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Structural elucidation of a novel mannogalactan isolated from the fruiting bodies of *Pleurotus geesteranus*

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ABSTRACT

A novel water-soluble polysaccharide named PGPB-1, with a molecular weight of 1.3×10^4 Da as determined by high-performance liquid chromatography (HPLC), was isolated from the fruiting bodies of *Pleurotus geesteranus* by boiling water as well as DEAE-Sepharose Fast Flow and High-Resolution Sephacryl S-300. On the bases of compositional analysis, methylation analysis and NMR spectroscopy (^{1}H NMR, ^{13}C NMR, 2D-COSY, TOCSY, HMQC, HMBC and NOESY spectra) analysis, the structural elucidation of PGPB-1 consisted of a α-D-(1 → 6)-galactopyranan and a α-D-3-*O*-Me-D-galactosyl unit backbone with a α-D-Mannosyl unit on *O*-2 of the 2,6-di-*O*-substituted-D-galactosyl units. The repeating unit of the polysaccharide was established as:

β-D-Man*p*
↓
2
→6)-α-D-Gal*p*-(1→6) - α-3-*O*-Me-D-Gal*p*-(1→

PGPB-1 also contains a minor proportion of 6-linked-D-Glc*p*.

1. Introduction

Polysaccharides from plant, animal and microbe extracts, together with proteins and polynucleotides, are essential biomacromoleules in the life activities and play important roles in cell–cell communication, cell adhesion and molecular recognition in the immune system (Dwek, 1996; F-Tischer, Talarico, Guimarães, Noseda, & Damonte, 2006). *Pleurotus* (P.) family, such as *P. sajor-caju*, *P. ostreatus*, *P. citrinopileatus* and *P. florida*, is one of the most widely cultivated and consumed edible mushroom genus in the world (Roy, Maiti, Mondal, Das, & Islam, 2008). Among different types of mushrooms of the genus *Pleurotus*, a β-glucan from the fruiting bodies of *P. eryngii* and *P. ostreatoroseus* is reported as a commonly available antitumor materials (Carbonero et al., 2006). A water soluble polysaccharide-protein complex extracted from *P. ostreatus* was found to inhibit Sarcoma S-180 tumor growth both in vivo and in vitro (Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006).

Although β-glucans are the most studied and characterized fungal polysaccharides, another class of polysaccharides having the same activities has stimulated interest (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007). It includes mannogalactans (Jakovljevic, Miljkovic-Stojanovic, Radulovic, & Hranisavljevic -Jakovljevic, 1998), xylomannans (Smiderle et al., 2006), fucogluco-galactans (Zhang et al., 2007) and fucomannogalactans. The partially 3-O-methylated mannogalactans have been isolated from the fruiting bodies of edible basidiomycetes *Pleurotus ostreatus* 'florida' Berk., *Pleurotus ostreatoroseus* Sing (Rosado et al., 2003) and *Pleurotus pulmonarius* (Smiderle et al., 2008).

P. geesteranus (PG) is a species that is getting the popularity lately because of its very pleasant flavor, richness of dietary fiber and high quality of proteins. It is also considered to have an immense potential as a valuable medicinal compounds. A high M_W fraction PG-2, extracted from *P. geesteranus*, is mainly composed of glucose appearing effective anti-tumor activity (Zhang et al., 2011). However, no detailed structural properties of purified polysaccharide from *P. geesteranus* have been investigated. Here, we report the detailed structural studies of a new polysaccharide fraction (PGPB-1) from the fruiting bodies of *P. geesteranus.*

2. Experimental

2.1. Materials

The fruiting bodies of *P. geesteranus* were obtained from Jiangshan in Zhejiang Province, China and identified by Professor Cai Weiming, Zhejiang Academy of Agricultural sciences. DEAE-Sepharose Fast Flow and High-Resolution Sephacryl S-300 were purchased from GE Healthcare. Dextrans, trifluoroacetic acids (TFA)

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and standard monosaccharide (D-galactose, D-arabinose, L-fucose, L-rhamnose, D-manonose, D-xylose and D-glucose) were purchased from Sigma–Aldrich Company. All other reagents were of A.R. grade. HPLC was carried out on a waters 1525 HPLC system (1525 HPLC pump, 2414 refractive index detector). GC was carried out using an Agilent 7890N instrument. GC–MS was carried out with a ThermoFinnigan TRACE MS and NMR spectra were determined by a Varian INOVA 500.

