ISOLATION AND STRUCTURE DETERMINATION OF *PACHYBASIUM* CEREBROSIDES WHICH POTENTIATE THE ANTIFUNGAL ACTIVITY OF ACULEACIN

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A set of four cerebrosides was isolated from a *Pachybasium* species and purified by preparative reversed-phase HPLC. All four products displayed activity in a natural product screen aimed at detecting novel cell wall-active antifungal agents based on synergy with the known glucan synthetase inhibitor, aculeacin. Based on degradation studies, fast atom bombardment mass spectrometry and ^{13}C and high field ^{1}H NMR techniques, the structure of the major cerebroside was determined to be (4*E*,8*E*)-*N*-D-2′-hydroxy-(*E*)-3′-hexadecenoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadiene. The other components were found to be the corresponding 2′-hydroxypalmitic acid analog with one less double bond and an analogous pair containing 2′-hydroxystearic acid with and without the 3′ double bond.

In the course of screening for novel antifungal agents in microbial fermentation mixtures, a product was obtained from a cell extract of a *Pachybasium* sp. which potentiated the antifungal activity of the cell wall-active antibiotic aculeacin against *Candida albicans*. Based on initialspect roscopic and TLC polarity data, this material appeared to be a mixture of cerebrosides containing α-hydroxy fatty acids. Since several commercial cerebroside preparations failed to potentiate the activity of aculeacin, we decided to determine the structures of the *Pachybasium* products in order to gain insight into the source of this unique activity. In order to assign structures unambiguously using mass spectral and NMR techniques and to relate the structures to bioactivity, it was necessary to resolve the mixture into its components. This paper describes the purification of the individual components by preparative HPLC and their subsequent structure determination by chemical and spectroscopic methods.

Materials and Methods

Isolation and Purification

Initial isolation of the cerebroside mixture from an acetone cell extract of *Pachybasium* sp. was performed on a column of Sephadex LH-20 (8×100 cm, Pharmacia, Inc.) using MeOH elution. Bioactive fractions were pooled, concentrated and chromatographed using a step gradient of MeOH (5~

50%) in methylene chloride on a column of Silica gel (Merck H-60, 1 kg) packed in a Jobin Yvon Chromatospac-100 Chromatograph (Instruments SA, Ltd.), to yield a mixture of cerebrosides, homogeneous on TLC. Separation of cerebrosides **A**, **B**, **C** and **D** was done on a reversed-phase column 2.5×50 cm (Altex; Berkeley, Calif.) packed with Merck Licroprep RP-18, (25~40 μm) packing, eluted with 90~95% MeOH - water and pumped with an FMI metering pump at 10 ml/minute. Detection was by refractive index (Waters 403, Waters Assoc., Milford, Mass.).

HPLC

Analytical HPLC was performed with a Beckman 100A pump on a Beckman Ultrasphere ODS column (5 μm, 4.6×250 mm) with a Beckman Model 155-10 variable wavelength detector set at 214 nm using MeOH at 3 ml/minute.

TLC

The separation of the cerebrosides was followed by analytical silica gel plates (Whatman LK5DF) eluted with methylene chloride - MeOH - water (90 : 10 : 1). Detection was by iodine vapor or by charring after spraying with 10% sulfuric acid in absolute EtOH. The fatty acid esters were monitored on silica gel plates (Whatman LK5DF) using methylene chloride - MeOH (98 : 2). Detection was by iodine vapor. The separation of the sphingosine bases was monitored on silica gel plates (Whatman LK5DF) using methylene chloride - MeOH (75 : 25) with ninhydrin detection.

GC

GC was performed on a Varian Model 3700 instrument equipped with flame ionization detector, a glass column (3% OV-17, 80/100 Supelcoport, 183 cm by 0.6 cm OD) and helium as carrier gas. For fatty acid esters, temperature programming was set at a range of 150~325°C and a rate of 17°C/minute. Methyl esters of 2-hydroxy fatty acids were obtained from Sigma and Supelco and used as standards. For 2-undecanone, temperature programming was set at a range of 100~325°C at a rate of 17°C/minute. Authentic 2-undecanone was purchased from Calbiochem.

GC-MS

GC-MS was performed on a Finnigan Model 3300 GC-MS equipped with an INCOS data system. The fatty acid ester samples were evaluated by chemical ionization using methane as both the GC carrier gas and the chemical ionization (CI) reagent gas. All samples were chromatographed on a 183×0.6 cm (OD) glass column packed with 3% OV-17 on Chromasorb WHP. The column temperature was programmed to increase from 125 to 325°C at 20°C/minute. The ion source pressure was 0.7 Torr and the ion source temperature was 150°C. A series of commercially available fatty acids, 2-hydroxy fatty acids and unsaturated fatty acids (from Sigma and Supelco) were evaluated as methyl esters to establish respective retention times and characteristic methane CI spectra using this system.

Field Desorption Mass Spectrometry (FD-MS)

FD spectra were obtained on a Varian MAT CH-5 DF mass spectrometer equipped with a combined FD/field ionization(FI)/electron impact (EI) source. Spectra were recorded and mass assignments made on a Varian SS-100 data system. Samples were loaded onto the emitter probe by dipping the probe into a MeOH - methylene chloride solution of the sample. The following instrument operating parameters were employed: Source temperature 60°C, emitter current 25 mA, extraction voltage 8 kV.

