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Note

Isolation and characterization of a mannoglucan from edible *Cordyceps sinensis* mycelium

Yalin Wu,[a] Nan Hu,[a] Yuanjiang Pan,[a,*] Lijun Zhou[b] and Xuxia Zhou[a]

[a]*Department of Chemistry, Zhejiang University, Hangzhou 310027, People's Republic of China*
[b]*Lishui Institute of Lianqu Cordyceps Sinensis, Zhejiang 323000, People's Republic of China*

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Abstract—*Cordyceps sinensis* is a well known tonic food or invigorant with broad-spectrum medicinal properties that is widely used in the People's Republic of China. A neutral mannoglucan **1** with a molar mass of $\sim 7.7 \times 10^3$ Da was obtained from the 0.05 M acetate buffer extract of *C. sinensis* mycelium. It had $[\alpha]_D^{20}$ +126 (*c* 0.2, H_2O) and consisted of Man and Glc units in the molar ratio of 1:9. A combination of chemical analysis and NMR and IR spectroscopy together with digestion with α-D-amylase showed **1** to have a α-D-glucan backbone with (1→4)- and (1→3)-linkages, and the side chains of α-D-(1→6)-Man*p* were attached to the backbone via O-6 of α-(1→3)-Glc*p* residues. Compound **1** showed weak cytotoxicity activity against SPC-I ($IC_{50} = 63$ μg/mL) cancer line, and no obvious cytotoxicity activities against BCAP37 ($IC_{50} > 100$ μg/mL) and SW480 ($IC_{50} > 100$ μg/mL) cancer lines.

Keywords: *Cordyceps sinensis*; Mannoglucan; Methylation; Periodate and chromium trioxide oxidation; NMR and IR spectroscopy; Antitumor activity

From ancient times, *Cordyceps sinensis* (Berk.) Sacc., a popular medicinal fungus belonging to the Hypocreales of the ascomycetes, has been widely used as a folk tonic food or invigorant by the Chinese as well as its usage as a crude drug preparation[1] for increased longevity, endurance and vitality. Several papers have reported its composition and biological activities.[2–6] The polysaccharide is believed to be one of the major active ingredients, as purified carbohydrates from this fungus contain activities in stimulating the function of phagocytes,[7] inhibiting tumor development,[8] protecting liver function,[9] and other activities.[10,11] In previous studies, we demonstrated an antitumor cordyglucans[12] without toxic effects in the test animals and two water-soluble polysaccharides[13,14] from it. In this work, our aim was to describe the structural analysis and antitumor activities against three cancer lines (SPC-I, BCAP37 and SW480) of another acetate buffer-extractable mannoglucan **1** from the same fungus. The polysaccharide–protein complexes extracted with 0.05 M acetate buffer (pH ~ 6.0) were obtained as a 85% ethanol (v/v) precipitate from *C. sinensis* mycelium, and deproteinized to afford CPs, which was fractionated by ion-exchange and gel-filtration chromatography to afford **1** (94.6 mg) with $[\alpha]_D^{20}$ +126 (*c* 0.2, H_2O). Compound **1** gave a single and symmetrical peak by DEAE-cellulose and was homogeneous by high-voltage paper electrophoresis. HPGPC showed only one symmetrical peak from which the weight-average molecular mass was estimated to be 7.7×10^3 Da by reference to standard dextrans (Fig. 1). No absorption at 280 nm and a negative response to the Lowry method[15] confirmed that **1** did not contain proteins. The total hexose content of **1** was determined by the anthrone method [16] and found to be 99.0%. It contained Glc and Man in the molar ratio of 9:1. Uronic acid was not detected by paper chromatography (Whatman No.1 paper, solvent **A**) or by a colorimetric method.[17] Determination of the absolute configurations of the sugar residues as acetylated (+)-2-butyl glycosides by GLC showed D-Glc and D-Man.

