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Structural elucidation of a novel fucogalactan that contains 3-*O*-methyl rhamnose isolated from the fruiting bodies of the fungus, *Hericium erinaceus*

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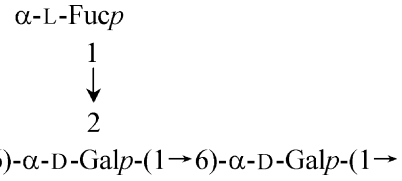
Abstract—A new heteropolysaccharide, HEPF3, was isolated from the fruiting bodies of *Hericium erinaceus*. HEPF3 has a molecular weight of 1.9×10^4 Da and is composed of fucose and galactose in a ratio of 1:4.12. Compositional analysis, methylation analysis, together with ^{1}H and ^{13}C NMR spectroscopy established that HEPF3 consists of a branched pentasaccharide repeating unit with the following structure:



HEPF3 also contains a minor proportion of 3-*O*-methylrhamnose that is thought to terminate the polymer main chain.

Keywords: Fucogalactan; *Hericium erinaceus*; Polysaccharide; Structural analysis

1. Introduction

Hericium erinaceus is a traditional Chinese medicinal fungus distributed throughout China. It is used to treat gastric ulcers, chronic gastritis and other digestive tract-related diseases. Both the fruiting bodies and the fungal mycelia have been reported to contain bioactive polysaccharides[1] that are reputed to exhibit various pharmacological activities including enhancement of the immune system, as well as anti-tumor, hypoglycemic, and anti-aging properties.[2,3] In order to identify correlations between structure and functionality, we have conducted structural studies on polysaccharides from *H. erinaceus*. In this paper, the structural elucidation of HEPF3, a novel neutral polysaccharide purified from the fruiting body of *H. erinaceus*, is described.

2. Results and discussion

High-performance liquid chromatography (HPLC) of HEPF3 produced a single symmetrical peak, indicating homogeneity. Its molecular weight was determined to be 1.9×10^4 Da. Sugar analysis and GC–MS revealed

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the presence of fucose and galactose in the polysaccharide in a ratio of 1:4.12, along with a small amount of 3-*O*-methylrhamnose. The latter was confirmed by comparison of retention times and mass spectra of partially O-methylated derivatives. The mass spectrum of 3-*O*-methylrhamnose is dominated by the cleavage of bonds between O-methylated carbons and adjacent O-acetylated carbons. For 3-*O*-methylrhamnose alditol acetate, this cleavage produced *m*/*z* 203 and 189 as the primary fragments, and *m*/*z* 87, 101, 129, and 143 as the other main fragments. This is in agreement with a previously published report[4] and was confirmed by methylation analysis. Absolute configuration analysis showed that fucose has the L configuration and galactose has the D configuration.

Methylation analysis of the polysaccharide revealed 2,3,4-tri-*O*-methylfucose, 2,3,4-tri-*O*-methylgalactose, and 3,4-di-*O*-methylgalactose in a ratio of 1:2.94:1.2, respectively, together with a minor proportion of 2,3,4-tri-*O*-methylrhamnose. Therefore, the polysaccharide is branched with a terminal fucopyranose residue and 2,6-disubstituted galactose residue at the branching point. Another galactose residue is substituted at the 6-position, and the 3-*O*-methylrhamnose is not glycosylated.

The ^{13}C NMR spectrum of the polysaccharide contained signals for three anomeric carbons at δ 100.6–104.2, one CH_3–C group (C-6 of Fuc) at δ 18.4, and sugar ring carbons linked to oxygen in the region of δ 63.12–80.7. In addition, there was a minor signal present at δ 59.1, which could be assigned to an *O*-methyl group, and a number of minor sugar signals that mostly belonged to 3-*O*-methylrhamnose. The ^{1}H NMR spectrum (Fig. 1) of the polysaccharide contained signals for three anomeric protons at δ 5.01–5.12, one CH_3–C group (H-6 of Fuc) at δ 1.28 ($J_{5,6}$ 6.42 Hz), other sugar protons in the region of δ 3.50–4.26, and a minor signal for an *O*-methyl group at δ 3.50 (data of the HMQC spectrum, Fig. 2).

