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Structural characterisation of a heteropolysaccharide by NMR spectra

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ABSTRACT

An undocumented water-soluble polysaccharide, LZ-C-1, was isolated from the fruiting bodies of the fungus, *Ganoderma lucidum*. The polysaccharide had a molecular weight of 7×10^3 Da, and was mainly composed of L-Fuc, D-Glc and D-Gal. LZ-C-1 had a sugar content of ~96.4% as measured using the phenol–sulphuric acid method. As a precondition to understand the bioactivity, structural features of LZ-C-1 were investigated by a combination of total hydrolysis, methylation analysis, FT-IR and NMR studies. The results indicated that LZ-C-1 had a backbone of 1,6-disubstituted-α-galactopyranosyl, 1,2,6-trisubstituted-α-galactopyranosyl, 1,3-disubstituted-β-glucopyranosyl and 1,4,6-trisubstituted-β-glucopyranosyl residues. The branches were mainly composed of 1-substituted-β-glucopyranosyl and 1-substituted-α-fucopyranosyl residues.

1. Introduction

The properties and functions of glycoconjugates and polysaccharides cover a wide range, from energy storage and structural blocks to the definition of the specificity of intercellular interactions in many immunological responses. Since the first applications of NMR techniques in structural analysis of polysaccharides from edible mushroom (Kemmelmeier & Zhang, 1981), the NMR spectrum has become a powerful tool used to elucidate the structures of polysaccharides from mushrooms. *Ganoderma lucidum* is a kind of fungi belonging to Ganodermataceae. As a traditional Chinese medicine, it has been used for the prevention and treatment of diseases for several thousand years (Lin, 2001). As an extract from *G. lucidum*, polysaccharides/glycoconjugates have showed strongly bioactive properties towards anti-tumor (Maruyama, Yamazaki, Murofushi, Konda, & Ikekawa, 1989; Zhang & Lin, 1999), inhibiting spontaneous and Fas-mediated apoptosis in human neutrophilis (Hsu, Lee, & Lin, 2002), anti-angiogenic activity (Cao & Lin, 2004) and immuno-modulating activities (Lin et al., 2006), etc. The determination of the structure of polysaccharides was necessary in order to establish a fundamental guide for assessing the bioactivity and pharmacological mechanism in vivo/in vitro. In previous work, the polysaccharide moiety structure of a glycopeptides (Ye et al., 2008) has been reported by our group. As a prelude to further investigations of structure–activity relationships, another polysaccharide was isolated from the fruiting bodies of *G. lucidum* strain *119*, and the chemical structure of this polysaccharide was investigated herein.

2. Experimental

2.1. Materials

The fruiting bodies of *G. lucidum* were collected by Dr. Jingson Zhang from Chongming district of Shanghai, People's Republic of China, and were authenticated by Prof. Taihui Li, Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, People's Republic of China. At the same time, a voucher specimen had been preserved in the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences (Accession No. LZ-2004-0119). BCA™ protein assay reagent kit was purchased from Pierce chemical company (Rockford, USA). The standard monosaccharides were from Sigma–Aldrich Company and Alamar Blue™ reagent was bought from Biosoure International Company. A Millipore Milli-Q Plus (Bedford, MA, USA) was used to generate deionized (18.2 mΩ/cm) water in-house.

2.2. Instrumentation

The ÄKTAprime plus and ÄKTA explorer equipped with a RID-10A refractive index detector (SHIMADZU) were bought from Amersham Biosciences. The Waters HPLC system consisted of a model

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2695 pump, a Waters 2410 RI detector, a Waters 2487 dual wavelength absorbance detector, an on-line degasser and two serially connected TSK PWXL 4000 and 3000 gel filtration columns in tandem. High-performance anion-exchange chromatography (HPAEC) were equipped with a CarboPac™ PA20 column for analyzing monosaccharides or an Amino Pac™ PA-10 column for analyzing amino acids and a pulsed amperometric detector purchased from Dionex Company. The 500M NMR instrument was purchased from Bruker Company, Germany. The 5mm BBI probe and XWIN-NMR 3.0 software were used to record the data.

