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Structural elucidation and immuno-stimulating activity of an acidic heteropolysaccharide (TAPA1) from *Tremella aurantialba*

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ABSTRACT

A novel acidic heteropolysaccharide (TAPA1) was purified from hot water extracts of *Tremella aurantialba* fruiting bodies by DEAE-Sepharose Fast Flow and Sephacryl S-500 High-Resolution Chromatography. The heteropolysaccharide had a molecular weight of ca. 1.35×10^6 Da, and a carbohydrate content estimated to be ~98.7% by the phenol–sulfuric acid method. It was composed mainly of D-mannose, D-xylose, and D-glucuronic acid in the ratio of ca. 5:4:1, along with trace amounts of D-galacturonic acid and D-glucose. Monosaccharide compositional analysis and GC–MS of methylated derivatives, combined with ^{1}H and ^{13}C NMR spectroscopies (including COSY, TOCSY, NOESY, HSQC, and HMBC spectra), revealed TAPA1 to consist of an α-(1→3)-linked mannopyranosyl backbone, partially substituted at position 4 with xylose side chains, and at position 2 with side chains composed of either xylose, mannose, and glucuronic acid or of xylose and mannose. Bioactivity testing showed that TAPA1 stimulated the proliferation of mouse spleen lymphocytes in vitro.

1. Introduction

The search for new immunomodulatory and anti-tumor compounds has intensified in recent years, and special interest has been focused on various polysaccharides and polysaccharopeptides produced by higher basidiomycetous fungi. Among the numerous mushrooms investigated in this context have been species of the genus *Tremella*,[1,2] which are reported to exhibit a range of pharmacological properties including enhancement of the immune system,[3] anti-diabetic,[4–6] anti-hyperlipidemic,[7,8] and anti-tumor activity, and anti-thrombotic effects.[9] However, relatively little is known about the composition and structure of bioactive agents from *Tremella* species, although an acidic heteropolysaccharide from *T. aurantia* (TAP) with hypoglycemic activity and composed of mannose, xylose, glucuronic acid and glucose (molar ratio 4:2:1:0.3), and containing 2.2% *O*-acetyl groups, has been reported.[10,11]

We now describe the isolation of a novel acidic heteropolysaccharide, TAPA1, from fruiting bodies of *Tremella aurantialba* Bandoni and Zang (known in China as Jin'er) another edible and medicinal basidiomycetous fungus belonging to the family Tremellaceae. TAPA1 strongly stimulated the proliferation of mouse spleen lymphocytes, and in view of its potential as an anti-tumor agent, we have undertaken a detailed structural analysis of the polysaccharide as a prelude to investigating structure–activity relationships.

2. Results and discussion

Polysaccharide TAPA1, purified according to the procedure outlined in Figure 1, appeared as a single symmetrical peak on Sephacryl S-500 High-Resolution Chromatography and had an estimated molecular weight of 1.35×10^6 Da. Total carbohydrate content was estimated to be ~98.7% by the phenol–sulfuric acid method.[12] The homogeneous heteropolysaccharide was composed of D-mannose, D-xylose, and D-glucuronic acid in the ratio of ca. 5:4:1, along with trace amounts of D-galacturonic acid and D-glucose.

The FTIR spectrum showed a strong broad absorption peak at 3388.4 cm^{-1} due to the hydroxyl stretching vibration of the polysaccharide. A C–H stretching vibration band at 2929.4 cm^{-1}, and absorption at 1730 cm^{-1} characteristic of the C=O stretching vibration, suggested an acidic polysaccharide and/or *O*-acetyl groups.

Absolute configuration determination showed that the mannose, xylose, and glucuronic acid residues all had the D configuration.[13] Methylation analysis detected 1-substituted, 1,2-disubstituted, 1,3-disubstituted, and 1,4-disubstituted xylopyranose residues, and 1-substituted, 1,3-disubstituted, 1,2,3-trisubstituted, and 1,2,3,4-tetrasubstituted mannopyranose residues. GC–MS analysis following methylation of reduced *T. auriantalba* polysaccharide

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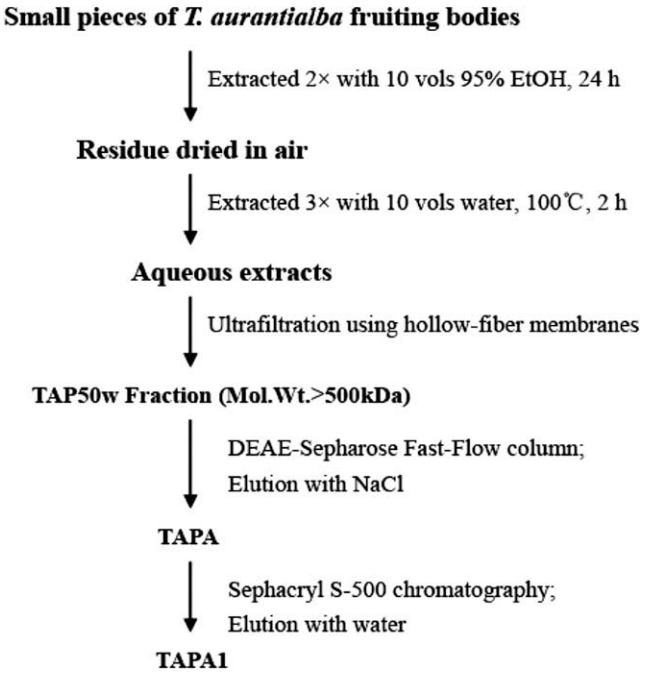

Figure 1. Scheme for purification of TAPA1 from *T. aurantialba*.