2.2. Isolation and purification

The dried fruiting bodies of *P. geesteranus* (0.5 kg) were chopped into slices and exhaustively reflux extracted with 95% ethanol for 12 h to remove lipids. The residue was air dried and extracted twice with 10 volumes of distilled water for 2 h at 100 °C. The combined aqueous solution was applied to an ultrafiltration membrane to separate it into four fractions according to different molecular weights, and were designated as fractions A, B, C and D. Fraction B was designated as PGPB, and was concentrated under vacuum under 40 °C using a rotary evaporator and freeze-dried. The crude extract from PGPB was dissolved in distilled water, applied to a DEAE-Sepharose Fast Flow column (XK 26 mm × 100 cm) and eluted by filtered (0.45 μm membrane) distilled water and then by 0–2 mol/L NaCl gradient solution. The fractions were collected by a fraction collector and detected by means of the phenol–sulfuric acid assay (Dubois et al., 1956) and a fraction eluted by distilled water forming a single peak (PGPBW) was collected. Fraction PGPBW was purified by gel-permeation chromatography on a high-resolution Sephacryl S-300 column (XK 26 mm × 100 cm) using filtered distilled water as eluate. Two absorption peaks were detected using a refractive index detector, and fraction forming the first peak was collected (PGPB-1) and verified as a homogenous polysaccharide by high performance liquid chromatography (HPLC).

2.3. Determination of purity and molecular weight

The homogeneity and molecular weight of the purified PGPB-1 was estimated by HPLC on an Agilent 1525 system equipped with a TSK-gel PWXL G4000 column and a refractive index detector (RID). The column and RI detector temperature were kept at 35 °C. 15 μL of sample solution (2.0 mg/mL) was applied to the machine in each run, with distilled water as the mobile phase at a flow rate of 1.0 mL/min. The molecular mass was estimated by the standard curve which was calibrated with T-series Dextrans standards (Mw 1000, 5000, 12,000, 80 k, 150 k, 270 k and 670 kDa).

2.4. Spectroscopic methods

The ultraviolet spectrum of PGPB-1 was recorded by UV-2450 UV-visible spectrophotometer. The infrared analysis was obtained by grinding a mixture of polysaccharide with dry KBr, and then pressing in a mold. The spectra were recorded on Nexus Euro FT-IR in the 4000–400 cm^{-1} region (Kumar, Joo, Choi, Koo, & Chang, 2004).

2.5. Sugar composition

The identification and quantification of the monosaccharide of PGPB-1 was achieved by GC analysis. The polysaccharide PGPB-1 (2 mg) was hydrolyzed in 2 mol/L trifluoroacetic acid (TFA, 4 mL) at 110 °C for 2 h. The released sugars were reduced by $NaBH_4$ (20 mg), followed by acidification with acetic acid. The solvent was then co-distilled with MeOH to remove excess boric acid and acetylated with acetic anhydride (Gerwig, Kamerling, & Vliegenthart, 1979). The results of alditol acetates were analyzed by gas chromatography (GC) using an Agilent 7890N instrument equipped with an HP-5 capillary column (30 m × 0.32 mm × 0.25 μm) and a flame-ionization detector (FID) at the temperature program as follows: the oven temperature was initially set at 120 °C, increasing to 240 °C at a rate of 10 °C/min and then held at 240 °C for 6.5 min. The heater temperatures of the injector and detector were both at 250 °C. Nitrogen was used as the carrier gas.

