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Chemical structure of a partially 3-*O*-methylated mannofucogalactan from edible mushroom *Grifola frondosa*

Gracy Kelly Faria Oliveira[a], Estefania Viano da Silva[a,b], Andrea Caroline Ruthes[c,d], Luciano Morais Lião[b], Marcello Iacomini[c], Elaine R. Carbonero[a,*]

[a] Departamento de Química, Universidade Federal de Goiás, Regional Catalão, 75704-020 Catalão, Brazil
[b] Laboratório de Ressonância Magnética Nuclear, Instituto de Química, Universidade Federal de Goiás, Campus Samambaia, 74001-970 Goiânia, Brazil
[c] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, 81531-980 Curitiba, Brazil
[d] Department of Entomology and Nematology, University of Florida, GCREC, 14625 County Road 672, Wimauma, FL 33598, United States

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ABSTRACT

An unusual heteropolysaccharide was isolated from the fruiting bodies of the medicinal mushroom *Grifola frondosa*, via successive cold aqueous extraction, followed by fractionation through freeze-thawing, precipitation with Fehling solution and dialysis using a membrane with a size exclusion cut-off of 500 kDa. Its chemical structure was determined based on total acid hydrolysis, methylation analysis and NMR studies. The mannofucogalactan had a molar mass of 15.9×10^3 g mol^{-1}, which was determinate by HPSEC-MALLS. This heteropolymer showed to have a main chain of (1 → 6)-linked α-D-Gal*p* partially substituted at O-2 by 3-*O*-α-D-mannopyranosyl-α-L-fucopyranosyl groups and in a minor proportion with α-L-Fuc*p* single-unit side chains. Moreover, the presence of 3-*O*-Me-Gal*p* units could also be observed in the main chain of the *G. frondosa* mannofucogalactan.

1. Introduction

Mushrooms have been valued as edible and medicinal resources. *Grifola frondosa* (*Maitake*), a basidiomycete belonging to the Polyporaceae family, may be one of the most versatile and promising medicinal mushroom used as a dietary supplement (Wu et al., 2006). It have been widely used in Japan, China and Korea as a traditional food additive (Gu et al., 2007) and is one of the most valuable and expensive mushrooms (Mayell, 2001).

Since the beginning of its cultivation in 1981, the study of its medicinal applications has been ongoing, and the activity of its purified polysaccharides has been highlighted (Mayell, 2001). Over the past three decades, many polysaccharides have been isolated from the fruiting bodies of *G. frondosa* and showed antitumor activity (Masuda et al., 2009), besides of antihypertensive (Konno, 2007; Talpur et al., 2002) anti-diabetic (Gu et al., 2007), and anti-hyperliposis effects (He et al., 2017; Minamino, Nagasawa, & Othtsuru, 2008).

Most of the polysaccharides from *G. frondosa* fruiting bodies were characterized as D-glucans with different linkage types, such as β-(1 → 3), β-(1 → 6) and α-(1 → 4) (He et al., 2017; Wasser, 2002). Grifolan (GRN) is the best known and most potent substances with antitumor and immunomodulating properties (Borchers, Keen, & Gershwin, 2004) isolated from the cultured fruiting bodies (Ohno et al., 1984), matted mycelia (Ohno et al., 1985) and liquid culture supernatant (Ohno et al., 1986) of *G. frondosa* (Fang et al., 2012). Grifolans are characterized as β-D-glucans (1 → 3)-linked in the backbone with a single (1 → 6)-linked β-D-glucosyl side branching unit on every third residue.

In addition to β-D-glucans, some heteropolysaccharides showing different compositions, most of them biologically active, have been obtained from *G. frondosa* (Cui et al., 2007; Masuda et al., 2009; Masuda, Ito, Konishi, & Nanba, 2010; Mizuno,Ohsawa, Hagiwara, & Kuboyama, 1986; Xu, Liu, Shen, Fei, & Chen, 2010; Wang et al., 2014). With the exception of the acid heteropolysaccharide, named GFPS1b, obtained from cultured mycelia of *G. frondosa* (Cui et al., 2007), and the water-soluble polysaccharide named GFPW from the fruiting bodies of this mushroom (Wang et al., 2014), the primary structures of the heteropolymers have not been unambiguously elucidated. GFPS1b showed to have a backbone consisting of (1 → 4)-linked α-D-Gal*p* and (1 → 3)-linked α-D-Glc*p* residues, the latter being partially substituted at O-6 by 4-*O*-α-L-arabinofuranosyl-α-D-glucopyranosyl groups, which showed to be effective in the inhibition of proliferation of mammary tumor MCF-7 cells in vitro (Cui et al., 2007). The other heteropolysaccharide chemically elucidate was the fraction GFPW, which had a main chain of (1 → 6)-linked α-D-Gal*p* residues, with branches of (1 → 3)-linked

* Corresponding author.
E-mail address: elainecarbonero@gmail.com (E.R. Carbonero).