(TAPA1-R) revealed one additional peak that was consistent with 2,3,6-tri-*O*-Me_3-glucitol, thereby indicating the presence of 1,4-substituted GlcA residues in TAPA1 (Table 1).

The ^{1}H NMR (600 MHz) spectrum of TAPA1 (Fig. 2) contained signals for seven anomeric protons at δ 5.18, 5.14, 5.10, 4.59, 4.52, 4.35, and 4.32 in a ratio of 1.00:1.00:2.02(2H):2.21(2H):1.96(2H):0.95:0.95. Eight signals were detected in the anomeric region of the ^{13}C NMR (125 MHz) spectrum (Fig. 3) at δ 106.56, 106.34, 106.15, 105.27, 105.01, 103.87, 103.39, and 103.05. Results from the ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, and ^{13}C–^{1}H HMQC (Fig. 4) experiments indicated that the repeating unit of the polysaccharide was composed of 10 sugar residues, which were designated as A–K in the ^{1}H NMR spectrum according to the decreasing chemical shifts of the anomeric protons. The ^{1}H NMR spectrum also contained a signal at δ 2.02 corresponding to the ^{1}H signal for the CH_3 moiety of an acetyl group.[14] Signals at δ 175.19 and 179.25 downfield in the ^{13}C NMR spectrum revealed two carboxyl groups, and the CH_3 moiety of an acetyl group was manifested by a signal at δ 23.79 upfield.[14] The identities of the monosaccharide residues A–K were established on the basis of 1D and 2D spectra as described in the following sections.

2.1. Identification of residues A, B, C, and D

Residue A was designated as a (1→2,3)-α-D-mannopyranose. Residue A had an anomeric signal at δ 5.18. Cross-peaks at δ 5.18/4.22 and δ 4.22/4.03 were detected in the COSY spectrum and, since δ 5.18 corresponded to H-1, the δ 4.22 and δ 4.03 signals were assigned to H-2 and H-3, respectively. The ^{1}H resonances for H-4 and H-5 in Residue A were assigned from the cross-peaks in the COSY, TOCSY, and NOSEY spectra. The H-6a and H-6b resonances were obtained from the COSY and TOCSY spectra. The carbon signals from C-1 to C-6 of residue A were identified from the HSQC spectrum (Figs. 4 and 5, Table 2). The relatively small coupling constant values $J_{H\text{-}1,H\text{-}2}$ and $J_{H\text{-}2,H\text{-}3}$ (<2 Hz), and large coupling constant values $J_{H\text{-}3,H\text{-}4}$ (>8 Hz), $J_{H\text{-}4,H\text{-}5}$ (>8 Hz), and $J_{C\text{-}1,H\text{-}1}$ (~166 Hz), showed that residue A was α-D-mannose.[15–17] From published ^{13}C chemical shift data for aldosides,[18,19] the downfield chemical shifts of the C-1 (δ 103.05), C-2 (δ 81.2), and C-3 (δ 80.5) carbon signals indicated that residue A was a (1→2,3)-α-D-mannopyranose. Residues B, C, and D had anomeric signals at δ 5.14, 5.10, and 5.10, respectively. Using a similar analysis as that adopted for residue A, ^{1}H resonances of residues B, C, and D were assigned by 2D NMR experiments, including COSY, TOCSY, NOESY, and HMBC spectra, and ^{13}C resonances were assigned from the HSQC spectrum (Figs. 4 and 5, Table 2). The linkage positions were determined from the high carbon chemical shift values.[18,19] Consequently, residues B, C, and D were identified as (1→3)-α-D-mannopyranose, 1-α-D-mannopyranose, and (1→2,3,4)-α-D-mannopyranose, respectively.

