New Glycosphingolipid Containing an Unusual Sphingoid Base from the Basidiomycete *Polyporus ellisii*

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ABSTRACT: A new 9-methyl-sphinga-4,8-dienine-containing glucocerebroside (**1**), together with two additional known analogs, cerebrosides B and D, was isolated from the chloroform-soluble lipid fraction of the ethanol and chloroform/methanol extract of the fruiting bodies of the basidiomycete *Polyporus ellisii* Berk. and characterized. The structure and relative stereochemistry of the new compound were identified as (2*S*,3*R*,4*E*,8*E*)-1-(β-D-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyheptadecanoyl]amino-9-methyl-4,8-octadecadiene by means of spectroscopic (^{1}H, ^{13}C, and two-dimensional nuclear magnetic resonance; mass spectrometry) and chemical methods.

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Two groups of sphingolipids in higher mushrooms, or basidiomycetes, are distinguished from one another by the relation of their carbohydrate to the ceramide moiety. Classical glycosphingolipids (GSL) of the first group have their sugar portion linked directly to the ceramide by a glycoside link. In the second group, the glyco-inositol-phospho-sphingolipids, carbohydrate is coupled to the lipophilic portion of the molecule *via* an inositol phosphate. Sphingolipids, e.g., ceramides, sphingomyelin, cerebrosides and gangliosides, are important building blocks of the plasma membrane of eukaryotic cells. Their function is to anchor lipid-bound carbohydrates to cell surfaces and to create an epidermal water permeability barrier, as well as to participate in antigen–antibody reactions and transmission of biological information (1,2). Some are also anti-ulcerogenic, ionophoretic, antihepatotoxic, antitumor, immunostimulatory or stimulatory to axon growth (3–7). In the course of our investigation on the sphingolipid composition of higher mushrooms collected from Yunnan Province of the People's Repulic of China, we recently reported the occurrence of two antifungal glucocerebrosides from *Russula ochroleuca* (8), and two ceramides containing C_{18}-phytosphingosine from *R. cyanoxantha* (9) and *Armillaria mellea* (10).

In continuing our studies on basidiomycete-derived bioactive secondary metabolites, we investigated the chemical constituents of the mushroom, *Polyporus ellisii* Berk. (Polyporaceae). A new glucocerebroside (**1**) has now been isolated and described.

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Abbreviations: CC, column chromatography; EI, electron impact; EI-MS, electron impact-mass spectrometry; FAB-MS, fast atom bombardment-mass spectrometry; GC–MS, gas chromatography-mass spectrometry; Glc, glucose; GSL glycosphingolipids; HMBC, heteronuclear multiple bond correlation; IR, infrared; LCB, long-chain base; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.

EXPERIMENTAL PROCEDURES

Chromatographic and instrumental methods. Melting points were obtained on an XRC-1 apparatus (Sichuan University, Sichuan, People's Republic of China). Optical rotations were taken on a Horiba SEPA-300 automatic polarimeter (Horiba, Tokyo, Japan). The nuclear magnetic resonance (NMR; ^{1}H, ^{13}C, and two-dimensional NMR) spectra were acquired on Bruker AM 400 (Rheinstetten, Germany) and DRX-500 NMR instruments (Karlsruhe, Germany); tetramethylsilane was used as an internal standard, and coupling constants were represented in Hertz. Mass spectra were measured with a VG Autospec3000 mass spectrometer (VG, Manchester, England). Infrared (IR) spectra were obtained in KBr pellets on a Bio-Rad FTS-135 IR spectrophotometer (Bio-Rad, Richmond, CA). Gas chromatography–mass spectrometry (GC–MS) was performed with a Finnigan 4510 GC–MS spectrometer (San Jose, CA) employing the electron impact (EI) mode (ionizing potential 70eV) and a capillary column (30 m × 0.25 mm) packed with 5% phenyl/95% methylsilicone on 5% phenyl-dimethylsilicone (HP-5) (Hewlett-Packard, Palo Alto, CA). Helium was used as carrier gas; column temperature 160–240°C (rate of temperature increase: 5°C/min).

Materials. Column chromatography (CC) was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Ltd., Qingdao, People's Republic of China) and Sephadex LH-20 gel (25–100 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Reversed-phase chromatography was carried out on LiChroprep^R RP-8 (40–63 μm) (Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) analysis was carried out on plates precoated with silica gel F_{254} (Qingdao Marine Chemical Ltd.), and detection was achieved by spraying with 10% H_2SO_4 followed by heating. All solvents were distilled before use.

Fresh fruiting bodies of *P. ellisii* were collected from Ailao Mountains in Yunnan Province in August 1998 and identified by Professor P.G. Liu, X.H. Wang, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, People's Republic of China, where a voucher specimen (no. HKAS 32905) has been deposited.

Extraction and isolation. Dried fruiting bodies (228 g) of *P.*

ellisii were first extracted twice with 95% ethanol (1 L × 24 h × 2) for 48 h and then four times with chloroform/methanol (1:1, vol/vol; 0.5 L × 36 h × 4) at room temperature. The combined organic phase was concentrated *in vacuo*. The residue was suspended in water and partitioned with chloroform. The crude chloroform extract (7.6 g) was chromatographed on a reversed-phase (RP)-8 column, eluted with a gradient of methanol in water. The fraction (1.1 g), eluted with 200 mL methanol, was passed through vacuum liquid chromatography with a chloroform/methanol mixture containing increasing amounts of methanol to provide six fractions. Of these, the fraction eluted with chloroform/methanol (5:1, vol/vol) was further purified by chromatography on an RP-8 column by elution with methanol/water (85:15, 90:10, 95:5, vol/vol) and followed by separation on Sephadex LH-20 using methanol to produce compounds **2** (6 mg), **1** (20 mg), and **3** (8 mg).

