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A Heterogalactan Elaborated by *Polyporus squamosus* (Huds.)

HÅKAN BJÖRNDAL and BARBRO WÅGSTRÖM

Institutionen för organisk kemi, Stockholms Universitet, S-113 27 Stockholm, Sweden

A heterogalactan has been isolated from the fruit-bodies of *Polyporus squamosus*. The heterogalactan consists of a chain of (1→6)-linked α-D-galactopyranose residues, about 90 % of which are substituted in the 2-position with either α-L-fucopyranose residues, 3-*O*-α-D-mannopyranosyl-α-L-fucopyranose residues or short side chains composed of α-(1→2)- and α-(1→3)-linked D-galactopyranose residues.

In previous publications,[1–5] we have reported studies on different polysaccharides isolated from wood-destroying fungi of the Basidiomycetes group. The present paper reports similar studies on a polysaccharide from *Polyporus squamosus*.

Fruit bodies of *P. squamosus* were harvested locally. The disintegrated material was extracted, first with hot water and then with 0.3 M potassium hydroxide at room temperature, and the extracts processed to give the polysaccharide fractions. Hydrolysates of the two extracts contained D-glucose as the dominant sugar, and smaller amounts of D-galactose, D-mannose, and L-fucose. Because of the similarity in the fractions only the hot water extracts from the fungus were further investigated.

The water-soluble fraction contained considerable amounts of glucans, probably both α- and β-glucans. Part of the latter was removed by precipitation with cetyltrimethylammonium hydroxide [6] (CTA-OH). The heteropolysaccharide fraction was then enriched by precipitation with boric acid and CTA-OH and obtained free from glucan by one further precipitation with this reagent. A hydrolysate of the fraction contained D-galactose and L-fucose in the relative proportion 7.5:1. Only small amounts of D-mannose were present (2—3 mole %). The polysaccharide showed $[\alpha]_{578}^{20}+138°$.

The polysaccharide was methylated by two treatments with methylsulphinyl sodium-methyl iodide in methyl sulphoxide, following the procedure devised by Hakomori.[7] The fully methylated polysaccharide was hydrolysed and the mixture of methylated sugars obtained reduced, acetylated and

Table 1. Methyl ethers from the hydrolysate of

A) methylated heterogalactan
B) methylated partially hydrolysed heterogalactan
C) detritylated methylated heterogalactan
D) remethylated alkali degraded heterogalactan
E) remethylated alkali degraded heterogalactan[b]

Sugars	T^a	mole % A	B	C	D	E[b]
2,3,4-Tri-*O*-Me-L-Fuc	0.64	8.0	2.3	5.7	13.6	8.7
2,3,4,6-Tetra-*O*-Me-D-Man	1.00	3.1	0.9	0.5	2.3	1.5
2,4-Di-*O*-Me-L-Fuc	1.15	3.0	1.0	5.7	3.5	2.3
2,3,4,6-Tetra-*O*-Me-D-Gal	1.25	20.3	32.9	7.8	12.4	8.0
Unknown	1.90	1.1	0.8	—	2.1	1.3
2,4,6-Tri-*O*-Me-D-Gal	2.28	13.5	13.1	4.4	8.5	5.5
3,4,6,Tri-*O*-Me-D-Gal	2.45	12.8	6.8	5.9	10.1	6.5
2,3,4-Tri-*O*-Me-D-Gal	3.41	4.7	10.0	15.3	19.2	12.4
Unknown	3.67	2.7	1.7	0.3	—	—
2,4-Di-*O*-Me-D-Gal	6.35	—	—	7.8	—	—
3,4-Di-*O*-Me-D-Gal	6.9	30.8	30.5	34.3	28.3	18.2
Unknown	9.1	—	—	8.0	—	—
Unknown	11.9	—	—	4.3	—	—

[a] Retention times of the corresponding alditol acetates on the ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
[b] Recalculation of the figures in column D (see the text).

analysed by GLC [8] — mass spectrometry.[9] The results are summarised in Table 1, column A.

