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Chemical analysis of a new (1→3)-, (1→6)-branched glucan from an edible mushroom, *Pleurotus florida*

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Abstract—A glucan that was soluble in aqueous sodium chloride was isolated from the aqueous extract of the fruiting bodies of *Pleurotus florida*. On the basis of total hydrolysis, methylation analysis, periodate oxidation, Smith degradation, and NMR studies (^{1}H, ^{13}C, TOCSY, DQF-COSY, NOESY, and HSQC), the structure of the repeating unit of the polysaccharide is established as:

→3)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→
6
↑
1
α-D-Glc*p*

This glucan stimulates the phagocytic activity of macrophages.

Keywords: *Pleurotus florida*; Polysaccharide; Glucan; Structure; Macrophage activation

1. Introduction

Among the different types of oyster mushrooms of the genus *Pleurotus*, *Pleurotus sajor-caju*,[1–3] *Pleurotus citrinopileatus*,[4] *Pleurotus ostreatus*,[5] and *Pleurotus florida*[6] are commonly available edible mushrooms that contain antitumor substances. The white oyster mushroom, *P. florida*, is commercially available at the local markets in India during December–January when the temperature remains about 28 °C. Nutritive values[7] of the fruit bodies of *P. florida* have been determined as 37.19% protein, 3.72% fat, and 10.98% ash on a dry weight basis. This mushroom contains 0.5% total lipid in which the content of neutral lipids, glycolipids, and phospholipids has been reported.[8] Fruit bodies of *P. florida* are reported to grow on rice straw supplemented with cotton-seed powder.[9] Extracellular insoluble glucans containing (1→3, 1→6)-β-D-linkages were isolated from the liquid cultures of the fungi genus *Pleurotus*.[10] Several mushroom polysaccharides have been developed, which are used as biological response modifiers (BRMs) or immunopotentiators.[11] BRMs are those materials that modify the host's biological response by the stimulation of the immune system and may be used for various therapeutic purposes. These polysaccharides also demonstrate remarkable antitumor activity against Sarcoma-180 in mice. Several polysaccharides with carcinostatic effects have been developed and commercialized using the fruit bodies of *Lentinus edodes*[12,13] (lantinan, Japan) and the liquid-cultured broth product of *Schizophyllum commune* (Sonifilan, SPG, schizophyllan).[14,15] But no work relating to the structural features of this polysaccharide from *P. florida* has been reported. In the present study, a NaCl-soluble glucan consisting of (1→3, 1→6)-linked D-glucose moieties with both α and β linkages has been

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isolated for the first time. This glucan shows strong activation of the phagocytic response in macrophages. In recent years, mushroom polysaccharides have drawn the attention of chemists and immunobiologists on account of their immunomodulatory[11] and antitumor properties.[16,17] Hence, detailed structural studies of this glucan were carried out and are reported herein.

2. Results and discussion

A mixture of polysaccharides (2.3 g) was isolated from the mushroom, *P. florida*, (2 kg) by hot water extraction, followed by precipitation in EtOH and centrifugation. It was then dissolved in minimum volume of water, and exhaustive dialysis was carried out to remove small carbohydrate molecules. The dialyzed material was freeze dried and collected (yield 2 g). The crude polysaccharide was then allowed to dissolve in 1% NaCl solution to give two fractions, a NaCl-soluble fraction and a NaCl-insoluble fraction. The NaCl-soluble fraction (30 mg) on fractionation through a Sephadex G-75 gel column yielded two polysaccharide fractions, Fraction-I (7 mg) and Fraction-II (12 mg). Total hydrolysis of Fraction-I showed that it is a glucan and Fraction-II a heteroglucan. Fraction-I was further purified by dissolving it in Me_2SO, with precipitation in EtOH.[5] Further work was carried out with Fraction-I (PS) and reported herein.