*(2*S*,3*R*,4*E*,8*E*)-1-(β-*D*-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyheptadecanoyl]amino-9-methyl-4,8-octadecadiene (**1**).* White amorphous powder (methanol); mp 154–156°C; $[\alpha]_D^{26}$ +4.9° (*c* 0.40, MeOH); IR (KBr) ν_{max} 3393 (OH), 2921, 2852 (C–H), 1650 (HNC=O), 1540 (NH), 1469, 1304, 1079 (C–O), 963 (*trans* C=C), 721 (methylenes) cm^{-1}; ^1H and ^{13}C NMR spectra are given in Table 1; EI–MS (70 eV) *m/z* (relative intensities, %) 724 $[M - OH]^+$ (0.5), 562 $[M - OH - 162]^+$ (4.8), 530 (2.8); negative fast atom bombardment-mass spectrometry (FAB-MS) *m/z*: 740 $[M - 1]^-$, 579 $[M - H - 162]^-$, 561 $[M - H - 179]^-$; negative high resolution FAB-MS *m/z* 740.5659 $[M - 1]^-$ (calcd. for $C_{42}H_{78}NO_9$, 740.5677).

*Acetylation of **1**.* Compound **1** (6.3 mg) was dissolved in pyridine (0.3 mL), and the mixture was treated with acetic anhydride (0.3 mL) and left standing overnight at room temperature. The reaction solution was then diluted with 2 mL of water and extracted with ethyl acetate (3 × 4 mL). The ethyl acetate extract was washed with brine and dried over Na_2SO_4, then evaporated to dryness under reduced pressure. The residue obtained was subjected to silica gel CC, with elution by petroleum ether/ethyl acetate (8:2, vol/vol) to give 7 mg of its peracetate derivative **1a**.

*(2*S*,3*R*,4*E*,8*E*)-1-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-3-*O*-acetyl-2-[(*R*)-2′-acetoxyheptadecanoyl]amino-9-methyl-4,8-octadecadiene (**1a**).* White powder solid. $[\alpha]_D^{26}$ +5.6° (*c* 0.36, $CHCl_3$); IR (KBr) ν_{max} 3369 (OH), 2925, 2856 (C–H), 1745 (C=O of ester), 1675 (C=O of amide), 1533

TABLE 1
^1H and ^{13}C Nuclear Magnetic Resonance (NMR) Data for Compound 1 in Pyridine-d_5

Atom no.	^1H (multiplicity, *J*, in Hz)	^{13}C (multiplicity)[a]	^1H-^1H COSY	HMBC
Long-chain base				
1a	4.71 (*dd,* 10.5, 6.0)	69.9 (CH_2)	H-1b, H-2	H-1″, H-2, H-4
1b	4.23 (*dd,* 10.4, 6.8)		H-1a, H-2	
2	4.81 (*m*)	54.5 (CH)	H-1a, H-1b, H-3, NH	H-1, H-3, H-4
3	4.75 (*m*)	72.3 (CH)	H-2, H-4	H-1, H-2, H-4
4	5.95 (*dt,* 15.3)	132.2 (CH)	H-3, H-5	H-3, H-6
5	5.99 (*dd,* 15.3, 5.8)	131.7 (CH)	H-4, H-6	H-3, H-7, H-8
6	2.15 (*m*)	32.9 (CH_2)	H-7	H-4, H-5
7	2.15 (*m*)	28.2 (CH_2)	H-8	H-5, H-8
8	5.25 (*br*)	124.0 (CH)	H-7	H-6, H-7, H-10, H-19
9		135.6 (C)		H-7, H-19
10	2.00 (*br t,* 7.5)	39.8 (CH_2)		H-8, H-19
11	1.38 (*m*)	28.1 (CH_2)		
12–17	1.25 (*br s*)	22.7–31.9 (CH_2)		
18-CH_3	0.86 (*t,* 7.0)	14.0 (CH_3)		
19-CH_3	1.61 (*s*)	15.9 (CH_3)		H-8, H-10
NHCO	8.36 (*d,* 8.6)		H-2	
N-acyl moiety				
1′		175.5 (C)		H-2, H-2′, H-3′
2′	4.57 (*dd,* 7.8)	72.4 (CH)	H-3′	NH, H-3′
3′	2.00, 2.14 (*m*)	35.5 (CH_2)	H-2′	H-2′
4′–16′	1.25 (*br s*)	22.7–31.9 (CH_2)		
17′-CH_3	0.86 (*t,* 7.0)	14.0 (CH_3)		
Sugar moiety				
1″	4.91 (*d,* 7.8)	105.4 (CH)	H-2″	H-1, H-2″
2″	4.03 (*m*)	74.9 (CH)	H-1″, H-3″	H-1″, H-3″, H-4″
3″	4.25 (*m*)	78.3 (CH)	H-2″, H-4″	H-1″, H-2″
4″	4.21 (*m*)	71.5 (CH)	H-5″, H-3″	H-3″, H-6″
5″	3.90 (*m*)	78.3 (CH)	H-6″, H-4″	H-3″, H-4″, H-6″
6″	4.36 (*dd,* 5.2, 11.8)	62.6 (CH_2)	H-5″	H-4″
	4.51 (*dd,* 2.2, 11.9)			

[a]Assignments were made by distortionless enhancement by polarization transfer (DEPT) and heteronuclear multiple quantum coherence (HMQC) analysis. HMBC, heteronuclear multiple bond coherence. COSY, correlation spectroscopy.

