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(1 → 2) and (1 → 6)-linked β-D-galactofuranan of microalga *Myrmecia biatorellae*, symbiotic partner of *Lobaria linita*

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ABSTRACT

A structural study of the cell wall polysaccharides of *Myrmecia biatorellae*, the symbiotic algal partner of the lichenized fungus *Lobaria linita* was carried out. It produced a rhamnogalactofuranan, with a (1 → 6)-β-D-galactofuranose in the main-chain, substituted at *O*-2 by single units of β-D-Gal*f*, α-L-Rha*p* or by side chains of 2-*O*-linked β-D-Gal*f* units. The structure of the polysaccharide was established by chemical and NMR spectroscopic analysis, and is new among natural polysaccharides. Moreover, in a preliminary study, this polysaccharide increased the lethality of mice submitted to polymicrobial sepsis induced by cecal ligation and puncture, probably due to the presence of galactofuranose, which have been shown to be highy immunogenic in mammals.

1. Introduction

From a chemical point of view, hexoses exhibit significant differences depending on whether they are present as pyranosides or as furanosides form. Because steric interactions are minimized in six-membered rings, they are thermodynamically more favored than their furanoside counterparts. Thus, hexoses in the pyranosidic configuration are much more widespread in nature, while only few of them (Gal, Fru, Glc, Fuc and Man) may be found in the furanose form (Peltier, Euzen, Daniellou, Nugier-Chauvin, & Ferrières, 2008). Among these, galactose is by far the most widespread hexose in the furanose form in naturally occurring polysaccharides and glycoconjugates, and β-linkages are most prevalent (Lowary, 2003; Peltier et al., 2008).

Importantly, Gal*f* has been shown to be present in numerous structures considered to be essential for virulence in many pathogenic organisms, such as mycobacteria, bacteria, protozoa and fungi. While Gal*p* is abundant in mammalian glycoconjugates, Gal*f* has not been reported; rather Gal*f*-containing epitopes have been shown to be highly antigenic in mammals. Thus, the search for molecules unique to pathogenic microorganisms has led Gal*f* and its metabolism as one potential chemotherapeutic target (Pedersen & Turco, 2003).

In addition to the above mentioned organisms, galactofuranose was also previously found in two microalgae involved in mutualistic relationship with terrestrial fungi (lichens). In one of them, namely *Trebouxia* sp., it was found a homopolymer (β-D-galactofuranan) and a galactofuranan-rich heteropolysaccharide, both with (1 → 5)-β-D-galactofuranosyl backbone, substituted in a small proportion at *O*-6 by β-D-Gal*f* units or by very complex branched structures, respectively (Cordeiro et al., 2005; Cordeiro, Oliveira, Buchi, & Iacomini, 2008; Ruthes et al., 2008). In the other, belonging to the genus *Asterochloris*, it was found a xylorhamnogalactofuranan having (1 → 3)-β-D-galactofuranosyl units in the main-chain. This was ramified at *O*-6 by sidechains containing 5-*O* and 6-*O*-substituted β-D-Gal*f* units, 2-*O*, 3-*O* and 2,3-di-*O*-substituted L-Rha*p* units, along with Xyl*p* and β-D-Gal*f* as nonreducing end units (Cordeiro, Sassaki, & Iacomini, 2007).

We now report the existence of polysaccharides of galactofuranose (galactofuranans) with unique structures in another non-pathogenic specie of green microalga, *Myrmecia biatorellae*, which is envolved in a symbiotic relationship with a terrestrial fungus to form the lichen *Lobaria linita*. Regarding lichen symbiosis, a discussion about polysaccharides found in the symbiotic thallus of *Lobaria*, as well in other aposymbiotically cultivated symbionts is also included.

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Due to the highly immunogenicity of galactofuranose in mammals, the effect of the galactofuranan of *M. biatorellae* was tested in a model of murine polymicrobial sepsis induced by cecal ligation and puncture (CLP). This model mimics the sepsis in humans, caused by pathogens derived from the intestinal tract and is considered to closely simulate clinical situation (Rittirsch, Huber-Lang, Flierl, & Ward, 2009). Furthermore, this biological model allows exploring both pro- and antiinflammatory agents throughout increasing or reduction of the lethality, since it reflects the severity of sepsis reached after treating animals.

Sepsis is a condition that results from a harmful or damaging host response to infection. It develops when the initial, appropriate host response to an infection becomes amplified, and then dysregulated, causing cell and tissue damage and hence multiple organ failure and death. This overactivation of the innate immune system caused by sepsis leads to the release of large amounts of inflammatory mediators including cytokines and chemokines. This proinflammatory storm causes the release of powerful secondary mediators, such as inflammatory enzymes and reactive oxygen species, which further amplify the inflammatory process and causes a multiple organ dysfunction syndrome (Cohen, 2002).