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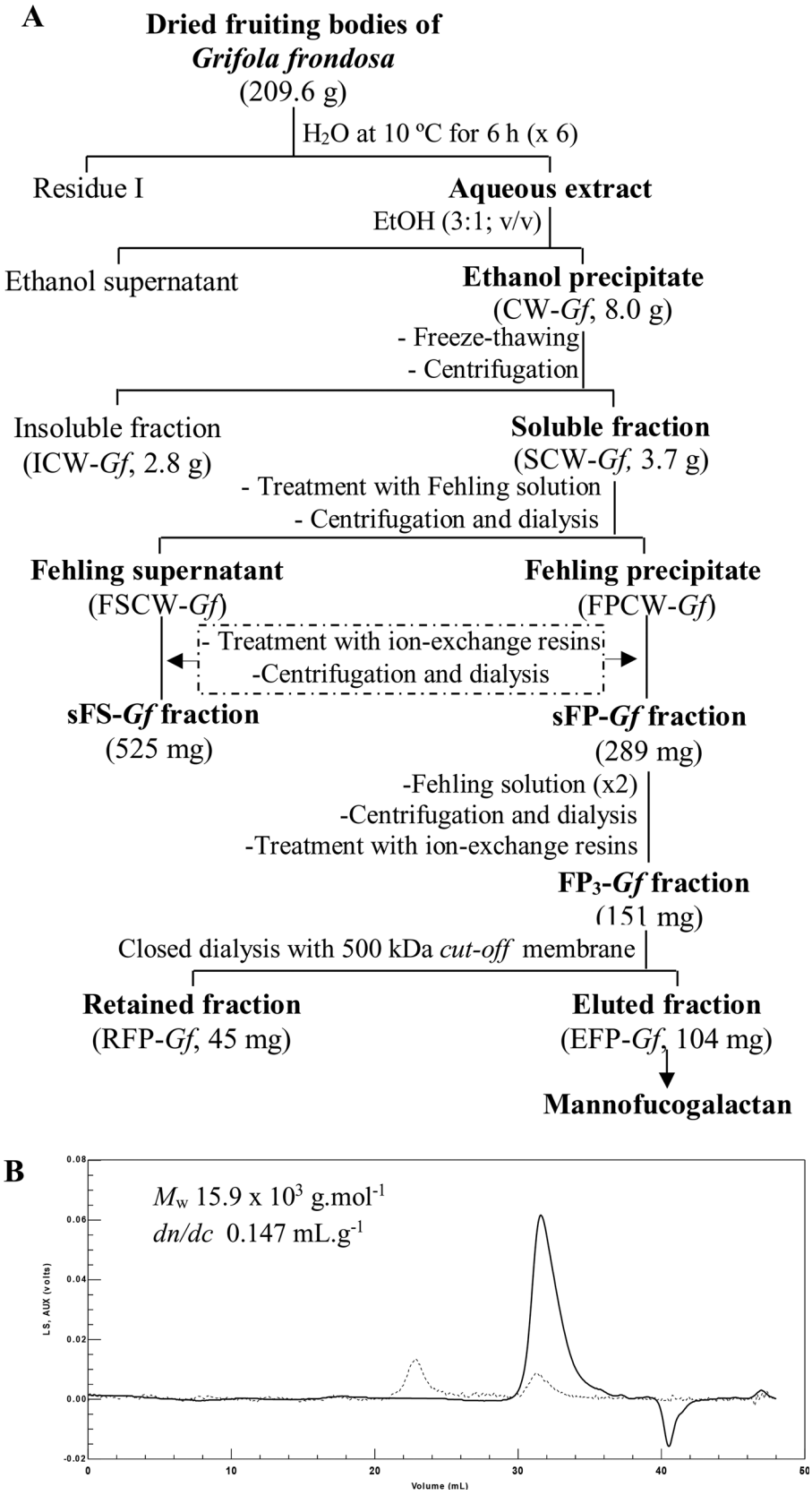


Fig. 1. (A) Scheme of extraction and purification of the heterogalactan from fruiting bodies of *G. frondosa*. (B) Elution profile of fraction EFP-*Gf* determined by HPSEC-MALLS using light scattering (—) and refractive index detectors (—).

fucose residues and α-terminal mannose substituting the O-2 position (Wang et al., 2014).

Novel polysaccharides from *G. frondosa* have been frequently isolated, purified and evaluated. As most of them have been shown to be bioactive, it is important to know the fine chemical structure of those compounds in an attempt to determine the structure-activity relationship. Thus, at the present study the isolation and structural characterization of a different heteropolysaccharide, a partially methylated

Table 1
Partially *O*-methylated alditol acetates formed on methylation analysis of the EFP-*Gf* fraction obtained from the fruiting bodies *of G. frondosa*.

Partially *O*-methylated alditol acetate[a]	Linkage type[b]	R_T (min)[c]	Fraction (mol%)	Mass fragmentation (*m/z*)
2,3,4-Me_3-Fuc	Fuc*p*-(1→	14.708	6.8	89,102,115,118,131,162,175
2,3,4,6-Me_4-Man	Man*p*-(1→	15.725	19.6	87,102,118,129,145,161,205
2,4-Me_2-Fuc	→3)-Fuc*p*-(1→	15.945	19.3	89,101,118,131,160,234
2,3,4-Me_3-Gal	→6)-Gal*p*-(1→	18.221	27.9	87,102,118,129,162,173,189,233
3,4-Me_2-Gal	→2,6)-Gal*p*-(1→	20.114	26.4	87,100,129,159,173,189,233

[a] Analyzed by GC–MS after methylation, total acid hydrolysis, reduction ($NaBD_4$) and acetylation.
[b] Based on derived *O*-methylalditol acetates.
[c] Retention time (minutes).

