Cerebrosides of Frog Brain. Structure of the Ceramide Part of the Cerebrosides

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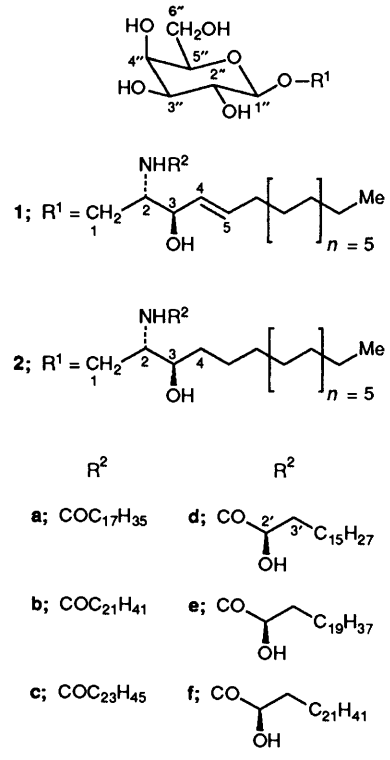
Twelve cerebrosides were isolated from the brain tissues of the bullfrog (*Rana catesbeiana*) and they were characterised as 1-*o*-β-D-galactopyranosyl ceramides. On the basis of chemical and spectral evidence, the ceramide parts of six of them were found to be composed of a sphingosine as a long-chain base and six fatty acids consisting of $C_{18:0}$, $C_{22:1}$ and $C_{24:1}$ acids and their 2-hydroxy derivatives. The ceramide parts of the others were found to be composed of a dihydrosphingosine and the six fatty acids. The configurations at C-2 and C-3 of the two long-chain bases were determined to be *S* and *R*, respectively, on the basis of the NMR spectra of their acetates and the exciton chiralities of the benzoate-benzamide derivatives. Further, the chiral centre of the three 2-hydroxy fatty acids was determined to be *R* in all cases by means of CD measurements. A different distribution of the cerebrosides was seen among the hemisphere, diencephalon and mixed tissue from the optic lobe, cerebellum and medulla oblongata of the brain.

Cerebrosides and gangliosides in the brain and other nervous systems of animals have a long-chain base moiety. (2*S*,3*R*)-2-Aminooctadecane-1,3-diol and (2*S*,3*R*,4*E*)-2-aminooctadec-4-ene-1,3-diol (C_{18}) and its homologues (C_{14} and C_{20}) are established as the long-chain bases of these materials of mammals and other higher vertebrates.[1] Also, the presence of these long-chain bases in amphibian and fish tissues has been proposed based on GLC and GLC-MS analyses.[1] However, further studies on the structures of the long-chain bases have not been performed. Recently, we isolated three gangliosides from the brain of bullfrog (*Rana catesbeiana* Shaw) and determined their structure, except for the stereochemistry of the long-chain base components.[2] The compositions of the long-chain bases, fatty acids and sugars in the cerebroside mixtures obtained from three species of frogs were analysed by means of GLC and GLC-MS.[3–5] We have now elucidated the structure of the cerebrosides present in the brain tissues of the bullfrog and the distribution of these compounds in brain hemisphere, diencephalon and mixed tissue from the optic lobe, cerebellum and medulla oblongata of the brain.

Results and Discussion

Isolation of Cerebrosides.—Following the procedure used for gangliosides,[2] fresh brain tissues of the bullfrog (*R. catesbeiana*) were treated to give neutral and acidic glycolipids; the latter gave three gangliosides. Repeated silica gel column chromatography of the neutral glycolipids gave two fractions, A and B. Each of the fractions A and B showed a single spot on normal-phase TLC, but exhibited six peaks on reversed-phase HPLC. Their secondary ionization (SI) mass spectra exhibited two sets of six molecular ion peaks at *m/z* 728, 730, 782, 784, 810 and 812 (for A) and at *m/z* 744, 746, 798, 800, 826 and 828 (for B). By means of reversed-phase HPLC on a preparative scale, six cerebrosides, **1a–1c** and **2a–2c**, were isolated from A, and six cerebrosides, **1d–1f** and **2d–2f**, were obtained from B. The twelve cerebrosides each exhibited a single peak on reversed-phase HPLC.

Structure of the Cerebrosides.—The structures of six of the twelve cerebrosides were determined to be 1-*o*-β-D-galactopyranosides of sphingosine-type ceramides, as shown in structures **1a–1f**. The structures of the other six cerebrosides were determined to be 1-*o*-β-D-galactopyranosides of dihydrosphingosine-type ceramides, as shown in structures **2a–2f**.



Cerebroside **1a** showed the molecular ion peak at *m/z* 728 $[M + H]^+$ in the SI mass spectrum. Compound **1a**, on hydrolysis with acid by the Gaver–Sweeley method,[6] yielded a fatty acid methyl ester (FAME) and a long-chain base (LCB) together with the methyl galactopyranoside **3**. The FAME was indicated to be methyl octadecanoate by GLC-MS analysis. The LCB was characterised as 2-aminooctadec-4-ene-1,3-diol by GLC-MS analysis of its acetate. Thus, cereboride **1a** was found to be 1-β-D-galactopyranosyloxy-2-(octadecanoylamino)-octadec-4-en-3-ol, which was confirmed by the ^1H and ^{13}C NMR spectra given in Tables 1 and 2.