The polysaccharide (2 mg) on acid hydrolysis, followed by GLC and paper chromatographic (PC) analysis, showed the presence of only glucose. The absolute configuration[18] of the glucose (Glc) was determined as D. The total sugar content of this polysaccharide was estimated by the phenol–sulfuric acid method,[19] and it was found to be 99.2%. Its molecular weight,[20] determined from a calibration curve prepared with standard dextrans, was found to be ~40,000 Da. The polysaccharide was methylated according to the method of Ciucanu and Kerek.[21] GLC and GLC–MS analyses of the alditol acetates obtained from the hydrolyzate of the methylated polysaccharide revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (*m*/*z* 43, 45, 71, 87, 101, 117, 129, 145, 161, 205), 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol (*m*/*z* 43, 45, 87, 101, 117, 129, 161, 233), and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol (*m*/*z* 43, 87, 117, 129, 189) in a ratio of nearly 1:2:1, respectively. These results indicate the presence of nonreducing-end D-glucopyranosyl, (1→3)-linked D-glucopyranosyl, and (1→3, 1→6)-linked D-glucopyranosyl (branch point) moieties in the glucan. Further, GLC analysis of the alditol acetates of the periodate-oxidized, reduced, methylated polysaccharide showed the presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol in a ratio of nearly 1:2. The absence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol indicates that the nonreducing end D-glucopyranosyl moiety is consumed during oxidation.

A Smith degradation[22,23] experiment was carried out with Fraction-I. The degraded polysaccharide was methylated, and the alditol acetates on GLC–MS analysis revealed the presence of only the 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucopyranosyl unit as the sole product. This result further confirms that the nonreducing end D-glucopyranosyl residue attached to the branching point at C-6 of glucan was completely consumed during oxidation, and a linear (1→3)-linked D-glucan is produced.

The FTIR spectrum of the polysaccharide showed bands at 850 cm^{-1} and 900 cm^{-1} corresponding to the presence of both α and β configurations, respectively. In addition to these characteristic bands, the spectrum showed the bands also at 1039, 1076, 1159, and 1120 cm^{-1} owing to the presence of (1→3)-di-*O*- substituted glucose residues.[10]

The 500-MHz ^{1}H NMR spectrum (Fig. 1) of the polysaccharide recorded in D_2O at 27 °C showed three anomeric proton signals at δ 5.09, δ 4.95, and δ 4.44 ppm that were assigned as (1→3)-α-D-Glc*p* (residue **I**), α-D-Glc*p*-(1→ (residue **II**), and (1→3, 1→6)-β-D-Glc*p* (residue **III**), respectively, in a ratio of nearly 2:1:1. All the proton signals of the polysaccharide (Table 1) were assigned on the basis of the correlation of TOCSY and DQF-COSY NMR experiments. This indicates that this glucan is composed of tetrasaccharide repeating units. The anomeric carbon signals for the **I**, **II**, and **III** residues appear at δ 100.1, 99.8, and 103.1 ppm, respectively, in a ratio of nearly 2:1:1. Thus, the ^{13}C NMR spectrum (Fig. 2) is also in agreement with the suggested tetrasaccharide repeating unit.

The large $^3J_{2,3}$, $^3J_{3,4}$, and $^3J_{4,5}$ coupling constant values of 8–10 Hz in residues **I**, **II**, and **III** support the presence of the D-glucopyranosyl configuration in the polysaccharide. Residue **I** (δ 5.09) was assigned to 3-*O*-substituted α-D-glucopyranose. The coupling constant values of

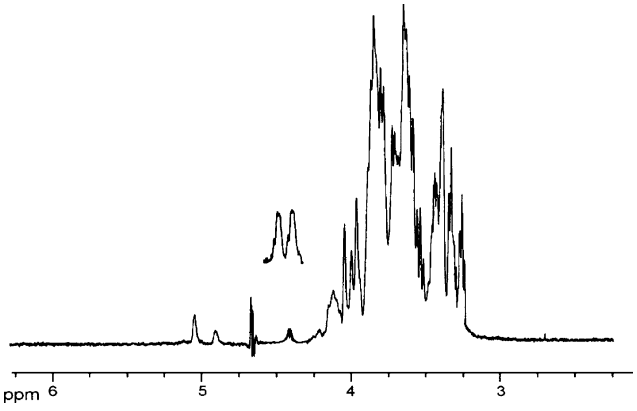

Figure 1. ^{1}H NMR (500 MHz, D_2O, 27 °C) spectrum of polysaccharide (Fraction-I) isolated from *Pleurotus florida*.

