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Structural analysis of leech galactocerebrosides using 1D and 2D NMR spectroscopy, gas chromatography–mass spectrometry, and FAB mass spectrometry[1]

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Abstract

Cerebrosides were isolated from the leech species, *Hirudo medicinalis*, and purified to homogeneity by silicic acid chromatography, followed by preparative thin-layer chromatography. Their structure was determined by spectroscopic and chemical methods. 1D and 2D ^{1}H NMR spectroscopy, DQF–COSY and HMQC indicated that the head group consists of a single galactose residue in the β configuration. The galacto configuration was determined by the characteristic chemical shift, the spin–spin splitting and the multiplicity of the characteristic resonance of its equatorial H-4 proton, as well as by the splittings of the other ring protons. GC, GC–MS and fast-atom-bombardment mass spectrometry studies indicated that $C_{24:0}$ and $C_{22:0}$ are the major saturated fatty acid species. Unsaturated fatty acids present were $C_{25:2}$, $C_{27:2}$, $C_{27:3}$, $C_{28:3}$, $C_{29:3}$, $C_{30:3}$, $C_{33:3}$. GC–MS indicated the presence of hydroxylated $C_{27:2}$ and one other unidentified hydroxylated fatty acid. The cerebroside contained an unusual polyunsaturated sphingosine analogue, namely 2-amino-1,3-dihydroxydocosatriene.

Keywords: Leech; Annelid; Galactocerebroside; Glycosphingolipids; Sphingosine; 2D NMR spectroscopy; DQF–COSY, HMQC, FABMS

1. Introduction

Cerebrosides are among the major lipids of the myelin sheath of vertebrate axons that are essential for the saltatory, high-velocity conduction of nerve impulses [1]. So far, the functions of galactoceramides have been largely explored in mammals.

[1] Abbreviations: NMR, nuclear magnetic resonance; HMQC, heteronuclear multiple-quantum coherence; DQF–COSY, double quantum filtered–correlated spectroscopy; GC–MS, gas chromatography–mass spectrometry; FABMS, fast-atom-bombardment mass spectrometry; TLC, thin-layer chromatography; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide.

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The high antibody titer to cerebrosides that accompanies sensory neuropathy [2] and multiple sclerosis [3] suggests that they play critical roles in myelin function. Cerebrosides are not necessary for the process of axon myelination, but rather for the maintenance of myelin, as is indicated by studies of transgenic mice [4]. The high-affinity binding of cerebrosides to laminin [5] suggests that they play an adhesive role, perhaps linking myelin to the extracellular matrix. Because cerebrosides have been not only isolated from glial cells, but also from a variety of other neural and epithelial tissues [6–9], they presumably play a number of different functions in vertebrates.

Recently, invertebrates lacking myelin, among them insects [10], mollusks [11] and annlids, were shown to possess galactose-containing glycosphingolipids. The galactose- and mannose-containing ceramides isolated from two earthworm species carry a phosphocholine headgroup [12,13], while trigalactosylceramides isolated from a leech species, *Hirudo nipponica*, are neutral [14].

Invertebrate models lacking myelin may serve to explore functions of cerebrosides other than those relating to the conduction of nerve impulses. That galactoceramides are important building blocks of the leech is suggested by the presence of ceramide glycanase in leeches [15]. In our physiological studies exploring the molecular mechanisms in neurogenesis in the leech embryo, we demonstrated the critical role of galactose-specific recognition in axonal targeting [16,17]. Perhaps galactocerebrosides participate in such developmental events.

In this paper we report on the isolation of a monogalactosylsphingolipid from the leech species, *Hirudo medicinalis*, with yields of 52 μg/g wet weight. Because bovine blood fed to these leeches possesses only trace amounts of glycosphingolipids, leeches presumably synthesize their own cerebrosides.

2. Materials and methods

General.—The leech species, *H. medicinalis*, was bred in the laboratory as previously reported [17] and fed with cow blood obtained from a slaughter yard. Sixty leeches maintained at room temperature were anesthetized in 8% ethanol, pinned out against wax, dorsal side facing upwards. Following a dorsal incision, their crops were emptied of blood and their hindguts were discarded. This yielded 250 g wet weight of leech tissue. Reference galactocerebrosides, sulfatides, and synthetic sphingosine were obtained from Sigma Chemical Co. (St Louis, MO, USA). BSTFA–1% Me_3SiCl was obtained from Pierce Chemical Co., Rockford, IL, USA, and silicic acid-n-hydrate was obtained from J.T. Baker Chemical Co., Phillipsburg, NJ, USA.

