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A GALACTOSPHINGOLIPID FROM THE LICHEN, *RAMALINA CELASTRI*

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Abstract—The characterization of a glycosphingolipid in a lichen is reported for the first time. That occurring in *Ramalina celastri* is an *O*-β-D-galactopyranosyl-(1 → 1′)-ceramide. Its main lipid components are (4*E*)-sphingenine, sphinganine and *icosa*-sphinganine, esterified with palmitic, oleic and 2-hydroxypalmitic acids.

INTRODUCTION

Glycolipids, as a group, are one of the major components of cell membranes in almost all living organisms but their functional significance remains largely undetermined [1–3]. Glycosphingolipids and glycoglycerolipids have been characterized in fungi, bacteria, yeasts, algae and animal tissues [4–8] but little is known concerning the detailed structure of those from lichens [9–13]. Several studies have been carried out on the fatty acid composition of lichens, namely those of *Evernia prunasti*, *Cladonia fimbriata*, *Hypogymnia physodes*, *Peltigera* sp. and *Xanthoria parietina* [10–12]. In the present work, the characterization of a glycosphingolipid from the lichen, *Ramalina celastri*, is described.

RESULTS AND DISCUSSION

The total lipids (yield 41.2 mg g^{-1} dry wt) extracted from *R. celastri* were fractionated by the method of Folch giving neutral and polar lipids (yield 18.1 mg g^{-1} dry wt), similar to yields obtained from other lichens [9, 10]. Those recovered from the lower Folch layer were examined by TLC, which detected four anthrone- and orcinol-positive spots with R_fs of 0.36, 0.60, 0.66 and 0.75. The R_f 0.60 component has already been characterized [13] as a digalactosyl glycerol esterified with long-chain fatty acids. Lepage [14] cited R_f 0.62 for a similar molecule isolated from plants, as well as R_f 0.77 for an esterified monogalactosyl glycerol. The possibility of a monohexosyl sphingolipid component was raised by the report of Fujino *et al.* [15], who found a glucosylceramide with R_f 0.78 from rice bran.

The mixture was fractionated on a silica gel 60-mesh column using chloroform containing increasing proportions of methanol, giving rise to fraction A (R_fs 0.75 and 0.66, 0.5 mg g^{-1}) fraction B (R_f 0.60, mg g^{-1}) and fraction C (R_fs 0.60 and 0.36, 0.41 mg g^{-1}). Fraction A was repeatedly chromatographed on silicic acid columns using various chloroform–methanol mixtures with increasing proportions of methanol, giving a pure fraction (G-1; R_f 0.66) with a 17:3 mixture. It was positive for carbohydrate and glycosphingolipid but negative for phosphorus. Aqueous acid hydrolysis gave rise to galactose (GC-mass spectrometry of alditol acetate) and methylation analysis to 2,3,4,6-Me_4-Gal (GC-mass spectrometry of derived OMe alditol acetate), corresponding with a single Gal*p* unit. Its glycosidic configuration is β, by virtue of a low field C-1 signal at δ 103.0 and a high field H-1 signal at δ 4.13 (*d*), J = 7.8 Hz. The complete ^{13}C and ^{1}H NMR spectra of G-1 closely resemble those reported by Dabrowski *et al.* for galactosyl ceramides from bovine brain [16] containing and *n*-fatty acid esters, some with hydroxy groups.

Bidimensional NMR examination of G-1 gave rise to unambiguous assignments. The linkage between C-1 of β-D-Gal*p* (δ 103.0) and C-1 of sphingosine was shown by an HMBC experiment, which showed correlation between the H-1 of β-Gal*p* (δ 4.13, *d*) and C-1 of ceramide (δ 68.0). Also found was a cross-peak correlating NH of sphingosine (δ 7.29, *d*) with C-2 of a hydroxy fatty acid (δ 73.6). The resonance at δ 52.5

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was from C-2 of sphingosine, since its shift is similar to that already assigned [4, 16]. In the carbonyl region of the ^{13}C NMR spectrum, there were two signals at δ 172.3 and 174.0 from the amide linkage of ceramide, one with and the other without hydroxy fatty acid [16]. In the methylene region of the ^1H NMR spectrum, the signal at δ 2.25 (*m*) arose from C-2 of non-hydroxylated fatty acids and that at δ 3.19 (*m*) from hydroxy fatty acids, similar to those found with fatty acids of glucosylceramide [4, 17] and gangliosides [18].