(N–H), 1467, 1376, 1234, 1125–1037 (glycosidic C–O), 977 (*trans* C=C), 889, 721 (nCH_2) cm^{-1}; ^1H NMR 500 MHz ($CDCl_3$), δ ppm 0.88 (6H, *t*-like, *J* = 6.7 Hz, 2 × Me), 1.25 (*s*, methylenes), 1.57 (3H, *s*, 19-Me), 2.05 (2H, *m*, H-3′), 1.95 (2H, *br t, J* = 7.8 Hz, H-10), 1.80 (2H, *m*, H-6), 2.06 (2H, *m*, H-7), 3.61 (1H, *dd, J* = 4.5, 10.3 Hz, H-1), 3.69–3.71 (1H, *m*, H-5″), 3.93 (1H, *dd, J* = 4.0, 10.3 Hz, H-1), 4.14 (1H, *dd, J* = 2.1, 12.3 Hz, H-6″), 4.24 (1H, *dd, J* = 4.6, 12.3 Hz, H-6″), 4.31 (1H, *m*, H-2), 4.48 (1H, *d, J* = 7.9 Hz, H-1″), 4.95 (2H, *m*, H-2″, H-3″), 5.08 (1H, *br t, J* = 9.7 Hz, H-8), 5.15 (1H, *dd, J* = 4.7, 7.1 Hz, H-2′), 5.19 (1H, *t, J* = 9.5 Hz, H-4″), 5.32 (1H, *dd, J* = 5.3, 6.8 Hz, H-3), 5.41 (1H, *dd, J* = 15.3, 7.4 Hz, H-4), 5.82 (1H, *dt, J* = 15.3 Hz, H-5), 6.36 (1H, *d, J* = 8.8 Hz, NHCO), 2.00 (*s*, MeCO), 2.02 (*s*, 2 × MeCO), 2.03 (*s*, MeCO), 2.09 (*s*, MeCO), 2.15 (*s*, MeCO); ^{13}C NMR 100 MHz ($CDCl_3$) δ ppm 14.0 (2 × Me), 15.9 (19-Me), 2 × 22.6, 24.7, 27.4, 28.0 (C-7), 29.3, 29.6 (all CH_2), 31.9 (C-6), 32.5 (C-3′), 39.7 (C-10), 67.2 (C-1), 50.8 (C-2), 74.0 (C-3), 73.2 (C-2′), 100.6 (C-1″), 71.3 (C-2″), 72.0 (C-3″), 68.4 (C-4″), 72.8 (C-5″), 62.2 (C-6″), 123.0 (C-8), 136.6 (C-9), 124.6 (C-4), 136.2 (C-5), 20.5 (4 × COMe), 20.9 (2 × COMe), 2 × 169.3, 3 × 169.6, 170.0, 170.4 (all COMe, NHCO); EI-MS (70 eV) *m/z* (relative intensities, %): 993 $[M]^+$ (2.0), 933 $[M - HOAc]^+$ (3), 874 $[M - HOAc - OAc]^+$ (1.0), 826 (1.5), 700 (0.5), 663 $[M - Glc(OAc)_4 + 1$, where glucose = $Glc]^+$ (0.5), 646 (2.8), 632 (1.2), 586 (2.2), 572 (2.2), 526 (1.0), 512 (0.5), 433 $[AcOCH^+CH_2OGlc(OAc)_4]$ (0.5), 390 $[H_2N^+{=}CHCH_2OGlc(OAc)_4]$ (15), 359 (1.8), 332 (17), 331 $[Glc(OAc)_4]^+$ (100), 276 $[C_{16}H_{28}CH{=}CH(NH_2)CH_2^+]$ (1.2), 271 (9), 229 (4), 211 (5), 170 (11), 169 (95.8), 145 (7.8), 139 (8.0), 127 (10.5), 109 (32.5), 81 (14), 60 (31.5).

*Methanolysis of **1***. Compound **1** (7.3 mg) was refluxed with 2.2 mL 0.9 M HCl in 82% aqueous methanol at 80°C for 18 h. The resultant reaction mixture was extracted with *n*-hexane, and the combined organic layer was dried over Na_2SO_4. Concentration of the hexane yielded a fatty acid methyl ester, which was purified by silica gel CC with *n*-hexane/ethyl acetate (9:1–7:3, vol/vol) to give a methyl ester of fatty acid **1b** (2.6 mg) and then analyzed by GC–MS.

*Methyl (2*R*)-2-hydroxyheptanoate (**1b**).* The retention time (t_R) of **1b** was 14.3 min; white solid. $[\alpha]_D^{25}$ –4.1° (*c* 0.061, $CHCl_3$) [lit. (11) $[\alpha]_D^{24}$ – 3.6° ($CHCl_3$)]; IR (KBr) ν_{max} 3400 (OH), 2934, 1740 (C=O), 1465, 1284, 720 (methylenes) cm^{-1}; ^1H NMR 400 MHz ($CDCl_3$) δ ppm 4.19 (1H, *dd, J* = 4.2, 7.4 Hz, H-2), 3.79 (3H, *s*, $COOCH_3$), 2.74 (1H, *bs*, OH), 1.76 (1H, *m*, H-3), 1.63 (1H, *m*, H-3), 1.25 (*br s*, methylenes), and 0.88 (3H, *t, J* = 7.0 Hz, terminal methyl); EI-MS (70 eV) *m/z* (relative intensities, %) 300 $[M]^+$ (2), 241 $[M - COOMe]^+$ (13.5), 189 (2.4), 149 (12.2), 83 (34), 69 (52.2), 57 (72.6), and 43 (100).

*2-Acetoamino-1,3-diacetoxy-9-methyl-4,8-octadecanediene (**1c**).* The aqueous methanolic layer was neutralized with saturated $NaHCO_3$, concentrated to dryness, and extracted with ether. The ether phase was dried over Na_2SO_4, filtered, and then concentrated to yield a long-chain base (LCB), which was heated with acetic anhydride/pyridine (1:1, vol/vol) for 1.5 h at 70°C. The reaction mixture was diluted with water and then extracted three times with ethyl acetate. The residue of the ethyl acetate fraction was chromatographed over silica gel using *n*-hexane/ethyl acetate (8:2, vol/vol) as eluents to furnish a peracetate of the LCB (**1c**, 1.4 mg) as white solid. ^1H NMR 500 MHz ($CDCl_3$) δ ppm 5.78 (1H, *m*, H-5), 5.67 (1H, *d, J* = 9.2 Hz, NHAc), 5.42 (1H, *m*, H-4), 5.29 (1H, *m*, H-3), 5.08 (1H, *m*, H-8), 4.42 (1H, *m*, H-2), 4.29 (1H, *dd, J* = 11.6, 6.0 Hz, H-1a), 4.05 (1H, *dd, J* = 11.6, 3.4 Hz, H-1b), 2.05, 2.08 (each 3H, *s*, 2 × OAc), 2.03 (3H, *s*, HNAc), 1.95–2.15 (6H, *m*, H-6, H-7, and H-10), 1.58 (3H, *s*, H-19), 1.21–1.63 (12H, *m*), 0.88 (3H, *t, J* = 6.1 Hz, CH_3); EI-MS (70 eV) *m/z* (relative intensities, %) 438 $[M + 1, 2]^+$, 396 $[M + 1 - Ac]^+$ (4), 378 $[M + 1 - HOAc]^+$ (5), 318 $[M + 1 - 2 \times HOAc]^+$ (7), 284 (5.2), 268 (38), 185 (24.5), 144 $[AcOCH_2CHNHAc + H]^+$ (43), 102 $[144 - Ac + 1]^+$ (48), 84 $[144 - HOAc]^+$ (97.5), 69 (68), 55 (100).

