



Rare 6-deoxy-D-altrose from the folk medicinal mushroom *Lactarius akahatsu*

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Abstract

A rare sugar, 6-deoxy-D-altrose, isolated from a polysaccharide extracted from an edible folk medicinal mushroom (*Lactarius akahatsu*) was identified using ¹H and ¹³C-NMR including 2D-COSY and 2D-HSQC spectroscopy, and specific rotation. The 6-deoxy-sugar isolated from the acid hydrolysate of the polysaccharide extracted from *L. akahatsu* was involved in four anomeric isomers (α - and β -pyranose, and α - and β -furanose) in aqueous solution due to mutarotation. Almost all of the signals from the 1D (¹H- and ¹³C-) and 2D (COSY and HSQC)-NMR spectra of the 6-deoxy-sugar agreed with the data from the authentic 6-deoxy-D-altrose. The specific rotation $[\alpha]_{589}^0$ of the 6-deoxy-sugar isolated from *L. akahatsu* was +17.6°. Thus, the 6-deoxy-sugar isolated from *Lactarius akahatsu* was identified as 6-deoxy-D-altrose.

Keywords: *Lactarius akahatsu*, folk medicinal mushroom, 6-deoxy-D-altrose, rare sugar, polysaccharide moiety

Introduction

In proceeding studies, we isolated a novel acetyl fucoidan from commercially marine cultured *Cladosiphon okamuranus* [1,2], and which has been patented [3]. The acetyl fucoidan has some biological activities, such as antitumor [4] and immune-enhancing [5]. Specifically, over-sulfated acetyl fucoidan, the sulfate content of which was 32.8%, showed significant antitumor activity [4]. The results suggested that the over-sulfated acetyl fucoidan was applicable to an anticancer drug.

The fruiting bodies of mushrooms have been used as foodstuffs and folk medicine throughout the world since ancient times, especially in Japan and China [6,7]. Many attempts have been made to explore the use of mushrooms and their metabolites for the treatment of various human ailments [8,9]. Certain polysaccharides (β -glucans) from mushrooms have been applied as anti-tumor and immune-enhancing agents for clinical use in Japan [7,10]. Consequently, mushroom polysaccharides have drawn the attention of chemists and immune-biologists in recent years because they possess antitumor and immune-enhancing properties.

The fruiting bodies of a *Lactarius lividatus* (previously *Lactarius hatsudake*) have historical use as antitumor and antiviral agents in Japanese and Chinese folk medicine [11]. We identified a rare 6-deoxy-D-altrose from the mushroom, which is widespread in natural environments within South-East Asia, East Asia, North America, and Europe [12]. Our previous report detailed the first complete identification of a 6-deoxy-D-altrose in nature.

Furthermore, we have previously reported the structure of

a novel polysaccharide, which was substituted with 6-deoxy-D-altrose, isolated from *L. lividatus* [13]. The polysaccharide was highly branched 1,2- and 1,6-linked α -D-glucan, and its terminal residues were 6-deoxy- β -D-altropyranose and α -D-galactopyranose. This report described the first identification of a rare 6-deoxy-D-altropyranose moiety on polysaccharides.

Lactarius akahatsu is widespread in natural environments throughout the world. In Okinawa Prefecture, Japan, its fruiting bodies have been eaten since ancient times alongside *L. lividatus*. The fruiting bodies of *Lactarius akahatsu* have also been used as folk medicine in Japan and China.

This paper describes identification of rare 6-deoxy-D-altrose from a polysaccharide isolated from *L. akahatsu*.

Materials and methods

The fresh fruiting bodies of *L. akahatsu* were collected in Onna Village, Okinawa, Japan in the February of 2009. The pilei of the fruiting bodies are 40-100 mm in diameter, and a light brownish orange in color. The lamellae and stipe are both orange. The fresh fruiting bodies were washed with distilled water and air dried at 40°C for 48 h before being ground into powder.

