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Structural elucidation of a 3-*O*-methyl-D-galactose-containing neutral polysaccharide from the fruiting bodies of *Phellinus igniarius*

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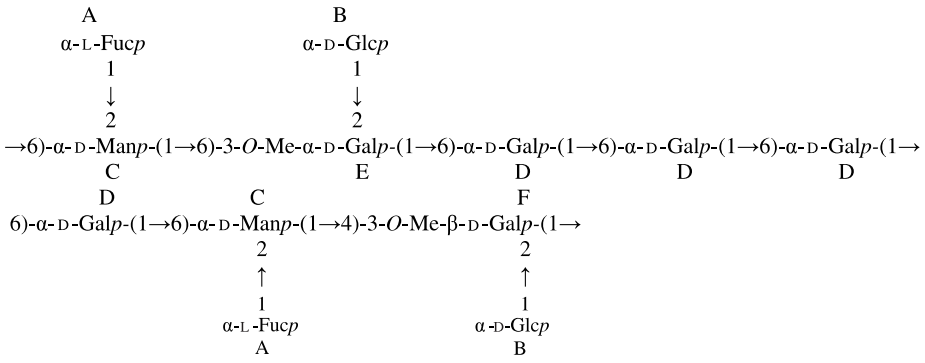
Abstract—PIP60-1, a novel heteropolysaccharide isolated from fruiting bodies of the medicinal fungus, *Phellinus igniarius*, has a molecular weight of 1.71×10^4 Da and is composed of L-fucose, D-glucose, D-mannose, D-galactose and 3-*O*-Me-D-galactose in a ratio of 1:1:1:2:1. A structural investigation of PIP60-1 carried out using sugar and methylation analyses, combined with ^{1}H and ^{13}C NMR spectroscopy, including COSY, TOCSY, NOESY, HSQC and HMBC experiments, established the repeating unit of the polysaccharide as the following:



Keywords: *Phellinus igniarius*; Polysaccharide; Structural analysis; NMR spectroscopy

1. Introduction

Phellinus igniarius (L.: Fr) Quel, is a medicinal basidiomycetous fungus belonging to the Hymenochaetaceae. The mushroom is mainly found in China, and has been well-known for many centuries in traditional Chinese medicine as 'Sanghuang (yellow polyporus)'.[1] *Phellinus linteus*, *Phellinus gilvus*, and *Phellinus rimosus*, species of *Phellinus* that are found predominantly in South Korea, have also been examined for pharmacological activity.[2–5] *P. linteus* has been used for the treatment of various diseases in northeast Asia, including gastroenteric disorders, lymphatic diseases and various cancers. Polysaccharides extracted from *P. linteus* are reported to stimulate cell-mediated and humoral immunity, and to inhibit tumor growth and metastasis.[6]

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Antitumor activity has also been attributed to polysaccharides of *P. igniarius* and *Phellinus robustus*.[7]

Relatively little is known about the composition and structure of bioactive agents from *Phellinus* species although an acidic proteoheteroglycan from *P. linteus* containing mannose, galactose, glucose, arabinose, and xylose, and shown by ^{13}C and ^{1}H NMR spectroscopy to be a novel biomolecule combining α and β linkages, has been described.[8] In this paper, we report the chemical and structural elucidation of PIP60-1, a novel neutral polysaccharide isolated from fruiting bodies of *P. igniarius*.

2. Results and discussion

PIP60-1, a purified neutral polysaccharide of *P. igniarius* under the conditions described in the experimental (Fig. 1), appeared as a single symmetrical peak on HPLC and had an estimated molecular weight of

