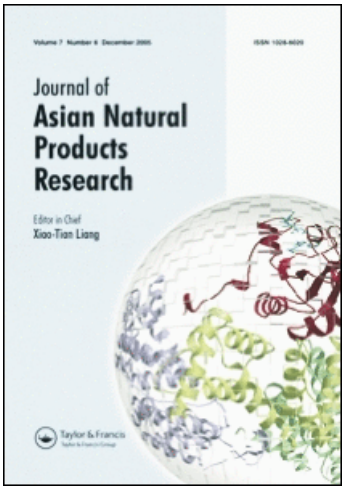

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Ting Jiang[a]; Tian Li[b]; Jun Li[a]; Hong-Zheng Fu[a]; Yue-Hu Pei[c]; Wen-Han Lin[a]
[a] State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing, China [b] Shangdong Key Laboratory of Marine Biological Active Substance, SOA, Qingdao, China [c] Department of Phytochemistry, Shenyang Pharmaceutical University, Shenyang, China

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CEREBROSIDE ANALOGUES FROM MARINE-DERIVED FUNGUS *ASPERGILLUS FLAVIPES*

TING JIANG[a], TIAN LI[b], JUN LI[a], HONG-ZHENG FU[a], YUE-HU PEI[c] and WEN-HAN LIN[a,*]

[a]*State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100083, China;*
[b]*Shangdong Key Laboratory of Marine Biological Active Substance, SOA, Qingdao, 266061, China;*
[c]*Department of Phytochemistry, Shenyang Pharmaceutical University, Shenyang, 110015, China*

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From the mycelium of the marine-derived fungus *Aspergillus flavipes*, isolated from the sea anemone *Anthopleura xanthogrammica*, two new cerebroside analogues, namely flavicerebrosides A (**1**): [(2*S*,2′*R*,3*R*,4*E*,8*E*)-*N*-2′-hydroxyoctadecanoyl-1-*O*-β-D-galactopyranosyl-9-methyl-4,8-sphingadienine], and B (**2**): [(2*S*,2′*R*,3*R*,3′*E*,4*E*,8*E*)-*N*-2′-hydroxy-3′-octadecenoyl-1-*O*-β-D-galactopyranosyl-9-methyl-4,8-sphingadienine], together with two known glycosphingolipids cerebrosides D (**3**) and C (**4**), were isolated. Their structures were identified by means of extensive spectroscopic analysis (IR, UV, 2D NMR, MS, CD) and chemical degradation. All four compounds showed cytotoxic activity against the KB cell line.

Keywords: Fungus; *Aspergillus flavipes*; Flavicerebrosides A and B

INTRODUCTION

The bioactive metabolites of the sea anemone *Anthopleura xanthogrammica* have been studied extensively, of which the polypeptides anthopleurin A and B [1–3] exerted a potent selective, positive inotropic effect on the mammalian heart without affecting heart rate or blood pressure [4–8]. So far, no chemical studies on sea-anemone-derived microorganisms have been reported. In continuation of our screening for bioactive components from marine organisms, the fungus *Aspergillus flavipes* was isolated from the sea anemone *Anthopleura xanthogrammica* collected in Qingdao Bay, eastern China Sea. *Aspergillus flavipes* isolates obtained from soil were reported to contain structurally diverse alkaloids [9,10], of which spiroquinazoline possessed substance P inhibitory properties, but until now no investigations of secondary metabolites from *A. flavipes* isolated from marine organisms have been published. By bio-assay guided fractionation of a culture of the marine-derived fungus *A. flavipes*, four cerebroside analogues were separated and purified from fractions displaying cytotoxic activity. Although there are several publications that describe cerebrosides from microorganisms [11–16], this is the first report of cerebrosides from a marine-derived

*Corresponding author. Tel.: +86-10-62062210. Fax: +86-10-62092724. E-mail: whlin@bjmu.edu.cn

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fungus. It is interesting to note that the cerebrosides from *A. flavipes* obtained in the present study are very similar to those isolated from the sea anemone *Metridium senile* [17], and all cerebrosides containing unusual 9-methyl-4,8-sphingadiene as long-chain base, which were discovered mainly in fungi [11,14–16]. Cerebrosides C (**4**) and D (**3**) were originally from *Pachybasium* sp. [16], and cerebroside C was also obtained from *Penicillium funiculosum* [18], *Magnaporthe grisea* [19], and *Fusarium solani* [20]. Flavicerebrosides A (**1**) and B (**2**) were shown to be new compounds. Here we report the isolation and structure elucidation of these novel cerebrosides.