Residue **a** →1)-α-L-Fuc*p*. The ^{1}H resonances for H-1, -2 and -3 of this residue were assigned from ^{1}H–^{1}H COSY cross-peaks. The assignment of H-4 relies on the TOCSY spectrum. H-5 and H-6 were assigned from the ^{1}H–^{1}H COSY spectrum. The cross-peaks of H-3 and H-6 in the NOESY spectrum, as well as H-6 and C-3 in the HMBC spectrum, unambiguously show that H-5 and H-6 are located on residue **a**. On the basis of the proton assignments, the chemical shifts of C-1 through C-6 were readily obtained from the ^{1}H–^{13}C HMQC spectrum (Table 1). Both the carbon and proton chemical shifts are typical of those of a 6-deoxyhexopyranose. Since L-Fuc was the only such sugar identified by GC–MS analysis, Residue **a** must be the fucose. Moreover, H-1 appears as a singlet ($J_{H\text{-}1,H\text{-}2} < 3$ Hz) in the ^{1}H NMR spectrum, and H-1/H-2 intra-residue correlations are present in the NOESY spectrum. Both values indicate an α-configuration at the anomeric center. Thus, residue **a** was identified as α-L-fucopyranoside.

Residue **b** →2,6)-α-D-Gal*p*. ^{1}H resonances for H-1 through H-4 of residue **b** were assigned from the

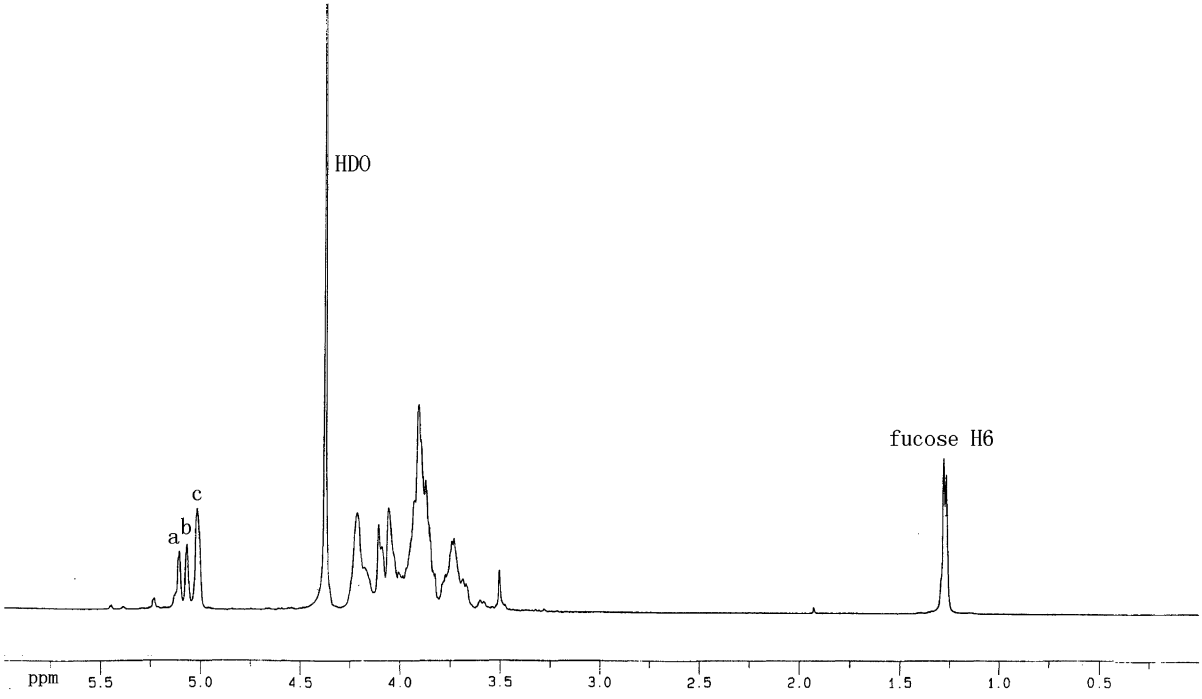


Figure 1. 500-MHz ^{1}H NMR spectrum of the HEPF3 polysaccharide isolated from *Hericium erinaceus* in D_2O at 70 °C. The anomeric protons are labeled a, b, c.