It has been demonstrated that for partially methylated alditol acetates, a unique mass spectrum is obtained for each substitution pattern. Isomeric alditol acetates with identical substitution patterns do not, however, give significantly different mass spectra. As the polysaccharide contains both D-galactose and D-mannose residues, it was therefore not possible to identify some of the methylated sugars from their mass spectra alone. When, however,

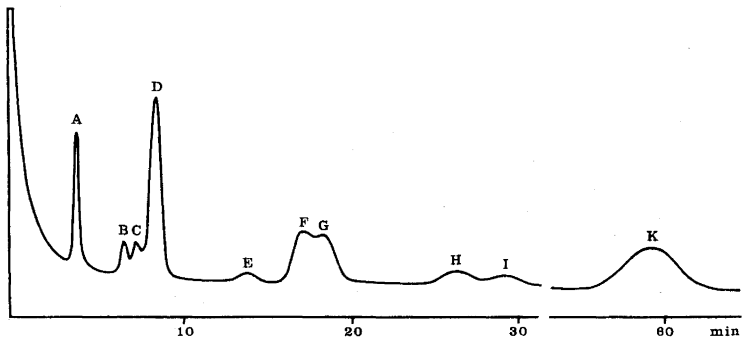

Fig. 1. GLC separation of the methylated sugars, as their alditol acetates, obtained from the hydrolysate of the fully methylated heterogalactan.

the mass spectrometric evidence was combined with the retention times (*T*-values), the different components could be identified.

The GLC for the alditol acetate mixture obtained from the heterogalactan is given in Fig. 1. The component in peak A had the same *T*-value and mass spectrum as the alditol acetate from 2,3,4-tri-*O*-methyl-L-fucose. Peak B from its *T*-value and mass spectrum was identified as the 2,3,4,6-tetra-*O*-methyl-D-mannose derivative, peak C as the 2,4-di-*O*-methyl-L-fucose derivative and peak D as the 2,3,4,6-tetra-*O*-methyl-D-galactose derivative. Peaks E and I were not identified. Peaks F and G were better resolved at lower temperature and were shown by their mass spectra to be 2,4,6- and 3,4,6-tri-*O*-methyl-hexose derivatives, respectively. Their *T*-values agree with those of the alditol acetates from 2,4,6- and 3,4,6-tri-*O*-methyl-D-galactose, respectively. Peak H was the 2,3,4-tri-*O*-methyl-D-galactose derivative and peak K was the 3,4-di-*O*-methyl-D-galactose derivative. From the result of the methylation analysis, the molar proportion of D-galactose to L-fucose were 7.5:1, in good agreement with the sugar analysis.

By subjecting part of the polysaccharide to a mild acid hydrolysis, most of the L-fucosidic linkages could be cleaved. The resulting material, $[\alpha]_{578}$ +173°, was recovered and subjected to methylation analysis (Table 1, column B). The decrease of 3,4,6-tri-*O*-methyl-D-galactose and the increase in 2,3,4,6-tetra-*O*-methyl-D-galactose in addition to evidence presented below demonstrates that a high percentage of D-galactose residues, most of which are linked to the 2-position of other galactose residues, have been hydrolysed. The percentage of methylated sugars derived from terminal and branched residues, respectively, indicates that the partially hydrolysed polysaccharide has an average DP of approximately 16.

In order to obtain further information, the polysaccharide was degraded by a procedure, devised by Lindberg and Lundström.[10] According to this procedure, hexose residues, with a free hydroxyl in the 6-position, are transformed into 6-deoxy-6-*p*-toluenesulphonylhexose residues. Because of the strongly electron attracting sulphonyl group, the glycosidic linkage in the latter is now sensitive to alkali. Although the reaction has only been studied with simple glucosides, it should be possible to apply it to polysaccharides and methylated polysaccharides. In the present polysaccharide, the L-fucosidic linkages and those of 6-substituted D-galactose residues, should be unaffected, all the other should be broken.

The polysaccharide was subjected to the following sequence of reactions, as exemplified for a terminal α-D-galactopyranose residue in Fig. 2. Reaction with triphenylmethylchloride, which is known to give etherification essentially in primary positions, was followed by methylation and detritylation. Part of the detritylated product was hydrolysed and the mixture of reducing sugars obtained analysed as above. The analysis (Table 1, column C) showed that about 60 % of the primary hydroxyl groups in the original polysaccharide had been tritylated. The presence of some unidentified components with high *T*-values, probably derived from mono-*O*-methyl-sugars, indicates that some secondary groups had also been tritylated.