2.2. Identification of residues F, H, J, and K

Residues F, H, J, and K had anomeric chemical shifts at δ 4.59, 4.52, 4.35, and 4.32, respectively, and the large coupling constant values $J_{H\text{-}1,H\text{-}2}$ (>7.8 Hz) and $J_{H\text{-}1,C\text{-}1}$ (~160 Hz) indicated that F, H, J, and K were β-linked residues. The appearance of two H-5 signals in each case (F: δ 3.22 and 3.24; H: δ 3.62 and 3.68; J: δ 3.80 and 3.70; K: δ 3.78 and 3.64) indicated the presence of a D-xylopyranosyl moiety.[20] β-Linkages were confirmed by the intra-residue NOESY signals between H-1 and H-3, and H-1 and H-5. Proton chemical shifts from H-2 to H-4 of residues F, H, J, and K were assigned from COSY, TOCSY, and NOESY spectra. H-5a and H-5b were assigned from the TOCSY and NOESY spectra, and carbon signals from C-1 to C-5 were assigned from the HSQC spectrum (Figs. 4 and 5, Table 2). The downfield chemical shifts of each residue indicated substituted positions.[18,19] The four residues were designated as follows: F: (1→2)-β-D-xylopyranose, H: (1→4)-β-D-xylopyranose, J: 1→β-D-xylopyranose, K: (1→3)-β-D-xylopyranose.

Table 1
Results of the methylation analysis of TAPA1 and TAPA1-R by GC–MS

Glycosyl residue	Methylated sugar (as alditol acetate)	Mode of linkage	Molar ratios[a]	
			TAPA1	TAPA1-R
Man	4,6-Me_2	→2,3[Man*p*]1→	1.08	1.04
	2,4,6-Me_3	→3[Man*p*]1→	0.94	0.98
	2,3,6-Me_3	→4[Man*p*]1→	0.21	0.18
	6-Me	→2,3,4[Man*p*]1→	1.02	1.00
	2,3,4,6-Me_4	[Man*p*]1→	1.72	1.67
Xyl	2,4-Me_2	→3[Xyl*p*]1→	1.01	1.17
	2,3,4-Me_3	[Xyl*p*]1→	0.99	1.00
	3,4-Me_2	→2[Xyl*p*]1→	1.13	1.02
	2,3-Me_2	→4[Xyl*p*]1→	0.94	0.89
Glc	2,3,6-Me_3	→4[Gl*pc*]1→	Trace	0.90
	2,3,4,6-Me_4	[Glc]1→	Trace	Trace

[a] The molar ratios of samples were calculated on the basis of the peaks areas of PMAAs.

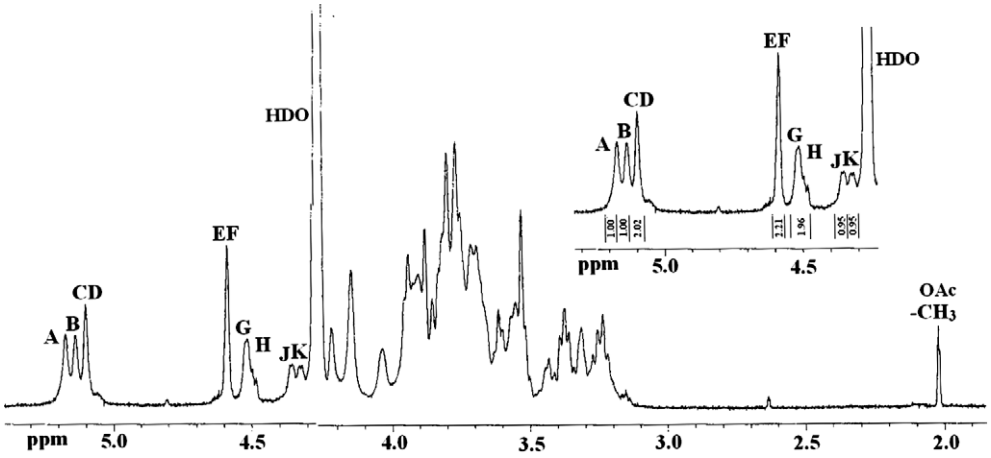

Figure 2. ^{1}H NMR (600 MHz) spectrum of *T. aurantialba* polysaccharide TAPA1. Determined in D_2O at 70 °C. The anomeric protons are labeled A–K.

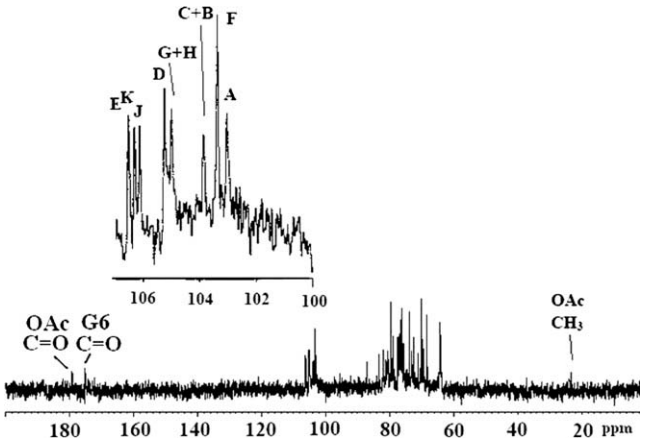

Figure 3. ^{13}C NMR (125 MHz) spectrum of *T. aurantialba* polysaccharide TAPA1. Determined in D_2O at 70 °C. The anomeric protons are labeled A–K.