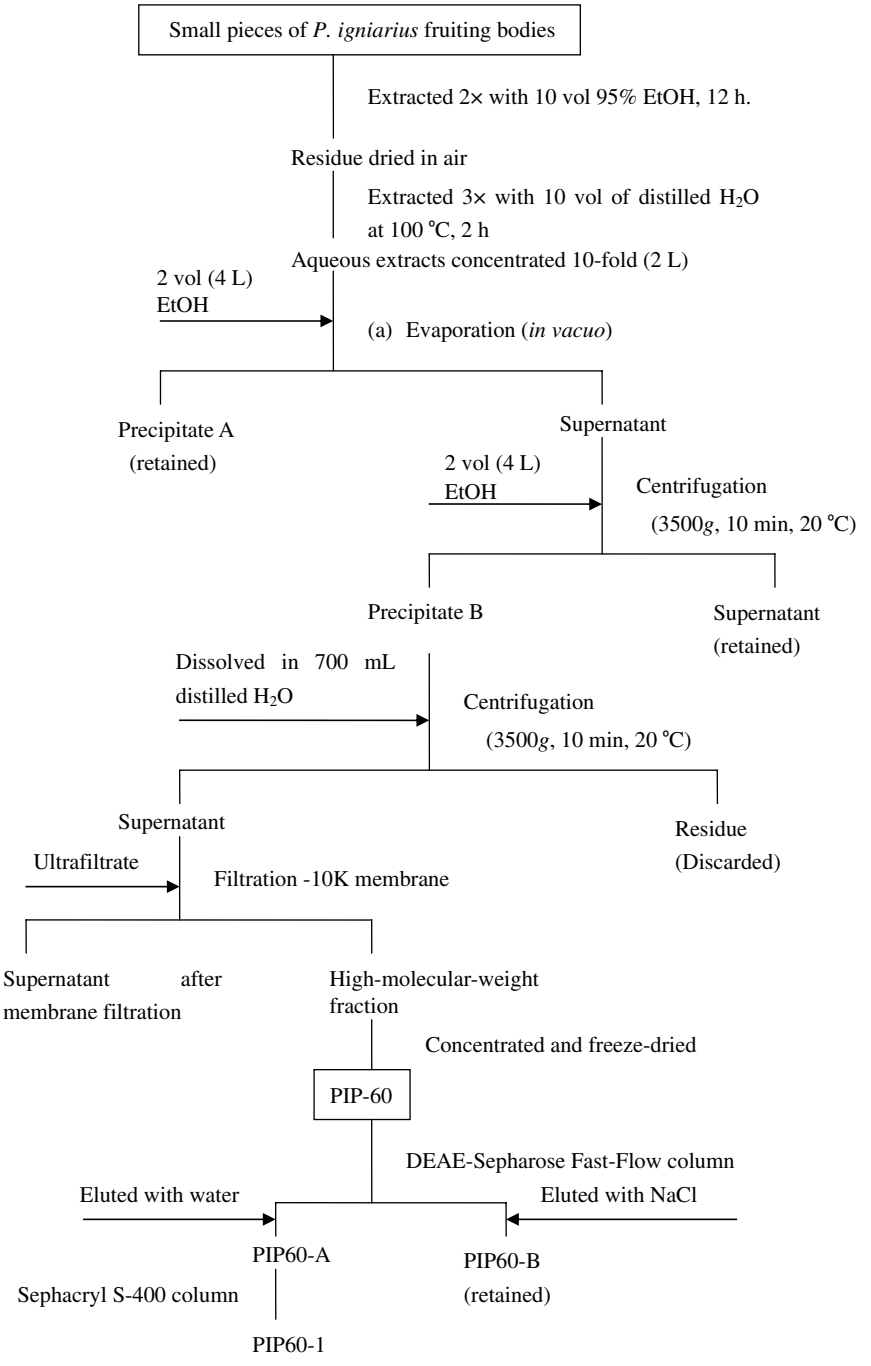


Figure 1. Scheme for isolation and purification of PIP60-1 fraction.

1.71×10^4 Da and a total carbohydrate content of 99.47% as determined by the phenol–sulfuric acid method.[9] TFA hydrolysis combined with high-performance anion-exchange chromatography (HPAEC)[10] revealed the presence of L-fucose, D-galactose, D-glucose, and D-mannose. In addition, another peak was observed eluting after L-fucose that did not match any of our monosaccharide standards. 3-(or 4)-*O*-Methylgalactose was shown to be present in PIP60-1 hydrolysates by gas chromatography–mass spectrometry (GC–MS) of the alditol acetates. Fragmentation of 3-(or 4)-*O*-methylgalactose alditol acetate reduced by $NaBH_4$ generated *m*/*z* 261 and 189 as the primary fragments, and *m*/*z* 85, 87, 99, 127, 129, 159 and 201 as the other main fragments.[11–13] 3-*O*-Methylgalactose was further confirmed by GC–MS of the alditol acetates in PIP60-1 hydrolysates reduced by $NaBD_4$ (*m*/*z* values 43, 85, 88, 99, 127, 130, 159, 190, 201, 261). The primary fragments, with *m*/*z* values of 261 and 190, indicated 3-*O*-methylgalactose and not 4-*O*-methylgalactose. Absolute configuration analysis showed that fucose had the L configuration and galactose, mannose, and glucose all had the D configuration.[14] Methylation analysis detected 2,3,4-tri-*O*-methylfucose, 2,3,4-tri-*O*-methylgalactose, 3,4-di-*O*-methylgalactose, 3,6-di-*O*-methylgalactose, 3,4-di-*O*-methylmannose, and 2,3,4,6-tetra-*O*-methylglucose in a ratio of 1.12:2.26:0.57:0.54:1.03:1.00, respectively.

IR spectroscopic analysis revealed signals at 1143, 1078, 1043, 917, 880, 813, and 778 cm^{-1} indicating that the sugar residues of PIP60-1 are in the pyranose form. The ^1H NMR (500 MHz) spectrum (Fig. 2) contained signals for six anomeric protons at δ 5.14, 5.11, 5.05, 5.00, 4.99, and 4.56 ppm in a ratio of 1.0:1.0:1.0:2.8 (2H):0.55. One CH_3–C group at δ 1.27 corresponded to the chemical shift of H-6 of Fuc,[15] with other sugar protons appearing in the δ 3.50–4.26 region along with a signal for an *O*-methyl group at δ 3.48. Sugar residues are designated A–F according to the decreasing chemical shifts of the anomeric configuration. The ^{13}C NMR spectrum (Fig. 3) contained signals for six anomeric carbons at δ 101.12–105.76 and one CH_3–C group (C-6 of Fuc) at δ 18.76. In addition, a strong signal at δ 59.49 was assigned to an *O*-methyl group which, based on GC–MS data, is probably due to 3-*O*-methyl-D-galactose.[16]