Table 1. ^{1}H and ^{13}C NMR data for the polysaccharide of *Pleurotus florida*[a]

Atoms	Residues		
	→3)-α-D-Glc*p* (1→ **I**	α-D-Glc*p* (1→ **II**	→3,6)-β-D-Glc*p* (1→ **III**
H-1	5.09	4.95	4.44
H-2	3.88	3.69	3.30
H-3	4.08	4.05	3.37
H-4	3.65	3.54	3.59
H-5	3.95	3.81	3.42
H-6	4.0, 4.27	3.97, 4.24	3.85, 4.16
C-1	100.1	99.8	103.1
C-2	70.0	72.1	73.0
C-3	80.4	74.0	85.2
C-4	69.3	71.0	68.8
C-5	72.5	72.5	76.4
C-6	61.1	60.6	67.0

[a] Spectra were recorded in D_2O at 27 °C. Values of ^{1}H chemical shifts were taken with respect to the HOD signal fixed at δ 4.70 ppm. Values of ^{13}C chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 ppm at 27 °C.

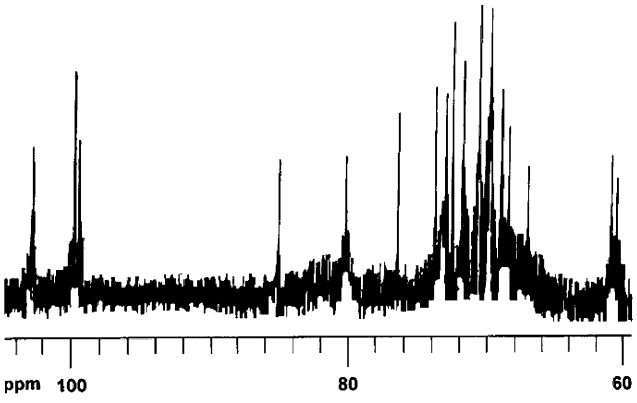

Figure 2. ^{13}C NMR (75 MHz, D_2O, 27 °C) spectrum of polysaccharide (Fraction-I) isolated from *Pleurotus florida*.

$^{3}J_{1,2} \sim 3.4$ Hz and $^{1}J_{C,H} \sim 176$ Hz indicate the α-configuration for **I**. Residue **II** (δ 4.95) was assigned to the terminal α-D-glucopyranosyl residue with coupling constants of $^{3}J_{1,2} \sim 3.1$ Hz and $^{1}J_{C,H} \sim 174$ Hz, indicating the α-configuration present in **II** also. Residue **III** (δ 4.44) was determined to be (1→3, 1→6)-linked β-D-glucopyranose. The coupling constant $^{3}J_{1,2} \sim 7.5$ Hz indicates a β-linkage, which is further corroborated by the $^{1}J_{C,H} \sim 160$ Hz. The 75-MHz ^{13}C NMR spectrum of the glucan at 27 °C shows the carbon signal at δ 85.2 ppm due to C-3 of the β-(1→3, 1→6)-linked D-glucopyranosyl moiety (residue **III**), which is shifted 8.4 ppm downfield compared to the resonance of standard methyl glycoside due to the α effect of glycosylation.[24,25] Similarly, the C-3 signal at δ 80.4 ppm of the α-(1→3)-linked D-glucopyranosyl moiety (Residue **I**) appears at 6.3 ppm downfield compared to that of the standard methyl glycoside value. The C-6 signal of (1→3, 1→6)-linked D-glucose appears at δ 67.0 ppm due to the α-glycosylation effect. The carbon signals at δ 61.1 ppm and δ 60.6 ppm are due to C-6 of the (1→3)-linked D-glucose and the nonreducing-end D-glucopyranosyl moieties, respectively. The C-2 (73.0 ppm), C-4 (68.8 ppm), and C-5 (76.4 ppm) signals of residue **III** appear at 2.2 ppm, 1.9 ppm, and 0.4 ppm upfield compared to the literature values of methyl glycosides due to the β glycosylation effect.[24,25] The C-2 (70.0 ppm) and C-4 (69.3 ppm) signals of residue **I** are observed at 2.4 and 3.0 ppm upfield for the same reason. The signals at 72.1, 74.0, 71.0, 72.5, and 60.6 ppm are due to C-2, C-3, C-4, C-5, and C-6 of the nonreducing-end α-D-glucopyranosyl unit. These values (Table 1) were further corroborated by the HSQC experiment.