The fruiting bodies of *L. akahatsu* were suspended in ethanol (90%) or acetone, and stirred for 3 h to extract the pigment or lipid, respectively. The sample (20 g) was suspended in distilled water and stirred at 90°C for 3 h to extract the polysaccharide. The extract was then centrifuged at 23000 g for 20 min, and the supernatant was filtered through Celite 545 (Nakarai, Japan). The filtrate was precipitated by adding 2 volumes of ethanol,

and the resulting solid was dried *in vacuo* [12].

The crude polysaccharide was dissolved in distilled water and 10% trichloroacetic acid was added to precipitate any protein. The solution was passed through Celite 545 and dialyzed at 4°C for 3 days. The dialysate was deionized by passage through a cation exchange column composed of Amberlite 120A H⁺ (Organo, Japan). The solution was dialyzed against distilled water for 24 h at room temperature and subsequently lyophilized [12].

Synthesis of the authentic 6-deoxy-D-altrose

The authentic 6-deoxy-D-altrose was synthesized from D-mannose *via* the D-rhamnoside [14]. The selective 3-O-benzoylation of the D-rhamnoside was accomplished using stannylation, in which an unprotected starting material was activated with dibutyltin oxide, benzylated in the presence of benzyl bromide and tetrabutylammonium iodide, and finally acetylated to afford the 3-O-benzylated derivative in 79% yield over two steps. The deprotection of benzyl group, which occurred without acetyl group migration, was accomplished in 98% yield by hydrogenolysis over palladium on carbon at atmospheric pressure. Next, the triflation was achieved using triflic anhydride and pyridine in dichloromethane to give the triflate, which was treated with tetrabutylammonium acetate in toluene to afford the expected 6-deoxy-D-altropyranoside in 71% over two steps. The ¹H NMR data of the compound confirmed that the desired structure with the signal at 5.17 (dd, 1 Hz, $J_{2,3} = 2.0$ Hz, $J_{3,4} = 3.5$ Hz, H-3) was synthesized. A dramatic change in the $J_{3,4}$ value from 9.6 Hz to 3.5 Hz indicated that the inversion of configuration had occurred at C-3. The oxidative removal of the *p*-methoxyphenyl group of the per-O-acetylated 6-deoxy-D-altropyranoside with ceric ammonium nitrate (CAN) afforded the hemiacetal in 68% yield, which was tentatively protected with a tetrahydropyranyl (THP) group to avoid any side reactions that may occur under the basic conditions in the next reaction. The removal of the acetyl groups under Zemplen conditions with subsequent acid hydrolysis of the THP group provided the desired free 6-deoxy-D-altrose in 78% over two steps.

Chemical procedures

The total carbohydrate content was determined with the phenol-sulfuric acid method using D-glucose as a standard [15]. The purified polysaccharide (70 mg) was dissolved in distilled water (20 mL) and sulfuric acid was added to reach a final concentration of 1.0 M. The mixture was subsequently heated to 100°C for 3 h. The hydrolysate was neutralized with BaCO₃.

Ascending paper chromatography of the acid hydrolysate from the polysaccharide, as well as authentic D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, L-fucose, and L-rhamnose, was performed on filter paper (No. 50; Advantec, Japan) using an eluent composed of butanol-ethanol-water (4:1:5, by volumes). Spots composed of reducing sugars were

stained by spraying with the aniline hydrogen phthalate reagent and subsequent heating at 105°C for 4 min.

The unknown sugar was separated by paper chromatography (Advantec Filter Paper No. 50; butanol-ethanol-water=4:1:5 as solvent). The band attributed to the unknown sugar (Spot 1: Rf 0.41) was cut off and extracted with distilled water at 4°C for 24 h. The extract was concentrated and freeze-dried.

Optical rotation was measured at 589 nm on a polarimeter (P-1100, Japan Spectroscopic Co., Ltd., Japan) for 0.2% (W/V) of polysaccharide, 6-deoxy-sugar (0.13%) and authentic 6-deoxy-D-altrose (0.11%) in distilled water at 25°C.