* Corresponding author. Tel.: +86 571 87951264; fax: +86 571 87951629; e-mail: cheyjpan@zju.edu.cn

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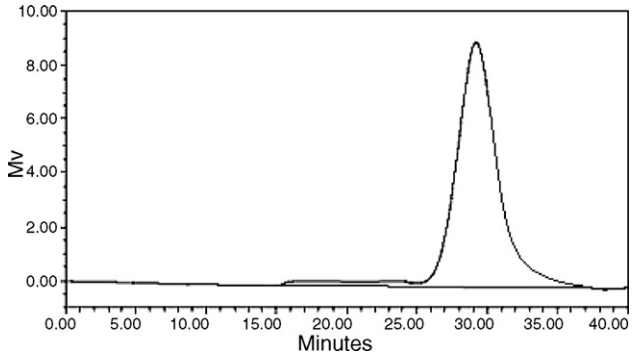

Figure 1. Elution profile of **1** with HPGPC, using a refractive index detector.

Compound **1** showed absorption bands at 3392, 2918, 1657 (br), 1420, 1160–1020, 933, 848, and 762 cm^{-1} in the IR spectrum. The band at 848 cm^{-1} was ascribed to α-type glycosidic linkages.[18] A combination of two bands at 848 and 933 cm^{-1} was characteristic of (1→4)-α-glucans. The broad band at 1657 cm^{-1} was principally associated with absorbed water,[19] since **1** had a strong affinity for water, and in the solid state this macromolecule might have a disordered structure which could easily be hydrated.[20] The bands between 1160 and 1020 cm^{-1} were dominated by the glycosidic linkage *ν*(C–O–C) and *ν*(C–O–H) contribution. An intensive band at 1420 cm^{-1} corresponded to C–O stretch and C–H or OH bending.

The results of methylation analysis of **1** were summarized in Table 1. As shown, **1** is a branched polysaccharide with a backbone composed mainly of (1→4)-(∼69%) and (1→3)-linked (∼31%) D-glucosyl residues. The side chains attached to C-6 of D-glucosyl residues contained single D-mannosyl groups.

Compound **1** was completely oxidized with 0.044 M sodium metaperiodate ($NaIO_4$) for 47 h at 4 °C in the dark. A total of 0.49 mol of $NaIO_4$ was consumed and 0.08 mol of formic acid was produced per mole of glycosyl residues. Supporting the results of methylation analysis, the composition analysis of polyalcohol resulting from periodate oxidation gave glycerol, erythritol and Glc. Detection of only D-glycerol but not of D-mannosyl glycerol in the soluble fraction confirmed the presence of single mannosyl groups as side chains. The anomeric configuration of the residues was determined by oxidation of the acetylated polysaccharide with chromium trioxide. Whereas >91% of Glc and >90% of Man residues survived, this suggested that D-Glc and D-Man were all α.

Compound **1** was hydrolyzed by the α-D-amylase, and the digest was shown by PC (Whatman No.1 paper, solvent **A**) to be composed of Glc and three unknown fractions, OG1-3, which were separated by Sephacryl S-200 chromatography, methylated by a modified Hakomori procedure,[21] purified by solid sorbent extraction and converted into their alditol acetates. GLC of alditol acetates from the partially methylated OG1 (AAPM-OG1) showed three peaks corresponding to 2,3,4,6-Me_4-Glc, 2,4-Me_2-Glc, and 2,3,4,6-Me_4-Man in the molar ratio of 1.00:2.04:1.97, GLC of AAPM-OG2 showed three peaks corresponding to 2,3,4,6-Me_4-Glc, 2,4-Me_2-Glc, and 2,3,4,6-Me_4-Man in the molar ratio of 1.00:1.06:0.98, and GLC of AAPM-OG3 showed only two peaks corresponding to 2,3,4,6-Me_4-Glc and 2,4,6-Me_3-Glc in the molar ratio of 1.00:1.89. These indicated OG1, OG2 and OG3 to have the structures shown in Figure 2 (**E**, **F**, and **G**). The absence of the large peak corresponding to 2,3,6-Me_3-Glc confirmed **1** to have α-(1→4)-D-glucosyl residues present in the main chain. On graded acid hydrolysis, **1** gave Glc, Man and four fractions (**f1**–**f4**), which were characterized conventionally (see Section 1). Fractions **f1**–**f4** were shown to have the structures indicated in Table 2.