The main part of the detritylated product was tosylated, the tosyl groups were replaced by iodide and iodide by *p*-toluenesulphonyl groups. NMR

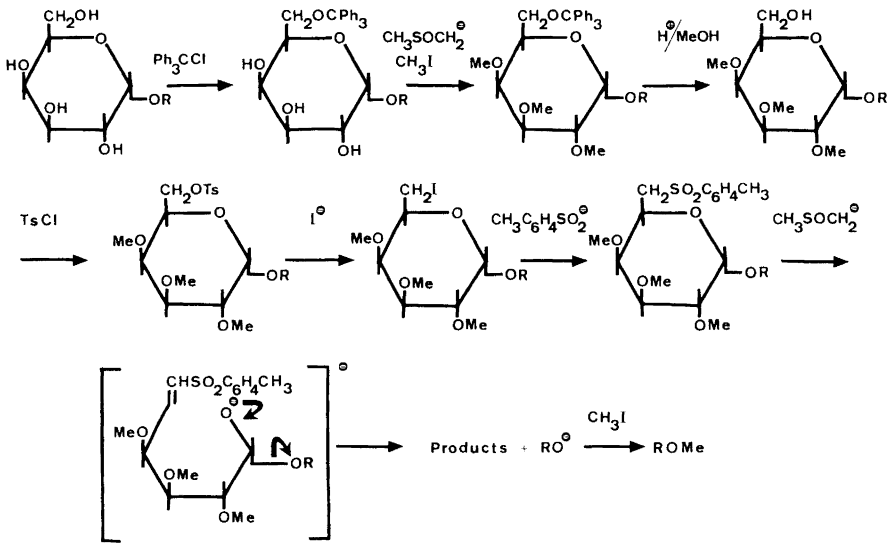

Fig. 2. Sequence of reactions in the selective degradation of the polysaccharide.

spectra of the tosyl- and toluenesulphonyl-derivatives showed the presence of the aromatic and C-methyl protons, which were virtually absent in the spectra of the iodo-derivative. On treatment with methylsulphinyl sodium in methyl sulphoxide the residues containing the 6-deoxy-6-*p*-toluenesulphonyl groups were eliminated and the released alkoxide ions were subsequently methylated by treatment with methyl iodide. The resulting, high molecular weight, material was recovered by gel filtration, hydrolysed and the mixture of methylated sugars obtained analysed as above (Table 1, column D). The percentage of methylated sugars derived from terminal and branched residues, respectively, indicates that the material has a high DP.

DISCUSSION

The methylation analysis of the original polysaccharide indicates that it is a highly branched heterogalactan, with D-galactose, D-mannose, and L-fucose as terminal residues.

The decrease of 2,3,4-tri-*O*-methyl-L-fucose and increase of 2,3,4-tri-*O*-methyl-D-galactose, in the methylation analysis of the partially hydrolysed material, indicates that the terminal L-fucose residues are linked to the 2-position of D-galactose residues, which are also substituted in the 6-position. This arrangement has been found in other fungal polysaccharides.[1–5] Some of these [1–4] also contain 3-*O*-α-D-mannosyl-α-L-fucopyranosyl residues linked to the 2-position of D-galactose. As essentially all the D-mannose in the original polysaccharide, and an equivalent amount of L-fucose, appear as 2,3,4,6-

tetra-*O*-methyl-D-mannose and 2,4-di-*O*-methyl-L-fucose, respectively, in the methylation analysis, it is reasonable to assume that a low percentage of such residues are present in the heterogalactan from *P. squamosus*.

From the high optical rotations of the original polysaccharide (+138°) and of the partially hydrolysed material, containing a low percentage of L-fucose residues, (+172°), it may be inferred that both the D-galactose and the L-fucose residues have the α-configuration.

Assuming that the alkali degraded, methylated material retains all the L-fucose residues of the original polysaccharide, since the L-fucose and 6-substituted D-galactose residues should not have been affected, the result of the methylation analysis (Table 1, column D) can be recalculated in percentage of methylated sugars derived from the latter (Table 1, column E). This calculation shows that the total loss of sugar residues is about 33 %. If all tritylated residues had reacted according to the scheme in Fig. 2, and no untritylated residue had been removed as a low molecular weight fragment during the degradation, from the methylation analysis of the tritylated polysaccharide the loss should have been 30 %. The good agreement between these figures indicates that the subsequent reactions in the degradation have proceeded in good yields.

The result of the methylation analysis of the polymeric material, obtained after the selective degradation, shows that most of the α-(1→2)- and α-(1→3)-linkages have been broken as expected. It also indicates that the L-fucose residues are linked directly to the main chain of α-(1→6)-linked D-galactose residues.

These results therefore strongly indicate that few if any D-galactose residues connected by (1→2)- and (1→3)-linkages are present in the main chain, which thus consists almost exclusively of α-(1→6)-linked D-galactose residues. About 90 % of these are substituted in the 2-position. In addition to α-L-fucopyranose and 3-*O*-α-D-mannopyranosyl-α-L-fucopyranose residues, there are also short side chains containing D-galactose residues with an average DP of 2.3. These chains contain α-(1→2)- and α-(1→3)-linked residues but probably no α-(1→6)-linked residues.

