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Carbohydrate, glycolipid, and lipid components from the photobiont (*Scytonema* sp.) of the lichen, *Dictyomema glabratum*

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Abstract—The photobiont of the lichen, *Dictyonema glabratum* (*Scytonema* sp.), was isolated and cultivated in a soil-extract medium and submitted to chemical analysis. Successive extractions with $CHCl_3$–MeOH, aqueous MeOH, and H_2O gave rise to solutions of lipids (25%), low-molecular-weight carbohydrates (22%), and polysaccharides (4%), respectively. TLC of the lipid extract showed the presence of glycolipids, which were further purified and examined by NMR spectroscopy and GC–MS. Monogalactosyldiacylglycerol (1%), digalactosyldiacylglycerol (0.8%), trigalactosyldiacylglycerol (0.4%), and sulfoquinovosyldiacylglycerol (0.5%) were identified. The most abundant fatty acid ester in each fraction was palmitic ($C_{16:0}$), but a great variation of the ester composition from one to another was found. Others present were those of $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:1}$, $C_{17:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{22:0}$, $C_{22:2}$, and $C_{24:0}$. The lipid extract was also subjected to acid methanolysis, which gave rise to dodecane, 2-Me-heptadecane, 2,6-Me_2-octadecane, and 8-Me-octadecane, methyl esters of $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{20:0}$, and $C_{24:0}$ fatty acids, and the dimethyl ester of decanedioic acid. The polysaccharide had mainly Glc, Gal, and Man, with small amounts of 3-*O*-methylrhamnose and 2-*O*-methylxylose, both found in plants, and unexpectedly, some of the units were β-galactofuranose, typical of fungal, but not cyanobacterial polysaccharides. The low-molecular-weight carbohydrates showed mannose as the main free reducing sugar, which differs from *Nostoc* sp. and *Trebouxia* sp. photobionts.

Keywords: *Scytonema* sp.; *Dictyonema glabratum*; Fatty acids; Glycolipids; Polysaccharides; Monosaccharides; NMR; GC–MS

1. Introduction

Studies have been carried out in our laboratory on comparison of polysaccharides present in ascomycetous and basidiomycetous lichens with those of free-growing ascomycetes and basidiomyctes. Surprisingly, *Dictyonema glabratum*, whose predominant mycobiont is a basidiomycete, contained a (1→6)-linked β-mannopyrananan and pseudonigeran, typical of an ascomycete.[1,2] Furthermore, a branched (1→3), (1→6)-linked β-glucan, typical of free basidiomycetes, was found in the ascomycetous lichens *Collema leptosporum* and *Teloschistes flavicans*.[3,4] Also unexpected was the presence of a fungus-type β-galactofuranan in the cultivated *Trebouxia* photobiont of *Ramalina gracilis*.[5] In terms of the carbohydrate components present in glycolipids of *D. glabratum*, the results obtained by Sassaki et al.[6–9] were consistent, being typical of plants and photobionts, namely monogalactosyldiacylglycerol (MGD), digalactosyldiacylglycerol (DGD), trigalactosyldiacylglycerol (TGD), *lyso*-TGD (Sn-1 and Sn-2), and the negatively charged glycolipids sulfoquinovosyldiacylglycerol (SQDG) and *lyso*-SQDG (Sn-1 and Sn-2). However, the fatty acid components of these glycolipids differed in their number and diversity from those obtained from many plants and other lichens.[6–11] As a continuation of these investigations, we now isolate from intact *D. glabratum* a *Scytonema* sp., which is its photobiont, and

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culture it in a minimum soil-extract medium. The differences in their lipids, glycolipids, derived fatty acid methyl esters (FAMEs), and carbohydrates,[9,12,13] present in the intact lichen and the photobiont, are determined and evaluated.

2. Experimental

2.1. Culture and isolation of the photobiont

Scytonema sp. was isolated from the fresh thallus of *D. glabratum*, which was collected in September 1999 from the embankment on the opposite side of the National Brazilian Highway, BR 277, 47 km, and at an altitude of 900 m near Curitiba, State of Paraná, Brazil.

The thallus was stirred ×3 in a PBS solution for 30 min and then in sterile water. Cells of the photobiont were scraped from the algal layer of the thallus and used as inoculum.

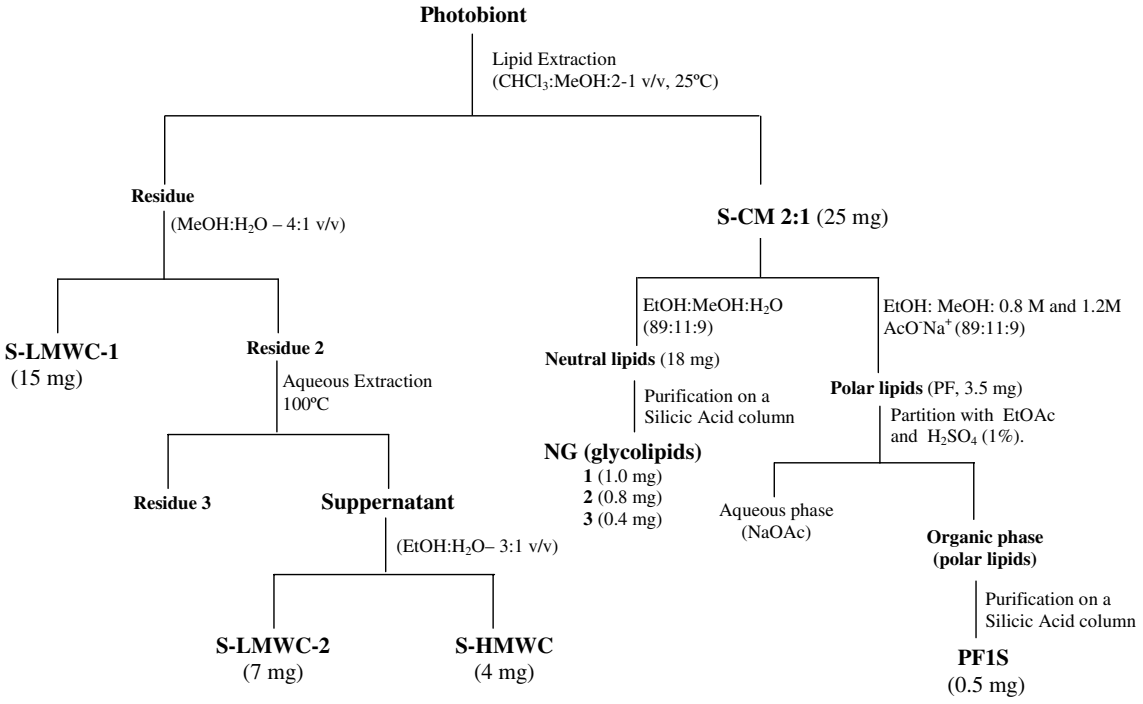
A minimum soil-extract medium was prepared using soil samples collected where the lichen was harvested. Equal parts of soil and water were autoclaved for 1 h (×2), and the mixture was allowed to cool and then filtered to give a clear filtrate, which was autoclaved and diluted 10-fold. Agar (2%) was then added to prepare a solid medium, as a modification of the method of Ahmadjian.[14]