Residue A was designated as α-L-fucopyranose. The ^1H resonances from the H-1 signal at δ 5.14 to H-2 at δ 3.92 and H-3 at δ 4.02 of this residue were assigned from ^1H–^1H COSY cross-peaks, and the assignment of H-4 is based on the TOCSY spectrum. Both H-3 and H-4 correlated with a signal at δ 4.22, and H-4 gave a NOE signal at δ 1.23. The cross-peaks of the proton with the signal at δ 1.23 and H-4 in the NOESY spectrum, the same proton and C-4 in the HMBC spectrum, and the correlation between the signals at δ 4.22 and δ 1.23 in both the COSY and TOCSY spectra, unambiguously show that δ 4.22 and δ 1.23 (H-6 of Fuc) are located on residue A. On the basis of the proton assignments, the chemical shifts of C-1–C-6 were readily obtained from the ^1H–^{13}C HMQC spectrum (Figs. 4 and 5, Table 1). Both carbon and proton chemical shifts are typical of 6-deoxyhexopyranose, and residue A can only be fucose since this sugar was the only deoxyglycose identified by GC–MS analysis. The appearance of H-1 as a singlet ($J_{H\text{-}1,H\text{-}2} < 3$ Hz) in the ^1H NMR spectrum, and H-1/H-2 intra-residue correlations in the NOESY spectrum, provide further support for this designation.

Residue B has an anomeric signal at δ 5.11, a $J_{H\text{-}1,H\text{-}2}$-value of <3 Hz, and H1/H2 intra-residue correlations in the NOESY spectrum, indicating an α-linked residue.

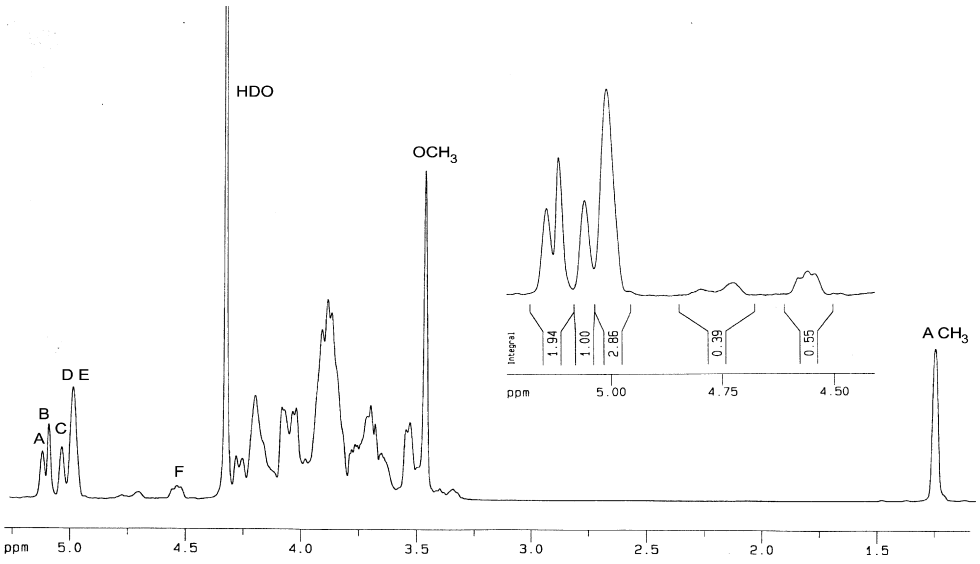


Figure 2. ^1H NMR spectrum of polysaccharide PIP60-1 of *P. igniarius*. Spectrum was determined in D_2O at 60 °C. The anomeric protons are labeled A–F.

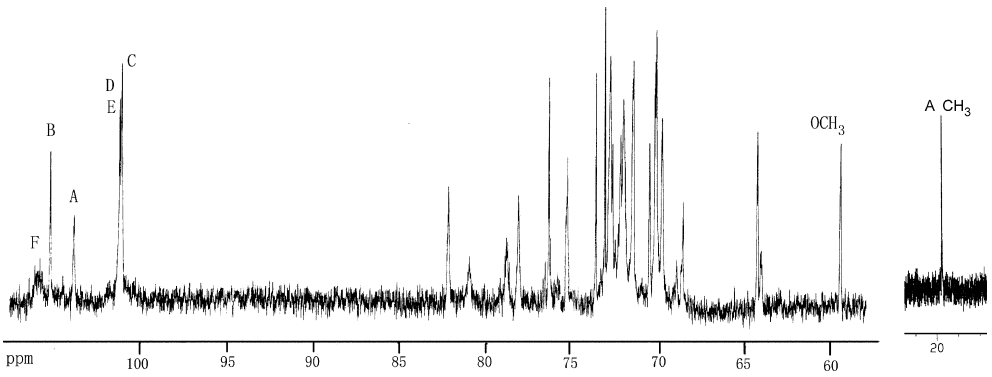


Figure 3. ^{13}C NMR spectrum of *P. igniarius* polysaccharide PIP60-1. Spectrum was determined in D_2O at 25 °C.

