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Structural investigation of a polysaccharide (Fr. I) isolated from the aqueous extract of an edible mushroom, *Volvariella diplasia*

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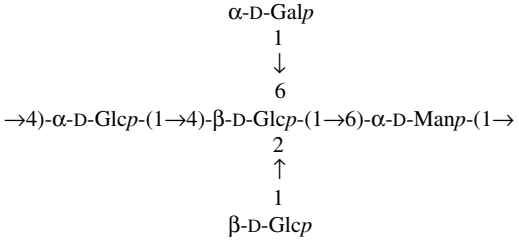
Abstract—A water-soluble polysaccharide, (Fr. I) isolated from the aqueous extract of an edible mushroom, *Volvariella diplasia*, is composed of D-glucose, D-mannose, and D-galactose in a molar ratio 3:1:1. Compositional analysis, methylation analysis, periodate oxidation study, Smith degradation, and NMR studies (^{1}H, ^{13}C, DQF-COSY, TOCSY, NOESY, ROESY, HMQC, and HMBC) revealed the presence of the following repeating unit in the polysaccharide:



Keywords: *Volvariella diplasia*; Polysaccharide; Structure; NMR spectroscopy

1. Introduction

Mushrooms of the genus *Volvariella*, namely *V. volvacea*, *V. bombycina*, and *V. diplasia*, are commonly available edible mushrooms with high nutritive value that grow abundantly in Southeast Asia. These belong to the well-known *Pluteaceae*[1] family, and are also known as straw mushrooms or paddy straw[1] mushrooms. Among the different species of *Volvariella, V. bombycina* and *V. diplasia* have been cultivated in India. The local people consume them as delicious food materials. These are available in local markets, especially in the town of Midnapur in large amounts. Nutritive values[2] of the fruit body *V. volvacea* have been analyzed and were reported to possess (92.41%) moisture, (2.19%) carbohydrate, (2.94%) protein, (0.40%) crude fat, (1.15%) crude fiber, (0.99%) ash, and (0.80%) nitrogen[3] on a dry weight basis. Ergosterol,[4] provitamin D2, was present in this mushroom (0.4% of dry material). The mycelium of *V. diplasia* was found to contain essential amino acids,[3] which are needed to build the proteins that make our bodies function. The local white straw mushroom *V. diplasia* is commercially available in June–July when the temperature remains nearly 30–35 °C.

Several glucans, including an α-(1→3)-glucan,[5–7] β-(1→3)-glucans,[8–13] β-(1→3)(1→6)-glucans,[14–17] a β-(1→6)-glucan,[18] an α-(1→4),β-(1→6)-glucan,[14,19] an α-(1→4)(1→6)-glucan,[14,19] an α-(1→3)(1→4)-glucan,[20]

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and an α-(1→3),β-(1→6)-glucan,[14] are widely used as antitumor and immunostimulating agents. Some linear glucans,[21–24] branched glucans,[25,26] and heteroglycans[27–29] have been reported by our group in *Carbohydrate Research*. Polysaccharides isolated from the medicinal[30] mushroom *V. volvacea* are used as dietary fiber.[31] The antitumor activity[32] of the polysaccharides was demonstrated in mice bearing sarcoma-180. In addition, these can reduce blood pressure,[33] exhibit a cardiovascular response,[34] and affect the biosorptions[35,36] of metal ions. No work has been reported on the polysaccharide, isolated from the aqueous extract of the mushroom *V. diplasia*. We are reporting herein the detailed structural studies of a polysaccharide (Fr. I) isolated from the hot water extract, followed by acetic acid treatment and gel filtration of *V. diplasia*.

2. Results and discussion

The fresh edible mushrooms *V. diplasia* (1.0 kg) were washed with distilled water and then extracted with hot water. The supernatant was collected by centrifugation and precipitated in EtOH (1:5, v/v). The precipitated material (polysaccharide) was lyophilized and then treated with 1% acetic acid solution to remove lipid material. The solution was centrifuged, dialyzed, and freeze dried, yielding 2.5 g of crude product. This material (30 mg) was fractionated through a Sepharose-6B column in aqueous medium in several lots, and two fractions (Fr. I, 12 mg and Fr. II, 10 mg) of polysaccharides were obtained by monitoring them through a spectrophotometer at 490 nm using the phenol–sulfuric acid method.[37] The molecular weight of Fr. I was estimated as $\sim 1.76 \times 10^5$ Da from a calibration curve prepared with standard dextrans.[38] We are reporting herein the structural characterization of Fr. I only.