The 500 MHz ^1H NMR spectrum of **1** contained three signals in the anomeric region suggesting three sugar-residue units, **A**–**C** shown in Table 3. Those anomeric protons at *δ* 5.40, 5.23, and 4.96 were, respectively, assigned to H-1 of **A**–**C**, [22] which confirmed that **A**–**C**

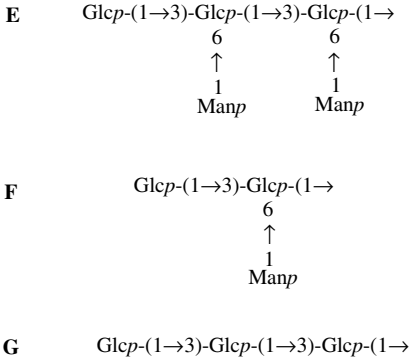

Figure 2. Proposed structures of OG1 (**E**), OG2 (**F**) and OG3 (**G**).

Table 1. Partial *O*-methylalditol acetates formed on methylation analysis of **1** from *C. sinensis* mycelium

Alditol acetates[a]	Fragments (*m*/*z*)	**1** (Fragments area %)	Deduced structural units
2,3,4,6-Me_4-Man	71, 87, 101, 117, 161, 205	10.7	Man*p*-(1→
2,4,6-Me_3-Glc	87, 113, 117, 129, 131, 161, 233	17.8	→3)-Glc*p*-(1→
2,3,6-Me_3-Glc	87, 99, 101, 113, 117, 129, 233	61.3	→4)-Glc*p*-(1→
2,4-Me_2-Glc	43, 87, 117, 129, 189	10.2	→3,6)-Glc*p*-(1→

[a] 2,3,4,6-Me_4-Man, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol, etc.

Table 2. Structural analysis of the resulting products of graded acid hydrolysis of **1** from *C. sinensis* mycelium

Partially methylated alditol acetates[a]	Mole ratios of methylated sugars in graded-hydrolysis product				Mode of linkage
	f1	f2	f3	f4	
2,3,4,6-Me_4-Glc	1.0	1.0	1.0	1.0	Glc(1→
2,3,4,6-Me_4-Man		1.0	0.9		Man(1→
2,3,6-Me_3-Glc	2.3	1.9		2.6	→4)Glc(1→
2,4,6-Me_3-Glc	1.1		2.8	1.8	→3)Glc(1→
2,4-Me_2-Glc		1.2	1.3		→ 3) Glc(1 → 6 ↑

[a] 2,3,4,6-Me_4-Glc, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, etc.

Table 3. ^{13}C NMR Data (ppm) for **1** of *C. sinensis* mycelium

Residue	C-1	C-2	C-3	C-4	C-5	C-6
1[a]						
→4)-α-D-Glc*p*-(1→	**100.3**	71.8	73.2	**78.2**	69.8	61.0
A	(7.5)			(7.6)		
α-D-Man*p*-(1→	**99.0**	72.9	74.4	71.8	73.6	61.1
B	(6.0)					
→3)-α-D-Glc*p*-(1→	**96.8**	70.6	**77.3**	72.8	76.4	60.7
C	(4.0)		(3.9)			
6-Substituted **C**	**96.8**	71.5	**77.9**	73.0	76.2	**68.3**
	(4.0)		(4.5)			(7.2)