2.3. Methods

2.3.1. Fractionation of fruiting bodies polysaccharides

To remove lipid, air-dried *G. lucidum* fruiting bodies (~5.0 kg) were chopped into slices and extracted for three 24 h periods with 20 l of 95% EtOH. The residue was extracted twice with 10 volumes of distilled water for 2 h at 100 °C and the aqueous extract was then divided into four parts based on molecular weight separation by ultra-filtration using a hollow fiber membrane: GLPA, GLPB, GLPC, and GLPD. Fraction GLPC was concentrated under vacuum under 40 °C using a rotary evaporator and freeze-dried. The crude extract from GLPC (17.5 g, 0.35% yield) was dissolved in 300 ml of distilled H_2O, applied to a DEAE-Sepharose Fast-Flow column (XK26 × 100 cm) and eluted by filtered (0.45 μm membrane) distilled water and then by 0–2 M NaCl gradient solution. Carbohydrate was determined using the phenol–sulfuric acid assay (Dubois, Gillis, Hamilton, Rebers, & Smith, 1956) and a fraction eluted by distilled water forming a single peak (GLPCW) was collected. Another fraction (GLPCS) appearing as a symmetrical peak was collected, too. Fraction GLPCW was purified by gel-permeation chromatography on a high-resolution Sephacryl S-300 column (XK26 × 100 cm) using filtered distilled water as eluate. Two carbohydrate peaks were detected using a refractive index detector (RID-10A, Shimadzu, Japan), and fraction forming the first peak was collected (LZ-C-1) and verified as pure polysaccharide by HPLC.

2.3.2. Molecular mass estimation

The molecular mass of the purified LZ-C-1 was estimated by HPLC. The column and RI detector temperature were kept at 35 °C. The mobile phase consisted of 0.1 mol/l NaH_2PO_4 and 0.3 mol/l $NaNO_3$, and the pH was adjusted to 7.0 with 0.1 mol/l NaOH. The flow rate of the mobile phase was 0.5 ml/min and 10 μl LZ-C-1 was applied to the machine. The molecular mass was estimated by the standard curve which was calibrated using dextrans (T-5, 12, 25, 50, 80, 150 and 270).

2.3.3. Linkage analysis

Methylation without a carboxyl reduction was performed according to the method of Ciucanu and Kerek (1984), modified by Needs and Selvendran (1993). The permethylated polysaccharide was hydrolyzed by treatment with HCO_2H (88%, 0.5 ml), MilliQ water (0.1 ml) and CF_3CO_2H acid (0.05 ml) for 16 h at 100 °C. The partially methylated sugars in the hydrolysate were reacted with $NaBH_4$ and acetylated by Ac_2O. Partially methylated alditol acetates were extracted into equal volumes of chloroform, washed with MilliQ water, and dried by vacuum evaporation. PMAAs were dissolved in chloroform at about 1 nmol/μl and 2 μl was injected into a gas chromatograph.

2.3.4. Infrared (IR) analysis

Infrared analysis of the samples was obtained by grinding a mixture of polysaccharide with dry KBr and then pressing in a mold. IR spectra were recorded on Nexus Euro FT-IR. Spectra were run in the 4000–400 cm^{-1} region (Kumar, Joo, Choi, Koo, & Chang, 2004).

2.3.5. Monosaccharide analysis

LZ-C-1 (2 mg) was hydrolyzed with 2 M (TFA) at 110 °C for 3 h, and the high-performance anion-performance chromatography equipped with a CarboPac™ PA20 column (3 mm × 150 mm) was applied to identify sugar composition. The column was eluted with 2 mmol/l NaOH with a flow rate of 0.45 ml/min. Monosaccharide components and percentage composition were determined using D-Gal, D-Glc, D-Ara, L-Fuc, L-Rha, D-Man and D-Xyl standards. The absolute configurations of the monosaccharides were investigated according to the method of Vliegenthart et al. using (+)-2-butanol (Gerwig, Kamerling, & Vliegenthart, 1978, 1979).