2.3. Identification of residues G and E

Residue G had the anomeric signal at δ 4.52 and a large $J_{H\text{-}1,H\text{-}2}$ coupling constant value (~8.5 Hz), indicating that G was a β-linked residue. The H-1 track (δ 4.52) of residue G in the TOCSY spectrum showed the complete spin system H-1,2,3,4,5, characteristic of a β-GlcA residue.[21,22] Cross-peaks between H-1 and H-2 (δ 4.52/3.38), and between H-2 and H-3 (δ 3.38/3.26), were observed in the COSY spectrum. The H-4 (δ 3.94) and H-5 (δ 3.47) resonances were assigned from the TOCSY and NOESY spectra. ^{13}C resonances were assigned from the HSQC spectrum (Figs. 4 and 5, Table 2). The downfield shifts of the C-1 (δ 105.01) and C-4 (δ 82.03) carbon signals indicated that residue G was a (1→4)-linked β-D-glucopyranuronic acid.

Residue E was designated as a (1→4)-β-D-mannopyranose. Cross-peaks between H-1 and H-2 (δ 4.59/3.88), and between H-2 and H-3 (δ 3.88/4.12), were observed in the COSY spectrum, but there was no H-4 and H-5 correlation. H-4 and H-5 were assigned by means of TOCSY, NOESY, and HMBC spectra. H-6a and

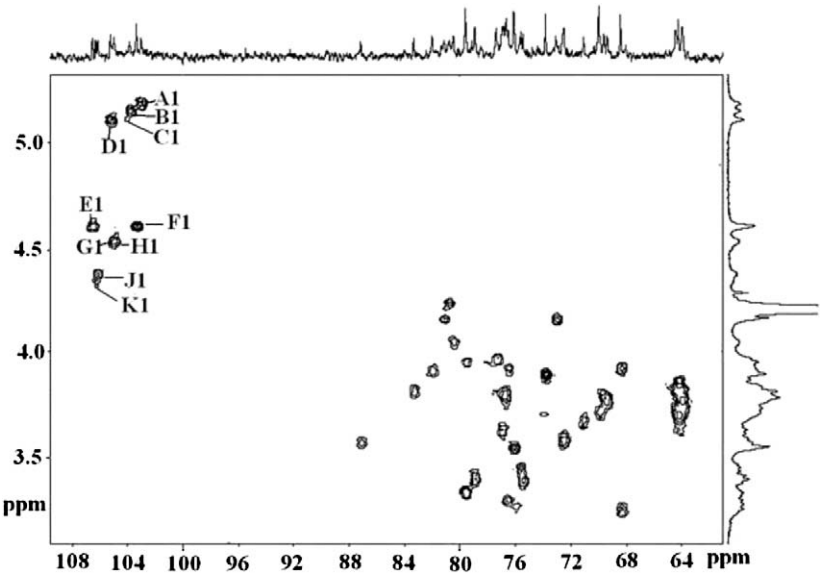

Figure 4. ^{1}H–^{13}C HSQC spectrum of *T. aurantialba* polysaccharide TAPA1 showing anomeric atom cross-peaks. Determined in D_2O at 70 °C.

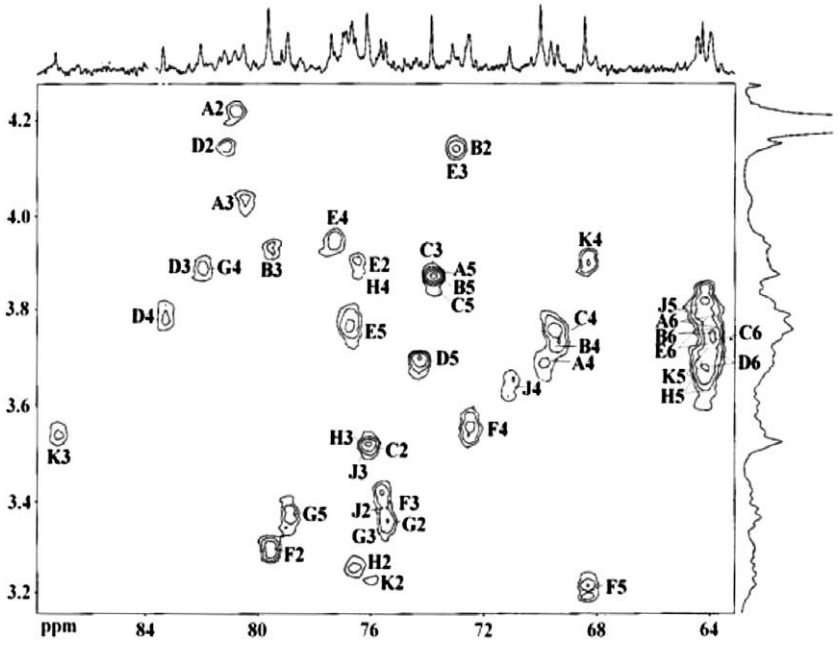

Figure 5. Part of the HSQC spectrum of *T. aurantialba* polysaccharide TAPA1. Determined in D_2O at 70 °C. Each cross-peak corresponds to a C–H pair.