Galactocerebroside isolation.—Batches of five leeches at a time were homogenized in 30 mL of ice-cold water using a polytron (PCU, Kinematica, Kriens, Switzerland; distributed by Brinkman, Westbury, NY) at the highest speed setting. Lipids were extracted by incubating the homogenate in 4:1:5 chloroform–methanol–water (12 L) for 24 h at room temperature on a shaker. Centrifuging the mixture at 8000 rpm (5 min) led to the accumulation of dense particulate matter at the interface between the hydrophobic and hydrophilic layer. The lower, chloroform-containing layer was recovered. The aqueous layer was combined with the particulate matter to be re-extracted. The combined organic layers were concentrated to dryness under N_2 (5 g of leech lipids). The lipids were divided into three aliquots that were separately fractionated by flash chromatography on a silicic acid column (4×2.5 cm) whose packing was equilibrated in chloroform. Cholesterol, diacylglycerol and other relatively nonpolar lipids were eluted, first with chloroform (100 mL) and then with 6:1 chloroform–acetone (100 mL). Subsequent elution with acetone (100 mL) yielded a mixture of galactocerebroside and cholesteroltriols [18]. Galactocerebroside was purified using preparative thin-layer chromatography (Analtech Uniplate, 20×20 cm, 1 mm Silica Gel G; Alltech Associates, Deerfield, IL, USA). All TLC plates were presaturated in 1:1 methanol–diethyl ether, preactivated by heating for 30 min at 110 °C and used immediately. Plates were developed in 6:4:1 chloroform–acetone–methanol. Individual bands (visualized with iodine vapors) were scraped off, and their lipids were eluted from the silica gel with acetone. A total of 13 mg of galactocerebroside was harvested (which amounts to about 60 nmol/g of wet weight leech, assuming an average MW of 840 for the mixture of galactocerebroside with different fatty acids). Leech and cow lipids were compared by TLC analysis (AL SiL GIUV254, 250 μm; Whatman Ltd, Maidstone, Kent, UK) using 6:4:1 chloroform–acetone–methanol. Leech and reference bovine galactocerebrosides and sulfatides were compared by TLC analysis using 60:25:2 chloroform–methanol–water.

To visualize lipid bands, the plates were sprayed with 10% phosphomolybdic acid or resorcinol and baked at 110 °C. The resorcinol reagent was prepared as described previously [19].

Isolation of lipids from cow blood.—Heparin was added to fresh cow blood to prevent coagulation. Lipids were extracted from cow blood using the same procedure as that employed for extracting leech lipids.

NMR spectroscopy.—^{1}H NMR spectra were recorded in methanol-d_4 at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a Varian VXR500 spectrometer. Experiments were conducted at 30 °C. The proton shifts were referenced relative to the methanol signal at 3.33 ppm. For the ^{1}H–^{13}C HMQC experiments [20], a total of 32 transients were acquired at 1024 points each. A total of 916 data sets was acquired. Data for the DQF–COSY experiment [21] were acquired using a total of 498 data sets with 16 transients at 2048 data points each.

Gas chomatography–mass spectrometry.—A sample of leech galactocerebroside was treated with 5% HCl in methanol at 70 °C for 24 h. After the solution was dried under N_2, one aliquot (100 μg) was treated at room temperature with acetic anhydride (100 μL) and pyridine (100 μL) for 18 h, after which time it was again dried. A mixture of chloroform and water was added to the sample, followed by vortexing and sonication. The organic phase containing the fatty acid methyl esters and the acetylated fatty acid methyl esters was harvested, filtered though a cotton plug in a Pasteur pipette and then concentrated under N_2. Another aliquot (100 μg) was treated with BSTFA–1% Me_3SiCl for 4 h at room temperature. The differently derivatized aliquots were analyzed on a JEOL AX-505 (JEOL USA, Peabody, MA, USA) double-focusing mass spectrometer coupled to a Hewlett-Packard 5890J gas chromatograph via a heated interface. GC separation employed a SPB^{Tm}-1 fused-silica column (12 m length, 0.25 mm i.d. with a 0.25 μm film coating (Supelco Inc., Bellefonte, CA, USA)). Direct (splitless) injection was used with a helium flow of approximately 1 mL/min. The oven temperature was programmed from 150 to 180 °C at 40 °C/min, to 230 °C at 20 °C/min, and then at 5 °C/min to 320 °C. The relative abundance of the fatty acid methyl esters was determined by GC analysis on a Hewlett-Packard 5890 gas chromatograph equipped with a DB-11 capillary column and flame-ionization detector (FID). The oven temperature was programmed from 150 to 300 °C at 3 °C/min. Helium was the carrier gas for both GC methods.

FABMS.—FAB mass spectra were obtained using a JEOL HX-110 double-focusing mass spectrometer (JEOL USA, Peabody, MA, USA) operating in the positive- or negative-ion mode. Ions were

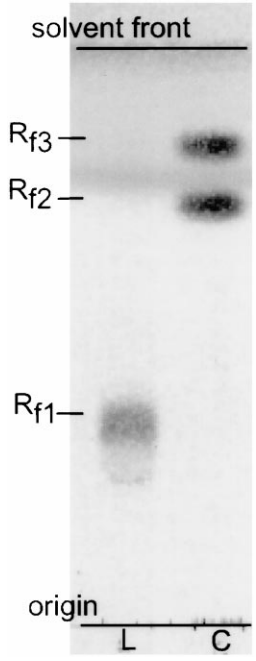


Fig. 1. TLC comparison of the mobility of leech sphingoglycolipid (Lane 'L') to that of lipids from cow blood (Lane 'C').