RESULTS AND DISCUSSION

The cultivated mycelium was extracted with 95% EtOH, and the extract was fractionated by silica gel vacuum column chromatography and eluted with a $CHCl_3$–MeOH gradient. A polar fraction which showed cytotoxicity (IC_{50} 21.3 $\mu g\,mL^{-1}$) against the KB cell line was subjected to extensive column chromatography and thus afforded four glycosphingolipids (**1**–**4**).

Analysis by IR, ^1H and ^{13}C NMR (Tables I and II), 2D NMR (^1H–^1H COSY, HMQC and HMBC), ESI-MS/MS spectral data (Fig. 1) as well as optical rotations of glycosphingolipids **3** and **4** showed that they were identical to cerebrosides D and C [16] respectively. Although previous work on the assignment of asymmetric centers in cerebrosides C and D has been reported [16], methanolysis of **3** and **4** respectively in

TABLE I ^1H NMR spectral data for compounds **1**–**4**

H	*3*	*4*	*1*	*2*
H-1A	3.89 (dd, 10.2, 3.1)	3.92 (dd, 10.4, 2.9)	3.89 (dd, 10.2, 3.1)	3.94 (dd, 10.2, 3.2)
H-1B	3.51 (dd, 10.2, 3.1)	3.50 (dd, 10.4, 2.9)	3.51 (dd, 10.2, 3.1)	3.50 (dd, 10.2, 3.2)
H-2	3.78 (dddd, 3.1, 3.1,7.9, 9.3)	3.76 (dddd, 2.9, 2.9,7.9, 9.3)	3.78 (dddd, 3.1, 3.1,7.9, 9.6)	3.76 (dddd, 3.2, 3.2, 7.9, 9.0)
H-3	3.95 (dd, 5.9, 7.9)	3.94 (dd, 6.0, 7.9)	3.95 (dd, 6.0, 7.9)	3.94 (dd, 6.0, 7.9)
H-4	5.38 (dd, 5.9, 16.8)	5.36 (dd, 6.0, 15.8)	5.39 (dd, 6.0, 16.8)	5.33 (dd, 6.0, 16.8)
H-5	5.54 (dt, 6.0, 16.8)	5.66 (m)	5.54 (dt, 5.9, 16.8)	5.66 (m)
H-6	1.93 (m)	1.94 (m)	1.93 (m)	1.93 (m)
H-7	1.95 (m)	1.95 (m)	1.95 (m)	1.95 (m)
H-8	5.07 (dd, 9.0, 3.2)	5.08 (dd, 9.1, 3.0)	5.07 (dd, 9.1, 3.2)	5.07 (dd, 9.2, 3.2)
H-10	1.87 (t, 5.3)	1.92 (t, 5.3)	1.87 (t, 5.4)	1.92 (t, 5.4)
H-11–16	1.20–1.60 (m)	1.21–1.60 (m)	1.20–1.61 (m)	1.20–1.61 (m)
H-17	1.22 (brs)	1.22 (brs)	1.21 (brs)	1.21 (brs)
H-18	0.84 (t, 6.9)	0.84 (t, 6.8)	0.83 (t, 6.9)	0.83 (t, 6.9)
H-19	1.52 (s)	1.52 (s)	1.51 (s)	1.51 (s)
H-2′	3.79 (m)	4.28 (m)	3.79 (m)	4.28 (m)
H-3′	1.30 (m)	5.45 (dd, 15.6, 6.0)	1.31 (m)	5.44 (dd, 5.8, 16.0)
H-4′	1.30 (m)	5.66 (m)	1.30 (m)	5.66 (m)
H-5′	1.51 (m)	1.98 (m)	1.53 (m)	1.97 (m)
H-6′–16′	1.20–1.40 (m)	1.42 (m)	1.20–1.42 (m)	1.41 (m)
H-17′	1.22 (brs)	1.22 (brs)	1.21 (brs)	1.21 (brs)
H-18′	0.84 (t, 6.9)	0.84 (t, 6.8)	0.83 (t, 6.9)	0.83 (t, 6.9)
N-H	7.40 (d, 9.3)	7.38 (d, 9.3)	7.40 (d, 9.6)	7.40 (d, 9.0)
H-1″	4.10 (d, 7.8)	4.11 (d, 7.8)	4.04 (d, 6.6)	4.05 (d, 6.6)
H-2″	2.93 (m)	2.94 (m)	3.30 (m)	3.30 (m)
H-3″	3.09 (m)	3.09 (m)	3.25 (m)	3.25 (m)
H-4″	3.06 (m)	3.05 (m)	3.60 (m)	3.60 (m)
H-5″	3.14 (m)	3.14 (m)	3.35 (m)	3.34 (m)
H-6a″	3.64 (m)	3.64 (m)	3.49 (m)	3.49 (m)
H-6b‴	3.33 (m)	3.33 (m)	3.46 (m)	3.45 (m)