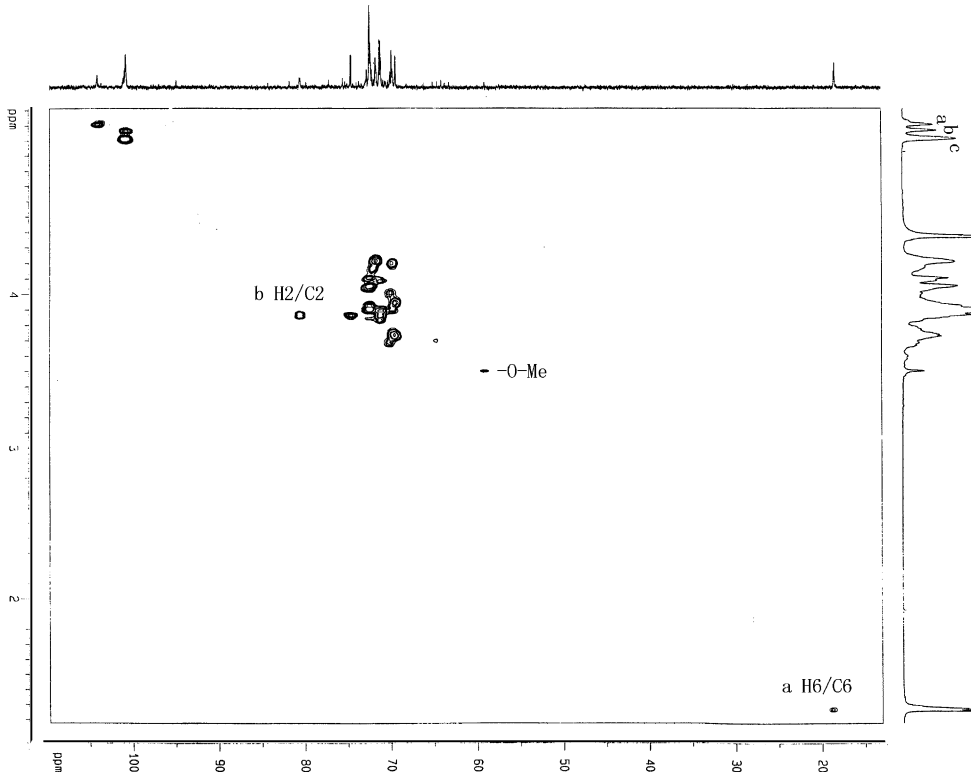


Figure 2. 500-MHz ^{1}H–^{13}C HMQC spectrum of HEPF3 polysaccharide isolated from *Hericium erinaceus* in D_2O at 70 °C. Each cross-peak corresponds to a C–H pair.

Table 1. Chemical shift data for HEPF3

Residue		Proton or carbon[a]						
		1	2	3	4	5	6a	6b
α-L-Fuc*p* (**a**)	H	5.10	3.85	3.91	3.82	4.19	1.28	
	C	104.2	71.3	71.2	71.5	72.3	18.4	
→2,6)-α-D-Gal*p* (**b**)	H	5.07	3.87	4.10	4.21	4.17	3.70	4.01
	C	100.7	**80.5**	71.3	69.9	72.4	**70.05**	
→6)-α-D-Gal*p* (**c**)	H	5.01	3.88	4.05	3.90	4.22	3.73	3.96
	C	100.6	74.6	72.4	72.3	72.1	**69.4**	

[a] Underlined bold numbers represent glycosylation sites.

^{1}H–^{1}H COSY and TOCSY spectra. H-5, H-6a and H-6b were assigned from the TOCSY spectrum. In the HMBC spectrum, the cross-peaks of H-1 and C-3, C-5 showed that H-5 and H-6 are located on residue **b**. The corresponding ^{13}C resonances were assigned from the HMQC spectrum. The H-4/5 coupling constant was small, as expected for a Gal-type residue. Furthermore, residue **b** adopted an α-configuration at its anomeric center, which is evident from the singlet of H-1 as well as the characteristic $J_{H\text{-}1,H\text{-}2} < 3$ Hz, the H-1/H-2 intra-residue correlations in the NOESY spectrum and the cross-peaks of H-1 and C-3, C-5 in the HMBC spectrum. The combination of these data identified residue **b** as →2,6)-α-D-Gal*p*.