1-O-*Methyl-*D*-glucopyranoside*. The remaining water layer was evaporated *in vacuo*. The residue was then chromatographed on silica gel using chloroform/methanol/water (7:3:0.5, by vol) to afford methyl glucopyranoside. $[\alpha]_D^{27}$ + 74.2° (*c* 0.01, methanol), [literature (12) $[\alpha]_D^{25}$ + 77.3° (*c* 0.1, methanol)]; negative FAB-MS *m/z* 193 $[M - 1]^-$.

*(2*S*,3*R*,4*E*,8*E*)-1-(β-*D*-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxypalmitoyl]amino-9-methyl-4,8-octadecadiene (= cerebroside B) (**2**)*. White amorphous powder. $[\alpha]_D^{27}$ +5.1° (*c* 0.3, methanol) IR (KBr) ν_{max}: 3380 (OH), 2960, 1650, 1540, 1000–1100, 720 cm^{-1}; negative FAB-MS *m/z* 726 $[M - 1]^-$, 564 $[M - 1 - 162]^-$; negative high resolution FAB-MS *m/z* 726.5561 $[M - 1]^-$ ($C_{41}H_{76}NO_9$, calcd. 726.55200). Methanolysis of **2** yielded a methyl 2-hydroxy palmitate (retention time 12.4 min) identified by GC–MS. The NMR (Table 2) and IR spectra of **2** were identical with those reported in the literature (8,18).

*(2*S*,3*R*,4*E*,8*E*)-1-(β-*D*-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyoctadecanoyl]amino-9-methyl-4,8-octadecadiene (= cerebroside D) (**3**)*. White amorphous powder. $[\alpha]_D^{27}$ +4.8° (*c* 0.2, methanol); IR (KBr) ν_{max}: 3385 (OH), 2960, 1650, 1541, 1000–1100, 721 cm^{-1}; negative FAB-MS *m/z* 754 $[M - 1]^-$, 592 $[M - 1 - 162]^-$. Methanolysis of **3** yielded a methyl 2-hydroxy stearate (retention time 16.2 min) identified by GC–MS. The NMR (Table 2) and IR spectra of **3** were identical with those reported in the literature (8,18).

RESULTS AND DISCUSSION

The chloroform-soluble part of the ethanol and chloroform/methanol extract from the fruiting bodies of *P. ellisii* was separated by normal-phase followed by reversed-phase CC to give compounds **1**, **2**, and **3**. The structural elucidation of new compound **1** was as follows.

Compound **1** was obtained as white amorphous powder, $[\alpha]_D^{26}$ +4.9° (*c* 0.40, methanol). The molecular formula of $C_{42}H_{79}NO_9$ for **1** was determined by negative high resolution FAB-MS at *m/z* 740.5659 $[M - H]^-$ (calcd. 740.5677). In the negative FAB-MS, compound **1** exhibited significant frag-

TABLE 2
^{1}H and ^{13}C NMR Data[a] for Compounds 2 and 3 in Pyridine-d_5

Atom no.	**2** δ ^{1}H (*J* in Hz)	δ ^{13}C (ppm)	**3** δ ^{1}H (*J* in Hz)	δ ^{13}C (ppm)
Long-chain base				
1	4.69 (*dd,* 5.4, 10.7)	70.05 *t*	4.69 (*dd,* 5.3, 10.8)	69.83 *t*
	4.20 (*m*)		4.20 (*m*)	
2	4.75 (*m*)	54.68 *d*	4.73 (*m*)	54.47 *d*
3	4.72 (*m*)	72.55 *d*	4.68 (*m*)	72.37 *d*
4	5.94 (*dd,* 15.3, 6.8)	131.85 *d*	5.93 (*dd,* 15.4, 5.8)	131.64 *d*
5	5.97 (*dt,* 15.3)	132.35 *d*	5.97(*dt,* 15.4)	132.22 *d*
6	2.14 (*m*)	33.04 *t*	2.15 (*m*)	32.86 *t*
7	2.14 (*m*)	32.12 *t*	2.15 (*m*)	31.93 *t*
8	5.25 (*m*)	124.17 *d*	5.23 (*m*)	123.99 *d*
9		135.51 *s*		135.64 *s*
10	2.00 (*br t,* 7.5)	39.99 *t*	1.98 (*br t,* 7.4)	39.80 *t*
11	1.36 (*m*)	28.35 *t*	1.35 (*m*)	28.17 *t*
12–15	1.25 (*br s*)	30.00–29.59 *t*	1.25 (*br s*)	29.80-29.41 *t*
16		32.12 *t*		31.93 *t*
17		22.91 *t*		22.73 *t*
18-CH_3	0.86 (*t,* 6.9)	14.24 *q*	0.84 (*t,* 6.4)	14.05 *q*
19-CH_3	1.61 (*s*)	16.12 *q*	1.59 (*s*)	15.93 *q*
NH	8.36 (*d,* 8.7)		8.33 (*d,* 8.7)	
N-acyl moiety				
1′		175.64 *s*		175.52 *s*
2′	4.57 (*m*)	72.40 *d*	4.55 (*dd,* 3.7, 7.4)	72.21 *d*
3′	1.74 (*m*), 2.14 (*m*)	35.66 *t*	1.75 (*m*), 2.15 (*m*)	35.46 *t*
4′–13′/15′	1.25 (*br s*)	30.00–29.59 *t*	1.25 (*br s*)	29.80–29.41 *t*
14′/16′		28.22 *t*		28.04 *t*
15′/17′		22.91 *t*		22.73 *t*
16′/18′-CH_3	0.86 (*t,* 6.9)	14.24 *q*	0.84 (*t,* 6.4)	14.05 *q*
Sugar moiety				
1″	4.90 (*d,* 7.6)	105.54 *d*	4.87(*d,* 7.8)	105.30 *d*
2″	4.03 (*m*)	75.07 *d*	4.00 (*m*)	74.87 *d*
3″	4.20 (*m*)	78.44 *d*	4.20 (*m*)	78.23 *d*
4″	4.19 (*m*)	71.63 *d*	4.18 (*m*)	71.45 *d*
5″	3.89 (*m*)	78.44 *d*	3.87 (*m*)	78.23 *d*
6″	4.48 (*br d,* 11.8)	62.75 *t*	4.47 (*dd,* 2.0, 11.9)	62.55 *t*
	4.33 (*dd,* 5.0, 11.8)			4.32 (*dd,* 5.3, 11.9)

