Isolation and identification of cerebrosides from the marine sponge *Chondrilla nucula*

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Abstract A cerebroside mixture has been isolated from the marine sponge *Chondrilla nucula*. Acid-catalyzed methanolysis of this cerebroside mixture afforded methyl glucosides, three long-chain bases, and a mixture of α-hydroxy fatty acid methyl esters. The bases were identified as saturated C_{17}, C_{18}, and C_{19} trihydroxy bases (1,3,4-trihydroxy-2-aminoalkanes) by gas–liquid chromatographic–mass spectrometric analysis of their corresponding trimethylsilyl derivatives. The methyl ester fraction consisted of a mixture of homologous C_{16} to C_{26} saturated straight-chain α-hydroxy esters plus a trace of saturated C_{25} iso α-hydroxy ester.

Supplementary key words 1,3,4-trihydroxy-2-aminoalkanes · α-hydroxy fatty acids · mass spectra · NMR spectra · tetrahydrofuran derivatives

Cerebrosides have long been recognized as lipid constituents of vertebrates, some invertebrates, and plants. The chemistry, biosynthesis, occurrence, and physiological function of these compounds have been reviewed (1, 2). In recent years it has been found that cerebrosides are also quite generally present in marine invertebrates. Vaskovsky et al. (3) examined the lipid extracts of 50 species of marine invertebrates from 11 phylla and detected cerebroside-like materials in 44 of these species; a variety of other glycolipids were also detected. Earlier, Rajagopal and Sohonie (4) reported the presence of considerable quantities of cerebroside in a sea anemone (*Gyrostoma* sp.), but the glycolipid was not purified and characterized. Karlsson (5) and Björkman et al. (6) have recently isolated a cerebroside fraction from the sea star *Asterias rubens* and characterized it by degradation. The cerebroside was found to contain glucose, saturated α-hydroxy fatty acids (C_{16}–C_{26}), and uncommon long-chain bases (mainly dihydroxy C_{18} and C_{22} bases with one and two double bonds). This appears to be the first cerebroside from a marine invertebrate to be studied in detail.

In the course of fractionating extracts of the marine sponge *Chondrilla nucula*[1] in search of a cardiovascular active component, we isolated a cerebroside mixture, and in this paper we report its structural elucidation based on degradative experiments. The lipid extracts of this sponge were examined by Bergmann and McTigue (7) in 1948; they found that the lipids contained a then-unknown sterol, which was named chondrillasterol (24[R]-ethylcholesta-7,22-dien-3β-ol).

EXPERIMENTAL

Melting points were determined on a Unimelt apparatus (A. H. Thomas) and are uncorrected. Infrared spectra were recorded on a Beckman IR-10 spectrophotometer. Nuclear magnetic resonance spectra were recorded on Varian T-60 and XL-100-15 spectrometers with tetramethylsilane as an internal standard. Elemental analyses were performed by the Microanalytical Service, Dept. of Chemistry, Stanford University. Thin-layer chromatography was carried out with 0.25-mm precoated silica gel plates and Merck silica gel H. The latter was also used for column chromatography.

Routine GLC analyses were carried out on a Varian Aerograph 1700 gas chromatograph with an 8 ft × ⅛ inch column containing 1% OV-1 on 100–120 mesh Gas-Chrom Q. Other conditions are described in the text. The combined GLC–MS analyses were carried out on an LKB-9000 instrument (LKB Instruments, Inc., Rockville, Md.). A 4 ft × ¼ inch glass column packed with 1% OV-1 on 100–120 mesh Gas-Chrom Q was used. The operating conditions were: injection port, 200°C; column, 190°C; molecular separators, 220°C; ion source, 250°C; and helium flow rate, 37 ml/min. Trimethylsilyl derivatives of methyl glycosides and sphingolipid bases were prepared by the method of Sweeley et al. (8). A reference mixture of α- and β-methyl glucosides was prepared from D-glucose by the same conditions used for the methanol-

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; MS, mass spectrometry; TMS, trimethylsilyl.