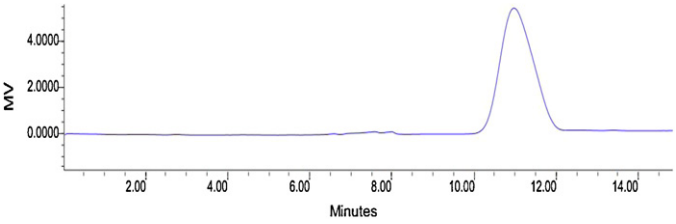

Fig. 1. The elution of the water-soluble PGPB-1 extracted from the fruiting bodies of *P. Geesteranus*.

2.6. Methylation analysis

PGPB-1 (2 mg) was dissolved in DMSO (2 mL) and methylated by treatment with NaOH/DMSO (0.2 mL) suspension and iodomethane (0.2 mL) by the modified method (Kalyan & Paul, 1992). The reaction mixture was extracted with $CHCl_3$ and the solvent was then removed by evaporation. Complete methylation was confirmed by the disappearance of the —OH band in the 3700–3200 cm^{-1} region of the IR spectrum. Hydrolysis of the methylated polysaccharide was then performed with 2 mol/L TFA at 100 °C for 6 h, and the partially methylated monosaccharides were reduced with $NaBH_4$ and acetylated with Ac_2O. The partially methylated alditol acetates were analyzed by GC–MS under the same chromatographic conditions as above.

2.7. Nuclear magnetic resonance (NMR) experiment

PGPB-1 (30 mg) was dried in a vacuum over P_2O_5 for 72 h, then deuterium exchanged three times by freeze drying from D_2O (Dueñas-Chasco et al., 1997). ^1H NMR (500 MHz, 25 °C, 60 °C) and ^{13}C NMR (60 °C) spectra were determined in 5-mm tubes using a Varian INOVA 500 NMR spectrometer. ^1H Chemical shifts were referenced to residual HDO at δ 4.78 ppm (25 °C) as the internal standard and DSS (δ_C 0.00 ppm) as external standard. COSY, TOCSY and HMQC spectra were used to assign the signals of chemical shifts. HMBC and NOESY spectra were used to assign inter-residue linkage sequence.

3. Results and discussion

The polysaccharide fraction PGPB-1, was purified by DEAE Sepharose anion-exchange chromatography and Sephacryl S-300 high resolution chromatography, gave a single symmetrical, narrow peak in HPLC (Fig. 1). Correlation with the calibration curve of Dextran standards indicated that its molecular weight was about 1.3×10^4 Da. Lack of absorption at 280 nm by UV scanning indicated that PGPB-1 contained no protein. No absorption peaks at 1730 cm^{-1} in the FT-IR spectrum indicated that there were no uronic acids.

Sugar analysis and GC–MS revealed that the monosaccharide composition consists of D-mannose, D-glucose and D-galactose, along with an amount of 3-*O*-methylgalactose, which did not match any of monosaccharide. The latter was confirmed by comparison of retention times and mass spectra of partially *O*-methylated derivatives. The mass spectrum of 3-*O*-methylgalocose is dominated by the cleavage of bonds between *O*-methylated carbons and adjacent *O*-acetylated carbons. For 3-*O*-methylgalactose alditol

Table 1
GC–MS data for methylation analysis of PGPB-1 isolated from the fruiting bodies of *P. geesteranus*.