The pure polysaccharide (Fr. I) has $[\alpha]_D^{25}$ +25.6 (*c* 0.68, water). The total sugar of Fr. I was estimated 98.08% using the phenol–sulfuric acid method.[37] On total hydrolysis with 2 M TFA, D-glucose, D-mannose, and D-galactose were detected by GLC analysis in a relative ratio of 3:1:1. The absolute configurations of the sugars were determined by the method of Gerwig et al.[39] Fr. I was methylated by the method of Ciucanu and Kerek[40] and then hydrolyzed. The alditol acetates of the methylated product were analyzed by GLC–MS. Fr. I showed 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol, and 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methyl-D-glucitol in a molar ratio 1:1: 1:1:1. These results indicated that terminal D-galactopyranosyl and D-glucopyranosyl, (1→6)-linked D-mannopyranosyl, (1→4)-linked D-glucopyranosyl, and (1→2,4,6)-linked D-glucopyranosyl moieties were present in Fr. I. The GLC analysis of alditol acetates of periodate-oxidized, reduced, and hydrolyzed product showed only the presence of D-glucose. The GLC analysis of periodate-oxidized, reduced, and methylated Fr. I showed the presence of 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methyl-D-glucitol. These results indicated that terminal D-galactopyranosyl and D-glucopyranosyl, (1→6)-linked D-mannopyranosyl and (1→4)-linked D-glucopyranosyl moieties were consumed during oxidation.

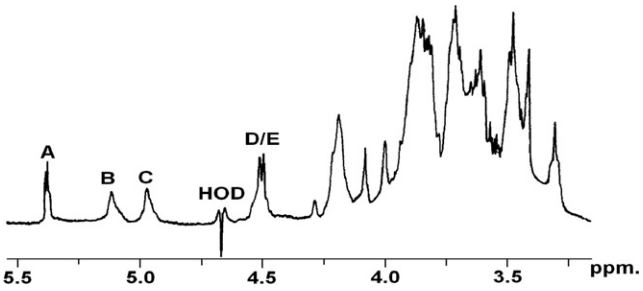


Figure 1. ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) of polysaccharide Fr. I, isolated from *Volvariella diplasia*.

The 500 MHz ^{1}H NMR spectrum of the polysaccharide Fr. I showed five anomeric proton signals at δ 5.38, 5.12, 4.97, 4.51, and 4.50 (Fig. 1, Table 1) in a

Table 1. ^{1}H NMR[a] and ^{13}C NMR[b] chemical shifts of the polysaccharide (Fr. I) isolated from *Volvariella diplasia* recorded in D_2O at 27 °C

Sugar residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a, H-6b/C-6
→4)-α-D-Glc*p*-(1→	5.38	3.57	3.42	3.66	3.60	3.92, 3.83
A	100.0	72.2	73.1	76.6	70.8	61.2
→6)-α-D-Man*p*-(1→	5.12	3.83	3.92	4.08	4.01	4.19, 3.86
B	99.5	71.8	72.1	68.7	71.9	69.2
α-D-Gal*p*-(1→	4.97	3.84	3.55	4.28	4.01	3.86, 3.82
C	98.4	69.2	70.8	70.4	71.8	61.4
β-D-Glc*p*-(1→	4.51	3.50	3.45	3.64	3.72	4.08, 3.88
D	103.2	74.6	76.6	70.0	76.1	61.2
→2,4,6)-β-D-Glc*p*-(1→	4.50	3.31	3.49	3.62	3.70	4.19, 3.84
E	103.3	79.9	75.4	77.4	74.5	69.2

[a] The values of chemical shifts were recorded keeping HOD signal fixed at δ 4.67.
[b] Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 31.05 at 27 °C.

