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Structure of a novel α-glucan substitute with the rare 6-deoxy-D-altrose from *Lactarius lividatus* (mushroom)☆

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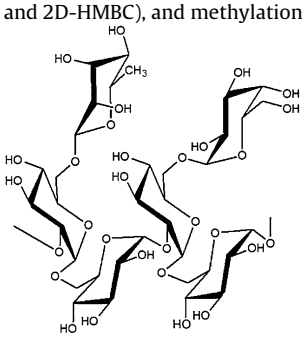
ABSTRACT

A novel α-glucan substituted rare 6-deoxy-D-altropyranose was isolated from edible fruiting bodies of a mushroom (*Lactarius lividatus*) grown in Okinawa, Japan. The polysaccharide consists of D-glucose, D-galactose and 6-deoxy-D-altrose in a molar ratio of 3.0:1.0:1.0. The specific rotation $[\alpha]_{589}$ was estimated as +64.3° (0.2% in water) at 25 °C. Based on results of IR, NMR (^{1}H, ^{13}C, 2D-COSY, 2D-HMQC, 2D-ROESY and 2D-HMBC), and methylation analyses, the structure of the polysaccharide was determined as



This work is the first demonstration of rare 6-deoxy-D-altropyranose moiety on polysaccharides.

1. Introduction

The fruiting bodies of mushrooms have been used in foods and folk medicines throughout the world (Wasser, 2002; Zhang, Cui, Cheung, & Wang, 2007). Many attempts have been made to explore the use of mushrooms and their metabolites for the treatment of various human ailments. Polysaccharides from mushrooms are regarded as useful antitumor and immunomodulating agents for clinical uses (Chihara, Maeda, Hamuro, Sasaki, & Fukuoka, 1969; Maeda & Chihara, 1971). In recent years, mushroom polysaccharides have drawn the attention of chemists and immunobiologists because of their immunomodulatory (Wasser, 2002) and antitumor (Zhang et al., 2007) properties.

The fruiting bodies of *Lactarius lividatus* (previously *Lactarius hatsudake*) have long been used as antitumor and antiviral agents in Chinese folk medicine (Gao et al., 2007). We previously identified a rare 6-deoxy-D-altrose from *L. lividatus* (Tako et al., 2012). That study was the first reported complete identification of 6-deoxy-D-altrose in nature. The present report describes structural features of a novel polysaccharide containing 6-deoxy-D-altrose.

2. Materials and methods

2.1. Preparation of polysaccharides

Fresh fruiting bodies of *L. lividatus* were collected from Onna Village, Okinawa, Japan in February 2008 and were washed with distilled water. The fruiting bodies were air-dried at 40 °C for 24 h and powdered. The powdered fruiting bodies were suspended in 90% ethanol and acetone. Then they were stirred for 2 h to extract pigment and lipids at room temperature. The sample (20 g) was suspended in distilled water and stirred at room temperature for 10 h to extract polysaccharides. The extract was then centrifuged

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at 23,000 × *g* for 20 min. The supernatant was filtered (Celite 545; Nakalai Tesque Inc.). Then the filtrate was precipitated by addition of two volumes of ethanol. The precipitate was dried in a vacuum chamber.

The crude polysaccharide was dissolved in distilled water. Then 10% lead acetate was added to precipitate proteins. The solution was passed through Celite 545 and dialyzed at 4 °C for 3 days. The filtrate was deionized by passage through a cation exchange column (50 mm × 200 mm, Amberlite IR-120A H+; Organo Co.) and was then neutralized with 0.05 M NaOH solution, dialyzed against distilled water, and freeze-dried.

The freeze-dried material (30 mg) was dissolved in distilled water (2 mL) and loaded on a column (20 mm × 600 mm) of DEAE-sepharose at a flow rate of 1 mL/1 min. Then it was eluted with distilled water. Based on the colorimetric test for total carbohydrate using the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), main fractions (No. 32-57) were collected, concentrated, and lyophilized to obtain a purified polysaccharide (23 mg). The purification process was conducted in three lots.

2.2. Chemical procedures

Total carbohydrates of the polysaccharide were determined using the phenol–sulfuric acid method (Dubois et al., 1956) with D-glucose as a standard. The purified polysaccharide was dissolved in distilled water. Sulfuric acid was added to a final concentration of 1.0 M. The mixture was then heated at 100 °C for 3 h. The hydrolysate was neutralized with $BaCO_3$.