Several studies have demonstrated that the proinflammatory mediators are strongly associated with sepsis severity. Therefore controlling inflammation and inhibiting the proinflammatory mediators overproduction during early sepsis may reduce organ injury and prevent death after septic insult (Cohen, 2002; Yun, Lee, & Lee, 2009). This was recently demonstrated by polysaccharides with anti-inflammatory activity (Meng, Pai, Liu, & Yeh, 2012; Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2012). In this study, we disclosed that rhamnogalactofuranan, a polysaccharide containing galactofuranose, is able to induce an opposite effect on lethality induced by sepsis, through increasing mortality of pretreated mice.

2. Materials and methods

2.1. Photobiont and culture conditions

The microalga *M. biatorellae* (Tschermak-Woess et Pessl) Petersen (strain 8.82), the symbiotic algal partner of the lichen *L. linita*, was purchased from Culture Collection of Algae (SAG), University of Göttingen (Germany) and was made axenic through the isolation of a pure colony on sucessive transference on solid Bolds Basal Medium (BBM) containing myconazol.

To obtain biomass, the microalga was cultivated on a modified BBM (1 L, in Erlenmeyers of 2 L), to which 1.5% glucose and 0.5% peptone were added. The cultures were illuminated with 45 mol/m^2 s for 12 h, followed by an interval of 12 h in the dark at 21 ± 2 °C. After 30 days, the algal cells were removed by filtration, washed with distilled water, and freeze-dried, to give 41.2 g of biomass.

2.2. Extraction and purification of polysaccharides

The algal cells were exhaustively extracted with EtOH at 80 °C for 4 h (9×, 1 L each), and then with 1:1 (v/v) $CHCl_3$–MeOH at 60 °C for 4 h (1×, 1 L), in order to remove lipids, pigments and other hydrophobic material. The polysaccharides were extracted from the residue with water at 100 °C for 4 h (4×, 1 L each). The aqueous extracts were obtained by centrifugation (3860 × *g*, 20 min at 25 °C), joined and concentrated under reduced pressure. The polysaccharides were precipitated with EtOH (3 vol.) and freeze-dried, giving fraction W. The remaining residue was then extracted with aq. 10% KOH, at 100 °C for 4 h (4×, 1 L each) and the alkaline extracts were neutralized with acetic acid, dialyzed for 48 h with tap water, concentrated under reduced pressure and freeze-dried, originating fraction K10.

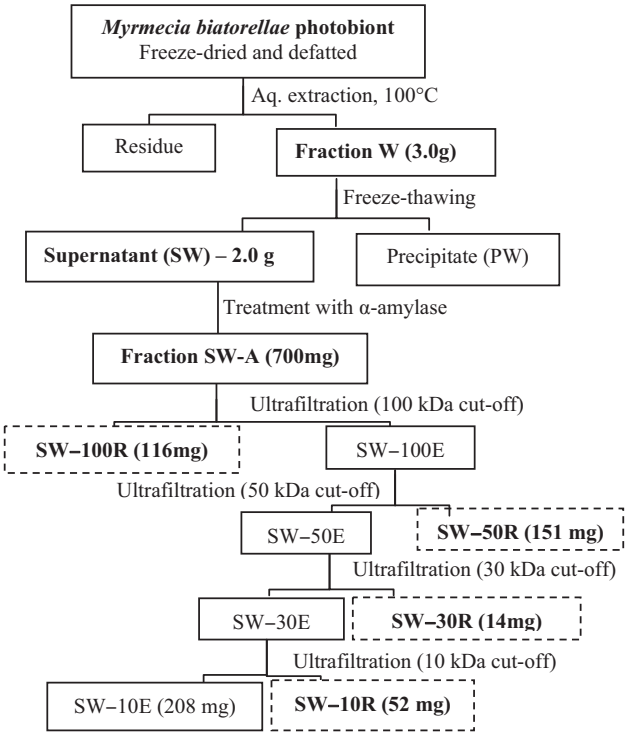


Fig. 1. Scheme of extraction and fractionation of polysaccharides from *Myrmecia biatorellae*. R indicates that the fraction was retained, while E indicate that the fraction was eluted in the ultrafiltration membrane.

Then, a freeze–thaw treatment was applied in these fractions, to give cold-water soluble fractions SW and SK10. In this procedure, the sample was frozen and then thaw at room temperature. Insoluble polysaccharides were recovered by centrifugation.

In order to remove starch, the supernatants of the freeze–thawing process (SW and SK10) were extensively treated with α-amylase (from *Bacillus licheniformis*, Sigma A3403) and dialyzed. The polysaccharides present were purified by sequential ultrafiltration through membranes (Fig. 1) with cut-offs of 100 kDa, 50 kDa, 30 kDa and 10 kDa (Ultracel, Millipore).

The yields were expressed as % based on the initial weight of freeze-dried algal biomass that were submitted to extraction (41.2 g).

2.3. Monosaccharide composition

Monosaccharide components of the polysaccharides and their ratio were determined by hydrolysis with 2 M TFA for 8 h at 100 °C, followed by conversion to alditol acetates by successive $NaBH_4$ or NaB_2H_4 reduction, and acetylation with Ac_2O-pyridine. The resulting alditol acetates were analyzed by GC–MS using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap model (ITD 800) mass spectrometer, with He as carrier gas. A capillary column (30 m × 0.25 mm i.d.) of DB-225, hold at 50 °C during injection for 1 min, then programmed at 40 °C/min to 220 °C and hold at this constant temperature for 19.75 min was used for the quantitative analysis.