Residue **c** →6)-α-D-Gal*p*. The ^{1}H resonances for H-1, -2, -3, and -4 were assigned from the cross-peaks in the ^{1}H–^{1}H COSY and TOCSY spectra. The H-5 resonance was assigned from the H-3/4 and H-4/H-5 cross-peaks in the NOESY spectrum. The H-5 and the H-6a and H-6b resonances were then obtained from the TOCSY spectrum. ^{13}C resonances were assigned from the HMQC spectrum. H-4 displays strong NOEs to both H-3 and H-5, which indicated that residue **c** is a Gal-type residue. H-1 appears as a singlet ($J_{H\text{-}1,H\text{-}2} < 3$ Hz) in the ^{1}H NMR spectrum, and the H-1/H-2 intra-residue correlations in the NOESY spectrum indicate that residue **c** is an α-configuration. Thus, residue **c** was identified as →6)-α-D-Gal*p*.

Table 2. Two- and three-bond ^{1}H–^{13}C correlations for the HEPF3[a]

Residue	Proton	correlation
α-L-Fuc*p* (**a**)	H-1	80.5 (**b**; C-2), 71.2 (**a**; C-3), 72.3 (**a**; C-5)
→2,6)-α-D-Gal*p* (**b**)	H-1	71.3 (**b**; C-3), 72.4 (**b**; C-5), 69.4 (**c**; C-6)
	H-2	104.2 (**a**; C-1)
→6)-α-D-Gal*p* (**c**)	H-1	69.4 (**c**; C-6), 70.05 (**b**; C-6), 72.4 (**c**; C-3), 72.1 (**c**; C-5)

[a] Inter-residue correlations are shown in bold font.

Table 3. NOE data for the HEPF3[a]

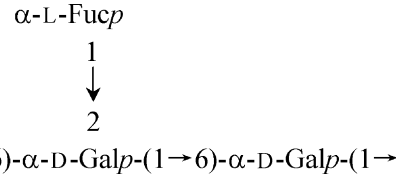
Residue	Proton	NOE to
α-L-Fuc*p* (**a**)	H-1	3.85 (**a**; H-2), 3.87 (**b**; H-2)
→2,6)-α-D-Gal*p* (**b**)	H-1	3.87 (**b**; H-2), 3.73 (**c**; H-6a)
	H-3	4.21 (**b**; H-4)
→6)-α-D-Gal*p* (**c**)	H-1	3.88 (**c**; H-2), 3.73 (**c**; H-6a), 3.96 (**c**; H-6b)
	H-3	4.22 (**c**; H-5)
	H-4	4.05 (**c**; H-3), 4.22 (**c**; H-5)

[a] Inter-residue NOEs are shown in bold font.

Comparison of the chemical shift data for residues **a**–**c** with those reported for glycosides[5] permitted identification of residue **a** as 1-linked α-L-Fuc*p*, residue **b** as 2,6-linked α-D-Gal*p*, residue **c** as 6-linked α-D-Gal*p*.

The sequence of the residues in the repeating unit was established from the HMBC spectrum, which showed clear correlations between H-1 of residue **a** and C-2 of residue **b**, between H-1 of residue **b** and C-6 of residue **c**, between H-1 of residue **c** and C-6 of residue **c**, and between H-1 of residue **c** and C-6 of residue **b**. The NOESY experiment confirmed this sequence information, showing clear inter-residue NOEs between H-1 of residue **a** and H-2 of residue **b**, between H-1 of residue **b** and H-6a of residue **c**, and between H-1 of residue **c** and H-6a and H-6b of residue **c**. HMBC correlations are presented in Table 2 and NOE data in Table 3.

The combined chemical and NMR data permit the structure of the pentasaccharide repeating unit of the HEPF3 to be written as



In most fungi examined, polysaccharides composed of β-(1→3)- and β-(1→6)-D-glucans, and α-(1→6)-D-mannans have been reported to be the major components of the cell wall and the intercellular matrix, with the latter found mainly in yeast cell walls.[6] In contrast, polysaccharides consisting of a α-(1→6)-linked α-D-galactan backbone and branches composed of glucose and rhamnose have been found in the fungus *H. erinaceus*.[7] The biological effects of these polysaccharides have been widely studied for their immunostimulating and anti-tumor activities.[8] However, structures containing an α-(1→6)-linked α-D-galactan backbone and branches composed of fucose have not been previously reported, and HEPF3 is therefore a novel fungal polysaccharide.

