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Note

The chemical structure of a mannofucogalactan from the fruit bodies of *Flammulina velutipes* (Fr.) Sing.

TAKAFUMI MUKUMOTO AND HARUKI YAMAGUCHI*

Department of Agricultural Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591 (Japan)

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The fruit bodies of fungi of the *Basidiomycetes* group generally contain (1→3)-β-D-glucans and mannofucogalactans. Structural investigations of these polysaccharides have been reported mainly for the *Polyporus* species[1–7], and only a few for other species of the *Basidiomycetes* group[8–10]. *Flammulina velutipes* (Fr.) Sing. is one of the edible mushrooms cultivated in Japan. Yoshioka *et al.*[11] showed recently the presence of a glucan and a neutral heteropolysaccharide in the fruit bodies of *Fl. velutipes* and reported on their antitumor activity. In a preliminary investigation, however, we observed that the fruit bodies contain at least two neutral heteropolysaccharides, in addition to a water-insoluble glucan. We now describe the isolation and structural investigation of a heterogalactan that is reactive with Concanavalin A.

Neutral polysaccharides were extracted from the fruit bodies of *Fl. velutipes* with cold water. Fraction A, precipitated at a concentration of 40% of acetone, was characterized as a mixture of a glucan and a xylomannan. Fraction B, precipitated at a concentration of 50% of acetone, gave galactose, mannose, fucose, and trace proportions of glucose and xylose on acid hydrolysis. This fraction was repeatedly precipitated at a concentration of 50% of acetone to remove the glucose- and xylose-containing components, and to give a purified polysaccharide containing D-galactose, D-mannose, and L-fucose in the molar ratio of 12:3:4. The polysaccharide gave a single symmetrical peak on electrophoresis and ultracentrifugation. The i.r. spectrum indicated that no *O*-acyl groups were present and that most of the glycosyl bonds had the α-D configuration. The polysaccharide formed readily a precipitate with Concanavalin A.

Only galactitol was found by g.l.c. analysis of the terminal alditol residue of the reduced polysaccharide, suggesting that the heteropolysaccharide may be terminated by a reducing galactose residue. From the molar proportion of the terminal galactitol to the total sugars, a DPn value of 6,000 (number-average molecular weight = 950,000) was calculated for the heteropolysaccharide, assuming that the polysaccharide

*To whom correspondence should be sent.

molecule has a galactose residue as reducing end. The absence of alditols other than galactitol is evidence for the homogeneity of the present polysaccharide preparation.

G.l.c. analysis[12–15] (see Table I) of the products of periodate oxidation, followed by borohydride reduction, indicated a preponderant proportion of glycerol and glycolaldehyde, and an absence of tetritol. This suggests that most of the hexose residues are linked at C-6 or are located at nonreducing ends, and none at C-4, or C-4 and C-6. Since no 1,2,3-butanetriol was found in the degradation products, 30% of the fucose residues are located at nonreducing ends or linked at C-2. The rest of the fucose residues were resistant to periodate oxidation, suggesting that they are linked at C-3 or present as branch points linked at C-2 and C-4. The production of a small amount of glyceraldehyde shows the presence of some sugar residues linked at C-2, or C-2 and C-6.

TABLE I

SMITH DEGRADATION PRODUCTS FROM THE HETEROGALACTAN

Degradation product	*Molar proportion*
1,2-Propanediol	[a]
1,2,3-Butanetriol	0
Glycerol	39
Erythritol	0
Threitol	0
Glycolaldehyde	32
Glyceraldehyde	8
Residual fucose	7
Residual mannose	0
Residual galactose	0

[a]Present, but could not be determined because of its volatility.

Gel filtration of the products of acetolysis gave two major fractions. The first fraction was a mixture of monosaccharides. The second fraction (obtained in a 40% yield, based on the mannose content) consisted of a disaccharide that was purified by paper chromatography and characterized as 3-*O*-α-D-mannopyranosyl-L-fucopyranose by methylation analysis. These results are consistent with those of the periodate oxidation method.