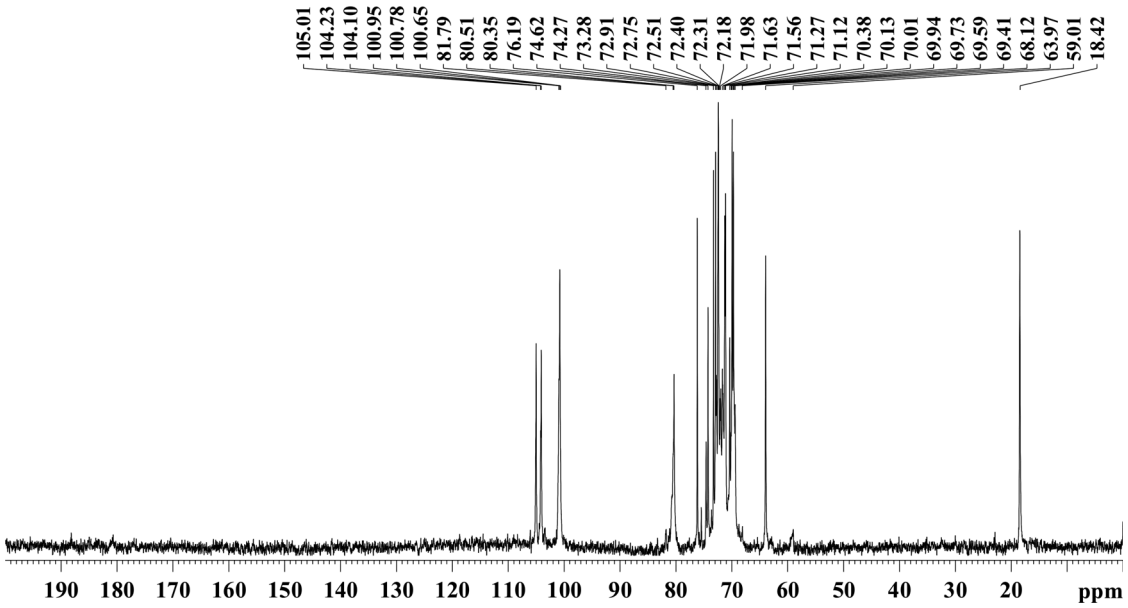


Fig. 2. ^{13}C NMR spectrum of mannofucogalactan (EFP-*Gf* fraction) from *G. frondosa*. EFP-*Gf*, analyzed in D_2O at 50 °C (chemical shifts are expressed in δ ppm).

mannofucogalactan from the fruiting bodies of *G. frondosa* is described.

2. Material and methods

2.1. Biological material

Fresh basidiocarps (fruiting bodies) of *Grifola frondosa (Dicks.) Gray* (1.03 kg) were provided by YURI Cogumelos (Owner: Iwao Akamatsu), located in Sorocaba, State of São Paulo, Brazil, in May of 2010.

2.2. Extraction and purification of polysaccharides

The fresh fruiting bodies of *G. frondosa* (1.03 kg) were freeze-dried, resulting in 209.6 g, which were pulverized and their polysaccharides were extracted with water at 10 °C for 6 h (×6, 2000 mL). The combined aq. extracts were evaporated to a small volume and added to excess ethanol (EtOH, 3:1; v/v) to precipitate polysaccharides, which were collected after centrifugation at 3000 rpm at 20 °C for 20 min. The precipitate was then dissolved in H_2O, dialyzed against distilled water for 20 h to remove low-molecular-weight carbohydrates, and freeze-dried (CW-*Gf* fraction). The fraction CW-*Gf* was then dissolved in distilled water and the solution submitted to a freeze-thawing process furnishing a cold water-soluble (SCW-*Gf*) and an insoluble fraction (ICW-*Gf*), which were separated under the same centrifugation conditions. The soluble portion (SCW-*Gf*) was treated with Fehling solution (Jones & Stoodley, 1965) and the precipitated material (FPCW-*Gf*) centrifuged off. Both fractions, FPCW-*Gf* (precipitate) and FSCW-*Gf* (supernatant) were neutralized with HOAc, dialyzed against tap water, deionized with mixed ion-exchange resins. During the treatment with ion-exchange resins, a part of these fractions became precipitated (pFP-*Gf* and pFS-*Gf* fractions, respectively), being separated by centrifugation (3000 rpm at 20 °C for 20 min). Fehling treatment was repeated two more cycles under fraction sFP-*Gf* to ensure that no residue of the supernatant was present in the precipitated fraction, giving the fraction FP_3-*Gf*.

FP_3-*Gf* fraction was further purified by closed dialysis through a membrane with a 500 kDa M_w cut-off (Spectra/Por® PVDF), giving rise to a retained (RFP-*Gf*) and an eluted (EFP-*Gf*) material (Fig. 1A).

2.3. Monosaccharide composition

Monosaccharide components of the polysaccharides were identified and their ratios were determined following hydrolysis with 2 M trifluoroacetic acid (TFA) for 8 h at 100 °C, and conversion to alditol acetates by successive $NaBH_4$ and/or $NaBD_4$ reduction, and acetylation with Ac_2O-pyridine (1:1, v/v) for 12 h at room temperature (Thompson, 1963a,1963b;). The resulting alditol acetates were analyzed by gas chromatography–mass spectrometry (GC–MS) using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, Model 810-R12 mass spectrometer. A DB-225 capillary column (30 m × 0.25 mm i.d.) held at 50 °C during injection and later programmed to 220 °C (constant temperature) at 40 °C min^{-1} was used for qualitative and quantitative analysis of alditol acetates. The alditol acetates were identified by their typical retention times and electron impact profiles.