2.3.6. Nuclear magnetic resonance (NMR) experiment

Exchangeable protons were replaced with deuterium by suspending LZ-C-1 in D_2O, and lyophilizing. This exchange process was repeated thrice. All spectra including ^{1}HNMR, ^{13}CNMR, COSY, DEPT, TOCSY, NOESY, HMQC and HMBC were recorded in D_2O on a Bruker Avance 500 spectrometer. Chemical shifts used the external DSS (δ 0.00) signal as reference for ^{13}C and the internal HDO (δ 4.32) as reference for ^{1}H at 57 °C (330 K). COSY was recorded using the Bruker standard pulse sequence. TOCSY experiment with mixing times of 100 ms was conducted, and NOESY dataset with mixing times of 250 ms was recorded. HMQC was recorded with carbon decoupling, for ^{13}C assignment. HMBC spectrum was recorded with a *J*-evolution time of 60 ms.

3. Results and discussion

The polysaccharide fraction, LZ-C-1, was obtained from DEAE-Sepharose anion-exchange chromatography and Sephacryl S-300 high resolution chromatography. The polysaccharide fraction was found as a symmetrical peak in HPLC, and had a molecular weight of 7×10^3 calibrated using standard dextrans T-5, 12, 25, 50, 80, 150 and 270. This homogeneous polysaccharide was identified as a heteropolysaccharide composed of L-fucose, D-galactose, D-glucose, and had a sugar content of ~96.4% as measured using the phenol–sulfuric acid method.

Three strong absorption bands at 1023.12 cm^{-1}, 1073.16 cm^{-1}, 1148.38 cm^{-1} in the range of 1200–1000 cm^{-1} in the IR spectrum suggested that the monosaccharide in LZ-C-1 had a pyrnaose ring (Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). The intensity of bands around 3376.76 cm^{-1} was due to the hydroxyl stretching vibration of the polysaccharide and as expected they were broad. The bands in the region of 2918.78 cm^{-1} were due to C–H stretching vibration, and the bands in the region of 1639.17 cm^{-1} were due to associated water (Cao et al., 2006; Park, 1971). The absorption at ca. 890 cm^{-1} indicated that LZ-C-1 had β-glycopyranosidic linkages (Barker, Bourne, Stacey, & Whiffen, 1954). Moreover, the characteristic absorptions at ca. 840 cm^{-1} indicated α-configurations existing in the polysaccharide, which was in good agreement with the anomeric proton signals at δ 5.13, δ 5.09, δ 5.04 in the ^{1}H NMR (500 MHz) spectrum.

Absolute configuration analysis showed that glucose, galactose and mannose residues had the D configuration and the fucose residue had the L configuration. Methylation analysis (Table 1) for LZ-C-1 proved that the galatose residues were 1,6-disubstituted, 1,2,6-trisubsituted, the glucose residues were 1-substituted, 1,3-disubstituted, 1,4,6-trisubstituted, and the fucose residue was 1-substituted.

LZ-C-1 showed mainly six anomeric signals in its ^{1}H NMR spectrum (Fig. 1). The very small values of $J_{H\text{-}1,H\text{-}2}$ and H-1 signals at low-fields confirmed that residue A, B and C, which gave anomeric proton signals at δ 5.13, δ 5.09, δ 5.04, were in a α-glycosidical conformation. H-1 signals at high-field in the ^{1}H NMR spectra and C-1 signals at low-fields (Hall & Johnson, 1969) in the ^{13}C NMR spectra, indicated that residue D, E and G were in β-glycosidical conformations,

Table 1
Linkage analysis of the LZ-C-1 isolated from *G. lucidum* fruiting bodies

Glycosyl residue	Methyl ether	Linkage	Molar ratio
Fuc*p*	2,3,4-Fuc	Terminal	0.93
Gal*p*	3,4-Gal[a]	1,2,6-	1.00
	2,3,4-Gal	1,6-	3.89
Glc*p*	2,3,4,6-Glc	Terminal	1.91
	2,3-Glc	1,4,6-	2.03
	2,4,6-Glc	1,3-	2.23
Man*p*	2,3,4-Man	1,6-	0.32
Other		Terminal	Trace

[a] 3,4-Gal = 1,2,6-tri-*O*-acetyl-3,4-di-*O*-methylgalactol.

which had been confirmed by the presence of an IR-band at ca. 890 cm^{-1}.