For confirming the linkages, an NMR experiment was carried out with Smith-degraded material. It was very interesting that the native polysaccharide was moderately water soluble, but the Smith-degraded polysaccharide was insoluble in water. So, in case of the native polysaccharide, ^{1}H and ^{13}C NMR experiments were performed with D_2O at 27°C, which produced a better ^{13}C spectrum than that run with Me_2SO-d_6 at the same temperature. The ^{13}C NMR spectrum (Fig. 3, Table 2) of Smith-degraded polysaccharide in Me_2SO-d_6 at 27 °C gave twelve well-defined signals. The signals present in a ratio of 1:2 at δ 103.4 and 100.2 ppm are due to C-1 of (1→3)-β- and (1→3)-α-linked D-glucopyranose units, respectively. No signal at 67.0 ppm was observed in the ^{13}C NMR spectrum of the Smith-degraded polysaccharide, which confirms that the nonreducing-end D-glucopyranosyl unit was attached at the C-6 position of the D-glucopyranosyl unit (unit **III**) in the native polysaccharide. The two other signals present in a ratio of 2:1 at 60.5 and 61.2 ppm appeared due to the unsubstituted C-6 of two α-D-glucosyl and one β-D-glucosyl units in the Smith-degraded polysaccharide. Further, the ^{13}C

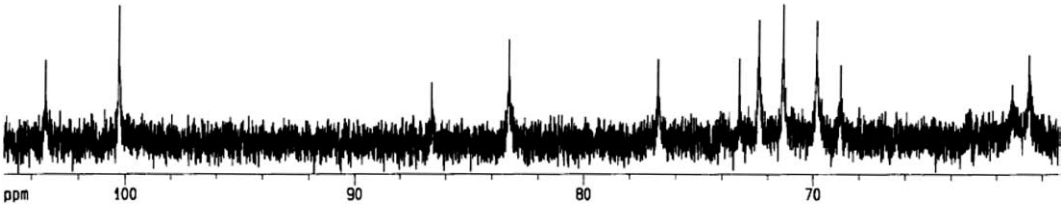

Figure 3. ^{13}C NMR (75 MHz, Me_2SO-d_6, 27 °C) spectrum of Smith-degraded polysaccharide.

Table 2. ^{13}C NMR data for Smith-degraded polysaccharide[a]

Atoms	Residues	
	→3)-α-D-Glc*p* (1→	→3)-β-D-Glc*p* (1→
C-1	100.2	103.4
C-2	71.3	73.2
C-3	83.2	86.6
C-4	69.8	68.8
C-5	72.3	76.7
C-6	60.5	61.2

[a] Spectra were recorded in Me_2SO-d_6 at 27 °C. Values of chemical shifts were referenced to acetone as the internal standard and fixed at δ 31.05 ppm at 27 °C.

spectrum of the Smith-degraded polysaccharide showed no anomeric signal at 99.8 ppm, which clearly indicates that the nonreducing-end D-glucopyranose residue in the native glucan is α-linked. In the degraded polysaccharide, all ^{13}C carbon signals of the (1→3)-α-linked D-glucopyranose moiety appeared nearly double that of the (1→3)-β-linked D-glucopyranose moiety. The appearance of a (1→3)-β-linked D-glucopyranosyl moiety in Smith-degraded glucan further supports the contention that the nonreducing-end D-glucopyranose moiety was attached to the C-6 position of the β-(1→3, 1→6)-linked D-glucopyranose moiety in the native polysaccharide. The C-3 signals of β-(1→3) and α-(1→3)-linked D-glucose of the Smith-degraded polysaccharide show downfield shifts due to the α-glycosylation effect, appearing at δ 86.6 and 83.2 ppm, respectively. Thus, from all these observations the structure of the repeating unit of the Smith-degraded glucan is established as

→3)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→

The sequence of glucopyranosyl residues was determined on the basis of a 2D-NOESY experiment. Residue **II** has an NOE contact from H-1 to H-6 of residue **III** in addition to intraresidue NOE contacts to H-2 and H-4. Hence, residue **II** is linked at the 6-position of residue **III**. Moiety **I** has a strong interresidue NOE contact from H-1 to H-3 of moiety **III** in addition to strong intraresidue contacts to H-2 and H-3, indicating that moiety **I** is linked to the 3-position of moiety **III**. Again, moiety **III** has a strong NOE contact from H-1 to H-3 of moiety **I** along with strong intraresidue contacts to H-3, H-5, and H-6, indicating that **III** is also linked to 3-position of moiety **I**. Thus, **III** is a 1,3,6-linked moiety.

