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Polysaccharides Elaborated by *Polyporus pinicola* (Fr)

R. N. FRASER, STEFAN KARÁCSONYI and BENGT LINDBERG

Träkemiska avdelningen, Svenska Träforskningsinstitutet och Institutionen för organisk kemi, Stockholms Universitet, Stockholm, Sweden

Two polysaccharides have been isolated from the fruit bodies of *Polyporus pinicola*. One was a fucomannogalactan of essentially the same structure as a polysaccharide previously isolated from *Armillaria mellea*. The other was a highly branched fucoxylomannan the side chains of which are terminated by D-xylose and L-fucose residues.

In previous publications we have reported studies on different polysaccharides isolated from higher fungi of the Basidiomycetes group. Fucomannogalactans were isolated from *Polyporus giganteus* [1] and *Armillaria mellea* [2] fruit bodies and a related polysaccharide, containing also 3-*O*-methyl-D-galactose as a component, was isolated from the mycelial growth of the latter fungus,[3] as well as a xylomannan. Ralph and Bender [4] report the isolation of a xylomannan from *P. tumulosus*. Rosík *et al.*[5] report the isolation of polysaccharides, containing glucose, mannose, xylose, and fucose from *Fomes marginatus*. The present paper reports studies on polysaccharides isolated from fruit bodies of *P. pinicola*.

Fruit bodies of *P. pinicola* were harvested locally, disintegrated and extracted with acetone and methanol. Water extraction yielded a polysaccharide material (A). Subsequent extraction with a 0.1 M potassium hydroxide solution yielded two polysaccharide fractions, one alkali-soluble, water-insoluble (B) and one alkali-soluble, water-soluble (C). Further extraction with 0.3 M potassium hydroxide solution yielded fraction D.

The heterogalactan. Crude fraction A had $[\alpha]_{578}$ + 122° and acid hydrolysis of a sample yielded glucose together with smaller amounts of galactose (7 %), mannose (3 %), fucose (2 %), and xylose (traces). The fraction was added to the top of a DEAE-cellulose column in the borate form, and this was then eluted batchwise with aqueous sodium borate of increasing concentrations, followed by 0.1 M and 0.3 M potassium hydroxide solutions. The polysaccharide material obtained from the first two fractions was a pure glucan, $[\alpha]_{578}$ + 175°. This material, which is probably a fungal glycogen, was not further investigated. The material eluted with 0.5 M sodium borate yielded on hydrolysis approximately equal amounts of glucose and galactose plus smaller amounts

of fucose and mannose. Enzymatic hydrolysis of this fraction, firstly with a commercial α-amylase preparation and secondly with a β-(1→3)-glucanase from Basidiomycete QM 806,[6] removed almost all of the glucose but did not release any of the other sugars. The remaining polysaccharide, which gave a single spot on electrophoresis in borate buffer on a glass fiber sheet, had $[\alpha]_{578}$ + 106° and on hydrolysis yielded D-galactose, L-fucose, and D-mannose in the relative proportions 5.9:1.7:1. These figures are similar to those obtained from the fucomannogalactan isolated from the *A. mellea* fruit bodies,[2] *viz.* $[\alpha]_{578}$ + 98° and D-galactose:L-fucose:D:mannose, 6.5:2:1, and indicate that the polysaccharides are related. This assumption was confirmed by the structural studies. The methylated sugars obtained on hydrolysis of the fully methylated polysaccharide (Table 1) are the same as those obtained from the *A. mellea*

Table 1. Methyl ethers from hydrolysed methylated heterogalactan.

Sugar	Retention times (T)[10]			
	column a		column b	
2,3,4,6-Me_4-Gal[a]	1.80		1.50	1.60
2,3,4,6-Me_4-Man	1.41		1.27	
2,3,4-Me_3-Fuc	1.72			
2,4-Me_2-Fuc	1.62	2.22		
2,3,4-Me_3-Gal	7.3		2.60	2.89
3,4-Me_2-Gal				
2,3-Me_2-Gal				
2(?)-Me-Fuc				
3-Me-Fuc				

[a] 2,3,4,6-Me_4-Gal = 2,3,4,6-tetra-*O*-methyl-D-galactose, *etc.*

heterogalactan. By visual inspection of the chromatograms and comparison of the intensity of the colour stains it was possible to show that the proportions of the methylated sugars were approximately the same as those from the hydrolysed methylated *A. mellea* heterogalactan.

