Glycosyldiacylglycerolipids from the Lichen *Dictyonema glabratum*

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Three glycolipids (**1**−**3**) were isolated from the basidiolichen *Dictyonema glabratum*. Their carbohydrate and lipid components were structurally characterized using 1D ^{1}H and ^{13}C and 2D NMR spectroscopy, complemented by mass spectrometry, as were the carbohydrate moieties formed on saponification. These were *O*-α-D-Gal*p*-(1″→6′)-*O*-*β*-D-Gal*p*-(1′↔1)-2,3-diacyl-D-glycerol (**2**) and two others not previously found in lichens, *O*-*β*-D-Gal*p*-(1′↔1)-2,3-diacyl-D-glycerol (**1**) and *O*-α-D-Gal*p*-(1‴→6″)-*O*-α-D-Gal*p*-(1″→6′)-*O*-*β*-D-Gal*p*-(1′↔1)-2,3-diacyl-D-glycerol (**3**). Each was saponified to give the free carbohydrates and its fatty acid methyl esters. The most abundant fatty acid esters in **1**−**3** was palmitic $C_{16:0}$, but there was a wide variation of ester composition. Others present were $C_{8:0}$ and $C_{14:0}$ in **1**, $C_{14:0}$, $C_{15:0}$, $C_{17:0}$, $C_{18:0}$, $C_{18:1}$ (oleic), $C_{18:2}$ (linoleic), $C_{22:0}$, and $C_{24:0}$ in **2**, and $C_{8:0}$, $C_{14:0}$, $C_{18:0}$, $C_{18:1}$ (oleic), $C_{18:2}$ (linoleic), and $C_{18:3}$ (linolenic) in **3**. As in ascolichens, the glycolipids appear to arise from the phycobiont.

Glycolipids are present in all living organisms and occur either as glycosphingolipids or acylated glycoglycerols. They are present in the cellular membrane, and their concentration depends on their biological function.[1] Glycosyldiacylglycerides are the major representatives present in chloroplasts and comprise about 50−80% of total lipids that constitute the membrane.[2] Relatively little attention has been paid by other groups to glycolipids from lichens, with many such compounds being analyzed only in terms of fatty acid characterization and quantification, and little importance has been given to the identification of the carbohydrate components.[3−5] The presence of glycosylglycerides in ascolichens has been observed in *Evernia prunastri*, *Hypogymnia physodes*, *Cetraria islandica*, and *Cladonia impexa*, but these were not totally characterized.[6] However, we have isolated *O*-α-D-Gal*p*-(1″→6′)-*O*-*β*-D-Gal*p*-(1′↔1)-D-glycerol via alkaline extraction from a *Sticta* sp., suggesting that it arose on saponification of a native acylated form, namely, a 2,3-diacylated digalactosyl-glycerol.[7] This type of compound was isolated from *Ramalina celastri* and examined in more detail, with its lipid components also being identified.[8] This lichen also contained *β*-galactopyranosyl ceramides.[9]

We describe herein the structures of three galactosyldiacylglycerides, obtained from *Dictyonema glabratum* (Sprengel) D. Hawks.[10] (family: Dictyonemataceae), which is one of the few basidiolichens in existence.

Results and Discussion

Lipid-containing material (3.2% yield, w/w, based on lichen) was obtained from *D. glabratum*, by successive homogenization of a cleaned sample with phosphate buffer and the residue extracted with organic solvents. It was then fractionated by Folch partition with 0.1 M KCl, which resulted in isolation of components (2.0%) of the organic layer, this yield being very similar to those found for *R. celastri*[9] and other lichens.[3,6] The reducing carbohydrate content was determined by the PhOH−H_2SO_4 method and shown to be 0.29% (w/w, based on lichen extract) for the lipid extract and 0.23% (w/w, based on organic layer) for the neutral lipids. The neutral lipids (organic layer) were examined by TLC, and six orcinol-positive spots were detected with R_f values of 0.19, 0.35, 0.60, 0.69, 0.77, and 0.83. These neutral lipids were fractionated on a column of silicic acid (particle size 0.1 mm) using as eluants $CHCl_3$ and $CHCl_3$−MeOH with increasing concentrations of MeOH, resulting in the following yields for the isolated compounds: A_1 (R_f 0.83, 0.004%), **1** (R_f 0.77, 0.186%), A_3 (R_f 0.69, 0.085%), **2** (R_f 0.60, 0.101%), **3** (R_f, 0.35, 0.202%), and D (R_f 0.19, 0.066%). All fractions obtained were rechromatographed under the same conditions until pure samples were obtained. Of these, compounds **1**−**3** were studied in more detail.