Fast Atom Bombardment Mass Spectrometry (FAB-MS)

The FAB spectra of components **A** and **B** were obtained at the Middle Atlantic Mass Spectrometry Laboratory through the courtesy of Mr. G. HANSEN. The instrument used was a Kratos MS-50 mass spectrometer equipped with a FAB ion source and a high field magnet. The samples were introduced in a matrix of thioglycerol and bombarded with a beam of 8 kV argon atoms.

Preparation of Reduced Cerebrosides

A sample of 20 mg of cerebroside **A** or **B** was dissolved in 8 ml of absolute MeOH. The solution was hydrogenated in the presence of 25 mg of 5% palladium on $BaSO_4$ under 2.1 kg/cm^2 of hydrogen.

After 2 hours, the catalyst was removed and the filtrate was evaporated *in vacuo* to dryness. The residue was chromatographed by preparative HPLC (E. Merck Hibar II, Licrosorb RP-18, 10 μm, 10×250 mm colum, using 97% MeOH as solvent at a flow rate of 10 ml per minute) to give chromatographically homogeneous product.

Oxidative Cleavages of Cerebrosides[1]

To a solution of 6 mg of each cerebroside in 3 ml of butanol (HPLC grade) was added 3 ml of a 0.25%-K_2CO_3 solution, followed by 3 ml of a permanganate-periodate solution (2.1 g $NaIO_4$ and 40 mg $KMnO_4$ in 100 ml of H_2O) and the purple mixture was vigorously stirred at room temperature. After 2 hours, 0.1 ml of concentrated sulfuric acid was added to the mixture, and the colorless solution was extracted twice with 9 ml of hexane (HPLC grade). The combined hexane extracts were dried over anhydrous Na_2SO_4 and then evaporated *in vacuo* to dryness. The residue was redissolved in 0.2 ml of hexane for GC analysis.

Preparation of Fatty Acid Methyl Esters (Small Scale)

A sample of 1 mg of the cerebroside was heated in 0.2 ml of 1 N anhydrous methanolic hydrogen chloride in a sealed vial for 3 hours at 100°C. The solution was cooled to room temperature, and evaporated to dryness *in vacuo*. The residue was partitioned twice between hexane and water, and the aqueous phase re-extracted with hexane. The combined hexane extracts were concentrated and subjected to TLC, GC and GC-MS analyses for hydroxy fatty acid methyl esters.

Preparation of the Sphingosine Base

A sample of 200 mg of the cerebroside was heated in 12 ml of 1 N anhydrous methanolic hydrogen chloride in a sealed tube for 4.5 hours at 100°C. After cooling, the solution was brought to a pH of 8.0 with 10% NaOH and evaporated to dryness *in vacuo*. The residue was partitioned between ethyl acetate and water and the ethyl acetate extract evaporated to dryness. The residue was chromatographed on a Florisil column (50 g) eluting with methylene chloride. Fractions were collected and combined according to analytical TLC (ninhydrin).

Preparation of the Reducing Sugar and D-α-Hydroxypalmitic Acid from Cerebroside **B**

A sample of 14 mg of the cerebroside **B** was heated in 3 ml of 2 N HCl in a sealed vial for 3 hours at 100°C. After cooling to room temperature, the solution was diluted with 8 ml of water, and passed through a C_{18} SepPAK (Waters Associates, Milford, MA). The SepPAK was washed with 4 ml of water and the combined aqueous solution was lyophilized. The residue was assayed by GC of its alditol acetate derivative[2] and by enzymatic analysis using a glucose oxidase kit (Statzyme, Worthington Diagnostics, Freehold, NJ). The SepPAK cartridge was further washed with 15 ml of MeOH to elute the fatty acids. The solvent was removed *in vacuo* and the residue was chromatographed on a Beckman Ultrasphere ODS column (10×250 mm) using 90% MeOH containing 0.2% trifluoroacetic acid at 4 ml/minute with monitoring at 210 nm. Fractions containing α-OH-palmitic acid were pooled and concentrated to 2.2 mg: $[\alpha]_D^{25}$ −6.4° (*c* 0.1, CH_2Cl_2); literature[3] $[\alpha]_D^{28}$ −8° (*c* 2.5, $CHCl_3$).

Aculeacin Potentiation Assay

Samples were assayed by depositing 20 μl of MeOH solutions at appropriate concentrations (Table 2) on duplicate 6.35 mm paper discs. After drying in air, the discs were placed on two Petri dishes inoculated with *Candida albicans* B311 in modified glucose agar, one as a control and one containing 0.05 μg/ml of aculeacin A[4]. Active compounds displayed a larger zone of inhibition in the aculeacin plate after overnight incubation at 37°C.

Spectroscopic Methods

IR spectra were taken in KBr pellets with a Perkin-Elmer Model 299B Spectrophotometer. UV spectra were obtained in MeOH with a Cary Model 15. ^{13}C NMR spectra were run in $CHCl_3$-*d*-MeOH-d_4 (1:1) in a Varian FT-80 spectrometer, ^1H NMR spectra were recorded in $CHCl_3$-*d*-MeOH-d_4 with a Brucker Instrument WM 360 spectrometer. TMS was used as an internal standard

and chemical shifts are reported in ppm (δ values). MP's were taken with a Thomas Hoover melting point apparatus and are uncorrected.