Partial acid hydrolysis yielded the disaccharide 3-*O*-α-D-mannopyranosyl-L-fucose and a homologous series of α-(1→6)-linked oligosaccharides containing D-galactose residues. On periodate oxidation the polysaccharide consumed

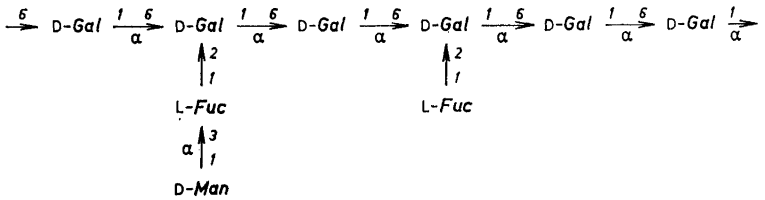

Fig. 1. Structure of the heterogalactan.

1.54 moles of periodate and released 0.76 moles of formic acid per hexose residue. These figures are in reasonably good agreement with the values calculated for a polysaccharide with the above composition and the general structure given in Fig. 1. This structure is the same as that proposed for the *A. mellea* fruit body heterogalactan, the two polysaccharides showing only a slight difference in the relative proportions of the sugar residues. As for the *A. mellea* heterogalactan,[2] the value of the optical rotation of the polysaccharide indicates that the L-fucopyranosyl residues have the α-configuration.

The fucoxylomannan. When the alkali-soluble, water-insoluble fraction (C) was dissolved in 2 % sodium hydroxide and saturated barium hydroxide solution was added, a precipitate was formed. By repeating this process twice, an essentially pure polysaccharide, composed of mannose, xylose, and fucose, was obtained. It still contained small amounts of glucan which was removed by treatment with β-(1→3)-glucanase. It was contaminated with some dark-coloured material which prevented the determination of its optical rotation.

The alkali-soluble, water-insoluble fraction (B) was also purified by precipitation with barium hydroxide to yield a fucoxylomannan. As a result of the insolubility of this material in water it was not treated with β-(1→3)-glucanase and still contained about 8 % glucose. The relative proportions of D-mannose, D-xylose, and L-fucose were 1.0:0.78:1.0, and the optical rotation, in 0.5 M sodium hydroxide, was $[\alpha]_{578}$ — 46°.

A sample from fraction B was exhaustively methylated, hydrolysed, and the methylated sugars were separated using preparative TLC. From the fractions obtained 3,4-di-*O*-methyl-D-xylose, 2,3,4-tri-*O*-methyl-D-xylose and 2,3,4-tri-*O*-methyl-L-fucose were identified with certainty. A di- and a tri-*O*-methyl-D-mannose were also obtained; the retention times of the latter on GLC indicated that it was either 2,3,4- or 3,4,6-tri-*O*-methyl-D-mannose. From these results it is possible to suggest that in the polysaccharide the mannose residues are present as chain units and as branching points, the xylose residues are present as chain units linked through C-1 and C-2 and as non-reducing end-groups and the fucose residues occur solely as non-reducing end-groups. The negative rotation of the polysaccharide indicates that the mannose and xylose residues are β-linked.

The fucoxylomannan is not related to the xylomannan from the *Armillaria mellea* mycelium [3] ($[\alpha]_{578}$ + 71°), which contains a backbone of α-(1→3)-linked D-mannopyranose residues, part of which are substituted in the 4-position with 4-*O*-α-D-xylopyranosyl-D-xylopyranose residues. A more detailed structural assignment to the fucoxylomannan must await further studies.

EXPERIMENTAL

General methods. Evaporations were carried out at reduced pressure at temperatures not exceeding 40°.