Forty-five days after inoculation, grown colonies were selected and ground in a mortar. The fragments were then washed with sterile water and filtered successively through membranes with 500- and 150-μm mesh to eliminate bacterial contaminants.[15] Fragments retained on the latter membrane were selected and inoculated onto the soil-extract–agar medium in order to obtain pure colonies.[16]

The isolated axenic culture was grown in a static soil-extract liquid medium with illumination at 45 mol m^{-2} s^{-1} and a photo-period of 12 h at 21 ± 2 °C. After 60 days, the algal cells were removed by filtration and freeze dried.

2.2. Extraction, fractionation, and isolation of glycolipids

Scheme 1 shows the purification steps carried out on the photobiont during the isolation of glycolipids. The freeze-dried product (95.1 mg) was extracted (×3) with 2:1 $CHCl_3$–MeOH (75 mL) under reflux for 2 h.[6] Following evaporation, the extract (22.1 mg) was fractionated on a column of fast-flow DEAE-Sepharose (1.8 × 10 cm), which was eluted with 80:11:9 EtOH–MeOH–0.8 M NaOAc (20 mL, solvent A) to give rise to the OAc^- form, and then 89:11:9 EtOH–MeOH–H_2O (20 mL, solvent B) to remove excess NaOAc.[8] The column was then successively eluted with solvent B (40 mL) and solvent A (30 mL), the last step being repeated until an eluate negative to PhOH–H_2SO_4 was obtained. The polar fraction (PF) was combined and partitioned between 0.5% v/v aq H_2SO_4 (0.5 mL) and EtOAc (9 mL). Fraction PF1 was present in the EtOAc



Scheme 1. Extraction, isolation, and purification of the components obtained from *Scytonema* sp.

layer, which was evaporated to dryness, dissolved in $CHCl_3$, and chromatographed on a column of silicic acid (1.8 × 10 cm), elution being carried out with $CHCl_3$ (50 mL), and then with increasing concentrations of MeOH in $CHCl_3$. A glycolipid (PF1S) was obtained with 25% MeOH in $CHCl_3$. The neutral glycolipids (NG) were obtained with solvent B and dissolved in $CHCl_3$, then chromatographed on a column of silicic acid as described above, and eluted with $CHCl_3$ (50 mL) and with mixtures of $CHCl_3$–MeOH (20 mL each), with increasing concentrations of MeOH. The neutral glycolipids, NG1, NG2, and NG3 were eluted with 10%, 20%, and 30% of MeOH, respectively. All the glycolipid fractions were stored in sealed tubes at below −10 °C.

2.3. Preparation of low-molecular-weight carbohydrates and polysaccharides

The residue remaining after $CHCl_3$–MeOH extraction (86 mg) was treated with 4:1 MeOH–H_2O (20 mL) under reflux for 2 h, giving soluble, low-molecular-weight carbohydrates (9 mg). The residue was then extracted with H_2O (20 mL) at 100 °C and EtOH (×3 v/v) added to give a precipitate of polysaccharide (4.0 mg) (Scheme 1).

2.4. Analytical procedures

2.4.1. Chromatography of lipids. TLC of glycolipid fractions was performed on silica gel G-60 plates (E. Merck) solvent: 65:25:4 $CHCl_3$–MeOH–H_2O, and their presence was detected by using a carbohydrate-specific orcinol–H_2SO_4 spray developed at 100 °C.

Lipid extracts and purified glycolipids (50 μg) were refluxed in 3% MeOH–HCl for 2 h, and the resulting FAMEs were partitioned between H_2O and *n*-hexane.[7] The upper layer was evaporated to dryness, and the residue analyzed by GC–MS using 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 constant temperature. Each FAME and hydrocarbon was characterized by its electron-impact mass spectrum (ESMS) and retention time, when compared to those of standards (Sigma Chemical Co. products for lipids).

2.4.2. Chromatography of carbohydrates. Hydrolysis of glycolipids and polysaccharide fractions was performed with 2 M TFA at 100 °C for 8 h. In the case of glycolipids, the hydrolyzate was partitioned between $CHCl_3$ and H_2O, and the $CHCl_3$ layer then discarded. These products and the low-molecular-weight carbohydrate fraction were reduced with $NaBH_4$, and the products acetylated with Ac_2O–pyridine at 25 °C for 15 h.[17] Resulting alditol acetates 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 constant temperature.