Cerebrosides **1b** and **1c** showed molecular ion peaks at *m/z* 782 and 810 $[M + H]^+$ in their SI mass spectra, respectively and exhibited ^1H and ^{13}C NMR spectra similar to those of cerebroside **1a** (Tables 1 and 2). Hydrolysis of compounds **1b**

Table 1 ^{1}H NMR spectra[a] of cerebrosides **1a–1f** and **2a–2f**. Coupling constants are given in parentheses

	Compounds											
H[b]	**1a**	**1b**	**1c**	**1d**	**1e**	**1f**	**2a**	**2b**	**2c**	**2d**	**2e**	**2f**
1_a	3.61dd (3, 10)	3.60dd (3, 10)	3.60dd (3, 10)	3.74dd (3, 10)	3.73dd (3, 11)	3.74dd (3, 10)	3.57dd (3, 11)	3.56dd (3, 11)	3.56dd (3, 10)	3.70dd (3, 11)	3.71dd (3, 11)	3.71dd (3, 10)
1_b	4.17dd (5, 10)	4.17dd (5, 10)	4.17dd (5, 10)	4.08dd (6, 10)	4.07dd (5, 11)	4.07dd (6, 10)	4.16dd (5, 11)	4.16dd (5, 11)	4.17dd (5, 10)	4.10dd (6, 11)	4.10dd (6, 11)	4.11dd (6, 11)
2	4.09m	4.08m	4.09m	4.00m	4.01m	4.01m	3.94m	3.95m	3.94m	3.96m	3.96m	3.96m
3	4.09t (7)	4.10t (7)	4.10t (7)	4.12t (7)	4.11t (7)	4.13t (7)	3.61td (6, 7)	3.60td (6, 7)	3.60td (6, 7)	3.62td (6, 7)	3.61td (6, 7)	3.62td (6, 7)
4	5.45dd (7, 15)	5.46dd (7, 15)	5.45dd (7, 15)	5.44dd (7, 16)	5.45dd (7, 15)	5.44dd (7, 16)	1.45m	1.44m	1.45m	1.43m	1.42m	1.42m
5	5.70td (6, 15)	5.71td (6, 15)	5.70td (6, 15)	5.71td (6, 16)	5.72td (6, 15)	5.72td (6, 15)	1.26[c]	1.26	1.26	1.26	1.26	1.26
NH	7.42d (9)	7.43d (9)	7.43d (9)	7.42d (9)	7.43d (9)	7.42d (9)	7.50d (9)	7.52d (9)	7.53d (9)	7.53d (9)	7.52d (9)	7.50d (9)
CH_2C=	2.03q (6)	2.02q (6)	2.03q (6)	2.02q (6)	2.03q (6)	2.04q (6)	1.26[c]	2.01q (6)	2.03 (6)	1.26	2.03q (6)	2.03q (6)
CH_2[c]	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26	1.26
Me[d]	0.89t (6)	0.89t (6)	0.89t (6)	0.89t (6)	0.89t (6)	0.89t (6)	0.89t (6)	0.89t (6)	0.89t (6)	0.89t (6)	0.89t (6)	0.89t (6)
2′	2.19t (6)	2.20t (6)	2.19t (6)	4.01dd (6)	4.02dd (4, 8)	4.01dd (4, 8)	4.01dd (4, 8)	2.20t (6)	2.20t (6)	4.02dd (4, 8)	4.03dd (4, 8)	4.05dd (4, 8)
3′	1.26[c]	1.26	1.26	1.56m, 1.76m	1.57m, 1.76m	1.56m, 1.76m	1.26	1.26	1.26	1.61m, 1.75m	1.62m, 1.76m	1.62m, 1.76m
CH=CH		5.34t[e] (6)	5.35t (5)		5.34t (5)	5.35t (6)		5.34t (6)	5.34t (6)		5.35t (6)	5.35t (6)

Galactopyranose moieties of the compounds[f]

1″	2″	3″	4″	5″	$6''_a$	$6''_b$
4.40–4.22d (7)	3.50–3.51dd (7, 9)	3.48–3.49dd (2–3, 9)	3.88–3.89d (2–3)	3.49–3.50dd (5–6, 6)	3.81–3.82dd (6, 12)	3.73–3.75dd (5–6, 12)

[a] Obtained in $CDCl_3$–CD_3OD (2:1). [b] Proton signals were assigned by the homonuclear ^{1}H-^{1}H shift-correlation spectra of cerebrosides **1c**, **1f**, **2c** and **2f**. [c] Proton signal for methylene groups of fatty acids and long-chain bases appeared at δ 1.26 (br s). [d] Proton signal for methyl group of fatty acids overlapped with that of the methyl group of long-chain bases. [e] Although the ^{1}H NMR spectra of the compounds showed the coupling constants (*J* 5–6 Hz) due to a *Z*-double bond [18] and the mass spectra of the FAMEs obtained from cerebrosides **1b**, **1c**, **2b**, and **2c** were identical with those of authentic methyl (*Z*)-docos-13-enoate and methyl (*Z*)-tetracos-15-enoate, the position of the double bond in the FAMEs could not be established due to lack of samples for chemical degradation. [f] Figures shown in this Table indicate the ranges of the chemical shifts and coupling constants in the ^{1}H NMR spectra of the compounds.

Table 2 ^{13}C NMR spectra[a] of cerebrosides **1a–1f** and **2a–2f**

	Compounds											
C[b]	**1a**	**1b**	**1c**	**1d**	**1e**	**1f**	**2a**	**2b**	**2c**	**2d**	**2e**	**2f**
1	68.5	68.5	68.5	68.3	68.5	68.5	68.4	68.3	68.5	68.2	68.2	68.1
2	53.0	52.8	52.9	52.8	53.0	52.8	52.9	52.9	53.0	52.9	52.8	52.9
3	71.7	71.8	71.7	71.7	71.7	71.7	70.8	70.7	70.8	70.8	70.8	70.8
4	128.8	128.7	128.8	128.5	128.6	128.5	25.6	25.4	25.6	25.4	25.6	25.6
5	134.0	134.0	134.1	134.0	134.0	134.1	[c]	[c]	[c]	[c]	[c]	[c]
Me	13.6	13.6	13.6	13.6	13.6	13.6	13.7	13.6	13.7	13.6	13.6	13.6
CH_2C=	26.9	26.9, 32.1	26.8, 31.8	26.9	26.9	26.9, 32.0	[c]	26.9, 31.6	26.9, 31.6	[c]	26.9, 32.0	26.8, 31.6
CO	174.3	174.3	174.3	175.5	175.6	175.7	174.2	174.3	174.2	175.6	175.7	175.6
2′	36.2	36.2	36.2	71.8	71.9	71.8	36.1	36.2	36.2	71.8	71.8	71.8
3′	[c]	[c]	[c]	34.1	34.1	34.2	[c]	[c]	[c]	34.2	34.1	34.2
CH=CH	[d]	129.6	129.6	[d]	129.6	129.6	[d]	129.6	129.7	[d]	129.6	129.5