Paper chromatograms were run on Whatman No. 1 and 3 MM papers, using the following systems (v/v):

a) ethyl acetate-acetic acid-water (3:1:1)
b) ethyl acetate-pyridine-water (8:2:1)
c) ethyl acetate-pyridine-water (2:1:2, upper phase)

d) butanol-ethanol-water (10:3:5)
e) butanone-water-conc. ammonia (200:17:1)

Electrophoretic analyses were performed on Whatman No. 1 papers in one of the following electrolytes:

a) 0.05 M germanate at 40°, pH 10.7[7]
b) 0.05 M sulphonated phenylboronic acid at 40°, pH 6.5[8]
c) 0.1 M borate at room temperature, pH 10

Components were detected with alkaline silver nitrate or *p*-anisidine hydrochloride. Sugar determinations were made using GLC of trimethylsilyl ethers.[9] GLC was carried out on a Perkin Elmer model 800 instrument, using the following columns:

a) 15 % butan-1,4-diol succinate on Chromosorb W at 175°
b) 15 % polyphenyl ether on Chromosorb W at 200°

The retention times of the methylated methyl glycosides are relative to that of methyl tetra-*O*-methyl-*β*-D-glucopyranoside.[10]

Optical rotations were determined at 578 mμ at room temperature (20—22°) in aqueous solutions unless otherwise stated.

TLC was performed on Kieselgel G (E. Merck AG) using benzene-acetone (1:1) as solvent.

Extraction of the polysaccharides.

P. pinicola fruit bodies (490 g) in ethanol were disintegrated in a Turmix blender and continuously extracted, first with acetone (29 h) and then with methanol (24 h). The air-dried residue was extracted with water (6 l) at 90° for 2 h, followed by extraction with water (3 l) at room temperature in a Philips L 368 ultrasonic bath for 3 h. The combined extracts were concentrated to 300 ml and the concentrate was poured into ethanol (1.8 l). The precipitate was worked up to give Fraction A (7.8 g).

The residue was treated further with 0.1 M potassium hydroxide solution (2 × 4 l) under nitrogen at room temperature for 2 h. The combined extracts were neutralised with acetic acid, and the water-insoluble precipitate was worked up to give Fraction B (42 g). A sample on hydrolysis yielded glucose (93 %), galactose (1 %), mannose (3 %), fucose (2 %), and xylose (2 %). The neutral solution was concentrated to 600 ml and the concentrate poured into ethanol (3 l) to give Fraction C (22 g).

Further extraction with 0.3 M potassium hydroxide (2 × 4 l) for 2 h gave Fraction D (10 g). Hydrolysates of C and D had about the same composition as that of B.

The heterogalactan

Isolation of the heterogalactan. Fraction A (3 g) in water (10 ml) was added to the top of a DEAE-cellulose column (40 × 4 cm) in the borate form. The column was irrigated with 2 l of each of the following solutions: water, 0.05 M, 0.1 M, and 0.5 M sodium borate and 0.1 M and 0.3 M potassium hydroxide. The progress of the fractionation was followed polarimetrically. The procedure was repeated with the remaining part of Fraction A. The results of the fractionation are given in Table 2.

Table 2. Composition of fractions from DEAE-cellulose column.

Fraction eluted by	Weight g	[α]	Gal	Glu	Man	Fuc
Original sample	3.0	+122°	+	++	+	+
0.05 M $NaBO_2$	0.64	+163°	—	++	—	—
0.1 M $NaBO_2$	0.21	+175°	—	++	—	—
0.5 M $NaBO_2$	0.82	—	++	++	+	+
0.1 M KOH	0.61	—	++	++	+	+
0.3 M KOH	0.47	+ 33°	+	++	+	+

++ Present in large amounts, + present, — absent.