Free monosaccharides from the aq MeOH–H_2O extraction and its ethanolic supernatant were combined, and the resulting mixture was examined by PC on a Whatman no. 1 filter paper (solvent: 5:3:3 *n*-BuOH–pyridine–H_2O) developed with the acetone–$AgNO_3$–NaOH dip reagent[18] and *p*-anisidine hydrochloride.[19]

2.4.3. Preparation of partially O-methylated alditol acetates. Standards of all partially O-methylated alditol standards, necessary for GC–MS identification, were prepared as described by Sassaki et al.[20] Rhamnose (10 mg Sigma Chemical Co.) was treated with refluxing 3% MeOH–HCl (1.0 mL) for 2 h. After neutralization ($AgCO_3$) and filtration, the filtrate was evaporated to dryness. The methyl aldopyranoside residue was slowly converted into partially O-methylated derivatives by stirring in a CH_3I–Ag_2O (1 mL, 250 mg) suspension. The formation of partially O-methylated derivatives was monitored by TLC (solvent: 9:1 chloroform–EtOH) and detected using an orcinol–H_2SO_4 spray. After 90 min, the CH_3I/Ag_2O suspension was filtered, and the filtrate was evaporated to a residue, which was hydrolyzed with M H_2SO_4 for 8 h at 100 °C, neutralized ($BaCO_3$), and converted into partially O-methylated alditol acetates, as described above but with $NaBD_4$ reduction. The product was then analyzed by GC–MS.

2.4.4. Methylation analysis. Per-O-methylation of the crude polysaccharide was carried out by agitation of the powdered NaOH in Me_2SO–MeI.[21] The products were converted into partially O-methylated aldose acetates by successive treatments with refluxing 2% MeOH–HCl for 2 h and total hydrolysis with 0.5 M H_2SO_4 for 14 h at 100 °C. The solution was neutralized ($BaCO_3$), filtered, and the filtrate was evaporated to dryness. The product was converted into partially O-methylated alditol acetates as described above and analyzed by GC–MS.

2.4.5. Colorimetric protein and carbohydrate determinations. Protein concentration was determined by the method of Bradford,[22] using bovine serum albumin as standard. Total sugar was determined by the phenol–sulfuric acid method[12] with D-glucose as standard.

2.4.6. ^1H and ^{13}C NMR spectroscopy. For NMR spectral examination, spectra were obtained with a Bruker DRX 400 MHz NMR spectrometer using a 5-mm inverse probe head. Solution samples were prepared as follows: PF1S and NG glycolipids were deuterium exchanged by repeated evaporations in 3:1:1 CD_3OD–$CDCl_3$–D_2O and the products were dissolved and examined in different proportions of $CDCl_3$–CD_3OD at 30 °C using Me_4Si as the external standard (δ, PPM = 0).

Fraction S-HMWC was dissolved in D_2O at 60 °C. Assignments in the 1D ^{13}C and ^{1}H NMR spectra of S-HMWC (4.0 mg), PF1S (0.5 mg), and NG glycolipids (NG1, 1 mg; NG2, 0.8 mg; NG3, 0.4 g) were carried out using HMQC, COSY, and TOCSY programs.

3. Results and discussion

3.1. Growth and isolation of the photobiont

In lichen thalli, whose photobionts are cyanobacteria, their microscopic identification is extremely difficult due to cell deformation, and since in its lichenized form the alga does not complete its life cycle, its isolation and cultivation are necessary for its identification.[24] We now isolate the photobiont of *D. glabratum* according to the method of Yamamoto et al.[15] and Ahmadjian.[16] The photobiont was first scraped from the thallus, and its colonies were grown in a soil-extract medium, then collected and ground prior to membrane filtration to eliminate bacterial contaminants present in the gelatinous sheaths. Fragments of the colony were re-inoculated, and after 45 days the resulting colonies were used as a source for submerged growth. The photobiont was identified as *Scytonema* sp. by Prof. Dr. Elfie Stocker-Wörgötter, Institute of Plant Physiology, University of Salzburg (personal communication).

After 60 days, the algal cells were isolated and extracted to give low-molecular-weight compounds in 47% yield based on their dry weight (combined S-CM 2:1, S-LMWC-1, and S-LMWC-2). The total lipid content of the S-CM 2:1 fraction (25%) was greater than that of intact *D. glabratum* which was only 5%.[6] These results are interesting since the isolated photobiont was cultured on a sterilized soil extract, without addition of nutrients or salts for two months, from soil obtained from the original harvest site of the lichen.

Soil extract components in culture media have been widely used in the isolation of lichen photobionts, but it usually has as a complementary role in BBM and BG-11 media, being added to stimulate cell growth. [25–27]

Our intention for using the soil-extract medium was to stimulate cell growth and to minimize contamination, which was possible since cyanobacteria fixes atmospheric carbon and nitrogen. The absence of carbohydrates and proteins was shown colorimetrically,[22,23] so that our overall objective was an attempt to grow the symbiont under nutritional conditions similar to those in the natural habitat. However, some factors such as thermal amplitude, light variation, and humidity, as well as nutrient cycling in a complex environment, are extremely difficult to mimic artificially.

3.2. Identification of hydrocarbons and glycolipids

The lipid extract (S-CM 2:1) obtained on $CHCl_3$–MeOH extraction was methanolyzed giving rise to hydrocarbons and FAMEs. The presence of 2-Me-heptadecane (3%), 2,6-Me_2-octadecane (4%), and 6-Me-octadecane 4%, each of which contains 17 CH_2 groups, agrees with previous results obtained with other cyanobionts, where these hydrocarbons were used as biomarkers[12] (Table 1). The FAME composition showed high concentrations of

Table 1. Fatty acid composition of S-CM 2:1 extract and glycolipid fractions from *Scytonema* sp.