Hence, from our chemical investigations and NMR spectral analysis of both native and Smith-degraded glucan, the following tetrasaccharide repeating unit of the polysaccharide is established as

I **III** **I**
→3)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→
6
↑
1
α-D-Glc*p*
II

Preliminary studies regarding the conformation of this polysaccharide were carried out by studying the changes of specific rotation and absorption spectra of the complexes formed with Congo Red[26,20] at various concentrations of alkali. Different types of glucan (AM-ASN, T-5-N) isolated from *Amanita muscaria*[27] and *Dictyophora indusiata* FISCH[20] showed abrupt changes in specific rotation values with increasing sodium hydroxide concentration. These also form complexes with Congo Red, changing λ_{max} from 490 to 511 nm in low concentration of alkali (0.05 M to <0.15 M) indicating that they have a triple-helical structure. The present polysaccharide shows a low value of specific rotation in neutral solution ($[\alpha]_D^{25}$ +0.064, *c* 0.8, water), and no abrupt change occurs with increasing the concentration of alkali. Furthermore, the polysaccharide does not show an appreciable absorption maximum with Congo Red. These results indicate that this polysaccharide may exist in either a random-coil or single-helix conformation.[28,29] Detailed conformational studies using spectroscopy and other physical methods are in progress.

This polysaccharide was found to activate the phagocytic response of macrophages (Table 3). A similar kind of material like lentinan (a β-glucan) inhibits tumor growth by stimulating the immune system[30] through activation of macrophages, T-helper, NK and other cells. Increased phagocytic activity was reported after incubating mice macrophages with this polysaccharide. The most effective dose of this glucan was observed at 1 mg/mL.

3. Experimental

3.1. Isolation of the polysaccharide

Two kilograms of fresh mushroom fruit bodies was collected from a local firm where they were grown on

Table 3. Stimulation of mice macrophages by the polysaccharide of *Pleurotus florida* by NO production assay at 550 nm[a]

Sample	Concentration of polysaccharide					
	10 ng/mL	100 ng/mL	1 μg/mL	10 μg/mL	100 μg/mL	1.0 mg/mL
PS	1.0145	1.135	1.158	1.190	1.290	1.435

[a] Control reading: 1.00.

rice straw supplemented with cottonseed powder at a temperature of 28 °C. After washing with distilled water and EtOH, the mushroom bodies were pulverized for extraction of polysaccharide by boiling with water for 4 h as applied in our earlier works.[3,31,32] The aqueous extract was filtered with linen cloth, and the filtrate was collected and kept overnight at 4 °C. The filtrate was centrifuged at 8000 rpm at 6 °C for 45 min to obtain a clear solution, and then the polysaccharide was precipitated with EtOH (80%, final concentration). After keeping the precipitate at 4 °C overnight, it was collected by centrifugation at 6 °C for 1 h. Then, it was dissolved in minimum volume of water and dialyzed against distilled water for 12 h to remove low-molecular-weight carbohydrate materials. The aqueous extract was then reprecipitated with EtOH (1:5, v/v) and centrifuged. The centrifugate was collected and freeze dried (yield, 2 g).

3.2. Fractionation of the polysaccharide

When this polysaccharide was dissolved in 1% sodium chloride solution, one portion became soluble while another portion remained insoluble. These two parts were separated by centrifugation. The soluble part was purified by gel-permeation chromatography. The polysaccharide (30 mg) was dissolved in 1% sodium chloride solution (3 mL). The soluble portion was separated and applied all at once to a Sephadex G-75 column (100 cm × 1 cm) saturated with 1% sodium chloride solution and fractionated (0.2 mL/min) using a Redifrac fraction collector. Each fraction (2 mL) was collected and analyzed with phenol–sulfuric acid reagent[21] at 490 nm using a Shimadzu UV–vis spectrophotometer, model 1601. Fractions (test tubes, 40–52 and 56–76) corresponding to two peaks were pooled together separately and freeze dried. After reducing the volume by freeze drying, the fractions were dialyzed against distilled water to remove NaCl and then again freeze dried (yield, Fraction-I, 7 mg and Fraction-II, 12 mg). This same procedure was followed several times. Fraction-I (30 mg) was further purified by dissolving it in 100 mL of Me_2SO by stirring for 24 h at room temperature. To the Me_2SO solution an equal volume of EtOH was added under vigorous stirring, and the mixture was kept overnight at 4 °C. A precipitate (6 mg) was collected by centrifugation at 8000 rpm for 30 min, and it was identified as (1→6)-α-D-glucan and reported.[6] The present polysaccharide was recovered from the supernatant by dialyzing against distilled water and then freeze dried (yield, 18 mg). The apparent molecular weight of Fraction-I was estimated using standard dextrans (T_{100}, T_{40}, and T_{10} from Pharmacia). Total carbohydrate of the polysaccharide (Fraction-I) was determined using the phenol–sulfuric acid reagent.[21]