High-performance anion exchange chromatography coupled with the pulse amperometric detector (HPAEC-PAD)

The monosaccharides in the hydrolysate of the polysaccharide were identified using a HPAEC (DX-500, Dionex Co., CA, USA), fitted with CarboPack PA1 column and a pulsed amperometric detector. The column was eluted at flow rate of 1 mL/min at 35°C with 10 mM NaOH.

¹H- and ¹³C-Nuclear magnetic resonance (NMR) spectroscopy

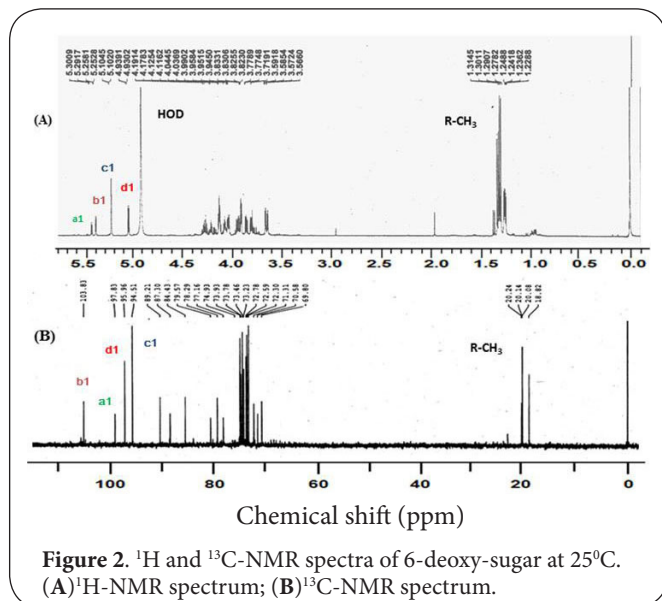
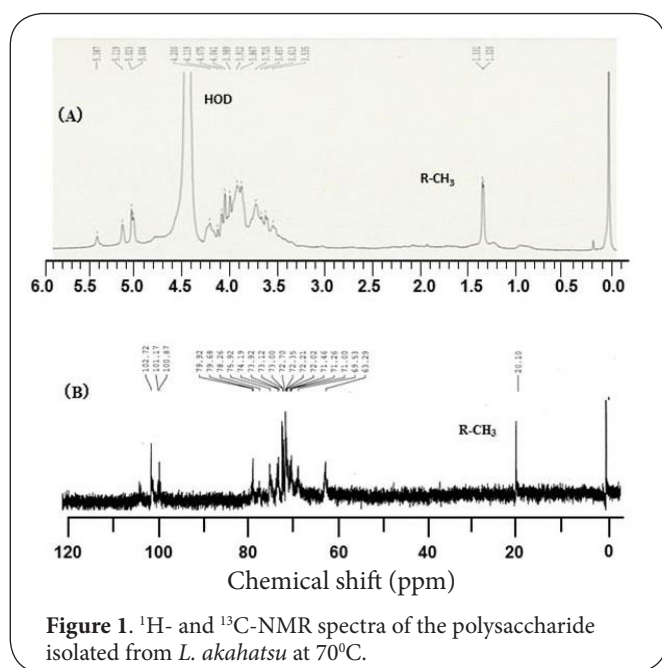
¹H- and ¹³C-NMR spectra were recorded on a FT-NMR spectrometer (AVANCE III 500 MHz, Bruker, Co., Ltd, Germany) at 500.00 and 125.65 MHz, respectively. The monosaccharide (2.0%, W/V), from the sample and the authentic 6-deoxy-D-altrose, as well as the polysaccharide (2%) were dissolved in D₂O and recorded at 25°C for the monosaccharide and at 70°C for polysaccharide. The ¹H- and ¹³C-NMR chemical shifts were expressed in parts per million (ppm) relative to sodium 3-(trimethylsilyl) propionic-2,2,3,3-d₄ acid (TSP, 0.00 ppm) as an internal standard. Two-dimensional spectra of ¹H-¹H COSY and ¹H-¹³C heteronuclear single quantum coherence (HSQC) were recorded using standard Bruker procedures [12,13].