Protein was measured using the method described by Lowry, Rosebrough, Farr, and Randall (1951).

2.3. High-performance anion exchange chromatography coupled using a pulse amperometric detector (HPAEC-PAD)

The monosaccharides in the hydrolysate of the polysaccharide were determined using HPAEC (DX-500; Dionex Corp., CA, USA) fitted with a column of Carbopack PA1 and a pulsed amperometric detector. The column was eluted at a flow rate of 1 mL/min at 35 °C with 15 mM NaOH.

2.4. Molecular mass

The molecular mass of the polysaccharide was determined using high-performance liquid chromatography (HPLC) (LC-6A; Shimadzu Corp., Japan) on a column of TSK-gel GMPW (7.8 mm × 300 mm, Tosoh Corp., Japan). HPLC was performed at room temperature at a flow rate of 0.3 mL/min, with refractive index detection (RID-6A; Shimadzu Corp.). The column was conditioned with 0.15 M sodium chloride in 0.05 M sodium phosphate buffer (pH 7.2). Elution was conducted with the same buffer. Standard pullulans (Showa Denko K.K., Japan) including P-400 (molecular mass, 4.04×10^5), P-100 (1.12×10^5), P-20 (2.28×10^4), and P-5 (5.9×10^3) were used as molecular mass markers (Tamaki, Teruya, & Tako, 2010).

2.5. Infrared spectra and specific rotation of the polysaccharide

The infrared spectrum of the polysaccharide was recorded using a spectrophotometer (FTS-3000; Bio-Rad Laboratories Inc., CA, USA) in transmittance mode from 4000 to 400 cm^{-1} in KBr discs (Tamaki, Teruya, & Tako, 2010).

Specific rotation was measured at 589 nm using a polarimeter (P-1010; JASCO Inc., Tokyo, Japan) at room temperature. The polysaccharide solution (0.2%) was prepared in distilled water.

2.6. ^{1}H-nuclear and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy

The polysaccharide was dissolved in 2 mL of D_2O and was then freeze-dried. The dried sample (2%) was dissolved in D_2O again. Then the solution was examined in 5 mm o.d. tubes. The ^{1}H NMR and ^{13}C NMR chemical shifts were expressed in δ (ppm), relative to the resonance of sodium 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid (TSP, 0.000 ppm) as an internal standard (Tamaki, Konishi, Fukuta, & Tako, 2008).

^{1}H NMR and ^{13}C NMR spectra were recorded using an FT-NMR spectrometer (AVANCE III 500 MHz; Bruker Analytik, Germany) at 500.00 and 125.65 MHz at 70 °C (Tako et al., 2012). The two-dimensional correlated spectroscopy ^{1}H–^{1}H COSY (homonuclear shift correlation), ^{1}H–^{1}H-ROESY, ^{1}H–^{13}C heteronuclear single quantum coherence (HMQC) and ^{1}H–^{13}C-heteronuclear multiple quantum coherence (HMBC) measurements were recorded using standard Bruker procedures and assigned signals to determine the sequences of sugar residues. A mixing time of 150 ms was used in the ROESY experiment.

2.7. Methylation analysis

The polysaccharide (5 mg) was methylated according to the procedure described by Ciucanu and Kerek (1984). The permethylated polysaccharide obtained was subjected to complete acid hydrolysis using 2 M TFA (2 mL) at 120 °C for 2 h. The hydrolysate was dissolved in 1 M NH_4OH (0.2 mL). DMSO (1 mL) containing 20 mg of $NaBH_4$ was added and the mixture was incubated at 40 °C for 90 min. Subsequently acetic anhydride (0.2 mL) was added to the mixture. Anhydrous 1-methylimidazole (0.2 mL) and acetic anhydride (1 mL) were then added, and the reaction mixture was incubated at ambient temperature for 10 min. After extraction with chloroform and washing with water, partially methylated alditol acetates were obtained (Tamaki, Teruya, & Tako, 2010; Teruya, Tatemoto, Konishi, & Tako, 2009).

The partially methylated alditol acetates were analyzed using a gas chromatograph (GC-14A; Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector using a capillary column (DB-1: 40 m × 0.25 mm, J&W Scientific Inc., CA, USA). The injector and detector temperature were, respectively, 210 °C and 270 °C. After injection, the oven temperature was maintained at 150 °C for 5 min, then raised at 5 °C/min to 250 °C. This temperature was maintained for 5 min. The identities of the peaks were confirmed using GC-MS (GCMS-QP 1000EX; Shimadzu Corp., Kyoto, Japan) under the same conditions.