3.3. Monosaccharide composition

The polysaccharide sample (3 mg) was hydrolyzed in 2 M trifluoroacetic acid (2 mL) at 100 °C for 16 h in a boiling water bath. The hydrolyzate was then converted into its respective alditol acetates and analyzed by gas–liquid chromatography (GLC) using a Hewlett–Packard model 5730 instrument equipped with a flame-ionization detector. Peaks were identified and estimated with arabinose as the internal standard. The alditol acetates were resolved on a glass column (1.8 m × 6 mm) containing 3% ECNSS-M on Gas Chrom Q (100–120 mesh) at 170 °C. Gas–liquid chromatography–mass spectrometric (GLC–MS) analysis was also performed on a Hewlett–Packard 5970A automatic GLC–MS system, using an HP-5 capillary column (25 m × 25 mm) and a temperature program starting at 150 °C (2 min), followed by an increase of 2 °C/min to 200 °C (5 min). Quantitation was carried out from the peak area, using response factors from standard monosaccharides.

3.4. Paper chromatographic studies

Paper partition chromatographic studies were performed on Whatmann nos. 1 and 3 mm sheets. Solvent systems used were: (X) BuOH–HOAc–H_2O (v/v/v, 4:1:5, upper phase) and (Y) EtOAc–pyridine–H_2O (v/v/v, 8:2:1). The spray reagent used was alkaline silver nitrate solution.[33]

3.5. Absolute configuration of the monosaccharides

The method used was based on Gerwig et al.[18] The polysaccharide (1 mg) was hydrolyzed by trifluoroacetic acid, and the acid was removed by co-distillation with water. A solution of 250 μL of 0.625 M HCl in (+)-2-butanol was added to it, and the mixture was heated at 80 °C for 16 h. The reactants were then evaporated, and per-*O*-TMS-derivatives were prepared with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The products were analyzed by GLC using a capillary column (SPB-1, 30 m × 0.26 mm) with a temperature program (3 °C/min) from 150 to 210 °C. The (+)-2-butyl 2,3,4,6-tetra-*O*-TMS-glycosides obtained were identified by comparison with those prepared from the D and L enantiomers of the monosaccharides.

3.6. Methylation analysis

The polysaccharide (4 mg) was methylated according to the methods of Ciucanu and Kerek,[21] and the products were isolated by partitioning between chloroform and water (5:2). The organic layer containing products was washed with 3 mL water for three times and dried. The methylated products were then hydrolyzed with 90% formic acid (1 mL) at 100 °C for 1 h, reduced with

$NaBH_4$, acetylated with 1:1 Ac_2O–pyridine and analyzed by GLC and GLC–MS using an HP-5 capillary column and the same temperature program indicated above. Quantitations were carried out from peak areas.

3.7. Periodate oxidation

The polysaccharide (10 mg) was oxidized with 0.1 M sodium metaperiodate (2 mL) at 27 °C in the dark during 48 h. The oxidation process was stopped by addition of 1,2-ethanediol, and the solution was dialyzed against distilled water. The dialyzed material was reduced with $NaBH_4$ for 15 h and neutralized with HOAc. The resulting material was obtained by co-distillation with MeOH. It was subjected to both hydrolysis and methylation by as usual procedures described above and analyzed by GLC–MS.