G.l.c. of the methylated alditol acetates obtained by methylation analysis revealed the presence of at least five major components (see Table II). All sugar residues are present in the pyranose form. D-Mannose residues are exclusively located at nonreducing terminal positions, whereas L-fucose residues are present at nonreducing ends and also as chain units linked at C-3. D-Galactose residues are present both as chain units linked at C-6, and as branch points linked at C-2 and C-6. These results are consistent with those obtained by the periodate oxidation method and by controlled acetolysis. Furthermore, the reactivity of the polysaccharide with

Concanavalin A may be explained by the presence of mannopyranose residues located at nonreducing terminals[16].

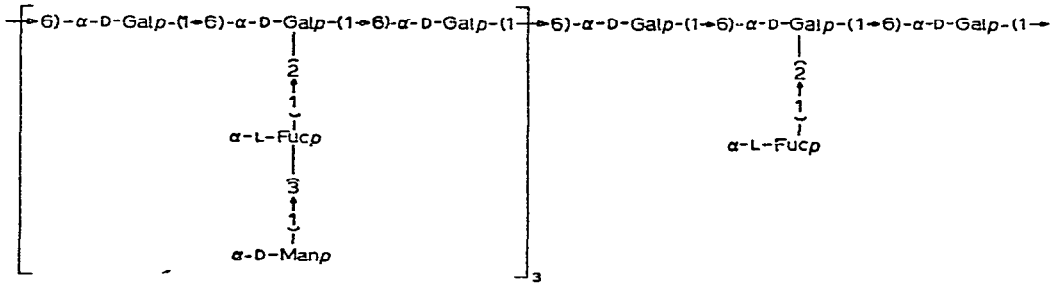
TABLE II

METHYL ETHERS FROM THE METHYLATED HETEROGALACTAN

Methylated sugar[a]	*Linkage indicated*	*Molar ratio*	T^b
2,3,4-Me_3-Fuc	Fuc*p*-(1→	1.1	0.65
2,3,4,6-Me_4-Man	Man*p*-(1→	2.8	1.00
2,4-Me_2-Fuc	→3)-Fuc*p*-(1→	2.8	1.15
2,3,4-Me_3-Gal	→6)-Gal*p*-(1→	8.0	3.24
3,4-Me_2-Gal	→6)-Gal*p*-(1→ 2 ↑	3.8	6.53

[a]Determined as alditol acetates. [b]Retention times of the corresponding alditol acetates on column (d) relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

On the basis of these results, we propose structure **1**. Every third (1→6)-linked α-D-galactopyranose residue is substituted with either a 3-*O*-α-D-mannopyranosyl-L-fucopyranose or an L-fucopyranose residue. The disaccharide unit and L-fucopyranose residue are present in a ratio of 3:1. From the optical rotation of the polysaccharide and the anomeric configuration of the other glycosidic linkages, it may be inferred that the L-fucopyranosyl residues have the α configuration. This structure is similar to those so far proposed for the mannofucogalactans obtained from other fungi of the *Basidiomycetes* group[2,3,8].



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EXPERIMENTAL

General methods. — All evaporations were conducted under reduced pressure at bath temperatures not exceeding 35°. Preparative paper chromatography was performed on Whatman 3MM paper by the ascending method with the solvent system 40:11:19 (v/v) butanol–ethanol–water[17]. Components were detected with

alkaline silver nitrate. Methanolysis of saccharides was performed by treatment with 2M hydrogen chloride in methanol for 8 h at 80° in a sealed tube. Hydrogen chloride was removed from the methanolyzate by neutralization with silver carbonate, followed by centrifugation. G.l.c. was conducted on a Shimadzu gas chromatograph, model 4APF, equipped with a hydrogen-flame ionization detector, and the following columns: (a) a glass column (200 × 0.4 cm i.d.) of Tabsorb (80–100 mesh, Regis Chem. Co., Chicago IL 60610) at 190° (alditol acetates), (b) a stainless-steel column (300 × 0.3 cm i.d.) of 5% of SE-30 on Chromosorb W (60–80 mesh) at 130–180° (4°/min) (Smith degradation products), (c) a glass column (200 × 0.4 cm i.d.) of 4% of SE-52 on Chromosorb W (80–100 mesh) at 175° (*O*-trimethylsilyl methyl glycosides), and (d) a glass column (200 × 0.4 cm i.d.) of 3% of ECNSS-M on Gaschrom Q (100–120 mesh) at 180° (partially methylated alditol acetates). G.l.c.–mass spectrometry was performed with chromatographic column (d) coupled to an LKB-9000 mass spectrometer. The mass spectra were recorded at an ionizing potential of 70 eV.