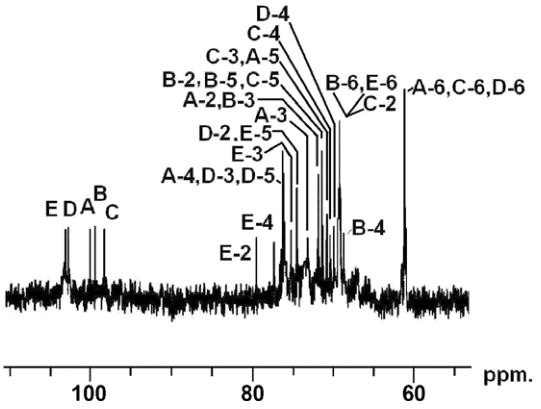


Figure 2. ^{13}C NMR (125 MHz, D_2O, 27 °C) spectrum of polysaccharide Fr. I, isolated from *Volvariella diplasia*.

molar ratio of 1:1:1:1:1. The sugar residues were designated as **A**, **B**, **C**, **D**, and **E** according to their decreasing ^{1}H anomeric chemical shifts. In the ^{13}C NMR (125 MHz) spectrum, five anomeric carbon signals appeared at δ 103.3, 103.2, 100.0, 99.5, and 98.4 (Fig. 2, Table 1) in a ratio of 1:1:1:1:1. All the ^{1}H and ^{13}C signals were assigned using DQF-COSY, TOCSY, HMQC, and HMBC (Fig. 3) NMR experiments. Since a small $J_{1,2}$ value for the D-mannosyl residue does not give information about the anomeric configuration, a proton-coupled ^{13}C NMR experiment was carried out.

Residue **A** has an anomeric proton chemical shift at δ 5.38 and $J_{H\text{-}1,H\text{-}2} \sim 3.9$ Hz, $J_{H\text{-}1,C\text{-}1} \sim 170$ Hz indicating that it is an α-linked residue. Large coupling constants $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ (~10 Hz) were observed for **A**, supporting that it is D-glucosyl moiety.[27,28] The ^{13}C signal for the anomeric carbon of the D-glucosyl moiety was observed at δ 100.0 and also confirmed by HMBC experiment, where a coupling corresponding to **A**C-1, **E**H-4 (Fig. 3, Table 3) is observed. The C-4 peak of residue **A** showed a downfield shift compared to that of the value of standard methyl glycosides[41,42] due to the effect of glycosylation, appearing at δ 76.6. The carbon values of residue **A** (Table 1) indicate that it is α-(1→4)-D-Glc*p*.

The anomeric signals of residue **B** at δ 5.12 (unresolved) and $J_{H\text{-}1,C\text{-}1} \sim 171$ Hz indicate that it is an α-linked residue. A relatively small coupling constant value of H-2, H-3 and a large coupling constant value of H-3, H-4 (~7.0 Hz) indicate that residue **B** is of the α-D-mannosyl configuration.[28] The anomeric carbon chemical shift of moiety **B** at δ 99.5 was confirmed by the presence of a cross peak **B**C-1, **A**H-4 in the HMBC experiment (Fig. 3, Table 3). The downfield shift of C-6 (δ 69.2) indicates that residue **B** is 6-linked to an α-D-Man*p* moiety. The carbon values of residue **B** (Table 1) indicate that it is (1→6)-linked α-D-mannopyranose.

Residue **C** was assigned to Gal*p* as it showed a large coupling constant $J_{H\text{-}2,H\text{-}3}$ (~8.9 Hz) and a relatively small coupling constant $J_{H\text{-}3,H\text{-}4}$ (~3.6 Hz). The anomeric proton chemical shift for residue **C** at δ 4.97 (unresolved) and a carbon chemical shift of δ 98.4 ($J_{H\text{-}1,C\text{-}1} \sim 170$ Hz) indicate that α-galactose is α-linked.[28] The C-1 signal of residue **C** at δ 98.4 was confirmed by the presence of two cross peaks **C**C-1, **E**H-6a and **C**C-1, **E**H-6b in an HMBC experiment (Fig. 3, Table 3). Thus, considering the results of methylation analysis and NMR experiments, it may be concluded that **C** is an α-glycosidically linked, terminal D-galactose moiety.