Aqueous acid hydrolysis of **1**−**3** gave galactose and glycerol, which were identified and shown to occur in 1:1, 2:1 and 3:1 molar ratios (GC−MS of derived alditol acetates), respectively.

Fatty acid compositions of the purified glycolipids **1**−**3** were determined by GC−MS, and the column retention times of the resulting methyl esters were compared with those of standards. The most abundant was that of palmitic acid $C_{16:0}$. Lesser amounts of other fatty acids were also found in the purified glycolipids (see Table 1). Glycolipid **2** was unusual since $C_{15:0}$ and $C_{17:0}$ esters were present, with these compounds not having been previously reported in lichen glycolipids.

The structures of glycolipids **1**−**3** were elucidated using NMR spectra obtained for the native structure (Table 2) and deacylated form of each one (Table 3). Compound **1** contained lipid signals from δ 22.8 to 34.6 with predominant signals of CH_2 at δ 29.65 and 29.7, of CH_3 at δ 14.1, and of ester carbonyl signals at δ 174.0 and 176.0. Also detected were lipid double bonds, with several signals from δ 122.9 to 133.0. ^{1}H and ^{13}C NMR spectra showed only one signal in the C-1 region corresponding to a single Gal*p* unit in the *β*-glycosidic configuration at low field (δ 104.1) and a typical high-field H-1 signal at δ 4.06, J = 7.05 Hz. TOCSY and COSY spectra of **1** allowed the assignment of proton signals and DEPT and HMQC spectra, which were then used for assigning ^{13}C NMR signals. Once it was known that the *β*-Gal*p* unit was linked (1′↔1) to glycerol, C-2′ to C-6′, H-2′ to H-6′ of *β*-Gal*p*, and C-1 to C-3 of glycerol could be assigned. The DEPT NMR spectrum of **1** showed inverted CH_2 signals in the carbohydrate region at δ 61.4,

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Table 1. Fatty Acid Compositions of Purified Glycolipids (**1**−**3**)

	glycolipid				
fatty acid[a]	t_R[b]	% in **1**[c]	% in **2**[c]	% in **3**[c]	main mass spectral fragments (*m*/*z*)
8:0 (caprylic)	4.33	22.7	–	4.5	158, 117, 99, 74, 59, 43, 41
14:0 (myristic)	8.14	–	2.8	2.75	242, 199, 143, 129, 74, 43, 41
15:0 (pentadecanoic)	8.52	–	7.2	–	256, 199, 143, 129, 74, 43, 41
16:0 (palmitic)	9.56	67.9	58	70.1	270, 227, 185, 143, 74, 55, 43, 41
17:0 (heptadecanoic)	10.39	–	1.8	–	284, 199, 143, 129, 74, 43, 41
18:0 (stearic)	11.27	8.6	4.3	3.0	298, 255, 199, 143, 74, 55, 41
18:1 (oleic)	11.38	–	12.8	7.3	296, 264, 222, 96, 55, 41
18:2 (linoleic)	11.59	–	10.5	7.1	294, 262, 217, 95, 81, 67, 41
18:3 (linolenic)	12.31	–	–	0.8	292, 263, 199, 173, 95, 79, 41
22:0 (behenic)	15.11	–	0.4	–	354, 338, 311, 269, 199, 143, 41
24:0 (lignoceric)	18.12	–	1.1	–	382, 339, 283, 199, 143, 74, 41

[a] Fatty acid methyl esters obtained after methanolysis and analyzed by GC−MS (column DB-23). [b] Retention time (t_R) in min, at 50 220 °C (40 °C min^{-1}). [c] **1**, galactosyldiacylglyceride; **2**, digalactosyldiacylglyceride; **3**, trigalactosyldiacylglyceride.