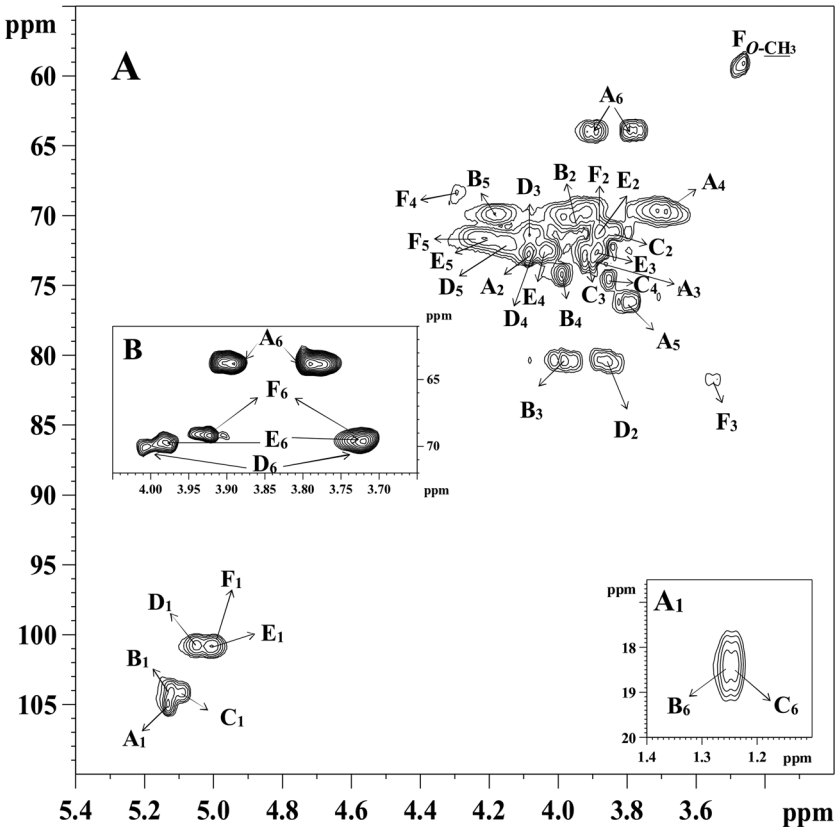


Fig. 3. HSQC (A) spectrum of mannofucogalactan (EFP-*Gf* fraction) from *G. frondosa*, with amplified inserts of the: C-6 region of Fuc*p* (A_1); HSQC-DEPT C-6 region (B). EFP-*Gf*, analyzed in D_2O at 50 °C (chemical shifts are expressed in δ ppm).
A = non reducing ends α-Man; B = α-Fuc*p* substituted at O-3 by α-Man*p*; C = non reducing ends α-Fuc*p*; D = 2,6-di-*O*- substituted α-Gal*p* units; E = 6-*O*- substituted α-Gal*p*; F = 6-*O*- substituted 3-*O*-Me-α-Gal*p* units.

2.4. Determination of homogeneity of polysaccharides and their molar mass (M_w)

The homogeneity and molar mass (M_w) of the fractions were determined using a Waters high-performance size-exclusion chromatography (HPSEC) apparatus coupled to a differential refractometer (RI) and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering detector (MALLS). The eluent was 0.1 M $NaNO_3$, containing 0.5 g L^{-1} NaN_3. The polysaccharide solutions were filtered through a membrane with 0.22 µm diameter pores (Millipore). The specific refractive index increment (*dn/dc*) was determined using a Waters 2410 detector, the samples being dissolved in the eluent, five increasing concentrations, ranging from 0.2 to 1.0 mg mL^{-1} being used to determine the slope of the increment.

2.5. Methylation analysis

Per-*O*-methylation of purified EFP-*Gf* fraction was carried out by the method of Ciucanu and Kerek (1984). Briefly, the sample (10 mg) was dissolved in dimethyl sulfoxide (Me_2SO; 500 µL), powdered NaOH (20 mg) and iodomethane (CH_3I; 500 µL) were added. After 30 min at 25 °C with vigorous stirring, the mixture was maintained overnight at 25 °C. The reaction was interrupted by addition of water, neutralization with HOAc, and the products were isolated by partition between $CHCl_3$ and water. The per-*O*-methylated derivatives from the lower layer were hydrolyzed with 1 M TFA (500 uL) for 4 h at 100 °C, followed by $NaBD_4$ reduction and acetylation as above (item 2.3), to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS using an Agilent 7820A gas chromatograph interfaced to an Agilent 5975E quadrupole mass spectrometer, fitted with split/splitless capillary inlet system, an Agilent G4513A autosampler, and a capillary HP-5MS column. The injector temperature was maintained at 250 °C, with the oven increasing from 75 °C (hold 1 min) to 100 °C (35 °C min^{-1}, then held for 5 min), 150 °C (45 °C min^{-1}, then held for 5 min), 200 °C (55 °C min^{-1}, then held for 15 min), 250 °C (65 °C min^{-1}, then held for 10 min), and to 270 °C (50 °C min^{-1} and held for 10 min). Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. Partially *O*-methylated alditol acetates were identified from *m/z* of their positive ions, by comparison with standards, the results being expressed as a relative percentage of each component (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.6. Partial acid hydrolysis of heterogalactan

Fraction EFP-*Gf* (60 mg) was partially hydrolyzed with 0.2 M TFA (2 mL) for 3 h at 100 °C. After neutralization with NaOH, the material was dialyzed (2 kDa *cut-off* membrane) against distilled water. The retained fraction (HEFP-*Gf*) was lyophilized and analyzed by NMR spectroscopy.