Two-dimensional Correlation Spectroscopy (2D-COSY) NMR Studies of Cerebroside A

The sample (5 mg) was dissolved in $CHCl_3$-*d* - MeOH-d_4 (2 : 1.5). 2D NMR spectra were obtained using a Bruker Instruments WM360 spectrometer operating at ambient temperature. The data were collected using the standard Bruker "COSYN" automated command sequence. 512 2K spectra comprised a data set and a spectral width of 3,012 Hz was measured. The data table was zero-filled in the F1 dimension and sine-bell apodized in both dimensions prior to double Fourier transformation. In experiments set to reveal long-range couplings, additional delays of 0.33 and 0.15 second were included prior to and following the observation pulse.

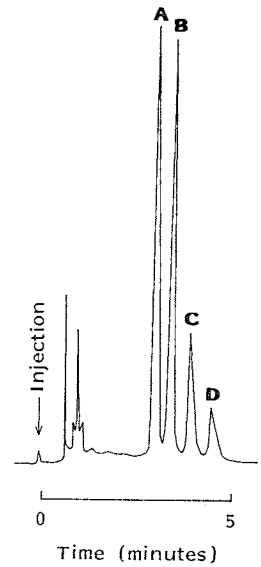
Results

Purification and Physical Characterization

Acetone cell extracts of *Pachybasium* sp. were chromatographed on Sephadex LH-20 and silica gel to yield a crude isolate. Although homogeneous on silica TLC, this preparation could readily be resolved into two major (**A** and **B**) and two minor components (**C** and **D**) by analytical reversed-phase HPLC (see Fig. 1) using methanol as an eluent and direct UV monitoring at 214 nm. This separation was readily scaled up on to a preparative column (2.5×50 cm) packed with Licroprep RP-18 (25~40 μm) using 92% methanol - water to yield four crystalline HPLC-homogeneous products. The physical properties of these compounds are listed in Table 1. Satisfactory combustion data could not be obtained for the two minor components in spite of several recrystallizations because they were available in limited quantities and co-crystallized with contaminants. All the products were equipotent in the bioassay as shown in Table 2. Each cerebroside displayed activity with aculeacin but not alone.

Based on the following chemical and spectroscopic data, the structures shown in Fig. 2 were assigned to the four cerebrosides. The products showed only end absorption in their UV spectra. Bands in their IR spectra were indicative of amide (1640 and 1530 cm^{-1}) and hydroxyl (3400 cm^{-1}) groups. The FD and FAB-MS coupled with the combustion data (assuming one nitrogen per molecule) implied a formula of $C_{41}H_{75}NO_9$ for the A component (see Table 1) and a dihydro-formula $C_{41}H_{77}NO_9$ for the **B** component. On catalytic hydrogenation, cerebrosides **A** and **B** both gave the same new reduced product as judged by HPLC retention time and FD-MS (*m/z* 732, M+H), consistent with a formula $C_{41}H_{81}NO_9$. The **C** ($C_{43}H_{79}NO_9$) and **D** ($C_{43}H_{81}NO_9$) components also differ from each other by one double bond and differ from the **A** and **B** pair in that they both contain two more methylene groups. Acid hydrolysis yielded D-

Fig. 1. Analytical HPLC chromatogram of cerebroside mixture.



Column: Beckman Ultrasphere ODS 4.6×250 mm, solvent: MeOH, flow: 3 ml/minute, detection: UV at 214 nm.

Table 1. Physical properties of cerebroside products.

	A	B	C	D
Molecular formula	$C_{41}H_{75}NO_9$	$C_{41}H_{77}NO_9$	$C_{43}H_{79}NO_9$	$C_{43}H_{81}NO_9$
C[a]	67.60 (67.83)	67.54 (67.64)	69.05 (68.49)	68.73 (68.31)
H	10.10 (10.41)	10.25 (10.66)	11.30 (10.56)	11.50 (10.80)
N	2.03 (1.93)	1.95 (1.92)	2.56 (1.86)	2.51 (1.85)
$[\alpha]_D^{20}$ [b]	−6.7°	+5.2°	−6.2°	+4.9°
FD-MS (*m/z*)	726 (M+H)	728 (M+H)	754 (M+H)	756 (M+H)
FAB-MS (*m/z*)	726 (M+H)	728 (M+H)	—	—
Fatty acid	α-OH Δ palmitic	α-OH palmitic	α-OH Δ stearic	α-OH stearic
MP[c] (°C)	170~178	180~190	159~169	178~189

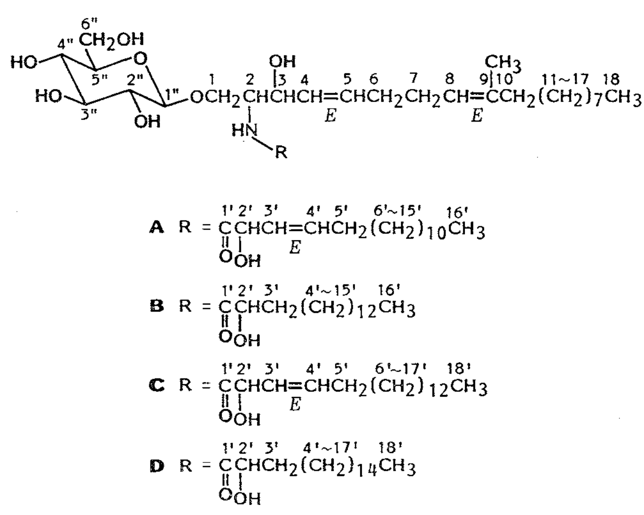
[a] Found (calcd).
[b] (*c* 1, MeOH).
[c] From EtOH - H_2O.
—: Not tested.

