ACTA CHEMICA SCANDINAVICA 13 (1959) 1299–1304

Structure of a Glucomannan from *Pinus silvestris* L.

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Investigation of a glucomannan from *Pinus silvestris* has shown that it is built up very largely of β-1,4-linked sugar residues with about three branch points per molecule, most of which are attached to glucose residues at $C_{(3)}$.

A fractionation of the hemicellulose constituents of *Pinus silvestris* L. (Scots Pine) was reported in a previous communication from this Department [1]. A relatively pure glucomannan has now been prepared by similar methods from wood meal delignified by the chlorite method [2]. The polysaccharide fraction contained galactose, glucose, mannose, arabinose and xylose in the relative proportions 3.9:20.6:72.5:1.5:1.5 and had a $\overline{DP}_n$, determined osmometrically on the nitrate ester, of 97. It showed a typical lignin spectrum in the UV, with a lignin content estimated from the extinction at 280 mμ as 3—4 %. This lignin did not affect the DP-determination as it was completely lost during preparation of the nitrate ester. The lignin may be linked to the polysaccharide, and it seemed desirable to remove it before undertaking the structural investigation. Treatment with chlorine and ethanolic ethanolamine in a homogeneous medium repeated three times reduced the lignin content to about 0.2 %. The $\overline{DP}_n$ was reduced only to 93 by this treatment, which is an indication of the merit of this delignification method. Timell and Jahn [3] found that it causes only slight depolymerisation of polysaccharides, and this has been further confirmed by Glaudemans and Timell [4] in a recent investigation of birch wood hemicelluloses. — The relative proportions of galactose, glucose, mannose, arabinose and xylose in our delignified glucomannan sample were 3.0:21.1:74.0:traces:1.9, and were thus practically unchanged. The specific rotation was −31° in 2 N sodium hydroxide.

The glucomannan was methylated, first with methyl sulphate in sodium hydroxide and then with methyl iodide and silver oxide in dimethyl formamide. The product had a methoxyl content of 43.9 % and a $\overline{DP}_n$ value of 44. The methylation thus involved considerable degradation, which might also account for the rather low yield (45 %) of the methylated product. In a previous study [5] of the glucomannans from spruce (*Picea abies*), the $\overline{DP}_n$-values of the methylated products were not determined, but it must be assumed that the methylation, done in the same way as above, involved a similar depolymerisation.

The methylated polysaccharide was hydrolysed and the monomers obtained were fractionated by chromatography on a carbon-Celite column. The di-*O*-methyl ethers were only partially resolved and were further fractionated by paper chromatography and paper electrophoresis. This gave 2,6-di-*O*-methyl-D--glucose, which was the main component, smaller amounts of unidentified di-*O*-methyl-D-mannoses and of 2,3-di-*O*-methyl-D-xylose. 2,3,6-Tri-*O*-methyl-D-mannose and 2,3,6-tri-*O*-methyl-D-glucose were obtained in a pure state, followed by a small fraction that on demethylation gave galactose only. This latter fraction appeared homogeneous on chromatography in ordinary solvent systems but was resolved into two components by chromatography with *iso*propyl ether on dimethylsulphoxide-impregnated paper [6]. These had the same R_F-values as 2,3,4- and 2,4,6-tri-*O*-methyl-D-galactose, the substance corresponding to the first of these giving a much stronger spot than that corresponding to the second. 2,3,4,6-Tetra-*O*-methyl-D-mannose and 2,3,4,6-tetra-*O*-methyl-D-glucose were obtained in separate fractions. In a previous investigation [5] these components were obtained as a mixture, the successful fraction in the present case is probably due to eluation with aqueous acetone, which gives more distinct fractions of highly methylated ethers than the aqueous ethanol previously used.

2,3-Di-*O*-methyl-D-xylose is the predominant xylose ether expected from a methylated xylan. The tri-*O*-methyl-D-galactose ethers are also normal products from the arabogalactans isolated from various coniferous woods. Polysaccharides of this type have recently been isolated from two pine species [7,8]. As these polysaccharides are highly branched, one should expect to find some di- and tetra-*O*-methyl-D-galactose ethers too, but as the percentage of galactose residues in the original material was quite low, these ethers might easily have been overlooked. We believe that the xylose and galactose ethers arose from contaminating polysaccharides and they are therefore not taken into account in Table 1, which gives the molar percentages of the ethers in the hydrolysates.

Table 1. Molar percentages of monomers in the hydrolysate of the methylated glucomannan.