Table 2. ^1H and ^{13}C NMR Spectroscopic Data of Glycolipids **1**−**3** (δ Values, ppm)

	1		**2**		**3**	
carbon	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
C-1‴	–	–	–	–	4.981	98.8
C-2‴	–	–	–	–	3.86	66.9
C-3‴	–	–	–	–	3.80	66.3
C-4‴	–	–	–	–	4.01	68.8
C-5‴	–	–	–	–	3.91	66.8
C-6‴	–	–	–	–	3.82[a]	61.8
	–	–	–	–	3.74[b]	
C-1″	–	–	4.88	100.32	4.917	99.1
C-2″	–	–	3.74	69.9	3.55	70.8
C-3″	–	–	3.63	70.3	3.51	73.9
C-4″	–	–	3.95	70.7	3.73	72.2
C-5″	–	–	3.89	72.1	4.18	72.9
C-6″	–	–	3.75[a]	62.5	3.85[a]	67.8
	–	–	3.73[b]		3.78[b]	
C-1′	4.06	104.1	4.27	104.9	4.281	104.2
C-2′	3.27	71.3	3.53	72.0	3.45	71.2
C-3′	3.26	71.13	3.53	74.3	3.45	74.2
C-4′	3.61	68.9	3.93	69.7	3.85	69.2
C-5′	3.46	75.16	3.74	74.1	3.72	74.2
C-6′	3.44[a]	61.4	3.74[a]	68.4	3.72[a]	67.9
	3.52[b]		3.76[b]		3.76[b]	
C-1, Gly	3.74[a]	68.56	3.98[a]	68.59	3.95[a]	67.9
	3.49[b]		3.72[b]		3.78[b]	
C-2, Gly	5.06	70.5	5.24	72.12	5.25	70.8
C-3, Gly	4.17[a]	63.2	4.19[a]	63.76	4.45[a]	63.3
	4.01[b]		4.18[b]		4.29[b]	
CH_2	1.1−2.3	22.8−34.6	1.2−2.3	23.4−35.0	1.2−2.3	22.8−34.4
CH=CH	5.12	122.8−133.0	5.23	128.8−130.8	5.35	126.0−133.3
C=O	–	173.9−176.0	–	174.6−175.0	–	174.5
CH_3	0.83	14.1	0.83	14.4	0.85	13.9

[a,b] Chemical shifts of protons linked directly to the carbon nucleus.

which corresponded to C-6′ of the Gal*p* unit, indicating that its carbon was not O-substituted and that those at δ 68.6, 70.5, and 63.2 respectively arise from C-1 (downfield *O*-glycosylation α-shift), C-2, and C-3 of the glycerol moiety. Examination of **1** using TOCSY and HMQC NMR techniques gave further information. Comparison of resonances in ^{13}C and DEPT NMR spectra confirmed the presence of lipid groups such as -CH_3, which in DEPT NMR spectra were positive and were distinguishable from inverted CH_2 signals. Each of these assignments was confirmed by HMQC, that indicated in a more evident way those that correspond to glycerol.

Glycolipid **2** showed lipid signals in the ^{13}C NMR spectrum at δ 14.4 (CH_3), signals from δ 23.4 to 35.0 of CH_2 with a predominant one at δ 30.2, and ester carbonyl signals at δ 174.6, 174.8, and 175.05. Lipids containing double bonds were indicated by signals at δ 128.8−130.85. The anomeric ^1H and ^{13}C NMR regions each contained two signals of equivalent size at δ 4.273/104.9 that corresponded to single β-Gal*p* units, and at δ 4.881/100.3 from single α-Gal*p* units. HMQC examination of **2** gave useful information for the ^1H and ^{13}C NMR assignments, and indicated signals that correspond to glycerol, an O-substituted C-6′ of the β-Gal*p* unit (δ 68.4) and a nonsubstituted C-6″ signal belonging to the α-Gal*p* unit (δ 62.51). The ^{13}C and ^1H NMR signals at δ 68.6 and [3.980 (a), 3.725 (b)]; 72.1 and 5.244; 63.8 and [4.195 (a), 4.186 (b)] corresponded to C-1, C-2, and C-3 and adjacent protons, respectively, of the glycerol moiety.

The ^{13}C NMR spectrum of glycolipid **3** contained lipid signals of CH_3 at δ 13.9 and of CH_2 from δ 22.8 to 34.4, with a predominant signal at δ 29.85. A carbonyl signal of an ester was present at δ 174.5, as were those of lipid double bonds at δ 126.1 to 133.3. The respective anomeric ^1H and ^{13}C NMR regions each contained three signals of equivalent size, namely at δ 4.281/104.2 corresponding to

Table 3. ^{1}H and ^{13}C NMR Spectroscopic Data of Saponified Glycolipids **1**−**3** (δ Values, ppm)