Table 2. Bioactivity of cerebroside products.

Compound	Bioactivity (zone size in mm)	
	Candida albicans B311	*C. albicans* B311 with 0.05 μg/ml aculeacin
Polyoxin B (100 μg/ml)	0	35
Cyclosporin A (25 μg/ml)	20	20
Amphotericin B (16 μg/ml)	15	16
Cerebroside **A** (250 μg/ml)	0	19
Cerebroside **B** (250 μg/ml)	0	19
Cerebroside **C** (250 μg/ml)	0	17
Cerebroside **D** (250 μg/ml)	0	23
Tetrahydrocerebroside **B** (250 μg/ml)	0	8
Bovin brain cerebrosides (Sigma)	0	0

Samples were assayed by depositing 20 μl of MeOH solutions at appropriate concentrations (Table 2) on duplicate 6.35 mm paper discs. After drying in air, the discs were placed on two Petri dishes inoculated with *C. albicans* B311 in modified glucose agar, one as a control and one containing 0.05 μg/ml of aculeacin A.

Fig. 2. Structures of cerebrosides **A**, **B**, **C** and **D**.



glucose as the only detectable carbohydrate in all of the cerebrosides. The D-configuration was confirmed by glucose oxidase enzymatic analysis.

Degradation Studies

GC-MS of the methyl esters isolated from the methanolysis of each compound indicated that the **B** and **D** components contained α-hydroxy palmitic (C_{16}) and α-hydroxy stearic (C_{18}) acids respectively. This was in both cases confirmed by comparison with authentic standards. A D-configuration was assigned to α-hydroxy palmitic acid isolated from cerebroside **B** based on its specific rotation of $-6.4°$ (literature[3] $-8°$). The **A** and **C** components also contained C_{16} and C_{18} fatty acids respectively, each with a double bond and a hydroxyl group, possibly allylic or homoallylic since the esters readily lost water in the mass spectrometer. After catalytic hydrogenation, the **A** and **C** components also yield α-hydroxy palmitic and α-hydroxy stearic acids, respectively, showing that the hydroxyl location is the same in the acid residue of all four cerebrosides. The location and stereochemistry of the double bond in the acid moieties of cerebrosides **A** and **C** were determined by NMR studies.

The sphingosine bases from the four cerebrosides were isolated as two ninhydrin positive materials by chromatography of their acidic methanolysis products. The major, more polar, product showed a molecular ion at m/z 312 (M+H) in its FD-MS. The minor component, a methylated analog, had an m/z of 326 (M+H) and was apparently an artifact of the methanolysis reaction[5] resulting from allylic exchange. A corresponding methylated byproduct was absent in the methanolysis products of the hydrogenated cerebrosides which all gave a single base four mass units larger (molecular ion at m/z 316, M+H). The sphingosine moiety of all four cerebrosides thus contains two double bonds. Subtraction of the elements of glucose and the relevant fatty acids from the formulae of the parent compounds gives a calculated formula of $C_{19}H_{37}NO_2$ for the sphingadiene base. The presence of an allylic methyl group in the base portion was suggested by the observation of a methyl singlet at 1.5 ppm in the ^{1}H NMR of the **A** cerebroside, absent in its hydrogenated product. Further evidence for the allylic methyl group was provided by the presence of an unprotonated olefin carbon peak in the ^{13}C NMR of each cerebroside. The location of the branched double bond at the 8-position was shown by the GC detection of 2-undecanone as a product of catalytic oxidation of the parent cerebrosides.

NMR Studies

^{13}C NMR (20.6 MHz, Table 3) and high field ^{1}H NMR (360 MHz, Figs. 3~6) spectra of the intact cerebrosides confirmed the above structural conclusions, and allowed the stereochemistry to be elucidated. Spectra were run in a mixture of chloroform-d and methanol-d_4, since chloroform alone gave broadened signals. This was probably attributable to micelle formation in the less polar solvent. In the ^{13}C NMR spectra, broadening was most pronounced for the signals of nuclei in or close to the sugar residue, consistent with aggregation of the polar ends of the molecules with consequent loss of mobility in these regions. The assignment of proton signals was assisted by spin-decoupling and 2D spectroscopy. Carbon assignments (Table 3) were confirmed, where possible, by comparison with model compounds and by off-resonance proton decoupling. However, there was extensive overlapping of multiplets in the off-resonance decoupled spectra and most of the ambiguities involved carbons bearing the same number of protons. Only the **A** and **B** cerebrosides are here discussed in detail. The ^{1}H and ^{13}C NMR spectra of the **C** and **D** cerebrosides were essentially indistinguishable from those of the **A** and **B** compounds, respectively, differences arising from the lengths of the methylene chains being hard to discern. Only the ^{1}H NMR spectrum of the **A** cerebroside is

Table 3. Chemical shifts and assignments for the **A** and **B** cerebrosides and model compounds. Carbon chemical shifts in ppm down-field from TMS[a].