Signals in the ^{1}H NMR and ^{13}C NMR spectra of polysaccharide LZ-C-1 were assigned as completely as possible according to 2D NMR analysis and on literature values (StØrseth, Kirkvold, Skjermoa, & Reitan, 2006; Tada et al., 2007).

Residue A had been confirmed to be an α-glycosidical residue. The ^{13}C signal for the anomeric carbon of residue A was observed at δ 104.0 according to HMQC spectrum (Fig. 2) analysis. The ^{1}H chemical shifts from the H-1 signal at δ 5.13 to H-2 at δ 3.86 and H-3 at δ 3.95 of this residue were clearly assigned from ^{1}H-^{1}H COSY cross-peaks and TOCSY cross-peaks. The H-4 at δ 3.90 was designated from the cross-peaks of H-4 and C-2 in the HMBC spectrum.

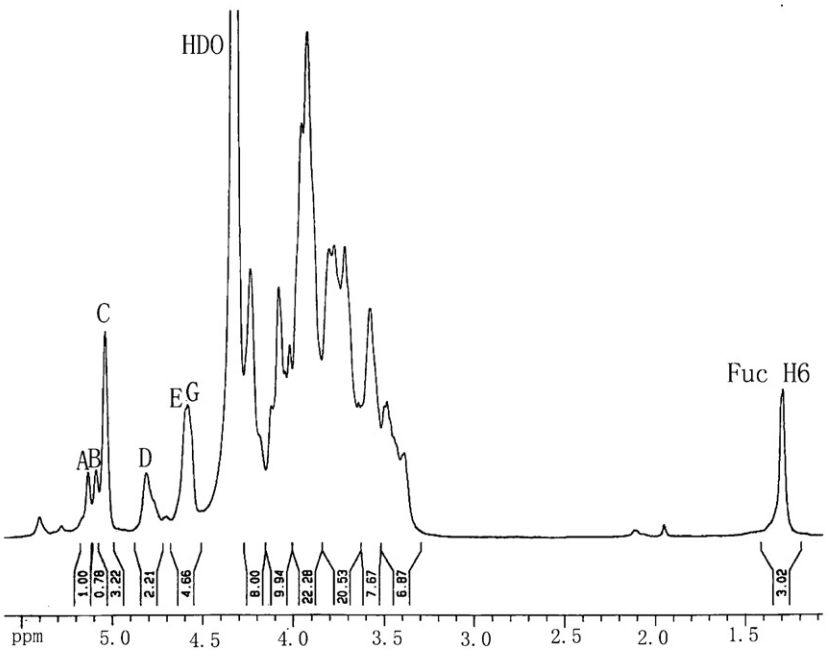

Fig. 1. ^{1}H NMR spectrum of *G. lucidum* polysaccharide LZ-C-1.

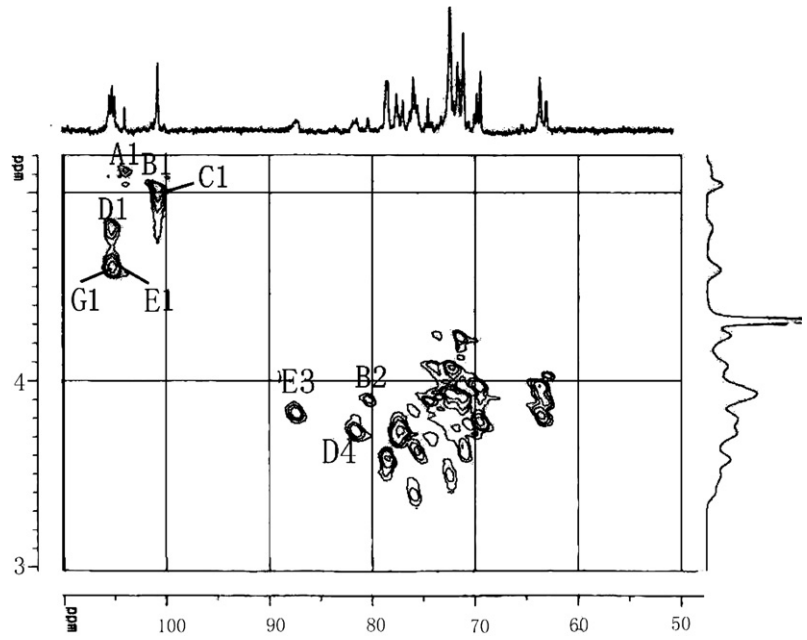

Fig. 2. HMQC spectrum of *G. lucidum* polysaccharide LZ-C-1.