Hydrocarbons, fatty acids[a]	t_R[b] (%) fatty acids						
	Min	S-CM 2:1	NG1	NG2	NG3	PF1S	Main mass spectral fragments (*m/z*)
C_{12} (Dodecane)	4.9	4	—	—	—	—	170, 141, 127, 112, 98, 85, 71, 57, 43
$C_{12:0}$ (Lauric)	6.5	—	1	—	2	5	214, 199, 143, 129, 74, 55, 43, 41
$C_{10:0}$ (Decanodioic)	7.2	2	—	—	—	—	230, 199, 166, 157, 125, 98, 74, 55
$C_{14:0}$ (Myristic)	8.2	3	2	5	9	10	242, 199, 143, 129, 74, 55, 43, 41
$C_{15:0}$ (Pentadecanoic)	9.2	4	1	3	4	1	256, 213, 199, 143, 129, 87, 74, 41
$C_{16:0}$ (Palmitic)	10.3	37	46	43	52	62	270, 227, 185, 143, 74, 55, 43, 41
$C_{16:1}$ (7-Hexadecenoic)	10.5	3	3	6	6	—	254, 236, 237, 192, 97, 69, 55, 41
$C_{17:0}$ (Heptadecanoic)	11.4	3	1	1	3	—	284, 253, 241, 143, 129, 87, 74, 41
2-Me-C_{17} (2-Me-heptadecane)	12.1	3	—	—	—	—	254, 239, 211, 113, 99, 85, 71, 57, 43
$C_{18:0}$ (Stearic)	12.6	13	12	15	12	12	298, 255, 199, 143, 129, 74, 55, 41
$C_{18:1}$ (Oleic)	13.2	8	4	7	5	—	296, 265, 222, 180, 74, 69, 55, 41
2,6-Me_2-C_{18} (2,6-Me_2-octadecane)	13.4	4	—	—	—	—	282, 267, 196, 113, 99, 85, 71, 57, 43
$C_{18:2}$ (Linoleic)	14.0	3	3	6	2	10	294, 263, 220, 178, 95, 81, 74, 67, 41
$C_{18:3}$ (γ)	14.3	—	23	9	2	—	292, 195, 194, 150, 107, 93, 79, 67
$C_{18:3}$	14.5	3	2	2	tr	—	292, 236, 191, 149, 108, 95, 79, 67
6-Me-C_{18} (6-Me-octadecane)	14.7	4	—	—	—	—	268, 253, 196, 113, 99, 85, 71, 57, 43
$C_{20:0}$ (Arachdic)	15.5	2	1	2	1	—	326, 263, 199, 171, 143, 95, 74, 41
$C_{20:2}$ (11,14-Eicosadienoic)	16.4	3	—	—	—	—	322, 291, 206, 109, 95, 81, 67, 41
$C_{22:0}$	19.1	—	—	1	1	—	354, 311, 255, 199, 143, 87, 74, 41
$C_{24:0}$ (Lignoceric)	24.2	3	1	—	1	—	382, 339, 283, 255, 199, 143, 74, 41

[a] FAMEs obtained after methanolysis and analyzed by GC–MS (DB-23 column).
[b] Retention time (t_R), at 50–220 °C (40 °C min^{-1}).

$C_{16:0}$ and $C_{18:0}$, although long- and short-chain fatty acids were also present, as well as saturated and unsaturated ones. Comparison between the intact lichen and its photobiont showed some differences, such as the absence of $C_{12:0}$ and $C_{16:2}$ in the photobiont, suggesting that these arise from the mycobiont, or could be synthesized under our culture conditions. $C_{16:1}$, $C_{18:1}$, and $C_{22:1}$ likely arose from the photobiont, since the first two are typical of cyanobionts.[12,28] These data confirm the hypotheses of Thiel et al.,[12] who suggested that lipids might be used as biomarkers and that FAME composition could aid in the pre-analysis of organisms present in the symbiotic association.

In order to investigate the glycolipids in S-CM 2:1, it was fractionated on a column of DEAE-Sepharose to give neutral glycolipids (NG) (18 mg) and a polar carbohydrate fraction (PF) (3.5 mg). Each fraction was examined on TLC, and we found four orcinol-positive spots with R_fs 0.35, 0.46, 0.59, and 0.76 using as solvent: 65:25:4 $CHCl_3$–MeOH–H_2O. The neutral fractions NG and PF1 were each chromatographed on silicic acid columns and rechromatographed under the same conditions, in order to obtain the pure glycolipids, NG1, NG2, and NG3 (R_f/yield: 0.76/1 mg; 0.59/0.8 mg; and 0.35/0.4 mg) and PF1S (R_f/yield 0.54/0.5 mg).

Neutral NG1, NG2, and NG3 gave on hydrolysis exclusively galactose and glycerol in different ratios, which showed that they could be galactolipids monogalactosyldiacylglycerol (MGD), digalactosyldiacylglycerol (DGD), and trigalactosyldiacylglycerol (TGD), previously detected in the intact lichen.[6] In contrast, PF1S only gave rise to glycerol on GC–MS detection of the alditol acetates. This confirmed its DEAE-Sepharose characteristics, which showed the presence of a negatively charged group. PF1S was examined on TLC and gave an R_f of 0.46, similar to that of a sulfoquinovosyldiacylglycerol (SQDG) standard.

The structures of the four glycolipids were confirmed by ^1H and ^{13}C NMR analysis, based on HMQC and TOCSY fingerprints previously obtained by Sassaki et al.[6–8] (see Fig. 1). The ^{13}C and ^1H shifts obtained by HMQC are shown in Table 3 and are discussed as follows.

The HMQC spectrum of glycolipid NG1 contained lipid signals from δ 22.8 to 34.6 with predominant CH_2 signals at δ 29.7 and CH_3 at δ 14.1, with double-bond signals of lipids from δ 122.8 to 133.0. The C-1 region contained a single anomeric signal at δ 4.05/104.1, suggesting a β-D-Gal*p*-(1′↔1)-glycerol linkage. Its HMQC spectrum also had doublets of CH_2 signals in the carbohydrate region at δ 61.4, which correspond to C-6′ of the Gal*p* unit, indicating that its carbon was not O-substituted, while those at δ 68.6, 70.5, and 63.2 arose, respectively, from C-1 (downfield O-glycosylation α-shift). C-2, and C-3 of the glycerol moiety esterified by acyl groups, mainly as esters of $C_{16:0}$ (46%) and $C_{18:3}$ (γ)

