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Structural elucidation of a neutral fucogalactan from the mycelium of *Coprinus comatus*

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Abstract—A water-soluble fucogalactan (CMP3), with a molecular mass of 1.03×10^4 Da as determined by high-performance size-exclusion chromatography (HPSEC), was obtained from the crude intracellular polysaccharide of *Coprinus comatus* mycelium. Its chemical structure was characterized by sugar and methylation analysis along with ^{1}H and ^{13}C NMR spectroscopy, including NOESY and HMBC experiments for linkage and sequence analysis. The polysaccharide is composed of a pentasaccharide repeating unit with the following structure:

α-L-Fuc*p*
1
↓
2
6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1

Keywords: *Coprinus comatus*; Mycelium; Fucogalactan; Polysaccharide; Structural analysis; NMR spectroscopy

1. Introduction

Mushroom-derived polysaccharides have emerged as an important class of bioactive substances, to which numerous medicinal and therapeutic properties have been attributed. So far, most of the polysaccharides isolated from the fruiting bodies of the basidiomycetes have been identified as either glucans or heterogalactans,[1] and various biological activities associated with the latter group have been demonstrated.[2,3]

The basidiomycete, *Coprinus comatus*, is a delicious and highly nutritious edible fungus, and is also considered to have an immense potential as a source of valuable medicinal compounds. Included among the various bioactivities ascribed to the mushroom are immunomodulation, together with hypoglycemic, hypolipidemic, antitumor, and antibacterial effects.[4,5] However, the structural properties of purified polysaccharides from *C. comatus* have not previously been investigated. As part of a continuing investigation of the chemical structures and biological activities of polysaccharides in this fungus, we now report the isolation of a fucogalactan

Abbreviations: NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; HMQC, heteronuclear multiple-quantum coherence spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HPLC, high-performance liquid chromatography; HPAEC-PAD, high-performance anion exchange-pulse amperometric detection chromatography.

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(CMP3) from the aqueous extracts of the fungal mycelium and its structural characterization.

2. Results and discussion

2.1. General observations

CMP3 gave a single symmetrical, narrow peak on elution from a Sephacryl S-300 column on HPLC. Its weight-average molar mass was estimated to be 1.03×10^4 Da by HPLC, using dextrans of known molecular weight as standards. CMP3 had an estimated total sugar content of 100% using the phenol–sulfuric acid method, and was protein-free according to the BCA™ protein assay (BCA™ protein assay kit, Pierce Chemical Co.). Hydrolysates of CMP3 contained galactose and fucose in the ratio of 4.02:1, and methylation analysis revealed 2,3,4-tri-*O*-methylfucose, 2,3,4-tri-*O*-methylgalactose, and 3,4-di-*O*-methylgalactose in the ratio of 1.15:2.88:1.00. Absolute configuration analysis[6,7] showed that the galactose residues had the D configuration and the fucose residue the L configuration.

The ^{1}H NMR spectrum (Fig. 1) contained signals for five anomeric protons at δ 5.12, 5.08, 5.02 (3H); the sugar residues are designated A–E according to the decreasing chemical shifts of the anomeric protons. The five low-field signals (A–E) with the *galacto* configuration all appear as singlets ($^3J_{1,2} < 3$ Hz) and represent sugars having the α-anomeric configuration.[9] The ^{13}C NMR spectrum contained signals for five anomeric carbons at δ 100.7–104.07, one CH_3–C group (C-6 of Fuc) at δ 18.33 and no signal for the unsubstituted hydroxymethylene protons in the region of δ 57.7–64.7 (all C-6 glycosylated).[10] As judged by the absence from the ^{13}C NMR spectrum of signals within δ 82–88, all sugar residues are in the pyranose form.[11]