3.8. Smith degradation

The polysaccharide (PS) (50 mg) was oxidized with 0.1 M sodium metaperiodate (10 mL) at 27 °C in the dark during 48 h. The oxidation was stopped by the addition of 1,2-ethanediol, and the solution was dialyzed against distilled H_2O. The dialyzed material was reduced with $NaBH_4$ for 15 h at 27 °C, neutralized with 50% HOAc and again dialyzed against distilled H_2O, and freeze dried. This portion was subjected to mild hydrolysis with 0.5 M trifluoroacetic acid for 15 h at 25 °C to eliminate the residues of oxidized sugars attached to the polysaccharide chain (Smith degradation). Acid was removed after repeated addition and evaporation of water at 37 °C. Finally, it was purified through exhaustive dialysis against distilled H_2O and freeze dried; yield 14 mg. A part of this polymeric material (2 mg) was methylated and analyzed as usual by GLC–MS. The remainder was used for ^{13}C NMR studies.

3.9. FTIR analysis

The IR spectrum was recorded with dried polysaccharide (1.1 mg) on a Jasco FTIR model 6200 using a solid-state ATR accessory.

3.10. NMR spectroscopy

The freeze-dried polysaccharide was kept over P_2O_5 in vacuum for several days and then deuterium exchanged three times, followed by lyophilization[34] with D_2O. The ^1H, TOCSY, DQF-COSY, and NOESY NMR spectra were recorded with a Bruker Avance DPX-500 spectrometer in D_2O at 27 °C. Chemical shifts were referred to the residual signal of HOD at δ 4.70 ppm. The TOCSY experiment was recorded with a mixing time of 150 ms, and complete assignment required several TOCSY experiments requiring several mixing times ranging from 60 to 300 ms. The NOESY mixing delay was 200 ms. A DQF-COSY spectrum was obtained using standard pulse sequences. The ^{13}C spectrum of native polysaccharide (PS) solution in D_2O was recorded with a Bruker Avance DPX-300 instrument, and that of the degraded polysaccharide solution in Me_2SO-d_6 was recorded with the same instrument at 27 °C. Acetone was used (δ 31.05 ppm) as an internal standard for both ^{13}C NMR spectra.

3.11. Interaction with Congo Red

The polysaccharide sample (2.5 mg) was dissolved in 2.5 mL of water containing Congo Red (91 μM). The concentration of alkali was increased from 0 to 0.35 M by addition of 4 M sodium hydroxide solution to the sample solutions. UV–vis absorption spectra were recorded with a Shimadzu model 1601 spectrophotometer.

3.12. Specific rotations in aqueous sodium hydroxide

The polysaccharide (8 mg) was dissolved in water (10 mL), and the concentration of alkali was increased from 0 to 0.35 M by stepwise addition of 4 M NaOH solution. Specific rotations were measured with a Jasco P 1020 automatic polarimeter at 25 °C at each concentration of alkali.

3.13. Test for macrophage activity by nitric oxide assay

Peritoneal macrophages (10^6 cell/mL) after harvesting were cultivated in complete RPMI medium in well plates.[35] The present polysaccharide was added to the wells at different concentrations (10 ng/mL to 1.0 mg/mL). The cells were cultured for 24 h at 37 °C in a humidified 5% CO_2 incubator. Production of NO was estimated by measuring the nitrite levels in the cell supernatant with the Greiss reaction.[36]. Equal volumes of Greiss reagent (1:1 of 0.1% in 1-napthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid) and sample cell supernatant were incubated together at room temperature for 10 min. Absorbance was read at 550 nm.