3. Results and discussion

3.1. Chemical components of the polysaccharide

The yield of purified polysaccharide was estimated to be 1.0% ($n = 5$) based on dried weight of fruiting bodies of mushroom. The polysaccharide contained 94.7% (W/W) of carbohydrate. The purified polysaccharide had a negative response to the Lowry test, indicating the absence of protein. A high-performance liquid chromatogram of hydrolysate of the polysaccharide is not shown, but presented in Fig. S1 (Supplementary data). Peaks 1 and 2 were in agreement with those of D-galactose and D-glucose in the molar ratio of 1.0:4.0. As reported previously (Tako et al., 2012), 6-deoxy-D-altrose was overlapped with D-glucose on HPAEC-PAD, but it was identified by paper chromatogram (not shown): Spot 1 (Rf, 0.40) was higher than that of standard L-rhamnosyl residue (0.37) and was identical to that of standard. The D-glucose and D-galactose was overlapped (0.12).

Table 1
The ^{1}H and ^{13}C NMR chemical shifts for the polysaccharide isolated from *Lactatius lividatus* in D_2O at 70 °C.

Glycosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a/C-6	H-6b
A	5.134	3.977	4.022	3.613	3.875	1.334	
6d-β-D-Alt*p*-(1→	101.08	73.08	71.25	72.29	70.92	20.72	
B	5.113	3.975	4.073	3.901	4.134	3.708	3.892
α-D-Gal*p*-(1→	101.12	72.65	72.19	72.18	72.06	63.61	
C	5.045	3.998	4.082	3.646	3.909	3.729	3.941
→6)-α-D-Glc*p*-(1→	102.53	71.18	72.86	72.29	70.90	69.10	
D	5.015	3.879	3.946	3.713	4.255	3.939	3.723
→2,6)-α-D-Glc*p*-(1→	100.78	79.65	72.13	70.07	71.35	69.54	

The polysaccharide molecular mass was estimated to be approximately 240 kDa according to a standard calibration curve obtained from the definite molecular mass of pullulan.

3.2. Specific rotation and infrared spectra (IR) of the polysaccharide

The specific rotation $[\alpha]_{589}$ of the polysaccharide (0.2%, W/V in water) at room temperature (25 °C) showed a value of +64.3°, indicating that α-configurations of the sugar residues are predominant (Cao et al., 2006; Das et al., 2008; Ishurd et al., 2010).

In the IR spectrum of the polysaccharide (not shown, but presented in Fig. 2S), the major absorption at around 3395 cm^{-1} was attributed to stretching of hydroxyl groups. Absorption at 2922 cm^{-1} resulted from C—H stretching of C—H groups. Absorption at 1648 cm^{-1} resulted from bound water. The strong absorption in the range of 1200–1000 cm^{-1} suggested that the monosaccharide of the polymer molecules consists of a pyranose form (Liu & Wang, 2007; Ishurd et al., 2010). Characteristic absorptions at 833 cm^{-1} was also observed, indicating that α-configuration of the sugar units were involved (Cao et al., 2006; Ishurd et al., 2010).

3.3. ^{1}H NMR and ^{13}C NMR spectra of the polysaccharide

The ^{1}H spectrum at 500 MHz of the polysaccharide is presented in Fig. 1a. Three chemical signals were observed in the anomeric region (δ 5.5–4.5) at 5.124, 5.033, and 5.008 ppm, but the former signal was overlapped two sugar residues (5.134 and 5.113 ppm: see Fig. 2 and Table 1), indicating that the polysaccharide consisted of a four-sugar repeating unit. The four sugar moieties were designated as residues A, B, C, and D according to their decrease in proton chemical shift. In addition, a major signal at 1.340 and 1.329 ppm was observed, indicating that a 6-deoxy-D-altrose was involved in the polysaccharide (Tako et al., 2012). From area of anomeric signals, molar ratio of residue A + B and residue C + D was estimated to be 1.0:1.9.