[a] Compound **1** fraction obtained by extracting with 0.05 M acetate buffer, and ion-exchange and gel-filtration chromatography. The calculated chemical shift changes ($\Delta\delta$) compared to the corresponding monomers are reported in parentheses (bold for positions of substitution).

were all linked α-glycosidically in accord with the presence of an IR band at 848 cm^{-1}. The chemical shifts from 3.4 to 4.3 ppm, showing overlapping peaks, were assigned to protons of C-2–C-6 of hexosyl glycosidic ring. The broad-band decoupled ^{13}C NMR spectrum (Fig. 3) confirmed the substitution pattern of Glc and Man revealed by the methylation studies. All of the resonances were resolved[14,23,24] and recorded in Table 3. The ^{13}C distortionless enhancement by polarization transfer (DEPT) 135 NMR spectrum of **1** was in agreement with the suggested three repeating units, since three signals were observed in the region of anomeric carbons. The signals at δ 61.1 (C-6) and 68.3 (C-6′) were, respectively, attributed to C-6 of O-unsubstituted Hex*p* and O-substituted Glc*p* residues.

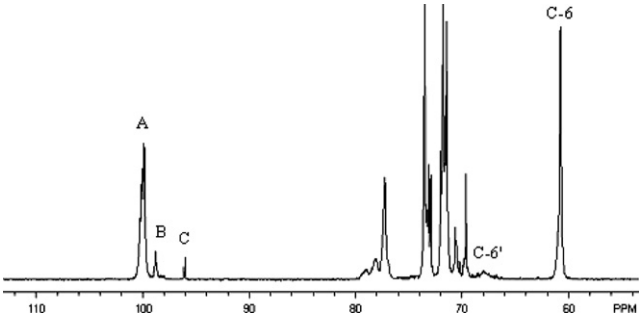

Figure 3. ^{13}C NMR Spectrum of **1** from *C. sinensis* mycelium in D_2O.

Thus, **1** isolated from *C. sinensis* mycelium had a backbone of predominantly (1→4)-linked α-D-Glc*p* (61.3%) together with a proportion of (1→3)-linked α-D-Glc*p* residues (28.0%), with single α-D-Man*p* units (10.7%) as the side chains attached to C-6 of (1→3)-linked D-Glc*p* residues (for every three or two D-Glc*p* residues, there is one α-D-Man*p* group).

The bioassay results of **1** showed a weak antitumor activity against SCP-I cancer line with IC_{50} value of 63 μg/mL, and no obvious antitumor activities against BCAP37 ($IC_{50} > 100$ μg/mL) and SW480 ($IC_{50} > 100$ μg/mL) cancer lines. The structure of **1** was not of the same type as that of cordyglucans[12] from this fungus. Some differences were found in the structures of main chains and branches, which contained the components and configurations of glycosyl type and linkage. Compound **1** showed a mixed-linkage (1→3)(1→4)-α-D-Glc*p* backbone with single α-D-Man*p* branches. Cordyglucans, different from **1**, had a (1→3)-linked β-D-Glc*p* main chains with (1→6)-linked β-D-Glc*p* (not α-D-Man*p*) branches. However, the latter possessed a strong antitumor activity against S-180 in vivo, and did not show toxic effects in the test animals, which remained in good physical condition throughout the testing period. It is interesting to note that cordyglucans and certain other glucans, although having very similar structures, show different levels of activity which may be due to small differences that influence physical properties.[25]

1. Experimental

1.1. General

IR Spectra were recorded with a Nicolet NEXUS-470 FT-IR spectrometer with KBr pellets. GLC of alditol acetates was analyzed using OV-225 capillary columns (0.25 mm × 30 m × 0.25 μm) linked to Hewlett–Packard Model 5713 gas chromatographs equipped with a flame-ionization detection. GLC–MS of partially O-methylated alditol acetates was conducted using a Thermo Quest Trace GC2000 linked to a Thermo Quest Trace mass spectrometer, using a HP-5 fused silica column. NMR spectra were recorded on a Bruker AVANCE DRX-500 NMR spectrometer at 500.13 MHz (^1H) and 125.75 MHz (^{13}C). Chemical shifts were given in ppm, using the D_2O signal (4.79 ppm, ^1H) and external Me_4Si (0 ppm, ^{13}C) as references. Data processing was performed using standard Bruker XWIN-NMR software. The NMR DEPT experiments were carried out using a polarization-transfer pulse of 135°.