Galactopyranose moieties of the compounds[e]

1″	2″	3″	4″	5″	6″
103.3–103.4	70.8–71.0	73.0–73.1	68.7–68.8	74.6–74.8	61.1–61.2

[a] Obtained in $CDCl_3$–CD_3OD (2:1). [b] Carbon signals were assigned by the heteronuclear ^{13}C–^{1}H shift-correlation spectra and ^{13}C-DEPT NMR spectra of cerebrosides **1c**, **1f**, **2c**, and **2f**. [c] Carbon signals overlapped with the signals due to the methylene groups of fatty acids and long-chain bases and appeared at δ_C 22.2, 22.3, 25.6, 29.0, 29.1, 29.2, 29.4 and 31.6. [d] Not seen. [e] Figures shown in this Table indicate the ranges of the chemical shifts in the ^{13}C NMR spectra of the compounds.

and **1c** in the same way as that for **1a** afforded an FAME and an LCB together with compound **3**. These hydrolysates were determined to be methyl docosenoate (for **1b**), methyl tetracosenoate (for **1c**) and 2-aminooctadec-4-ene-1,3-diol (for

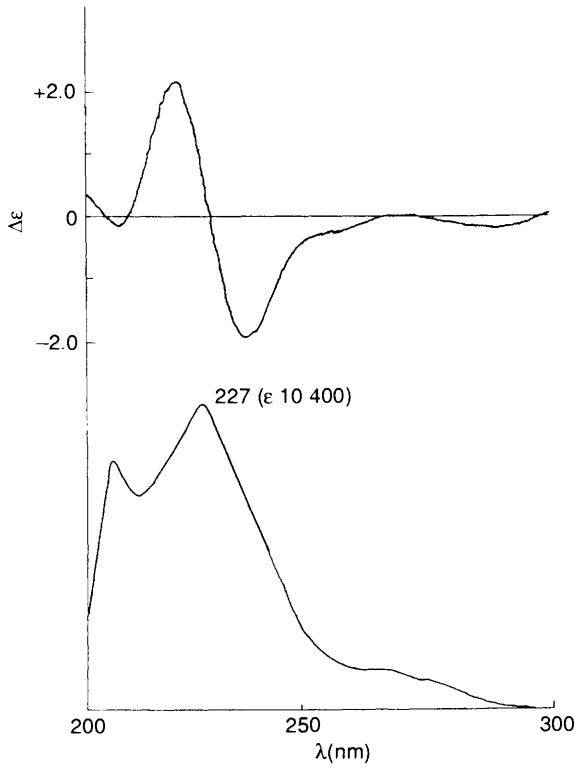
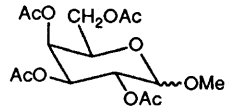


Fig. 1 CD and UV spectra of compound **6**

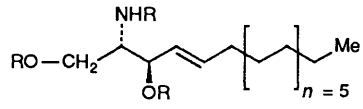
1b and **1c**) by GLC-MS analysis. Accordingly, the structures of cerebrosides **1b** and **1c** were found to be 1-(β-D-galactopyranosyloxy)-substituted 2-(docosenoylamino)octadec-4-en-3-ol and 2-(tetracosenoylamino)octadec-4-en-3-ol, respectively.

Cerebrosides **1d**, **1e** and **1f** showed molecular ions at *m*/*z* 744, 798 and 826 $[M + H]^+$, respectively, in their SI mass spectra and exhibited ^1H and ^{13}C NMR spectra similar to those of cerebrosides **1a**–**1c**, except for the proton signals due to an oxygenated CH group (Tables 1 and 2). These findings suggest that the three cerebrosides are 1-*o*-β-D-galactopyranosides of sphingosine-type ceramides possessing hydroxy fatty acids. Their fatty acids and LCB components were determined to be 2-hydroxyoctadecanoic acid (for **1d**), 2-hydroxydocosenoic acid (for **1e**), 2-hydroxytetracosenoic acid (for **1f**), and 2-aminooctadec-4-ene-1,3-diol (for **1d**–**1f**) in the same manner as used for the structure determination of compounds **1a**–**1c**. Thus, cerebrosides **1d**–**1f** were elucidated as 1-(β-D-galactopyranosyloxy)-substituted 2-(2- hydroxyoctadecanoylamino)-octadec-4-en-3-ol, 2-(2-hydroxydocosenoylamino)octadec-4-en-3-ol, and 2-(2-hydroxytetracosenoylamino)octadec-4-en-3-ol, respectively.