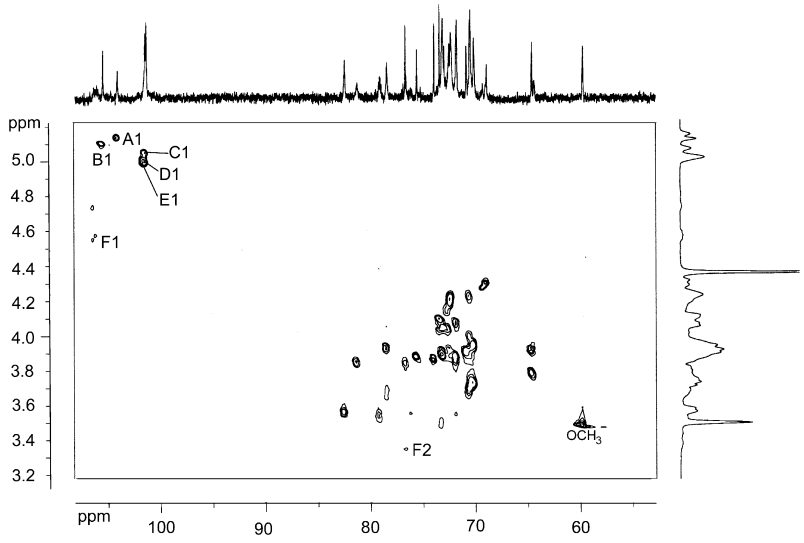


Figure 4. ^{1}H, ^{13}C HMQC spectrum of *P. igniarius* polysaccharide PIP60-1 showing anomeric atom cross-peaks. Determined in D_2O at 60 °C (65–110 ppm).

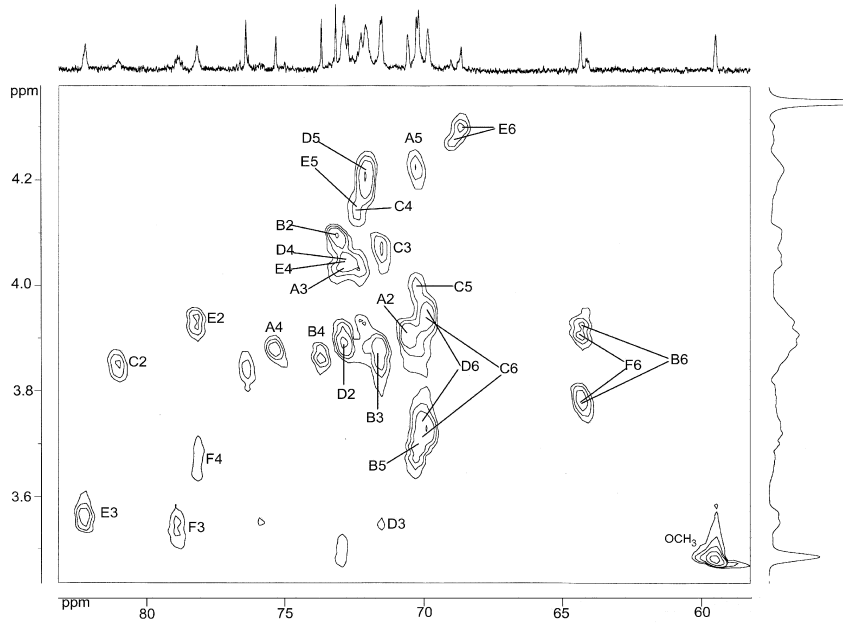


Figure 5. ^{1}H, ^{13}C HMQC spectrum of *P. igniarius* polysaccharide PIP60-1. Determined in D_2O at 60 °C (60–80 ppm). Each cross-peak corresponds to a C–H pair.

Table 1. ^{1}H and ^{13}C NMR chemical shift data (δ, ppm) for *P. igniarius* polysaccharide PIP60-1

Residue	$^{1}H/^{13}C$						
	1	2	3	4	5	6	3-*O*-Me
α-L-Fuc*p*-(1→ (A)	5.14	3.92	4.02	3.87	4.22	1.23	
	103.86	70.59	72.57	75.71	70.29	18.76	
α-D-Glc*p*-(1→ (B)	5.11	4.09	3.88	3.86	3.69	3.78, 3.94	
	105.27	73.25	72.91	73.69	70.20	64.32	
→2,6)-α-D-Man*p*-(1→ (C)	5.05	3.84	4.07	4.13	4.00	3.72, 3.90	
	101.12	81.03	71.52	72.31	70.28	69.52	
→6)-α-D-Gal*p*-(1→ (D)	5.00	3.89	3.55	4.04	4.20	3.76, 3.95	
	101.22	72.86	71.57	72.73	72.26	69.51	
→2,6)-3-*O*-Me-α-D-Gal*p* (E)	4.99	3.93	3.56	4.03	4.14	4.26, 4.28	3.48
	101.22	78.41	82.22	72.64	72.38	68.95	59.48
→2,4)-3-*O*-Me-β-D-Gal*p* (F)	4.56	3.36	3.52	3.63	3.98	3.91, 3.78	3.48
	105.76	76.47	78.89	78.38	70.21	64.16	59.48