Carbon No.	A Cerebroside			B Cerebroside			Model compounds	
	Sugar	Acid	Sphingosine	Sugar	Acid	Sphingosine	Sugar[b]	Sphingosines[c]
C-1	104.0	174.8	69.1	103.7	176.4	68.9	104.3	69.1
C-2	74.3*	73.6*	54.1	74.0*	72.6*	53.8	74.2	53.9
C-3	77.2	134.3†	72.5*	77.0††	↑	72.4*	76.9	72.6
C-4	70.9	128.1	134.6†	70.6	|	134.3	70.8	134.6
C-5	77.2	↑	130.1	76.9††	|	129.7	76.9	129.7
C-6	62.2	|	↑	62.0	|	↑	61.9	
C-7		|	28.3～33.3		25.6～35.1	25.6～35.1		
C-8		28.3～33.3	↓ 124.2		|	↓ 123.8		
C-9		|	136.4		|	136.3		
C-10		|	40.3		|	40.1		
C-11～C-14		↓	↑		↓	↑		
C-15		23.3	28.3～33.3		23.1	25.0～35.1		
C-16		14.3	↓		14.2	↓		
C-17			23.3			23.1		
C-18			14.3			14.2		
C-19			16.1			16.1		

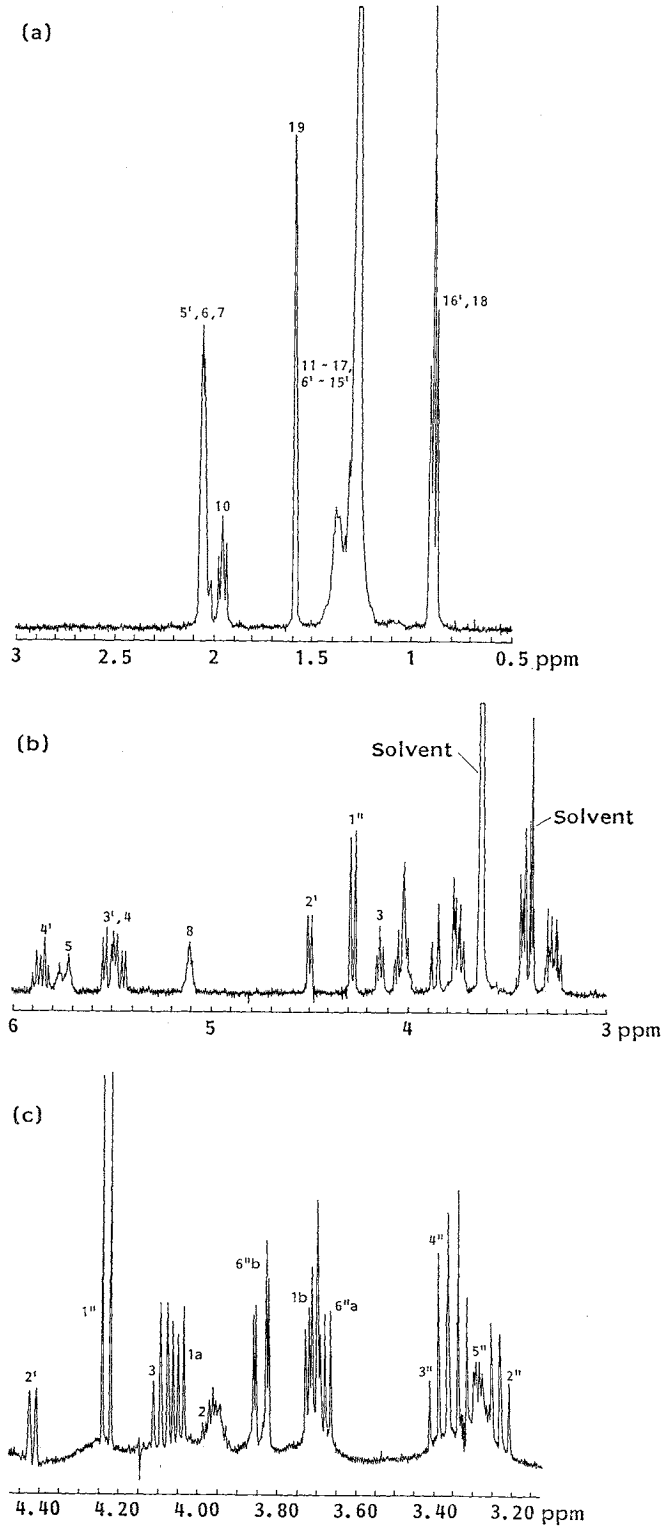
[a] Signals marked with the same symbol (*, † or ††) may be interchanged.
[b] β-Glucopyranoside from ref 7.
[c] From ref 7.

here presented in its entirety (Fig. 3). Apparent coupling constants are reported obtained by treating the spectra as first order.