Table 2
^1H and ^{13}C NMR chemical shift data (δ, ppm) for *T. aurantialba* polysaccharide TAPA1 determined in D_2O at 70 °C

Residue	$^1H/^{13}C$						
	1	2	3	4	5/5a	6a/5b	6b
A	**5.18**	**4.22**	**4.03**	3.77	3.89	3.78	3.75
→2,3)-α-D-Man*p*-(1→	**103.05**[a]	**81.2**	**80.5**	69.4	73.85	63.99	
B	**5.14**	4.15	**3.94**	3.83	3.86	3.75	3.71
→3)-α-D-Man*p*-(1→	**103.87**	73.12	**79.63**	69.4	73.85	63.99	
C	**5.10**	3.43	3.94	3.80	3.83	3.66	3.62
α-D-Man*p*-(1→	**103.87**	75.48	73.85	69.64	73.85	63.20	
D	**5.10**	**4.15**	**3.88**	**3.80**	3.76	3.72	3.77
→2,3,4)-α-D-Man*p*-(1→	**105.27**	**81.40**	**82.03**	**83.36**	73.88	63.99	
E	**4.59**	3.88	4.12	**3.91**	3.53	3.75	3.77
4-β-D-Man*p*-(1→	**106.58**	73.85	73.12	**77.41**	76.88	64.46	
F	**4.59**	**3.26**	3.38	3.55	3.22	3.24	
→2)-β-D-Xyl*p*-(1→	**103.39**	**79.63**	75.48	72.57	68.44		
G	**4.52**	3.38	3.26	**3.90**	3.47		
→4)-β-D-GlcA *p*-(1→	**105.01**	75.48	78.84	**82.03**	76.88	175.19	
H	**4.52**	3.26	3.53	**3.80**	3.62	3.68	
→4)-β-D-Xyl*p*-(1→	**105.01**	76.45	75.48	**77.40**	64.46		
J	**4.35**	3.41	3.55	3.65	3.80	3.70	
β-D-Xyl*p*-(1→	**106.15**	75.48	76.88	71.10	64.46		
K	**4.32**	3.22	**3.59**	3.85	3.78	3.64	
→3)-β-D-Xyl*p*-(1→	**106.34**	75.64	**87.19**	68.50	64.26		

[a] Values in bold font indicate the substituted positions.

H-6b were assigned from the TOSCY spectrum. Carbon signals from C-1 to C-6 were assigned from the HSQC spectrum (Figs. 4 and 5, Table 2). Residue E had an anomeric signal at δ 4.59 with a relatively small H-1/H-2 coupling constant (~1 Hz). Large H-4/H-5 (~9 Hz) and C-1/H-1 (~156 Hz) coupling constants indicated that residue E was β-mannose. Furthermore, the downfield shift of the C-5 (δ 76.88) by approximately 4.0 ppm and the upfield shift of the H-5 (δ 3.53) by approximately 0.4 ppm were typical for a β-mannose unit.[23,24] ^{13}C and ^1H resonances of residue E were assigned using similar procedures as adopted above. The downfield shifts of the C-1 (δ 106.58) and C-4 (δ 77.41) carbon signals indicated that residue E was a (1→4)-β-D-mannopyranose.[18,19]

Following the virtually complete assignment of the ^1H and ^{13}C spectral signals, the sequence of the glycosyl residues in TAPA1 was determined using long-range ^{13}C–^1H correlations obtained from HMBC and NOESY spectra.

HMBC spectral analysis (Fig. 6) revealed inter-residue connectivities between H-1 of residue D and C-3 of residue B, between C-1 of residue D and H-3 of residue B, between H-1 of residue B and C-3 of residue A, between C-1 of residue B and H-3 of residue A, between H-1 of residue A and C-3 of residue D, and between C-1 of residue A and H-3 of residue D, which indicated that the sequence of residues A, B, and C (Sequence I) was as follows:

→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→

D B A (I)

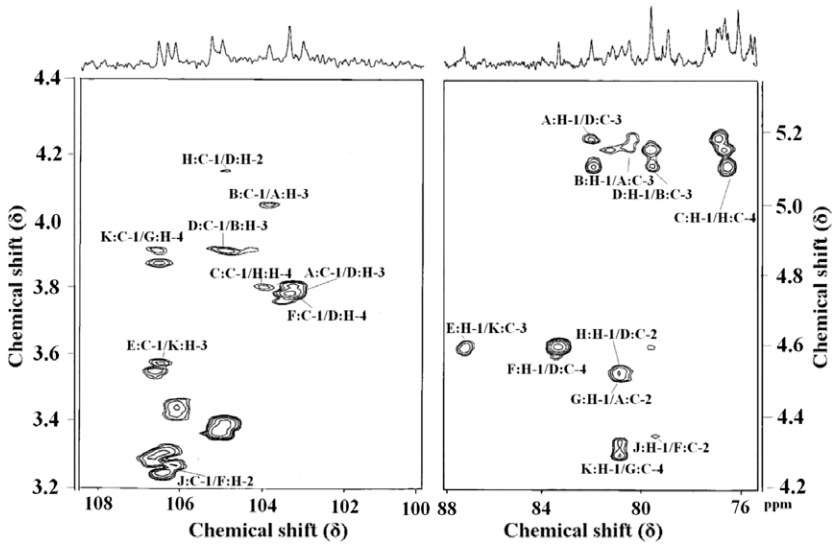

Figure 6. HMBC spectrum of *T. aurantialba* polysaccharide TAPA1 showing inter-residue connectivity. Anomeric proton region (right panel); anomeric carbon region (left panel).