Results and discussion

The isolated yield of the polysaccharide was estimated to be 1.3% (n=5), based on the dried weight. The polysaccharide contained 93.5% (W/W) of carbohydrate. The specific rotation [α]₅₈₉ of the polysaccharide (0.2%, W/V) was +23.8°, indicating that the α -configurations are predominated. Using high performance anion-exchange chromatography coupled with a pulse amperometric detector (HPAEC-PAD) to separate the hydrolysate of the polysaccharide, not shown in Figure, D-galactose and D-glucose were identified in a molar ratio 1.0:3.0.

The ¹H-NMR spectrum of the polysaccharide collected at 500 MHz was shown in (Figure 1A). The four signals were observed in the anomeric region (δ 5.5–4.5) at 5.387, 5.119, 5.023 and 5.004 ppm, respectively. In addition, a major signal at 1.331 and 1.320 ppm was also observed, indicating that a 6-deoxy-sugar was integrated into the polysaccharide.

The ¹³C-NMR spectrum of the polysaccharide was shown



in (Figure 1B). The three anomeric signals were observed at 103.17, 101.68, and 101.32 ppm, respectively. A methyl signal at 20.54 ppm was observed, indicating that a 6-deoxy-sugar was part of the polysaccharide.

Although the HPAEC-PAD data suggested that the polysaccharide consisted only of D-galactose and D-glucose, an unknown 6-deoxy-sugar, which would have overlapped with the peak of D-galactose or D-glucose in the HPAEC-PAD data, was detected in the ¹H- and ¹³C-NMR spectra. These results agreed with the data from the polysaccharide isolated from *L. lividatus* [12].

Paper chromatography of the acid hydrolysate of the polysaccharide was conducted and is displayed in (Supplement figure S1). Spot 1 (R_f, 0.41) was higher than the spot attributed to the standard L-rhamnosyl residue (0.37). Although D-glucose and D-galactose were detected with HPAEC-PAD, both sugars overlapped on the paper chromatogram (spot 2 and 3).

Using paper chromatography on a preparative scale, 6-deoxy-sugar (spot 1) was isolated from the mixture obtained by hydrolyzing the polysaccharide with acid and extracting the product with distilled water.

The ¹H-NMR of the isolated 6-deoxy-sugar was presented in (Figure 2A). As reported previously [12], the 6-deoxy-D-altrose existed in aqueous solution as a mixture of the α and β conformers of the pyranose and furanose forms. This phenomenon was also observed with 6-deoxy-L-altrose [16]. The spectrum of the 6-deoxy-sugar (Figure 2A) indicated four forms were present (a, b, c and d signals for H-1 at 5.3009 and 5.2917, 5.2581 and 5.2581, 5.1045 and 5.1020, and 4.9391 and 4.9302 ppm; for H-6 at 1.3145 and 1.3011, 1.2907 and 1.2782, 1.2488 and 1.2418, and 1.2326 and 1.2283 ppm, respectively). All of the signals agreed with the data collected from the authentic 6-deoxy-D-altrose, (not shown), as well as the sample of the 6-deoxy-D-altrose isolated from *L. lividatus*, previously reported [12].

The ¹³C-NMR spectrum of the 6-deoxy-sugar (Figure 2B) indicated the presence of four forms (signals for C-1 at 103.83, 97.83, 95.96, and 94.5 ppm, respectively; for C-6 at 20.24, 20.14, 20.01 and 18.82 ppm, respectively). All of the signals (H-1–H-6) for the 6-deoxy-sugar agreed with the data collected from the authentic 6-deoxy-D-altrose, (not shown), as well as the 6-deoxy-D-altrose isolated from *L. lividatus*.

