

# DOUGLAS-FIR BARK. II. ISOLATION AND CHARACTERIZATION OF A GLUCOMANNAN<sup>1,2</sup>

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## ABSTRACT

A glucomannan has been isolated from the holocellulose fraction of the inner bark of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco], and its structure and physical constants have been determined. The molar ratio of D-mannose, D-glucose, and D-galactose was 2:1:0.3. The glucomannan was completely methylated and hydrolyzed. Gas-liquid chromatographic and mass spectrometric analyses showed the presence of 2,3,4,6-tetra-, 2,3,6-tri-, and 2,3-di-*O*-methyl-D-glucopyranose, 2,3,6-tri- and 2,3-di-*O*-methyl-D-mannopyranose, and 2,3,4,6-tetra-*O*-methyl-D-galactopyranose. The proposed glucomannan structure consists of a linear chain of  $\beta$ -D-(1  $\rightarrow$  4)-linked mannose and glucose units with  $\alpha$ -D-(1  $\rightarrow$  6)-linked galactose as intermittent, single-unit branches. The structural and physical properties of this glucomannan place it within the alkali-soluble family of polysaccharides known to be contained in the wood of all gymnosperms.

*Keywords:* Douglas-fir bark, hemicellulose, glucomannan, holocellulose.

## INTRODUCTION

Carbohydrates are the major constituents (50 to 55%) of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] inner bark (Laver et al. 1974). As such they represent a large reservoir of materials similar to those used to make pulp and paper. Although the pulping of tree barks is not yet common, increasing amounts of bark are being pulped, particularly with the increasing use of whole-tree chipping. Because raw materials to provide carbohydrates for pulp and paper are becoming scarcer and more expensive, the large supply of carbohydrates in tree barks is of economic interest to supplement the supply of carbohydrates currently isolated from wood.

Laver et al. (1974) showed that the polysaccharides comprised in a chlorite holocellulose prepared from Douglas-fir inner bark were a xylan, a galactoglucomannan, a glucomannan, and a glucan-rich fraction with characteristics of

<sup>1</sup> First in series: Laver, M. L., C.-H. Chen, J. V. Zerrudo, and Y.-C. Lai. 1974. Carbohydrates of the inner bark of *Pseudotsuga menziesii*. *Phytochemistry* 13:1891-1896.

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cellulose. In the present study, we isolated a glucomannan hemicellulose from this inner bark and determined its structure and physical constants. We refer to the polysaccharide as a glucomannan rather than a galactoglucomannan, although it contains 7.7% galactose units, because it is similar in structure and physical properties to the group of glucomannans discussed by Timell (1965). Most of these glucomannans also contained small numbers of galactose units. It has been known for many years that a close association exists between cellulose and glucomannan in wood cell walls because of the early difficulties in isolating pure glucomannans. This association is further evident from investigations on the acid sulfite cooking of wood (Timell 1965). Doubling the amount of glucomannan retained produced a 5% increase in pulp yield (Hansson and Hartler 1970). If glucomannans could be retained with the cellulose-like fraction in carbohydrate recovery operations, pulp yields might be significantly increased.

The forest products industry in Oregon generates several million tons of Douglas-fir bark each year. This paper reports part of the results of a continuing program in our laboratory to make maximum use of this natural resource.

#### CHROMATOGRAPHIC METHODS

We used three chromatographic methods to ensure proper identification and characterization of the glucomannan isolated.

Sugars were identified by paper chromatography (descending method) on Whatman No. 1 paper. The simple sugars were separated with solvent system A, ethyl acetate-pyridine-water (8:2:1 v/v) (Hough and Jones 1962). The methylated sugars were separated with solvent systems B, 2-butanone saturated with water (Boggs et al. 1950); C, *n*-butanol-ethanol-water (5:1:4 v/v) (Hirst and Jones 1949); and D, 2-butanone-water-ammonium hydroxide (30% ammonia in water) (100:50:3 v/v, upper layer) (Kooiman and Adams 1961). After irrigation, the paper chromatograms were sprayed with aniline hydrogen phthalate reagent (1.66 g of phthalic acid dissolved in 100.0 ml of water-saturated *n*-butanol containing 0.92 g of freshly distilled aniline) and heated at 100 C (Partridge 1949) so we could locate the sugars.