The HMBC spectrum revealed inter-residue cross-peaks between H-1 of residue E and C-3 of residue K, between C-1 of residue E and H-3 of residue K, between H-1 of residue K and C-4 of residue G, between C-1 of residue K and H-4 of residue G, between H-1 of residue G and C-2 of residue A, and between C-1 of residue G and H-2 of residue A, thereby establishing the following sequence (Sequence II):

4-β-D-Man*p*-(1→3)-β-D-Xyl*p*-(1→4)-β-D-GlcA *p*-(1→2)-α-D-Man*p*-(1→

E K G A (II)

Cross-peaks were observed in the HMBC spectrum between H-1 of residue C and C-4 of residue H, between C-1 of residue C and H-4 of residue H, between H-1 of residue H and C-2 of residue D, and between C-1 of residue H and H-4 of residue D, thereby establishing the following sequence (Sequence III):

α-D-Man*p*-(1→4)-β-D-Xyl*p*-(1→2)-α-D-Man*p*-(1→

C H D (III)

Cross-peaks were also observed in the HMBC spectrum between H-1 of residue J and C-2 of residue F, between C-1 of residue J and H-2 of residue F, between H-1 of residue F and C-4 of residue D, and between C-1 of residue F and H-4 of residue D, thereby establishing the following sequence (Sequence IV):

β-D-Xyl*p*-(1→2)-β-D-Xyl*p*-(1→4)-α-D-Man*p*-(1→

J F D (IV)

Confirmation of Sequences I–IV was provided by the inter-residue proton cross-peaks observed in the NOESY spectrum (Table 3). NOESY analysis revealed a moderate strength NOE cross-peak between H-1 of residue D and H-3 of residue B, a strong signal between H-1 of residue B and H-3 of residue A, and a moderate contact between H-1 of residue A and H-3 of residue D, thereby confirming the presence of Sequence I. Sequence II was confirmed by the strong cross-peak between H-1 of residue E and H-3 of residue K, the weak cross-peak between H-1 of residue K and H-4 of residue G, and the moderate strength cross-peak between H-1 of residue G and H-2 of residue A. Moderate NOE contacts between H-1 of residue C and H-4 of residue H and between H-1 of residue H and H-2 of residue D supported Sequence III. The weak NOE contact between H1 of residue J and H-2 of residue F and the strong NOE contact between H-1 of residue F and H-4 of residue D confirmed the presence of Sequence IV. Therefore, the NOESY data were in complete accord with the HMBC experiments, and NMR spectral analysis indicated that TAPA1 had a regular chemical structure consisting of a decasaccharide repeating unit.

Furthermore, the presence of a small number of *O*-acetyl groups in TAPA1 was indicated by signals for the $-CH_3$ group of an acetyl moiety at δ 2.02 in the ^{1}H NMR spectrum (Fig. 1) and at δ 23.79 in the ^{13}C NMR spectrum (Fig. 2). Location of the *O*-acetyl groups in TAPA1 was determined by a modification[25] of the method described by Bouveng,[26] whereby the substitution positions of the *O*-methyl groups in the derivative corresponded to the positions of the *O*-acetyl groups in the original polysaccharide. GC–MS data for the *O*-methyl alditol acetate mixture revealed mono-substitution of mannose residues with *O*-acetyl groups at position O-4 (1.87 M%) and di-substitution at positions O-2 and O-3 (trace). The weak NOE contact between the ^{1}H signal of the acetyl CH_3 group and H-6a of residue E at δ 2.02/3.75 indicated probable substitution by O-acetyl groups at residue E (β-D-Man*p*).[22] Accordingly, it was concluded that the location of *O*-acetyl substitution in TAPA1 was mainly at the 4-position of residue E.