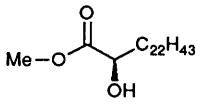
The stereochemical structure of the ceramide parts of the six cerebrosides was determined as follows. The predominant cerebroside **1f**, on hydrolysis, gave methyl 2-hydroxytetracosenoate **4** and an LCB together with methyl D-galactopyranoside **3**. The CD spectrum of the FAME **4** exhibited a negative Cotton effect which thus indicated the *R*-configuration.[7] The LCB, on acetylation in the usual way, gave 2-acetamido-1,3-diacetoxyoctadec-4-ene **5**. The ^1H and ^{13}C NMR spectra of compound **5** were identical with those of synthesized (2*S*,3*R*,4*E*)-2-acetamido-1,3-diacetoxyoctadec-4-ene.[8] This indicated that compound **5** is in an *erythro* form (2*S*,3*R*,4*E* or 2*R*,3*S*,4*E*). Furthermore, the LCB was treated with benzoyl chloride under mild conditions to give compound **6**, which was characterised as (4*E*)-2-benzoylamino-1,3-dibenzoyloxyoctadec-4-ene by its ^1H NMR and chemical ionisation (CI) mass spectra. The CD spectrum of compound **6** showed,



3a; (1-H^{ax})
3b; (1-H^{eq})



5; R = Ac
6; R = Bz



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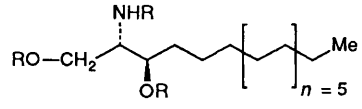

7; R = Ac
8; R = Bz

first a negative, and second a positive Cotton effect (Fig. 1). The absolute configuration at C-2 of compound **6** was thus determined to be *S* on the basis of the 1,2-dibenzoate chirality rule;[9] this is known to be extendable to the acyclic benzoate-benzamide derivatives.[10] From these findings, the LCB of cerebroside **1f** was determined to be (2*S*,3*R*,4*E*)-2-amino-octadec-4-ene-1,3-diol. Accordingly, the ceramide part of cerebroside **1f** was established to be (2*S*,3*R*,4*E*)-2-[(2*R*)-2-hydroxytetracosenoylamino]octadec-4-ene-1,3-diol.

The absolute configuration of the LCB in cerebroside **1c** was elucidated in the same way as that used for cerebroside **1f**. The absolute configuration of the ceramide parts of cerebrosides **1a**, **1b**, **1d** and **1e** could not be determined for lack of samples. However, these four cerebrosides were thought to have the same stereochemistry as those of compounds **1c** and **1f**, since they exhibited similarities in their NMR spectra (Tables 1 and 2).

The other six cerebrosides, **2a**–**2f**, showed molecular ion peaks at *m*/*z* 730, 784, 812, 746, 800 and 828 $[M + H]^+$, respectively, in their SI mass spectra. These mass spectral data suggest that these cerebrosides have dihydrosphingosine-type ceramides. This finding was supported by the ^1H and ^{13}C NMR spectra of compounds **2a**–**2f** (Tables 1 and 2). The fatty acids and LCB components of the six cerebrosides **2a**–**2f** were thus characterised as octadecanoic acid (for **2a**), docosenoic acid (for **2b**), tetracosenoic acid (for **2c**), 2-hydroxyoctadecanoic acid (for **2d**), 2-hydroxydocosenoic acid (for **2e**), 2-hydroxytetracosenoic acid (for **2f**) and 2-aminooctadecane-1,3-diol (for **2a**–**2f**) in the same way as that used for the elucidation of compound **1a**. Furthermore, the configurations at C-2 and C-3 of the LCB in cerebrosides **2c** and **2f** were determined to be *S* and *R*, respectively, in a similar manner to that used for compound **1f**. Also, the *R*-configuration of 2-hydroxytetracosenoic acid in compound **2f** was established. Thus, the stereochemical structures **2a**–**2f** were established to be 1-(β-D-galactopyranosyloxy)-substituted (2*S*,3*R*)-2-(octadecanoylamino)octadecan-3-ol, (2*S*,3*R*)-2-(docosenoylamino)-octadecan-3-ol, (2*S*,3*R*)-2-(tetracosenoylamino)octadecan-3-ol, (2*S*,3*R*)-2-[(2*R*)-2-hydroxyoctadecanoylamino]octadecan-3-ol, and (2*S*,3*R*)-2-[(2*R*)-2-hydroxytetracosenoylamino]octadecan-3-ol, respectively.

Consequently, it was found that the ceramide parts of the twelve cerebrosides **1a**–**1f** and **2a**–**2f** isolated from the bullfrog's brain have the same stereochemistry as those of the corresponding moieties in cerebrosides from mammals and other higher vertebrates.[1,11,12]

Distribution of the Cerebrosides in the Brain.—The brain tissue of the bullfrog was divided into hemisphere, diencephalon and mixed optic lobe, cerebellum and medulla oblongata tissue, due to the difficulty in separation of these three from each other. Table 3 gives compositions of the cerebrosides present in these three parts. The same cerebrosides as those in the total brain

Table 3 Compositions of cerebrosides **1a**–**1f** and **2a**–**2f** in bullfrog brain tissue and its constituent parts

	Relative content (%)[a]											
	1d	**1a**	**2d**	**2a**	**1e**	**1b**	**2e**	**2b**	**1f**	**1c**	**2f**	**2c**[b]
Total[c]	2.4	5.2	1.6	3.8	7.1	9.3	5.6	4.6	12.6	27.4	9.2	11.2
Tissue	(4.8)	(1.0)	(4.4)	(0.9)	(10.7)	(4.3)	(5.7)	(1.5)	(17.9)	(21.6)	(13.5)	(13.7)
Hemisphere	14.0	2.2	2.3	50.8	1.9	1.1	2.6	3.4	4.1	9.4	3.6	4.6
Diencephalon	3.9	3.9	tr.[d]	32.0	11.8	3.6	3.6	3.0	9.9	17.9	5.2	5.2
Optic lobe, Cerebellum, and Medulla oblongata	5.9	4.6	2.3	26.0	4.3	6.1	8.4	1.0	5.7	25.1	5.6	5.0

[a] Determined by reversed-phase HPLC analyses using a Wako Pak C_{18} column (7.5 mm i.d. × 250 mm) with methanol [flow rate 1.0 cm^3/min; monitored at 210 nm]. [b] Described in order of elution from the HPLC column. [c] Figures shown in parentheses indicate the relative content on the basis of the isolated yields of the twelve cerebrosides. [d] Trace.