Gas-liquid chromatography (GLC) was performed on a Hewlett-Packard 5750B instrument fitted with flame-ionization detectors. The conditions were: column, 6.5% ECNSS-M on Gas Chrom Q 100/120 mesh, 180 cm in length  $\times$  0.3 cm outside diameter stainless steel; column temperature 170 C isothermal; injection port 270 C; detector 280 C; helium carrier gas flow 30 ml/min.

GLC-mass spectrometry (GLC-MS) was performed on a Varian Aerograph Series 1200 gas chromatograph attached to a CH 7 Mass Spectrometer Varian Mat. The GLC column was the same as that used with the Hewlett-Packard GLC, but column temperature was lowered to 160 C.

#### ISOLATING THE GLUCOMANNAN

Inner bark was taken from a freshly cut Douglas-fir tree<sup>3</sup> in Dunn Forest, Benton County, Oregon. The bark, from which the cambium had been removed, was immersed in 95% ethanol and water added to give a 4:1 v/v ethanol-water solution.

<sup>3</sup> A voucher specimen (no. 142702; branch and trunk section) is deposited at the Herbarium of the Department of Botany, Oregon State University, Corvallis, OR.

After 2 days, the solids were recovered by decantation. The residue was then air-dried, ground in a Wiley-mill, and screened for material between  $-20$  and  $+40$  mesh size. A 12,000.0-g sample of material of this mesh size was extracted in a Soxhlet apparatus with benzene-ethanol (2:1 v/v) resulting in 11,060.1 g of insoluble residue. That residue was extracted with hot water (50–60 C) to yield 9,609.4 g of insoluble residue, which in turn was extracted with 0.5% aqueous ammonium oxalate solution (70–80 C), leaving 8,418.7 g of unsolubilized, extracted bark meal. The extracted bark meal was delignified by two treatments with acidified sodium chlorite reagent (Whistler et al. 1948). Holocellulose insolubles were recovered by filtration, dialyzed for 1 week against running water, and freeze-dried; yield was 4,100.8 g, or 24.3% of the original ethanol-water insoluble bark.

The glucomannan was isolated from the holocellulose by modification of the procedures described by Beelik et al. (1967). Part (50.0 g) of the holocellulose insolubles was slurried in 782.0 g of aqueous barium hydroxide solution containing 64.0 g of barium hydroxide octahydrate. After intermittent stirring for 20 min, 925.0 g of 18.5% aqueous potassium hydroxide was added to the slurry and stirring continued for an additional 20 min. The mixture was separated by filtration with a sintered glass funnel, and the residual solids were washed with an aqueous solution (250.0 ml) of barium hydroxide and potassium hydroxide having the same concentration as the extracting liquor. The insoluble solids were washed thoroughly with distilled water, then dispersed in 700.0 ml of 3.0% aqueous acetic acid and allowed to stand overnight. These solids were recovered by filtration, washed to neutrality with distilled water, dialyzed for 3 days against running water, and freeze-dried; yield was 44.0 g of white powder.

Part (40.0 g) of the white powder was dispersed in 990.0 g of 1.0% aqueous sodium hydroxide and stirred for 20 min. The slurry was separated by filtration and the insoluble residue washed with 250.0 ml of 1.0% aqueous sodium hydroxide solution followed by 1.0 liter of distilled water. The insoluble residue was then dialyzed for 3 days against running water and freeze-dried; yield was 39.8 g of white powder.