Residue **D** has an anomeric proton signal at δ 4.51 and the $J_{H\text{-}1,H\text{-}2} \sim 8.29$ Hz, $J_{H\text{-}1,C\text{-}1} \sim 160$ Hz indicate that it is a β-linked moiety. Large coupling constants $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ (~10 Hz) were observed from the DQF-COSY spectrum for residue **D**, supporting that it is a D-glucosyl moiety. The C-1 signal of residue **D** at δ 103.2 was confirmed by the appearance of cross peak **D**C-1, **E**H-2 in an HMBC experiment (Fig. 3, Table 3). From the results of methylation analysis and NMR experiments, it may be concluded that **D** is terminal β-D-glucose.

The anomeric proton chemical shift for residue **E** is δ 4.50. A large coupling constant $J_{H\text{-}1,H\text{-}2}$ value (~8.29 Hz) and $J_{H\text{-}1,C\text{-}1}$ value (161 Hz) indicate that it is a β-linked residue. The $J_{H\text{-}2,H\text{-}3}$ value (~9.8 Hz) and the $J_{H\text{-}3,H\text{-}4}$ value (~10 Hz) for residue **E** indicate that it is a β-D-glucosyl residue. The anomeric carbon chemical shift of moiety **E** at δ 103.3 was confirmed by the presence of two cross peaks **E**C-1, **B**H-6a and **E**C-1, **B**H-6b in the HMBC experiment (Fig. 3, Table 3). The downfield shifts of the C-2 (δ 79.9), C-4 (δ 77.4), and C-6 (δ 69.2) signals with respect to the standard value of methyl glycosides[41,42] are due to the effect of glycosylation. The carbon values of residue **E** (Table 1) indicate that it is a 1,2,4,6-linked D-glucose.

The sequence of glycosyl residues of the polysaccharide (Fr. I) was determined from NOESY (Fig. 4, Table 2) as well as ROESY experiments, followed by confirmation with an HMBC experiment. From inter-residue NOE contacts (Fig. 4, Table 2), the following sequences were established:

α-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→ ; →4)-β-D-Glc*p*-(1→6)-α-D-Man*p*-(1→
A **E** **E** **B**

→6)-α-D-Man*p*-(1→4)-α-D-Glc*p*-(1→ ; α-D-Gal*p*-(1→6)-β-D-Glc*p*-(1,4→
B **A** **C** 2 **E**
↑
1
β-D-Glc*p*
D