2.7. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra (^1H, ^{13}C, COSY, HSQC-DEPT, HSQC-TOCSY, HMBC, HSQC-NOESY and coupled HSQC) were obtained using a 500 MHz Bruker Avance spectrometer incorporating Fourier transform. Analyses were performed at 50 °C on samples dissolved in D_2O or Me_2SO-d_6. Chemical shifts are expressed in δ relative to Me_4Si (TMS; $\delta = 0$) or Me_2SO-d_6 ($\delta = 39.70$ and 2.50 for ^{13}C and ^1H signals, respectively).

3. Results and discussion

G. frondosa was shown to contain 79.1% moisture on desiccation in a freeze dryer, and the product was submitted to aqueous extraction at 10 °C.

The extracted polysaccharides were recovered by ethanol precipitation, dialyzed against tap water, and the solution freeze-dried,

giving CW-*Gf* fraction (8.0 g) (Fig. 1A), which showed to be composed by glucose (Glc, 44%) as its main component, in addition to fucose (Fuc, 10%), mannose (Man, 24%), and galactose (Gal, 22%), according to GC–MS of derived alditol acetates. Fractionation of the CW-*Gf* by freeze/thawing process furnished water-soluble (SCW-*Gf*, 3.7 g) and insoluble (ICW-*Gf*, 2.8 g) polysaccharidic fractions, which were separated by centrifugation. SCW-*Gf* was composed of Fuc (7%), Man (39%), 3-*O*-methyl-galactose (3-*O*-Me-Gal, 2%) (confirmed by GC–MS ions at *m/z* 130 and 190 after reduction with $NaBD_4$ and acetylation), galactose (27%), and glucose (25%), and its HPSEC–MALLS analysis showed heterogeneity.

In order to obtain a purified sample, the soluble fraction (SCW-*Gf*) was treated with Fehling solution three times, sequentially, giving rise to a precipitate (FP_3-*Gf*; 151 mg), which was further fractionated by dialysis (500 kDa M_w cut-off membrane).

The eluted fraction (EFP-*Gf*, 104 mg) was homogeneous on HPSEC-MALLS (Fig. 1B), had M_w 15.9×10^3 g mol^{-1} (dn/dc = 0.147 mL g^{-1}) and contained fucose (25.5%), mannose (20.3%), 3-*O*-methyl-galactose (10.8%) and galactose (43.4%) as monosaccharide components, suggesting the presence of a mannofucogalactan.

In order to characterize the glycosidic linkages of EFP-*Gf*, it was submitted to methylation analysis, which showed a branched structure, containing non-reducing end units of Fuc*p* (2,3,4-Me_3-Fuc; 6.8%), and Man*p* (2,3,4,6-Me_4-Man; 19.6%), in addition to 6-*O*-substituted (2,3,4-Me_3-Gal; 27.9%) and 2,6-di-*O*-substituted units (3,4-Me_2-Gal; 26.4%) of galactopyranose. The presence of the 2,4-Me_2-Fuc*p* (19.3%) derivative indicates that Fuc*p* was substituted at O-3 (Table 1).

Spectroscopic analysis [^{1}H-, ^{13}C- (Fig. 2), HSQC (Fig. 3A), HSQC-DEPT (Fig. 3B), HSQC-TOCSY (Fig. 4) and coupled HSQC NMR] were also helpful to elucidate the structure of EFP-*Gf*, since the coupling of protons observed in COSY and TOCSY 2D-NMR spectra, made possible the assignments of EFP-*Gf* respective units carbons using HSQC analysis (Fig. 3; Table 2), which were confirmed by connectivities observed in HSQC-TOCSY spectrum (Table 3).

The ^{1}H NMR spectrum recorded in D_2O at 50 °C showed the presence of mainly six signals in the anomeric region at δ 5.13, 5.12, 5.09, 5.05, 5.00, and 4.99. The sugar residues were designated as A–F according to their decreasing anomeric proton chemical shift values, which were attributed to non-reducing end groups of Man*p* (δ 5.13) and Fuc*p* (δ 5.09), 3-*O*-substituted units of Fuc*p* (δ 5.12), 6-*O*-substituted 3-*O*-Me-Gal*p* (δ 4.99), and 6-*O*- (δ 5.00) and 2,6-di-*O*-substituted Gal*p* (δ 5.05), respectively.

HSQC spectrum (Fig. 3) showed signals (C-1/H-1) at δ 105.01/5.13 and 104.23/5.09, and 104.10/5.12 corresponding to non-reducing end groups of Man*p* and Fuc*p*, and 3-*O*-substituted Fuc*p* residues, respectively. Anomeric signals (C1/H1) at δ 100.78/5.05 and 100.95/5.00, were from 6-*O*- and 2,6-di-*O*-substituted Gal*p* residues, respectively, and that at δ 100.65/4.99 were from 6-*O*-substituted 3-*O*-Me-Gal*p* units. All units showed α-configurations due to the value of $J_{C\text{-}1,H\text{-}1}$ = 171.6 Hz found in ^{1}H/^{13}C coupled HSQC spectrum (Perlin & Casu, 1969).