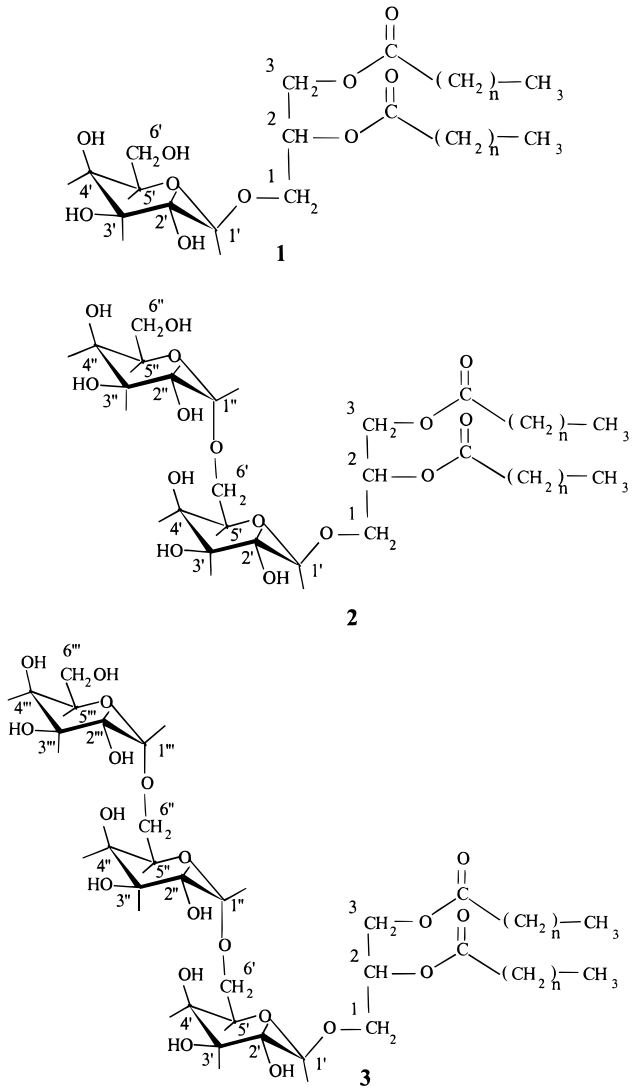
	1		**2**		**3**	
carbon	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
C-1‴	–	–	–	–	5.01	99.4
C-2‴	–	–	–	–	3.86	70.5
C-3‴	–	–	–	–	3.86	69.8
C-4‴	–	–	–	–	3.95	70.4
C-5‴	–	–	–	–	3.89	71.5
C-6‴	–	–	–	–	3.77[a]	62.7
	–	–	–	–	3.75[b]	
C-1″	–	–	5.00	99.7	4.92	99.7
C-2″	–	–	3.86	69.8	3.61	72.5
C-3″	–	–	3.86	71.0	3.63	72.5
C-4″	–	–	4.01	70.8	3.78	70.2
C-5″	–	–	3.79	72.5	4.05	70.7
C-6″	–	–	3.77[a]	62.7	3.93[a]	67.5
	–	–	3.75[b]		3.88[b]	
C-1′	4.35	104.0	4.44	104.5	4.44	104.5
C-2′	3.61	73.7	3.57	72.3	3.57	72.3
C-3′	3.58	71.2	3.68	70.2	3.68	70.2
C-4′	3.89	71.5	3.99	70.2	3.99	70.2
C-5′	3.64	75.7	3.95	74.2	3.96	74.2
C-6′	3.79[a]	61.5	3.92[a]	67.9	3.92[a]	67.9
	3.74[b]		3.73[b]		3.73[b]	
C-1, Gly	3.72[a]	71.2	3.89[a]	72.4	3.79[a]	72.9
	3.79[b]		3.79[b]		3.79[b]	
C-2, Gly	3.92	69.3	3.94	71.8	3.94	72.2
C-3, Gly	3.67[a]	63.0	3.69[a]	64.0	3.68[a]	63.5
	3.62[b]		3.63[b]		3.63[b]	

[a,b] Chemical shifts of protons linked directly to the carbon nucleus.

a β-Gal*p* unit, and those at δ 4.917/99.1, and 4.981/98.8 both arising from α-Gal*p* units.