2.2. Assignment of residue A

The COSY spectrum revealed stepwise connectivities from the H-1 (A) signal at δ 5.12 to H-3 at δ 3.93, but there was no H-3, H-4 correlation. The assignment of H-4 relies on the total correlation originating from H-1. Since the first two originate from H-2 and H-3, the third resonance must arise from H-4. In the NOESY spectrum of CMP3, both H-3 and H-4 correlate with a signal at δ 4.20, and H-2 shows a NOE to a signal at δ 1.28. Thus, these two protons (δ 4.20 and 1.28) are also located on residue A. Furthermore, because the signals at δ 4.20 and 1.28 (H-6 of Fuc) correlate with each other in both the COSY and TOCSY spectra, they were assigned to H-5 and H-6, respectively. Once the protons had been identified, the chemical shifts of their corresponding carbons were readily determined from the HMQC spectrum between carbon and proton in C–H pairs (Fig. 2, Table 1). Both carbon and proton chemical shifts (Table 1) are typical of a 6-deoxyhexopyranose. Since L-Fuc was the only such sugar identified by GC–MS analysis, residue A was assigned as the fucose residue, specifically as α-L-fucopyranoside.

2.3. Assignment of residue B

Using similar approaches, the spin system with the H-1 (B) signal at δ 5.08 was obtained from the complete proton and carbon chemical shifts (Table 1). The H-3 and H-4 signals of residue B overlapped due to similar chemical shifts. The linkage positions were determined from

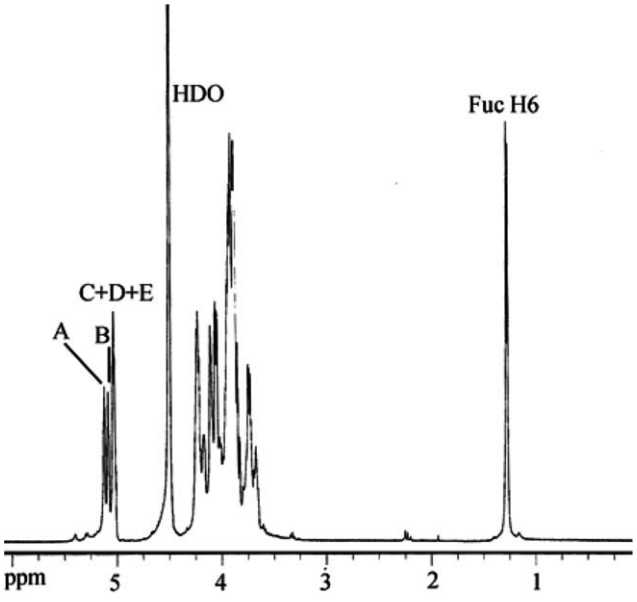


Figure 1. ^{1}H NMR spectrum of fucogalactan CMP3 from *Coprinus comatus* mycelium in D_2O at 60 °C. Anomeric protons are labeled A–E. The chemical shifts are expressed in δ ppm.

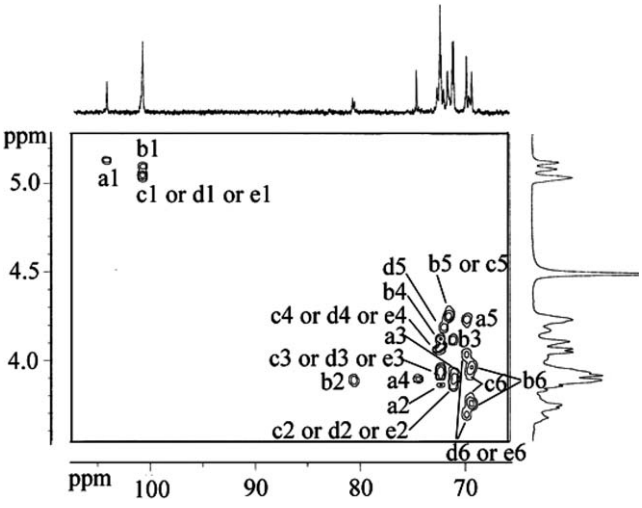


Figure 2. HMQC spectrum of fucogalactan CMP3 from *Coprinus comatus* mycelium in D_2O at 60 °C. Cross-peaks marked by lowercase letters indicate the correlations between H and C atoms.