The Acid Portion

The ^{13}C NMR spectra of the cerebrosides showed a single low-field resonance assignable to the amide carbonyl of the acid moiety and a resonance at about 73 ppm appropriate for the hydroxylated C-2′. The ^{1}H NMR spectrum of the **A** cerebroside displayed a signal for the 2′-H at δ 4.49 (d, slightly br, J=6 Hz), the low-field position being consistent with its location on an oxygenated carbon lying between a carbonyl group and a double bond. In the **B** compound the corresponding proton resonance was moved upfield and was lost in the δ 3.2～4.1 region. The presence of the 3′:4′ double bond in the **A** cerebroside was evidenced in the carbon spectrum by signals for two monoprotonated olefinic carbons not present in the **B** compound. The ^{1}H NMR spectrum of the **A** cerebroside showed the 3′-H at δ 5.52 (dd, slightly br, J=15 and 6 Hz) and the 4′-H at δ 5.85 (dd, slightly br, J=15 and 7 Hz). Coupling constants for the 3′-H, whose signals overlap that of the 4-H of the sphingosine base, were conveniently measured with decoupling of the allylic methylene protons by irradiating at δ 2.04 to remove broadening by long-range interactions (Fig. 4). The large coupling between the 3′-H and 4′-H established their *trans* disposition about the double bond[6]. Irradiation of the 2′-H in the **A** cerebroside caused the signal at δ 5.52 to collapse to a doublet, substantiating placement of the hydroxyl of the acid residue next to the double bond[6]. The resonance of the allylic 5′-methylene protons of the **A** cerebroside was shown to lie within a multiplet centered at δ 2.04 (with the 6- and 7-methylenes of the sphingosine) by the collapse of the 4′-H signal to a doublet (J=15 Hz) when this multiplet was irradiated. The C-6′ through C-15′ methylene protons of the **A** cerebroside and the C-4′ through C-15′ methylene protons of the **B** compound gave signals within an envelope between δ 1.2 and δ 1.5, with the non-allylic methylenes of the sphingosine. The diastereotopic 3′-methylene protons of the

Fig. 3. 360 MHz ^{1}H NMR spectrum of cerebroside A in $CHCl_3$-*d* - MeOH-d_4.
(a) 0.5~3 ppm, (b) 3~6 ppm, (c) expansion of 3.1~4.2 ppm.



B cerebroside appear as broad signals centered at δ 1.55 and δ 1.75. The terminal methyl protons of the acid and sphingosine chains coincided in a signal at δ 0.90 (t, J=6.5 Hz). The methylene and methyl carbons of the acid showed resonances within the expected high-field range.

The Sphingosine Base Portion

The ^{1}H and ^{13}C NMR spectra of the **A** and **B** cerebrosides confirmed that they contain identical sphingosine bases. Most of the discussion which follows refers to the **B** compound, since this was free from interfering proton signals from the olefinic and allylic groups of the unsaturated acid residue present in the **A** compound. However, for reasons of availability the **A** compound was used in the COSY study. The usual sphingosine substitution pattern of a glycosylated hydroxyl at C-1, an acylamino group at C-2 and a free hydroxyl at C-3 was evidenced by the chemical shifts of these three carbons. All three agree within 1.4 ppm with values reported for the corresponding carbons in a cerebroside of known structure and configuration[7]. Indeed the signals for C-1 and C-2 agree within 0.2 ppm with the published values. The deviation of 1.4 ppm for C-3 was obtained by using the worst match out of the three resonances possibly assignable to this carbon. The close agreement of these values, and also that of the C-4 resonance (within 0.3 ppm), with the data for the known cerebroside are consistent with attribution of the same relative stereochemistry, *R*S** or *erythro*, to the C-2 and C-3 asymmetric centers. The congested region from δ 3.1 to δ 4.2 in the ^{1}H NMR spectrum contained signals arising from the 1-H$_2$, 2-H and 3-H of the sphingosine base along with protons from the pyranoside ring. The spectrum was assigned using 2D COSY, which was interpreted as follows. The 3-H (δ 4.08, dd, J=7.9 Hz) was identified by its coupling to the 4-H (δ 5.43) and to the 2-H (δ 3.95, m) which was coupled in turn to methylene protons at δ 4.05 and δ 3.67 and to a slowly exchanged amide NH at δ 7.51. The remaining signals in this region were similarly assigned as individual pyranoside resonances. Fig. 3c shows the assigned, expanded region. The large coupling constant displayed by the C-4 olefinic proton (dd, slightly br, J=15 and 7 Hz) measured with decoupling of the allylic protons at δ 2.04, (Fig. 6) demonstrated its *trans* relationship to the 5-H (δ 5.75, two unresolved bands, $w_{1/2}$ *ca.* 12 Hz, separated by *ca.* 15 Hz, collapsing to a single broad band on irradiation of the 4-H). The complexity of the 5-H signal contrasted strongly with the sharpness of the pattern of the 4-H, whose four discrete peaks were only slightly broadened by allylic coupling (Fig. 5). The explanation for the complexity of the 5-H signals

Fig. 4. Olefinic proton region of 360 MHz ^{1}H NMR spectrum of cerebroside **A** in $CHCl_3$-*d* - MeOH-d_4, with decoupling frequency set at δ 2.04.

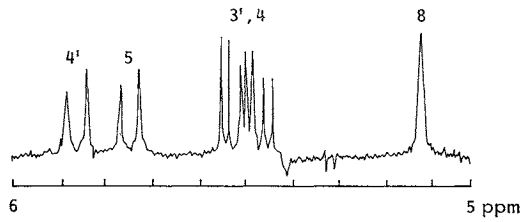


Fig. 5. Olefinic proton region of 360 MHz ^{1}H NMR spectrum of cerebroside **B** in $CHCl_3$-*d* - MeOH-d_4.