Ether	%
Di-*O*-methyl-D-mannoses	2.6
2,6-Di-*O*-methyl-D-glucose	5.3
2,3,6-Tri-*O*-methyl-D-mannose	67.6
2,3,6-Tri-*O*-methyl-D-glucose	19.5
2,3,4,6-Tetra-*O*-methyl-D-mannose	4.2
2,3,4,6-Tetra-*O*-methyl-D-glucose	0.8

These percentages are quite similar to those found in studies on the glucomannans from *Picea abies* [5], and it can be concluded that the polysaccharides have a similar structure with chains of 1,4-β-linked mannose and glucose residues. The high percentage of tetra-*O*-methyl-hexoses indicates that the molecules are branched, containing about 3 branches per 100 hexose residues. The percentage of di-*O*-methyl-hexoses found is certainly too high, due to incomplete methylation and possibly also to some demethylation during the

hydrolysis. As 2,6-di-*O*-methyl-D-glucose predominates among the di-*O*-methyl-hexoses, it is reasonable to assume that most of the branches start from 3-positions in glucose residues. Studies on partially methylated celluloses [9] have shown, however, that the hydroxyl in the 3-position is methylated at a slower rate than those in the 2- and 6-positions, so that 2,6-di-*O*-methyl-D-glucose is the only di-*O*-methyl-D-glucose which should occur in significant amounts in the hydrolysate of a slightly undermethylated product. It can also be seen from Table 1 that most of the non-reducing end groups are mannose residues, as would be expected if a statistical distribution of mannose and glucose residues in the polysaccharide is assumed.

On periodate oxidation the glucomannan consumed 0.90 moles of periodate per hexose residue, with the formation of 0.12 moles of acids. This result indicates a higher branching than was found by the methylation study, and most probably is due to over-oxidation. The hydrolysate of the oxidised polysaccharide contained more glucose than mannose, their relative proportions being about 2:1. This provides further support for the assumption that the polysaccharide is branched and that most branches start from glucose residues.

The chain of 1,4-β-linked glucose and mannose residues seems to be a common feature of glucomannans isolated from coniferous woods [5,10–14]. These show glucose to mannose ratios of between 1:4 and 1:2. The proportion of glucose is increased in the partially hydrolysed polysaccharides [5]. A high glucose content in a glucomannan might indicate that its isolation from the wood has involved degradation or that the material is contaminated with cellulose. It seems fairly certain that the glucomannans from pine and spruce [5,12] have a slightly branched structure. The partially degraded polysaccharide from a hemlock sulphite pulp [13] showed no such branching. The undegraded glucomannan from spruce was amorphous but the partially hydrolysed product obtained from it was highly crystalline [15]. This difference is easily understood if the crystalline material is made up of linear fragments of the branched polysaccharide.

Indications have been recorded that coniferous woods contain galactoglucomannans as well as glucomannans [14,16–18]. Studies here on hemicellulose fractions isolated from holocellulose from pine and spruce wood have not given any support for this hypothesis. It is true that glucomannan fractions often contain considerable amounts of galactose and xylose residues which cannot be removed by further fractionation. We found, however, that these samples always contained a small percentage of lignin. After further delignification the contaminating polysaccharides could usually be separated from the glucomannans. A typical example of such a fractionation is given in the experimental part. A glucomannan that had been extracted with alkali from a pine holocellulose, after fractionation with barium hydroxide, contained 11 % galactose- and 5.5 % xylose residues. After delignification of the sample the galactose residues could be reduced to about 1 to 2 % and the xylose residues to traces by precipitation with barium hydroxide followed by precipitation twice with Fehling's solution. At the same time an arabogalactan or galactan was obtained which was free from glucose and mannose residues and had not been precipitated on the addition of Fehling's solution. We therefore believe that different polysaccharides, glucomannans and xylans as well as galactans

or arabogalactans may be linked to the same lignin molecule, which of course would make fractionation prior to further delignification impossible. The existence of linkage between lignin and hemicelluloses in woods has been strongly indicated by recent investigations by Björkman [19] and Lindgren [20]. The small amounts of galactose and xylose residues in our glucomannan used for methylation could be explained in the same way. The isolation, after partial hydrolysis of the polysaccharide, of oligosaccharides containing galactose and either glucose or mannose residues would of course prove the presence of galactoglucomannans but such proof has not yet been obtained*.

EXPERIMENTAL

Paper chromatography was carried out on Whatman No. 1 and on 3 MM filter papers. Chromatographic solvents: (*a*) ethyl acetate-acetic acid-water (3:1:3)
(*b*) butan-2-one, saturated with water
(*c*) 4 % dimethyl sulphoxide in benzene

Paper electrophoresis was carried out on Whatman No. 3 MM filter paper in borate buffer at pH 10.

Solutions were always concentrated under reduced pressure at a bath temperature of about 40°C.