1.2. Fungal material

The *C. sinensis* mycelium was donated by Lishui Institute of Lianqu *C. sinensis* (Zhejiang Province, People's Republic China), and authenticated by Dr. Shifeng Ni (Department of Botany, Nanjing University). A voucher specimen (DFC 0423) is deposited in the Herbarium of Department of Chemistry, Zhejiang University, People's Republic of China.

1.3. Isolation and purification

C. sinensis mycelium (400 g), pulverized by a commercial grinder, was extracted with refluxing ethanol (95%, 2 h, three times), and acetone (1 h, twice) for removing lipids and pigments, and then dipped in 75% ethanol for 24 h. The residue was dried in air, and then extracted with 0.05 M acetate buffer (pH ~6.0) for 5 h at 85 °C, the process of extraction was repeated once. The extracting liquids were combined, neutralized with ammonia, concentrated, and dialyzed against distilled water for 48 h. The non-dialyzable phase was poured with vigorous stirring into three volumes of ethanol (85%), and the resulting precipitate was collected by squeezing in a fine gauze, and washed with absolute ethanol and acetone, and finally dried in a vacuum desiccator at room temperature. The obtained product was deproteinated five times by neutroenzyme-Sevag method[26] to afford crude polysaccharides (CPs, 1.84%, yield). A portion (458 mg) of CPs was eluted from a column (2.6 × 40 cm) of DEAE-Sepharose Fast flow with 0.02 M sodium acetate–acetic acid buffer (pH 4.8, 400 mL), followed by distilled water (300 mL) to afford two fractions (**I** and **II**). Compound **I** was further subjected to a column (1.6 × 60 cm) of Sephadex G-100 eluted with 0.1–1 M sodium chloride. Compound **1** was obtained and used in the subsequent studies. Fractions (4 mL each) were collected and combined in accord with the results of analysis by the phenol–sulfuric acid procedure.[27]

1.4. High-performance gel permeation chromatography (HPGPC) and molecular weight

A solution of **1** (4 mg) in distilled water (1 mL) was applied to HPGPC system incorporating in a Waters 515 instrument fitted with two columns in series (ultrahydrogel 250 and ultrahydrogel 2000, Waters). The eluent was distilled water and monitored with Waters 2410 refractive index detector.

1.5. Homogeneity criteria

(a) Anion-exchange chromatography. A solution of **1** (5 mg) in 0.1 M sodium chloride (1 mL) was applied to a column (1.6 × 60 cm) of DEAE-cellulose and eluted with 0.1–0.5 M sodium chloride at 4.5 mL/h. Fractions (1 mL) were analyzed by the phenol–sulfuric acid method.[27]

(b) High-voltage paper electrophoresis.[28] Compound **1** was conducted at 2 °C, 450 V (voltage) and against the borate buffer (pH 9.5). After 2 h, the paper (Whatman No. 1, 8 cm × 25 cm) was fixed in ethanol, and then immersed in periodic acid. After 8 min, the paper was washed by 70% acetic acid, and then immersed in fuchsin–sulfurous acid for 35 min. The paper was washed by sulfite flushing fluid for three times. Finally, the paper was dried in air on the glass-plate after removing water by ethanol. Only a homogeneous maroon spot was shown in this paper.