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References

1. Dong, Y.; Xi, J. *The Edible Mushrooms in China*; China Tourist Publishing House: Beijing, 1988; pp 202–216.
2. Zhuang, C.; Mizuno, T.; Shimada, A.; Ito, H.; Suzuki, C.; Mayuzumi, Y.; Okamato, H.; Ma, Y.; Li, J. *Biosci. Biotechnol. Biochem.* **1993**, *57*, 901–906.
3. Pramanik, M.; Mondal, S.; Chakraborty, I.; Rout, D.; Islam, S. S. *Carbohydr. Res.* **2005**, *340*, 629–636.
4. Zhang, J.; Wang, G.; Li, H.; Zhuang, C.; Mizuno, T.; Ito, H.; Suzuki, C.; Okamato, H.; Li, J. *Biosci. Biotech. Biochem.* **1994**, *58*, 1195–1201.
5. Yoshioka, Y.; Tabeta, R.; Satio, H.; Uehara, N.; Fukuoka, F. *Carbohydr. Res.* **1985**, *140*, 93–100.
6. Rout, D.; Mondal, S.; Chakraborty, I.; Pramanik, M.; Islam, S. S. *Med. Chem. Res.* **2004**, *13*, 509–517.
7. Kwon, Y. J.; Uhm, T. B. *Hanguk Yongyang Siklyong Hakhoechi* **1984**, *13*, 175–180.
8. Rajarathnam, S.; Shashirekha, M. N.; Bano, Z. *World J. Microbiol. Biotechnol.* **2001**, *17*, 221–227.
9. De, C. R.; Marcia, R. T.; Sturion, G. L. *Arch. Latinoam. Nutr.* **1998**, *48*, 339–348.
10. Gutiérrez, A.; Prieto, A.; Mertínez, A. T. *Carbohydr. Res.* **1996**, *281*, 143–154.
11. Wasser, S. P.; Weis, A. L. *Int. J. Med. Mushroom* **1999**, *1*, 31–62.
12. Chihara, G.; Maeda, Y. Y.; Hamuro, J.; Sasaki, T.; Fukuoki, F. *Nature* **1969**, *222*, 667–668.
13. Sasaki, T.; Taksuka, N. *Carbohydr. Res.* **1976**, *47*, 99–104.
14. Kikumoto, S. T.; Miyajina, T.; Yoshijumi, S.; Fujimoto, S.; Kimura, K. *Nippon Nogei Kagaku Kaishi (Jpn. J. Agric. Chem.)* **1971**, *44*, 337–342.
15. Kikumoto, S. T.; Miyajina, T.; Kimura, K.; Okubo, S.; Kamatsu, N. *Nippon Nogei Kagaku Kaishi (Jpn. J. Agric. Chem.)* **1971**, *45*, 337–342.
16. Kishida, E.; Sone, Y.; Misaki, A. *Carbohydr. Res.* **1989**, *193*, 227–239.
17. Maeda, Y. Y.; Chihara, G. In *Immunomodulatory Agents from Plants*; Wagner, H., Ed.; Birkhäuser: Basel, 1999; pp 203–221.
18. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. *Carbohydr. Res.* **1978**, *62*, 349–357.
19. Chaplin, M. F.; Kenedy, J. F. *Carbohydrate Analysis: A Practical Approach*; IRL Press: Oxford, 1986.
20. Hara, C.; Kiho, T.; Tanaka, Y.; Ukai, S. *Carbohydr. Res.* **1982**, *110*, 77–87.
21. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
22. Datta, A. K.; Basu, S.; Roy, N. *Carbohydr. Res.* **1999**, *322*, 219–227.
23. Abdel-Akher, M.; Hamilton, J. K.; Montgomery, R.; Smith, F. *J. Am. Chem. Soc.* **1952**, *74*, 4970–4971.
24. Agrawal, P. K. *Phytochemistry* **1992**, *31*, 3307–3330.
25. Rinaudo, R.; Vincendon, M. *Carbohydr. Polym.* **1982**, *2*, 135–144.
26. Tabata, K.; Ito, W.; Kojima, T. *Carbohydr. Res.* **1981**, *89*, 121–135.
27. Kiho, T.; Katsuragawa, M.; Nagai, K.; Ukai, S. *Carbohydr. Res.* **1992**, *224*, 237–243.
28. Harada, T.; Misaki, A.; Saito, H. *Arch. Biochem. Biophys.* **1968**, *124*, 292–298.
29. Rees, D. A. *J. Chem. Soc. B* **1969**, 217–226.
30. Chihara, G. *Mushroom Sci.* **1978**, *9*, 797–814.
31. Mondal, S.; Chakraborty, I.; Pramanik, M.; Rout, D.; Islam, S. S. *Carbohydr. Res.* **2004**, *339*, 1135–1140.
32. Chakraborty, I.; Mondal, S.; Pramanik, M.; Rout, D.; Islam, S. S. *Carbohydr. Res.* **2004**, *339*, 2249–2254.
33. Hoffman, J.; Lindberg, B.; Svensson, S. *Acta Chem. Scand.* **1972**, *26*, 661–666.
34. Ensley, H. E.; Tobias, B.; Pretus, H. A.; McNamee, R. B.; Jones, E.; Browder, I. W.; Williams, D. L. *Carbohydr. Res.* **1994**, *258*, 307–311.
35. Ohno, N.; Hasimato, T.; Adachi, Y.; Yadomae, T. *Immunol. Lett.* **1992**, *52*, 1–7.
36. Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. *Anal. Biochem.* **1982**, *126*, 131–138.