Table 1. ^{1}H and ^{13}C NMR chemical shift data for fucogalactan CMP3 from *C. comatus* mycelium (δ, ppm)

Residue	$^{1}H/^{13}C$					
	1	2	3	4	5	6
α-$Fuc_{5.12}$	5.12	3.85	3.93	3.89	4.20	1.28
V (A)	104.07	72.2	71.02	74.48	69.82	18.33
α-$Gal_{5.08}$	5.08	3.87	4.10	4.12	4.23	3.74, 3.95
III (B)	100.7	80.65	71.14	72.29	71.40	69.32
α-$Gal_{5.03}$	5.03	3.89	3.92	4.07	4.23	3.74, 3.95
II (C)	100.7	71.02	72.29	72.20	71.50	69.58
α-$Gal_{5.02}$	5.02	3.89	3.92	4.05	4.17	3.68, 4.01
IV (D)	100.70	71.02	72.29	72.59	71.94	69.82
α-$Gal_{5.01}$	5.01	3.89	3.92	4.04	4.17	3.68, 4.01
I (E)	100.70	71.02	72.29	72.59	71.94	69.82

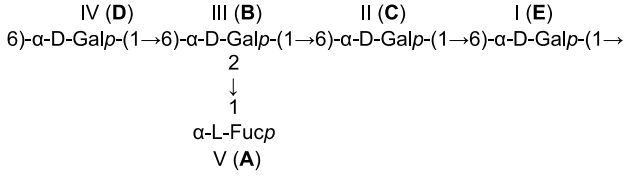
the downfield displacements of the C-2 and C-6 signals. Hence, residue B was identified as a 2,6-disubstituted α-D-galactopyranoside.

2.4. Assignments of remaining residues

The TOSCY spectrum demonstrated correlations between the H-1 signal at δ 5.02 and all protons from H-2 to H-4 for all sugar residues. The COSY spectrum showed most of the correlations between the neighbouring protons within each spin system, but there were no H-4, H-5 correlations. The signals from H-1–H-3 of residues C, D, and E are coincident and diverge from H-4. The NOESY spectrum showed the H-4, H-5 intraresidue cross-peaks C, D, and E at δ 4.07/4.23, 4.05/4.17 and 4.04/4.17, respectively. H-6a and H-6b were assigned from the TOCSY spectrum. The linkage positions were determined from the high chemical shift value for C-6. The remaining residues were assigned to a 6-substituted α-D-galactopyranoside.

Once the ^{1}H and ^{13}C spectra had been virtually completely assigned, the sequence of the glycosyl residues in the pentasaccharide was determined by the observed interresidue connectivities generated by NOESY and HMBC experiments. NOE connectivities were observed between A H-1 and B H-2, B H-1 and C H-6a/b, C H-1 and E H-6a/b, D H-1 and B H-6a/b, and E H-1 and D H-6a/b. The assignment of the disaccharide elements was further supported by the interresidue $J_{C,H}$-connectivities (Fig. 3) observed in the HMBC spectrum.

The results demonstrate that the polysaccharide consists of pentasaccharide repeating units with the following structure:



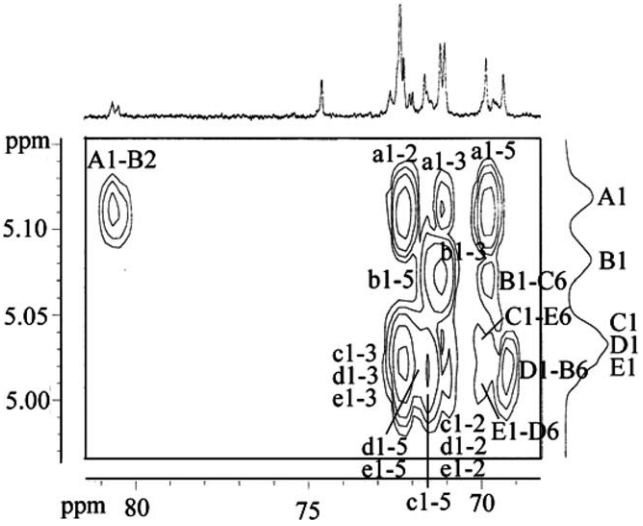


Figure 3. HMBC spectrum of fucogalactan CMP3 from *Coprinus comatus* mycelium in D_2O at 60 °C. The selected region shows the long-range ^{1}H–^{13}C connectivities of the anomeric protons. Cross-peaks marked by uppercase letters indicate three bond couplings ($^{3}J_{CH}$) across the glycosidic linkages between pertinent residues; those marked by lowercase letters refer to $^{2}J_{CH}$ and $^{3}J_{CH}$ couplings within the glycosyl rings.