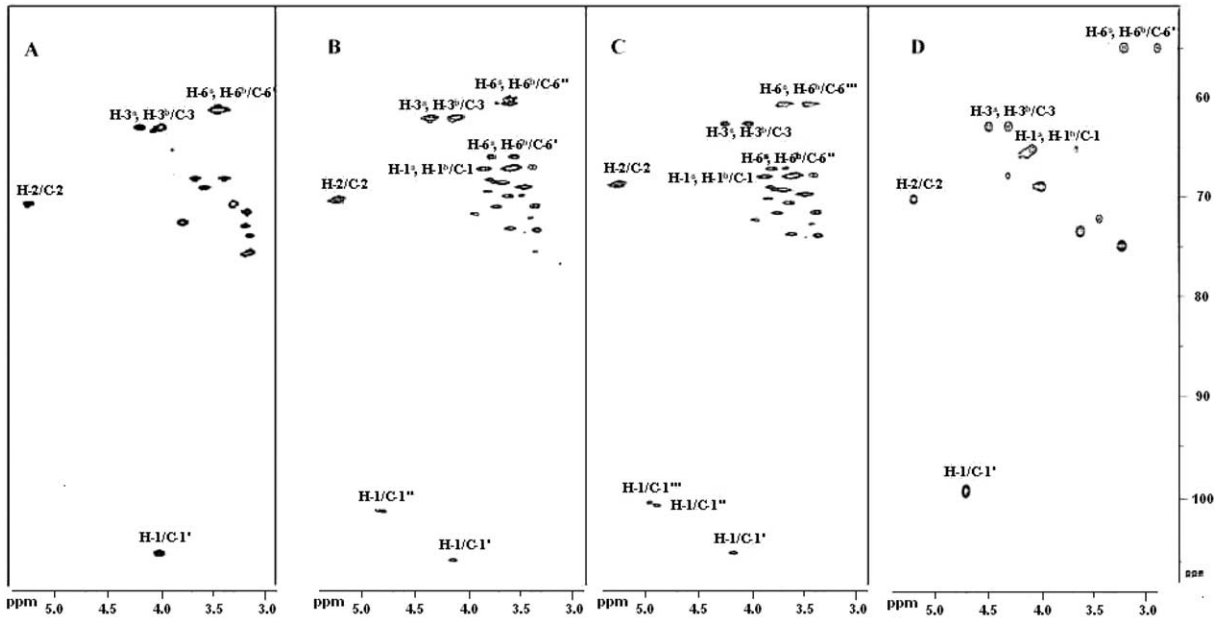


Figure 1. Partial HMQC (^{1}H(obsd)^{13}C NMR spectra) of glycolipids (NG1) A; (NG2) B; (NG3) C; and (PF1S) D; isolated from *Scytonema* sp. (carbohydrate and glycerol regions), the proton signals at δ 5.12–5.27 (H-2), H-1^a/H-1^b (3.74–4.00 and 3.49–3.78) and H-3^a/H-3^b (4.17–4.47 and 4.01–4.29), assignable signals from glycerol moiety for glycosylglycerolipids. Proton and ^{13}C signals in the anomeric region of the HMQC spectra show a number of units present in the glycoconjugates, and the type of glycosidic configuration; as we observed for (NG3) C; two high-field signals at δ 4.98/98.9 and 4.91/99.1, corresponding to α-D-Gal*p*-(1‴→6″) and α-D-Gal*p*-(1″→6′) units, respectively, and the downfield signal at δ 4.28/104.2 to a β-D-Gal*p*-(1′↔1)-glycerol linkage.

(23%). Glycolipid NG2 contained in its HMQC spectrum similar lipid signals of ^{13}C at δ 14.4 (CH_3) and at δ 23.4–35.0 of CH_2 with a predominant one at δ 30.2 and those of double bonds at δ 128.8–130.85. The anomeric ^1H and ^{13}C NMR regions each contained two signals of equal intensity at 4.87/100.3 and 4.28/104.8, which indicated α-D-Gal*p* and β-D-Gal*p*-(1′↔1)-glycerol groups, respectively. The spectrum also gave information on the O-substituted C-6′ of the α-Gal*p* unit (δ 68.4) and a non-substituted C-6″ signal belonging to the β-Gal*p* unit (δ 62.5). The ^{13}C and ^1H NMR doublet signals at δ 68.6/3.95 (a), 3.72 (b); 72.1/5.24; and 63.8/4.19 (a), 4.18 (b); correspond to C-1, C-2, and C-3 and adjacent protons of glycerol esterified mainly by $C_{16:0}$ (43%) and $C_{18:0}$ (15%).

The HMQC spectrum of glycolipid NG3 gave rise to lipid signals of CH_3 at δ 13.9 and at CH_2 from δ 22.8 to 34.4, with a predominant one at δ 29.8; lipid double bonds at δ 126.1–133.3 were also present. The anomeric ^1H and ^{13}C NMR regions contained two high-field signals (δ 4.98/98.9 and 4.91/99.1) corresponding to two α-Gal*p* units, respectively, and a downfield one (δ 4.28/104.2) characteristic of β-Gal*p*. The carbohydrate region also contained an O-substituted C-6′ signal of the β-Gal*p* unit at δ 67.9 and the C-6″ of the O-substituted α-Gal*p* unit at δ 67.8, and that of a non-substituted C-6‴ signal of the terminal α-Gal*p* unit (δ 61.8). The presence of a D-glycerol moiety was also confirmed by correlated doublet and singlet signals of $^1H/^{13}C$, which were determined by HMQC and TOCSY experiments, namely C-1 at δ 4.00/67.9; 3.52, β-D-Gal*p*-(1′↔1)-glycerol linkage 4.28/104.2, and C-2(a) 70.8/5.27, and C-3 63.3/4.47 (a), 4.25 (c) O-acylated carbons, which were mainly esterified by $C_{16:0}$ (52%) and $C_{18:0}$ (12%).