[1] *Chondrilla nucula* ("chicken liver sponge") is a small, fleshy, dark brown or olive sponge, aptly described by its common name, found in the waters of the Caribbean and around Florida.

ysis of the cerebrosides. Mannitol-TMS ether was used as an internal standard for GLC analyses of the glycoside and sphingolipid base TMS ethers.

Extraction and isolation of cerebrosides

Alcohol-preserved sponge (5 kg, drained and air-dried) was shredded in a blender and extracted with benzene[2] for 30 hr in a continuous percolator–extractor (9). Concentration of this extract gave a thick dark oil (28.2 g). The sponge was subsequently extracted with chloroform and then methanol in a similar manner. The latter extracts were not studied for their cerebroside content.

A portion of benzene extract (19.2 g) was chromatographed (as outlined in the tabulation below) on a 7.5-cm (ID) column containing 500 g of silica gel (60–200 mesh grade H; W. R. Grace, Davison Chemical Div., Baltimore, Md.). Fractions 12 and 13 (2.1 g) contained the crude cerebroside, which was obtained as a white solid. Examination of these fractions by TLC (silicic acid; $CHCl_3$–CH_3OH 80:20) revealed the presence of a single major slow-moving component with minor amounts of faster-moving impurities (visualization with 10% H_2SO_4 spray followed by development at 110°C). Fractions 14 and 15 appeared by TLC to contain some cerebroside in addition to larger amounts of slower-moving components. TLC analysis of fraction 11 indicated that it contained a small amount of cerebroside in addition to other faster-moving components. The infrared spectrum of fraction 11 showed broad ester carbonyl absorption (centered at 1725 cm^{-1}) but had no distinct absorption at the cerebroside amide position, 1630 cm^{-1}. The infrared spectrum of fraction 14 showed two carbonyl absorptions of approximately equal intensities, 1725 and 1630 cm^{-1}, thus confirming the TLC evidence for the presence of some cerebroside in this fraction in addition to other components.

Fraction	Solvent	(500 ml/ fr.)	Eluate (g)
1	$CHCl_3$		0.35
2	"		1.02
3	"		2.22
4	"		0.92
5	$CHCl_3$–CH_3OH	95:5	2.0
6	"	"	1.8
7	"	95:10	2.2
8	"	"	1.3
9	"	95:15	1.41
10	"	"	1.0
11	"	95:25	1.3
12	"	"	1.0
13	Acetone		1.1
14	"		0.6
15	"		0.5

The crude cerebroside (fractions 12 and 13) was purified further by chromatography on a 4-cm (ID) column containing 40 g of silica gel H, (TLC mesh, E. Merck, Darmstadt, distributed by Brinkmann Instruments, Inc., Westbury, N.Y.). Elution with $CHCl_3$–CH_3OH 80:20 afforded a cerebroside mixture that appeared as a single spot by TLC analysis (silicic acid; $CHCl_3$–CH_3OH 80:20); mp (after recrystallization from 95% ethanol) 150–200°C, slow, gradual softening; 200–209°C melting. The crude cerebroside was also purified via acetylation with acetic anhydride–sodium acetate (100°C, 2 hr) followed by chromatography and deacetylation of the purified acetate derivative with sodium methoxide–methanol to give a white solid, mp 160–210°C.

Analysis: $C_{46}H_{91}NO_{10}$ (avg mol wt);
calculated: C, 67.52; H, 11.21; N, 1.71
found: C, 66.11; H, 10.96; N, 1.57

Methanolysis of cerebrosides

The cerebroside mixture (20 mg) in anhydrous methanolic HCl (60 ml, 0.8 N) was heated at 80°C for 24 hr in a Pyrex glass tube fitted with a 4-mm-bore Fisher threaded glass needle valve with a Teflon stem. The methanolic solution was extracted with hexane (4 × 50 ml) and the combined hexane extracts were washed with methanol (50 ml). Evaporation of the solvent gave a mixture of methyl hydroxy esters as a white solid (5.6 mg) (62% based on avg mol wt of $C_{23}H_{46}O_3$).