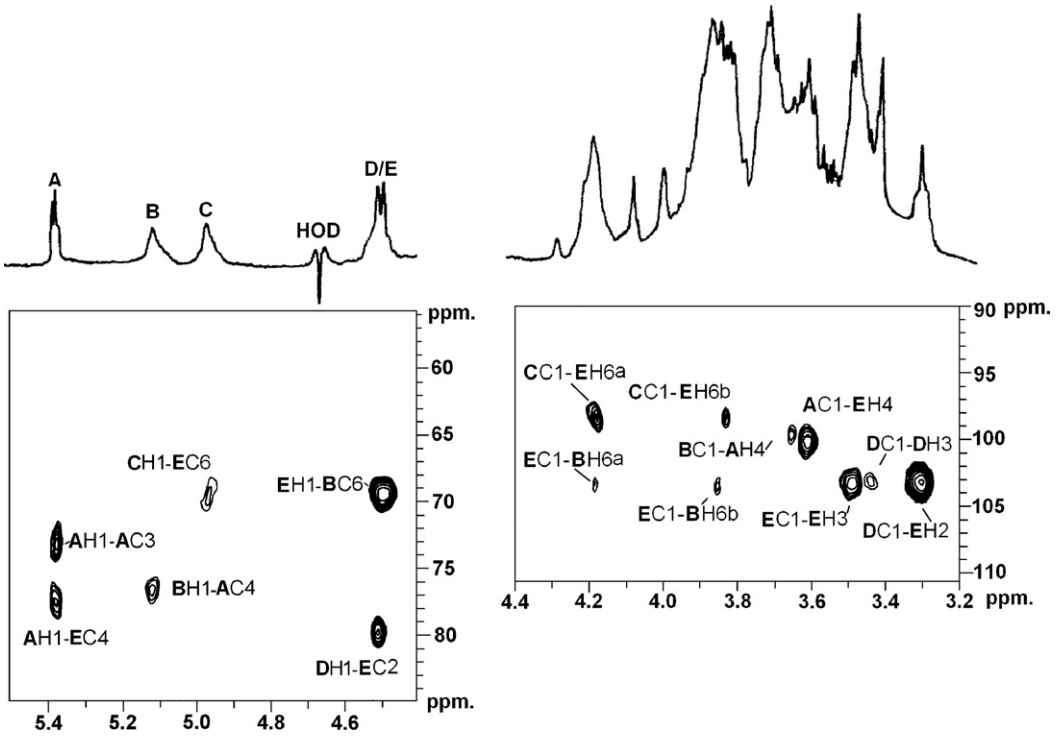
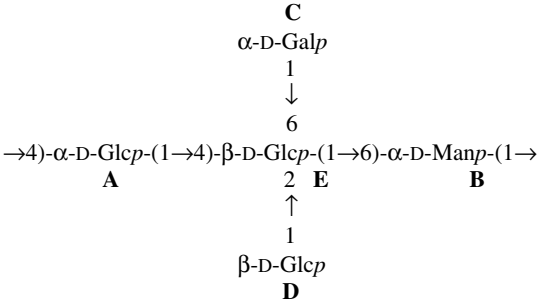


Figure 3. HMBC spectrum (anomeric proton region in the left panel and anomeric carbon region in the right panel) of polysaccharide Fr. I, isolated from *Volvariella diplasia*. The delay time in the HMBC experiment was 80 ms.

The above sequences of glycosyl residues of the polysaccharide were also determined from a ROESY experiment. Since residues **A**, **B**, **C**, **D**, and **E** are present in a molar ratio of 1:1:1:1:1, therefore, the following pentasaccharide repeating unit for the polysaccharide (Fr. I) is assigned:



Long-range ^{13}C–^{1}H correlations obtained from the HMBC experiment (Fig. 3) corroborated the assigned pentasaccharide repeating unit as deduced from both NOESY and ROESY experiments. From the HMBC experiment, the cross peaks of both anomeric proton and carbon of each of the sugar moieties were examined, and intra- and inter-residual connectivities were observed (Table 3). Cross peaks were observed between H-1 of residue **A** and C-4 of residue **E** (**A**H-1, **E**C-4); C-1 of residue **A** and H-4 of residue **E** (**A**C-1, **E**H-4), with other intra-residual coupling between H-1 of residue **A** with its own C-3 atom (**A**H-1, **A**C-3). The cross peaks between H-1 of residue **B** and C-4 of residue **A** (**B**H-1, **A**C-4), C-1 of residue **B** and H-4 of residue **A** (**B**C-1, **A**H-4), were observed. Similarly, the cross peaks between H-1 of residue **E** and C-6 of residue **B** (**E**H-1, **B**C-6), C-1 of residue **E** and H-6a and H-6b of residue **B** (**E**C-1, **B**H-6a and **E**C-1, **B**H-6b), were observed with other intra-residual coupling (**E**C-1, **E**H-3) between C-1 of residue **E** with its own H-3 position. The cross peaks between H-1 of residue **C** with C-6 of residue **E** (**C**H-1, **E**C-6) and C-1 of residue **C** with H-6a and H-6b of residue **E** (**C**C-1, **E**H-6a and **C**C-1, **E**H-6b) were observed. The cross peaks between H-1 of residue **D** with C-2 of residue **E** (**D**H-1, **E**C-2) and C-1 of residue **D** with H-2 of residue **E** (**D**C-1, **E**H-2) were observed. Hence, the appearance of these cross peaks supports the presence of the pentasaccharide repeating unit in the polysaccharide (Fr. I) as indicated above.