The fraction eluted with 0.5 M sodium borate (1.8 g) was dissolved in 0.02 M phosphate buffer of pH 6.9 (50 ml) and treated with α-amylase (*Bacillus subtilis*, 4 mg) for 64 h at 20°. The recovered polysaccharide, which still contained some glucan, was dissolved in 0.025 M citrate buffer of pH 4.2 (30 ml) and treated with β-(1→3) D-glucanase (20 mg) at 30° for 72 h. The recovered polysaccharide (570 mg) had [α] + 106°. A hydrolysate contained galactose, mannose, and fucose in the molar ratios 5.9:1.0:1.7, together with traces of glucose. No sugars other than glucose containing oligosaccharides and glucose were released during the enzymatic treatments.

Methylation analysis of the heterogalactan. The polysaccharide (151 mg) was dissolved in hot dimethylformamide (7.5 ml) and the solution, after cooling, was treated with acetic anhydride (25 ml) and pyridine (4 ml) overnight at room temperature and then at 45° for 2 h. The solution was poured into ice-water and the precipitate formed was collected by filtration and dried. Precipitation from chloroform solution by light petroleum gave a white powder (188 mg) with [α] + 106° (*c* 0.46, chloroform). The acetylated polysaccharide was methylated as described for the α-glucan from *Pullularia pullulans* [11] to give a syrup. After precipitation from chloroform solution with light petroleum a white powder (50 mg) with [α] + 97° (*c* 0.30, chloroform) and methoxyl content 41.2 % (theoretical value 44%) was obtained. IR examination indicated the absence of hydroxyl groups.

A sample was methanolysed and the methyl glycosides so produced were examined by GLC on columns a and b. The remainder of the material was treated for 1 h with 90 % formic acid at 100° and then hydrolysed with 0.25 M sulphuric acid at 100° for 18 h. The hydrolysate was deionised and concentrated to a syrup (39 mg). The methylated sugars obtained were compared using paper chromatography in solvents b, d, and e with the hydrolysate of methylated *Armillaria mellea* fruit body heterogalactan and authentic samples. The methylated sugars detected are listed in Table 1, with the retention times when available.

Partial acid hydrolysis of the heterogalactan. The polysaccharide (460 mg) was given successive hydrolytic treatments at 100° for 1 h, first twice with 0.05 M hydrochloric acid, secondly twice with 0.1 M hydrochloric acid, and finally once with 0.25 M hydrochloric acid. The hydrolysis mixture was neutralised after each treatment (Dowex 2, free base) and the part to be hydrolysed further precipitated with ethanol. The low molecular weight fragments were examined by paper chromatography in solvents a, b, and c. The two disaccharides, having the highest mobilities, were separated using thick filter paper and were obtained pure in low yields. They were characterised as follows. *3*-O-α-D-*Mannopyranosyl*-L-*fucose*, [α] + 1.2° (*c* 0.21) gave equimolecular amounts of mannose and fucose on acid hydrolysis. It was chromatographically and electrophoretically indistinguishable from the mannosyl-fucose isolated from the *P. giganteus* [1] and *Armillaria mellea* [2] fruit body heterogalactans. *6*-O-α-D-*Galactopyranosyl*-D-*galactose*, [α] + 144° (*c* 0.13), gave only galactose on acid hydrolysis and was chromatographically and electrophoretically indistinguishable from authentic α-(1→6)-galactobiose. This disaccharide was shown to be the lowest member of a homologous series of α-(1→6)-linked galactose containing oligosaccharides, indistinguishable from those obtained from the *Armillaria mellea* fruit body heterogalactan.

Periodate oxidation. A sample of the polysaccharide consumed 1.54 moles of periodate and released 0.76 moles of formic acid per anhydrohexose unit. The periodate consumption was followed spectrophotometrically [12] and the released acid titrated potentiometrically with sodium hydroxide solution. After completion of the oxidation sodium borohydride was added, and on acid hydrolysis of the deionised residue, fucose and glycerol were the only fragment that could be detected.