Hydrochloric acid was removed from the methanolic solution by percolation through Amberlite IR-4B ion-exchange resin. Evaporation of the methanol left a mixture of methyl glycosides and sphingolipid bases as a clear gum (12.8 mg).

Cerebroside acetates

The cerebroside mixture (120 mg) was treated with acetic anhydride (1 ml) and anhydrous sodium acetate (50 mg) and heated at 100°C for 2 hr. The excess of acetic anhydride was hydrolyzed with aqueous sodium bicarbonate solution, and the cerebroside acetates were extracted with chloroform. The chloroform extracts were washed with water and dried over magnesium sulfate, and the solvent was removed on a rotary evaporator to give 120 mg (73% yield) of crude peracetate mixture. The acetate mixture was purified by chromatography on a silica gel column to

[2] Since the isolation of cerebrosides from *C. nucula* was incidental to another objective, the solvent extraction procedure described is not ideal for cerebroside isolation. Benzene extraction of the sponge was carried out as a routine method for defatting the animal prior to extracting it with more polar solvents. Sequential solvent extractions using increasingly polar solvents were carried out for the purpose of effecting partial resolution of the entire mixture of sponge metabolites into groups of compounds varying in polarity. A better procedure for isolation of the total cerebroside content is that of Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.

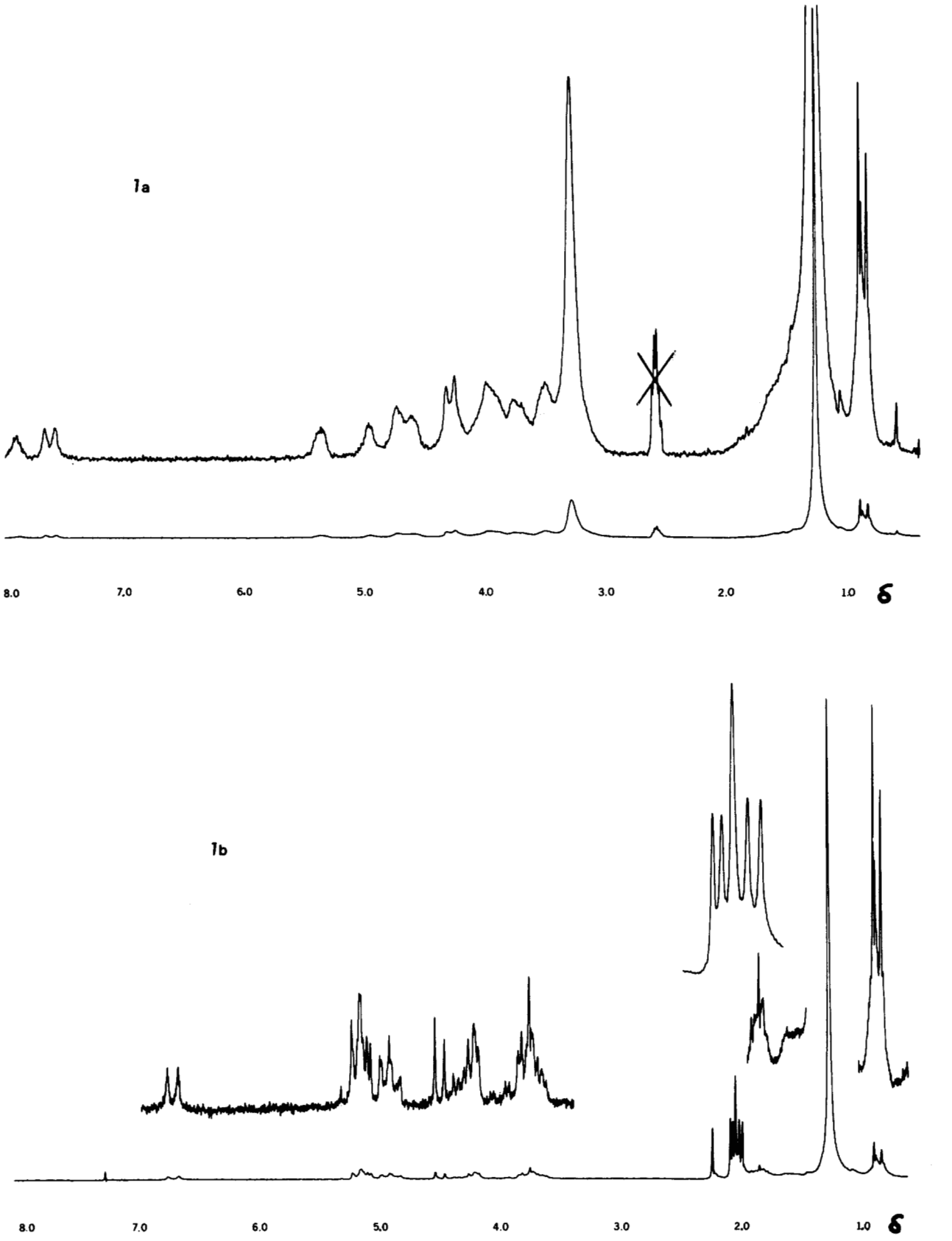


Fig. 1 (*a*) 100-MHz NMR spectrum of sponge cerebroside mixture ($CDCl_3$–DMSO-d_6 2:1); (*b*) 100-MHz NMR spectrum of acetylated sponge cerebroside mixture ($CDCl_3$).

yield an amorphous solid (single spot on TLC), mp 57–60°C.

Analysis: $C_{50}H_{105}NO_{17}$ (avg mol wt);
calculated: C, 64.77; H, 9.51; N, 1.26
found: C, 64.83; H, 9.54; N, 1.24

RESULTS AND DISCUSSION