Furthermore, because the signals at δ 4.22 and 1.29 correlate with each other in both the COSY and TOCSY spectra, they were assigned to H-5 and H-6, respectively. The ^{13}C NMR spectrum identified signals of the C-methyl of Fuc*p* at 18.6, which corresponded to a C-methyl resonance proton at δ 1.29 in the ^{1}H NMR spectrum. The carbon signals from C-1 to C-6 were identified from ^{1}H/^{13}C heteronuclear single quantum correlation (HMQC) spectroscopy (Fig. 2). The downfield shift of C-1 with reference respect to standard value and no carbon signal was evident in the δ 76–82 indicating that residue A was substituted at C-1.

The anomeric ^{13}C chemical shift at δ 100.7 for the residues B and the H-1 (δ 5.09) tracks of the resonances in the TOCSY spectrum giving correlation up to H-3 (δ 4.12) showed that residue B was α-Gal-type residue (Jachymek, Czaja, & Niedziela, 1999). The chemical shift of H-2 at δ 3.89 was assigned from ^{1}H-^{1}H COSY. The cross-peak of H-3/H-4 in NOESY indicated that the chemical shift of H-4 was δ 3.92, which was confirmed by the signal peak of H-4/C-2 in HMBC. The H-5 resonance was assigned from the H-3/H-4 and H-4/H-5 cross-peaks in NOESY spectra. Then, the chemical shifts of H-6a at 3.90 and H-6b at 4.03 were obtained from the cross-peaks of H-5/H-6a and H-5/H-6b in COSY spectra. Based on the chemical shifts of protons, the carbon chemical shifts from C-1 to C-6 of residue B were identified from the HMQC spectrum (Fig. 2). The downfield shifts of the C-1 (δ 100.7), C-2 (δ 80.4) and C-6 (δ 70.0) indicated that residue B was a 1,2,6-trisubstituted α-D-galactopyranose.

Residue C had an anomeric chemical shift at δ 5.04. The chemical shifts of H-2 and H-3 at δ 3.92 and δ 4.08 were assigned according to the cross-peaks in ^{1}H-^{1}H COSY and TOCSY spectra. H-3 of residue C displayed strong NOE signals to both H-4 and H-5 at δ 4.08/3.87 and δ 4.08/4.24 indicating an α-Gal-type residue. The chemical shifts of H-6a and H-6b at δ 3.78 and δ 3.98 were designated from the cross-peaks in ^{1}H-^{1}H COSY spectra. On the basis of the proton assignments, the carbon resonances (Table 2) of residue C were assigned from the HMQC spectrum (Fig. 2). The downfield shifts of the C-1 (δ 100.7) and C-6 (δ 69.4) carbon signals and no carbon signal evident in the δ 76–82 range indicated that residue C was a 1,6-disubstituted α-D-galactopyranose.

The chemical shifts of H-2 at δ 3.48 of residue D was easily obtained from the cross-peak at δ 4.82/3.48 in the ^{1}H-^{1}H COSY spectrum. The resonances of H-3 to H-6 at δ 3.74 (H-3), δ 3.72 (H-4), δ 3.56 (H-5), δ 3.68 (H-6a) and δ 3.80 (H-6b) were assigned according to the cross-peaks in COSY, TOCSY and NOESY spectra. The H-1 tracks of the resonances in the TOCSY spectrum for this residue giving the correlation up to proton H-6 (Chakraborty, Mondal, Prananik, Rout, & Islam, 2004), and the typical H-1, H-3 and H-5 intra-correlations in NOESY spectra (Rout, Mondal, Chakraborty, Pramanik, & Islam, 2005) indicated that residue D was of a β-pyranose glucose configuration. On the basis of proton signals, the carbon signals from C-1 to C-6 were identified from HMQC spectroscopy (Fig. 2). The chemical shifts from C-1 to C-6 for residue D corresponded nearly to the documented reference values (Zhang, 1999), and the downfield shifts of the C-1 (δ 105.5), C-3 (δ 81.5) and C-6 (δ 70.2) carbon signals confirmed that residue D was a 1,4,6-trisubstituted-β-D-glucopyranose.