The fucoxylomannan

Isolation of the fucoxylomannan. Fraction C (18 g) was dissolved in 2 % aqueous potassium hydroxide and saturated aqueous barium hydroxide was added dropwise until no more precipitate was formed. The precipitate was collected by centrifugation, washed twice with dilute barium hydroxide solution, dissolved in acetic acid, and precipitated with ethanol. This procedure was repeated twice. The polysaccharide was then

treated with β-(1→3)-glucanase (20 mg) as described above. The resulting product (1.5 g) contained mannose, xylose, fucose, and only trace amounts of glucose.

The alkali-soluble, water-insoluble fraction (B, 30 g) was fractionated by precipitation with barium hydroxide as described above. Because of the insolubility of the product in water the enzymatic treatment was omitted. The material obtained (1.7 g) had [α] — 46° (*c* 1.0, 0.5 M sodium hydroxide). A hydrolysate contained 8 % glucose and mannose, xylose and fucose in the relative molar proportions 1.0:0.78:1.0. The sugars in the hydrolysate were isolated by preparative paper chromatography (solvent b) and characterised. D-*Mannose*, [α] + 16°, phenylhydrazone, m.p. and mixed m.p. 198—200°. D-*Xylose*, [α] + 18°, osazone, m.p. and mixed m.p. 160—162°, L-*Fucose*, [α] — 64°, 1-methyl-1-phenylhydrazone, m.p. and mixed m.p. 175—177°.

Methylation analysis of the fucoxylomannan. The water soluble polysaccharide (713 mg) was dissolved in hot dimethylsulphoxide (70 ml) and, after cooling, was treated with dimethyl sulphate (24.5 ml) and powdered sodium hydroxide [13] (35 g), first at room temperature for 24 h and then on a steam bath for 1 h. After neutralisation (5 M sulphuric acid), filtration to remove precipitated sodium sulphate, and washing of the precipitate with chloroform, the filtrate was extracted continuously with chloroform for 16 h. The combined chloroform solutions were dried over sodium sulphate, concentrated, and the methylation procedure was repeated once. The partially methylated polysaccharide was refluxed with methyl iodide (20 ml) and silver oxide (20 g) for 16 h, and this procedure was repeated twice. The product was precipitated by the addition of a chloroform solution to light petroleum to give an almost colourless powder (460 mg), having [α] —67° (*c* 0.5, chloroform) and a methoxyl content of 38.7 % (theoretical value 41.1 %). An IR examination of the product showed the absence of hydroxyl absorption.

The methylated polysaccharide (216 mg) was dissolved in 90 % formic acid (20 ml), kept at 100° for 90 min, cooled, concentrated, and then treated with 0.25 M. sulphuric acid (20 ml) at 100° for 18 h. The product was neutralised with barium carbonate, filtered, deionised, and concentrated to a syrup (205 mg). The mixture of methylated sugars was fractionated by preparative TLC (see Table 3).

Di-O-*methyl*-D-*mannose*. The sugar had the chromatographic mobility of a di-*O*-methyl mannose and gave only mannose on demethylation with boron trichloride. Its electrophoretic mobility coupled with other evidence suggested that it might be 2,6-di-*O*-methyl-D-mannose.

3,4-Di-O-*methyl*-D-*xylose*, $[\alpha]_D$ + 20°, on demethylation gave only xylose. It gave a positive reaction with 2,3,5-triphenyltetrazolium chloride. On GLC the retention times of its methyl glycosides corresponded to those of the methyl glycosides of 3,4-di-*O*-methyl-D-xylose and were distinguishable from those prepared from 2,3- and 2,4-di-*O*-methyl-D-xylose.

Tri-O-*methyl*-D-*mannose*, was mixed with 2,4,6-tri-*O*-methyl-D-glucose, the latter deriving from contaminating glucan. Demethylation yielded a mixture of mannose and glucose. GLC of the methyl glycoside mixture indicated that the mannose derivative

Table 3. Methylated sugars from the methylated fucoxylomannan (205 mg).