Retent time	Methylated sugars	Substituted sugar unit	Molar ratios	Major mass fragments (*m*/*z*)
9.754	2,3,4,6-Me_4-Man*p*	Reducing end Man*p* unit	1.00	43,71,87,101,117,129,145,161,205
10.712	2,3,4-Me_3-Glc*p*	6-*O*-Substituted Glc*p* unit	0.25	43,71,87,101,117,129,147,161,175,189,233
11.321	2,3,4-Me_3-Gal*p*	6-*O*-Substituted 3-*O*-Met-Gal*p* unit	1.14	43,71,87,99,101,117,129,161,173,189,233
12.184	3,4-Me_2-Gal*p*	2,6-di-Substituted Gal*p* unit	0.94	43,71,87,99,129,159,173,189,233

acetate, this cleavage produced *m*/*z* 189 and 161 as the primary fragments, and *m*/*z* 87, 99, 129, 159, 201 and 231 as the other main fragments. This is in agreement with a previously published report (Stepan, Bleckmann, Geyer, Geyer, & Staudacher, 2010) and was confirmed by methylation and NMR analysis.

Methylation analysis (Table 1) of the polysaccharide revealed 2,3,4,6-tera-*O*-methylmannose, 2,3,4-tri-*O*-methylgalactose and 3,4-di-*O*-methylgalactose in a ratio of 1.00:1.14:0.94, respectively, together with a minor proportion of 2,3,4-tri-*O*-methylglucose. Therefore, the polysaccharide is branched with a terminal mannopyranose residue and 2,6-disubsitituted galactopyranose residue at the branching point. Another 3-*O*-methylgalactopyranose residue is substituted at 6-position, and a minor of 6-subsitituted glucopyranose.

The ^{1}H NMR spectrum (Fig. 2) of the polysaccharide mainly contained signals for three anomeric protons at δ 4.81–5.13, other sugar protons in the region of δ 3.39–4.30, and one *O*-CH_3 group signal at δ 3.46. The ^{13}C NMR spectrum (Fig. 3) of the polysaccharide contained mainly three anomeric carbons signals at δ 98.62–101.75, and sugar ring carbons linked oxygen in the region of δ 61.20–79.10. In addition, there was a signal present at δ 56.10, which could be assigned to an *O*-CH_3 group signal.

Residue **a** →2, 6)-α-D-Gal*p*. ^{1}H resonances for H-1 through H-4 of residue **a** were assigned from the cross-peaks in ^{1}H–^{1}H COSY and TOCSY spectra. The H-5 resonance was assigned from the

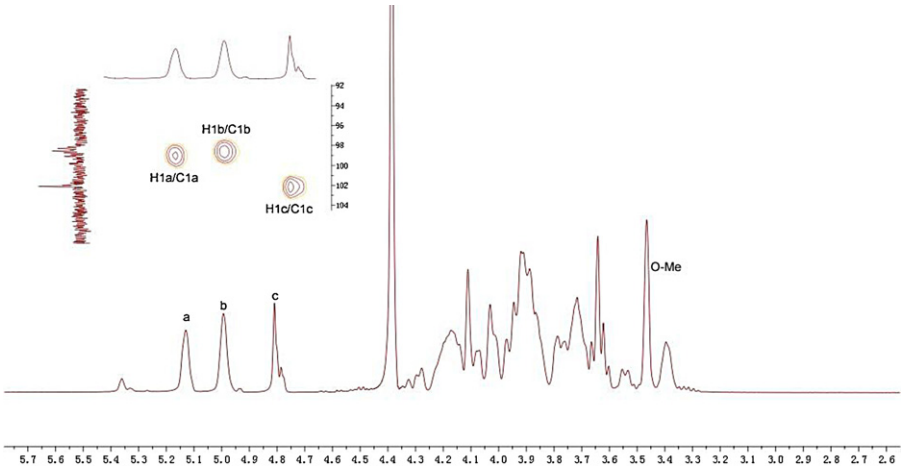

Fig. 2. 500 MHz ^{1}H NMR spectrum of PGPB-1 isolated from the fruiting bodies of *P. Geesteranus*.