[a]For abbreviation see Table 1.

ment peaks at *m/z* 740 $[M - H]^-$, 579 [M – H – 162 (glucosyl)]$^-$, and 561 $[M - 1 - 179]^-$. The IR spectrum of **1** showed absorption bands ascribable to hydroxyl at 3393 cm^{-1}, glycosidic (C–O) at 1037 cm^{-1}, a secondary amide at 1540 and 1650 cm^{-1}, and long aliphatic chains at 2921, 1469, and 721 cm^{-1}. The ^{1}H and ^{13}C NMR spectral data of **1** indicated the presence of a sugar, an amide, and long-chain aliphatic moieties, strongly suggesting the glycolipid nature of the molecule (Table 1).

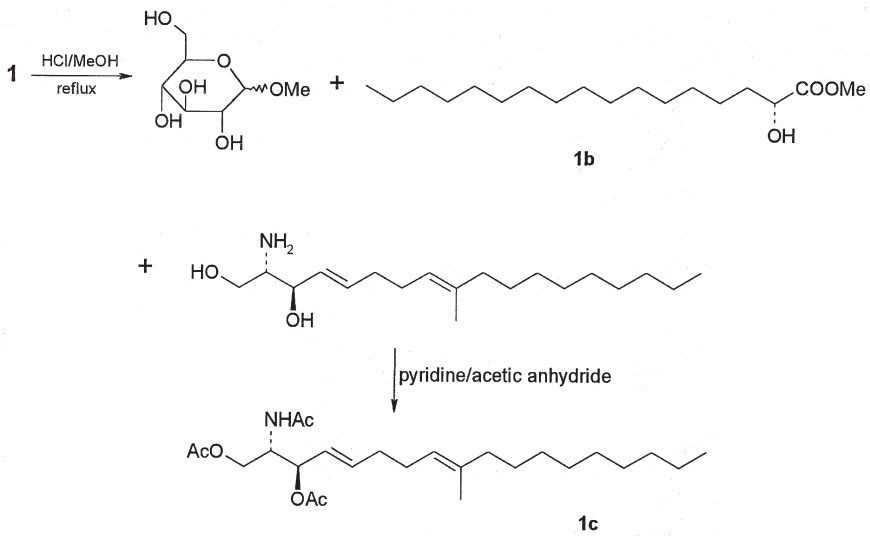
To determine the number of hydroxyl groups, compound **1** was acetylated with acetic anhydride/pyridine at room temperature to give its peracetate derivative **1a**, which showed a molecular ion peak at *m/z* 993 $[M]^+$ in its EI-MS, consistent with the composition $C_{54}H_{91}NO_{15}$ for **1a**. The existence of a fragment ion peak at *m/z* 663 [M – 331 (tetraacetyl hexose)]$^+$ confirmed hexose as the sugar residue. Meanwhile, the EI-MS data of **1a** also displayed the diagnostic fragments of the sugar moiety at *m/z* 331 (base peak), 271, 229, 211, 169, and 109, due to an acetylated glucopyranoside (13). Compound **1a** showed six acetyl signals at δ 2.15 (3H, *s*), 2.09 (3H, *s*), 2.03 (3H, *s*), 2.02 (6H, *s*), and 2.00 ppm (3H, *s*) in the ^{1}H NMR spectrum and at δ 20.5 (four *C*H_3CO), 20.9 (two *C*H_3CO) and at δ ppm 169.3 (two CH_3*C*O), 169.6 (three CH_3*C*O) and 170.0 (one CH_3*C*O) in the ^{13}C NMR spectrum, respectively. In addition to a fragment ion at *m/z* 663 [M – Glc(OAc)$_4$ + 1]$^+$ **1a** also provided typical fragment ions at *m/z* 933 $[M - HOAc]^+$ and 874 $[M - HOAc - OAc]^+$, thereby confirming the presence of six hydroxyl groups in the original structure of **1**.

On methanolysis (6,9), compound **1** yielded a fatty acid methyl ester, a mixture of α- and β-anomers of methyl glucoside, and an LCB (Scheme 1). The methyl ester **1b** was identified as methyl 2′**-**hydroxyheptanoate by the help of GC–MS analysis, with a molecular ion peak at *m/z* 300, corresponding to the composition $C_{18}H_{36}O_3$. Comparison of the ^{1}H NMR and optical rotation data ($[\alpha]_D^{27}$ – 4.5°) with those reported in the literature (11) led us to propose that the relative stereochemistry at C-2′ of the fatty acid methyl ester was *R*. That the optical rotation of the methyl glucoside, $[\alpha]_D^{27}$ +74.2° (determined on the methanolysis product from **1**), was close

to that of the authentic sample, $[\alpha]_D^{25}$ + 77.3° (12), indicated that glucose was present as its D-isomer.