tissues were uniformly present in the three parts (mixed tissues). However, the compositions of the cerebrosides in these parts differed from each other; the main cerebrosides in the hemisphere were **1d** and **2a**, the main cerebrosides in the diencephalon were **1c**, **1e** and **2a** and those in the mixed tissues were **1c** and **2a**. This difference in composition was not observed in the case of the gangliosides present in the three parts of the bullfrog's brain.[2] Further, the structures of the ceramide parts of bullfrog brain cerebrosides clearly differed from that of the brain gangliosides which have hexadecanoic acid ($C_{16:0}$) and octadecanoic acid ($C_{18:0}$) as predominant fatty acids and 2-aminooctadec-4-ene-1,3-diol and 2-aminoicos-4-ene-1,3-diol as long-chain bases.[2] Also, the total brain tissue of the bullfrog was characterised by the presence of cerebrosides having fatty acid components with a C_{22}-carbon chain, such as compounds **1b**, **1e**, **2b** and **2e**, since these are known to be only minor constituents of cerebrosides in other animal species.[4,5,13,14]

Experimental

M.p.s were measured on a Yanaco hot-stage microscope apparatus and were not corrected. Optical rotations were measured with a JASCO DIP-360 digital polarimeter. CD Spectra were recorded on a JASCO J-600 spectropolarimeter.

Normal-phase TLC was performed on precoated silica gel plates (Merck GF_{254}, 0.25 mm). Reversed-phase TLC was carried out on Whatmann KC_{18} plates (thickness 0.25 mm). Cerebrosides were visualised by spraying of the plates with anthrone–H_2SO_4 reagent. GLC was performed on Shimadzu GC-15A equipped with flame ionisation detection (FID) and 2.6 mm (i.d.) × 3 m glass column packed with either 2% OV-17 or 2% OV-225 on Chromosorb W-AWDMCS (80–100 mesh). GLC-MS was performed on a Shimadzu QP-1000 equipped with the above column. Electron impact (EI) mass spectra were obtained at 70 eV and 200 °C (ion-source temp.). SI mass spectra were obtained by detection of positive ions with a Hitachi M-80B double-focusing mass spectrometer equipped with an M-8086 xenon-beam-generating system and an M-0101 data processing system. Analytical conditions were as follows: a sample of cerebroside (5 μg) dissolved in chloroform–methanol (2:1; 1 mm^3); matrix: mixture of glycerol and 0.1% $MeCO_2Na$ in methanol (1:1, 1 mm^3); accelerating voltage 3 keV. Assignment of mass number was achieved by comparison of the spectra with the mass spectrum of Fomblin.[2] EI mass spectra were measured by a direct probe with the same instrument under the above described conditions and CIMS were obtained by using isobutane as reactant. ^1H and ^{13}C NMR spectra were recorded on a JEOL GSX-500 spectrometer with Me_4Si as internal standard.

Isolation of Cerebrosides.—Fresh bullfrog brain (1047 specimens, 330 g) was ground in a mortar after being frozen with liquid N_2. The ground brain tissue was treated with acetone (2000 cm^3) to remove fats. After filtration, the insoluble residue was extracted with chloroform–methanol (1:1; 2000 cm^3) followed with hot ethanol (60 °C; 1000 cm^3). The combined extracts, after removal of solvent under reduced pressure, gave crude glycolipids (5.1 g). These were subjected to chromatography on DEAE-Sephadex A-25 (acetate form). Elution with chloroform–methanol–water (3:7:1; 700 cm^3) gave an eluate containing neutral glycolipids. This eluate was evaporated under reduced pressure to give neutral glycolipids (3.55 g). The neutral glycolipids were then subjected to column chromatography on silica gel with chloroform–ethyl acetate–methanol–water (8:2:2:1) to give two fractions, A (164 mg) and B (144 mg). The two fractions showed one spot on TLC developed with the above solvent (A: R_f 0.32; B: R_f 0.26). However, each fraction showed six peaks on HPLC using a Wako Pak C_{18} column (4.6 mm i.d. × 150 mm) with methanol as eluent [A: **1a** (7.2%), **2a** (5.1%), **1b** (10.3%), **2b** (8.4%), **1c** (43.7%) and **2c** (25.3%); B: **1d** (9.0%), **2d** (5.3%), **1e** (24.7%), **2e** (11.5%), **1f** (32.0%) and **2f** (17.5%)]. Separation of each component was carried out by means of HPLC on a Wako Pak C_{18} column (7.5 mm i.d. × 250 mm) with methanol as eluent. Each component was repeatedly purified on the same column to give 1.4 mg, 1.2 mg, 6.0 mg, 2.1 mg, 30.0 mg, 19.0 mg, 6.6 mg, 6.1 mg, 14.9 mg, 7.9 mg, 24.9 mg and 18.7 mg of pure compounds **1a**, **2a**, **1b**, **2b**, **1c**, **2c**, **1d**, **2d**, **1e**, **2e**, **1f** and **2f**, respectively.

Cerebroside **1a**; m.p. 194–195 °C (from MeOH); (SIMS) *m/z* 728 $[M + H]^+$, 710 $[(M + H) - H_2O]^+$, 549 $[(M + H) -$ 179 (galactose residue)] and 264 $\{^+CH_2–C(NH_2)=CH–CH=CH[CH_2]_{12}Me$ (a fragment due to long-chain base component[15]$\}$.

Cerebroside **1b**; m.p. 188–190 °C (from MeOH); (SIMS) *m/z* 782 $[M + H]^+$, 764 $[(M + H) - H_2O]^+$, 603 $[(M + H) -$ $179]^+$ and 264.