The 2D COSY spectrum of the 6-deoxy-sugar was shown in (Figure 3). From the Figure, it was possible to correlate H-1 of a (δ 5.29) with H-2 (δ 4.07), H-2 with H-3 (δ 4.18), H-3 with H-4 (δ 3.71), H-4 with H-5 (δ 3.94) and H-5 with H-6 (δ 1.22); H-1 of b (δ 5.24) with H-2 (δ 4.01), H-2 with H-3 (δ 4.09), H-3 with H-4 (δ 3.76), H-4 with H-5 (δ 3.95) and H-5 with H-6 (δ 1.24); H-1 of c (δ 5.08) with H-2 (δ 3.80), H-2 with H-3 (δ 4.03), H-3 with H-4 (δ 3.56), H-4 with H-5 (δ 3.84) and H-5 with H-6 (δ 1.26); H-1 of d (δ 4.91) with H-2 (δ 3.75), H-2 with H-3 (δ 3.94), H-3 with H-4 (δ 3.70), H-4 with H-5 (δ 4.14) and H-5 with H-6 (δ 1.31). Almost all of the signals for the 6-deoxy-sugar agreed with the data from the authentic 6-deoxy-D-altrose and the 6-deoxy-D-altrose from *L. lividatus* [12]. The coupling constants for the 6-deoxy-sugar that were determined from COSY experiment were summarized in (Table 1). Almost all of the coupling constants for the 6-deoxy-sugar were identical with those of the authentic, (Supplement figure S2) and the 6-deoxy-D-altrose from *L. lividatus*. The results of the COSY experiment and the analysis of the HSQC spectrum (Figure 4) helped to assign residue a of the C-1, C-2, C-3, C-4, C-5 and C-6 peaks in the 6-deoxy-sugar to δ 95.4, δ 77.0, δ 74.2, δ 84.6, δ 68.1 and δ 20.2 ppm, respectively. The other residues (b, c and d) were

Table 1. ^1H and ^{13}C NMR data for mutarotating 6-deoxy-D-altrose from *L. akahatsu*.

Sample		C-1	C-2	C-3	C-4	C-5	C-6	H-1	H-2	H-3	H-4	H-5	H-6
6-Deoxy-sugar													
6-Deoxy-D-altp(d)^a	a	95.0	71.0	69.9	70.3	68.5	16.1	4.91	3.75	3.94	3.70	4.14	1.31
	24.7 ^{b%}	--	--	--	--	--	--	--	$J_{1,2}=4.1$	$J_{2,3}=6.2$	$J_{3,4}=3.2$	$J_{4,5}=6.2$	$J_{5,6}=6.2$
6-Deoxy-D-altp(c)^a	b	91.6	70.9	75.5	72.11	72.56	19.97	5.08	3.80	4.03	3.56	3.84	1.26
	49.1 ^{b%}	--	--	--	--	--	--	--	$J_{1,2}=1.1$	$J_{2,3}=3.4$	$J_{3,4}=3.2$	$J_{4,5}=8.8$	$J_{5,6}=6.3$
6-Deoxy-D-altf(b)^a	a	101.3	81.8	75.5	86.4	67.0	17.3	5.24	4.01	4.09	3.76	3.95	1.24
	16.8 ^{b%}	--	--	--	--	--	--	--	$J_{1,2}=2.7$	$J_{2,3}=4.0$	$J_{3,4}=5.3$	$J_{4,5}=5.2$	$J_{5,6}=6.2$
6-Deoxy-D-aktf(a)^a	b	95.4	77.0	74.2	84.6	68.1	20.2	5.29	4.07	4.18	3.71	3.94	1.22
	9.4 ^{b%}	--	--	--	--	--	--	--	$J_{1,2}=4.3$	$J_{2,3}=7.0$	$J_{3,4}=5.8$	$J_{4,5}=7.9$	$J_{5,6}=6.5$

^aThe four isomers were designated as residues a, b, c, and d according to their decreasing proton chemical shifts. ^bCalculated from the height of signals of the anomeric protons in **Figure 2**.