Coupling constants (*J*) in Hz are indicated in parentheses.

TABLE II ^{13}C NMR spectral data for compounds **1**–**4**

C	*3*	*4*	*1*	*2*
C-1	68.8 (t)	68.7 (t)	68.6 (t)	68.6 (t)
C-2	52.8 (d)	52.9 (d)	52.8 (d)	52.9 (d)
C-3	70.6 (d)	70.5 (d)	70.6 (d)	70.6 (d)
C-4	131.1 (d)	130.9 (d)	131.1 (d)	130.8 (d)
C-5	131.0 (d)	130.9 (d)	131.1 (d)	130.9 (d)
C-6	32.2 (t)	32.2 (t)	32.2 (t)	32.2 (t)
C-7	27.4 (t)	27.4 (t)	27.4 (t)	27.4 (t)
C-8	123.5 (d)	123.5 (d)	123.5 (d)	123.5 (d)
C-9	134.9 (s)	134.9 (s)	134.9 (s)	134.9 (s)
C-10	39.5 (t)	39.5 (t)	39.5 (t)	39.5 (t)
C-11–16	28.0–32.1 (t)	28.2–32.1 (t)	28.1–32.2 (t)	28.1–32.2 (t)
C-17	22.1 (t)	22.1 (t)	22.1 (t)	22.1 (t)
C-18	13.9 (q)	13.9 (q)	13.9 (q)	13.9 (q)
C-19	15.7 (q)	15.7 (q)	15.7 (q)	15.7 (q)
C-1′	173.8 (s)	172.0 (s)	173.7 (s)	172.0 (s)
C-2′	71.0 (d)	71.9 (d)	71.0 (d)	71.9 (d)
C-3′	32.4 (t)	129.1 (d)	32.4 (t)	129.1 (d)
C-4′	29.3 (t)	130.9 (d)	29.4 (t)	131.0 (d)
C-5′–16′	28.5–32.3 (t)	27.2–31.5 (t)	28.4–32.3 (t)	27.1–32.7 (t)
C-17′	22.1 (t)	22.1 (t)	22.1 (t)	22.1 (t)
C-18′	13.9 (q)	13.9 (q)	13.9 (q)	13.9 (q)
C-1″	103.5 (d)	103.5 (d)	104.1 (d)	104.2 (d)
C-2″	73.4 (d)	73.4 (d)	70.6 (d)	70.5 (d)
C-3″	76.9 (d)	76.9 (d)	73.3 (d)	73.3 (d)
C-4″	70.0 (d)	70.0 (d)	68.1 (d)	68.1 (d)
C-5″	76.6 (d)	76.6 (d)	75.3 (d)	75.3 (d)
C-6″	61.1 (t)	61.1 (t)	61.4 (t)	60.4 (t)

Multiplicity is deduced by DEPT.