The proposed structure (Fig. 3) is to some extent based upon analogy with other fungal galactans which also contain a backbone of (1→6)-linked α-D-galactopyranose residues, part of which are substituted in the 2-position.

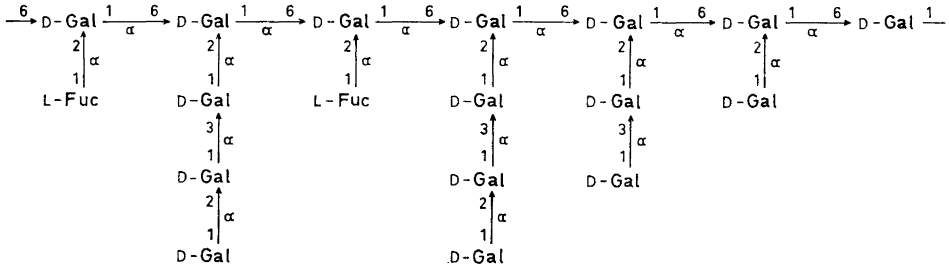

Fig. 3. Proposed structure for the heterogalactan from *P. squamosus*.

From the present results, related structures, with different chain length distribution and mutural order of the (1→2)- and (1→3)-linkages in the side chains, are not excluded.

EXPERIMENTAL

General methods. Paper chromatograms were run on Whatman No. 1 and 3 MM papers using the following solvent systems (v/v): a) Ethyl acetate-acetic acid-water, 3:1:1. b) Ethyl acetate-pyridine-water, 8:2:1. The components were detected with *p*-anisidine hydrochloride. Other general methods were the same as in a previous investigation.[11]

Isolation of the polysaccharide. The fungus was cut in small pieces, disintegrated in a Turmix blender and washed with acetone. The dried material (280 g) was extracted with boiling water (2 l) for 1 h and the procedure repeated twice. The combined extracts were concentrated and the polysaccharide material (16.5 g) isolated by precipitation with ethanol. The fungal material was then extracted, under nitrogen, for 17 h with 0.3 M aqueous potassium hydroxide (2 l) and this extraction repeated once. The extract was neutralised with acetic acid, concentrated and precipitated with ethanol. The fraction weighed 12.7 g. The water-soluble fraction was dissolved in water (1 l) and 0.25 M CTA-OH (80 ml) was added. The precipitate (9.5 g) on hydrolysis yielded D-glucose only. The supernatant solution was deionised, concentrated and precipitated with ethanol to give a polysaccharide fraction (5.4 g), which on hydrolysis yielded D-glucose, D-galactose, D-mannose, and L-fucose. This fraction in 0.1 M boric acid (200 ml) was precipitated with 0.25 M CTA-OH (150 ml). The precipitate was treated with acetic acid-ethanol to yield a fraction (3.5 g) which on hydrolysis yielded D-galactose, L-fucose, a small amount of D-mannose and traces of D-glucose. The polysaccharide material from the supernatant solution on hydrolysis yielded essentially D-glucose. The glucose in the heteropolysaccharide fraction was removed by one further precipitation with the same reagent. The final fraction weighed 2.9 g and had $[\alpha]_{578}^{20}+138°$ (*c* 0.5, water).

For the sugar analysis, the polysaccharide was dissolved in 0.25 M sulphuric acid, kept at 100° for 12 h, and neutralised with barium carbonate. Part of the sugar mixture was converted into alditol acetates and analysed by GLC.[12] Another part of the sugar mixture was fractionated on thick filter paper, using solvent systems (a) and (b). The following components were isolated as syrups: D-galactose, $[\alpha]_{578}^{20}+60°$, and L-fucose, $[\alpha]_{578}^{20}-54°$.