Fraction	weight, mg	R^a (solvent d)	R^a (TLC)	sugar
1	57	0.71	0.15	Me_2-Man
2	35	0.79	0.54	3,4-Me_2-Xyl[b]
3	21	0.82	0.63	Me_3-Man 2,4,6-Me_3-Glu
4	59	0.94	0.85	2,3,4-Me_3-Fuc
5	7	0.99	0.97	2,3,4-Me_3-Xyl
		1	1	2,3,4,6-M_4e-Glu

[a] Relative to 2,3,4,6-tetra-*O*-methyl-D-glucose.
[b] 3,4-Di-*O*-methyl-D-xylose, *etc.*

Table 4. GLC analysis of methyl ethers from methylated fucoxylomannan.

Sugar	molar %	*T* (column a)		*T* (column b)	
2,6(?)-Me_2-Man[a]	29.6			3.00	
3,4-Me_2-Xyl	25.5	1.29	1.53	0.71	0.76
2,4,6-Me_3-Glu	4.0	3.08	6.32	1.69	2.29
2,3,4- or 3,4,6-Me_3-Man	6.2	3.31			
2,3,4-Me_3-Fuc	30.7	0.76		0.65	
2,3,4-Me_3-Xyl	3.4	0.50	0.61	0.42	0.50
2,3,4,6-Me_4-Glu	0.5	1	1.43	1	1.31

[a] 2,6-Di-*O*-methyl-D-mannose, *etc.*

was either 2,3,4-or 3,4,6-tri-*O*-methyl-D-mannose,[14] the retention times of these two sugars being indistinguishable.

2,3,4-Tri-O-*methyl*-L-*fucose*, $[\alpha]$ −120°, on demethylation yielded fucose. On GLC a single peak, with the expected retention time, was observed.

2,3,4-Tri-O-*methyl*-D-*xylose* was contaminated with traces of 2,3,4,6-tetra-*O*-methyl-D-glucose. The sugars were identified by GLC and demethylation.

For quantitative analysis the methylated polysaccharide (2 mg) was treated with 3 % methanolic hydrogen chloride (0.2 ml) at 100° for 18 h and the product was analysed by GLC. The relative responses of the various methylated sugars to the flame ionisation detector were determined with synthetic mixtures. The results are given in Table 4. *Periodate oxidation* of the fucoxylomannan was carried out as described above for the heterogalactan. The polysaccharide consumed 1.04 moles of periodate and released 0.44 moles of formic acid.

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REFERENCES

1. Bhavanandan, V. P., Bouveng, B. O. and Lindberg, B. *Acta Chem. Scand.* **18** (1964) 504.
2. Fraser, R. N. and Lindberg, B. *Carbohydrate Res.* **4** (1967) 12.
3. Bouveng, B. O., Fraser, R. N. and Lindberg, B. *Carbohydrate Res.* **4** (1967) 20.
4. Ralph, B. J. and Bender, V. J. *Chem. Ind. (London)* **1965** 1181.
5. Rosík, J., Zitko, V., Bauer, Š. and Kubala, J. *Collection Czech. Chem. Commun.* **31** (1966) 3751.
6. Reese, E. T. and Mandels, M. *Can. J. Microbiol.* **5** (1959) 173.
7. Lindberg, B. and Swan, B. *Acta Chem. Scand.* **14** (1960) 1043.
8. Garegg, P. J. and Lindberg, B. *Acta Chem. Scand.* **15** (1961) 1913.
9. Sweeley, C. C., Bentley, R., Makita, M. and Wells, W. W. *J. Am. Chem. Soc.* **85** (1963) 2499.
10. Aspinall, G. O. *J. Chem. Soc.* **1963** 1676.
11. Bouveng, H. O., Kiessling, H., Lindberg, B. and McKay, J. *Acta Chem. Scand.* **16** (1962) 615.
12. Aspinall, G. O. and Ferrier, R. J. *Chem. Ind. (London)* **1957** 1216.
13. Srivastava, H. C., Singh, P. P., Harske, S. N. and Virk, K. *Tetrahedron Letters* **1964** 493.
14. Bouveng, H. O., Bremner, I. and Lindberg, B. *Acta Chem. Scand.* **19** (1965) 967.

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