MeOH–HCl gave rise to the same long-chain base (LCB), and the CD spectrum of the tribenzoylated derivative of the LCB (CE: λ 236 nm, Δε −6.12; λ 221 nm, Δε +2.98) revealed the 2*S*,3*R* stereochemistries [12,21], which are opposite to that reported in the literature.

Flavicerebroside A (**1**) showed the same molecular formula as **3** by HR-FABMS. Its IR, ^{1}H, ^{13}C NMR and MS spectra were almost superimposable on those of **3**. However, **1** differed from **3** in the hexose moiety, which was identified as galactose based on 1D TOCSY and ^{13}C NMR spectra as well as TLC of an acidic hydrolysate in comparison with authentic galactose. It was reported that cerebrosides give characteristic fragments of $[LCB + H - H_2O]^+$ and $[LCB + H]^+$ in positive mode FABMS [12,22] and lithiated ESIMS measurement [23]. The ESI-MS/MS spectra of **1**–**4** afforded the same fragment ions of *m/z* 294 $[LCB + H]^+$ and *m/z* 276 $[LCB + H - H_2O]^+$ attributed to $[C_{19}H_{36}NO]^+$ and $[C_{19}H_{34}N]^+$ respectively, indicating that **1**–**4** possess the same LCB moiety. In the HMBC spectrum, the anomeric proton H-1″ (δ 4.04, d, $J = 6.6$ Hz) correlated to oxygenated carbon (C-1, δ 68.6 (t)). Thus the sugar was attached to C-1 and adopted a β-configuration. Moreover, two double bonds were proved to be located at C-4 and C-8 of LCB due to the correlations of H-2 (δ 3.78, dddd, $J = 3.1$, 3.1, 7.9, 9.6 Hz) and H-3 (δ 3.95, dd, $J = 6.0$, 7.9 Hz) with C-4 (δ 131.1, d), as well as correlations of H-5 (δ 5.54, dt) and H-8 (δ 5.07, dd) with C-6 (δ 32.2, t) and C-7 (δ 27.4, t). The absolute configuration of **1** was assumed to be the same as that of **3** by comparison of the optical rotation of FAM [12,24], the CD spectrum of the tribenzoylated LCB as well as the ^{1}H and ^{13}C NMR data (Tables I and II). Thus, the structure of flavicerebroside A (**1**) was determined as