2.4. Determination of homogeneity of polysaccharides and molecular weight of components

The homogeneity and average molar mass (M_w) of soluble polysaccharides were determined by high performance steric

exclusion chromatography (HPSEC), using a differential refractometer (Waters) as detection equipment. Four columns were used in series, with exclusion sizes of 7×10^6 Da (Ultrahydrogel 2000, Waters), 4×10^5 Da (Ultrahydrogel 500, Waters), 8×10^4 Da (Ultrahydrogel 250, Waters) and 5×10^3 Da (Ultrahydrogel 120, Waters). The eluent was 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 ml/min. The sample, previously filtered through a membrane (0.22 μm, Millipore), was injected (250 μl loop) at a concentration of 1 mg/ml. The specific refractive index increment (dn/dc) was determined and the results were processed with software ASTRA provided by the manufacturer (Wyatt Technologies).

2.5. Methylation analysis of polysaccharide

The purified polysaccharides were *O*-methylated according to the method of Ciucanu and Kerek (1984), using powdered NaOH in DMSO-MeI. The per-*O*-methylated derivatives were pre-treated with 72% (v/v) H_2SO_4 for 1 h at 0 °C and then hydrolyzed for 16 h at 100 °C after dilution of the H_2SO_4 to 8%. This was then neutralized with $BaCO_3$ and the resulting mixture of partially *O*-methylated monosaccharides was successively reduced with $NaBD_4$ and acetylated with Ac_2O-pyridine. The products (partially *O*-methylated alditol acetates) were examined by capillary GC–MS. A capillary column (30 m × 0.25 mm i.d.) of DB-225, held at 50 °C during injection for 1 min, then programmed at 40 °C/min to 210 °C and held at this temperature for 31 min was used for separation. The partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005; Sassaki, Iacomini, & Gorin, 2005).

2.6. Nuclear magnetic resonance (NMR) spectroscopy

^{13}C NMR spectra and DEPT-135 experiment (Distortionless Enhancement by Polarization Transfer) were obtained with a Bruker DRX 400 MHz AVANCE III NMR spectrometer (Bruker Daltonics, Germany), according to standard Bruker procedures. Analyses were performed with a 5 mm inverse gradient probe, at 50 °C, the water soluble samples being dissolved in D_2O and the water-insoluble ones in Me_2SO-d_6.

For ^1H NMR and bidimensional experiments (COSY, coupled and decoupled HSQC and TOCSY), the fraction SW-50RM was deuterium-exchanged three times by freeze-drying of D_2O solutions, finally dissolved in Me_2SO-d_6 and transferred into 5 mm NMR sample tube. The experiments were performed using conditions provided by the Bruker manual.

Chemical shifts are expressed as δ PPM, using the resonances of CH_3 groups of acetone internal standard (^1H at δ 2.224; ^{13}C, δ 30.2), or Me_2SO-d_6 (^1H at δ 2.60; ^{13}C, δ 39.7). The spectra were handled using the computer program Topspin® (Bruker).

2.7. Experimental animals

Male albino Swiss mice (3 months old, weighing 25–30 g), from the Universidade Federal do Paraná colony were used for biological tests. They were maintained under standard laboratory conditions, with a constant 12 h light/dark cycle and controlled temperature (22 ± 2 °C), Standard pellet food (Nuvital®, Curitiba/PR, Brazil) and water were available *ad libitum*. All experimental procedures were previously approved by the Institutional Ethics Committee of the University (authorization number 430).

2.8. Procedure to induce sepsis by cecal ligation and puncture (CLP)

Mice were randomly divided into three groups with 10 mice/group: sham-operation, CLP plus saline (10 ml kg^{-1} s.c.) and CLP plus rhamnogalactofuranan of *M. biatorellae* (50 mg kg^{-1}, dissolved in saline, s.c.). Mice were anesthetized with ketamine (80 mg kg^{-1}, i.p.) and xylazine (20 mg kg^{-1}, i.p.) before the surgical procedures. Polymicrobial sepsis was induced by CLP as previously described (Rittirsch et al., 2009). Briefly, a midline incision (approx. 1.5 cm) was performed on the abdomen, the cecum was carefully isolated and the distal 50% was ligated. The cecum was then punctured twice with a sterile 20-gauge needle and squeezed to extrude the fecal material from the wounds. The cecum was replaced and the abdomen was closed in two layers. In the group named Sham, the animals were treated identically, but no cecal ligation or puncture was carried out. After surgery, each mouse received subcutaneously 1 ml of sterile saline for fluid resuscitation and were then placed on a heating pad until they recovered from the anesthesia. Food and water *ad libitum* were provided throughout the experiment. Survival was monitored twice a day (each 12 h), for 7 days and during this period, saline and polysaccharide were subcutaneously administered daily at the doses mentioned above.