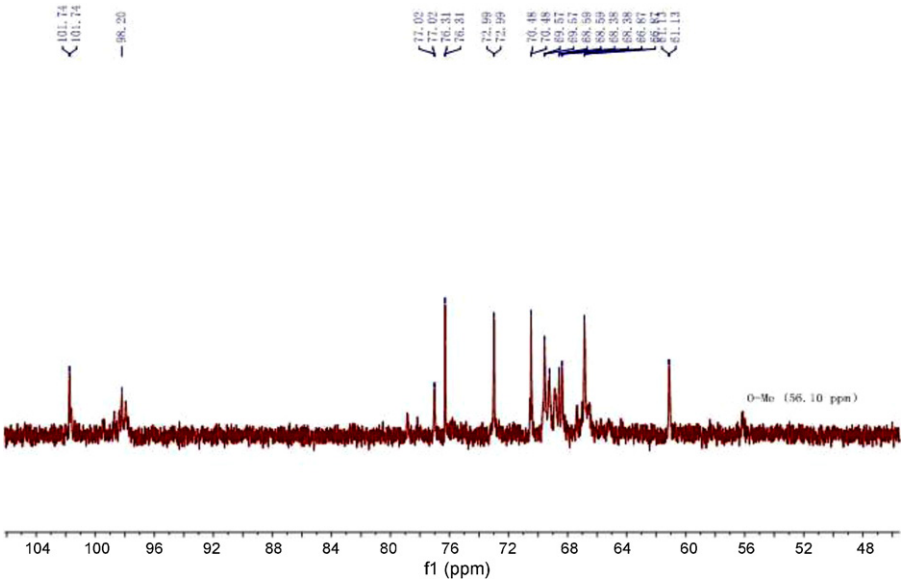

Fig. 3. ^{13}C NMR spectrum of PGPB-1 isolated from the fruiting bodies of *P. Geesteranus*.

Table 2
Chemical shifts data for PGPB-1 isolated from the fruiting bodies of *P. geesteranus*.

Residue		Proton or carbon							
		1	2	3	4	5	6a	6b	*O*-Me
→2,6)-α-D-Gal*p* (**a**)	H	5.13	**3.97**	3.68	4.30	4.02	**3.72**	**3.91**	
	C	98.62	**77.11**	70.30	65.52	68.72	**67.11**		
→6)-α-3-*O*-Me-D-Gal*p* (**b**)	H	4.99	3.86	**3.54**	4.27	3.88	**3.71**	**3.93**	3.46
	C	98.20	68.59	**79.10**	65.64	69.58	**66.85**		58.10
β-D-Man*p* (**c**)	H	4.81	4.12	3.65	3.62	3.39	3.78	3.92	
	C	101.75	70.42	73.15	67.12	76.36	61.20		

Bold numbers represent glycosylation sites.

H-3/H-4 and H-4/H-5 cross-peaks in the NOESY spectrum. The H-5 and the H-6a and H-6b resonances were then obtained from the TOCSY spectrum and validated by HMQC(Fig. 4) spectrum. The cross-peaks of H-2 and H-6a and H-6b in TOCSY spectrum showed H-6a and H-6b are located on residue **a**. ^{13}C resonances were assigned from the HMQC spectrum (Table 2). The H-4/H-5 coupling constant was small in the ^{1}H–^{1}H COSY spectrum (Staaf, Urbina, Weintraub, & Widmalml, 1999) and H-4 displays strong NOE signals H-3 and H-5 in the NOESY spectrum, which indicated that residue **a** is Gal-type residue. H-1 appears as a singlet ($J_{H\text{-}1,\ H\text{-}2} < 3$ Hz) in the ^{1}H NMR spectrum, and the H-1/H-2 intra-residue correlation in the NOESY spectrum indicate the residue **a** is an α-configuration. The downfield shifts of the C-2 and C-6 carbon signals with respect to the standard values for glycopyrannoses indicated that residue **a** was identified as →2, 6)-α-D-Gal*p*.