Cerebroside **1c**; m.p. 181–182 °C (from MeOH) (Found: C, 69.6; H, 11.7; N, 1.6. Calc. for $C_{48}H_{91}NO_8 \cdot H_2O$: C, 69.6; H, 11.3; N, 1.7%); $[\alpha]^{25}_D$ −2.7° (*c* 0.6, pyridine); (SIMS) *m/z* 810 $[M +$ $H]^+$, 792 $[(M + H) - H_2O]^+$, 631 $[(M + H) - 179]^+$ and 264.

Cerebroside **1d**; m.p. 209–210 °C (from MeOH); (SIMS) *m/z* 744 $[M + H]^+$, 726 $[(M + H) - H_2O]^+$, 565 $[(M + H) -$ $179]^+$ and 264.

Cerebroside **1e**; m.p. 190–191 °C (from MeOH); $[\alpha]^{25}_D$ +7.4° (*c* 0.3, pyridine); (SIMS) *m/z* 798 $[M + H]^+$, 780 $[(M + H) -$ $H_2O]^+$, 619 $[(M + H) - 179]^+$ and 264.

Cerebroside **1f**; m.p. 185–186 °C (from MeOH) (Found: C, 68.0; H, 11.5; N, 1.6. Calc. for $C_{48}H_{91}NO_9 \cdot H_2O$: C, 68.3; H, 11.1; N, 1.7%); $[\alpha]^{25}_D$ +4.8° (*c* 0.3 pyridine); (SIMS) *m/z* 826 $[M +$ $H]^+$, 808 $[(M + H) - H_2O]^+$, 646 $[(M + H) - 179]^+$ and 264.

Cerebroside **2a**; m.p. 196–197 °C (from MeOH); (SIMS) *m/z* 730 $[M + H]^+$ and 551 $[(M + H) - 179]^+$.

Cerebroside **2b**; m.p. 184–185 °C (from MeOH); (SIMS) *m/z* 784 $[M + H]^+$ and 605 $[(M + H) - 179]^+$.

Cerebroside **2c**; m.p. 185–186 °C (from MeOH) (Found: C, 68.9; H, 11.8; N, 1.6. Calc. for $C_{48}H_{93}NO_8 \cdot H_2O$: C, 69.4; H, 11.5; N, 1.7%); $[\alpha]^{25}_D$ +1.6° (*c* 0.4, pyridine); (SIMS) *m/z* 812 $[M + H]^+$ and 633 $[(M + H) - 179]^+$.

Cerebroside **2d**; m.p. 214–215 °C (from MeOH); $[\alpha]^{25}_D$ +4.9° (*c* 0.1 pyridine); (SIMS) *m/z* 746 $[M + H]^+$ and 567 $[(M + H) - 179]^+$.

Cerebroside **2e**; m.p. 188–189 °C (from MeOH); $[\alpha]^{25}_D$ + 7.6° (*c* 0.2, pyridine); (SIMS) *m/z* 800 $[M + H]^+$ and 621 $[(M + H) - 179]^+$.

Cerebroside **2f**; m.p. 199–200 °C (from MeOH) (Found: C, 67.9; H, 11.5; N, 1.6. Calc. for $C_{48}H_{93}NO_9 \cdot H_2O$: C, 68.1; H, 11.3; N, 1.7%); $[\alpha]^{25}_D$ +7.3° (*c* 0.3, pyridine); (SIMS) *m/z* 828 $[M + H]^+$ and 649 $[(M + H) - 179]^+$.

The ^1H and ^{13}C NMR spectra of cerebrosides **1a**–**1f** and **2a**–**2f** are given in Tables 1 and 2, respectively.

Acid Hydrolysis of Cerebrosides (Gaver–Sweeley Method[6]*).*—A typical experimental procedure is described below. Cerebroside **1f** (15 mg) was hydrolysed with 1 mol dm^{-3} HCl in 82% MeOH at 70 °C for 18 h. The reaction mixture was extracted with hexane and the extract was washed, dried and evaporated to give the fatty acid methyl ester (FAME) (*ca.* 4 mg).

The residual reaction mixture free from the FAME was treated with Dowex-WGR. The neutralised solution thus obtained was extracted with diethyl ether (10 cm^3 × 3) to give the long-chain base (LCB) (*ca.* 4 mg).

The residual aqueous solution free from the LCB was lyophilised and then treated with acetic anhydride–pyridine (1:1) (0.5 cm^3) at 70 °C for 2 h. The reaction mixture was partitioned between $CHCl_3$–water (1:1) to give sugar acetates (*ca.* 4 mg) in the organic layer.

Analysis of Fatty Acid Components.—The FAMEs obtained from 12 cerebrosides by acid hydrolysis were analysed by GLC and GLC-MS (column 2% OV-17; column temp. 200–250 °C at 2 °C/min for non-hydroxy-FAME and 250 °C for 2-hydroxy-FAME). The 2-hydroxy-FAME obtained from cerebroside **1f** was then subjected to column chromatography on silica gel with $CHCl_3$–MeOH (99:1) to give pure compound **4** (3.6 mg).

The FAMEs of the 12 cerebrosides were identified by mass spectral comparison and co-injection analyses on GLC with authentic materials.

Analysis of the Acetates of Long-chain Base Components.—A portion of the LCB obtained from 12 cerebrosides was acetylated in the same way as that used for the sugar components. The LCB acetates thus obtained were analysed by GLC and GLC-MS operated under the same conditions as those used for analysis of 2-hydroxy-FAMEs. The LCB acetate obtained from cerebrosides **1f** was subjected to silica gel column chromatography with $CHCl_3$–MeOH (95:5) to give compound **5** (1.6 mg).

The LCB acetates of the 12 cerebrosides were identified by their EI mass spectral fragmentation patterns.