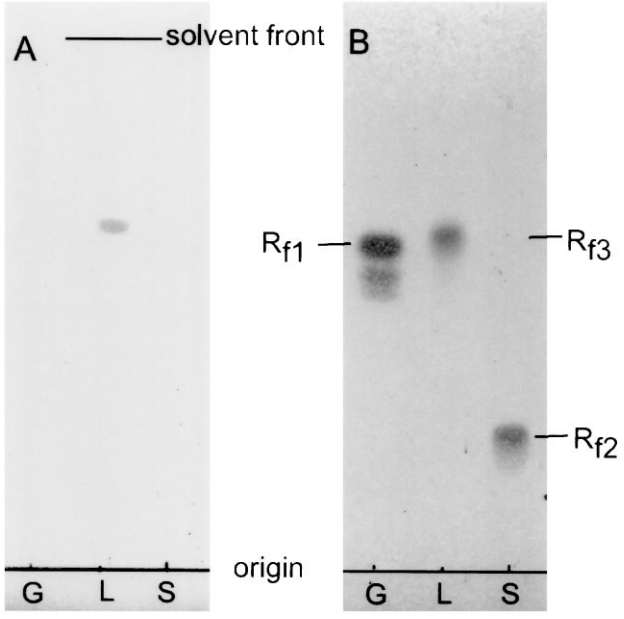


Fig. 2. TLC comparison of the mobility of leech sphingoglycolipids (Lane 'L') to that of galactocerebrosides (Lane 'G') and sulfatides (Lane 'S') isolated from bovine brain.

produced by bombardment with a beam of argon atoms (3 kV). The accelerating voltage was 10 kV and the resolution set at 1000. The instrument was scanned in 14 s with an interscan delay of 3 s from m/z 0 to 1500. Spectra presented were from four averaged scans. Emission current was 5 mA. The matrix used was *p*-nitrobenzyl alcohol for fatty acid methyl esters and glycerol for the intact lipids.

3. Results and discussion

The blood-sucking leech species, *H. medicinalis*, was bred in the laboratory and fed on cow blood. To determine whether cerebrosides isolated from this leech species are a significant constituent in cow blood, we compared the mobility of leech glycosphingolipids to that of lipid moieties extracted from cow blood using TLC analysis (Fig. 1). Leech