Chromatography of the lipid extracts of *C. nucula* on silica gel gave a white solid, mp 160–210°C, which appeared as a single spot on TLC; chemical analysis indicated the presence of 1.57% nitrogen. The infrared spectrum (KBr disc) of the solid showed absorption at 3200–3600 cm^{-1} and also a broad peak centered at 1635 cm^{-1}, which was attributed to the carbonyl of an amide. Acetylation of the natural product gave a peracetate whose infrared spectrum (KBr disc) showed absorption at 1740–1755 cm^{-1} (acetates) and 3450, 3390, 1680, and 1660 cm^{-1}, indicative of a secondary amide. The presence of a secondary amide was further indicated by NMR data. The NMR spectra of the natural product (Fig. 1*a*) and its peracetate (Fig. 1*b*) exhibited doublet signals at δ 7.62 and 6.72 ppm, respectively (J 9 Hz; exchangeable with D_2O), similar to the signal due to the amide proton in the NMR spectrum of the peracetate prepared from *threo*-dihydrosphingosine.

The NMR spectrum of the lipid peracetate (Fig. 1*b*) also contained resonance signals for 7 acetate units (δ 1.97–2.5 ppm), a long methylene chain (δ 1.3), a terminal methyl group (δ 0.85), and unresolved multiplets at δ 3.6–5.25, typical of protons on acetylated sugars and amines. Since only 7 acetyl units were incorporated upon acetylation (ratio of amide H signal vs. acetate methyl signals = 1:21) it could be concluded that the lipid contained only one sugar residue (four free hydroxyls) in addition to three other hydroxyl groups elsewhere in the molecule. On the basis of the molecular features suggested by the foregoing data, i.e., secondary amide, single sugar moiety, and long *n*-alkyl residues, it could be concluded that the polar sponge lipid was a cerebroside. This conclusion was supported by the mass spectrum of the lipid peracetate, which exhibited a series of prominent ions separated by 14 mass units from m/e 694 to 806 and another series extending from m/e 634 to 746. These two series of ions correspond to M − (O—glycoside), M − (CH_2—O—glycose), and M − (CH_2—O—glycose − 60) ions noted in the spectrum of certain acetylated cerebrosides (10). The lipid peracetate mixture also exhibited intense ions at m/e 331 (acetylated glycose residue) and 390 ([$H_2N{=}^{+}_{A}CH$—CH_2—O—glycose]$^+$), typical of other cerebroside peracetates (10). The nature of the component parts of the cerebroside were ascertained from established degradative procedures described below.