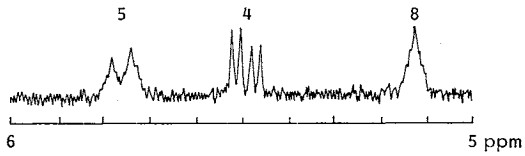
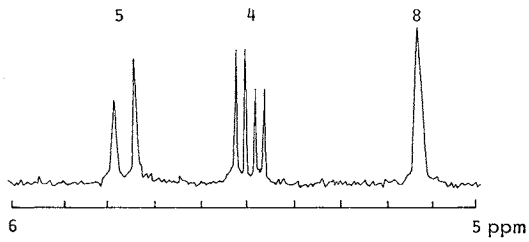


Fig. 6. Olefinic proton region of 360 MHz ^{1}H NMR spectrum of cerebroside **B** in $CHCl_3$-*d* - MeOH-d_4, with decoupling frequency set at δ 2.04.



is found in the concept of "virtual coupling"[8]. If the 6-H_2 and 7-H_2 resonances coincide in the compact multiplet at δ 2.04, then these isochronous protons are very strongly coupled to one another ($J\sim 7$ Hz, frequency difference ~ 0) and the 5-H is coupled to the 6-H_2 and virtually coupled to the 7-H_2. The C-5 through C-8 protons constitute, in fact, an A_2B_2MX partial spin system. This situation can arise only if the 7-H_2 have the shift appropriate for an allylic location. If the 7-H_2 are allylic, then the second double bond of the sphingosine must occupy the 8:9 position, as deduced from oxidation studies. The assignment of the protons on C-6 and C-7 was confirmed by observation of appropriate cross peaks in the COSY spectrum with the 5-H and 8-H. A further very broad olefinic proton signal at δ 5.13 was assigned to the 8-H, coupled to the 7-H_2 and virtually coupled to the 6-H_2. Irradiation of the allylic region at δ 2.04 (Fig. 6) converted the 5-H signal to a slightly broadened doublet, (J=15 Hz) confirming the *trans* disposition of 4-H and 5-H; at the same time the 8-H signal was sharpened, its residual width being attributable to allylic coupling to the 10-methylene and the 19-methyl protons. The signal for the latter (δ 1.58, s, slightly br) was consistent with its being attached to the unprotonated C-9 site.

Since the 8:9 double bond is trisubstituted, no information as to its stereochemistry could be derived from interproton couplings. The configuration of the substituents was deduced from the carbon spectrum. The three methyl carbons of the cerebroside are represented by signals at δ 14.2 and δ 16.1, both of which split into quartets in the off-resonance proton decoupling experiment. Of these two signals, the one at δ 14.2 is assigned to the two terminal methyls, C-18 and C-16′, on the basis of its intensity and chemical shift. The resonance of C-19 is therefore at δ 16.1. BOHLMANN *et al.*[9] have shown, in a comparison of the terpenes nerol and geraniol, that a similarly situated methyl carbon appears at δ 16.0 when it lies *cis* to an alkyl chain and at δ 23.5 when *cis* to a hydrogen. The difference in shifts may be rationalized in terms of steric compression. It follows that the 19-methyl must be *cis* to the 7-methylene, giving the double bond at the 8 position the *E*-configuration. Apart from the protons of the allylic 10-methylene group (δ 1.98, t, slightly br, J=7 Hz) the remaining carbons and protons of the sphingosine chain resonate within the expected high-field range.

The Sugar Portion

The carbon spectra of both the **A** and **B** cerebrosides showed all the peaks expected for glucose in a β-pyranoside linkage. None of the shifts found differed from reported values[10] by more than 1.7 ppm, even taking the worst match where the assignments were ambiguous. The ^{1}H NMR spectra showed the anomeric hydrogen resonance at δ 4.28 (d, J=8 Hz), further supporting the β-pyranoside configuration[11]. The rest of the sugar proton resonances lie in the congested region of the spectrum described above. The 2″-H (δ 3.21, dd, J=8 Hz) is identified from the COSY spectrum based on its coupling to the 1″-H and 3″-H (δ 3.36), overlapped with the 4″-H (δ 3.34). The 5″-H is a multiplet (δ 3.26) which is coupled to the methylene protons on C-6″ (δ 3.66 and 3.84). The large couplings for the protons on C-2″ and C-3″ confirm the *trans* diaxial relationship among the protons on C-1″ to C-4″ required by the glucopyranoside structure.

Discussion

The data discussed above for **B** and similar studies for **A**, **C** and **D** agree with the chemical findings and are consistent with the assigned structures for the four cerebrosides as shown in Fig. 2, cerebroside **A** being (4*E*,8*E*)-*N*-D-2′-hydroxy-(*E*)-3′-hexadecenoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-

sphingadiene, **B** being the corresponding 2′-hydroxypalmitic acid analog and **C** and **D** being the corresponding 2′-hydroxystearic acid analogs.

On biological evaluation, all four cerebrosides and their hydrogenated derivatives potentiated the activity of aculeacin, although none of the compounds were active in the absence of aculeacin. This test system was originally developed as a presumptive screen for cell wall-active antifungal agents. It was based on the observations that polyoxin, a known chitin synthetase inhibitor, displayed synergy with aculeacin[4], a known glucan synthetase inhibitor, both chitin and glucan being cell wall constituents of fungi. Unfortunately, no significant chitin synthetase inhibition could be demonstrated for the purified cerebrosides.