1.6. Paper chromatographic studies

Descending paper chromatography (PC) was done on Waterman No. 1 paper for qualitative analysis, and Whatman 3 MM paper for large amounts, using (**A**) 6:4:3 *n*-butanol–pyridine–water and (**B**) 4:1:5 *n*-butanol–acetic acid–water as the developing solvents, and detection was made with aniline/diphenylamine/phosphoric acid.[29]

1.7. Optical rotation

Optical rotation was measured on a Perkin–Elmer 341 MC spectropolarimeter at 25 °C.

1.8. Sugar analysis

Compound **1** was hydrolyzed with 2 M TFA at 100 °C for 4 h. The monosaccharides were reduced with sodium borohydride in aqueous 0.1% sodium hydroxide at room temperature for 4 h, acetylated with a 1:1 (v/v) mixture of pyridine and acetic anhydride at 25 °C for 6 h and at 85 °C for 1 h, and analyzed by GLC. The absolute configurations of the monosaccharides were determined by GLC of acetylated (*S*)-(+)-2-butyl glycosides according to the published methods.[30,31]

1.9. Methylation analysis

According to the published literature,[21] **1** (8.5 mg) was performed with methyl iodine in dimethyl sulfoxide in the presence of barium hydroxide. After hydrolysis with 2 M TFA, the methylated sugar residues were converted into their alditol acetates, and analyzed by GLC–MS.[32]

1.10. Periodate-oxidation and Smith degradation

Periodate-oxidation, reduction, and mild hydrolysis with acid were performed by the procedure of Johnson et al.[33] A solution of **1** (18.5 mg) was mixed with

44 mM sodium metaperiodate (40 mL) and kept at 4 °C in the dark. The oxidation was stopped by addition of ethylene alcohol and the solution was dialyzed against distilled water for 48 h. The dialyzed material was reduced with sodium borohydride overnight, neutralized with acetic acid, dialyzed, and freeze-dried. The obtained polysaccharide polyol was partially hydrolyzed in 1 M trifluoroacetic acid, and converted into the alditol acetate, and analyzed by GLC.

1.11. Oxidation with chromium trioxide

A mixture of **1** (11 mg) and *myo*-inositol (5 mg) in formamide (3.0 mL) was acetylated with acetic anhydride (2.5 mL) and pyridine (2.5 mL) under constant stirring for 24 h at room temperature. The mixture was dissolved in chloroform (20 mL), and a portion (0.5 mL) of this solution was taken as a control. The remaining solution was conducted according to the published procedure.[34] Sugar analysis was carried out before and after oxidation.

1.12. Treatment with α-D-amylase

To a solution of **1** (16.7 mg) in 0.05 M sodium acetate buffer (pH 5, 3 mL) was added 50 μL of α-D-amylase (6 mg/mL). The mixture was stirred at 37 °C for 48 h and dialyzed against the same buffer overnight. The dialyzed solution was heated at 100 °C for 10 min to destroy the enzyme, concentrated and centrifugated. The supernatant solution was dialyzed against distilled water and concentrated. The digest (3.5 mL) was applied to a column (2.6 × 45 cm) of Sephacryl S-200 and eluted with distilled water.

1.13. Graded acid hydrolysis

A solution of **1** (85 mg) in 40% aqueous formic acid (40 mL) was heated for 3 h at 100 °C. The formic acid was removed under reduced pressure by co-distillation with water. Paper chromatography (Whatman 3 MM paper, solvent **B**) of the hydrolysate gave fractions **f1**–**f4**, which were all found to be homogeneous. Fractions **f1**–**f4** were separately methylated, isolated by extraction with chloroform, washed with distilled water, removed the solvents under reduced pressure, and dried over P_2O_5 in vacuo. The methylated **f1**–**f4** were analyzed by the method described for methylation analysis of **1** to establish their sequences of linkages.

1.14. Cytotoxicity assay

Bioassay against SPC-I, BCAP37, and SW480 cancer cells was based on the reported procedure.[35] The IC_{50} values were defined by a comparison with the untreated cells as the concentration of test sample resulting in 50% reduction of absorbance.

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