Preparation of the Benzoate-benzamide Derivative of the Long-chain Base Component.—A portion of the LCB (*ca.* 2 mg) obtained from cerebroside **1f** was treated with benzoyl chloride (5 mm^3) and pyridine (40 mm^3) at room temperature overnight. The reaction mixture was partitioned between $CHCl_3$–water (1:1) to give the benzoate-benzamide derivative **6** in the organic layer. Purification of **6** was carried out by preparative reversed-phase TLC with MeOH–water (97:3) followed by preparative HPLC [column Wako Pak C_{18} (4.6 mm i.d. × 150 mm); eluent MeOH–water (9:1)] to give a pure sample (1.0 mg).

Analysis of Sugar Acetates.—The sugar acetates obtained from 12 cerebrosides were analysed by GLC and GLC-MS [column 2% OV-225; column temp. 200 °C]. Five peaks appeared on GLC [t_R (min) 24.1 (65–68%), 26.1 (1–3%), 30.3 (24–25%), 45.2 (2–3%), and 50.8 (3–4%)] and they were assigned as methyl 2,3,4,6-tetra-*O*-acetyl-α-galactopyranoside, methyl 2,3,4,6-tetra-*O*-acetyl-α-glucopyranoside, methyl 2,3,4,6-tetra-*O*-acetyl-β-galactopyranoside, 1,2,3,4,6-penta-*O*-acetyl-α-galactopyranose and 1,2,3,4,6-penta-*O*-acetyl-β-galactopyranose, respectively, by the EI mass spectral fragmentation patterns and co-injection analyses with authentic materials. The sugar acetates from cerebrosides **1f** were then subjected to preparative reversed-phase TLC with MeOH–water (3:2) to give major products **3a** (1 mg) (R_f 0.56; 89% pure on GLC) and **3b** (3 mg) (R_f 0.43; 98% pure on GLC). Compound **3b** was finally purified by means of preparative HPLC using the same column as above with MeOH–water (4:1) to give a pure sample (1 mg) (> 99% pure on GLC).

Methyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **3a**; $[\alpha]^{25}_D$ −10.0° ± 5° (*c* 0.02, $CHCl_3$) (lit.,[16] $[\alpha]_D$ −14.0°); $\delta_H(CDCl_3)$ 2.04, 2.05, 20.6 and 2.15 (each 3 H, s, OAc × 4), 3.66 (3 H, s, OMe), 3.91 (1 H, dt, *J* 1.0, 6.4 Hz, 5-H), 4.14 (1 H, dd, *J* 6.4, 11.2 Hz, 6-H^a), 4.17 (1 H, dd, *J* 6.4, 11.2 Hz, 6-H^b), 4.40 (1 H, d, *J* 7.8 Hz, 1-H), 5.11 (1 H, dd, *J* 3.4, 10.3 Hz, 3-H), 5.20 (1 H, dd, *J* 7.8, 10.3 Hz, 2-H) and 5.39 (1 H, dd, *J* 1.0, 3.4 Hz, 4-H). The specific rotation of this compound is not accurate because of the presence of a small amount of contaminant.

Methyl 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranoside **3b**; $[\alpha]^{25}_D$ +110.5° ±10° (*c* 0.01, $CHCl_3$) (lit.,[16] $[\alpha]_D$ +133°); $\delta_H(CDCl_3)$ 1.98, 2.05, 2.09 and 2.15 (each 3 H, s, OAc × 4), 3.41 (3 H, s, OMe), 4.10 (1 H, dt, *J* 1.9, 6.4 Hz, 5-H), 4.11 (2 H, d, *J* 6.4 Hz, 6-H_2), 5.00 (1 H, d, *J* 3.4 Hz, 1-H), 5.16 (1 H, dd, *J* 3.9, 10.7 Hz, 3-H), 5.35 (1 H, d, *J* 3.4, 10.7 Hz, 2-H) and 5.45 (1 H, dd, *J* 1.9, 3.9 Hz, 4-H).

Methyl (2*R*)-2-hydroxytetracosenoate **4**; $[\alpha]^{25}_D$ −12.5° ±3° (*c* 0.08, $CHCl_3$) [Found: M^+ (EI), 396.3630. $C_{25}H_{48}O_3$ requires M, 396.3601]; $\Delta\varepsilon_{263}$ −0.002 (*c* 0.01, $CHCl_3$); ν_{max} 3500 (OH), 2900 (CH) and 1740 cm^{-1} (CO); $\delta_H(CDCl_3)$ 0.88 (3 H, t, *J* 6.8 Hz, Me), 1.26 (16 H, br s, $[CH_2]_8$), 2.02 (4 H, dt, *J* 5.8, 6.3 Hz, $[CH_2]_2C$=), 2.66 (1 H, d, *J* 5.9 Hz, OH), 3.79 (3 H, s, OMe), 4.19 (1 H, dt, *J* 5.9, 6.3 Hz, CHO) and 5.35 [2 H, dt, *J* 4.4, 5.8 Hz, CH=CH (*Z*)].

Compound **4** (3.4 mg) was also obtained from cerebroside **2f** (15 mg) by acid hydrolysis.