To confirm the immune activity of the rhamnogalactofuranan, the fraction was tested for LPS contamination using the method recently described by Santana-Filho et al. (2012). It was not observed the presence of LPS contamination and thus, its influence on the experiments could be disregarded.

3. Results and discussion

The freeze-dried biomass of *M. biatorellae* (41.2 g) was exhaustively extracted with EtOH and $CHCl_3$–MeOH (1:1), giving soluble material in a 36.6% combined yield. This value is in agreement with values previously reported for symbiotic microalgae, such as, 23% for *Trebouxia* sp. (Cordeiro et al., 2005), 35% for *Asterochloris* sp. (Cordeiro et al., 2007) and 38.6% for *Coccomyxa mucigena* (Cordeiro, Sassaki, Gorin, & Iacomini, 2010). The defatted photobiont cells were then submitted to successive extraction with water and 10% aq. KOH, both at 100 °C, and the extracted polysaccharides (fraction W and K10, respectively) recovered by EtOH precipitation and dialysis, respectively. The fraction K10 was obtained in 13.4% yield, while fraction W was a polysaccharide obtained in only 7.4% yield. Both fractions were submitted to freeze–thawing treatment, giving rise to precipitates (PW and PK10) and supernatants (SW, 5.1% yield and SK10, 10.3% yield).

A sugar composition analysis showed that the cold-water soluble fractions consisted of Rha:Ara:Man:Gal:Glc in the molar ratio of 4.2:1:5:29.8:60 for fraction SW and of 2.3:1.7:3.7:48:44.3 for fraction SK10. As previously observed for lichen photobionts (Cordeiro et al., 2005, 2007, 2010), the glucose content was due to the polysaccharide amylose, which is a very common storage polymer found in trebouxioid algae. To remove this polysaccharide, a α-amylase digestion was performed. Then, the resulting fractions SW-A and SK10-A were composed mainly by galactose (Table 1). Moreover, a comparison of their ^{13}C NMR spectra demonstrated the presence of identical peaks, and thus, only one of these fractions was further purified.

The homogeneity of fraction SW-A was determined by high performance steric exclusion chromatography (HPSEC), which gave rise to a main peak (peak I) and three others of smaller intensities (peaks II–IV, Fig. 2A). This fraction was submitted to purification by sequential ultrafiltration through membranes (Fig. 1) with cut-offs of 100 kDa, 50 kDa, 30 kDa and 10 kDa (Millipore). All the retained fractions (SW-100R, SW-50R, SW-30R and SW-10R) showed an

Table 1
Monosaccharide composition of fractions obtained from the microalga *Myrmecia biatorellae*.

Fractions	Monosaccharide composition (%)[a]				
	Rha	Ara	Man	Gal	Glc
SW-A	10.0	2.0	14.0	70.0	4.0
SW-100R	6.4	–	–	93.6	–
SW-50R	6.0	–	–	94.0	–
SW-30R	5.0	–	–	95.0	–
SW-10R	10.0	–	–	90.0	–
SK10-A	6.6	3.6	9.6	80.2	–

[a] % of peak area relative to total peak areas, determined by GC–MS.

homogeneous elution profile on HPSEC analysis, with molar mass of 647.600 Da, 211.000 Da, 170.000 Da and 108.000 Da, respectively (Fig. 2B). A sugar composition analysis showed that the purified fractions consisted mainly of galactose, with small amounts of rhamnose (Table 1). ^{13}C NMR analysis demonstrated that they have similar spectra, thus only fraction SW-50R, which was obtained in higher yield, was further analyzed.

Methylation analysis of fraction SW-50R showed the presence of a rhamnogalactofuranan, where all the rhamnose units were present as non-reducing end units, together with Gal*f* (7.8%). All the galactose units were present in the furanosidic conformation and 2-*O*-(28.8%), 6-*O*-(44.6%) and 2,6-di-*O*-substituted (12.7%), due to the presence of the derivatives 3,5,6-Me$_3$-Gal-ol-acetate, 2,3,5-Me$_3$-Gal-ol-acetate and 3,5-Me$_2$-Gal-ol-acetate, respectively. Its ^{13}C NMR spectrum (Fig. 3A) contained five anomeric signals which were designated A–E according to the decreasing shifts of the anomeric carbon. The signals at δ 108.2, δ 107.7, δ 106.3, δ 106.2 were atributed to C-1 of β-D-galactofuranose units, due to their low-field C-1 resonances (Ahrazem et al., 2007; Sassaki, Iacomini, et al., 2005), while the signal at δ 100.4 is corresponding to C-1 of