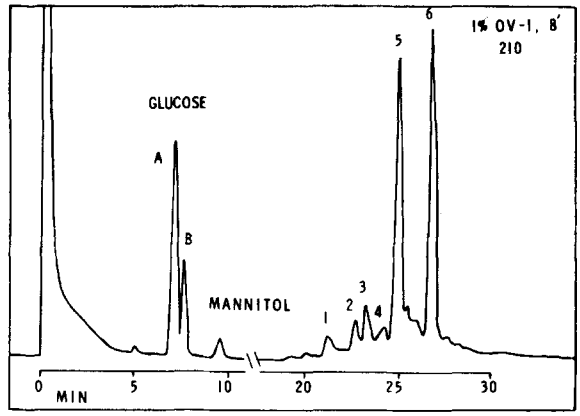


Fig. 2. Gas–liquid chromatogram of TMS methyl glycosides (*A* and *B*) and TMS trihydroxy bases plus added TMS mannitol. Identification of numbered components: *1* and *2*, tetrahydrofuran artifacts from C_{17} and C_{18} trihydroxy bases, respectively; *3*, *5*, and *6*, C_{17}, C_{18}, and C_{19} trihydroxy bases, respectively; *4*, unidentified.

Methanolysis of the cerebroside mixture by the procedure of Vance and Sweeley (11) afforded a fatty acid ester fraction and a methyl glycoside–long-chain base mixture. The sugar–base mixture was silylated and analyzed by GLC using the TMS ether derivative of mannitol as an internal standard (see Fig. 2). Peaks A and B in the chromatogram (Fig. 2) corresponded, respectively, to the TMS ether derivatives of α- and β-methyl glucosides as judged by peak enhancement experiments using a reference sample prepared from D-glucose. Thus, glucose appears to be the predominant, if not exclusive, sugar in the sponge cerebroside.

The gas–liquid chromatogram of the long-chain base mixture (as TMS ethers) derived from the cerebroside is shown in Fig. 2 (peaks 1–6). By GLC–MS analysis, components 3, 5, and 6 were identified, respectively, as C_{17}, C_{18}, and C_{19} 1,3,4-trihydroxy-2-aminoalkanes. The principal ions present in the spectra of these compounds are recorded in Table 1, and they correspond well to the fragmentation pattern found in the published spectrum of 1,3,4-trihydroxy-2-aminooctadecane tris-trimethylsilyl ether (12). However, it has been noted elsewhere (13) that branching in long-chain bases probably cannot be detected in the mass spectra of the intact long-chain bases and hence the possibility that these three long-chain bases are branched cannot unambiguously be ruled out. A plot of log retention time vs. number of carbon atoms in the long-chain base for components 3, 5, and 6 of Fig. 2 does yield a straight line, and this indicates that the long-chain bases are either all straight-chain or all branched.