Residue E had an anomeric proton signal at δ 4.59 and a cross-peak at δ 4.59/3.60 easily found in ^{1}H-^{1}H COSY spectrum, implying that the chemical shift of H-2 was δ 3.60. Other proton signals of the residue E were assigned according to the resonances in the ^{1}H-^{1}HCOSY, NOESY and TOCSY spectra. The H-1 tracks of the resonances in the TOCSY spectrum for this residue giving the correlation up to proton H-6 (Chakraborty et al., 2004), and the typical H-1 (δ 4.59), H-3 (δ 3.82) and H-5 (δ 3.72) intra-correlations in NOESY spectra (Rout et al., 2005) indicated that residue E was in a β-pyranose glucose configuration. Based on the chemical shifts of protons, the carbon chemical shifts of residue E was assigned from HMQC spectroscopy (Fig. 2). The chemical shifts from C-1 to C-6 for residue E with reference to the documented NMR data (Zhang, 1999), and the downfield shifts of the C-1 (δ 105.1) and C-3 (δ 87.3) carbon signals indicated that residue E was a 1,3-disubstituted-β-D-glucopyranose.

The cross-peak δ 4.56/3.40 was detected in the ^{1}H-^{1}H COSY spectrum and, since δ 4.56 corresponded to anomeric proton, the δ 3.40 was assigned to H-2 of residue G. The H-3 chemical shift at δ 3.55 was determined according to another clear cross-peak δ 3.40/3.55 in ^{1}H-^{1}H COSY spectra. The chemical shifts of H-4, H-5, H-6 were assigned from COSY, TOCSY and NOESY spectra. A self-spin system from H-1 to H-6 indicated that residue G was a glucopyranose (Cao et al., 2006). H-1 of residue C displayed strong NOE signals to both H-3 and H-5 at δ 4.56/3.55 and δ 4.56/3.49 indicating a β-Glc-type residue. The carbon chemical shifts of residue G were easily assigned from the HMQC spectrum (Fig. 2). The large $J_{H\text{-}1,H\text{-}2}$ coupling constant (broad peak) and the absence of other inter-correlated protons apart from H-1 in HMBC spectra, indicated that residue G was a nonreducing end D-glucopyranose (Mondal, Chakraborty, Rout, & Islam, 2006; Dominaika, Philip, & Nikolay, 2002).

All chemical shifts for residues in ^{13}C NMR and ^{1}H NMR spectra are summarized in Table 2.

To deduce the sequence of polysaccharide LZ-C-1 and to confirm the assignments made from the HMQC and TOCSY spectra, the heteronuclear multiple-bond coherence (HMBC) experiment was used. Residue A had inter-residue correlations from H-1 to C-2 of residue B and from C-1 to H-2 of residue B indicating that residue A was linked at the 2-position of residue B. Residue B had inter-residue correlations from H-1 to C-6 of residue C and from C-1 to H-6 of residue C, indicating that residue B was linked at the 6-position of residue C. Residue C had inter-residue correlations from H-1 to C-6 of residue C and from C-1 to H-6 of residue C, indicating that there were linkages of two C residues. Similarly, residue C had inter-residue correlations from H-1 to C-6 of residue D and C-1 to H-6 of residue D indicating that residue C was linked at the 6-position of residue D. Moreover, residue C had inter-residue correlations H-1 to C-6 of residue B and C-1 to H-6 of residue B, indicating that residue C was linked at the 6-position of residue B. Residue D had an inter-residue correlation from H-1 to C-3 of residue E indicating that residue D was linked at the 3-position of residue E. Residue E had an inter-residue correlation from H-1 to C-6 of residue D, indicating that residue E was linked at the 6-position of residue D. Furthermore, residue E had an inter-residue correlation from C-1 to H-6 of residue B, indicating that residue E was linked at the 6-position of residue B. Residue G had an inter-residue correlation from H-1 to C-4 of residue D, indicating that residue G was linked at the 4-position of residue D.