Residue **b** →6)-α-3-*O*-Me-D-Gal*p*. The ^{1}H resonances for H-1 to H-4 of residue **b** were assigned from the cross-peaks in the ^{1}H–^{1}H COSY and TOCSY spectra. The H-5 and the H-6a and H-6b resonances were then obtained from the TOCSY spectrum and validated by HMQC spectrum. The cross-peaks of H-1 and H-2, H-3, H-4 and H-5 in TOCSY spectrum showed H-5 and H-6 are located on residue **b**. The carbon chemical shifts from the C-1 to C-6 were assigned from the HMQC spectrum (Table 2). The chemical shifts of *O*-CH_3 (δ 3.46/56.10) were assigned from the HMQC spectrum. ^{1}H resonances for *O*-CH_3 correlated with C-3 (δ 3.46/79.10) of residue **b** in the HMBC spectrum showed that *O*-CH_3 is located on residue **b**. The H-4/H-5 coupling constant was small in the ^{1}H–^{1}H COSY spectrum and H-4 and H-3, H-5 displays strong NOE signals in the NOESY spectrum, as expected for a Gal-type residue. H-1 appears as a singlet ($J_{H\text{-}1,\ H\text{-}2} < 3$ Hz) in the ^{1}H NMR spectrum, and the H-1/H-2 intra-residue correlation in the NOESY spectrum indicate the residue **c** is an α-configuration. The downfield shift of C-6 carbon signal with respect to the standard values for glycopyrannoses indicated that residue **b** was identified as →6)-α-3-*O*-Me-D-Gal*p*.

Residue **c** β-D-Man*p*. The ^{1}H resonances for H-1 to H-4 of residue a were assigned from the cross-peaks in the ^{1}H–^{1}H COSY spectrum, and the resonance for H-5 was assigned according to the cross-peaks in the TOCSY and NOESY spectra. H-6a and H-6b resonances were assigned from TOCSY spectrum. The carbon signals from C-1 to C-6 were identified from HMQC spectrum (Table 2). The manno-configuration for residue a was supported from a relatively small coupling constant value of $J_{H\text{-}1,\ H\text{-}2}$ (~1.0 Hz) and a large coupling constant value of $J_{H\text{-}4,\ H\text{-}5}$ (~9.0 Hz). A small $J_{H\text{-}1,\ H\text{-}2}$ values for D-mannosyl residue did not give information about the anomeric configuration (Paramonov et al., 2001). The β configuration of residue **c** was inferred by the H-5 and C-5 chemical shifts at δ_H 3.39 and δ_C 76.36 (compare published data δ_H 3.82 and δ_C 73.34 for α-mannopyranose, δ_H 3.38 and δ_C 77.00 for β-mannopyranose (Jansson, Kenne, & Widmalm, 1989). The combination of these data identified residue **c** as β-D-Man*p*.

Comparison of the chemical shifts data for residues **a**–**c** with those reported for glycosides (Agrawal, 1992) permitted identification of residue **a** as 2,6-linked α-D-Gal*p*, residue **b** as 6-linked α-3-*O*-Me-D-Gal*p*, residue **c** as 1-linked α-D-Man*p* and a minor of 6-linked-D-Glc*p*.

The sequence of glycosyl residues was determined from NOESY spectrum studies followed by confirmation with HMBC experiments. Inter-residue NOEs correlations (Table 3) were observed between H-1 of residue **a** and H-6b of residue **b**, between H-1 of residue **b** and H-6a and H-6b of residue **b**, between H-1 of residue **c** and H-2 of residue **a**. HMBC experiments (Table 4) showed clear correlations between H-1 of residue **a** and C-6 of residue **b**, between H-1 of residue **b** and C-6 of residue **a** and **b**, between H-1 of residue **c** and C-2 of residue **a**.

The combined chemical and NMR data permit the structure of the trisaccharide repeating units of the PGPB-1 to be written as follows, and it also contains a minor of 6-linked-D-Glc*p*.