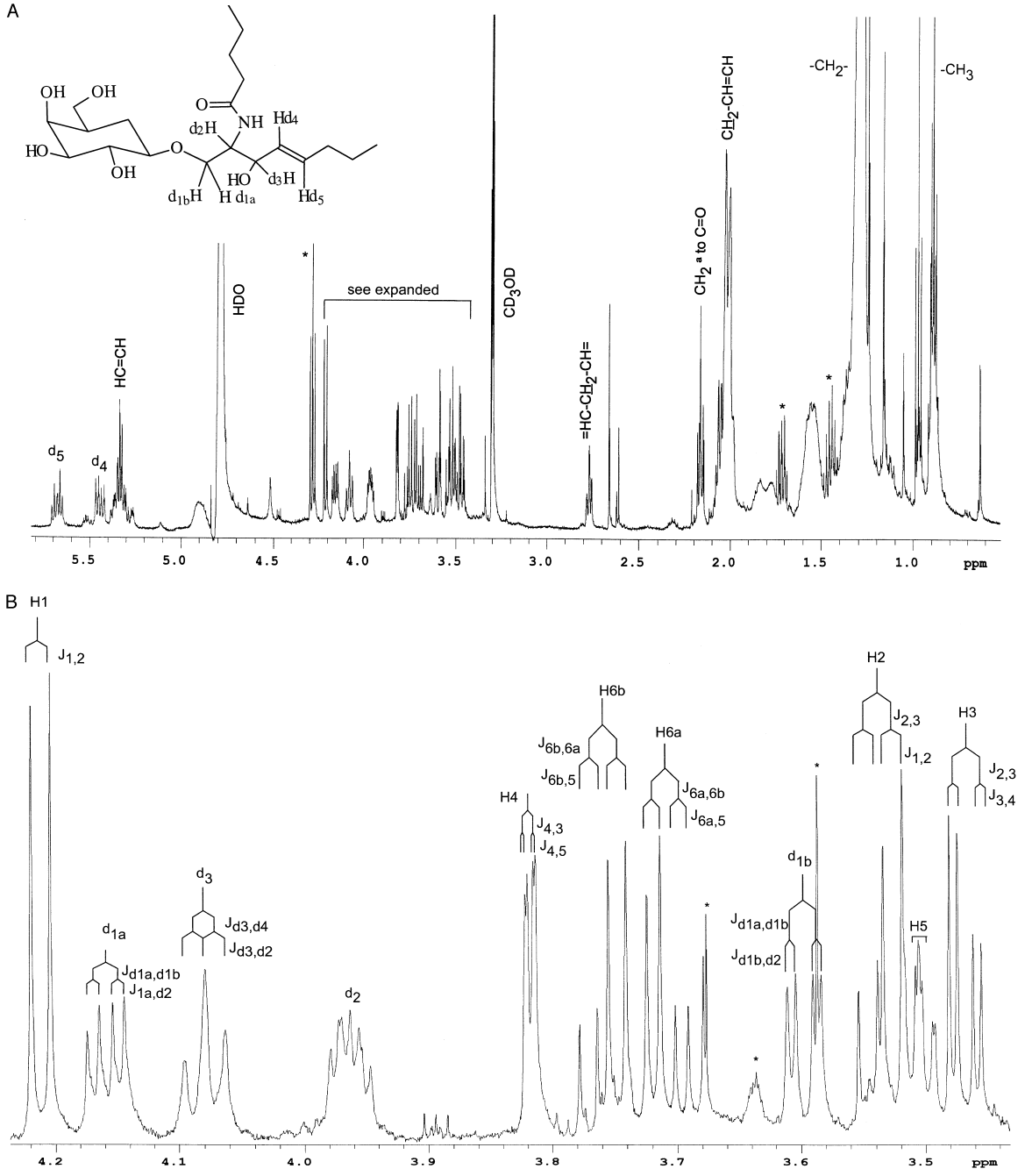


Fig. 3. (A) 500 MHz ^{1}H NMR spectrum of leech galactocerebroside. Resonances labeled * are attributed to a contaminating alcohol. (B) Expanded region between 3.2 and 4.2 ppm. Assignments are marked in the spectrum and shown in Table 1.

glycosphingolipids occur in a broad band that migrates slowly in the solvent system 6:4:1 chloroform–acetone–methanol (Lane 'L', R_{f1} 0.36). In contrast, cow blood lipids migrate faster, indicating their lesser polarity (Lane 'C', R_{f3} 0.83, R_{f2} 0.74). Applying 2, 5, 10 and 100 times higher concentrations of cow lipids did not reveal any polar lipids with mobilities comparable to those of leech glycosphingolipids. Because the glycosphingolipids that we purified from *H. medicinalis* are not major constituents of bovine blood, we conclude that *H. medicinalis* synthesizes its own galactocerebrosides.

In a separate TLC analysis, using the solvent system 60:25:2 chloroform–methanol–water, the mobility of leech glycosphingolipids was compared to that of bovine galactose cerebroside and sulfatides from isolated from cow brain (Fig. 2B). The mobility of leech glycosphingolipids (R_{f3} 0.62) was similar to that of bovine galactocerebrosides (R_{f1} 0.61) but differed from that of bovine sulfatides (R_{f2} 0.26). Leech cerebroside, unlike bovine cerebroside or sulfatide, could be visualized by resorcinol (Fig. 2A). The color of the leech cerebroside band was sky blue.

We studied the composition and the structure of leech glycosphingolipids with 1D (Fig. 3) and 2D NMR spectroscopy, DQF–COSY (Fig. 4) and ^{1}H–^{13}C HMQC (Fig. 5). The ID ^{1}H NMR spectrum shows resonances for vinyl protons at 5.45 and 5.68 ppm with a splitting pattern that suggested the presence of sphingosine, d_4 and d_5, respectively, in Fig. 3A. In the HMQC (Fig. 5), this assignment is supported by the correlation of these protons with carbons at 132 and 135 ppm, respectively. Tracing the spin connectivities of the d_4 and d_5 protons by DQF–COSY (Fig. 4) shows that d_4 has a cross peak with a methine proton signal at 4.08 ppm (d_3) which is, in turn, coupled to another signal (d_2) at 3.96 ppm. These latter two signals are established as protons connected to an oxygenated and aminated carbon, respectively, by virtue of cross peaks at 72 and 54 ppm, respectively, in the HMQC spectrum. The proton signal at 3.96 ppm is further coupled to another pair of signals, one at 4.16 and the other at 3.60 ppm (d_{1a} and d_{1b}, respectively). The HMQC spectrum (Fig. 5) shows that both these proton signals correlate with the same oxygenated carbon resonance at 70 ppm thus establishing the sphingosine headgroup structure. Further assignments are given in Table 1.

The carbohydrate proton resonances appear in the region of 3.4–4.3 ppm (Fig. 3B). Signals