The ^{13}C NMR of the polysaccharide is presented in Fig. 1b. The four anomeric signals were observed at 102.69, 101.17, 100.86, and 100.62 ppm. The methyl signal at 20.11 ppm was observed, indicating that a 6-deoxy-D-altrose was involved in the polysaccharide (Tako et al., 2012). The absence from the ^{13}C NMR spectrum of signals within the δ 82–88 region suggested that all the sugar residues were in the pyranose form (Harding et al., 2005; Ye et al., 2008) which was in agreement with that of IR spectrum, as mentioned above.

The 2D COSY spectrum of the polysaccharide was shown in Fig. 2. It was possible to correlate H-1 of A (δ 5.134) with H-2 (δ 3.977), H-2 with H-3 (δ 4.022), H-3 with H-4 (δ 3.613), H-4 with H-5 (δ 3.875), and H-5 with H-6 (δ 1.334); H-1 of B (δ 5.113) with H-2 (δ 3.975), H-2 with H-3 (δ 4.073), H-3 with H-4 (δ 3.901), H-4 with H-5 (δ 4.134), H-5 with H-6a (δ 3.708), and H-5 with H-6b (δ 3.892); H-1 of C (δ 5.045) with H-2 (δ 3.998), H-2 with H-3 (δ 4.082), H-3 with H-4 (δ 3.646), H-4 with H-5 (δ 3.909), H-5 with H-6a (δ 3.729), and H-5 with H-6b (δ 3.941; H-1 of D (δ 5.015) with H-2 (δ 3.879), H-2 with H-3 (δ 3.946), H-3 with H-4 (δ 3.713), H-4 with H-5 (δ 4.255), and H-5 with H-6a (δ 3.939), and H-5 with H-6b (δ 3.723).

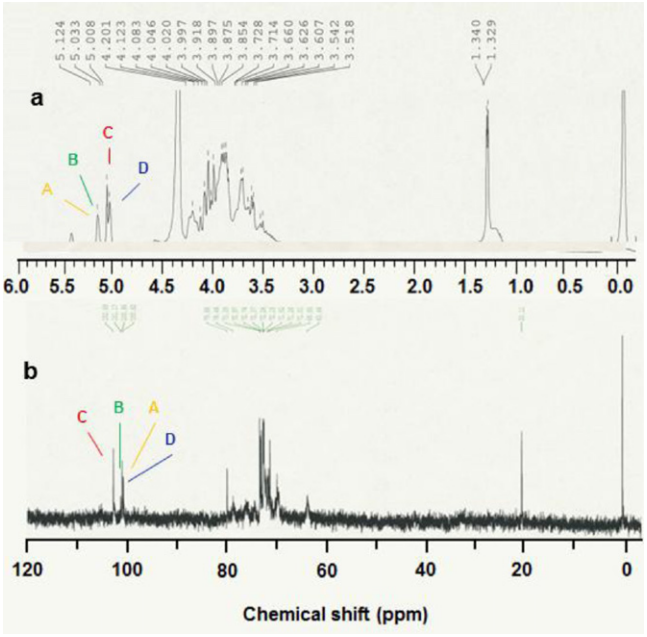

Fig. 1. ^{1}H and ^{13}C NMR spectra of the polysaccharide in D_{2O} at 70 °C.

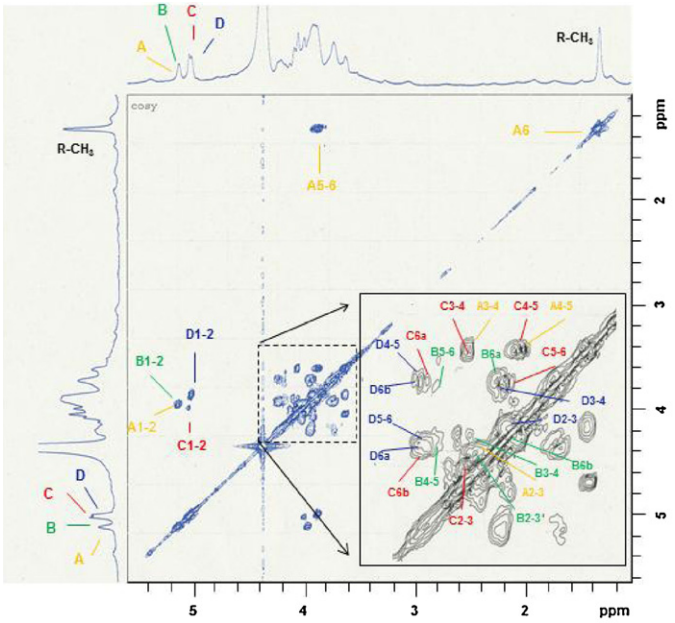

Fig. 2. COSY spectrum of the polysaccharide at 70 °C.