The major fatty acid methyl esters found on methanolysis of G-1 (GC-mass spectrometry) were 14:0 (2.5%), 16:0 (36%), 18:0 (13%), 18:1 (28%) and 18:2 (10%). A hydroxylated 16:0 (11%) was present, as indicated by fragment ions at *m/z* 90 and 227 $[M\text{-}59]^+$, characteristic of 2-hydroxy *n*-fatty acids [19]. The presence of unsaturated fatty acids was also indicated by signals at δ 125.0–131.0 in the ^{13}C NMR spectrum of G-1 and, that of hydroxy fatty acids, by an HMBC cross-peak correlating the NH of sphingosine (δ 7.29, *d*) with C-2 of an hydroxy fatty acid (δ 73.6). The resonance at δ 52.5 was from C-2 of sphingosine. Several hydroxylated fatty acids, as well as other types, have been reported in lipids from lichens [9–12].

A negative FAB-mass spectrum of G-1 contained several ion peaks (Table 1) corresponding with galactosylceramide with a ceramide moiety having a variety of fatty acids and long-chain base components. Appropriate fragment ions corresponding with the dominant ceramide portion were present at *m/z* 536, 552, 562, 565, 582 and 591, as well as respective $[M-162]^-$ ions.

The precise physiological functions of lichen glycolipids are not yet clear. Thus, an important task in the future is to determine their biological significance, if any. One possibility is that they maintain the structural integrity of membranes, as proposed for other organisms [20, 21], or perhaps they have a role in symbiosis between alga and fungi, as suggested for the bacterium, *Rhizobium trifolii* [22].

Table 1. Major ion peaks (negative FAB-mass spectrum) of galactosylceramide and corresponding molecular species of ceramide moiety

		Ceramide species	
$[M-H]^-$ (%)	$[M-162]^-$ (%)	FA*	LCB†
698 (25)	536 (28)	16:0	18:1
714 (10)	552 (13)	OH-16:0	18:1
724 (13)	562 (17)	18:1	18:1
727 (5)	565 (3)	16:0	20:0
		18:1	18:0
743 (11)	582 (15)	OH-16:0	20:0
753 (5)	591 (8)	18:1	20:0

* FA = fatty acid.

† LCB = long-chain base [18:1 = (4*E*) sphingenine; 18:0 = sphinganine; 20:0 = *icosa*-sphinganine].

EXPERIMENTAL

Plant material. The lichen was harvested from November to January (in spring to early summer), from roadside trees in the residential district of Jardim Social, Curitiba, PR., Brazil. Extraneous material was removed carefully and the clean tissue ground to a powder.

General. All evapns were carried out below 40° under red. pres. Total carbohydrate contents were determined by the PhOH–H_2SO_4 method [23]. PC was performed as previously described [13] using Whatman No. 1 paper with *n*-BuOH–pyridine–H_2O (5:3:3), sugars being detected with alk. $AgNO_3$. TLC was performed on silica gel plates (Merck) with $CHCl_3$–MeOH–H_2O (65:25:4). Glycolipids on TLC plates were detected with I_2 vapour (lipids) and by spraying with orcinol–H_2SO_4 (carbohydrates), sodium molybdate–hydrazine HCl (phospholipids) and chlorox–benzidine (glycosphingolipids) [24]. Carbohydrate and fatty acid derivatives were identified by GC-MS by matching their R_fs and EIMS with those of standards. Analyses were carried out using a GC linked to an ion-trap MS. For alditol acetates and partially methylated alditol acetates, an OV-225 capillary column (30 m × 0.25 mm i.d.) was used, programmed from 50° to 220° (40° min^{-1}), then held. For fatty acid Me esters, a capillary column of DB-23 (30 m × 0.25 mm i.d.) was used, programmed from 50° to 212° (40° min^{-1}), then held.