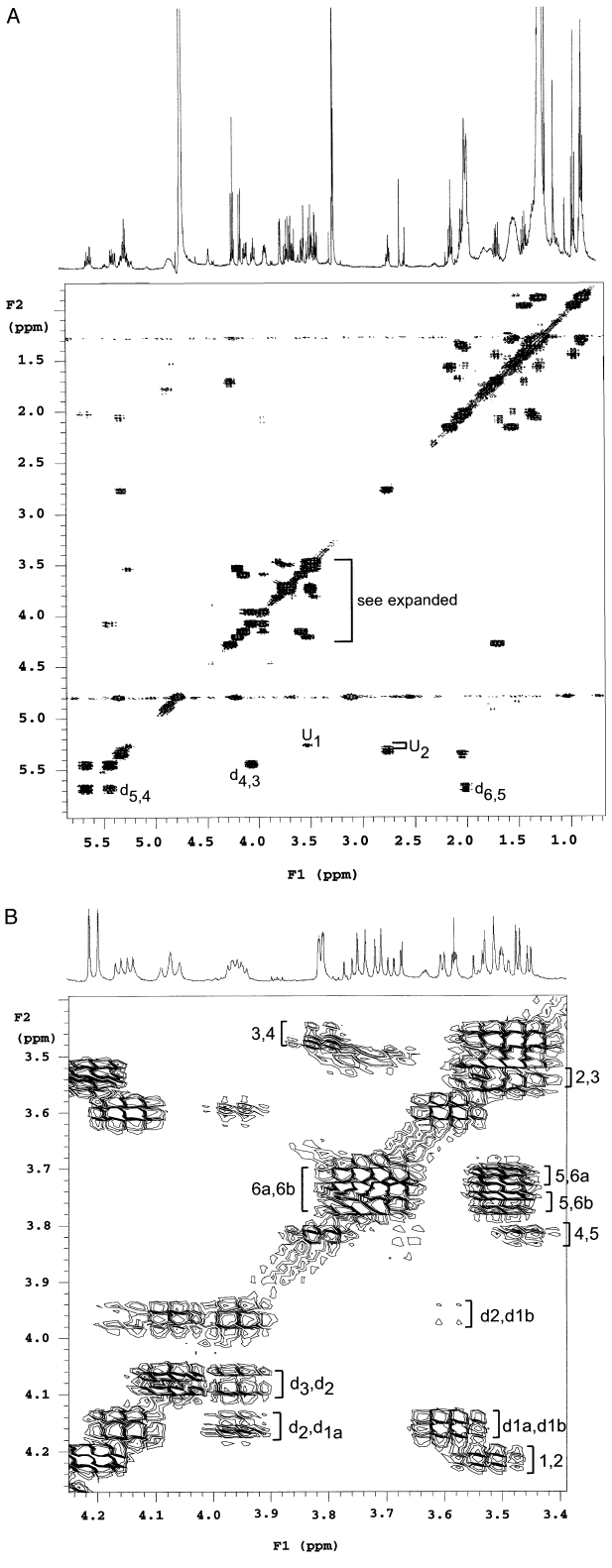


Fig. 4. (A) DQF–COSY spectrum of leech galactocerebroside. (B) Expanded region between 3.4 and 4.2 ppm. The assignments for characteristic connectivities are indicated. For instance, 1,2 denotes the cross peak correlating the signals for H-1 and H-2 of galactose, respectively.