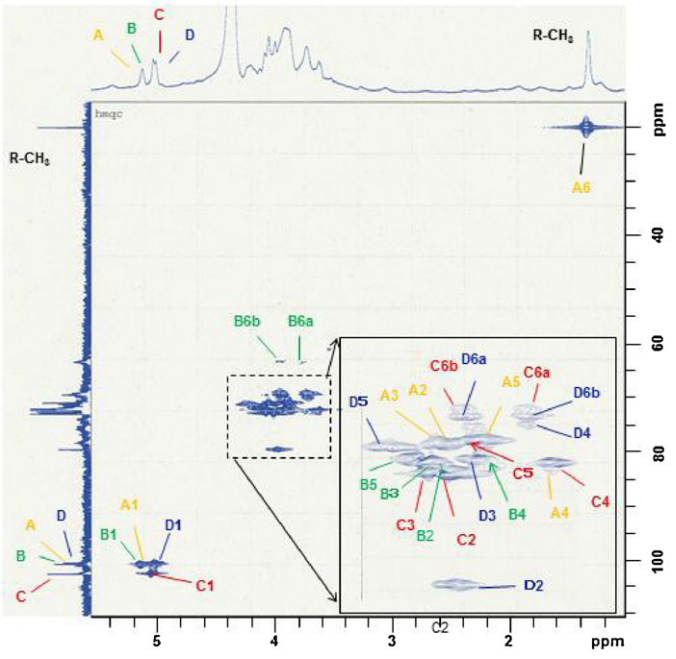

Fig. 3. HMQC spectrum of the polysaccharide at 70 °C.

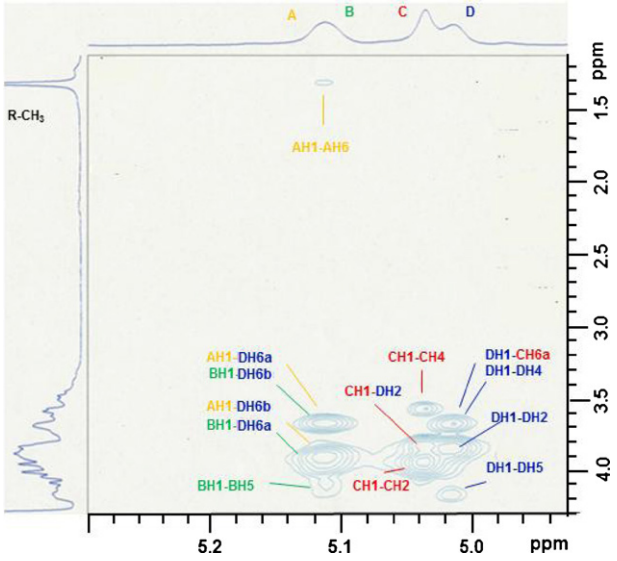

Fig. 4. ROESY spectrum of the polysaccharide at 70 °C.

Based on the COSY spectrum, residue A was assigned to 6-deoxy-β-D-altropyranose (Hanniffy et al., 1999; Tako et al., 2012). From the chemical shift of anomeric proton, residue B (δ 5.113), C (δ 5.045), and D (δ 5.015) were reported to adopt the α-configuration (Cao et al., 2006; Das et al., 2008; Harding et al., 2005; Roy, Maiti, Mondal, Das, & Islam, 2008; Ye et al., 2008).

The result (COSY) helped the assignment in the HMQC spectrum (Fig. 3) of residue A (6-deoxy-β-D-altropyranose) of C-1, C-2, C-3, C-4, C-5, and C-6 to δ 101.08, δ 73.08, δ 71.25, δ 72.29, δ 70.92, and δ 20.72 ppm. Residue B of C-1, C-2, C-3, C-4, C-5, and C-6 was assigned to δ 101.12, 72.65, 72.19, 72.18, 72.06, and 63.61. Residue C of C-1, C-2, C-3, C-4, C-5, and C-6 was assigned to δ 102.53, 71.18, 72.86, 72.29, 70.90, and 69.10. Residue D was δ 100.78, 79.65, 72.13, 70.07, 71.35, and 69.54. They are presented in Table 1. From the COSY and HMQC spectra, residue A was identified as terminal 6-deoxy-β-D-altropyranose (Hanniffy et al., 1999; Tako et al., 2012). Residue B was α-linked terminal D-hexopyranose (Das et al., 2008; Harding et al., 2005; Mondal, Chakraborty, Rout, & Islam, 2006; Roy et al., 2009). Residue C was (1 → 6)-linked α-D-hexopyranose (Cao et al., 2006; Das et al., 2008; Roy et al., 2008; Ye et al., 2008). In addition, residue D was (1 → 2)- and (1 → 6)-linked α-D-hexopyranose (Ye et al., 2008).