The above methylation analysis indicated the presence of 3-*O*, 6-*O*- and 2-*O*-substituted linkages (Table 1), these being confirmed by NMR spectroscopy. *O*-substituted C-3 signals for 3-*O*-substituted Fuc*p* and C-2 signals from 2,6-di-*O*-substituted Gal*p* units were at δ 80.35 and 80.50, respectively (Figs. 2–4), and substituted $-\underline{C}H_2$ groups of the 6-*O*- and 2,6-di-*O*-substituted units of the main chain were at δ 69.59 (6-*O*-substituted Gal*p*); 69.41 (6-*O*-substituted 3-*O*-Me-Gal*p*) and δ 70.00 (2,6-di-*O*-substituted Gal*p*), respectively, giving rise to inverted signals in the HSQC-DEPT spectrum (Fig. 3B).

The presence and position of *O*-methyl groups of the heteropolysaccharide were confirmed by δ 59.01/3.46 and δ 81.80/3.56 (C/H) signals corresponding to $-O\underline{C}H_3$ and *O*-substituted C-3 substituted/H-3, respectively (Figs. Figure 3A and Figure 4; Table 3).

The signals at δ 72.90/4.09, 73.28/3.92, 69.73/3.69, 76.19/3.80, and 63.97/3.90;3.78 arose from C-2/H-2 to C-6/H-6 of Man*p* units,

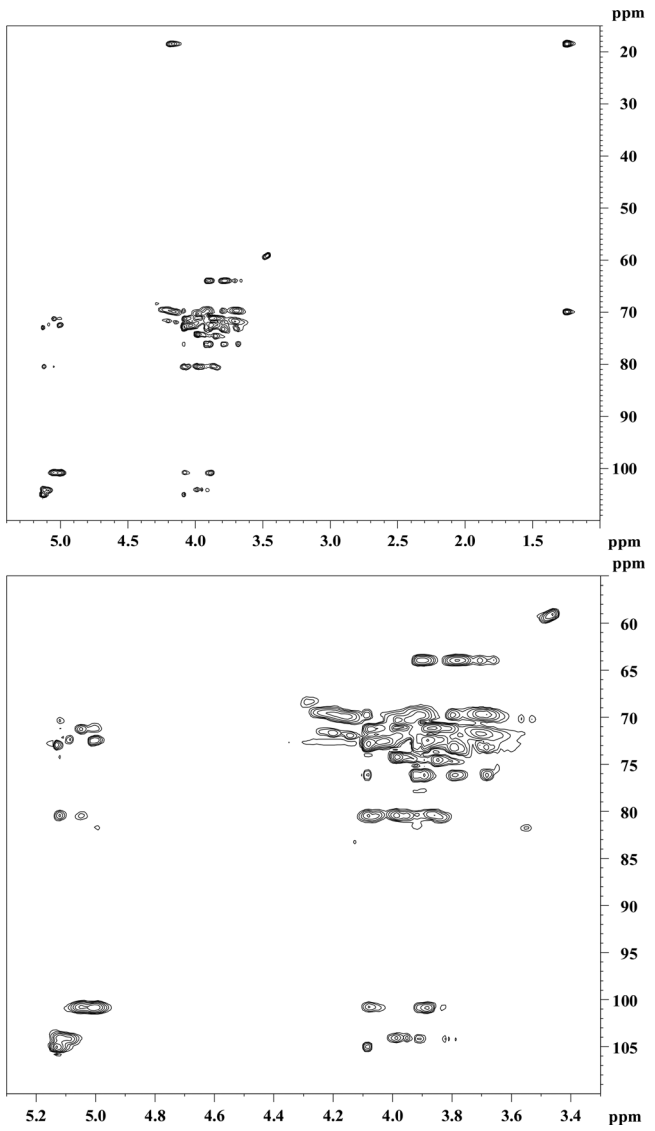


Fig. 4. HSQC-TOCSY spectrum of mannofucogalactan (EFP-*Gf* fraction) from *G. frondosa*. EFP-*Gf*, analyzed in D_2O at 50 °C (chemical shifts are expressed in δ ppm).

respectively, while those at δ 71.12/3.83, 72.31/3.91, 74.62/3.85, 69.95/4.18, and 18.42/1.24 were from similar C-2/H-2 to C-6/H-6 correlations of Fuc*p* residues.

In order to elucidate the core of the heterogalactan, a partial acid hydrolysis was carried out. The product of partial hydrolisys gave a HSQC-DEPT spectrum (Fig. 5) with signals characteristics of a linear partially 3-*O*-methylated (1 → 6)-linked α-galactopyranan (Carbonero, Gracher, Rosa et al., 2008), showing that side groups were removed from main chain.

Interresidues correlations observed in the HSQC-NOESY and HMBC experiments were important to confirm the glycosidic linkages between monosaccharides, but due to the overlapping signals from substituted $-CH_2$ groups of Gal and 3-*O*-Me-Gal*p* units of the main chain, it was not possible to determine the sequence of all units of in this polymer. The units of α-Man*p* (residue A) have an interresidue correlation with H-1 (δ 5.13) to C-3 (δ 80.35) of 3-*O*-substituted Fuc*p* units (residue B). The *O*-substituted C-2 signals (δ 80.50) from 2,6-di-*O*-Gal*p* units of the main chain (residue D) showed interresidue correlations with C-1/H-1 at δ 104.10/5.12 of 3-*O*-substituted Fuc*p* units (residue B) and 104.23/5.09 of non-reducing ends of Fuc*p* (residue C).