Components 1 and 2 of Fig. 2 appear to be tetrahydrofuran by-products derived from the major cerebroside long-chain bases, the C_{18} and C_{19} trihydroxy bases. The formation of such tetrahydrofuran artifacts in the acid cleavage of glycolipids containing trihydroxy bases is well established (5, 14–16). The mass spectrum of component

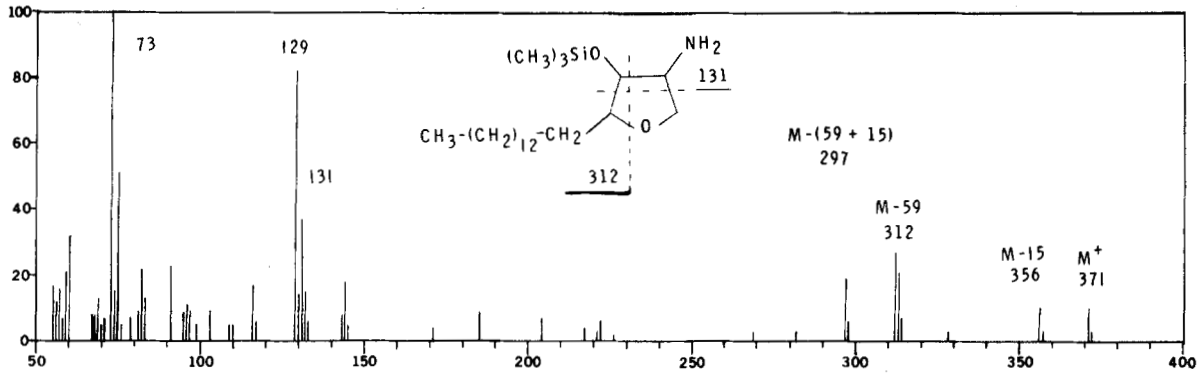


Fig. 3. Mass spectrum of the component numbered 1 in the gas–liquid chromatogram shown in Fig. 2: tetrahydrofuran artifact from C_{18} trihydroxy base.

1 in Fig. 2 is shown in Fig. 3 along with suggested fragmentations that could account for the principal ions observed. The mass spectrum of component 2 is very similar to the spectrum shown in Fig. 3, except that the higher mass ions (e.g., 297, etc.) are shifted 14 mass units to the right.

Proposed structures for the ions observed in the spectrum of the C_{18} tetrahydrofuran by-product (component 1, Fig. 2) are outlined in Fig. 4. The ion of mass 312 can be visualized as arising from a molecular ion with a positive charge on the TMS ether oxygen by cleavage of the tetrahydrofuran ring to give a vinyl ether ion and a neutral fragment. The *m/e* 312 ion can give rise to the *m/e* 297 peak by loss of a silyl methyl group and alternatively to the intense *m/e* 129 ion by cleavage at the allylic carbon. An alternate molecular ion in which the charge is borne by the amino group could undergo tetrahydrofuran ring cleavage with expulsion of a neutral alkyl epoxide (or its equivalent, formaldehyde plus an olefin) to form the stabilized *m/e* 131 ion.

The nature of component 4 of the sphingosine base mixture (Fig. 2) has not been established because its mass spectrum was still significantly contaminated with ions

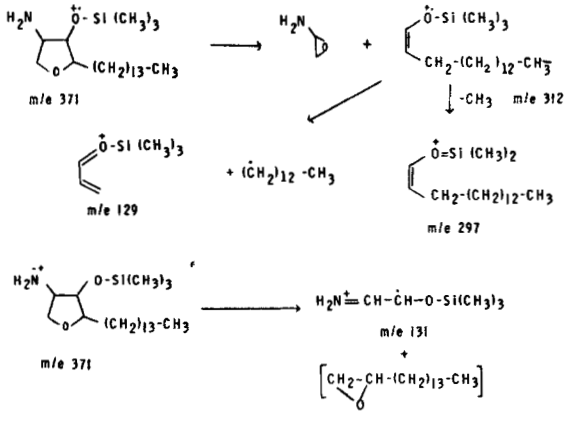


Fig. 4. Proposed structures for major ions in mass spectrum of tetrahydrofuran artifacts (component 1, Fig. 2) from C_{18} trihydroxy base.

from component 3 (C_{17} trihydroxy base), and other ions did not readily identify it as one of the anticipated base by-products.