This was confirmed by the relatively large $J_{C\text{-}1,H\text{-}1}$ value (171 Hz)[17] from the correlated C-1 signal at 105.27. ^{1}H resonances for H-1–H-4 were assigned from the ^{1}H–^{1}H COSY and TOCSY spectra. The cross-peak between H-2 and H-5 at δ 4.09/3.86 in the NOESY spectrum is evidence for locating H-5 on residue B. Correlation between H-5 and C-6 in the HMBC spectrum demonstrated that H-6a/6b at δ 3.78 and δ 3.94 are also located on residue B. Corresponding ^{13}C resonances were assigned from the HMQC spectrum (Figs. 4 and 5, Table 1). The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constants (9–10 Hz), the typical H-1, H-2, and H-4 intra-correlations in the NOESY spectrum (Table 2), and the absence of other inter-correlated protons apart from H-1 in the NOESY and HMBC spectra, indicated that residue B was a nonreducing-end D-glucopyranosyl residue.[18,19]

Residue C was designated as a (1→2,6)-linked α-D-mannopyranose. The ^{1}H resonances for H-1, H-2, H-3, and H-4 were assigned from the cross-peaks in the 2D-COSY and TOCSY spectra, and the H-5 resonance was assigned from NOESY and HMBC spectra. H-6a and H-6b resonances were then obtained from TOCSY (Table 1). ^{13}C Resonances were assigned from the HMQC spectrum (Figs. 4 and 5). Residue C had an anomeric signal at δ 5.03, $J_{C\text{-}1,H\text{-}1} \sim 170$ Hz and relative small H-1/H-2 and H-2/H-3 coupling constants and large H-3/H-4 (~7.5 Hz) and H-4/H-5 (~10 Hz) coupling constants, indicating that residue C was α-D-mannose.[18,20,21] The anomeric carbon signal at 101.12 ppm was confirmed by the presence of a cross-peak between C-1 of residue C and H-4 of residue F in the HMBC spectrum (Fig. 6). The downfield shifts of the C-2 (δ 81.03) and C-6 (δ 69.52) carbon signals with respect to standard values for methyl glycosides[25] indicate that residue C is a (1→2,6)-linked α-D-mannopyranose.

Residue D has an anomeric signal at δ 5.00, and H-2, H-3, and H-4 protons were observed in the TOSCY spectrum. Cross-peaks between H-1 and H-2, and between H-2 and H-3, were seen in the COSY spectrum, but there was no H-4 and H-5 correlation. The NOESY spectrum showed an H-4 and H-5 intra-residue cross-peak at δ 4.04/4.20. H-6a and H-6b were assigned from the TOSCY spectrum (Table 1). ^{13}C Resonances were assigned from the HMQC (Figs. 4 and 5) and ^{13}C NMR DEPT-135 spectra. The singlet of H-1 and the characteristic $J_{H\text{-}1,H\text{-}2}$ coupling constant value of <3 Hz, the H-1/H-2 intra-residue correlation in the NOESY spectrum and the cross-peaks between H-1 and C-3 and between H-1 and C-5 in the HMBC spectrum, and the lower-field location of the H-4 resonance showed the residue D was α-D-Gal*p*.[20–23] The cross-peak between H-1 of residue D and H-6 of residue C in the NOESY spectrum (Table 2), and between H-1 of residue D and C-6 of residue C in the HMBC spectrum (Fig. 6), and the downfield shift of C-6 (δ 69.51) carbon signal, indicated that residue D is a (1→6)-linked α-D-galactopyranose.

^{1}H resonances for H-1, H-2, H-3, and H-4 in residue E were assigned from the cross-peaks in the 2D-COSY and TOCSY spectra. H-3 of residue E displays strong NOE signals to both H-4 and H-5 at δ 3.56/4.24 and δ

Table 2. NOE effects of PIP60-1 observed in the NOESY spectrum

Residue	NOE signals		
	From	Intra-correlation	Inter-correlation
α-L-Fuc*p*-(1→ (A)	H-1	A H-2, A H-3	C H-2
α-D-Glc*p*-(1→ (B)	H-1	B H-2, B H-4	F H-2, E H-2
→2,6)-α-D-Man*p*-(1→ (C)	H-1	C H-2, C H-3, C H-5	F H-4, E H-6a/b
→6)-α-D-Gal*p*-(1→ (D)	H-1	D H-2, H-5, H-6a/b	C H-6a/b
→2,6)-3-*O*-Me-α-D-Gal*p*-(1→ (E)	H-1	E H-2, E H-3,	D H-6a/b
→2,4)-3-*O*-Me-β-D-Gal*p*-(1→ (F)	H-1	F H-3, F H-6	C H-6a/b