Isolation of the glucomannan. Wood meal (18—35 mesh) from *Pinus silvestris* was extracted with acetone in a Soxhlet-type extractor and the residue was delignified by the chlorite method [8]. The product remaining after delignification amounted to 67.2 % of the acetone-extracted wood and contained less than 1 % Klason lignin. The holocellulose (297 g) was extracted successively under nitrogen with dimethyl sulphoxide, boiling water, 14 % potassium hydroxide and finally with 24 % potassium hydroxide and 3 % boric acid. The hemicelluloses from the last extract after acidification of the solution were precipitated with ethanol, dissolved in water and a glucomannan was precipitated by the addition of barium hydroxide [21]. The glucomannan was further purified by redissolving in water and precipitating once more with barium hydroxide. A sample (5.3 g) on hydrolysis gave 3.9 % galactose, 20.6 % glucose, 72.5 % mannose, 1.5 % arabinose and 1.5 % xylose. It contained between 3 and 4 % lignin (determined by UV-absorption [1]) and was therefore dissolved in water, and delignified by three successive chlorine-ethanolamine treatments as described earlier [1]. This reduced the lignin content to about 0.2 %. The delignified product used in the following methylation study had $[\alpha]_D^{20}$ −31° (c = 0.8, in 2.5 N sodium hydroxide) and on hydrolysis gave 3.0 % galactose, 21.1 % glucose, 74.0 % mannose, a trace of arabinose and 1.9 % xylose.

Determination of the $\overline{DP}_n$-values. The $\overline{DP}_n$-values were determined osmometrically on the nitrate-esters before and after the final delignification of the glucomannan and also on the methylated product. These gave values of 97.6, 93.0 and 43.7, respectively. The determinations were carried out in a Zim and Myerson osmometer and butyl acetate was used as solvent. The procedure has been described in an earlier paper [15].

Fractionation of a galactose-rich glucomannan. A glucomannan fraction which had been extracted with alkali from a pine holocellulose was purified by two precipitations with barium hydroxide [21] and then contained galactose, glucose, mannose, arabinose and xylose in the proportions 11.0:21.3:59.7:2.5:5.5. The lignin content was 5.3 %. After delignification by the chlorine-ethanolamine method, the sample was precipitated once with barium hydroxide and then contained galactose, glucose, mannose, arabinose and xylose in the proportions 10.1:20.6:64:2.6:2.7. The lignin had been reduced to 0.3 %. Part of the fraction (800 mg) was dissolved in water (100 ml), Fehling's solution (30 ml)

* *Added in proof.* From a partial hydrolysate of an easily soluble spruce hemicellulose fraction, rich in galactose residues, we have now isolated a galactosyl-mannose and a galactosyl-mannobiose. From these results, the presence of either a galactoglucomannan or a galactomannan, in addition to the previously known glucomannan is indicated.

was added and the precipitate was centrifuged. After washing once with water it was dissolved in 1 N hydrochloric acid, the hemicelluloses were precipitated with ethanol and the whole procedure was repeated. This sample (420 mg) was then hydrolysed and was found to be an almost pure glucomannan containing 1—2 % galactose and only traces of arabinose and xylose. After centrifuging the first precipitate with Fehling's solution, the solution was acidified with hydrochloric acid and dialysed, acidified again and then precipitated with ethanol, yielding a polysaccharide fraction (140 mg) which, on hydrolysis, gave about 50 % galactose, 10 % glucose, 30 % mannose, 5 % arabinose and 5 % xylose. This hemicellulose in aqueous solution was again treated with Fehling's solution. The precipitate formed was found to be a rather pure glucomannan but the centrifugate was free from mannose- and glucose residues and contained a galactan or arabogalactan contaminated with an araboxylan. A synthetic mixture of a pure glucomannan and a galactan from spruce wood [22] was easily separated into pure components by the same method.

Methylation of the glucomannan. The glucomannan (2 g) was dissolved in 22.5 % sodium hydroxide (50 ml), under nitrogen, and methyl sulphate (10 ml) was added over 1 h with vigorous stirring while the temperature was kept at 20° by external cooling. After 6 h the solution was neutralised with 6 N sulphuric acid and the polysaccharide was recovered by dialysis. Yield 1.98 g (OCH_3, 26.1 %).

The partially methylated product was dissolved in dimethyl formamide (30 ml) and methyl iodide (6 ml) was added in one portion. Silver oxide (6 g) was added over 1 h with vigorous stirring. After 20 h the insoluble material was centrifuged off and washed with dimethyl formamide (10 ml) and chloroform (10 ml). The combined centrifugates were concentrated. The resulting syrup was treated with benzene-chloroform (1:1, 20 ml) and the undissolved salts were centrifuged off. The solid residue was washed with benzene (20 ml). The combined centrifugates were evaporated to dryness and then methylated again three times using the procedure described above. The product finally obtained (0.89 g) had a OCH_3-content of 43.9.