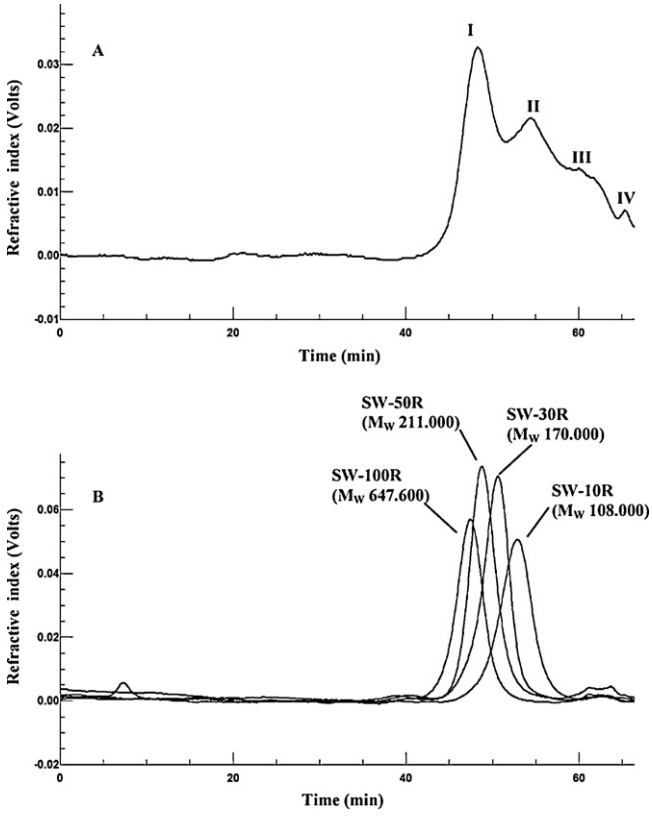


Fig. 2. HPSEC elution profile of (A) fraction SW-A and (B) purified fractions SW-100R, SW-50R, SW-30R and SW-10R. Refractive index detector.

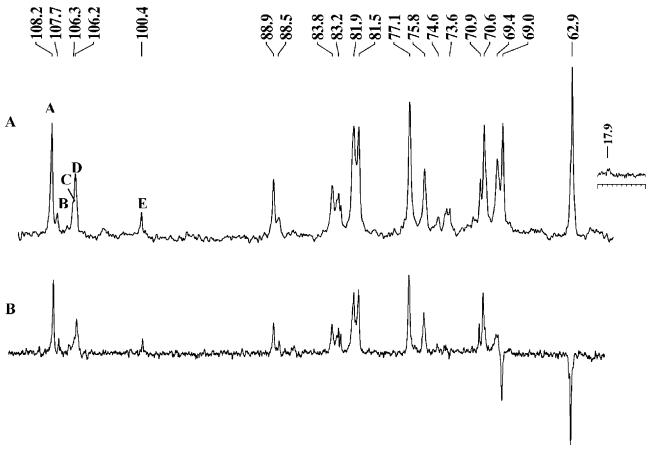


Fig. 3. (A) ^{13}C NMR spectrum and (B) DEPT experiment of fraction SW-50R, in DMSO-d_6 at 50 °C (chemical shifts are expressed as δ PPM) obtained from *Myrmecia biatorellae*.

α-L-rhamnopyranose units. The anomericity of the L-Rha*p* units was confirmed by its coupling constant $^{1}J_{C\text{-}1,\ H\text{-}1}$ (172.3 Hz) observed in coupled HSQC (Ahrazem et al., 2007; Molinaro, Evidente, Lanzetta, Parrilli, & Zoina, 2000). The coupling of these anomeric carbons with their hydrogens was seen in the HSQC spectrum (Fig. 4A). Moreover, the COSY experiment (Fig. 5) demonstrated the coupling of the H-1/H2 of unit C at δ 5.03/4.07, and H-1/H2 of unit D at δ 5.19/4.02. In the HSQC spectrum, these H-2 signals coupled with their C-2 signals at δ 4.07/88.5 (unit C) and δ 4.02/88.9 (unit D). The downfield shifts of these C-2s as compared with the respective signals of methyl β-D-galactofuranoside (Gorin & Mazurek, 1975) confirmed that units C and D are β-D-Gal*f* residues that carry a 2-*O*-substituted carbon. The inverted DEPT-135 signal at δ 68.9 (Fig. 3B) confirmed the presence of 6-*O*-substituted β-D-Gal*f* units. The non-substituted C-6 of β-D-Gal*f* and α-L-Rha*p* units corresponded to signals at δ 62.9 and δ 17.9, respectively.

All the carbons and hydrogens present in the rhamnogalactofuranan of fraction SW-50R were assigned by 1D (^{13}C and DEPT-135) and 2D NMR spectroscopy (HSQC, COSY, and TOCSY), as well by comparison with literature data (Ahrazem, Leal, Prieto, Jiménez-Barbero, & Bernabé, 2001; Ahrazem et al., 2006, 2007; Khatuntseva, Shashkov, & Nifant'ev, 1997; Parra et al., 1994a; Shashkov et al., 2012; Sorum, Robertsen, & Kenne, 1998) and are shown in Table 2. These analysis revealed that **A** was a 6-*O*-substituted β-D-Gal*f*, **B** a terminal β-D-Gal*f*, **C** a 2,6-di-*O*-substituted β-D-Gal*f*, **D** a 2-*O*-substituted β-D-Gal*f* and **E**, a terminal α-L-Rha unit.