In summary, the results of monosaccharide composition, methylation and NMR spectroscopic analysis of EFP-*Gf*, showed it to be a

Table 2
The significant connectivities observed in an HSQC-TOCSY spectrum for the protons/carbons of the residues of the polysaccharide of *G. frondosa*.

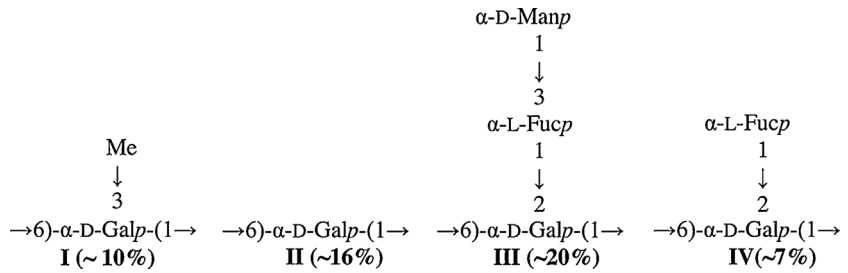
Units	H/C δ_H/δ_C	Observed cross peaks δ_H/δ_C
α-D-Man*p*-(1→ (Residue A)	105.01(C1)	5.13 (H1); 4.09 (H2)
	72.90 (C2)	5.13 (H1); 4.09 (H2); 3.92 (H3)
	73.28 (C3)	4.09 (H2); 3.92 (H3); 3.69 (H4); 3.80 (H5)
	69.73 (C4)	4.09 (H2); 3.69 (H4)
	76.19 (C5)	4.09 (H2); 3.92 (H3); 3.69 (H4); 3.80 (H5); 3.78 (H6a); 3.90 (H6b)
	63.97 (C6)	3.69 (H4); 3.80 (H5); 3.78 (H6a); 3.90 (H6b)
→3)-α-L-Fuc*p*-(1→ (Residue B)	104.10 (C1)	5.12 (H1); 3.95 (H2); 3.98 (H3)
	70.38 (C2) 80.35 (C3) 74.27 (C4)	5.12 (H1); 3.95 (H2); 3.98 (H3); 3.99 (H4)
	69.95 (C5) 18.42 (C6)	4.18 (H5); 1.25 (H6)
α-L-Fuc*p*-(1→ (Residue C)	104.23 (C1)	H1 (5.09); 3.82 (H2); 3.91 (H3); 3.85 (H4)
	71.12 (C2)	3.82 (H2); 3.91 (H3); 3.85 (H4)
	72.31 (C3) 74.62 (C4)	H1 (5.09); 3.82 (H2); 3.91 (H3); 3.85 (H4)
	69.95 (C5) 18.42 (C6)	4.18 (H5); 1.24 (H6)
→2,6)-α-D-Gal*p*-(1→ (Residue D)	100.78 (C1) 80.50 (C2) 71.27 (C3) 72.35 (C4)	5.05 (H1); 3.87 (H2); 4.08 (H3); 4.06 (H4)
	71.89 (C5) 70.00 (C6)	4.14 (H5); 4.00 (H6a); 3.71 (H6b)
→6)-α-D-Gal*p*-(1→ (Residue E)	100.95 (C1) 71.12 (C2) 72.40 (C3) 72.51 (C4)	5.00 (H1); 3.86 (H2); 3.89 (H3); 4.04 (H4)
	71.63 (C5)	4.20 (H5); 3.72 (H6a)
	69.59 (C6)	4.20 (H5); 3.72 (H6a); 3.98 (H6b)
→6)-3-*O*-Me-α-D-Gal*p*-(1→ (Residue F)	100.65 (C1)	4.99 (H1); 3.86 (H2); 3.56 (H3)
	70.13 (C2)	3.86 (H2); 3.56 (H3)
	81.80 (C3)	4.99 (H1); 3.56 (H3)
	68.12 (C4)	3.56 (H3); 4.29 (H4)
	71.56 (C5) 69.41 (C6)	4.24 (H5); 3.71 (H6a); 3.92 (H6b)

Table 3
^{1}H and ^{13}C NMR chemical shifts [expressed as δ (ppm)] of mannofucogalactan (EFP-Gf fraction) from *G. frondosa*.

Units		1	2	3	4	5	6		-*O*-CH_3
							6a	6b	
α-Man*p*-(1→ (Residue A)	^{13}C	105.01	72.90	73.28	69.73	76.19	63.97	–	–
	^{1}H	5.13	4.09	3.92	3.69	3.80	3.78	3.90	–
→3)-α-Fuc*p*-(1→ (Residue B)	^{13}C	104.10	70.38	80.35	74.27	69.95	18.42	–	–
	^{1}H	5.12	3.95	3.98	3.99	4.18	1.25	–	–
α-Fuc*p*-(1→ (Residue C)	^{13}C	104.23	71.12	72.31	74.62	69.95	18.42	–	–
	^{1}H	5.09	3.83	3.91	3.85	4.18	1.24	–	–
→2,6)-α-Gal*p*-(1→ (Residue D)	^{13}C	100.78	80.50	71.27	72.35	71.98	70.00	–	–
	^{1}H	5.05	3.87	4.08	4.06	4.14	3.71	4.00	–
→6)-α-Gal*p*-(1→ (Residue E)	^{13}C	100.95	71.12	72.40	72.51	71.63	69.59	–	–
	^{1}H	5.00	3.86	3.89	4.04	4.20	3.72	3.98	–
→6)-3-*O*-Me-α-Gal*p*-(1→ (Residue F)	^{13}C	100.65	70.13	81.80	68.12	71.56	69.41	–	59.01
	^{1}H	4.99	3.86	3.56	4.29	4.24	3.71	3.92	3.46