The methyl esters obtained from methanolysis of the cerebroside were recognized as α-hydroxy esters from the absorptions at 3460 cm^{-1} (broad) and 1740 cm^{-1} in the infrared spectrum and the prominent peak at *m/e* 90 in the mass spectrum of the crude ester mixture (17). After silylation the α-hydroxy methyl ester mixture was more fully characterized by GLC and combined GLC–MS analysis. The gas–liquid chromatogram of the ester mixture is shown in Fig. 5. All of the components except the first two minor ones were identified by mass spectra, each of which exhibited a large M − 59 peak characteristic (18) of the TMS ethers of α-hydroxy acid methyl esters. The molecular ions observed established that components

TABLE 1. Principal ions in mass spectra of sponge cerebroside long-chain bases

	GLC Component in Fig. 2					
	3 (t 17:0)[a]		5 (t 18:0)[a]		6 (t 19:0)[a]	
	m/e	%	*m/e*	%	*m/e*	%
M^+	519	*b*	533	*b*	547	*b*
M−15	504	5	518	5	532	4
M−103	416	3	430	3	444	2
M−132	387	1–2	401	1–2	415	1–2
M− (90+103)	326	26	340	23	354	17
	298	19	312	19	326	17
M−234	285	19	299	19	313	15
	204	45	204	43	204	39
	147	13	147	7	147	11
	132	100	132	100	132	100
	129	39	129	21	129	26
	116	27	116	25	116	31
	103	11	103	9	103	7
	75	32	75	20	75	13
	73	95	73	73	73	69

[a] In this notation, t means trihydroxy; the figure before the colon indicates carbon-chain length and the figure after the colon indicates number of double bonds (12).

[b] M^+ usually not observable at the amplification used.

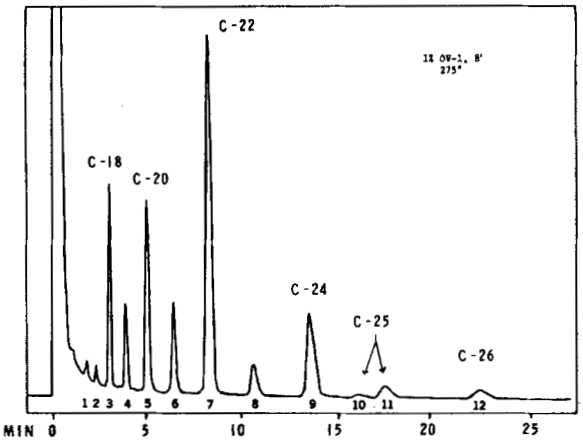


Fig. 5. GLC of TMS α-hydroxy fatty acid methyl esters from methanolysis of the sponge cerebroside mixture. Identification of components: peak nos. 1–9, 11, and 12 correspond to a homologous series of straight-chain α-hydroxy acids with carbon numbers from C_{16} to C_{26}; peak 11 corresponds to C_{25} iso α-hydroxy fatty acid.

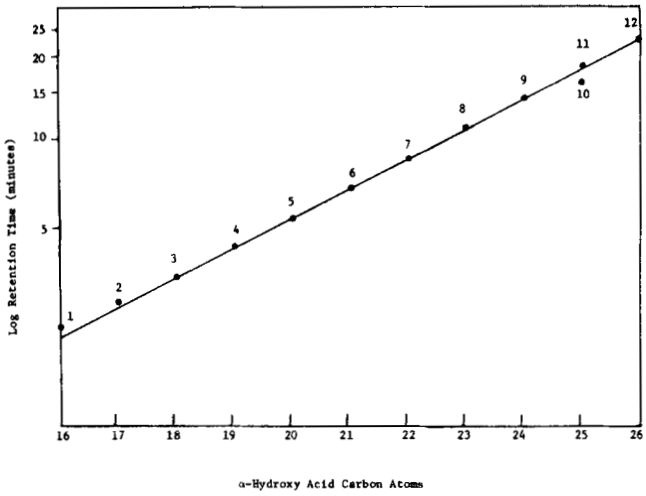


Fig. 6. Plot of log retention time of TMS α-hydroxy fatty acid methyl esters as a function of acid carbon-chain length. Nos. 1–12 correspond to peak numbers in gas–liquid chromatogram, Fig. 5. Straight line represents homologous series of α-hydroxy straight-chain esters. Lower point corresponding to C_{25} is assigned C_{25} iso acid structure.