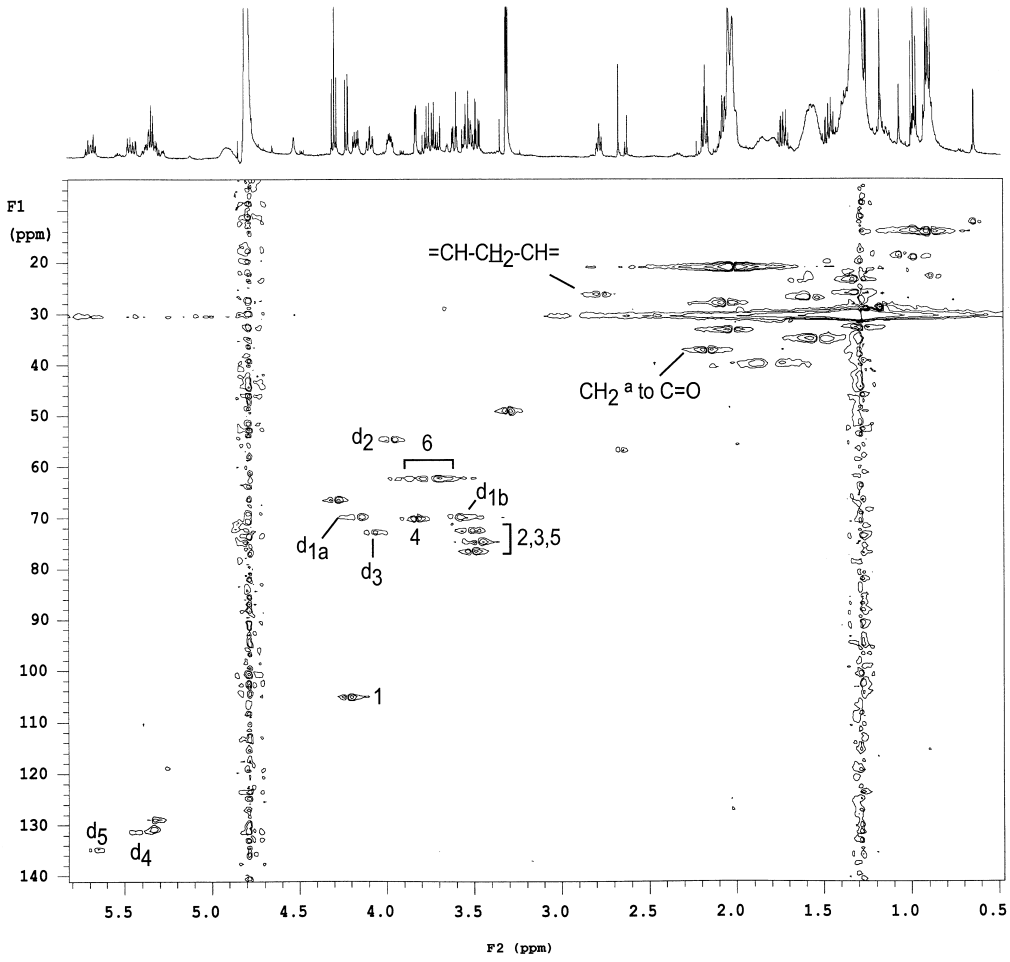


Fig. 5. ^{1}H–^{13}C HMQC spectrum of leech galactocerebroside. The assignments for characteristic connectivities are indicated. The cross peak designated * is attributed to the contaminating alcohol.

belonging to the contiguous carbohydrate spin system were traced by DQF–COSY (Fig. 4B). The doublet at 4.21 (J 7.51 Hz) is assigned to the β-anomeric proton H-1. Its large coupling constant arises from vicinal diaxial coupling with H-2. The ^{1}H–^{13}C HMQC spectrum (Fig. 5) confirms this assignment, since the signal correlates with a carbon resonance at 105 ppm. That this carbohydrate is galactose is shown by the characteristic resonance of its equatorial H-4, a doublet of doublets at 3.82 ppm, in the ^{1}H NMR spectrum (Fig. 3B). The resonances of the ABX system of the H-6 are between 3.71 and 3.76 ppm. The exact assignments are shown in the spectra and given in Table 1. The occurrence of just one cross peak in the anomeric proton region and the absence of substantial other signals in the carbohydrate region confirms the presence of only a single glycosyl component.

The ^{1}H NMR spectrum also contains signals that indicate the presence of a hydroxylated unsaturated fatty acid chain. There are low-intensity signals obscured by the prominent ones assignable to the methylene protons between two vinyl groups at 2.78 ppm. The cross peaks for these signals are readily discernible in the DQF–COSY spectrum and are labeled U_1 and U_2 (Fig. 4A). The U_2 cross peak overlaps with that for the methylene protons between two vinyl groups, and the U_1 signal appears at 3.52 ppm as expected for a proton on a carbon bearing a hydroxyl group.

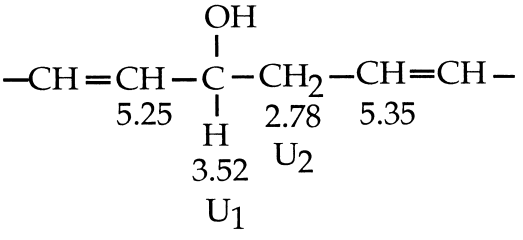
Other resonances that are typical for the fatty acid component of the cerebroside are the triplet at 2.18 ppm which is assigned to the methylene protons adjacent to a carbonyl group. The dominant signals between 0.9 and 1.3 ppm in the ^{1}H NMR spectrum (Fig. 3A) are assigned to methyl and methylene protons of the hydrocarbon tails of sphingosine and fatty acids. Signals at about 2.0 ppm are assigned to methylene protons adjacent to one vinyl group.

Leech glycosphingolipids were subjected to methanolysis and then to GC and GC–MS analysis to determine fatty acid species. The principal saturated fatty acid species were $C_{24:0}$ (49%) and $C_{22:0}$ (30%). Of lesser abundance were $C_{18:0}$ (10%), $C_{23:0}$ (7%) and $C_{16:0}$ (4%). There was only a trace of $C_{26:0}$. Unsaturated fatty acid species are present as well. The most abundant unsaturated fatty acids are $C_{25:2}$, $C_{27:2}$. The GC–MS analyses indicate that small amounts of hydroxylated fatty acids, the major one of which is a $C_{25:2}$ fatty acid, are also present (Fig. 6). The presence of such fatty acids was indicated earlier by the NMR spectroscopic analyses.

Table 1
NMR data for the galactocerebroside from leech

	Chemical shift assignmnts (ppm)[a]	
	^{1}H	^{13}C
Galactose		
1 (H-1)	4.21 (d; $J_{1,2}$ 7.51)	105
2 (H-2)	3.54 (dd; $J_{2,3}$ 9.72, $J_{1,2}$ 7.51)	73
3 (H-3)	3.47 (dd; $J_{2,3}$ 9.72, $J_{3,4}$ 3.32)	73
4 (H-4)	3.82 (dd; $J_{4,3}$ 3.32, $J_{4,5}$ 0.88)	70
5 (H-5)	3.50 (m)	70
6a (H-6a)	3.71 (dd; $J_{6b,6a}$ 11.40, $J_{6a,5}$ 5.16)	62
6b (H-6b)	3.76 (dd; $J_{6a,6b}$ 11.40, $J_{6b,5}$ 6.95)	
Sphingosine		
d_{1a}	4.16 (dd; $J_{d1a,d1b}$ 10.16, $J_{1a,d2}$ 4.86)	70
d_{1b}	3.60 (dd; $J_{d1b,d1a}$ 10.16, $J_{d1b,d2}$ 3.31)	70
d_2	3.96 (m)	54
d_3	4.08 (t; $J_{d3,d4}$ 8.07, $J_{d3,d2}$ 8.06	72
d_4	5.45 (m)	132
d_5	5.68 (m)	135
Fatty acid		
$-COCH_2-$	2.18 (t)	37
Hydrocarbon chains		
$=CH-CH_2-CH=$	2.78	24
$-HC=CH-$	5.35	130
$-CH_2-$	1.3	30
$-CH_3$	0.9	12