To our knowledge, this is the first time that a neutral fucogalactan (CMP3) has been obtained from *C. comatus*. However, fungal heterogalactans have been reported previously, some of which are similar to CMP3, including a fucogalactan from *Ganoderma applanatum*,[12] mannofucogalactans from *Fomitopsis fraxinea* (Imaz.),[2] *Flammulina velutipes* (Fr.) Sing.,[3] *Polyporus pinicola* (Fr.),[13] *Polyporus fomentarius* (Fr.), and *Polyporus igniarius* (Fr.),[14] and fucomannogalactans from *Laetiporus sulphureus* (Bull.:Fr.) Murr.[15] These structures consist mainly of a backbone of (1→6)-linked α-D-galactopyranosyl residues, and are substituted at O-2 either by α-L-fucopyranosyl or 3-*O*-α-D-mannopyranosyl-α-L-fucopyranosyl residues. It is noteworthy that these structures have so far only been

reported in fungi, where it has been suggested that they may function as a type of storage material. Possible immunodulatory properties associated with CMP3 are currently under investigation.

3. Materials and methods

C. comatus mycelium was produced in a 30-L Biostat® E bioreactor (B. Braun Biotech International, Germany). The growth medium consisted of the following (w/v): 4% maize powder, 2% sucrose, 4% wheat bran, 0.1% KH_2PO_4, and 0.1% $MgSO_4$. Fermentation process conditions were as follows: growth temperature 26 °C, operational volume factor 2/3, inoculation 10%, gas throughput 10 L air min^{-1}, agitation rate 150–200 rpm, culture time 5 days. The mycelium was harvested, centrifuged (1687*g* for 15 min), washed with distilled water and dried in an oven at 60 °C.

3.1. Isolation and purification of polysaccharide

Dried *C. comatus* mycelium (325.15 g) was extracted 3× with 95% of EtOH for 1 h under reflux to remove lipid, and the residue was then extracted 4× with 10 vol of distilled water for 1 h at 100 °C. After centrifugation (1687*g* for 10 min, at 20 °C), the supernatant was concentrated 10-fold, and the crude polysaccharide fraction was precipitated with 2 vol of 95% EtOH. The precipitate was collected by centrifugation, dissolved in a minimum volume of distilled water and dialyzed in a DEAE cellulose bag against distilled water for 2 days to remove low-molecular-weight materials. The dialyzed solution was collected and freeze dried.

The crude polysaccharide (32.74 g, 10.07% yield) dissolved in water was purified using a DEAE-Sepharose Fast Flow column (Cl^- form, 26 × 100 cm). After applying the sample, the column was washed with 200 mL of distilled H_2O, and a fraction designated CMW was eluted (713.8 mg, yield: 2.18% of the crude polysaccharide). This fraction was freeze dried, reconstituted in distilled water (10 mg/mL), and applied to a Sephacryl S-300 High-Resolution column (26 × 100 mm), and purified CMP3 (108.6 mg, yield: 15.21% of fraction CMW) was eluted with 700 mL of distilled water.

3.2. Homogeneity and molecular mass

The homogeneity and molecular mass of CMP3 were determined by high-performance size-exclusion chromatography (HPSEC), using a Waters 2695 HPLC system fitted with two serially linked TSK PWXL 4000 and 3000 columns, a Model 2695 pump, a Waters 2410 RI detector, a Waters 2487 dual wavelength absorbance detector and an on-line de-gasser. The mobile phase was 0.3 M $NaNO_3$ and 0.1 M NaH_2PO_4, and the flow rate was 0.5 mL min^{-1} at 30 °C. A sample (3 mg) was dissolved in the mobile phase (1.5 mL) and centrifuged (1687*g*, 10 min), and 10 μL of supernatant was injected in each run. The molecular mass was estimated by reference to a calibration curve prepared using a set of Dextran T-series standards of known molecular mass (T-670, 410, 270, 150, 80, 50, 25, 12, 10).