[(a)]Assignments are based on ^{1}H, ^{13}C, HSQC-DEPT, HSQC-TOCSY, and COSY examination. [(b)] The values of chemical shifts were recorded with reference to TMS as internal standard.

branched mannofucogalactan containing a (1 → 6)-linked main chain, composed of 3-*O*-Me-α-D-galactopyranosyl (I), and α-D-galactopyranosyl units (II), partially substituted at O-2 by 3-*O*-α-D-mannopyranosyl-α-L-fucopyranosyl groups (III) and in a minor proportion with α-L-Fuc*p* single-unit side chains (IV). However, the presence of few percentage of α-D-Man*p* non-reducing end units were not completely excluded due to the possibility of signals overlapping on NMR analyzes.



There have been several other reports dealing with the isolation and characterization of heterogalactans of basidiomycetes. Most of these compounds have a common structure consisting of a backbone of (1 → 6)-linked, α-D-Gal*p* residues, and may present variations in the side chains, being named fucogalactans, mannogalactans, mannofucogalactans or fucomannogalactans. Such structures are mainly substituted at O-2 only by α-L-Fuc*p* or by α-L-Fuc*p* in addition to α- or β-Man*p*, β-Gal*p* single units, or 3-*O*-α/β-D-mannopyranosyl-α-L-fucopyranosyl side chains.

Polysaccharides resembling the heterogalactan found at fraction EFP-*Gf* have been previously described for *G. frondosa* (Wang et al., 2014), *Laetiporus sulphureus* (Alquini et al., 2004), *Fomitella fraxinea*

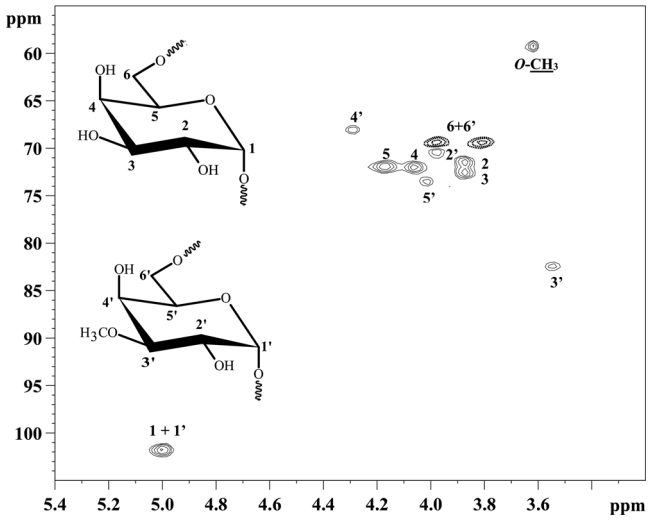


Fig. 5. HSQC-DEPT spectrum of partially degraded mannofucogalactan, in Me_2SO-d_6 at 50 °C, chemical shifts are expressed in ppm.

(Cho, Koshino, Yu, & Yoo, 1998; Cho, Yun, Yoo, & Koshino, 2011), *Flammulina velutipes* (Mukumoto & Yamaguchi, 1977; Smiderle, Carbonero, Sassaki, Gorin, & Iacomini, 2008), *Polyporus pinicola* (Fraser, Karacsonyi, & Lindberg, 1967), *Polyporus fomentarius* (Björnal & Lindberg, 1969), *Polyporus giganteus* (Bhavanandan, Bouveng, & Lindberg, 1964), *Polyporus squamosus* (Bjorndal & Wagstrom, 1969). However, none of these heterogalactans have 3-*O*-Me-Gal*p* in their structures, different from what was observed in the present study. The presence of 3-*O*-Me-Gal*p* units have only been described in fucogalactans, such as those from *Agaricus bisporus* var. *hortensis* (Komura et al., 2010) and *Agaricus bisporus* (Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2012; Ruthes et al., 2013), and in mannogalactans, all from *Pleurotus* species: *P. pulmonarius* (Smiderle, Olsen et al., 2008), *P. ostreatus* (Jakovlević, Miljković-Stojanović, Radulović, & Hranisavljević-Jakovlević, 1998), *P. ostreatoroseus* and *P. ostreatus* var. *florida* (Rosado et al., 2003), and *P. geesteranus* (Zhang, Xu, Fu, & Sun, 2013).

In addition to presenting well-known chemical structures, heterogalactans are also recognized for their relevant biological activities, whether antitumor (Cho et al., 1998), immunomodulatory (Fan et al., 2006), or concerned to anti-inflammatory and antinociceptive effects (Carbonero, Gracher, Komura et al., 2008; Fan et al., 2006; Komura et al., 2010; Ruthes et al., 2012, 2013). Thus, the mannofucogalactan (EFP-*Gf*) obtained from *G. frondosa* could present itself as a good candidate to be evaluated for its biological potential, taking into account the results obtained for other heterogalactans.

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