Assignment of intra-residual and inter-residual cross-peaks in the 2D ROESY spectrum (Fig. 4) and in the HMBC spectrum (Fig. 5) allowed the identification of sugar residues and the determination of sequence and linkage positions of the residues within the repeating unit of the polysaccharide. On the ROESY spectrum, residue A H-1 (δ 5.134) and residue B H-1(δ 5.113) tracks were overlapped. They showed very strong resonance with residue D H-6a (δ 3.939) and D H-6b (δ 3.723), suggesting the existence of A (1 → 6) D and B (1 → 6) D linkages. Residue A was also confirmed as 6-deoxy-D-altropyranose from the intra-residual cross-peak between A H-1 and A H-6 (δ 1.334: CH_3). The inter-cross peak between C H-1 (δ 5.045) and D H-2 (δ 3.879) was assigned, suggesting that residue C substituted at C-2 of residue D. Furthermore, the inter-cross-peak between D H-1 (δ 5.015) and C H-6a (δ 3.729) was assigned, suggesting that residue D substituted at C-6 of residue C. Because the intra-cross peak between residue C H-1 and C H-4 (δ 3.646), and between residue D H-1 and D H-4 (δ 3.713) was observed in ROESY spectrum, residues C and D was identified, respectively, as α-linked D-glucopyranosyl moiety (Cao et al., 2006; Harding et al., 2005; Roy et al., 2008). Consequently, residue B was identified as α-linked D-galactopyranosyl residue (Das et al., 2008; Harding et al., 2005; Roy et al., 2008) because no assignment was observed between residue B H-1(δ 5.113) and B H-4 (δ 3.901: see Table 1) in the ROESY spectrum, the chemical shift of which was higher than that of residue C H-4 (δ 3.646) and D H-4 (δ 3.713) (Harding et al., 2005; Roy et al., 2008; Ye et al., 2008).

The cross peaks of both anomeric protons and carbons from HMBC experiment (Fig. 5) of each sugar moiety were examined. Both intra-residual and inter-residual connectivities were observed. Cross peaks were found between residue A H-1 (δ 5.134) and residue D C-6 (δ 69.54), between residue B H-1 (δ 5.113) and residue D C-6 (δ 69.54), between residue A C-1 (δ 101.08) and residue D H-6a (δ 3.939), between residue A C-1 and residue D H-6b (δ 3.723), between residue B C-1 (δ 101.12) and residue D H-6a, and between residue B C-1 and residue D H-6b. Results show that

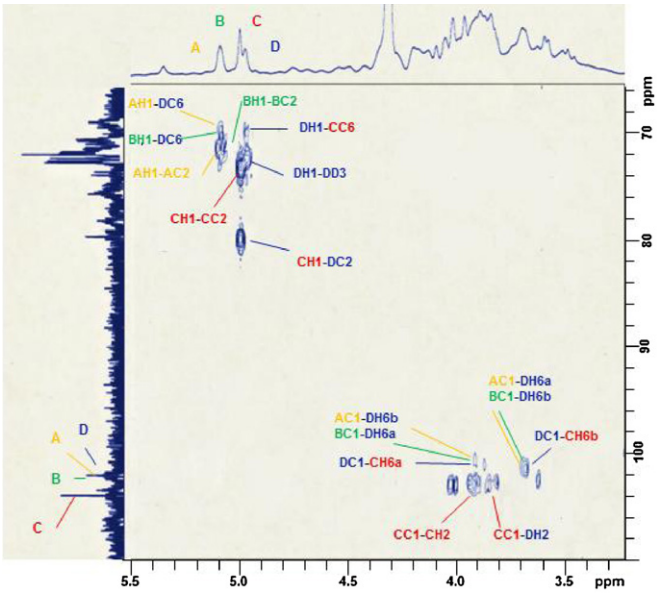

Fig. 5. HMBC spectrum of the polysaccharide at 70 °C.