In the ^{1}H NMR spectrum of **1** an anomeric signal indicative of the sugar moiety was observed at δ 4.91, and the coupling constant (*d, J* = 7.8 Hz) of this signal suggested the β-configuration of a glucoside linkage. The six oxygenated carbon signals at δ 105.4 (CH), 78.3 (CH), 78.3 (CH), 74.9 (CH), 71.5 (CH), and 62.6 (CH_2) in the ^{13}C NMR spectrum also supported the presence of the β-glucopyranoside moiety in **1** by comparison of the observed and reported chemical shifts (14). In addition, from the heteronuclear multiple bond correlation spectrum, the correlation between H-1″ [δ 4.91 (1H, *d*)] and C-1 [δ 69.9 (CH_2)] suggested that the glucose was attached to the C-1 position of the LCB.

The ^{1}H NMR data (Table 1) of **1** revealed the presence of two terminal methyls at δ 0.86 (6H, *t, J* = 7.0 Hz), an allylic methyl group at δ 1.61 (3H, *s*, H-19), methylene protons at δ 1.25 (*br s*), an amide proton signal at δ 8.36 (*d, J* = 8.6 Hz), an anomeric proton at δ 4.91 (*d, J* = 7.8 Hz), and carbinol protons appearing as multiplets between δ 3.90 and 4.75. A signal appearing at δ 4.81 (*m*, H-2) was assigned as a methine proton vicinal to the nitrogen atom, clearly suggesting a branched cerebroside containing a 2-hydroxy fatty acid (6,15). Furthermore, **1** was considered to possess a normal type of side chains since the carbon signals due to the terminal methyl groups were observed at δ 14.0 (normal form) in the ^{13}C NMR spectrum (16). The ^{13}C NMR spectrum of **1** exhibited carbon signals at δ 175.5 (carbonyl carbon), 54.5 (CHNH, C-2), 22.7–31.9 (methylene carbons), 14.0 (two terminal methyls, C-18 and C-17′), and 15.9 (an allylic methyl group, C-19), which further support the branching glycolipid nature of the molecule. Four olefinic carbon signals observed at δ 124.0 (CH), 131.7 (CH), 132.2 (CH), and 135.6 (quaternary carbon) suggested that **1** possessed two double bonds. In the ^{1}H-^{1}H homonuclear correlation spectroscopy spectrum, the correlation between H-4 and H-3, H-4 and H-5, H-5 and H-6, H-6 and H-7, H-7 and H-8 was observed. The above correlation analysis has thus unambiguously assigned the position of the two double bonds at C-4 and C-8, respectively. The analysis was further supported by HMBC spectrum of **1**, which displayed the correlation between H-6 and C-4, H-3 and C-5, H-7 and C-9, H-10 and C-8. On the other hand, the presence of an allylic methyl group (C-19) in the branched LCB was also confirmed by the HMBC spectrum in which the correlation between H-19 and C-8 was observed. The geometry of the C-4/C-5 alkene bond was determined to be *E* by the large vicinal coupling constant (*J* = 15.3 Hz) displayed between H-4 and H-5, as also evidenced by the ^{13}C NMR chemical shift of the methylene carbon C-6 (δ 32.9) next to the olefinic carbon (15) and the signals of olefinic protons (H-4 and H-5) that appear in the vicinity of δ 5.95 as a multiplet (17). When C-7 methylene protons were irradiated in an nuclear Overhauser effect (NOE) difference experiment, an NOE enhancement of the C-19 methyl protons was observed, arguing that the C-8/C-9 double bond was also assigned *E*. Furthermore, the ^{13}C NMR chemical shift of the C-19 methyl group (δ 15.9) in turn supported the assignment of this *trans* isomer, as demonstrated by comparison with the chemical shifts of the C-3 methyl groups in *E* (δ 15.4) and *Z* (δ 22.7) isomers of 3-methyl-3-hexene (18). It is thus clear that **1** possesses a branched sphingoid moiety with (4*E*,8*E*) geometry, 2-amino-1,3-dihydroxy-9-methyl-4,8-octadecanediene. In addition, treatment of the methanolysis product of **1** with acetic anhydride/pyridine at 70°C afforded production of a triacetyl LCB **1c**, which we suggest is 2-acetoamino-1,3-diacetoxy-9-methyl-4,8-octadecanediene on the bases of the molecular ion at *m/z* 438 and the ^{1}H NMR spectrum, which are consistent with those of the synthetic model compound (19). All of the above spectral evidence further supported that **1** is a cerebroside composed of a (4*E*,8*E*)-2-amino-1,3-dihydroxy-9-methyl-4,8-octadecanediene, (2*R*)-2-hydroxy fatty acid, and β-D-glucopyranose.



SCHEME 1

TABLE 3
^{1}H NMR Data and Optical Rotations of Cerebroside 1, Natural 4, Synthetic 5, and Two Derivatives 1a and 6[a]

