tables 1 and 2.

Acetylation of 2 Compound **2** (5 mg) was acetylated with acetic anhydride-pyridine (0.3 ml, 1 : 1) at room temperature for 1 d to give **2a** (6 mg). **2a**: ^{1}H-NMR (CD_3OD–$CDCl_3$, 1 : 1, 400 MHz) δ: 1.96, 1.97, 2.05, 2.06, 2.06, 2.14, 2.15 (21H, s, $COCH_3$). Cer unit: 3.55 (dd, J=7.0, 9.6 Hz, H_2-1), 3.93 (dd, J=5.0, 9.6 Hz, H_2-1), 4.30 (ddd, J=5.0, 7.0, 7.0 Hz, H-2), 5.25 (dd, J=7.0, 7.0 Hz, H-3), 5.37 (dd, J=7.0, 15.0 Hz, H-4), 5.79 (ddd, J=7.0, 7.0, 15.0 Hz, H-5), Gal unit: 4.47 (d, J=8.0 Hz, H-1), 5.10 (dd, J=8.0, 8.0 Hz, H-2), 5.01 (dd, J=3.0, 8.0 Hz, H-3), 5.34 (dd, J=0.7, 3.0 Hz, H-4), 3.91 (ddd, J=0.7, 6.2, 5.5 Hz, H-5), 3.76 (m, H_2-6), Gal′ unit: 4.56 (d, J=8.0 Hz, H-1), 5.12 (dd, J=8.0, 8.0 Hz, H-2), 4.96 (dd, J=3.0, 8.0 Hz, H-3), 5.47 (dd, J=3.0, 1.0 Hz, H-4), 4.02 (m, H-5), 3.84, 4.06 (m, H_2-6), Cho unit: 4.22 (H_2-1″), 3.58 (H_2-2″).

Fatty Acid Analysis Five milliliters of HF (47%) was added to each sample (3—9 mg) in 1 ml of $CHCl_3$–MeOH (1 : 10) at 0 °C in a polyethylene tube fitted with a cap, and the reaction mixture was allowed to stand at 0 °C for 36 h. The pH of the solution was then brought to 6.5 with a saturated LiOH solution. The precipitate was filtered off and the filtrate was passed through a Sephadex LH-20 column using MeOH to give a product (2—8 mg). This product was heated with 7.5% methanolic HCl (0.5 ml) at 90 °C for 1 h. The fatty acid methyl ester formed was extracted with *n*-hexane. The *n*-hexane layer was analyzed by GC (fused silica capillary column bonded MPS-50 (Quadrex), 0.25 mm × 50 m; column temperature, 230 °C (hold 12 min)→240 °C at 1 °C/min; carrier gas, N_2 at 2.0 kg/cm^2), t_R (min): 21.44 (methyl *n*-docosanoate) from **1**, **2**, **3**, and **4**, 26.02 (methyl *n*-tricosanoate) from **5** and **6**, and 32.39 (methyl *n*-tetracosanoate) from **7**, **8**, and **9**. Each peak substance was identified as the corresponding methyl ester by EI-MS comparison with an authentic sample.

Analysis of the Sugar Residue The MeOH layer obtained above was neutralized with Ag_2CO_3. After centrifugation, the supernatant was evaporated to dryness under a nitrogen stream at room temperature. The residue was subjected to column chromatography on silica gel using $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5) to give a methyl glycoside and a LCB. The former was treated with *N*-trimethylsilylimidazole and the derivative was analyzed by GC according to the method described in the preceding paper.[3] The peaks (t_R; 11.0, 12.2, 12.6, 13.7 min) were identical with those of TMS derivatives obtained from methyl galactoside.

Analysis of the LCB Parts Each LCB part of **1**—**9** was acetylated with acetic anhydride-pyridine (0.3 ml, 1 : 1) at room temperature for 1 d to give a product. The product was identified as a known sphingenine unit by comparisons of FAB-MS and ^{1}H-NMR spectral data with those of authentic samples[3]: D-*erythro*-16-methylheptadecasphingenine (from **2**, **5**, **7**). D-*erythro*-17-methyloctadecasphingenine (from **4**, **6**, **9**). D-*erythro*-octadecasphingenine (from **3**, **8**). D-*erythro*-15-methylhexadecasphingenine triacetate (**1a**). **1a**: $[\alpha]_D$ −14.3° (c=0.1, $CHCl_3$). Positive ion FAB-MS *m/z*: 434 $(M+Na)^+$. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (6H, d, J=7.0 Hz, H_3-16 and 17), 1.94, 1.96, 2.06 (3H, s, $COCH_3$), 3.98 (dd, J=4.0, 12.0 Hz, H-1), 4.16 (dd, J=6.0, 12.0 Hz, H-1), 4.33 (m, H-2), 5.29 (t, J=7.0 Hz, H-3), 5.39 (m, H-4), 5.65 (m, NH), 5.78 (ddd, J=7.0, 8.0, 15.0 Hz, H-5).

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