TAPA1 stimulated the proliferation of mouse spleen lymphocytes (MSLs) in vitro in a dose-dependent manner, and at 500 μg/mL concentration, exhibited a similar potency to 6 μg/mL phytohemagglutinin, which served as the positive control (Fig. 7). Immunoregulation is one of several functions associated with polysaccharides.[27] A crude polysaccharide preparation from *T. aurantialba* fruit bodies was previously shown to exhibit immuno-enhancing activity,[3] but the structure of the active component was not determined. Various bioactivities, including immuno-modulatory, anti-diabetic, and anti-hyperlipidemic properties, have also been assigned to polysaccharides isolated from the related species, *T. aurantia*.[3–8]

In conclusion, our NMR spectral data, monosaccharide compositional analysis, and GC–MS of methylated derivatives demonstrated that TAPA1 consisted of an α-(1→3)-linked mannopyranosyl backbone, partially substituted at position 4 with

Table 3
NOE effects of *T. aurantialba* polysaccharide TAPA1 observed in the NOESY spectrum

Residue	NOE signals		
	From (^{1}H:δppm)	Inter-correlation (Residue:Atom/^{1}H:δppm)	Intensity[a]
A: →2,3)-α-D-Man-(1→	H-1(5.18)	D:H-3(3.88)	m
B: →3)-α-D-Man-(1→	H-1(5.14)	A:H-3(4.03)	s
C: α-D-Man-(1→	H-1(5.10)	H:H-4(3.80)	m
D: →2,3,4)-α-D-Man-(1→	H-1(5.10)	B:H-3(3.94)	m
E: 4-β-D-Man*p*-(1→	H-1(4.59)	K:H-3(3.59)	s
F: →2)-β-D-Xyl-(1→	H-1(4.59)	D:H-4(3.80)	s
G: →4)-β-D-GlcA-(1→	H-1(4.52)	A:H-2(4.22)	m
H: →4)-β-D-Xyl-(1→	H-1(4.52)	D:H-2(4.15)	m
J: β-D-Xyl-(1→	H-1(4.35)	F:H-2(3.26)	w
K:→3)-β-D-Xyl-(1→	H-1(4.32)	G:H-4(3.90)	w

[a] Intensities were estimated following visual inspection of the NOESY spectrum (not shown) and designated as follows: s = strong, m = moderate, w = weak.

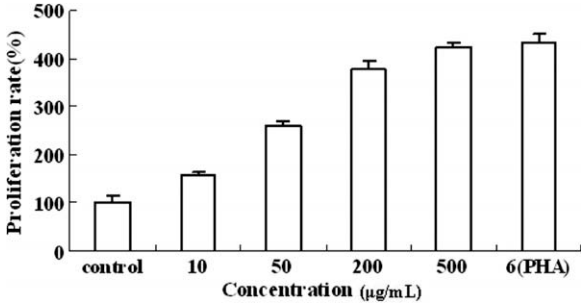

Figure 7. Effect of *T. aurantialba* polysaccharide TAPA1 on the proliferation of mouse splenocytes in vitro. (PHA: phytohemagglutinin (positive control)).

xylose side chains, and at position 2 with side chains composed of either xylose, mannose, and glucuronic acid or of xylose and mannose. TAPA1 contains a non-reducing terminal α-D-mannopyranosyl residue, which has also been reported in polysaccharides from other species of *Tremella* and which appears to contribute to hypoglycemic activity.[11] TAPA1 has also been shown to stimulate the proliferation of mouse spleen lymphocytes in vitro. Further research is underway to determine if this polysaccharide can be assigned other bioactivities.

3. Materials and methods

3.1. Materials

Fruiting bodies of *T. aurantialba* were provided by Kunming Edible Fungi Institute of General National Supply and Marketing Cooperative, China. DEAE-Sepharose Fast-Flow and High-Resolution Sephacryl S-500 were purchased from Amersham Pharmacia Company. Dextrans and the monosaccharide standards D-Gal, D-Glc, D-Ara, L-Fuc, L-Rha, D-Fru, D-Man, D-Xyl, D-GlcA, and D-GalA were from Sigma–Aldrich. All other reagents were from Chinese sources and were of analytical reagent (AR) grade.

3.2. Isolation and purification of the polysaccharide TAPA1

T. aurantialba fruiting bodies were cut into small pieces and extracted twice with 10 vol of 95% (v/v) EtOH for 24 h under reflux to remove lipid material. The residue was dried in air and extracted three times with 10 vol of distilled water at 100 °C for 2 h. The combined aqueous extracts were then separated on the basis of molecular weight into four fractions by ultrafiltration using hollow-fiber membranes of different pore size. The >500 kDa fraction, TAP50w, was concentrated under vacuum at 65 °C using a rotary evaporator and then lyophilized. Crude TAP50w extract was dissolved in distilled water (5 mg/mL), applied to a DEAE-Sepharose Fast-Flow column (XK26 × 100 cm), and eluted first with filtered (0.45-μm membrane) distilled water and then with a NaCl gradient (0–2.0 M). Polysaccharide in the eluate fractions was detected using the phenol–sulfuric acid method.[12] No polysaccharide was present in the distilled water eluate, but five separate peaks (TAPA, TAPB, TAPC, TAPD, and TAPE) were eluted by the NaCl gradient. Fraction TAPA was further purified by gel-permeation chromatography on a High-Resolution Sephacryl S-500 column (XK16 × 100 cm) eluted with 0.2 M $NaNO_3$ (at a flow rate of 0.5 mL/min). Two polysaccharide peaks were detected using a refractive index detector (RID-10A, Shimadzu, Japan), and the fractions forming the symmetrical first peak were collected and designated as TAPA1.