Table 2
^{1}H and ^{13}C NMR chemical shift data (δ, ppm) for polysaccharide LZ-C-1

Residues	1	2	3	4	5	6a	6b
(A) α-L-Fuc*p*-(1→	**5.13**	3.86	3.95	3.90	4.22	1.29	
	104.0	71.6	72.4	74.8	69.8	18.6	
(B) →2,6)-α-D-Gal*p*-(1→	**5.09**	**3.89**[a]	4.12	3.92	4.23	**3.82**	**4.01**
	100.7	**80.4**	73.2	72.3	72.2	**70.0**	
(C) →6)-α-D-Gal*p*-(1→	**5.04**	3.92	4.08	3.87	4.24	**3.78**	**3.98**
	100.7	71.1	72.3	71.6	72.4	**69.4**	
(D) →4,6)-β-D-Glc*p*-(1→	**4.82**	3.48	3.74	**3.72**	3.56	**3.68**	**3.80**
	105.5	72.3	77.6	**81.5**	72.5	**70.2**	
(E) →3)-β-D-Glc*p*-(1→	**4.59**	3.60	**3.82**	3.78	3.72	3.90	4.03
	105.1	75.7	**87.3**	69.7	77.9	63.1	
(G) β-D-Glc*p*-(1→	**4.56**	3.40	3.55	3.62	3.49	3.82	3.98
	105.3	76.0	78.7	71.1	78.4	63.7	

[a] Values shown in bold font indicate linkage positions.

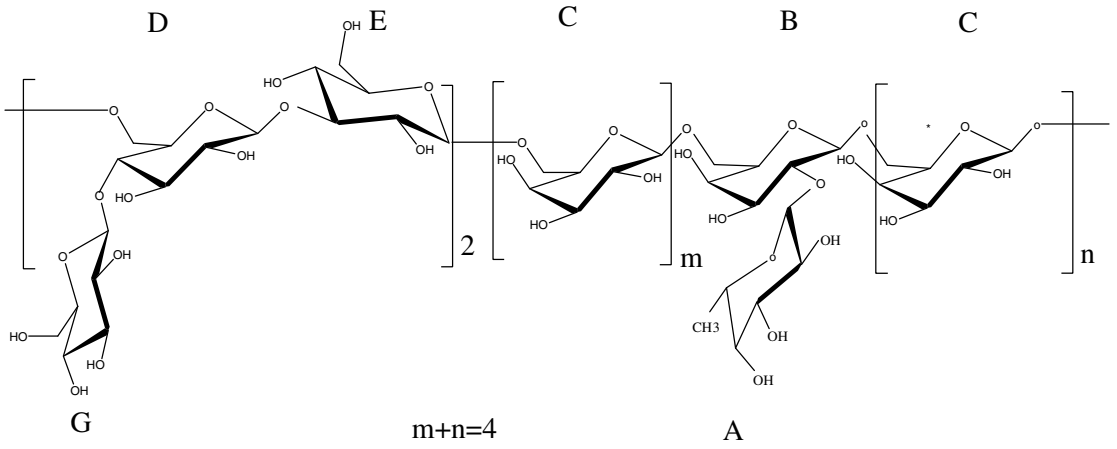

Fig. 3. Predicted chemical structure of polysaccharide LZ-C-1.

Therefore, according to the monosaccharide composition, methylation data, NMR spectra analysis for fraction LZ-C-1, the predicted primary structure of the polysaccharide was established as shown in Fig. 3.

The polysaccharide/glycoconjugate fractions of *G. lucidum* have been widely investigated and reported, but mainly focused on their bioactivity. For the first time, the structure of polysaccharide LZ-C-1 isolated from the fruiting bodies of *G. lucidum* was identified. The bioactivities including antioxidant properties and structure-relationships are currently under investigation.

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