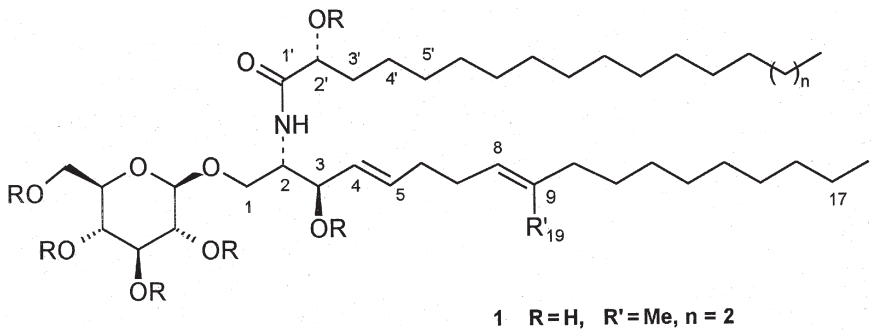
δ ^{1}H	**1** (pyridine-d_5)	**4** (pyridine-d_5)	**5** (pyridine-d_5)	**1a** ($CDCl_3$)	**6** ($CDCl_3$)
1-Ha	4.71 (*dd,* 6.0, 10.5)	4.71 (*dd,* 5.9, 10.3)	4.69 (*dd,* 5.4, 10.7)	3.93 (*dd,* 4.0 10.3)	4.04 (*dd,* 3.7, 10.2)
1-Hb	4.23 (*dd,* 6.8, 10.4)	4.23 (*m*)	4.20 (*m*)	3.61 (*dd,* 4.5, 10.3)	3.64 (*dd,* 4.4, 10.0)
2-H	4.81 (*m*)	4.80 (*m*)	4.76 (*m*)	4.31 (*m*)	4.31 (*m*)
3-H	4.75 (*m*)	4.75 (*m*)	4.76 (*m*)	5.32 (*dd,* 5.3, 6.8)	5.28 (*m*)
2′-H	4.57 (*dd,* 5.2, 7.8)	4.57 (*m*)	4.57 (*m*)	5.15 (*dd,* 4.7, 7.1)	4.95 (*dd,* 5.4, 6.3)
$[\alpha]_D$	+4.9° (MeOH)	+7.0° (*n*-PrOH)	+5.4° (MeOH)	+5.6° ($CHCl_3$)	+8.8° ($CHCl_3$)

[a]*J* is given in Hz, in parentheses. δ are in ppm. For abbreviation see Table 1

The relative stereochemistry at C-2 and C-3 in **1** was presumed as 2*S*,3*R* (*erythro*) which was shown to be the same as that of the natural cerebrosides **4**, which are phallusides isolated from the ascidian *Phallusia fumigata* (11) and of synthetic glucosyl-(2*S*,3*R*)-sphingadienine **5** (20). The chemical shifts and coupling constants of H-1, H-2, H-3, and H-2′ in **1** and **1a** are in agreement with those of natural **4** and synthetic **5**, synthetic precursor of **5**, (2*S*,3*R*,4*E*,8*E*, 2′*R*)-2-(2′-acetoxyhexadecanoyl)amino-3-*O*-acetyl-1-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-4,8-octadecadien-1,3-diol **6** (20) (Table 3). Moreover, the specific rotations of **1** ($[\alpha]_D^{26}$ +4.9°) and **1a** ($[\alpha]_D^{26}$ +5.6°) are also in accordance with those of natural **4** ($[\alpha]_D^{26}$ +7.0°) and synthetic **5** ($[\alpha]_D^{20}$ +5.4°) and **6** ($[\alpha]_D^{26}$ +8.8°). These data suggest that **1** has the same absolute configuration as that of natural **4** and synthetic **5** for the core structure at chiral centers 2, 3, and 2′. On the basis of the above evidence, the structure of **1** was therefore established as (2*S*,3*R*,4*E*,8*E*)-1-(β-D-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyheptadecanoyl]amino-9-methyl-4,8-octadecadiene (Scheme 2).

Compounds **2** and **3** have the same ^{1}H and ^{13}C NMR (Table 3) data in addition to IR absorptions as **1**, indicating that both **2** and **3** are 9-methyl-sphinga-4,8-dienine-type cerebrosides possessing 2-hydroxy fatty acid and β-D-glucopyranose moieties. The molecular formulas of **2** and **3** were determined as $C_{41}H_{77}NO_9$ and $C_{43}H_{81}NO_9$, respectively, by negative high-resolution FAB-MS and ^{13}C NMR data. Further methanolysis of both yielded the corresponding fatty acid methyl esters, namely, methyl 2-hydroxy palmitate and methyl 2-hydroxy stearate, which were identified by GC–MS. From the above evidence and comparison of the physicochemical properties with the reported data, compounds **2** and **3** were characterized as (2*S*,3*R*,4*E*,8*E*)-1-(β-D-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxypalmitoyl]amino-9-methyl-4,8-octadecadiene (= cerebroside B), and (2*S*,3*R*,4*E*,8*E*)-1-(β-D-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyoctadecanoyl] amino-9-methyl-4,8-octadecadiene (= cerebroside D) (Scheme 2), respectively, which were previously obtained from a basidiomycete, *R. ochroleuca* (8) and an imperfect fungus *Pachybasium* sp. (18); both had antifungal activity.

Sphingolipids are ubiquitous membrane constituents of animals, plants, and also lower forms of life, the principal component of which is the LCB or sphingoid base. In nature, the most widely occurring sphingoid base is D-*erythro*-4(*E*)-sphyingenine, whereas branched (4*E*,8*E*)-sphingadienines having two double bonds in the hydrocarbon chain are minor sphingoid bases. The present study has demonstrated the presence in *P. ellisii* of a previously unrecognized cerebroside and two known cerebrosides, consisting of 9-methyl-4,8-sphingadienine in amide linkage with a hydroxy fatty acid and in β-glycosidic bond with glucose, respectively. The new cerebroside belongs to the first class of GSL (which have their sugar portion linked directly to the ceramide by a glycoside), and contains a fairly unusual dienic LCB with a methyl branch at C-9. This cerebroside has been found in a unique marine microorganism *Thraustochytrium globosum* (15), an



1 R = H, R' = Me, n = 2
1a R = Ac R' = Me, n = 2
2 R = H, R' = Me, n = 1
3 R = H, R' = Me, n = 3
5 R = H, R' = H, n = 1

SCHEME 2

imperfect fungus *Pachybasium* sp. (18), a pathogenic fungus *Fusicoccum amygdali* (21), and a marine animal sea anemone *Metridium senile* (22). The natural occurrence of molecules of this species from higher fungi has also been reported (8). Thus, the branched nonadecasphingadienine is presumed to be a characteristic component in cerebrosides from lower organisms. From the viewpoint of comparative biochemistry, it will be of considerable interest to elucidate fully its distribution and also to investigate the physiological significance of the 9-methyl branch, as well as the biosynthetic pathway.

It should be noted that the occurrence of structurally closely related sphingolipid derivatives in taxonomically remote species is very intriguing and may indicate the connection with a common producer, probably symbiotic microorganisms.