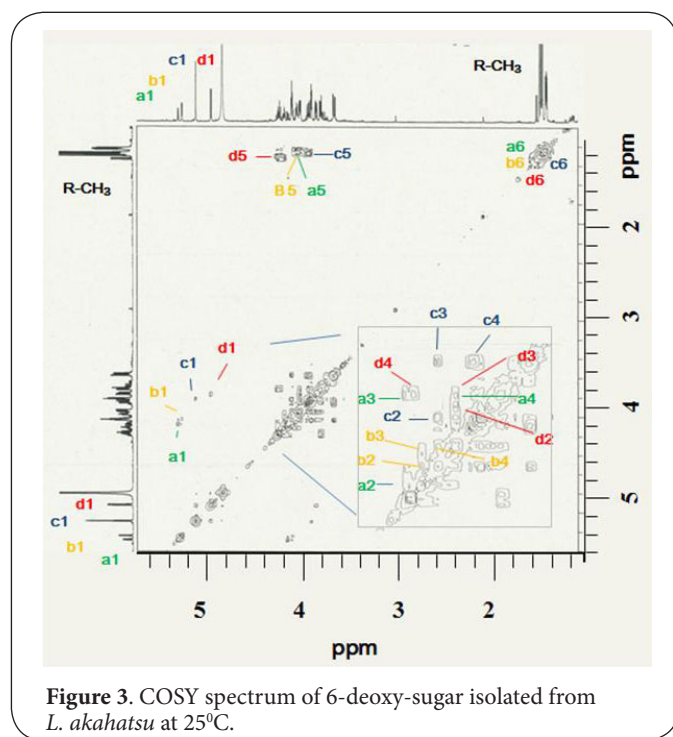


Figure 3. COSY spectrum of 6-deoxy-sugar isolated from *L. akahatsu* at 25°C.