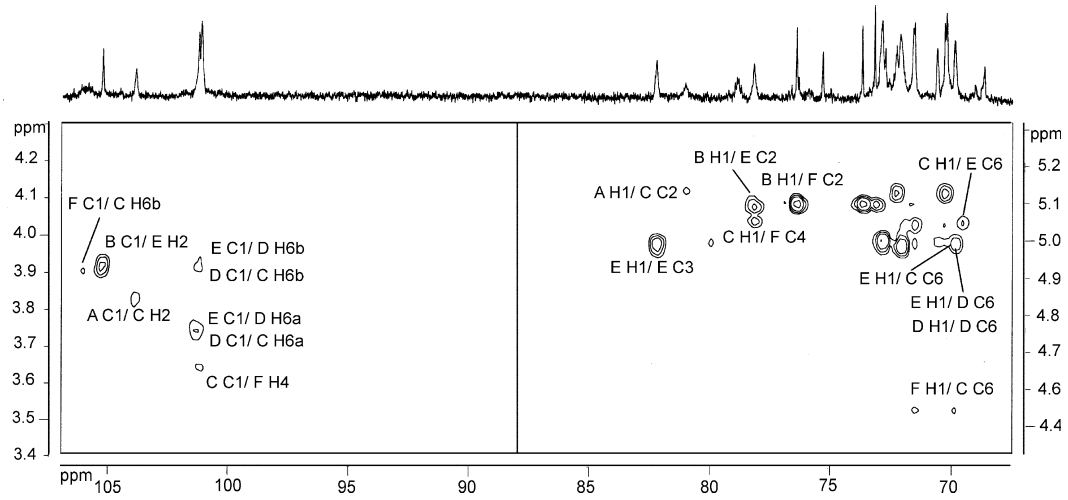


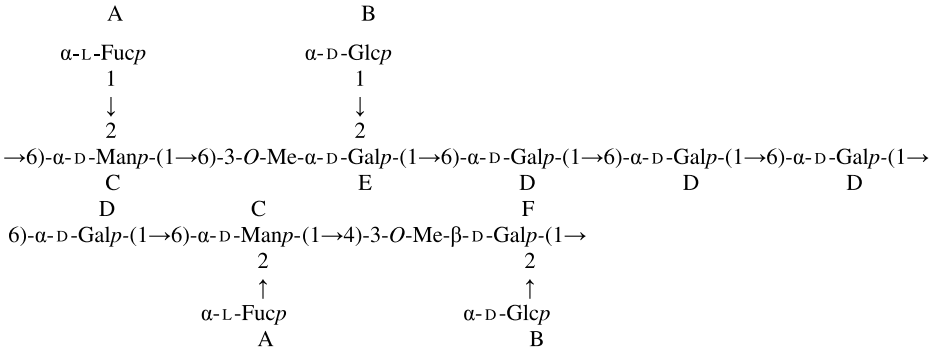
Figure 6. HMBC spectrum of *P. igniarius* polysaccharide PIP60-1 showing inter-residue connectivities. Determined in D_2O at 60 °C.

3.56/4.16 indicating a Gal-type residue.[24] The appearance of H-1 as a singlet ($J_{H\text{-}1,H\text{-}2} < 3$ Hz) in the ^1H NMR spectrum, and H-1/H-2 intra-residue correlations in the NOESY spectrum, support an α configuration. ^1H resonances for OCH_3 correlated with C-3 at δ 3.48/82.22, the cross-peak between H-1 of residue E and H-6 of residue D in the NOESY spectrum (Table 2) and between H-1 of residue E and C-6 of residue D in the HMBC spectrum (Fig. 6). The downfield shift of the C-2 (δ 78.41) and the C-6 (δ 68.95) carbon signals, indicated that residue E is (1→2,6)-linked 3-*O*-Me-α-D-galactopyranose.

Residue F has an anomeric chemical shift at δ 4.56 and a large coupling constant $J_{H\text{-}1,H\text{-}2}$ value (~8.3 Hz), indicating a β-linked residue.[25] Chemical shifts from H-1 to H-6 were assigned from 2D-COSY and TOCSY spectra. The $J_{H\text{-}3,H\text{-}4}$ value (<5 Hz) is evidence for a β-D-galactosyl residue, and the correlations between the ^{13}C resonances for OCH_3 with H-3, H-2, and H-4 at δ 59.48/3.52, δ 59.48/3.36 and δ 59.48/3.63 in the HMBC spectrum are indicative of 3-*O*-methyl-D-galactose. The cross-peak between H-1 of residue F and H-6 of residue C in the HMBC spectrum (Fig. 6), and the downfield shifts of the C-2 (δ 76.47) and C-4 (δ 78.38) carbon signals, further support the designation of residue F as (1→2,4)-linked 3-*O*-Me-β-D-galactopyranose.

The sequence of the glycosyl residues in PIP60-1 was determined from NOESY and HMBC experiments. Inter-residue NOE correlations (Table 2) were observed between H-1 of residue A and H-2 of residue C, between H-1 of residue B and H-2 of residue E, between H-1 of residue B and H-2 of residue F, between H-1 of residue C and H-4 of residue F, between H-1 of residue D and H-6a/b of residue C, between H-1 of residue D and H-6a/b of residue D, between H-1 of residue E and H-6a/b of residue D, and between H-1 of residue F and H-6a/b of residue C. These correlations were further confirmed by the inter-residue $J_{C,H}$-connectivities (Fig. 6) observed in the HMBC spectrum.

According to methylation analysis and ^1H NMR spectral data, residues A, B, C, D, E, and F are present in the approximate ratio of 1:1:1:2:0.5:0.5, indicating that the repeat unit of polysaccharide PIP60-1 is made up of these residues in a ratio of 2:2:2:4:1:1. We also undertook a partial degradation experiment with PIP60-1 and found only Glc, Fuc and a little Gal were present in the first hydrolysate following hydrolysis with 1 M TFA. This indicated that Gal, 3-*O*-Me-Gal and Man was present in the main chain of PIP60-1. Based on data presented above, PIP60-1 has the repeat unit:



Plant polysaccharides present in elm bark,[26] sassafras twigs,[27] and hydrolysates of the leaves of various dicotyledon trees[28] have previously been reported to contain 3-*O*-Me-D-galactose, and 3-*O*-Me-D-galactose residues have also been detected in polysaccharide components of the fungi *Armillaria mellea* and *Lamperomyces japonicus*.[29] However, PIP60-1 represents the first 3-*O*-Me-D-galactose-containing neutral polysaccharide to be identified in the mushroom, *P. igniarius*, and is structurally unique compared to polysaccharides previously isolated from mushrooms including other *Phellinus* spp. Antitumor and cell-mediated immuno-enhancing activities of *P. igniarius* extracts using a mouse model have been reported earlier,[30] and possible links between PIP60-1 and these immunodulatory and antitumor properties of *P. igniarius* are currently under investigation.