3.3. Sugar and methylation analysis

Samples (1 mg) were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 °C for 6 h, and the monosaccharides were identified by high-performance anion-exchange pulsed-amperometric detection chromatography (HPAEC–PAD). The absolute configurations of the monosaccharides were determined by GLC of the acetylated glycosides with (+)-2-octanol 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^{-1}, increasing to 215 °C at 2 °C min^{-1}, and finally to 280 °C at 20 °C min^{-1}.[6,7]

Samples (2 mg) were methylated twice according to the modified NaOH–DMSO method.[8] The product showed no band in the 3600–3300 cm^{-1} region of the IR spectrum. Hydrolysis of the methylated polysaccharide was then performed with 2 M TFA at 100 °C for 6 h, and the partially methylated monosaccharides were reduced with $NaBH_4$ and acetylated with Ac_2O. The partially methylated alditol acetates were analyzed by GC–MS under the same chromatographic conditions as above.

3.4. Nuclear magnetic resonance (NMR) spectroscopy

Prior to the measurements, samples were deuterium-exchanged three times by freeze drying from D_2O. ^1H NMR (500 MHz, 25, 60 °C) and ^{13}C NMR (125 MHz, 25 °C) spectra were recorded with a Bruker AV-500 spectrometer. Chemical shifts are referenced to the HDO resonance (δ_H 4.78) at 25 °C as internal standard and DSS (δ_C 0.00 ppm) as external standard.

3.5. Chemicals and reagents

DEAE-Sepharose Fast Flow and Sephacryl™ S-300 High-Resolution reagents were purchased from the Amersham–Pharmacia Company. Dextrans and the monosaccharide standards, D-Glc, D-Man, D-Gal, L-Fuc, L-Rha, D-Ara, D-Xyl, were from Sigma–Aldrich. All other reagents were of analytical grade.

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References

1. Rosado, F. R.; Carbonero, E. R.; Claudino, R. F.; Tischer, C. A.; Kemmelmeier, C.; Iacomini, M. *FEMS Microbiol. Lett.* **2003**, *221*, 119–124.
2. Cho, S. M.; Koshino, H.; Yu, S. H.; Yoo, I. D. *Carbohydr. Polym.* **1998**, *37*, 13–18.
3. Mukumoto, T.; Yamaguchi, H. *Carbohydr. Res.* **1977**, *59*, 614–621.
4. Bailey, C. J.; Turner, S. L.; Jakeman, K. J.; Hayes, W. A. *Planta Med.* **1984**, *50*, 525–526.
5. Liu, Y. F.; Zhang, J. S. *Acta Edulis Fungi* **2003**, *10*, 60–63.
6. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1978**, *62*, 349–357.
7. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1979**, *77*, 1–7.
8. Needs, P. W.; Sevendran, R. R. *Carbohydr. Res.* **1993**, *245*, 1.
9. Harding, L. P.; Marshall, V. M.; Hernandez, Y.; Gu, Y.; Maqsood, M.; McLay, N.; Laws, A. P. *Carbohydr. Res.* **2005**, *340*, 1107–1111.
10. Agrawal, P. K. *Phytochemistry* **1992**, *31*, 3307–3330.
11. Bushmarinov, I. S.; Ovchinnikova, O. G.; Kocharova, N. A.; Blaszczyk, A.; Toukach, F. V.; Torzewska, A.; Shashkov, A. S.; Knirel, Y. A.; Rozalski, A. *Carbohydr. Res.* **2004**, *339*, 1557–1560.
12. Usui, T.; Iwasaki, Y.; Mizuno, T. *Carbohydr. Res.* **1981**, *92*, 103–114.
13. Fraser, R. N.; Karácsonyi, S.; Lindberg, B. *Acta Chem. Scand.* **1967**, *21*, 1783–1789.
14. Björndal, H.; Lindberg, B. *Carbohydr. Res.* **1969**, *10*, 79–85.
15. Alquini, G.; Carbonero, E. R.; Rosado, F. R.; Cosentino, C.; Iacomini, M. *FEMS Microbiol. Lett.* **2004**, *230*, 47–52.