(2*S*,3*R*,4*E*)-2-Acetamido-1,3-diacetoxyoctadec-4-ene **5**; [Found: M^+ (EI), 424.9669. $C_{24}H_{43}NO_5$ requires M, 425.3130]; $\delta_H(CDCl_3)$ 0.88 (3 H, t, *J* 6.4 Hz, Me), 1.25 (22 H, br s, $[CH_2]_{11}$), 2.05 (2 H, br s, CH_2C=), 2.00, 2.10 and 2.12 (each 3 H, s, OAc × 3), 4.04 (1 H, dd, *J* 3.9, 11.7 Hz, 1-H^a), 4.30 (1 H, dd, *J* 6.3, 11.7 Hz, 1-H^b), 4.42 (1 H, m, 2-H), 5.28 (1 H, t, *J* 7.3 Hz, 3-H), 5.38 (1 H, dd, *J* 7.3, 15.0 Hz, 4-H), 5.65 (1 H, d, *J* 8.8 Hz, NH) and 5.80 (1 H, td, *J* 6.8, 15.0 Hz, 5-H); δ_C 14.0 (Me), 20.7, 22.6 and 23.0 (CO*Me*), 28.7, 28.8, 29.0, 29.2, 29.4, 29.5, 29.6, 31.8 and 32.1 (CH_2), 50.7 (C-2), 62.6 (C-1), 73.8 (C-3), 124.2 (C-4), 137.4 (C-5) and 169.6 and 169.9 (CO); (EIMS) *m/z* 425 (M^+, 1%), 366 $[(M - OCOCH_3)^+$, 5], 338 (10), 331 (10), 264 (20), 236 (20), 169 (45), 61 (85), 55 (79) and 43 (100).

(2*S*,3*R*,4*E*)-2-Benzamido-1,3-dibenzoyloxyoctadec-4-ene **6**; λ_{max} 227 nm (ε 10 400); Δε 236 (−1.79), 227 (0.0) and 217 nm (+2.09) (Fig. 1); $\delta_H(CDCl_3)$ 0.84 (3 H, t, *J* 6.4 Hz, Me), 1.25 (22 H, br s, $[CH_2]_{11}$), 2.05 (2 H, q, *J* 6.4 Hz, CH_2C=), 4.61 (1 H, dd, *J* 4.6, 11.4 Hz, 1-H^a), 4.72 (1 H, dd, *J* 6.4, 11.4 Hz, 1-H^b), 4.93 (1 H, m, 2-H), 5.66 (1 H, dd, *J* 7.3, 15.2 Hs, 4-H), 5.87 (1 H, t, *J* 7.3 Hz, 3-H), 5.96 (1 H, dd, *J* 6.0, 15.2 Hz, 5-H), 6.60 (1 H, d, *J* 8.3 Hz, NH), 7.40–7.57 (9 H, m, *m*-, *p*-ArH), and 8.02 (6 H, m, *o*-ArH); (CIMS) *m/z* 612 $[(M + H)^+$, 1%], 507 $[(M + H - C_6H_5CO)^+$ 21, 386 (12), 368 (10), 268 (3), 146 (20), 123 (100) and 105 (80).

(2*S*,3*R*)-2-Acetamido-1,3-diacetoxyoctadecane **7**; [Found: M^+ (EI), 427.3196. $C_{24}H_{45}NO_5$ requires M, 427.3296]; $\delta_H(CDCl_3)$ 0.88 (3 H, t, *J* 6.8 Hz, Me), 1.25 (26 H, br s, $[CH_2]_{13}$], 2.00, 2.06 and 2.07 (each 3 H, s, OAc × 3), 4.06 (1 H, dd, *J* 3.9, 11.6 Hz, 1-H^a), 4.25 (1 H, dd, *J* 5.8, 11.6 Hz, 1-H^b), 4.35 (1 H, m, 2-H), 4.90 (1 H, td, *J* 6.6, 7.3 Hz, 3-H) and 5.84 (1 H, d, *J* 8.8 Hz, NH); δ_C 14.0 (Me), 21.0, 22.7 and 23.4 (CO*Me*), 20.8, 25.3, 29.3, 29.4, 29.5, 29.7, 31.5 and 31.9 (CH_2), 50.5 (C-2), 62.6 (C-1), 74.1 (C-3) and 169.6 (CO); (EIMS) *m*/*z* 427 (M^+, 3%), 368 [$(M - COCH_3)^+$, 14], 331 (10), 266 (10), 264 (12), 169 (45), 61 (100) and 43 (90).

Compound **7** (1.8 mg and 1.6 mg) was obtained from a portion of the LCB of cerebrosides **2c** and **2f** (each 15 mg) in the same way as was compound **5**. The ^1H and ^{13}C NMR spectra of these samples of compound **7** were identical with those of the authentic sample.[17]

(2*S*,3*R*)-2-Benzamido-1,3-dibenzoyloxyoctadecane **8**; λ_{max} 227 (ε 10 200); Δε 235 (− 1.95), 227 (0.0) and 217 nm (+ 1.06); $\delta_H(CDCl_3)$ 0.88 (3 H, t, *J* 6.4 Hz, Me), 1.25 (26 H, br s, $[CH_2]_{13}$), 4.62 (1 H, dd, *J* 4.6, 11.4 Hz, 1-H$_a$), 4.71 (1 H, dd, *J* 6.4, 11.4 Hz, 1-H^b), 4.91 (1 H, m, 2-H), 5.46 (1 H, td, *J* 6.6, 7.3 Hz, 3-H), 6.40 (1 H, d, *J* 9.3 Hz, NH), 7.40–7.57 (9 H, m, *m*-, *p*-ArH) and 8.02 (6 H, m, *o*-ArH); (CIMS) *m*/*z* 614 [$(M + H)^+$, 1%], 5.09 [$(M + H - C_6H_5CO)^+$, 40], 388 (15), 370 (10), 268 (3), 147 (22), 123 (20) and 105 (100).

Compound **8** (1.1 mg and 1.0 mg) was obtained from a portion of the LCB of cerebrosides **2c** and **2f** in the same way as was compound **6**.

Distribution of Cerebrosides in the Brain.—The bullfrog brain tissue was divided into hemisphere (18 g), diencephalon (5 g) and mixed (38 g) optic lobe, cerebellum and medulla oblongata tissue. Following the procedure for the total brain tissue, the three parts were each treated to give 27 mg, 21 mg and 81 mg, respectively, of the mixtures of neutral glycolipids and cerebrosides. The compositions of the cerebrosides in these mixtures are given in Table 2.

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