According to monosaccharide composition, methylation analysis and spectral data, fraction SW-50R is a rhamnogalactofuranan, probably with a (1 → 6)-β-D-galactofuranose in the main-chain, substituted at *O*-2 by single units of β-D-Gal*f*, α-L-Rha*p* or by side chains of 2-*O*-linked β-D-Gal*f* units (Structure 1a and b). The relative proportions of Structure 1a and b, estimated by methylation analysis, are ~78% and 22%, respectively. To our knowledge this structure is new among natural polysaccharides.

Galactofuranose has attracted interest in the last two decades due to its presence in various microorganisms and due to their absence in mammals. It has been shown to be present in numerous structures considered to be essential for the viability or pathogenicity of many microorganisms (mycobacteria, protozoa, fungi and bacteria), and thus, the metabolism of Gal*f* has become a very attractive candidate as a target for new antimicrobial drugs (Pedersen & Turco, 2003). Most of these structures containing Gal*f* are glycoconjugates, and among them, few have Gal*f* *O*-glycosidically-linked to another Gal*f* unit by (1 → 2),

Table 2
NMR chemical shifts of the rhamnogalactofuraran present in fraction SW-50R of *Myrmecia biatorellae*.

Units	Nucleus	1	2	3	4	5	6
→6)-β-D-Gal*f*-(1→	^{13}C	108.1	81.5	77.0	81.9	70.5	**68.9**[a]
A	^{1}H	4.91	3.935	3.94	3.86	3.67	3.75/3.50
β-D-Gal*f*-(1→	C	107.7	81.8	77.0	81.9	70.5[c]	62.8
B	H	5.04	3.94	4.09	3.68	3.84[c]	3.57
→2,6)-β-D-Gal*f*-(1→	C	106.2	**88.5**[a]	74.2	83.3	70.6	**68.9**[a]
C	H	5.03	4.07	4.10	3.90	3.57	3.75/3.50
→2)-β-D-Gal*f*-(1→	C	106.1	**88.9**[a]	75.6	83.7	69.3	62.8
D	H	5.19	4.02	4.11	3.86	3.81	3.57
α-L-Rha*p*-(1→	C	100.4	na[b]	na[b]	73.5	70.5[c]	17.8
E	H	5.00	3.93	na[b]	3.50	3.84[c]	1.24

[a] Underlined bold numbers represent glycosylation sites.
[b] Not assigned due to overlapping.
[c] The assignments may be interchanged.

(1 → 3), (1 → 5) or (1 → 6)-linkages, while others are attached to glycopyranoside entities, as for example, Man*p*, Glc*p*, Glc*p*NAc, Gal*p*, GalpNAc, Fru*p* and Rha*p* (Peltier et al., 2008). Polymers of galactofuranose, such as galactofuranans, have been extracted mainly from cell walls of various species of fungi, such as *Penicillium* (Parra et al., 1994b), *Eupenicillium* (Leal, Prieto, Gómez-Miranda, Jiménez-Barbero, & Bernabé, 1993), *Neosartorya* (Leal et al., 1995), *Aspergillus* (Leal, Guerrero, Gómez-Miranda, Prieto, & Bernabé, 1992) and *Guignardia* (Sassaki et al., 2002). These fungal β-galactofuranans had a variety of structures, being linear chains constituted by only (1 → 5)- or alternate (1 → 5), (1 → 6)-β-D-Gal*f* units, as well branched structures. In lichenized fungi, a complex heteropolysaccharide (thamnolan) consisting mainly of α-L-Rha*p* and β-D-Gal*f* units was isolated from the lichen *Thamnolia subuliformis*. It has a predominant structure of (1 → 3)-β-D-galactofuranosyl units with branches on C-6, complex rhamnopyranosyl sidechains (3-*O*-, 2,3-*O*- and 2,4-di-*O*-substituted) and terminal Glc*p*, Man*p*, Gal*f* and Xyl*p* units (Olafsdottir, Omarsdottir, Paulsen, Jurcic, & Wagner, 1999).

It is noteworthy that galactofuranans were also found in symbiotic microalgae, the lichen photobionts of the genera *Trebouxia*