Hydrocarbon of hydroxylated fatty acid: ^{1}H (ppm)



[a] Data were obtained at 500 MHz for ^{1}H and 100 MHz for ^{13}C on samples in MeOH-d_4. Chemical shifts are on the δ-scale (ppm), measured downfield from internal standard Me_4Si. Spin–spin coupling values are in Hz.

Perhaps the most important result to come out of the GC–MS analyses is the characterization of the sphingosine moiety (Fig. 7A). This was done by trimethylsilylating the organic-soluble fraction obtained by partitioning the total methanolysate between water and chloroform. The signals due to the derivatized headgroup were readily observed in both leech sphingosine (Fig. 7A) and synthetic sphingosine (2-amino-1,3-dihydroxy-4-octadecene) (Fig. 7B). Hence the ion at m/z 73 is due to the Me_3Si group, the one at m/z 116 has the structure $CH_2=CHO^+SiMe_3$, the one at m/z 132 corresponds to $^+NH_2=CH–CH_2OSiMe_3$, and the signal at m/z 147 can be assigned to the 132 fragment to which a methyl group has migrated. The striking difference was in the fragments for the rest of

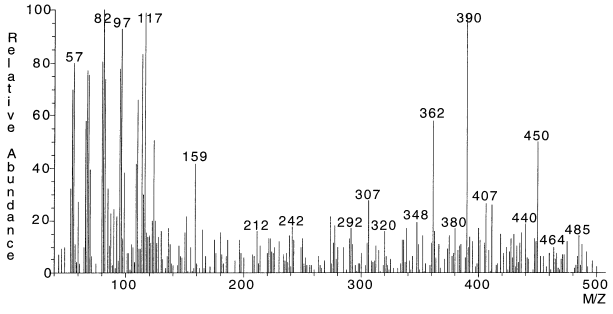


Fig. 6. GC–MS spectrum of acetylated fatty methyl ester of $C_{25:2,0H}$. Note the loss of 60 daltons (acetic acid) from the ion at 450 amu.

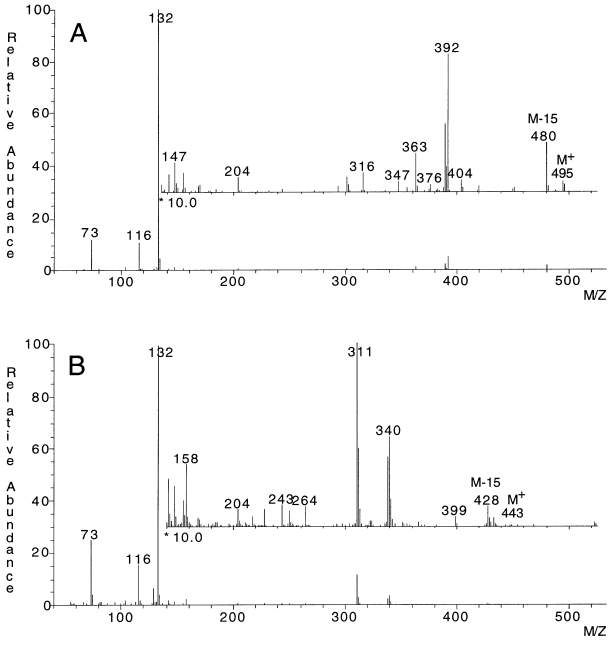


Fig. 7. (A) GC–MS spectrum of leech sphingosine, 2-amino-1,3-dihydroxydicosatriene. (B) GC–MS spectrum of synthetic sphingosine, 2-amino- 1,3-dihydroxy-4-octadecene.

the chain. The predominant α-cleavage ion from synthetic sphingosine (corresponding to $CH_3(CH_2)_{12}CH=CHOSiMe_3CH=NH_2^+$) that appeared at m/z 340 (Fig. 7B) was replaced by one at m/z 392 in the spectrum of the derivative from the leech lipid (Fig. 7A). This indicated that the chain was somewhat longer and contained an additional four carbons and two double bonds. The other ions that were derived from the hydrocarbon chain (including the molecular ion) consistently showed this difference of 52 daltons. An FABMS analysis (Fig. 8) of the total chloroform-soluble extract from the methanolysate contained ions for all of the fatty acids, as well as those for the protonated 2-amino-1,3-dihydroxydocosatriene after the expected loss of water, because of a hydroxyl group at the allylic position. This ion appears at m/z 334. The other two predominant ions at m/z 355 and 383 correspond to the protonated forms of the methyl esters of $C_{22:0}$ and $C_{24:0}$, respectively. The molecular weights of the expected predominant cerebroside species were clearly seen in the FAB mass spectrum of the intact lipid (Fig. 9). Hence the ion at m/z 863 corresponds to the cerebroside with a $C_{24:0}$ fatty acid, and the ones at m/z 835 and 817 correspond to $C_{22:0}$ and $C_{20:0}$ species, respectively. Ions for triunsubstituted fatty acid derivatives, which were minor or undetectable in the GC–MS analysis, were also observed.