3. Materials and methods

3.1. Materials

Fruiting bodies of *P. igniarius* were purchased from Shangzhi City in Heilongjiang Province, China. A stock specimen of the fungal material has been deposited at the Herbarium of Edible Fungi Culture Collection Center, Branch of China Culture Collection Center of Agricultural Microorganisms (Accession No. 1400). DEAE-Sepharose Fast-Flow and High-Resolution Sephacryl S-400 were purchased from Amersham Pharmacia Company. Dextrans and the monosaccharide standards, D-Gal, D-Glc, D-Ara, L-Fuc, L-Rha, D-Man, and D-Xyl were from Sigma–Aldrich. All other reagents were from Chinese sources and were reagent grade.

3.2. Isolation and purification of the polysaccharide

Fruiting bodies of *P. igniarius* were cut into small pieces and dried at 50–55 °C for 48 h. Dried material (2000 g) was extracted twice with 10 vol 95% (v/v) EtOH under reflux for 12 h to remove lipids. The residue was dried in air and then extracted three times with 10 vol of distilled H_2O for 2 h at 100 °C. The combined aqueous extracts were filtered, concentrated 10-fold, and 95% (v/v) EtOH added to a concentration of 30%. Precipitated material was removed by centrifugation (3500*g*, 10 min, 20 °C), and 95% EtOH again added to 60% final concentration. Precipitated polysaccharide (crude PIP-60 fraction) was collected by centrifugation as above, dissolved in 700 mL of distilled H_2O, and insoluble material was removed by centrifugation as above. Low-molecular-weight compounds in the supernatant were removed by passage through a hollow-fiber cartridge (membrane pore size 10k) after which the high-molecular-weight fraction was concentrated and freeze-dried.

Crude polysaccharide, PIP-60 (64.71 g, 3.23% yield) was dissolved in 330 mL of distilled H_2O, applied to a DEAE-Sepharose Fast-Flow column (XK26 × 100 cm), and the column was eluted with water to yield fraction PIP60-A. Polysaccharide in the eluted fractions was detected using the phenol–sulfuric acid method.[9] Fraction PIP60-A was further purified by gel-permeation chromatography on a High-Resolution Sephacryl S-400 column (XK26 × 100 cm) attached to a refractive index detector. Purified polysaccharide, fraction PIP60-1, eluted as a single peak.

3.3. Determination of purity and molecular weight

Homogeneity and the molecular weight of PIP60-1 were determined by HPLC using a Waters 2695 HPLC system fitted with two serially linked TSK PWXL 4000 and 3000 gel filtration columns, a Model 2695 pump, a Waters 2410 RI detector, a Waters 2487 dual wavelength absorbance detector and an on-line de-gasser. Columns were eluted with 0.1 M NaH_2PO_4 and 0.3 M $NaNO_3$ (pH 7.0) at a flow rate of 0.5 mL/min, and calibrated using Dextrans T-700, 580, 300, 110, 80, 70, 40, 12 and 4.

3.4. Sugar analysis

PIP60-1 (2 mg) were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 °C for 2 h, and the monosaccharides were identified by high-performance anion-exchange chromatography (HPAEC) using a Dionex LC30 equipped with a CarboPac™PA20 column (3 mm × 150 mm). The column was eluted with 2 mM NaOH (0.45 mL/min), and the monosaccharides were monitored using a pulsed amperometric detector (Dionex).[10] In order to identify all the component monosaccharides of PIP60-1, sugars in the hydrolysates were converted into their alditol acetates[31] by reduction with $NaBH_4$ and $NaBD_4$, respectively, then analyzed by GC–MS using a DB-5MS column (30 m × 0.25 mm × 0.25 μm) and a temperature program of 180 °C to 270 °C at 20 °C/min, with holding at 270 °C for 25 min. The absolute configurations of the monosaccharides were determined as described by Vliegenthart and co-workers[14] using (+)-2-butanol.

3.5. Methylation analysis

Freeze-dried samples (2 mg) were dissolved in DMSO (2 mL), and methylated four times according to an improved NaOH–DMSO method.[32] Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. The methylated polysaccharide was treated with formic acid (88%,

3 mL) for 3 h at 100 °C, evaporated to dryness on a rotary evaporator and hydrolyzed with 2 M TFA (0.05 mL) for 6 h at 100 °C. The partially methylated sugars in the hydrolysate were reduced with $NaBH_4$ and acetylated by AC_2O, and the methylated alditol acetates were analyzed by GC–MS using the same conditions as described above.