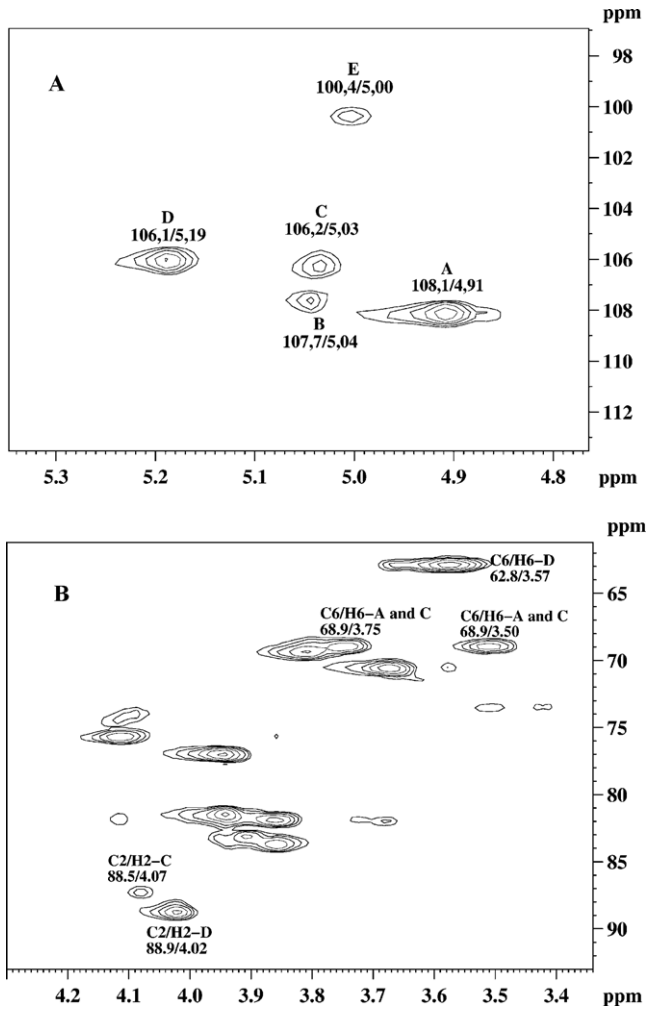


Fig. 4. 2D HSQC spectrum of fraction SW-50R isolated from *Myrmecia biatorellae*, (A) anomeric region. The cross-peaks are labeled with letters A–E as explained in the text and (B) C-2-C6 region. The cross-peaks showing relevant connections have been labeled. Sample was dissolved in DMSO-d_6 and data collected at probe temperature of 70 °C.

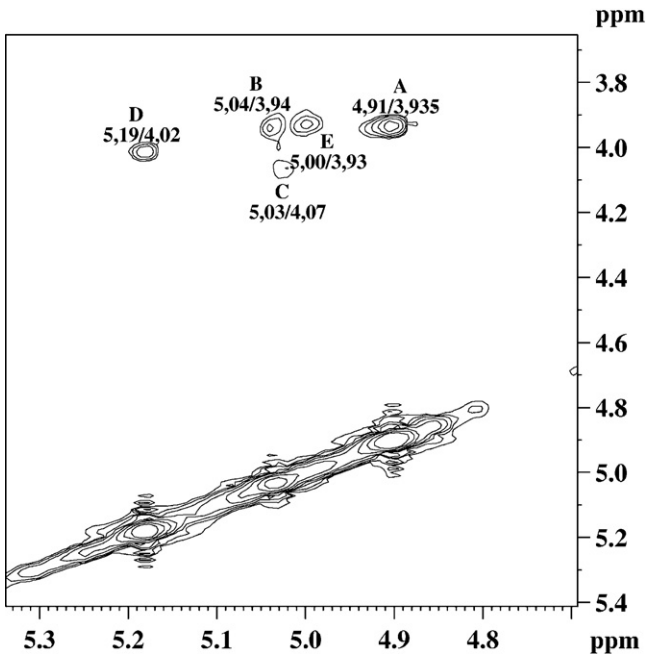
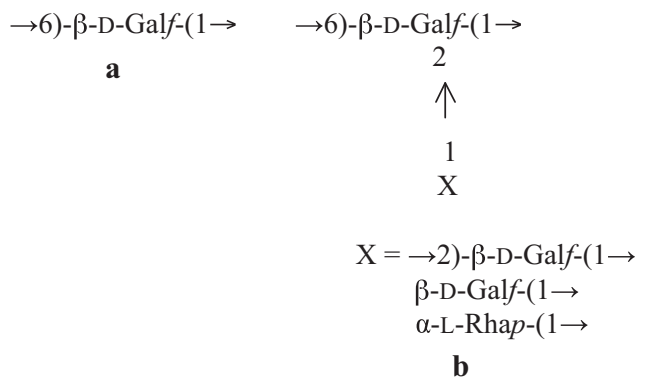


Fig. 5. Anomeric region of the 2D COSY spectrum of fraction SW-50R isolated from *Myrmecia biatorellae*. Sample was dissolved in DMSO-d_6 and data collected at probe temperature of 70 °C.



Structure 1. Rhamnogalactofuranan.

and *Asterochloris* (Cordeiro et al., 2005, 2007, 2008; Ruthes et al., 2008). However, the *Trebouxia* galactofuranans contained (1 → 5)-β-D-galactofuranosyl backbone, while the galactofuranan from *Asterochloris* was predominated by (1 → 3)-β-D-galactofuranosyl units.

Interestingly, these galactofuranan-containing photobionts (*Trebouxia*, *Asterochloris* and now *Myrmecia*) belongs to the order Trebouxiales, within the family Trebouxiophyceae (Friedl & Büdel, 2008). It seems that galactofuranans could be typical polysaccharides of photobionts of this order, once the carbohydrates produced by others trebouxiophyceae photobionts studied up to now have different structures. An *O*-methylated mannogalactan was found in the symbiotic microalgae *Coccomyxa* (Cordeiro et al., 2010), while a cyclic (1 → 2)-β-D-glucan was described for microalgae of the genus *Chlorella* (Suárez et al., 2008). Further studies, involving more photobiont genera should be performed to reinforce this hypothesis.