Methylation analysis of the polysaccharide. The polysaccharide (10 mg) in a 5 ml serum bottle, sealed with a rubber cap, was dissolved in dry methyl sulphoxide (1.5 ml). Nitrogen gas was flushed through the bottle and a solution of 2 M methylsulphinyl sodium in methyl sulphoxide (1.0 ml) was added dropwise, using a syringe. The resulting, gelatinous solution was agitated in an ultrasonic bath (40 kc/s) for 1 h and kept at room temperature for 6 h. Methyl iodide (0.1 ml) was then added dropwise, cooling externally with tap-water and the resulting turbid solution agitated for 20 min in the ultrasonic bath, to give a clear solution. A second portion of 2 M methyl sulphinyl sodium in methyl sulphoxide (1.0 ml) was added and the procedure above repeated, except that an excess of methyl iodide (1.0 ml) was added. The solution was then poured into water (25 ml), dialysed overnight against tap-water and evaporated to dryness. The methylated polysaccharide was treated with 90 % formic acid (3 ml) at 100° for 3 h, concentrated and then hydrolysed in 0.25 M sulphuric acid (3 ml) at 100° for 12 h. The hydrolysate was neutralised with barium carbonate and the sugars were converted into alditol acetates and analysed by GLC [8] — mass spectrometry.[9] As is evident from Fig. 1 the separation of some components on the ECNSS-M column at 175° was not very good. The separation was slightly improved when the separation was performed at 160° on the same column. The results are summarised in Table 1, column A.

Partial acid hydrolysis of the polysaccharide. The fucogalactan (30 mg) was dissolved in 0.025 M sulphuric acid (3 ml) and kept at 100° for 2 h. The hydrolysate was dialysed against tap-water overnight and lyophilysed. The hydrolysate (20 mg) showed $[\alpha]_{578}^{20}+172°$ (*c* 0.5, water). Part of this product (5 mg) was subjected to methylation analysis. The results are summarised in Table 1, column B.

Preparation and degradation of the partially methylated sulphone derivative of the polysaccharide

Tritylation.[13] The polysaccharide (100 mg) and triphenylmethylchloride (700 mg) were heated in pyridine (3 ml) and formamide (3 ml) at 100° for 45 min until complete solution was attained and the solution was kept at room temperature for 5 days. Addition of methanol (8 vol.) followed by reprecipitation furnished the polysaccharide derivative (135 mg).

Methylation. The tritylated product was dissolved in dry methyl sulphoxide (3 ml) and methylated according to the method described above. This gave the methylated *O*-triphenylmethyl derivative (170 mg). In this and the following steps, the lipophilic polysaccharide derivative was purified from low-molecular weight material by chromatography, in chloroform solution, on a Sephadex LH-20 column.

Detritylation.[13] The tritylated methylated derivative was dissolved in a mixture of chloroform (7 ml) and methanol (1 ml), containing 1 % of hydrogen chloride. After 3 h the solution was neutralised furnishing partially methylated fucogalactan (95 mg). Part of this product (5 mg) was treated as above and analysed by GLC.[8] The results are summarised in Table 1, column C.

Tosylation.[14] The partially methylated polysaccharide was dissolved in pyridine (3 ml) and *p*-tolylsulphonylchloride (1.2 g) in pyridine (3 ml) was added. The bottle was sealed and kept at 55° for 28 h after which the solution was poured out into water and extracted with chloroform. The chloroform solution was washed with 0.25 M sulphuric acid, aqueous sodium hydrogen carbonate and water. Concentration to dryness afforded the tosylated derivative (102 mg) of the partially methylated polysaccharide.

Replacement with iodide.[14] The tosyl derivative was dissolved in methyl formamide (5 ml) and sodium iodide (150 mg) was added. The solution was heated on a steam bath overnight and after concentration partitioned between water and chloroform. The chloroform phase was washed with aqueous sodium thiosulphate and water and concentrated to dryness to give the 6-deoxy-6-iodo-derivative (78 mg) of the partially methylated fucogalactan.

Replacement with sulphinate.[10] The 6-deoxy-6-iodo-derivative and anhydrous sodium-*p*-tolylsulphinate (150 mg) in methyl formamide was kept at 100° overnight. After concentration, the solution was partitioned between water and chloroform. The chloroform phase was washed with aqueous sodium thiosulphate and water and concentrated to dryness furnishing the 6-deoxy-6-sulphone derivative (68 mg) of the partially methylated polysaccharide.

Alkaline degradation and remethylation. The 6-deoxy-6-sulphone derivative was dissolved in dry methyl sulphoxide (3 ml). Nitrogen gas was flushed through the sealed bottle and a solution of 2 M methylsulphinyl sodium in methyl sulphoxide (6 ml) was added. The resulting solution was agitated in an ultrasonic bath (40 kc/s) for 1 h and kept at room temperature overnight. Then an excess of methyl iodide (3 ml) was added. The solution was poured out into water, extracted with chloroform, concentrated and the polymeric material separated from the degradation products. The polymeric material (27 mg) was hydrolysed, reduced and acetylated as above and analysed by GLC.[8] The results are summarised in Table 1, column D.

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