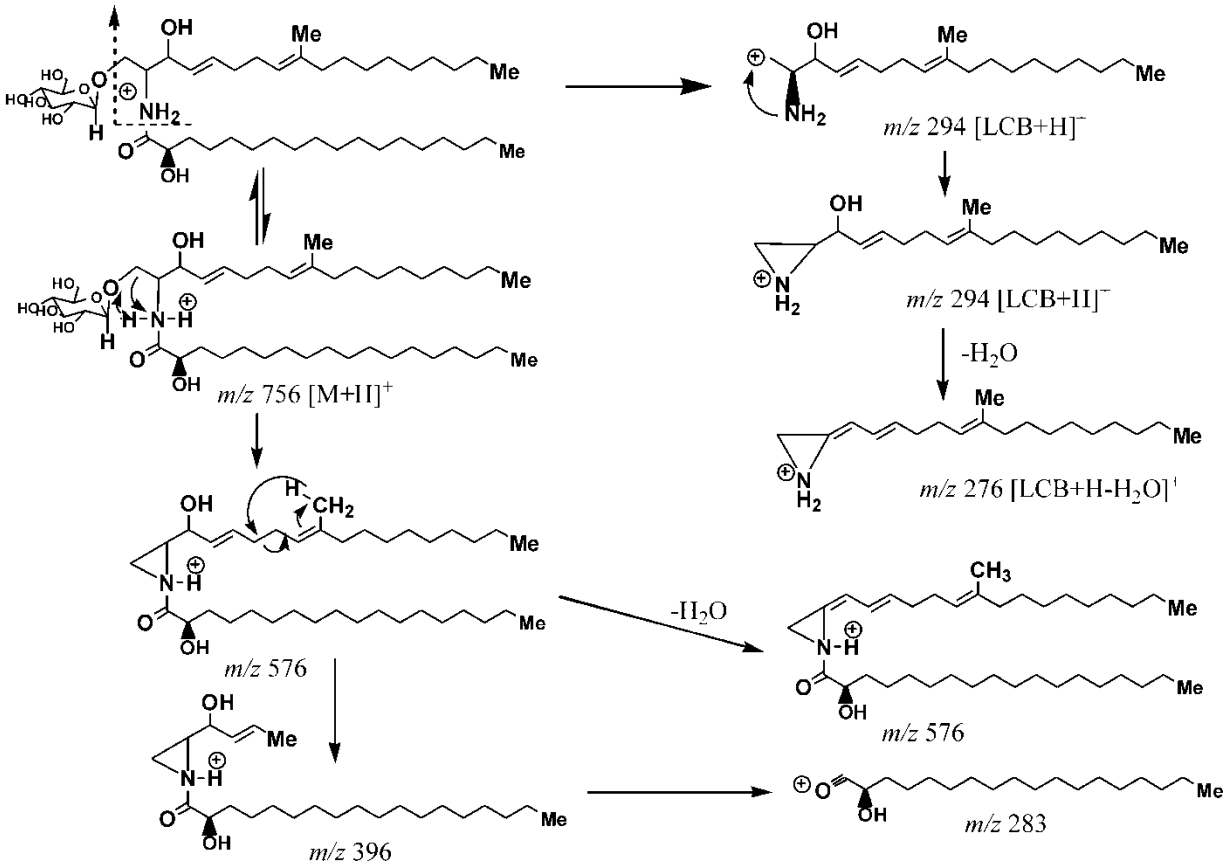
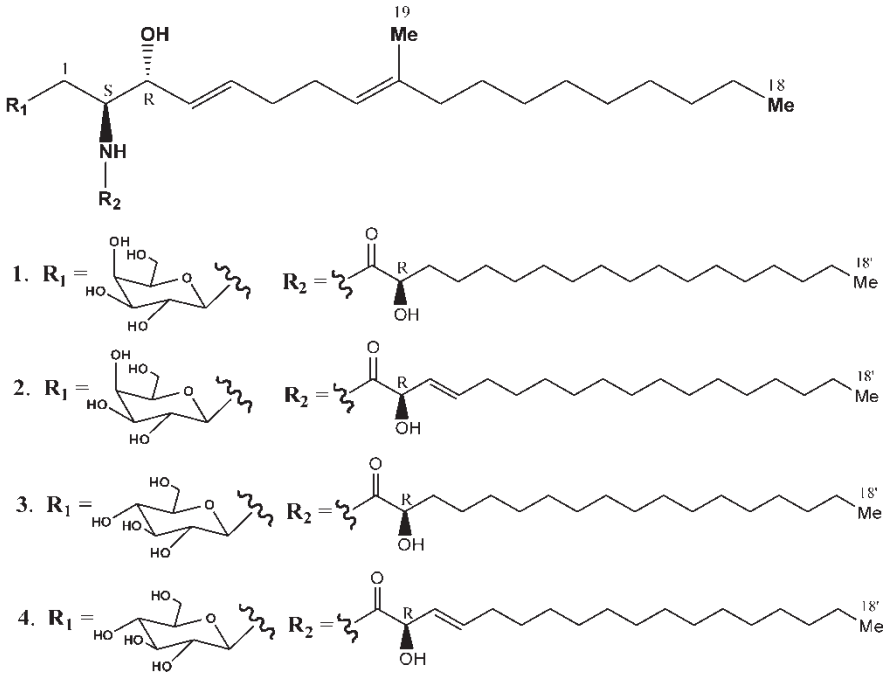


FIGURE 1 Proposed main MS cleavage pathway of cerebroside D (**3**).

(2*S*,2′*R*,3*R*,4*E*,8*E*)-*N*-2′-hydroxyoctadecanoyl-1-*O*-β-D-galactopyranosyl-9-methyl-4,8-sphingadienine.