The polar fraction PF1S was also analyzed by NMR spectroscopy, and its HMQC spectrum contained lipid signals at δ 14.4 (CH_3), from δ 23.3 to 35.0 of CH_2, with a predominant one at δ 30.3. The presence of lipids containing double bonds was shown by signals at δ 126.4–131.5. Its $^{13}C/^1H$ NMR anomeric regions contained one signal at δ 4.71/99.8 identical to that of a unit of α-quinovopyranose *p*-6-sulfonate (1′↔1)-linked to glycerol.[8] It also provided key information in terms of ^1H and ^{13}C NMR assignments, which indicated signals belonging to the glycerol unit, which was confirmed by TOCSY. The presence of doublets of CH_2 signals of *S*-substituted C-6′, corresponded to an α-Qui*p* unit in the high-field region at δ 55.8 (3.04, 2.69) and the presence of the signals at δ 66.0/4.00 (a), 3.52 (b); 72.1/5.244; and 63.7/4.47 (a), 4.25 (b) arising from C-1, C-2, and C-3. These assignments were very similar to those we found in the previous works for sulfonoglycolipids,[8] which, respectively, can be attributed to the glycerol unit mainly esterified by $C_{16:0}$ (61.5%) and $C_{18:0}$ (12%).

The presence of these four glycolipids in the *Scytonema* sp., obtained as a free-living photobiont, can be related to a previous study by Reshef et al.,[29] who cultured it in a BG-11 medium for blue green algae, which differs from ours. They showed that their glycolipids had a high antiviral (HIV-1) activity, and also suggested the presence of palmitic esters occurring at C-3′ of the quinovose unit of SQDG and in the external α-Gal*p* at C-3″ of DGD. Their conclusion appears to be erroneous, however, since the ^{13}C NMR signals for these carbons are at δ 71.6 and δ 72.7, respectively, which correspond to free CHOH groups, according to many authors.[6–9,11,30]

The FAME composition of the glycolipids NG1, NG2, NG3, and PF1S (Table 1) shows a great diversity of fatty acid esters, from saturated and unsaturated fatty acids, although $C_{16:0}$ and $C_{18:0}$ are present in high amounts in each one. The former is very common in photosynthetic tissues as well as in cyanobacteria.[31] An interesting datum was the increase of the unsaturated fatty acids $C_{18:1}$, $C_{18:2}$, $C_{18:3}$ (γ-linolenic), and $C_{18:3}$ (linolenic), which are commonly found in glycolipids of algae, plants, and cyanobacteria.[28] The presence of unsaturated fatty acids as well as $C_{16:0}$ in photosynthetic glycolipids commonly occurs in cyanobacteria.[31] Our PF1S isolated from *Scytonema* sp. shows a similar behavior as that of the sulfonolipids from *D. glabratum*.[8]

3.3. Structure of monosaccharides and polysaccharide components

After extraction of the photobiont with $CHCl_3$–MeOH the residue was then extracted with MeOH–H_2O to give S-LMWC (low-molecular-weight carbohydrates), and then with H_2O to give S-LMWC: after treatment with excess EtOH the resulting precipitate consisted of polysaccharides (S-HMWC).

S-LMWC was hydrolyzed, and its component monosaccharides are shown in Table 2. The crude polysaccharide fraction S-HMWC had a carbohydrate content of 90% and its monosaccharide composition showed high amounts of Gal, Glc, and Man (Table 2), with 3% 3-Me-Rha and 2% 2-Me-Xyl, confirmed by $NaBD_4$ reduction. These were characterized using partially O-methylated alditol acetate standards. These were prepared starting from the methyl aldopyranosides and graded methylation using the Purdie reagent, followed by hydrolysis, reduction with $NaBD_4$, and acetylation. The products were examined by GC–EIMS in the range of *m/z* 80–220 and compared with alditol acetates from the hydrolyzed polysaccharide (Fig. 2). In both, chromatograms and EIMS spectra (Fig. 2A and B), were obtained with an identical retention time and MS spectrum for 3-*O*-methylrhamnitol acetate (2C) with t_R at 8.657 min, and the following main fragments at *m/z* 129 (100%), 143 (59%), 87 (38%), 101 (28%), 189 (8%), 117 (5%), 159 (4%), 111 (4%), and 203 (3%).

The presence of rhamnose and 3-*O*-Me rhamnose-containing lipopolysaccharides have been described for

Table 2. Monosaccharide composition of some fractions obtained from *Scytonema* sp.

Fraction	Monosaccharide (%)											
	Gly	Thr	Rha	Rha[b]	Fuc	Rib	Ara	Xyl[c]	Xyl	Man	Gal	Glc
S-CM 2:1[a]	tr	21	3	—	—	5	3	—	2	4	39	23
S-HMWC[a]	—	—	6	3	5	2	13	2	5	17	30	17
S-LMWC[a]	1	—	1.5	—	0.5	tr	8	—	6	3	8	72
S-LMWC[d]	26	—	—	—	—	1	17	—	1	28	3	24
S-LMWC[e]	25	—	4	—	3	—	—	—	1	—	—	18
S-LMWC[f]	—	—	—	—	—	1	2	—	—	42	—	4

tr: traces, S-CM 2:1 $CHCl_3$–MeOH extract, S-HMWC: ethanolic precipitate, S-LMWC: low-molecular-weight carbohydrates.
[a] Alditol acetates obtained on hydrolysis, followed by successive borohydride reduction and acetylation and analyzed by GC–MS (column DB-225).
[b] 3-*O*-Methylrhamnose.
[c] 2-*O*-Methylxylose.
[d] Alditol acetates obtained after reduction, then acetylation.
[e] Alditol acetates obtained after acetylation.
[f] Monosaccharide acetates detected by the characteristic fragment at *m/z* 140 for unreduced ketoses and aldoses.