A Smith-degradation[43,44] experiment was also carried out to further prove these linkages. The degraded product (SDPS) was identified as β-D-Glc*p*-(1→3)-Gro. GLC analysis of the alditol acetates of methylated, reduced SDPS showed the appearance of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and 1-*O*-acetyl-2,3-di-*O*-methyl-D-glycerol, which indicates that 1,2,4,6-linked D-glucose is present in the backbone of the polysaccharide (Fr. I). The 125-MHz ^{13}C NMR experiment showed only one anomeric carbon signal at δ 103.3 that corresponds to β-D-Glc*p*. The carbon signals of C-2 to C-6

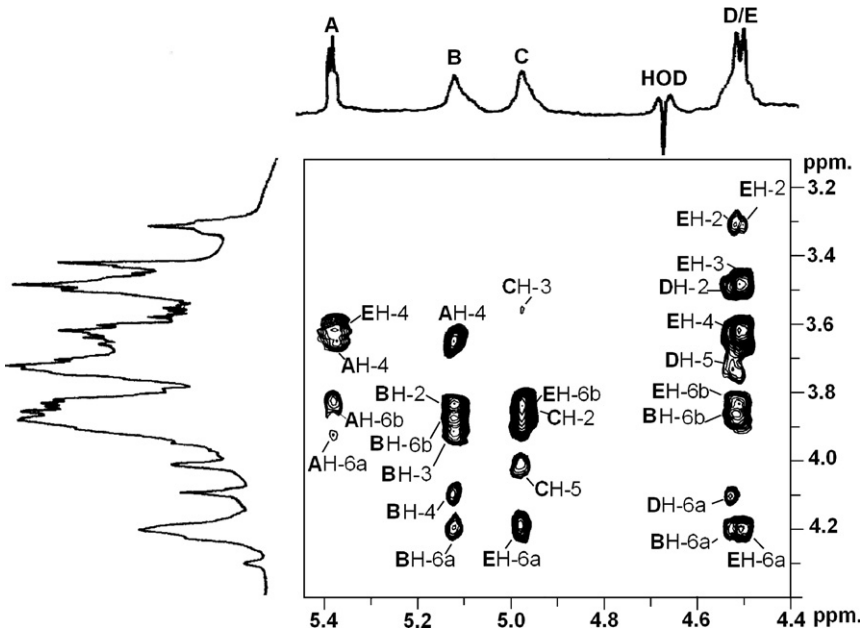


Figure 4. NOESY spectrum of polysaccharide Fr. I, isolated from *Volvariella diplasia*. The NOESY mixing time was 300 ms.

Table 2. NOE data for the polysaccharide (Fr. I) isolated from *Volvariella diplasia*

Anomeric proton Glycosyl residue	δ_H	δ_H	NOE contact to proton Residue, atom
→4)-α-D-Glc*p*-(1→	5.38	3.62	**E**H-4
A		3.66	**A**H-4
		3.92	**A**H-6a
		3.83	**A**H-6b
→6)-α-D-Man*p*-(1→	5.12	3.66	**A**H-4
B		3.83	**B**H-2
		3.92	**B**H-3
		4.08	**B**H-4
		4.19	**B**H-6a
		3.86	**B**H-6b
α-D-Gal*p*-(1→	4.97	4.19	**E**H-6a
C		3.84	**E**H-6b
		3.84	**C**H-2
		3.55	**C**H-3
		4.01	**C**H-5
β-D-Glc*p*-(1→	4.51	3.31	**E**H-2
D		3.50	**D**H-2
		3.72	**D**H-5
→2,4,6)-β-D-Glc*p*-(1→	4.50	4.19	**B**H-6a
E		3.86	**B**H-6b
		3.31	**E**H-2
		3.49	**E**H-3
		3.62	**E**H-4
		4.19	**E**H-6a
		3.84	**E**H-6b

appeared at δ 74.1, 76.7, 70.7, 76.3, and 61.5 for the β-D-glucosyl moiety (**E**) and the carbon signals C-1, C-2, and C-3 of glycerol moiety were assigned as δ 67.5, 73.7 and 61.9, respectively. Therefore, on the basis of the above results, it is confirmed that the polysaccharide (Fr. I) has the structure depicted as above.