The molecular formula of **2** was afforded by HR-FABMS measurement and was the same as that of **4**. The ^{1}H and ^{13}C NMR spectra of **2** closely resembled those of **4**, except for the ^{1}H and ^{13}C NMR data of the sugar moiety, which were comparable to the galactopyranose as described in **1**. The $^{1}H-^{1}H$ COSY, TOCSY, HMQC and HMBC spectra indicated that **2** was the corresponding galactose analogue of **4**. The locations of two double bonds at LCB were confirmed to be identical to those of **1** since the HMBC spectrum of **2** showed similar correlations as that of **1**. A double bond in FAM was considered to be at C-3′ based on the HMBC correlations of H-3′ (δ 5.44, dd) with C-1′ (δ 172.0, s) and C-2′ (δ 71.90, d) as well as correlation of H-4′ (δ 5.66, m) with C-2′. The absolute configuration of the ceramide partial structure in **2** was identified as being the same as that of **4** due to their almost identical optical rotations, NMR data, as well as similar CD spectra of the tribenzoylated LCB derivative. Flavicerebroside B (**2**) was therefore determined as (2*S*,2′*R*,3*R*,3′*E*,4*E*,8*E*)-*N*-2′-hydroxy-3′-octadecenoyl-1-*O*-β-D-galactopyranosyl-9-methyl-4,8-sphingadienine.

The antitumour activities of compounds **1**–**4** were tested *in vitro* using the KB cell line derived from a human oral epidermoid carcinoma as a preliminary screening for their biological activity. The test results showed that the IC_{50}s of the compounds **1** to **4** were 20.7, 14.3, 17.8 and 12.9 $\mu g\,mL^{-1}$ respectively. The bioassay results implied that the double bond in the fatty acid moiety increased the potency of tumour cell inhibition.

EXPERIMENTAL

General Experimental Procedures

Optical rotations were measured at 25°C with a WZZ-15 automatic polarimeter. Melting points (uncorrected) were measured using a Kober apparatus. All ^{1}H and ^{13}C NMR spectra

were recorded on a Bruker AC-300 spectrometer at 300 and 75 MHz in DMSO-d_6 respectively. The 2.49 ppm resonance of residual DMSO and 39.5 ppm of DMSO-d_6 were used as internal references. All 2D NMR data were acquired using a Bruker AMX-500 spectrometer at 500 MHz. IR spectra were acquired using a Perkin-Elmer 559B spectrophotometer. HR-FABMS were measured on a KYKY-2HP-5# spectrometer. EIMS spectra were measured on a VG-MM7070 spectrometer and an AEI MS-50 spectrometer. ESI-MS/MS spectra were measured on a MDS SCIEX QSTAR spectrometer (ion source: Turbo Ion Spray; needle voltage: +3.8 kV; DP: 40 eV; CE: 35 eV; collision gas: N_2); FABMS spectra were obtained in the positive mode using glycerol as a matrix. CD spectra were recorded on a JASCO J-500C spectropolarimeter. Silica gel H (10–40 μm, 200–300 mesh) for column chromatography was purchased from Qingdao Chemical Industry in Qingdao, China. Preparative HPLC was performed using an ODS column (Alltech ODS C_{18}, 10 × 250 mm, 5 μm, UV detector at 210 nm).

Fungal Material

The fungus *Aspergillus flavipes* was isolated from fresh tissue of the sea anemone *Anthopleura xanthogrammica*, collected in Qingdao beach, China. Under sterile conditions, tissue samples were taken from inside of the anemone body and were inoculated at 28°C. From the growing cultures, pure strains of *Aspergillus flavipes* were isolated by re-inoculation on malt agar plate (yeast extract 0.4%, malt extract 1%, glucose 0.4%, agar 2%, prepared in 50% artificial seawater). The fungus was identified by Dr Li Tian of the Qingdao Institute of Marine Bioactive Materials, and a voucher strain (no. QA-1) was deposited at the State Key Laboratories of Natural and Biomimetic Drugs, Peking University. The fungal isolate was cultured in 18 replicate 1 L Fernbach flasks in a marine fungal medium (PDA: 200 mL potato extract, 10 g glucose, 1 g yeast extract, 2 g pepton, 15 g NaCl, 1.2 g $MgCl_2 \cdot 6H_2O$, 0.15 g KCl in 1000 filtrate water, and the pH of the medium was adjusted to 6.5). The flasks were cultured on a rotary shaker for 6 days at 25°C.