This study reports on the isolation of cerebrosides from the leech species, *H. medicinalis*. The structure of these glycosphingolipids is characterized by ^{1}H NMR spectroscopy, DQF–COSY and HMQC, as well as GC–MS, ESIMS, and FABMS. The major unusual feature of the structure is that it contains an unusual 2-amino-1,3-dihydroxydocosatriene chain and very long fatty acids. There are also significant amounts of unsaturated fatty acyl chains and minor amounts of hydroxylated unsaturated chains. It is clear that the blue color in the resorcinol test for this lipid stems from the uniqueness of the hydrocarbon chains, and this may serve as a way of quickly determining whether this unusual moiety appears in other biological systems. *H. medicinalis* is a useful model system for studying the developmental role of carbohydrate

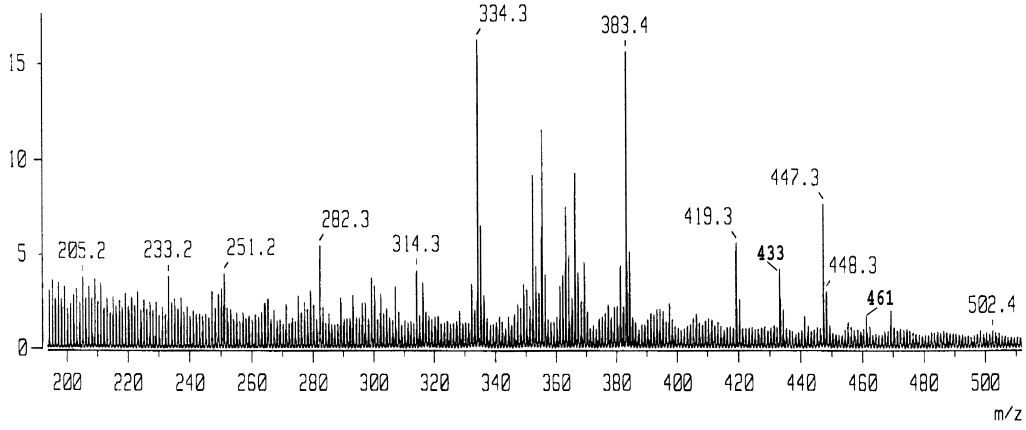


Fig. 8. Positive-ion FABMS of the total chloroform-soluble extract from the methanolysate of leech cerebroside. The peak at m/z 333.4 represents the protonated 2-amino-1,3-dihydroxydicosatriene after loss of water. The remaining peaks represent the following fatty acids: m/z 355.5 ($C_{22:0}$), 383.4 ($C_{24:0}$), 419 ($C_{27:3}$), 433 ($C_{28:3}$), 447 ($C_{29:3}$), 461 ($C_{30:3}$), and 503 ($C_{33:3}$).

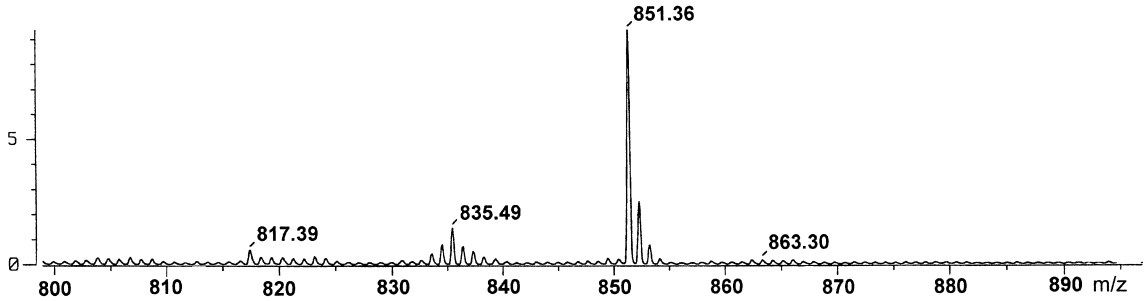


Fig. 9. Negative-ion FABMS of leech galactocerebroside. The ions at m/z 863, 835 and 817 correspond to leech cerebrosides with $C_{24:0}$, $C_{22:0}$ and $C_{20:0}$ fatty acid species, respectively. The intense ion at m/z 851 is from the glycerol matrix.

recognition in the generation of neuronal patterns [16,17,22]. For example, experimentally manipulating the cultured, virtually intact, embryo demonstrated the critical role of galactose-specific recognition in axonal targeting. Perhaps the embryonic leech will also lend itself to an in situ analysis of the function of galactocerebrosides.

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