Extraction and fractionation of glycolipids. Powdered lichen (100 g) was extracted × 3 with 10 vols of $CHCl_3$–MeOH (2:1) and then with $CHCl_3$–MeOH (1:1), each step under reflux for 2 hr. Extracts were then combined and evapd to small vol. (10 ml). The crude lipid extract was then partitioned with 2 ml of 0.1 M aq. KCl [25]. The upper aq. phase was removed and the lower $CHCl_3$ phase washed × 2 with Folch's theoretical upper phase (0.1 M aq. KCl–MeOH–$CHCl_3$ 4:3:1). Both phases were dried *in vacuo*. The lower phase was evapd to dryness, dissolved in $CHCl_3$ and partially purified by passage through a column (5 × 50 cm) of silica gel (Merck, 70–230 mesh). The column was eluted successively with $CHCl_3$ (700 ml) and portions (250 ml each) of $CHCl_3$ containing 2, 5, 10, 12, 15, 20, 25, 30 and 50% MeOH. Appropriate frs were combined based on TLC analysis, dried, dissolved in 2 ml of $CHCl_3$ and rechromatographed on a small column of silicic acid (40 × 3 cm) using as eluants (250 ml each) $CHCl_3$ containing increasing concs of MeOH (5, 10, 12, 15, 20, 25 and 30%). Purified glycolipids were stored in a sealed tube under N_2 below −5°.

Acid hydrolysis of glycolipid. This was carried out with 2 M TFA at 100° for 8 hr, followed by evapn to dryness and successive reduction with $NaBH_4$ and acetylation with Ac_2O–pyridine at room temp for 18 hr [26]. The resulting alditol acetates were then examined by GC-MS.

Methylation analyses of glycolipids. Samples of gly-

colipid (5 mg) were dissolved in DMSO (2 ml) and methylated with methylsulphinyl carbanion (0.5 ml) and MeI (1 ml) [27]. The methylation procedure was repeated and the product gave no absorbance of OH groups (3600–3400 cm^{-1}) in its IR spectrum. The permethylated product was then treated with refluxing 3% MeOH–HCl for 3 hr, which was neutralized ($AgCO_3$), filtered, the filtrate evapd and the residue treated with MTFA at 100° for 4 hr. The products were converted to mixts of partially *O*-methylated alditol acetates, which were analyzed by GC-MS.

Analyses of fatty acid components of glycolipids. Purified glycolipids (5 mg) were methanolyzed by refluxing with 3% MeOH–HCl for 2 hr. The resulting fatty acid Me esters were extracted with $CHCl_3$ and analyzed by GC-MS.

NMR. This was carried out on DMSO-$^2H_6$ solns at 33° using a Bruker 500-MHZ NMR spectrometer. Chemicals shifts of the signals of G-1 are reported in δ based on a standard of TMS (δ 0). β-D-Galp: δ 4.13 (*d*, H-1′). *Sphingosine*: δ 3.88 (H-1a), 3.55 (H-1b), 3.82 (H-2), 7.29 (NH), 4.07 (H-3), 5.40 (H-4), 5.59 (H-5), 1.95 (H-6).

Sphingolipid fatty acid-chain. δ 3.19 (H-2 of 2-OH 16:0), 1.45–2.30 (aliphatic CH_2s), 0.82 (CH_3). ^{13}C NMR data. β-D-Galp: δ 103.0 (C-1′), 73.0 (C-2′), 76.2 (C-3′), 69.7 (C-4′), 76.4 (C-5′), 60.8 (C-6′). *Sphingosine*: δ 68.0 (C-1), 52.5 (C-2), 70.6 (C-3), 127.0 (C-4), 130.0 (C-5), 34.1 (C-6).

Sphingolipid fatty acid-chain. δ 172.3 and 174.0 (C=Os), 73.6 (C-2 of 2-OH 16:0), 28.0–30.5 (aliphatic CH_2s), 13.5 (CH_3).