Table 3. Assignment of some ^1H and ^{13}C NMR signals of glycosylglycerides (in δ ppm)

Carbon	NG1		NG2		NG3		PF1S	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
C-1‴	—	—	—	—	4.98	98.9	—	—
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‴	—	—	—	—	3.82[a]	61.8	—	—
	—	—	—	—	3.74[b]		—	—
C-1″	—	—	4.87	100.3	4.91	99.1	—	—
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″	—	—	3.75[a]	62.5	3.85[a]	67.8	—	—
	—	—	3.73[b]		3.78[b]			
C-1′	4.05	104.1	4.28	104.8	4.28	104.2	4.71	99.8
C-2′	3.61	73.7	3.57	72.3	3.57	72.3	3.30	72.9
C-3′	3.58	71.2	3.68	70.2	3.68	70.2	3.49	74.3
C-4′	3.89	71.5	*3.99*	*70.2*	*3.99*	*70.2*	3.05	75.8
C-5′	3.64	75.7	*3.95*	*74.2*	*3.96*	*74.2*	3.91	69.7
C-6′	3.44[a]	61.4	3.74[a]	68.4	3.72[a]	67.9	3.04[a]	55.8
	3.52[b]		3.76[b]		3.76[b]		2.69[b]	
C-1, Gly	3.74[a]	68.56	3.98[a]	68.59	3.95[a]	67.9	4.00[a]	66.0
	3.49[b]		3.72[b]		3.78[b]		3.52[b]	
C-2, Gly	5.12	70.5	5.24	72.12	5.25	70.8	5.27	71.0
C-3, Gly	4.17[a]	63.2	4.19[a]	63.76	4.45[a]	63.3	4.47[a]	63.7
	4.01[b]		4.18[b]		4.29[b]		4.25[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	1.1–2.3	23.3–35.0
CH=CH	5.12	122.8–133.0	5.23	128.8–130.8	5.35	126.0–133.3	7.1–7.8	126.4–131.5
CH_3	0.83	14.1	0.83	14.4	0.85	13.9	0.85	14.6

[a,b]Chemical shifts of protons linked directly to the carbon nucleus (HMQC and TOCSY experiments) used as fingerprints for detection of glycosylacylglycerides and sulfonoglycolipids (Refs. 6–10). Tentative assignments are in italics.

some cyanophytae of the genera *Anacystis*, *Anabeana*, and *Agmenellum*. For that of *Anabeana variabilis*, in particular, the O-somatic side chains were composed of ~48% rhamnosyl and 23% of 3-*O*-methylrhamnosyl units, with those of glucose representing 44% of its side chains.[32] 2-*O*-Methylxylopyranosyl units have been reported the occur in plant polysaccharides, such as a rhamnogalactouronan (RGII type), which contained 2-Me-β-Xyl*p* branches of a long side chain that is (1→3)-linked to α-Fuc*p* units.[33]

The high proportion of Glc, Gal, and Man in our polysaccharide is consistent with other cyanophyceae[34]

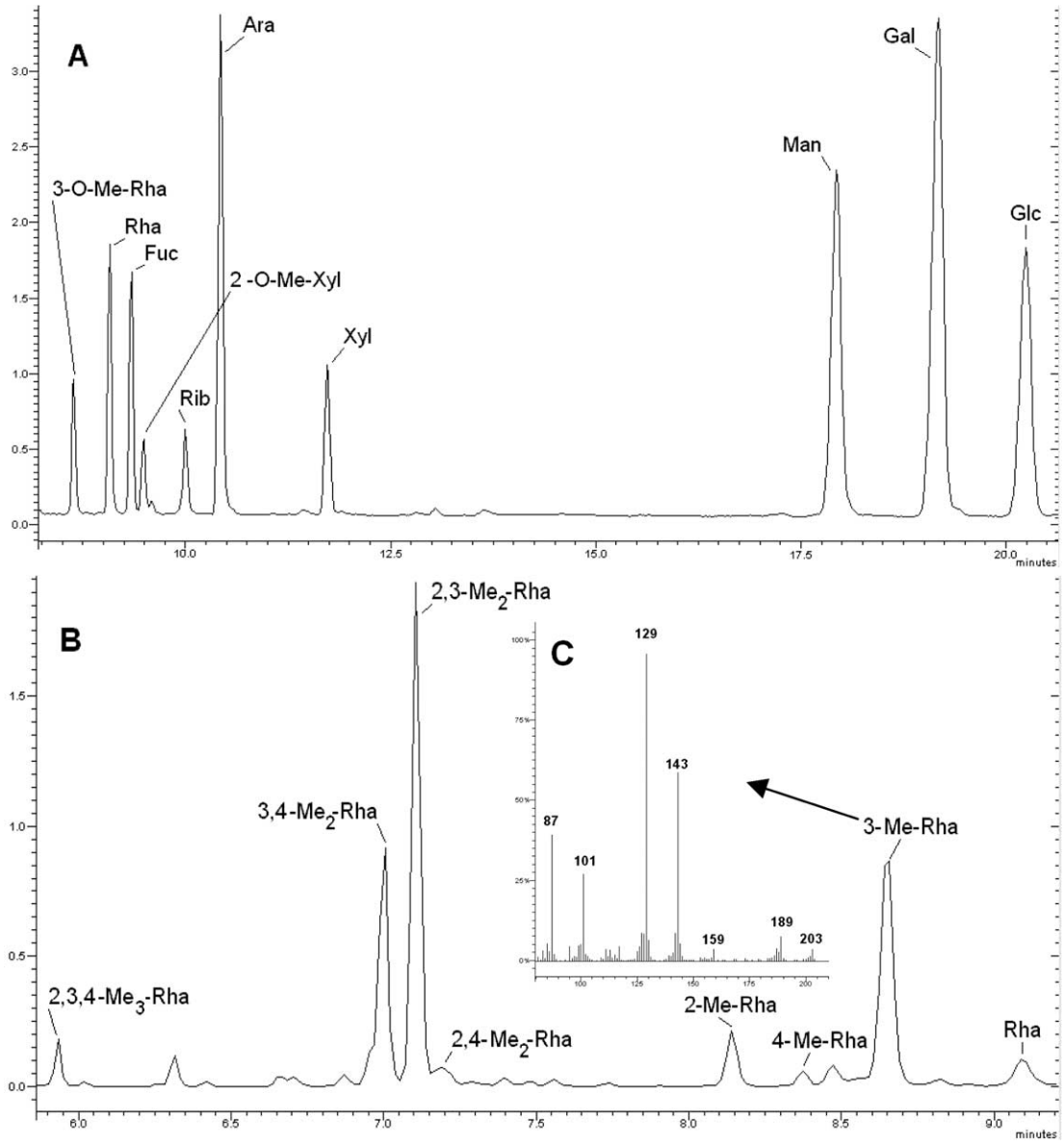


Figure 2. GC–MS chromatograms: (A) polysaccharide fraction S-HMWC; (B) pool of partially O-methylated rhamnitol acetate derivatives; (C) EIMS spectrum of 3-*O*-methylrhamnitol acetate.