3. Experimental

3.1. Materials

Fruiting bodies were purchased from Qing'an in Zhejiang Province, China. DEAE-Sepharose Fast Flow and Sephacryl S-300 High Resolution were purchased from Amersham Pharmacia Biotech. Dextrans and the monosaccharides, D-Gal, D-Ara, L-Fuc, L-Rha, D-Man, D-Xyl, and D-Glc, were from Sigma. All other reagents were of A.R. grade and made in China. HPLC was carried out on a Waters 2695 HPLC system (2695 HPLC Pump, 2414 Refractive Index Detector). GC–MS was carried out using a ThermoFinnigan TRACE MS, and NMR spectra were determined with a Varian INOVA 500.

3.2. Isolation and purification

The total fruiting bodies of *H. erinaceus* were first exhaustively extracted with EtOH under reflux for 12 h to remove lipids. This step was repeated three times. After filtration, the residue was dried in air at room temperature, and then extracted with boiling distilled water thrice (2 h for each); the liquid extracts were combined. The combined aqueous filtrate was concentrated into one-tenth of the original volume, and 95% EtOH was added to the aqueous filtrate until the final alcohol concentration reached 30%. The resulting precipitate was separated out, defined as HEPF30. A portion of HEPF30 was dissolved in water, and the insoluble residue was removed by centrifugation. The supernatant was applied to a DEAE-Sepharose Fast Flow column (XK26 × 100 cm), eluted first with then with a 0–2 M gradient of NaCl. The fractions were collected by an auto-collector, and components were detected by means of the phenol–sulfuric acid assay.[9] HEPF30-A was

obtained from the water elute, and HEPF30-B and HEPF30-C were obtained from the 0–2 M gradient NaCl eluate. HEPF3 was purified by gel-permeation chromatography on a column of Sephacryl S-300 High Resolution (XK26 × 100 cm) from HEP30-A whose molecular weight range was first determined on a linked columns of TSK PWXL 4000 and 3000.

3.3. Determination of purity and molecular weight

Determination of the homogeneity and the molecular weight of the samples was done by HPLC on a linked gel-filtration column of TSK PWXL 4000 and 3000, eluting with 0.1 M phosphate buffer solution (PBS) and 0.3 M $NaNO_3$ at pH 7.0 with a flow rate of 0.6 mL/min. The column was kept at 30.0 ± 0.1 °C. The linear regression was calibrated by dextrans (T-700, 580, 300, 110, 80, 70, 40, 9.3, 4). All samples were prepared as 0.2% (w/v) solutions, and 10 μL of solution was analyzed in each run.

3.4. Sugar analyses

HEPF3 (2 mg) was hydrolyzed in 4 mL of 2 M trifluoroacetic acid (TFA) at 110 °C for 2 h. The monosaccharides were conventionally converted into the alditol acetates[10] and analyzed by GC–MS using a DB-5 column (30 m × 0.25 mm × 0.25 μm) and a temperature program consisting of 80–200 °C at 5 °C/min, increasing to 215 °C at 2 °C/min, and finally to 280 °C at 20 °C/min. The absolute configurations of the monosaccharides were determined as described by Vliegenthart and co-workers using (+)-2-butanol.[11]

3.5. Methylation analysis

Vacuum-dried polysaccharide (2 mg) was dissolved in DMSO (2 mL) and methylated by treatment with an NaOH–DMSO (0.2 mL) suspension and iodomethane (0.2 mL) by the method of Kalyan and Paul.[12] The reaction mixture was extracted with $CHCl_3$, and the solvent was then removed by evaporation. Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. The permethylated polysaccharide was hydrolyzed by treatment with HCO_2H (88%, 0.5 mL), H_2O (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, and the resulting mixture of alditol acetates was analyzed by GC–MS.

3.6. NMR analysis

HEPF3 (30 mg) was lyophilized three times in D_2O (0.5 mL). The ^1H NMR (25, 70 °C) and ^{13}C NMR (25 °C) spectra were determined in 5-mm tubes using a Varian INOVA 500 NMR spectrometer. ^1H chemical shifts were referenced to residual HDO at δ 4.78 ppm (25 °C) as the internal standard. ^{13}C chemical shifts were determined in relation to DSS (δ 0.00 ppm) calibrated externally. ^1H–^1H correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY), and heteronuclear multiple quantum coherence (HMQC) were used to assign signals. Two-dimensional heteronuclear multiple-bond correlation spectroscopy (HMBC) and two-dimensional Overhauser effect spectroscopy (NOESY) were used to assign inter-residue linkages and sequences.

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