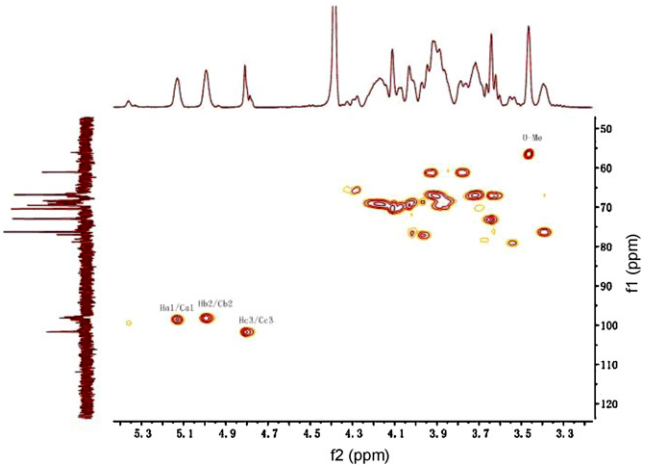

Fig. 4. 500 MHz ^{1}H–^{13}C HMQC spectrum of PGPB-1 polysaccharide isolated from the fruiting bodies of *P. geesteranus*. Each cross-peak corresponds to a C–H pair.

Table 3
Interglycosidic correlations from NOESY spectrum of PGPB-1 isolated from the fruiting bodies of *P. geesteranus*.

Residue	Proton	Intra-correlation[a]
→2,6)-α-D-Gal*p* (a)	H-1	3.97 (**a**; H-2), 3.68 (**a**; H-3), 4.30 (**a**; H-4), **3.93** (**b; H-6b**)
	H-4	3.68 (**a**; H-3), 4.02 (**a**; H-5)
→6)-α-3-*O*-Me-D-Gal*p* (b)	H-1	3.86 (**b**; H-2), 3.54 (**b**; H-3), 4.27 (**b**; H-4), 3.88 (**b**; H-5), **3.71** (**b; H-6a**), **3.93** (**b; H-6b**)
	H-4	3.54 (**b**; H-3), 3.88 (**b**; H-5)
(-D-Man*p* (c)	H-1	4.12 (**c**; H-2), 3.97 (**a; H-2**), 3.65 (**c**; H-3), 3.39 (**c**; H-5)

[a] Inter-residue NOEs are showed in bold font.

Table 4
Two-and three-bond ^{1}H–^{13}C correlations for the PGPB-1 in the HMBC spectrum isolated from the fruiting bodies of *P. geesteranus*.

Residue	Proton	Proton correlation[a]
→2,6)-α-D-Gal*p* (**a**)	H-1	77.11 (**a**; C-2), 68.72 (**a**; C-5), **66.85** (**b; C-6**)
→6)-α-3-*O*-Me-D-Gal*p* (**b**)	H-1	79.10 (b; C-3), **66.85** (**b; C-6**), **67.11** (**a; C-6**)
	H (*O*-Me)	**79.10** (**b; C-3**)
(-D-Man*p* (**c**)	H-1	70.42 (**c**; C-2), **77.11** (**a; C-2**)

[a] Inter-residue correlations are shown in bold font.

4. Conclusion

The polysaccharide fractions of *Pleurotus* have been widely investigated and reported, but mainly focused on *P. sajor-caju*, *P. ostreatus*, *P. eryngii* and *P florida*. For the first time, the novel water-soluble polysaccharide PGPB-1, isolated from the fruiting bodies of *P. geesteranus* with boiling water extraction and purified by DEAE Sepharose Fast Flow column and High-Resolution Sephacryl S-300, was identified. The structural elucidation of PGPB-1 identified by compositional analysis, methylation analysis, together with NMR analysis consists of a α-D-(1 → 6)-galactopyranan and a α-D-3-*O*-Me-D-galactosyl unit backbone with a α-D-Mannosyl unit on *O*-2 of the 2,6-di-*O*-substituted-D-galactosyl units and it also contained a minor of 6-linked-D-Glc*p*. The results also confirmed the conclusion that the presence of partially 3-O-methylated mannogalactan appears to be typical of *Pleurotus* spp.

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