Optical rotations were measured at 23° with a Jasco Polarimeter DIP-SL. Electrophoretic examination (Hitachi HTB-2) was made on a solution of 0.5% of polysaccharide in 0.1M borate buffer (pH 9.2). The sedimentation pattern was obtained with a Beckman Ultracentrifuge model E, on a solution (0.5%) of the polysaccharide in 1% of sodium chloride, at 20°, for 100 min at a rotor speed of 52 000 r.p.m. I.r. spectra were measured on a KBr disk (polysaccharide), or on a chloroform solution (methylated polysaccharide) with a Jasco Infrared Spectrophotometer IRA-2.

The saccharides were permethylated by the Hakomori method[18] with methylsulfinylsodium (prepared according to the procedure of Sandford and Conrad[19]) and methyl iodide. Partially methylated alditols were converted into acetates for g.l.c. and g.l.c.–mass spectrometry. Peracetylation[20] of alditols and partially methylated alditols was performed by heating with anhydrous pyridine (0.1 ml) and freshly distilled acetic anhydride (0.1 ml) at 100° for 2 h. The excess reagents were removed, and the acetylated products dissolved in chloroform (50 μl) and chromatographed. Per-*O*-trimethylsilylation of the methyl glycosides obtained by methanolysis and of the compounds obtained by the periodate oxidation method was performed by treatment with 5:3:10 (v/v) hexamethyldisilazane–chlorotrimethylsilane–pyridine[21].

Isolation and purification of the heterogalactan. — The fresh fruit bodies of *Fl. velutipes* (600 g, obtained commercially in a supermarket, immediately after being harvested) were cut into small pieces and disintegrated in a Waring Blendor with cold water (1 l). The resulting slurry was centrifuged, and the precipitate separated from the supernatant was extracted with cold water (500 ml). Proteins were removed from the combined extracts by addition of trichloroacetic acid (100 g), followed by centrifugation. The supernatant was concentrated to 500 ml, and a polysaccharide material was precipitated with acetone (330 ml). The precipitate was centrifuged off, washed successively with ethanol and ether, and dried under reduced pressure (yield 660 mg, Fraction A). On hydrolysis, Fraction A yielded glucose, mannose, xylose, and trace amounts of galactose and fucose. The supernatant was treated with additional

acetone (170 ml), and the resulting precipitate was separated and dried as just described (yield 510 mg, Fraction B). On hydrolysis, Fraction B gave galactose, mannose, fucose, and trace amounts of glucose and xylose. To the supernatant was further added acetone, and the precipitate formed at the final acetone concentration (80%) was separated as just described (yield 70 mg, Fraction C). The hydrolyzate of this fraction contained glucose, galactose, and small amounts of mannose and fucose.

Fraction B was purified by dissolution in water (60 ml), the insoluble material being centrifuged off. To the supernatant solution in a 300-ml centrifuge bottle, immersed in an ice-water bath, was added acetone (60 ml) dropwise with stirring. The resulting turbid solution was kept overnight in a refrigerator. The precipitate was centrifuged off, and the same precipitation procedure was repeated twice. The precipitate was dissolved in water, dialyzed for 3 days against distilled water, and lyophilized (yield 460 mg); $[\alpha]_D^{23}$ +54° (*c* 1, water). The acid hydrolyzate, $[\alpha]_D^{23}$ +31° (*c* 0.5, water), contained galactose, mannose, and fucose.