The microalga *M. biatorellae* here studied was isolated from specie of *Lobaria* (*L. linita*). Regarding polysaccharides of this lichen genus, there is only an early report where an antitumor active glycopeptide (LOF-1) was identified from *L. orientalis*. The main carbohydrate components of LOF-1 were shown to be a (1 → 6)-glucan and (1 → 3)-mannan, which was linked to serine and threonine of the peptide part by *O*-glycosyl linkages (Takahashi, Takeda, Shibata, Inomata, & Fukuoka, 1974). Thus, taking in account this report, there is no similarities between the rhamnogalactofuranan of the photobiont *Myrmecia* and those polysaccharides of the thallus of *Lobaria*. In previous studies carried out by our research group, we observed that the polysaccharides extracted from the symbiotic thallus were produced by the aposymbiotically cultured mycobiont (Cordeiro, Messias, Sassaki, Gorin, & Iacomini, 2011; Cordeiro, Stocker-Wörgötter, Gorin, & Iacomini, 2004), while any polysaccharides with a photobiont origin could be detected in it. On the other hand, the symbiotic microalgae have been shown a source of unique and unusual polysaccharide structures for algae. Some questions are fascinating and still unresolved: (a) why symbiotic microalgae produce these polysaccharides containing an unusual monosaccharide that is the galactofuranose; (b) what are the functions of these galactofuranans for the microalgae, and (c) have these molecules a role in the symbiosis?

Due to their absence in mammals, galactofuranose-containing epitopes have been shown to be highly immunogenic (Pedersen & Turco, 2003). In a preliminary study, we tested the effect of the rhamnogalactofuranan of *M. biatorellae* in a model of murine polymicrobial sepsis induced by cecal ligation and puncture (CLP). This model mimics the sepsis in humans, caused by pathogens derived from the intestinal tract and is considered to closely simulate clinical situation (Rittirsch et al., 2009). It was observed an increase of 30% in the lethality of mice receiving subcutaneously the rhamnogalactofuranan (50 mg/kg) when compared with the control (treated subcutaneously with saline), 24 h after the induction of the sepsis. No death was observed in the sham-operated mice.

An uncontrolled hyperinflammatory response and inappropriate cytokine response during early sepsis is proposed to be the main cause of multiple organ dysfunction syndrome (MODS) during early sepsis. Several studies have demonstrated that some cytokines, specially TNF-α, IL-1β and IL-6 are strongly associated with sepsis syndrome, therefore controlling inflammation and inhibiting the proinflammatory cytokine overproduction during early sepsis may reduce organ injury and prevent death after septic insult (Cohen, 2002; Yun et al., 2009). This was recently demonstrated by polysaccharides with anti-inflammatory activity. They were able to reduce the mortality in septic mice, and this was related to the decreasing in the serum levels of pro-inflammatory mediators (TNF-α, IL-1β, IL-6 and monocyte chemotactic protein-1) caused by the polysaccharides (Meng et al., 2012; Ruthes et al., 2012).

As could be seen above, the rhamnogalactofuranan of *M. biatorellae* had an opposite effect when compared with these anti-inflammatory polysaccharides, since it increased the lethality of septic mice. The mechanism of this action requires further investigation however, the rhamnogalactofuranan must be amplifying the systemic inflammatory response and thus contributing to multiple organ failure and death. Curiously, a pro-inflammatory effect is also described for carrageenan, a linear sulfated polysaccharide extracted from red seaweeds and widely used as a food additive. It was demonstrated that carrageenan induces proinflammatory responses in animal models and human cells. The carrageenan-induced inflammatory cascades stimulate important proinflammatory components as toll-like receptor (TLR) 4 and activation of the transcription factor NF-κB, leading to increased cytokine production (Borthakur et al., 2012).

We believe that this biological effect observed for rhamnogalactofuranan from *M. biatorellae* may be related to its chemical structure (rich in galactofuranose) and reinforce the fact that the determination of the fine structure of polysaccharides is significant in understanding the relation between their structure and biological activity.

4. Conclusions

The aposymbiotically cultivated *M. biatorellae* produced a rhamnogalactofuranan, probably with a (1 → 6)-β-D-galactofuranose in the main-chain, substituted at *O*-2 by single units of β-D-Gal*f*, α-L-Rha*p* or by side chains of 2-*O*-linked β-D-Gal*f* units (Structure 1a and b). Regarding the symbiosis, there are no similarities between this rhamnogalactofuranan with polysaccharides previously found in the lichen of the genus *Lobaria*. Indeed, this rhamnogalactofuranan has a unique structure, and a similar polysaccharide has not been described in the literature. Finally, this polysaccharide increased the lethality of mice submitted to polymicrobial sepsis induced by cecal ligation and puncture, probably due to the presence of galactofuranose, which have been shown to be highly immunogenic in mammals.

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