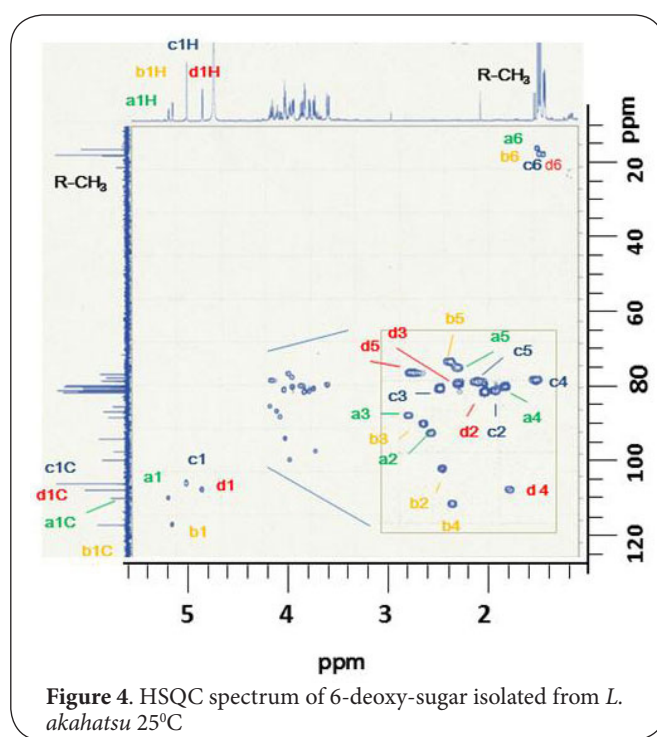


Figure 4. HSQC spectrum of 6-deoxy-sugar isolated from *L. akahatsu* 25°C

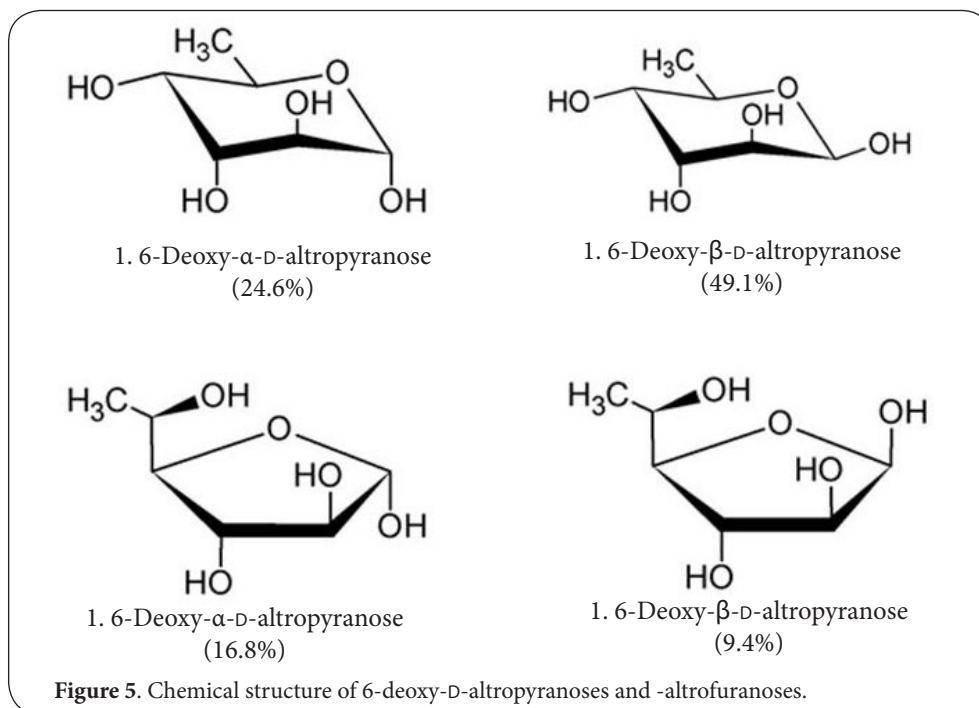
also assigned by a similar procedure and summarized in **Table 1**. Almost all of the signals of the 6-deoxy-sugar agreed with the signals of the authentic 6-deoxy-D-altrose, (**Supplement figure S3**), and the 6-deoxy-D-altrose isolated from *L. lividatus* [12]. Therefore, from the results, the 6-deoxy-sugar isolated from *L. akahatsu* was identified as 6-deoxy-D or L-altrose.

The specific rotation of the 6-deoxy-D or L-altrose isolated from *L. akahatsu* was estimated to be +17.6° (0.13%, at 25°C in H₂O). The values for the chemically synthesized authentic sample, as well as the material isolated from *L. lividatus*, were

+18.4° (0.13%, at 25°C) and +18.2° (0.11%), respectively. The specific rotation of the 6-deoxy-L-altrose has been reported as a negative value [17-19]. Therefore, this result indicates that the 6-deoxy-sugar isolated from *L. akahatsus* was identified as 6-deoxy-D-altrose.

Conclusions

In conclusion, by combining the results obtained in the current study with the data from a previous paper [12], four isomers of 6-deoxy-D-altrose (a, b, c and d) were identified as 6-deoxy-β-D-furanose (9.4%), 6-deoxy-α-D-furanose (16.8%),



6-deoxy- β -D-pyranose (49.1%) and 6-deoxy- α -D-altropyranose (24.6%) residue, respectively, as presented in (Figure 5). The signals for the 6-deoxy- α - and β -altropyranosyl residues were significantly more intense than the corresponding signals of α - and β -altrifuranosyl residues (Figure 2).

Finally, (Supplement figure S1) indicates that a large amount of the rare sugar, 6-deoxy-D-altrose, is available for isolation from the hydrolysate of the polysaccharide extracted from the fruiting bodies of *L. akahatsu* and *L. lividatus* [12]. Structural and biological investigations of the 6-deoxy-D-altrose and the polysaccharide from *L. akahatsu* are current in progress.

Additional files

Supplement figure S1
 Supplement figure S2
 Supplement figure S3

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	MT	JS	WJ	MY	HI	MK
Research concept and design	✓	--	--	--	✓	✓
Collection and/or assembly of data	✓	✓	✓	✓	--	--
Data analysis and interpretation	✓	--	--	--	✓	✓
Writing the article	✓	--	--	--	--	--
Critical revision of the article	✓	--	--	--	--	--
Final approval of article	✓	--	--	--	--	--
Statistical analysis	✓	--	--	--	--	--

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