For quantitative analysis, the heteropolysaccharide (0.1 mg) was dissolved in 0.5M sulfuric acid (0.2 ml) in a small glass tube and hydrolyzed *in vacuo* for 4–12 h at 100°. To the hydrolyzate was added D-arabinitol (25 μg) as an internal standard. The sulfuric acid was removed with barium carbonate (50 mg), followed by centrifugation. The neutral supernatant solution was passed through a small column of Dowex 50 X8 (H^+, 0.2 ml) to remove barium ions. The resin was eluted with water (twice 0.1 ml, and then 0.5 ml). The effluent was evaporated to dryness, and the residue was treated with 0.05M sodium hydroxide (0.1 ml) containing sodium borohydride (0.5 mg) for 3 h at room temperature. The excess of sodium borohydride was decomposed by adding 0.2M acetic acid (0.1 ml), and the resulting acidic solution was passed through Dowex 50 X8 (H^+, 0.2 ml) as just described. The effluent was evaporated to dryness in a glass tube with a Teflon-lined screw cap. The residual boric acid was removed by repeated additions and evaporations of methanol under reduced pressure. The products obtained were analyzed by g.l.c. as the acetates.

Analysis of the reducing end-group of the polysaccharide. — The reducing-end sugar residue of the polysaccharide was analyzed by the previously described method[22,23] with some modifications: The polysaccharide (15 mg) was reduced with sodium borohydride and the nonreducing polysaccharide obtained was hydrolyzed *in vacuo* with 0.5M sulfuric acid (2 ml) for 10 h at 100°. The volume of the hydrolyzate was adjusted to 5.0 ml with water.

(*a*). *Separation and determination of the terminal alditol of the reduced polysaccharide.* A large portion (4.5 ml) of the hydrolyzate was transferred to a centrifuge tube. To the acidic solution was added 2-deoxy-D-*arabino*-hexitol (3 μg) prepared by reduction of 2-deoxy-D-*arabino*-hexose (Sigma Chem. Co., Saint Louis, Missouri 63178) with sodium borohydride as an internal standard. Sulfuric acid was removed by addition of 0.5M barium hydroxide, followed by centrifugation. The slightly alkaline supernatant was passed through a small column of Dowex 50 X8 (H^+, 0.2 ml), and the resin was eluted with water (0.7 ml). The effluent was evaporated to a syrup, and the residue was subjected to repeated additions and evaporations of methanol

to remove trace amounts of boric acid. The residue was dissolved in water (0.3 ml), and the resulting solution was applied to a column of Dowex 1 X8 (OH^-, 0.8 ml) to remove reducing sugars. After washing the resin with water (2.2 ml), the effluent was evaporated to a small volume, and then again passed through Dowex 50 X8 (H^+, 0.2 ml), as just described. The effluent was evaporated to dryness in a small glass tube with a Teflonlined screw cap, and the last traces of boric acid were removed by repeated additions and evaporations of methanol under reduced pressure. The alditols were acetylated and analyzed by g.l.c., as just described.

(*b*) *Determination of all carbohydrate components.* A small portion (0.2 ml) of the hydrolyzate was diluted to 25 ml with water. To a part (0.3 ml) of the resulting solution was added the same internal standard as that used for determination of the terminal alditol. The mixture was passed through a small column of Dowex 1 X8 (AcO^-, 0.2 ml) to remove sulfuric acid, and the resin was eluted with water (0.7 ml). The effluent was evaporated to dryness, and the residue was dissolved in water (50 μl) and then treated for 3 h at 30° with 0.05M sodium hydroxide (30 μl) containing sodium borohydride (0.42 mg). The excess of sodium borohydride was decomposed by adding 0.2M acetic acid (0.1 ml), and the resulting acidic solution was passed through Dowex 50 X8 (H^+, 0.2 ml). The resin was eluted with water (0.7 ml), and the combined effluents were evaporated to dryness in a glass tube with a Teflon-lined screw cap. The residual boric acid was removed by repeated additions and evaporations of methanol under reduced pressure. The products obtained were acetylated and analyzed by g.l.c.