FAB-MS. Samples were suspended on the target in a glycerol–nitrobenzyl alcohol matrix. Negative ion mass range: *m/z* 500–2500 every 10 secs delay, 5 secs.

REFERENCES

1. Hakomori, S. I., *Annual Review of Biochemistry*, 1981, **50**, 733.
2. Barreto-Bergter, E., Vermelho, A. B., Hartmann, R., Pohlentz, G., Klein, R. and Egge, H., *Molecular Biochemistry and Parasitology*, 1992, **51**, 263.
3. Villas Boas, M. H., Silva, M. C. F., Oliveira, T. G., Travassos, L. R. and Barreto-Bergter, E., *Journal of Clinical Laboratory Analysis*, 1994, **8**, 260.
4. Villas Boas, M. H., Egge, H., Pohlentz, G., Hartmann, R. and Barreto-Bergter, E., *Chemistry and Physics of Lipids*, 1994, **70**, 11.
5. Vermelho, A. B., Hogge, E. L. and Barreto-Bergter, E., *Journal of Protozoology*, 1986, **33**, 208.
6. Travassos, L. R., Almeida, I. C. and Takahashi, H., *Ciência e Cultura*, 1993, **46**, 242.
7. Stults, C. L. M., Sweeley, C. C. and Macher, B. A., *Methods in Enzymology*, 1989, **179**, 167.
8. Kates, M., *Glycolipids, Phosphoglycolipds and Sulfoglycolipids*, ed. M. Kates. Plenum Press, New York, 1990, p. 235.
9. Dertien, B. K., Kok, L. J. and Kuipier, P., *Physiology of Plants*, 1977, **40**, 175.
10. Dembitsky, V. M., *Progress in Lipid Research*, 1992, **31**, 373.
11. Dembitsky, V. M., Rezanka, T. and Bychek, I., *Phytochemistry*, 1994, **36**, 601.
12. Dembitsky, V. M., Rezanka, T. and Bychek, I., *Journal of Experimental Botany*, 1994, **45**, 403.
13. Machado, M. J., Gorin, P. A. J., Torri, G. and Iacomini, M., *Brazilian Journal of Medical and Biological Research*, 1994, **27**, 253.
14. Lepage, M., *Journal of Chromatography*, 1964, **13**, 99.
15. Fujino, Y., Ohnishi, M. and Ito, S., *Agricultural and Biological Chemistry*, 1975, **49**, 2753.
16. Dabrowski, J., Egge, H. and Hanfland, P., *Chemistry and Physics of Lipids*, 1980, **26**, 187.
17. Koerner, T. W. Jr., Cary, L. W., Li, S. and Li, Y., *Journal of Biological Chemistry*, 1979, **254**, 2326.
18. Koerner, T. A., Prestegard, J. H., Demou, P. C. and Yu, R. K., *Biochemistry*, 1983, **22**, 2676.
19. Ryage, R. and Stenhagen, E., *Journal of Lipid Research*, 1960, **1**, 5.
20. Curatolo, W., *Biochimica et Biophysica Acta*, 1987, **906**, 137.
21. O'Sullivan, J. N. and Dalling, M., *Journal of Plant Physiology*, 1989, **134**, 504.
22. Spaink, H. P., Sheeley, D. M., van Brussel, A. A. N., Glushka, J., York, W., Tak, T., Kennedy, E. P., Reinhold, V. N. and Lugtenberg, B. J., *Nature*, 1991, **354**, 125.
23. Kushwaha, S. C. and Kates, M. *Lipids*, 1981, **16**, 372.
24. Skipki, V. P., *Methods in Enzymology*, 1985, **35**, 396.
25. Folch, J., Lees, M. and Stanley, G. H. S., *Journal of Biological Chemistry*, 1957, **226**, 497.
26. Corradi da Silva, M. L., Iacomini, M., Jablonski, E. and Gorin, P. A. J., *Phytochemistry*, 1993, **33**, 547.
27. Morrison, I. M., In *Carbohydrate Analysis*, ed. M. F. Chaplin and J. Kennedy. IRL-Press, Washington, 1986, p. 205.