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REFERENCES

1. Kolter, T., and Sandhoff, K. (1999) Sphingolipids—Their Metabolic Pathways and the Pathobiochemistry of Neurodegenerative Diseases, *Angew. Chem. Int. Ed. Engl. 38*, 1532–1568.
2. Hannun, Y.A., and Bell, R.M. (1989) Functions of Sphingolipids and Sphingolipid Breakdown Products in Cellular Regulation, *Science 243,* 500–507.
3. Okuyama, E., and Yamazaki, M. (1983) The Principles of *Tetragonia tetragonoides* Having Anti-ulcerogenic Activity. II. Isolation and Structure of Cerebrosides, *Chem. Pharm. Bull. 31*, 2209–2211.
4. Shibuya, H., Kawashima, K., Sakagami, M., Kawanishi, H., Shimomura, M., Ohashi, K., and Kitagawa, I. (1990) Sphingolipids and Glycerolipids. I. Chemical Structures and Ionophoretic Activities of Soyacerebrosides I and II from Soybean, *Chem. Pharm. Bull. 38*, 2933–2938.
5. Kim, S.Y., Choi, Y.-H., Huh, H., Kim, J., Kim, Y.C., and Lee, H.S. (1997) New Antihepatotoxic Cerebroside from *Lycium chinense* Fruits, *J. Nat. Prod. 60*, 274–276.
6. Natori, T., Morita, M., Akimoto, K., and Koezuka, Y. (1994) Agelasphins, Novel Antitumor and Immunostimulatory Cerebrosides from the Marine Sponge *Agelas mauritianus*, *Tetrahedron 50*, 2771–2784.
7. Yamada, K., Harada, Y., Miyamoto, T., Isobe, R., and Higuchi, R. (2000) Constituents of Holothuroidea. Isolation and Structure of a New Ganglioside Molecular Species from the Sea Cucumber *Holothuria pervicax*, *Chem. Pharm. Bull. 48*, 157–159.
8. Gao, J.M., Dong, Z.J., Yang, X., and Liu, J.K. (2001) Constituents of the Basidiomycete *Russula ochroleuca, Acta Bot. Yunnanica 23*, 86–91.
9. Gao, J.M., Dong, Z.J., and Liu, J.K. (2001) A New Ceramide from the Basidiomycete *Russula cyanoxantha*, *Lipids 36*, 175–180.
10. Gao, J.M., Dong, Z.J., and Liu, J.K. (2001) A New Ceramide from the Basidiomycete *Armillaria mellea*, *Chin. Chem. Lett. 12*, 139–140.
11. Duran, R., Zubia, E., Ortega, M.J., Naranjo, S., and Salva, J. (1998) Phallusides, New Glucosphingolipids from the Ascidian *Phallusia fumigata*, *Tetrahedron 54*, 14597–14602.
12. Jin, W., Rinehart, K.L., and Jares-Erijman, E.R. (1994) Ophidiacerebrosides: Cytotoxic Glycosphingolipids Containing a Novel Sphingosine from a Sea Star, *J. Org. Chem. 59*, 144–147.
13. Biemann, K., DeJongh, D.C., and Schnoes, H.K. (1963) Application of Mass Spectrometry to Structure Problems. Acetates of Pentoses and Hexoses, *J. Am. Chem. Soc. 85*, 1763–1768.
14. Walker, T.E, London, R.E., Whaley, T.W., Barker, R., and Matwiyoff, N.A. (1976) Carbon-13 Nuclear Magnetic Resonance Spectroscopy of [1-^{13}C] Enriched Monosaccharides. Signal Assignments and Orientational Dependence of Geminal and Vicinal Carbon-Carbon and Carbon-Hydrogen Spin-Spin Coupling Constants, *J. Am. Chem. Soc. 98*, 5807–5813.
15. Jenkins, K.M., Jensen, P.R., and Fenical, W. (1999) Thraustochytrosides A–C: New Glycosphingolipids from a Unique Marine Protist, *Thraustochytrium globosum*, *Tetrahedron 40*, 7637–7640.
16. Higuchi, R., Natori, T., and Komori, T. (1990) Isolation and Characterization of Acanthacerebroside B and Structure Elucidation of Related, Nearly Homogeneous Cerebrosides, *Liebigs Ann. Chem.,* 51–55.
17. Higuchi, R., Inagaki, M., Togawa, K., Miyamoto, T., and Komori, T. (1994) Isolation and Structure of Cerebrosides of the Sea Cucumber *Pentacta australis*, *Liebigs Ann. Chem.,* 653–658.
18. Sitrin, R.D., Chan, G., Dingerdissen, J., DeBrosse, C., Mehta, R., Roberts, G., Rottschaefer, S., Staiger, D., Valenta, J., Snader, K.M., Stedman, R.J., and Hoover, J.R.E. (1988) Isolation and Structure Determination of *Pachybasium* Cerebrosides Which Potentiate the Antifungal Activity of Aculeacin, *J. Antibiot. 41*, 469–480.
19. Wang, X.-Z., Wu, Y.-L., Jiang, S., and Singh, G. (1999) Synthesis of (2*S*,3*R*,4*E*,8*E*)-9-Methyl-4,8-sphingadienine *via* a Novel S_N2 Type Reaction Mediated by a Thioether Carbanion, *Tetrahedron Lett. 40*, 8911–8914.
20. Murakami, T., Shimizu, T., and Taguchi, K. (2000) Synthesis of Sphingadienine-type Glucocerebrosides, *Tetrahedron 56*, 533–545.
21. Ballio, A., Casinovi, C.G., Framondino, M., Marino, G., Nota, G., and Santurbano, B. (1979) A New Cerebroside from *Fusicoccum amygdali* Del., *Biochim. Biophys. Acta 573*, 51–60.
22. Karlsson, K.-A., Leffler, H., and Samuelsson, B.O.E. (1979) Characterization of Cerebroside from the Sea Anemone, *Metridium senile*, *Biochim. Biophys. Acta 574*, 79–93.

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