3.3. Determination of purity and molecular weight

Homogeneity and the molecular mass of the TAPA1 were determined by gel-permeation chromatography on a High-Resolution Sephacryl S-500 column (XK16 × 100 cm) calibrated using dextrans T-150, 270, 410, 670, and 2000. The column and RID detector temperature was maintained at 30 °C, the flow rate of the mobile phase (0.2 M $NaNO_3$) was 0.5 mL/min, and 150-μL sample was applied to the column.

3.4. Monosaccharide analysis

TAPA1 (2 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 110 °C for 5 h, and the monosaccharide composition was determined by high-performance anion-exchange pulsed-amperometric detection chromatography (HPAEC-PAD), using a Dionex LC30 equipped with a CarboPac™ PA20 column (3 mm × 150 mm). The column was eluted with 2 mM NaOH (0.45 mL/min), and the monosaccharides were monitored using a pulsed amperometric detector (Dionex).[28] D-Gal, D-Glc, D-Ara, L-Fuc, L-Rha, D-Fru, D-Man, D-Xyl, D-GlcA, and D-GalA were employed as standards to determine monosaccharide components and percentage composition. The absolute configurations of the monosaccharides were determined as described by Vliegenthart and co-workers[13] using (+)-2-butanol.

3.5. Carboxyl reduction of the acidic polysaccharide TAPA1

The reduction of uronic acid residues in TAPA1 was accomplished according to the method of Taylor et al.[29] The procedure was repeated three times until the carboxyl group was completely reduced (disappearance of the C=O band at 1730 cm^{-1} in FTIR) and the product TAPA1-R was obtained.

3.6. FTIR analysis

FTIR spectroscopy of TAPA1 mixed with dry KBr was performed in the 4000–400 cm^{-1} region (Nexus Euro FTIR instrument).

3.7. Methylation analysis

Since TAPA1 was insoluble in Me_2SO, it was first acetylated. A vacuum-dried sample (4 mg) suspended in 1.0 mL Me_2SO was acetylated with a mixture of Ac_2O and pyridine at 100 °C for 1 h, dialyzed against double-distilled H_2O for 72 h, and then lyophilized to yield TAPA1-acet.

TAPA1-acet and TAPA1-R samples were methylated three times according to the method of Needs and Selvendran,[30] and the disappearance of the OH band (3200–3700 cm^{-1}) in the FTIR spectrum was used to confirm complete methylation. The permethylated polysaccharide was hydrolyzed by treatment with HCO_2H (88%), distilled water, and TFA in the ratio of 3:1:2 for 5 h at 100 °C. The partially methylated sugars in the hydrolysate were then reduced with $NaBH_4$, acetylated with Ac_2O, and the partially methylated alditol acetates were analyzed by GC–MS as described by Ye et al.[31]

3.8. Nuclear magnetic resonance (NMR) experiments

TAPA1 (30 mg) was deuterium-exchanged three times with 0.5 mL of D_2O. ^1H NMR, ^{13}C NMR, COSY, TOCSY, NOESY, HMQC, and HMBC spectra were recorded in D_2O at 600 MHz (^1H NMR) or 125 MHz (^{13}C NMR) using a Bruker Avance 600 spectrometer. ^{13}C chemical shifts were acquired in relation to DSS (δ 0.00 ppm) calibrated externally, and HDO (δ 4.32) was used as the internal reference signal for ^1H at 70 °C (343 K).

3.9. Location of *O*-acetyl groups in individual monosaccharide units of TAPA1

Location of the *O*-acetyl groups was carried out using a modification[25] of the method described by Bouveng,[26] whereby the partially O-methylated polysaccharide (8.8 mg) was hydrolyzed with aqueous H_2SO_4 and analyzed by GC–MS as described above.

3.10. Spleen cell proliferation assay

C57 BL/6 mice (ca. 18 g) were killed by cervical dislocation. The spleens were removed and cut into small pieces, and then pressed through a stainless steel mesh (100 mesh) to obtain a suspension of isolated spleen cells. Red cells in the suspension were lysed with a solution of Tris–HCl–NH_4Cl (pH 7.2).[32] The cell suspension was further diluted with a fivefold excess of medium, and after mixing and centrifugation, cell pellets were finally resuspended in RPMI-1640 medium and adjusted to a concentration of 2×10^6 cells/mL. Aliquots (180 μL) of the cell suspension and 20 μL of different TAPA1 concentrations (dissolved in PBS) were added to each well of a 96-well plate. Negative controls were treated only with PBS, and positive controls with 6 μg/mL phytohemagglutinin (PHA). After incubation at 37 °C in a 5% CO_2 atmosphere for 72 h, 20 μL of Alamar Blue reagent (Biosource, Nivelles, Belgium) was added to each well, and the incubation was continued for another 6 h. The extinction was measured at 570 nm and 600 nm using a micro ELISA auto-reader, and the proliferation rate was calculated according to the Biosource protocol.

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