3.6. Nuclear magnetic resonance (NMR) spectroscopy

Samples of PIP60-1 (30 mg) were deuterium-exchanged three times by lyophilization from D_2O. ^1H NMR (500 MHz, 60 °C) and ^{13}C NMR (125.8 MHz, 25 °C) spectra were recorded using a Varian INOVA 500 NMR spectrometer. ^1H chemical shifts were referenced to residual HDO at δ 4.78 ppm (25 °C) as internal standard. ^{13}C chemical shifts were acquired in relation to DSS (δ 0.00 ppm) calibrated externally. The 2D-COSY NMR experiment was performed using standard Bruker software. Total correlation spectroscopy (TOCSY) and heteronuclear multiple quantum coherence (HMQC) were used to assign signals. Two-dimensional heteronuclear multiple-bond correlation spectroscopy (HMBC) and two-dimensional Overhauser effect spectroscopy (NOESY) were used to assign inter-residue linkages and sequences.

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References

1. Song, L.; Sun, P. L.; Guo, B. B. *Edible Fungi of China* **2005**, *24*, 7–15.
2. Song, K. S.; Cho, S. M.; Lee, J. H.; Kim, H. M.; Han, S. B.; Ko, K. S. *Chem. Pharm. Bull.* **1995**, *43*, 2105–2108.
3. Rew, Y. H.; Jo, W. S.; Jeong, K. C.; Yoon, J. T.; Choi, B. S. *Korean J. Mycol.* **2000**, *28*, 6–10.
4. Han, S. B.; Lee, C. W.; Jeon, Y. J.; Hong, N. D.; Yang, K. H.; Kim, H. M. *Immunopharmacology* **1999**, *41*, 157–164.
5. Ajith, T. A.; Janardhanan, K. K. *J. Ethnopharmacol.* **2003**, *84*, 157–162.
6. Kim, H. M.; Han, S. B.; Oh, G. T.; Kim, Y. H.; Hong, D. H.; Hong, N. D.; Yoo, I. D. *Int. J. Immunopharmacol.* **1996**, *18*, 295–303.
7. Ohtsuka, S.; Uneo, S.; Yoshikumi, C.; Hiroshi, F.; Ohmura, Y.; Wada, T.; Fujii, T.; Takahashi, E. U.S. Patent 4,051,314, 1977.
8. Kim, G. Y.; Park, H. S.; Nam, B. H.; Lee, S. J.; Lee, J. D. *Bioresource Technol.* **2003**, *89*, 81–87.
9. Dubios, M.; Gillis, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
10. Yang, R. Z.; Zhang, J. S.; Tang, Q. J. *Edible Fungi China* **2005**, *24*, 42–44.
11. Gerard, S.; Paolo, T.; Sergio, L. *J. Microbiol. Methods* **1991**, *14*, 185–191.
12. Chen, W. F.; Jiang, L. Y.; Xu, L. H. *Microbiology* **2000**, *27*, 416–420.
13. Watt, D. K.; O'Neill, S. A.; Percy, A. E. *Carbohydr. Polym.* **2002**, *50*, 283–294.
14. Gerwig, G. J.; Karmerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1979**, *77*, 1–7.
15. Fan, J. M.; Zhang, J. S.; Tang, Q. J. *Carbohydr. Res.* **2006**, *341*, 1130–1134.
16. Popper, Z. A.; Sadler, I. H.; Fry, S. C. *Phytochemistry* **2001**, *57*, 711–719.
17. Pramanik, M.; Mondal, S.; Chakraborty, I. *Carbohydr. Res.* **2005**, *340*, 629–636.
18. Mondal, S.; Chakraborty, I.; Rout, D. *Carbohydr. Res.* **2006**, *341*, 878–886.
19. Dominaika, D.; Philip, V. T.; Nikolay, P. A. *Carbohydr. Res.* **2002**, *337*, 1535–1540.
20. Rout, D.; Mondal, S.; Chakraborty, I. *Carbohydr. Res.* **2006**, *341*, 995–1002.
21. Grigorij, K.; Dusan, U.; Jean, R. B. *J. Biochem.* **1996**, *271*, 8786–8790.
22. Jones, C.; Lemercinier, X. *Carbohydr. Res.* **2005**, *340*, 403–409.
23. Mikael, S.; Felipe, U.; Andrej, W. *Eur. J. Biochem.* **1999**, *262*, 56–62.
24. Prabhakar, G. R.; Unaiza, H.; Xu, Q. W. *Eur. J. Biochem.* **1998**, *255*, 279–288.
25. Agrawal, P. K. *Phytochemistry* **1992**, *31*, 3307–3330.
26. Barsett, H.; Paulsen, B. S. *Carbohydr. Polym.* **1991**, *17*, 137–144.
27. Springer, G. F.; Takahashi, T.; Desai, P. R.; Kolecki, B. J. *Biochemistry* **1965**, *4*, 2012–2099.
28. Bacon, J. S. D.; Cheshire, M. V. *Biochem. J.* **1971**, *124*, 555–562.
29. Bouveng, H. O.; Fraser, R. N.; Lindberg, B. *Carbohydr. Res.* **1967**, *4*, 20–31.
30. Che, H. L.; Meng, F. Y.; Du, J. *Chin. J. Pub. Health* **2005**, *21*, 79–81.
31. Albersheim, P.; English, P. D.; Karr, A. *Carbohydr. Res.* **1967**, *5*, 340–345.
32. Needs, P. W.; Sevendran, R. R. *Carbohydr. Res.* **1993**, *245*, 1–10.