3. Experimental

3.1. Isolation and purification of polysaccharide

The fruit body of the mushroom, *V. diplasia* (1.0 kg), was collected from the local market and washed with water. It was swollen in 250 mL of distilled water and boiled for 4 h. The whole mixture was kept overnight at 4 °C and then filtered through a linen cloth. The filtrate was centrifuged at 10,000 rpm (using a Heraeus Biofuge stratos centrifuge) for 45 min at 4 °C. The supernatant was collected and precipitated in EtOH (1:5, v/v). It was kept overnight at 4 °C and again centrifuged as above. The precipitated material (polysaccharide) was freeze dried. It was dissolved in 1% HOAc solution and boiled for 30 min on a water bath. The solution was centrifuged, and the filtrate was again precipitated in EtOH (1:5, v/v) and collected through centrifugation. Then, it was dissolved in water and dialyzed through dialysis tubing (cellulose membrane, Sigma–Aldrich, retaining MW > 12,400) for 10 h to remove acid and low molecular weight materials. It was freeze dried; yield, 2.5 g. The purity of the polysaccharide was determined by gel-permeation chromatography on a Sepharose-6B column (65 × 2 cm) loading 30 mg crude polysaccharide for each run. Test tubes (95 containing 2 mL eluant each) were collected using a Redifrac fraction collector and monitored by the

Table 3. The significant $^{3}J_{H,C}$ connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide (Fr. I), isolated from *Volvariella diplasia*

Residue	Sugar linkage	H-1/C-1	Observed connectivities		
		δ_H/δ_C	δ_H/δ_C	Residue	Atom
A	→4)-α-D-Glc*p*-(1→	5.38	77.4	**E**	C-4
			73.1	**A**	C-3
		100.0	3.62	**E**	H-4
B	→6)-α-D-Man*p*-(1→	5.12	76.6	**A**	C-4
		99.5	3.66	**A**	H-4
C	α-D-Gal*p*-(1→	4.97	69.2	**E**	C-6
		98.4	4.19	**E**	H-6a
			3.84	**E**	H-6b
D	β-D-Glc*p*-(1→	4.51	79.9	**E**	C-2
		103.2	3.31	**E**	H-2
E	→2,4,6)-β-D-Glc*p*-(1→	4.50	69.2	**B**	C-6
		103.3	4.19	**B**	H-6a
			3.86	**B**	H-6b
			3.49	**E**	H-3

phenol–sulfuric acid procedure[37] at 490 nm. Fractions (test tube numbers 20–45 and 68–89) were collected and freeze dried; yield, Fr. I (12 mg), Fr. II (10 mg).

3.2. Monosaccharide analysis

The polysaccharide Fr. I (3.0 mg) was hydrolyzed with 2 M CF_3CO_2H (2 mL) at 100 °C for 18 h in a boiling water bath. The excess acid was completely removed by co-distillation with water. It was reduced with $NaBH_4$ (9 mg), followed by acidification with dilute HOAc, and then co-distilled with pure CH_3OH to remove excess boric acid. The reduced sugars (alditols) were acetylated with 1:1 pyridine–Ac_2O in a boiling water bath for 1 h to give the alditol acetates, which were analyzed by GLC using column (A) 3% ECNSS-M on Gas Chrom Q (100–120 mesh) and column (B) 1% OV-225 on Gas Chrom Q (100–120 mesh) at 170 °C. Gas–liquid chromatography–mass spectrometric (GLC–MS) analysis was also performed on Hewlett–Packard 5970A automatic GLC–MS system, using an HP-5 capillary column (25 m × 25 m). The program was isothermal at 150 °C; hold time 2 min, with a temperature gradient of 4 °C min^{-1} up to a final temperature of 200 °C. Quantitation was carried out from the peak area, using response factors from standard monosaccharides.