Hydrolysis of the methylated glucomannan. The methylated glucomannan (0.89 g) was dissolved in anhydrous formic acid and kept at 100°C for 6 h. The solution was concentrated and the resulting syrup was dissolved in 0.5 N sulphuric acid and kept at 100°C for 15 h. This solution was then neutralised with barium carbonate, giving finally a syrup (0.75 g). Chromatographic examination of the hydrolysate (Solvent *a*) revealed the presence of di-, tri- and tetra-*O*-methyl hexoses but no glucose, mannose or mono-*O*-methyl hexoses.

Examination of the hydrolysis products from methylated glucomannan. The hydrolysate from the methylated glucomannan (753 mg) was added to the top of a carbon-Celite column (43 × 3.5 cm) which was then developed by gradient elution with the following solvents:

10 % → 30 % aqueous ethanol	5 000 ml
30 % aqueous ethanol → 30 % aqueous acetone	3 000 »

The eluate was divided into fractions (30 ml) which were examined on paper chromatograms (Solvent *a*). Those fractions which were indistinguishable were combined and taken to dryness.

Fraction 1. (54 mg.) Five components were isolated by paper electrophoresis. The principal component on demethylation gave glucose only and was chromatographically and electrophoretically indistinguishable from authentic 2,6-di-*O*-methyl-D-glucose. The components were fractionated, in two separate experiments, on paper chromatograms (Solvent *b*) and on paper electrophoretograms, and quantitative determinations of the principal components were made by hypoiodite oxidation [23]. Three of the components isolated gave mannose only on demethylation but were not further investigated. The fifth component was shown to be 2,3-di-*O*-methyl-D-xylose (17 mg).

Fraction 2. (395 mg.) This fraction on demethylation yielded mannose only. An aniline derivative was prepared, m. p. 125—126°, alone or in admixture with authentic 2,3,6-tri-*O*-methyl-*N*-phenyl-D-mannosylamine. This fraction was chromatographically homogeneous.

Fraction 3. (99 mg.) The material crystallised when the solvent was evaporated. One crystallisation from ethyl acetate yielded the pure substance, m. p. 117—119°, undepressed on admixture with authentic 2,3,6-tri-*O*-methyl-D-glucose. Chromatograms of the mother liquor indicated traces of an unknown substance with a slightly higher

R_F-value (Solvent *a*) than 2,3,6-tri-*D*-methyl-D-glucose. This was probably the same substance as was previously isolated [5] and that had been shown to be a tri-*O*-methyl-D-glucose, distinguishable from 2,3,4- and 2,3,6-tri-*O*-methyl-D-glucose.

Fraction 4. (17 mg) on demethylation yielded galactose only. Chromatography on dimethyl sulphoxide treated paper with solvent *c* separated the fraction into two components with the same R_F-values as 2,3,4-tri-*O*-methyl-D-galactose and 2,4,6-tri-*O*-methyl-D-galactose. The spot corresponding to the former had the lowest R_F-value and was the principal substance in this fraction.

Fraction 5. (26 mg) gave mannose only on demethylation. The aniline derivative, m. p. 142—144°C, was not depressed on admixture with the aniline derivative of 2,3,4,6-tetra-*O*-methyl-D-mannose.

Fraction 6. (5 mg) on demethylation yielded glucose only. It was chromatographed on dimethyl sulphoxide treated paper using *iso*propyl ether as solvent, and gave an elongated spot with the same R_F-values as authentic 2,3,4,6-tetra-*O*-methyl-D-glucose (0.56) but higher than 2,3,4,6-tetra-*O*-methyl-D-mannose (0.47).

Periodate oxidation studies. The glucomannan (373 mg) was kept in water (50 ml) overnight in the refrigerator (2—4°). Sodium metaperiodate solution (0.32 M, 25 ml) was then added and the volume was adjusted to 125 ml. The solution was kept in the refrigerator and samples (5 ml) were withdrawn at intervals and analysed for periodate consumption and for the formation of acid. The results are summarised in Table 2.

Table 2. Periodate oxidation of the glucomannan.

Time, h	Moles of periodate consumed per $C_6H_{10}O_5$	Acid equivalents per $C_6H_{10}O_5$
60	0.71	0.061
72	0.86	0.076
100	0.89	0.108
140	0.92	0.136
200	0.95	0.144
350	0.97	0.148

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Received December 23, 1958.