Table 2
Methylation analysis of the polysaccharide from *L. lividatus*.

No.	Methylated sugar	[a]Retention time	Molar ratio	Mode of linkage
(1)	2,3,4-Tri-*O*-methyl-6d-D-altrose	0.83	1.0	6d-D-altrose-β-1→
(2)	2,3,4,6-Tetra-*O*-methyl-D-galactose	1.00	0.4	D-galactose-α-1→
(3)	2,3,4-Tri-*O*-methyl-D-glucose	1.14	1.4	→6)-D-glucose-α-1→
(4)	3,4-Di-*O*-methyl-D-glucose	1.26	2.0	→2.6)-D-glucose-α-1→

[a] Relative retention time to 2,3,4,6-tetra-*O*-methyl-D-galactose.

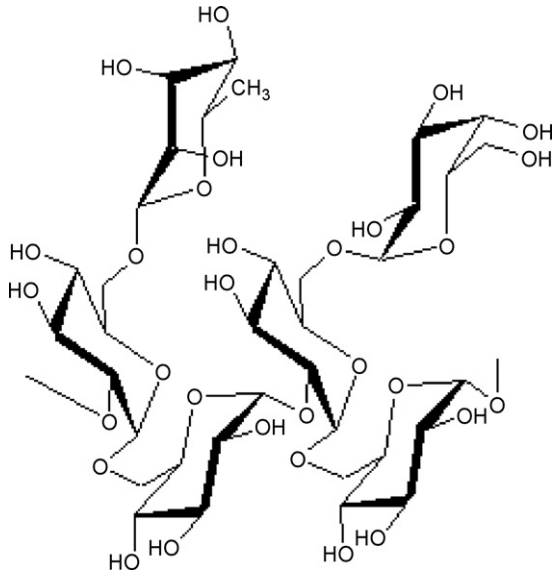

Fig. 6. The chemical structure of the polysaccharide isolated from *Lactarius lividatus*.

terminal residue A substituted at C-6 of residue D, and terminal residue B substituted at C-6 of residue D. Assignments were made between residue C C-1 (δ 102.53) and residue D H-2 (δ 3.879), and between residue D H-1 (δ 5.015) and residue C C-6 (δ 69.10), indicating that C (1 → 2) D and D (1 → 6) C linkages were also involved.

3.4. Methylation analysis

The polysaccharide was methylated according to the procedure described by Ciucanu and Kerek (1984). The obtained permethylated polysaccharide was subjected to complete acid hydrolysis to furnish mixtures of the methylated sugars, which were analyzed as the corresponding alditol acetates using gas–liquid chromatography (GC) and combined gas–liquid chromatography/mass spectroscopy (MS). The chromatogram was shown in Fig. S3. Partially methylated alditol acetates were identified using published data (Jansson, Kenne, Liedgren, Lindberg, & Lonngren, 1976; Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005). They are summarized in Table 2, which showed correspondence to terminal 2,3,4-tri-*O*-methyl-6-deoxy-D-altrose, terminal 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucose and branching 3,4-di-*O*-methyl-D-glucose with respective molar ratios of 1.0:0.4:1.4:2.0. Results show that the polysaccharide consists of terminal 6-deoxy-β-D-altropyranosyl, α-D-galactopyranosyl, 1,6-linked D-glucosyl, and 1,2,6-linked D-glucosyl residues.

4. Conclusions

Based on the NMR and methylation analysis, in addition to chemical shifts reported in the literatures (Cao et al., 2006; Das et al., 2008; Harding et al., 2005; Roy et al., 2008; Tako et al., 2012; Ye et al., 2008), residue A was assigned as terminal 6-deoxy-β-D-altropyranose, residue B as terminal α-D-galactopyranose, residue C as 1,6-linked α-D-glucopyranose, and residue D as 1,2,6-linked α-D-glucopyranose.

Consequently, the physicochemical and structural results showed that the polysaccharide extracted from *L. lividatus* was a novel 1,6-linked and 1,2,6-linked α-D-glucan substituted at C-6 with terminal 6-deoxy-β-D-altrose and α-D-galactose moiety, respectively, the primary structure of which is depicted in Fig. 6. An examination of the biological activity of the polysaccharide is now in progress.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.11.010.

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