3.3. Methylation analysis

The polysaccharide Fr. I (4.0 mg) was methylated using the procedure described by Ciucanu and Kerek.[40] The methylated products were isolated by partition between $CHCl_3$ and H_2O (5:2, v/v). The organic layer containing products was washed with water (3 × 3 mL) and dried. The methylated products were then hydrolyzed with 90% HCO_2H (1 mL) at 100 °C for 1 h, reduced with $NaBH_4$, acetylated with (1:1) Ac_2O–pyridine and analyzed by GLC (using columns A and B) and GLC–MS (using HP-5 fused silica capillary column) and the same temperature program as indicated above.

3.4. Periodate oxidation

The polysaccharide Fr. I (5 mg) was oxidized with 0.1 M $NaIO_4$ (2 mL) at 27 °C in the dark during 48 h. The oxidation process was stopped by the 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. The periodate-reduced material was divided into two portions. One portion was hydrolyzed with 2 M CF_3COOH for 18 h, and alditol acetates were prepared as usual. Another portion was methylated by the method of Ciucanu and Kerek,[40] and the alditol acetates of this methylated product were prepared. Alditol acetates were analyzed by GLC using columns A and B.

3.5. Smith degradation[43,44]

The polysaccharide Fr. I (32 mg) was oxidized with 0.1 M $NaIO_4$ (5 mL) at 27 °C in the dark during 48 h. The excess periodate was destroyed by adding 1,2-ethanediol, and the solution was dialyzed against distilled water for 45 min. The dialyzed material was reduced with $NaBH_4$ for 15 h at 27 °C, neutralized with 50% HOAc followed by dialysis against distilled water and freeze drying. This portion was subjected to mild hydrolysis with 0.5 M CF_3CO_2H for 18 h at 27 °C to eliminate the residues of oxidized sugars attached to the polysaccharide chain (Smith degradation). The

excess acid was removed after repeated addition and evaporation of water. It was further purified by passing through a Sephadex G-25 column, and one fraction was obtained and freeze dried; yield, 9 mg. A part of this material was methylated and analyzed as usual by GLC and GLC–MS. The remainder was used for ^{13}C NMR studies.

3.6. Absolute configuration of monosaccharides

The method used was based on that of Gerwig et al.[39] After CF_3CO_2H (TFA) hydrolysis of 1.0 mg of polysaccharide, the acid was removed by co-distillation with water. A solution of 250 μL of 0.625 M HCl in *R*-(+)-2-butanol was added and heated at 80 °C for 16 h. Then, the reactants were evaporated, and TMS-derivatives were prepared with *N*,*O*-bis(trimethylsilyl)trifluroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30 m × 0.26 mm), a temperature program (3 °C min^{-1}) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

3.7. Optical rotation

Optical rotation was measured on a Perkin–Elmer model 241 MC spectropolarimeter at 25 °C.

3.8. Determination of molecular weight

The molecular weight of polysaccharide (Fr. I) was determined by a gel-chromatographic technique. Standard dextrans[38] T-200, T-70, and T-40 were passed through a Sepharose-6B column and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of Fr. I was then plotted on the same graph and the molecular weight of Fr. I was determined.

3.9. NMR studies

The freeze-dried polysaccharide was kept over P_2O_5 in vacuum for several days and then deuterium exchanged[45] four times followed by lyophilization with D_2O (99.96 atom% ^2H, Aldrich). The ^1H and ^{13}C NMR spectra (both ^1H coupled and decoupled) were recorded at 27 °C. With a Bruker Avance DPX-500 spectrometer,^1H, TOCSY, DQF-COSY, NOESY, ROESY, HSQC, and HMBC NMR spectra were recorded in D_2O at 27 °C. The ^1H NMR spectrum was recorded by suppressing the HOD signal (fixed at δ 4.67) using the WEFT pulse sequence.[46] The 2D-DQF-COSY experiment was carried out using standard Bruker software at 27 °C. The TOCSY experiment was recorded at a mixing time of 150 ms, and complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY and ROESY mixing delay was 300 ms. The ^{13}C NMR spectrum of the polysaccharide as a solution in D_2O was recorded at 27 °C using acetone as the internal standard, fixing the methyl carbon signal at δ 31.05. The delay time in the HMBC experiment was 80 ms.

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