Isolation and Separation

The culture broth (18 L) was filtered and the mycelium (400 g wet weight) was extracted with 95% EtOH (500 mL × 3) and evaporated under reduced pressure to give a residue (32 g). The extract was then subjected to silica gel VLC using 10% stepwise elution with a $CHCl_3$ and MeOH gradient to yield 10 crude fractions (1–10). Fraction 7 (1 g) was chromatographed on silica gel with $CHCl_3$–MeOH–H_2O (90:10:0.5) as eluent to give 10 fractions, and the collected fractions 7 to 10 (150 mg) were combined and chromatographed on a Sephadex LH-20 column with MeOH. The second collected fraction (120 mg) was subjected to silica gel column chromatography with EtOAC–EtOH–H_2O (20:2:1) as eluent to obtain two fractions (A and B) each of which showed single spots on normal-phase thin-layer chromatography (TLC). Each fraction could readily be resolved into two major components by analytical reversed-phase HPLC using methanol as an eluent and monitoring UV absorption at 214 nm. The separation was scaled up by preparative reversed-phase HPLC (97% MeOH–H_2O), 2.0 mL min^{-1}, UV detection at 210 nm) to yield compounds **4** (12.5 mg) and **2** (7.8 mg) from fraction A, and compounds **1** (6.7 mg) and **3** (6.9 mg) from fraction B.

Degradation of 3

Compound **3** (5 mg) was refluxed in a solution of 12 M HCl (0.2 mL) and 82% MeOH (5 mL) for 24 h. The reaction mixture was extracted with cyclohexane (3 × 4 mL), and

the cyclohexane layer was washed with water, dried, and purified by silica gel column, eluting with EtOAc–hexane (1:3) to give 2-hydroxystearic acid methyl ester (FAM) (1.0 mg): $[\alpha]_D^{25}$ −3.8 (*c* 0.10, $CHCl_3$); ^{1}H NMR δ in $CDCl_3$: 4.85 (m, 1H, H-2), 3.30 (s, 3H, OMe), 1.75 (m, 1H, H-3A), 1.65 (m, 1H, H-3B), 1.26 (m, 28H, methylenes), 0.80 (t, $J = 6.5$ Hz, Me-17). EI-MS *m/z*: 314 $[M + H]^+$, 255, 236, 149, 111, 97, 83, 57, 43. The aqueous MeOH layer was neutralized with dilute ammonia and then concentrated. The residue was dissolved in H_2O (3 mL) and extracted with EtOAc (2 × 2 mL), and the EtOAc phase was concentrated *in vacuo*. The EtOAc extracts were chromatographed on a silica gel column, eluting with CH_2Cl_2, to afford free LCB (1.1 mg) which was benzoylated with benzoyl chloride (20 mL) and DMAP (1.5 mg) by stirring in dry pyridine (2.0 mL) for 6 h at 50°C. The reaction was quenched by adding H_2O (1.0 mL) and stirring for 5 min, and then concentrating *in vacuo*. The residue was separated and purified by preparative TLC (silica gel, hexane–acetone = 10:1) to afford 2-benzoylamido-1,3-dibenzoyloxy-9-methyl-4,8-sphingadienine (0.5 mg): Cotton effect (λ 236 nm, $\Delta\varepsilon$ −6.12; λ 221 nm, $\Delta\varepsilon$ +2.98). FABMS *m/z*: 624 $[M + H]^+$.

Degradation of 4

Methanolysis of **4** (4.5 mg) as described above yielded 0.8 mg FAM, $[\alpha]_D^{25}$ +7.3 (*c* 0.2, $CHCl_3$); ^{1}H NMR δ in $CDCl_3$: 4.34 (m, H-2), 3.36 (s, OMe), 5.25 (dd, $J = 16.0, 6.2$ Hz, H-3), 5.50 (m, H-4), 2.10 (m, 2H, H_2-5), 1.26 (m, 24H, methylenes), 0.80 (t, $J = 6.5$ Hz, Me-17). EIMS *m/z*: 312 $[M]^+$. The free LCB of **4** was confirmed to be identical to that of **3** according to its comparable TLC and the CD data of the benzoylated derivative with those of **3**.