The cerebrosides reported here are similar to a single cerebroside, reported by BALLIO *et al.*[12] isolated from another fungus, *Fusicoccum amygdali* Del. The major physico-chemical characteristics reported for that compound (2.17% nitrogen; mp 160~165°C; $[\alpha]_D^{25}$ −8.4° (*c* 1.2, MeOH); *m/z* 753, M^+) were very similar to those found for our minor component **C**, and, like component **C**, BALLIO's material was reported to contain glucose, an α-hydroxy unsaturated C_{18} acid and a C_{19} branched sphingadiene. However, BALLIO assigned the double bond stereochemistry in the base as *cis* at the 4-position bond and left the stereochemistry at the branched double bond at position 8 unassigned. Other investigators have also reported the isolation of similar cerebrosides from fungal sources. FUJINO and OHNISHI[13] reported a mixture of glucose containing cerebrosides from *Aspergillus oryzae* which also contained a branched sphingadiene as well as a mixture of saturated and unsaturated α-hydroxystearic acids. No biological properties were ascribed to either of these materials.

While this work was in progress, KAWAI and IKEDA[14~16] described the isolation and chemical characterization of a cerebroside which appears identical to our component **B**. This material, one of several products isolated from the fungus *Schizophyllum commune*, was reported to be a stimulant of fruiting body formation in fungi. In their hands, BALLIO's cerebroside, its ceramide (derivative with glucose removed), and, to some extent, hydrogenated derivatives all showed fruiting-inducing activity, whereas a selection of other cerebrosides was devoid of activity. This parallels our results where the four *Pachybasium* cerebrosides and their hydrogenated derivatives, but not other cerebrosides, displayed activity in the aculeacin potentiation assay. This suggests that the fruiting-inducing activities reported elsewhere and the aculeacin potentiation in *C. albicans* reported here may be operating by a similar mechanism.

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References

1) KNAPP, D.: Handbook of Analytical Derivatization Reactions. *Ed.*, D. KNAPP, pp. 227~228, Wiley Interscience, New York, 1979
2) SITRIN, R. D.; G. W. CHAN, J. J. DINGERDISSEN, W. HOLL, J. R. E. HOOVER, J. R. VALENTA, L. WEBB & K. M. SNADER: Aridicins, novel glycopeptide antibiotics. II. Isolation and characterization. J. Antibiotics 38: 561~571, 1985
3) HITCHCOCK, C.; L. MORRIS & A. JAMES: The stereochemistry of α-oxidation of fatty acids in plants. The configuration of biosynthetic long chain 2-hydroxy acids. J. Biochem. 3: 473~475, 1968
4) MIZUNO, K.; A. YAGI, S. SATOI, M. TAKADA, M. HAYASHI, K. ASANO & T. MATSUDA: Studies on aculeacin. I. Isolation and characterization of aculeacin A. J. Antibiotics 30: 297~302, 1977
5) JUNGALWALA, F.; J. EVANS, E. BREMER & R. MCCLUER: Analysis of sphingoid bases by reversed-phase high performance liquid chromatography. J. Lipid Res. 24: 1380~1388, 1983
6) JACKMAN, L. M. & S. STERNHELL: Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry. 2nd Ed. *Eds.*, L. M. JACKMAN & S. STERNHELL, p. 302, Pergamon Press, London, 1969
7) KOERNER, T. A. W.; L. W. CARY, S.-C. LI & Y.-T. LI: Carbon 13 NMR spectroscopy of a cerebroside. Proof of the β-pyranosyl structure of D-glucosylceramide. J. Biol. Chem. 254: 2326~2328, 1979

8) MUSHER, J. I. & E. J. COREY: Virtual long-range spin-spin couplings in nuclear magnetic resonance (N.M.R.). The linear 3-spin system and qualitative implications of higher systems. Tetrahedron 18: 791~809, 1962
9) BOHLMANN, F.; R. ZEISBERG & E. KLEIN: C-NMR-Spektren von monoterpenen. Org. Magn. Reson. 7: 432~436, 1975
10) STODDART, J.: Stereochemistry of Carbohydrates. *Ed.*, J. STODDART, pp. 129~135, Wiley Interscience, New York, 1971
11) GORIN, P. A. J. & M. MAZUREK: Further studies on the assignment of signals in ^{13}C magnetic resonance spectra of aldoses and derived methyl glycosides. Can. J. Chem. 53: 1212~1223, 1975
12) BALLIO, A.; C. CASINOVI, M. FRAMONDINO, G. MARINO, G. NOTA & B. SANTURBANO: A new cerebroside from *Fusicoccum amygdali del.* Biochem. Biophys. Acta 573: 51~60, 1979
13) FUJINO, Y. & M. OHNISHI: Structure of cerebroside in *Aspergillus oryzae.* Biochem. Biophys. Acta 486: 161~171, 1977
14) KAWAI, G. & Y. IKEDA: Fruiting inducing activity of cerebrosides observed with *Schizophyllum commune.* Biochem. Biophys. Acta 719: 612~618, 1982
15) KAWAI, G. & Y. IKEDA: Chemistry and functional moiety of fruiting inducing cerebroside in *Schizophyllum commune.* Biochem. Biophys. Acta 754: 243~248, 1983
16) KAWAI, G. & Y. IKEDA: Structure of biologically active and inactive cerebrosides prepared from *Schizophyllum commune.* J. Lipid Res. 26: 338~343, 1985