and could suggest the presence of a glucan, which is commonly found as a (1→4)-linked α-glucan, similar to glycogen, amylose, and amylopectin found in the genera *Lyngbya*, *Phormidium*, and *Plectonema*.[35,36] In order to test for their presence, S-HMWC was treated with iodine solution but no characteristic colorations were detected.

^{13}C NMR analysis of a small amount (4.0 mg) of the crude polysaccharide gave rise to at least seven anomeric signals, suggesting either a complex structure or a mixture of polyssacharides. The main signal at δ 98.9 can be assigned to α-Glc*p* units and those at δ 107.9 and 107.8 to those of β-Gal*f*, which also appeared as a component of a β-galactofuranan in the cultured *Trebouxia* sp. (chlorophyta) photobiont of *Ramalina gracilis*.[5] Methylation analysis of S-HMWC also showed the presence of a complex structure (see Table 4) with low amounts of nonreducing ends (14% of Fuc*p*, Rha*p*, and Gal*p*). The 4-*O*-substituted-Glc*p* units (16%) must arise from a non-starch component. The presence of Gal*f* units was confirmed by the 2,3,5-Me_3-Gal*f* derivative (4%), arising from 6-*O*-substituted units, and the presence of 2,3,6-Me_3-Gal (5%) and 2,6-Me_2-Gal (16%) derivatives does not exclude 5-*O*- and 3,5-di-*O*-substituted units. Fractions S-CM (2:1 $CHCl_3$–MeOH) and S-LMWC (combined ethanolic and 4:1 MeOH–H_2O, supernatants) contained polyalcohols and monosaccharides (Table 2). Fraction S-LMWC was submitted

Table 4. Analysis of O-methylated alditol acetates obtained on methylated analysis of polysaccharide fraction S-HMWC, obtained from *Scytonema* sp.

OMe-alditol acetate[a]	t_R % of OMe-alditol acetate		
	Min	%	Linkage type
2,3,4-Me_3-Rha	9.8	6	Rha*p*-(1→[b]
2,3,4-Me_3-Fuc	9.9	5	Fuc*p*-(1→
3,4-Me_2-Rib	10.0	2	→2)-Rib*p*-(1→
2,4-Me_2-Ara	10.4	7	→3)-Ara*p*-(1→
2,3,4,6-Me_4-Gal	10.7	6	Gal*p*-(1→
2,3-Me_2-Xyl	10.9	1	→4)-Xyl*p*-(1→[c]
3,4,6-Me_3-Man	12.9	7	→2)-Man*p*-(1→
2-Me-Ara	13.1	4	3,4/5→)-Ara-(1→
2,3,6-Me_3-Man	13.5	8	→4)-Man*p*-(1→
2,3,6-Me_3-Gal	14.0	6	→4/5)-Gal-(1→
2,3,6-Me_3-Glc	14.3	18	→4)-Glc*p*-(1→
2,3,5-Me_3-Gal	15.7	5	→6)-Gal*f*-(1→
2,6-Me_2-Gal	17.0	16	→3,4/5)-Gal-(1→
3,4-Me_2-Man	20.4	7	→2,6)-Man*p*-(1→
3,4-Me_2-Gal	23.1	3	→2,6)-Gal*p*-(1→

[a] The exopolysaccharide was methylated as described by Ciucanu and Kerek (Ref. 21).
[b] Mixture of nonreducing end-units of Rha*p* and 3-Me-Rha*p*.
[c] Mixture of nonreducing end-units of Xyl*p* and 2-Me-Xyl*p*.

to different hydrolytic processes of reduction and acetylation as described above. Mannose was the main free reducing sugar (yield 42%), although ribose, arabinose, and glucose were present in low amounts. Polyalcohols such as glycerol (21%) and glucitol (18%) were also detected, and are probably involved in the metabolic process as sources of carbon skeletons. Total acid hydrolysis of S-LMWC gave a high yield of glucitol (72.5%), from glucose and/or glucitol, which suggests the presence of also oligosaccharides. Glucose is the free reducing sugar observed for the genus *Nostoc*, which is utilized by the symbiotic fungus and converted to mannitol as the carbon source. In the genus *Trebouxia*, the nonreducing ribitol is converted to mannitol and arabinitol.[14] Our present results suggest that *Scytonema* sp. provides mannose to be consumed in *D. glabratum*, although more specific studies are needed to arrive at a definite conclusion.

Analysis of the *Scytonema* extracts, especially the lipid-containing ones, show specific roles for each of the bionts. The glycolipids are probably synthesized by the photobiont, both alone and in the intact *D. glabratum*. Some lipids have been used as biomarkers as well as hydrocarbons, which contain 17 CH_2 and branched CH_3 groups for identification of the cyanobiont.[12] Our study now shows us significant differences, mainly in the fatty ester composition of the glycolipids of the fresh thallus and that of the isolated photobiont, suggesting that these compounds could serve as an aid in the identification of bionts involved in symbiosis. Clearly, experiments should be carried out especially in relation to the lichen thallus, growing under conditions similar to that of its photobiont, as well as with the photobiont isolated mechanically from the lichenized fungus.

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