Degradation of 1 and 2

By the same way as mentioned above, the FAM of **1** $[[\alpha]_D^{25}$ −4.2 (*c* 0.14, $CHCl_3$); EIMS *m/z*: 314 $[M]^+$] and the FAM of **2** $[[\alpha]_D^{25}$ +6.8 (*c* 0.10, $CHCl_3$); EIMS *m/z*: 312 $[M]^+$] were identical to the FAMs derived from **3** and **4** respectively; and the free LCB of both **1** and **2** correspond with that of **3**, having the same molecular weight in the MS spectra and similar CD data for the tribenzoylated LCB derivative, as well as favourable TLC comparisons.

Flavicerebroside A (1)

A colorless amorphous powder, mp 190–192°C; $[\alpha]_D^{25}$ +5.3 (*c* 0.5, MeOH); Molish reaction positive; IR (KBr) ν_{max} (cm^{-1}): 3407, 2918, 1636, 1528, 1400, 1084, 1031, 611; ^{1}H and ^{13}C NMR data, see Tables I and II; FABMS (positive) *m/z* 778 $[M + Na]^+$. HR-FABMS (positive) *m/z* 778.5800 (calcd for $C_{43}H_{81}NO_9Na$, 778.5803).

Flavicerebroside B (2)

A colorless amorphous powder, mp 188–189°C; $[\alpha]_D^{25}$ −6.2 (*c* 0.43, MeOH); Molish reaction positive; IR (KBr) ν_{max} (cm^{-1}): 3400, 2915, 1636, 1528, 1452, 1054, 600; ^{1}H and ^{13}C NMR data, see Tables I and II; FABMS (positive) *m/z* 778 $[M + Na]^+$. HR-FABMS (positive) *m/z* 776.5648 (calcd for $C_{43}H_{79}NO_9Na$, 776.5647).

Cerebroside D (3)

Colorless amorphous powder, mp 186–188°C; $[\alpha]_D^{25}$ +7.8 (*c* 0.25, MeOH); Molish reaction positive; IR (KBr) ν_{max} (cm^{-1}): 3407, 2907, 1636, 1528, 1463, 1044, 961; ^{1}H and ^{13}C NMR

data, see Tables I and II; FABMS (positive) *m/z* 778 $[M + Na]^+$, HR-FABMS (positive) *m/z* 778.5799 (calcd for $C_{43}H_{81}NO_9Na$, 778.5803).

Cerebroside C (4)

Colorless amorphous powder, mp 175–177°C; $[\alpha]_D^{25}$ −9.3 (*c* 0.23, MeOH); Molish reaction positive; IR (KBr) ν_{max} (cm^{-1}): 3400, 2915, 1636, 1528, 1456, 1031, 952; ^1H and ^{13}C NMR data, see Tables I and II; FABMS (positive) *m/z* 776 $[M + Na]^+$. HR-FABMS (positive) *m/z* 776.5643 (calcd for $C_{43}H_{79}NO_9Na$, 776.5647).

In Vitro Antitumour Assay

Antitumour activity was assayed using the human epidermoid KB cell line [25] with the described methods [26,27]. The cells were maintained in Eagle's minimum essential medium (MEM) supplemented with glutamine, non-essential amino acids (1%), and newborn calf serum (10%), and the cell population doubling time was 24 h. For the cytostatic assay, a cell culture in the exponential growth phase was used. The compounds were dissolved in sterile DMSO, and these solutions were diluted with the growth medium to the desired concentrations. The final DMSO concentration in the culture medium was 0.5% and showed no cytostatic effect in preliminary tests. Five concentrations (100, 50, 25, 10 and 1 $\mu g\,mL^{-1}$) of each compound were used and each agent was assayed in duplicate. The incubation time was 72 h and the cell growth was calculated by the sulforhodamine B (SRB) method [28]. The cytostatic activity was detected from the inhibition of cell growth in the treated cultures with respect to the controls. The activity was expressed as the concentration of the compounds at which tumour cell growth showed 50% inhibition (IC_{50}), and was calculated by linear regression analysis [29].

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