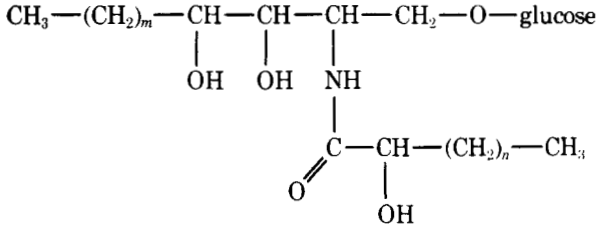
3–9 plus 11 and 12 constituted a homologous series of esters having acid carbon numbers ranging from C_{18} to C_{26}.

The minor ester components 1 and 2 (Fig. 5) are tentatively identified as the normal C_{16} and C_{17} α-hydroxy fatty acid methyl esters by virtue of their GLC retention time relative to that of the other esters in the mixture on a plot of retention time vs. acid carbon number (Fig. 6). Thus, it can be seen from Fig. 6 that components 1 and 2 fall very close to their expected positions (19) for the C_{16} and C_{17} acids on the straight-line plot formed by the other esters (except component 10; see below).

Components 10 and 11 in the gas–liquid chromatogram (Fig. 5) of the α-hydroxy fatty acid ester mixture gave nearly identical mass spectra with molecular ions of 484 corresponding to C_{25} TMS α-hydroxy acid methyl esters. Because the retention time of component 11 falls on the straight-line plot of retention time vs. acid carbon number, this component can be identified as the saturated straight-chain C_{25} α-hydroxy fatty acid ester (19). The ester corresponding to component 10 is assigned a saturated C_{25} isocarbon skeleton because its slightly shorter retention time compared with component 11 is consistent with other branched vs. straight-chain pairs of esters (19). Furthermore, it was noted that the mass spectrum of component 10 possesses a very weak fragment ion at m/e 419, which is not observed in the spectrum of the C_{25} straight-chain ester (component 11). The m/e 419 ion corresponds to the M − 65 ion, which is reported to be characteristic of iso acid esters (20).

The absence of any significant amounts of normal fatty acid residues in the cerebroside mixture was established by gas–liquid chromatographic analysis. Specifically, a temperature-programmed analysis (100°C → 280°C @ 4°C/min) of the total silylated mixture of methyl esters obtained by methanolysis of the cerebroside revealed the absence of any significant components (>0.01–0.1% of the total) of shorter retention time than peak 1 of Fig. 5. Under these same conditions, methyl stearate had a retention time essentially the same as that of peak 1. Thus, although the graphical data of Fig. 6 indicate that the minor components 1 and 2 are α-trimethylsilyloxy acid methyl esters, it is also possible that components 1 and 2 of Fig. 5 represent small amounts of normal acid esters.

On the basis of the foregoing evidence, the structure of the cerebroside mixture isolated from *C. nucula* may be formulated as shown below. The ring size (pyranoside or furanoside) of the sugar residue in the cerebroside cannot be specified from the data presented, but a pyranoside structure seems likely because the furanose form of hexoses in nature is rare (6). The anomeric configuration of the glucose group was not determined, but it may be noted that in most glycolipids the base is joined to the glycoside via a β linkage at the anomeric carbon (1).



$m = 12, 13, 14$
$n = 13$–23; also trace of $n = 22$ iso acid

Investigation of polar lipids of other marine animals are underway in our laboratory. Glycolipids are apparently

widely distributed in marine organisms (3), and a knowledge of their occurrence and structure is of interest in the areas of comparative biochemistry, taxonomy, and biochemical evolution.

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