Smith degradation of the polysaccharide and g.l.c. analysis of the products. — The polysaccharide (12 mg) was oxidized for 10 days at 18° in the dark with 0.1M sodium periodate (3 ml). At suitable intervals, the consumption of periodate was followed by measuring[25] the absorbance at 290 nm. The excess of periodate was decomposed by addition of 1,2-ethanediol, and the mixture was subsequently treated for 5 h at room temperature with sodium borohydride (42 mg). The excess of borohydride was decomposed with acetic acid, and the solution was evaporated to a small volume and applied to a column (160 × 2 cm) of Sephadex G-25. The polyalcoholic derivative was eluted with water at a flow rate of 6 ml/h. Carbohydrates in the fractions were detected by the orcinol–sulfuric acid reaction[26]. The appropriate fractions were combined and lyophilized (yield 10 mg).

The compounds produced by periodate oxidation of the polysaccharide, followed by borohydride reduction, were determined by the method of Yamaguchi *et al.*[12–15]. To the aqueous solution (0.5 ml) containing the polyalcoholic derivative (200 μg) was added 0.175M hydrochloric acid (0.2 ml) containing hydroxylamine hydrochloride (5 mg). The solution was heated at 80° in a sealed tube for 90 min, and then evaporated to dryness under reduced pressure. The products were *O*-trimethylsilylated in anhydrous pyridine (0.1 ml) with 2-ethyl-2-(hydroxymethyl)-1,3-propanediol (50 μg) as an internal standard and analyzed by g.l.c.

The residual sugars in the polyalcoholic derivative were also determined by g.l.c., as indicated for the analysis of the polysaccharide.

Controlled acetolysis of the polysaccharide, and isolation and characterization of an oligosaccharide. — The polysaccharide (20 mg) was mixed with 1:1 (v/v) acetic anhydride and anhydrous pyridine (0.8 ml). The dispersion was kept for 24 h at room temperature and then heated for 1 h at 100°. The acetylated polysaccharide was freed from the acetylating reagents by repeated additions and evaporations of water. Acetolysis of the acetylated polysaccharide was performed by the procedure described by Stewart *et al.*[27], but with some modifications. The acetylated polysaccharide was dissolved in the acetolysis medium (2 ml) and kept for 16 h at 30°. The yellowish solution was poured into 5 ml of water, and the pH of the solution was adjusted to about 5 by addition of sodium hydrogencarbonate. The acetolysis products were repeatedly extracted with chloroform. The chloroform extracts were combined and washed with water, and then evaporated to dryness. The products were deacetylated by dissolution in absolute methanol (1 ml) and addition of a catalytic amount of sodium methoxide in methanol. After 1 h, the precipitate formed was dissolved by adding water, and the solution was immediately neutralized with dilute hydrochloric acid.

The deacetylated products were filtered through a column (180 × 2 cm) of Bio-gel P-2 (100–200 mesh) and eluted with water at a flow rate of 6 ml/h. The fractions were examined by the orcinol–sulfuric acid reaction[26], and the appropriate fractions were combined and evaporated to dryness. The oligosaccharide was purified by preparative paper chromatography and methylated. The permethylated oligosaccharide, recovered by partition between chloroform and water, was hydrolyzed and analyzed by g.l.c.–mass spectrometry, as described for the polysaccharide.

Methylation analysis of the polysaccharide. — The polysaccharide (30 mg), dried *in vacuo*, was dissolved in dimethyl sulfoxide (3 ml) under an atmosphere of nitrogen. To the clear solution was added 2M methylsulfinylsodium in dimethyl sulfoxide (3 ml), and the solution was stirred at room temperature for 5 h. Methyl iodide (1.5 ml) was added at 18–20° with external cooling (ice–water), and the turbid solution was stirred overnight. The reaction mixture was poured into water (20 ml), and the methylated polysaccharide was repeatedly extracted with chloroform. The chloroform extracts were combined, washed with water, dried (sodium sulfate), and evaporated to a syrup. The methylation procedure was repeated once again as just described.

The permethylated polysaccharide (0.3 mg) was hydrolyzed with 90% formic acid (0.5 ml) for 2 h at 100°, and then with 0.5M sulfuric acid (0.2 ml) *in vacuo* for 5 h at 100°. After neutralization with barium carbonate (50 mg) and centrifugation, the neutral hydrolyzate was passed through a small column of Dowex 50 X8 (H^+, 0.2 ml) to remove barium ions. The partially methylated sugars were converted into alditol acetates as previously described, and analyzed by g.l.c.–mass spectrometry.