The carbohydrates obtained after saponification of **1**−**3** were identified by NMR techniques (Table 3) identical to those used above for the glycolipids (Table 2), and which confirmed their carbohydrate structures. The ^{13}C NMR spectrum of saponified **1** contained nine signals. Its H-1 and C-1 resonances were typical of β-Gal*p* units and its HMQC NMR spectrum showed ^{1}H and ^{13}C signals of CH_2 groups at δ 3.74 and 3.79/61.5 (β-Gal*p*), δ 3.72 and 3.79/71.2 (H-1 and C-1, Gly), and 3.62 and 3.67/63.0 (H-3 and C-3, Gly), characteristic of a (1′↔1)-glycerol linkage. The ^{13}C NMR spectrum of saponified **2** contained fifteen signals. Its H-1 and C-1 signals indicated β-Gal*p* and α-Gal*p* units (1:1 ratios). CH_2 resonances were present at δ 3.73 and 3.92/67.9 (β-Gal*p*), 3.55 and 3.77/62.7 (α-Gal*p*), 3.79 and 3.89/72.4 (H-1 and C-1, Gly), and 3.63 and 3.69/64.0 (H-3 and C-3, Gly), indicating (1″→6′) and (1′↔1) linkages. The ^{13}C NMR spectrum of saponified **3** contained twenty one signals and its C-1 and H-1 resonances arose from β-Gal*p* and α-Gal*p* units (1:2 ratios). CH_2 resonances appeared at δ 3.73 and 3.92/67.9 (β-Gal*p*), 3.88 and 3.93/67.5 (α-Gal*p*), 3.75 and 3.77/62.7 (α-Gal*p*), 3.79/72.9 (H-1 and C-1, Gly), and 3.63 and 3.68/63.5 (H-3 and C-3).

With these NMR results, the structures of the glycolipids were elucidated as **1**−**3**. Glycolipid **1** is a *O*-β-D-Gal*p*-(1′↔1)-2,3-diacyl-D-glycerol, a galactosylglyceride, whose main fatty ester is $C_{16:0}$. Glycolipid **2** is a *O*-α-D-Gal*p*-(1″→6′)-β-D-Gal*p*-(1′↔1)-2,3-diacyl-D-glycerol, a digalactosyldiacylglyceride, whose carbohydrate moiety was reported in a lichen for the first time in *R. celastri*[8] and whose main ester is $C_{16:0}$ with the unusual $C_{15:0}$ and $C_{17:0}$. Glycolipid **3** is a *O*-α-D-Gal*p*-(1‴→6″)-*O*-α-D-Gal*p*-(1″→6′)-*O*-β-D-Gal*p*-(1′↔1)-2,3-diacyl-D-glycerol, a trigalactosyldiacylglyceride, with the major lipid ester is also that of $C_{16:0}$.



The amount of lipids present in lichens of the lichen lipids is related to two factors, namely, the particular species and the weather conditions.[3,6]

The presence of acylated galactosylglycerols in the basidiomycetous lichen *D. glabratum*, as well as the ascomycetous *R. celastri* is of interest in terms of their respective origins, namely, myco- or phytobiont. This type of compound has not been completely characterized in fungi, because of the very small amounts in their tissues.[11] It appears that they arise respectively from the phycobionts *Stigonema* or *Scytonema* (M. Marcelli, personal communication) and *Trebouxia*. The presence of the trigalactosylglyceride **3** in *D. glabratum* reinforces this argument, since this compound has been described in algae,[12] but is now described for the first time in a lichen. The presence of high amounts of the $C_{16:0}$ fatty acid in the glycolipids is very common in photosynthetic tissues, such as in blue-green algae,[13] but it is interesting that $C_{15:0}$ and $C_{17:0}$ esters are present in **2**, since they occur in basidiomycetes and $C_{15:0}$ appears with more frequency in spores.[11] This agrees with the observations that these galactolipids are associated with photosynthetic membranes and that the galactosylglyceride is the most abundant among them, with there also being a considerable amount of unsaturated lipids.[14−16] From these data, it can be suggested that the carbohydrate moiety of the glycolipids arises from the phytobiont and some of the fatty acids from the mycobiont, showing a symbiosis from the lichen *D. glabratum*.

Experimental Section

General Experimental Procedures. Extracts of lipid-containing compounds, obtained from *D. glabratum* (see below), were evaporated at <40 °C under reduced pressure. Total carbohydrate contents were determined by the PhOH−H_2SO_4 method,[17] the solutions were evaporated to dryness and to the residues dissolved in a water (1 mL) and aqueous 5% phenol (0.5 mL) solution. Concentrated H_2SO_4 (2.5 mL) was added and the mixture maintained in a boiling water bath for 5 min. The mixtures were cooled for 30 min and their absorbances read at 490 nm and compared with that of a galactose standard. To show the purity of glycolipids **1**−**3**, TLC was performed on silica gel plates (DC-Alufolien Kieselgel 60 from Merck) solvent: $CHCl_3$−MeOH−H_2O, 65:25:4 (v/v). The homogeneity of saponified **1**−**3** was determined by paper chromatography using Whatman No. 1 paper (solvent, phenol−water, 5:2) and detected with alkaline $AgNO_3$.[18]

The presence of the glycolipids on TLC plates was detected by spraying with carbohydrate-specific orcinol−H_2SO_4.[19] Carbohydrates and fatty acids, obtained from the glycolipids by saponification, were analyzed by GC−MS incorporating EI, and compared with those of standards (lipid obtained from Sigma Chemical Co.). To analyze alditol acetates, obtained on successive hydrolysis, $NaBH_4$ reduction, and acetylation, the samples were examined by GC−MS on a DB-225 capillary column (30 m × 0.25 mm i.d), programmed from 50 to 220 °C (40 °C min^{-1}), then held at 220 °C. Fatty acid Me esters were analyzed on a DB-23 capillary column (30 m × 0.25 mm i.d.), programmed from 50 to 220 °C (40 °C min^{-1}), then held at 220 °C. NMR spectra were determined in $CDCl_3$−CD_3OD (3:1) at 30 °C, using acetone (δ_H 2.224, δ_C 30.2) as internal standard (δ 0). Signal assignments in the 1D ^1H and ^{13}C NMR spectra of the glycolipids (Table 2) and carbohydrates formed on saponification (Table 3) were carried out using COSY, TOCSY, HMQC, and DEPT programs. All spectra were obtained with a Bruker DRX 400 MHz NMR spectrometer.

Plant Material. The lichen *D. glabratum* was collected in March 1997 from the embankment on the opposite side of the National Highway (BR) 277 from the 47 km sign, at an altitude of 900 m, near Curitiba, PR, Brazil.

Extraction and Isolation. The lichen (367 g) was cleaned and homogenized in a blender with a 0.1 M phosphate buffer at pH 7.2, to extract most of the proteins, and the resulting residue (231 g) extracted three times each with $CHCl_3$−MeOH (2:1; 1 L), $CHCl_3$−MeOH (1:1; 1 L), and Me_2CO. Each organisc extraction was carried out under reflux for 2 h. Each lipid-containing extract was examined on TLC and since they were found to have spots with identical R_f values, they were combined and evaporated. The residue in $CHCl_3$−MeOH (100 mL; 2:1)] was partitioned by the Folch procedure,[20] the aqueous layer was removed (polar lipids), and the organic layer (neutral lipids) was washed twice with Folch's theoretical upper phase (0.1 M aqueous KCl−MeOH−$CHCl_3$, 4:3:1). Both the organic and aqueous layers were evaporated to dryness. The former was then dissolved in $CHCl_3$ and chromatographed on a column of silicic acid (particle size 0.1 mm, column dimensions 5.6 × 12 cm). Elution was carried out using $CHCl_3$ (600 mL) and then with mixtures of $CHCl_3$−MeOH (600 mL each), with successive MeOH concentrations of 1, 2, 3, 5, 7, 10, 12, 15, 20, 25, 30, 35, 40, and 50%. Glycolipids **1**, **2**, and **3** were eluted with concentrations of MeOH of 10, 20, and 30%, respectively. These were stored in sealed tubes below −10 °C.

Acid Hydrolysis of Glycolipids. Hydrolyses (2 mg) of purified glycolipids were performed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness, the residual material being successively reduced with aqueous $NaBH_4$ (5 mg) and acetylated with Ac_2O−pyridine at 25 °C for 15 h.[7] The resulting alditol acetates were analyzed by GC−MS as described above.

Analysis of Fatty Acid Components of Glycolipids. Purified glycolipids (5 mg) were methanolyzed by refluxing in 3% MeOH−HCl for 2 h.[21] The resulting fatty acid Me esters were extracted from water with $CHCl_3$ and analyzed by GC−MS as described above (Table 1).

Glycolipid Saponification. Samples **1-3** (30 mg) were each treated with 0.5 M NaOH in $CHCl_3$−MeOH (1:1; 5 mL) at 40 °C for 90 min[13] and following evaporation, the residue was deionized using mixed bed ion-exchange resins Dowex-50 resin (H^+ form) and Amberlite resin IR-45 (HCO_3^- form). Filtration and evaporation gave rise to the free carbohydrate.

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