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REFERENCES

1 V. P. BHAVANANDAN, H. O. BOUVENG, AND B. LINDBERG, *Acta Chem. Scand.*, 18 (1964) 504–512.
2 R. N. FRASER, S. KARACSONYI, AND B. LINDBERG, *Acta Chem. Scand.*, 21 (1967) 1783–1789.
3 H. BJÖRNDAL AND B. LINDBERG, *Carbohydr. Res.*, 10 (1969) 79–85.
4 H. BJÖRNDAL AND B. WÅGSTRÖM, *Acta Chem. Scand.*, 23 (1969) 1560–1566.
5 K. AXELSSON, H. BJÖRNDAL, AND B. LINDBERG, *Acta Chem. Scand.*, 23 (1969) 1597–1600.
6 K. AXELSSON AND H. BJÖRNDAL, *Acta Chem. Scand.*, 23 (1969) 1815–1842.
7 H. BJÖRNDAL AND B. WÅGSTRÖM, *Acta Chem. Scand.*, 23 (1969) 3313–3320.
8 R. N. FRASER AND B. LINDBERG, *Carbohydr. Res.*, 4 (1967) 12–19.
9 M. SHIDA, K. HARYU, AND K. MATSUDA, *Carbohydr. Res.*, 41 (1975) 211–218.
10 S. UKAI, K. HIROSE, T. KIHO, AND C. HARA, *Chem. Pharm. Bull.*, 22 (1974) 1102–1107.
11 Y. YOSHIOKA, T. SANO, AND T. IKEKAWA, *Chem. Pharm. Bull.*, 21 (1973) 1772–1776.
12 H. YAMAGUCHI, T. IKENAKA, AND Y. MATSUSHIMA, *J. Biochem. (Tokyo)*, 63 (1968) 553–554.
13 H. YAMAGUCHI, T. IKENAKA, AND Y. MATSUSHIMA, *J. Biochem. (Tokyo)*, 68 (1970) 253–254.
14 H. YAMAGUCHI, T. IKENAKA, AND Y. MATSUSHIMA, *J. Biochem. (Tokyo)*, 68 (1970) 843–848.
15 K. FUKAGAWA, H. YAMAGUCHI, O. UOTANI, T. TSUJIMOTO, AND D. YONEZAWA, *Agric. Biol. Chem.*, 39 (1975) 1703–1710.
16 I. J. GOLDSTEIN, *Methods Carbohydr. Chem.*, 6 (1972) 106.
17 P. A. J. GORIN AND J. F. T. SPENCER, *Can. J. Chem.*, 46 (1968) 3407–3411.
18 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
19 P.A. SANDFORD AND H. E. CONRAD, *Biochemistry*, 5 (1966) 1508–1517.
20 W. F. LEHNHARDT AND R. J. WINZLER, *J. Chromatogr.*, 34 (1968) 471–479.
21 C. C. SWEELEY, R. BENTLEY, M. MAKITA, AND W. W. WELLS, *J. Am. Chem. Soc.*, 85 (1963) 2497–2507.
22 H. YAMAGUCHI, S. INAMURA, AND K. MAKINO, *J. Biochem. (Tokyo)*, 79 (1976) 299–303.
23 H. YAMAGUCHI AND K. MAKINO, *J. Biochem. (Tokyo)*, 81 (1977) 563–569.
24 M. ABDEL-AKHER, J. K. HAMILTON, AND F. SMITH, *J. Am. Chem. Soc.*, 73 (1951) 4691–4692.
25 T. IKENAKA, *J. Biochem. (Tokyo)*, 54 (1963) 328–333.
26 M. ANAI, T. IKENAKA, AND Y. MATSUSHIMA, *J. Biochem. (Tokyo)*, 59 (1966) 57–62.
27 T. S. STEWART, P. B. MENDERSHAUSEN, AND C. E. BALLOU, *Biochemistry*, 7 (1968) 1843–1854.