All the white powder was dispersed in 920.0 g of 15.0% aqueous sodium hydroxide solution and stirred for 20 min. The mixture was separated by filtration and the residue washed with 250.0 ml of 15.0% aqueous sodium hydroxide followed by 1.0 liter of distilled water. The filtrate plus washings were combined, and 500.0 ml of saturated aqueous barium hydroxide was added to the solution to form a precipitate. The precipitate was recovered by centrifugation and then dissolved in 400.0 ml of 2 N aqueous acetic acid. Methanol (1,200.0 ml) was added, resulting in the formation of a precipitate, which was recovered by centrifugation. The precipitate was redispersed in 700.0 ml of 70.0% aqueous methanol and then recovered by centrifugation. The dispersion procedure was repeated 3 times. The final precipitate was slurried in distilled water and freeze-dried; yield was 1.43 g of crude glucomannan solids. Five additional portions of holocellulose insolubles were similarly treated to obtain a sufficient quantity of crude glucomannan.

Part (5.10 g) of the glucomannan solids was dialyzed against running water for 6 days and freeze-dried; yield was 4.63 g of purified glucomannan. Its ash content was 3.27% as sulfate (Humphries 1956); this was assumed to be barium sulfate

because barium cations had been used to precipitate the glucomannan for purification. The glucomannan fraction was thus calculated to contain 1.92% barium. Its Klason lignin content of 1.05% (TAPPI Standard 1954) and acid-soluble lignin content of 0.94% (Goldschmid 1971) showed that the glucomannan was quite free of lignin contamination. Its specific rotation was  $[\alpha]_D^{25} - 35.9^\circ$  ( $c$  0.43 g/100 ml, 10% aqueous sodium hydroxide). Analysis of the reducing end-groups by the Somogyi method (Hodge and Hofreiter 1962) showed reducing end-groups equivalent to 0.09 g of a mannose–glucose mixture (2:1 w/w) per glucomannan sample weight of 795 mg. The method of Henley (1960) showed that the intrinsic viscosity  $[\eta]$  of the glucomannan in cadoxen at  $25.0 \pm 0.1$  C was 0.46 dl/g.

#### CONFIRMING THE IDENTITY OF THE GLUCOMANNAN

By isolating and preparing crystalline derivatives, Laver et al. (1974) positively established the presence and configurations of D-glucose, D-mannose, and D-galactose in the acid hydrolyzates of the holocellulose of Douglas-fir inner bark. These structures are therefore assigned to the sugars in this study.

#### *Hydrolysis*

Once isolated, the glucomannan (250 mg) was dissolved in 4.5 g of 77.0% sulfuric acid. Water (84.5 ml) was added with stirring to give a 3% sulfuric acid solution. Hydrolysis was accomplished by refluxing for 9.0 h. Upon cooling, the solution was neutralized with saturated aqueous barium hydroxide, and the solids were removed by centrifugation.

We subjected the resulting hydrolyzate to paper chromatography using solvent A. Aniline phthalate indicator produced spots, estimated visually, that corresponded to mannose (strong), glucose (medium), galactose (weak), xylose (trace), and arabinose (trace). The simple sugars were converted to their respective alditols with sodium borohydride and acetylated with acetic anhydride and concentrated sulfuric acid. We quantitatively analyzed the resulting alditol acetates with GLC (Borchardt and Piper 1970) and, on the basis of the anhydromonosaccharide units in the original polysaccharide, found 56.4% anhydromannose units, 26.2% anhydroglucose units, 7.7% anhydrogalactose units, 1.5% anhydroxylose units, and 1.4% anhydroarabinose units. The small quantities of anhydroxylose, anhydroarabinose, and anhydrogalactose residues demonstrated that essentially all of the xylan and the readily soluble galactoglucomannan hemicelluloses had been removed.

The total sugar analysis (93.2%) plus the ash (1.92% barium) and lignin (1.05% and 0.94%) contents indicated a total recovery of 97.11%, which is exceptionally good for this type of material. Once the purified glucomannan was shown to be essentially free of ash, lignin, and contaminating polysaccharides, its structure could be characterized.

#### *Methylation*

Although there are limitations inherent in elucidating the types of linkages in polysaccharides by methylation analysis, the method has been widely applied for many years. Sandford and Conrad (1966) enumerate some of the limitations as incomplete methylation, destruction and demethylation of the methylated sugars during hydrolysis, recovery of the methylated fragments, and precise determi-

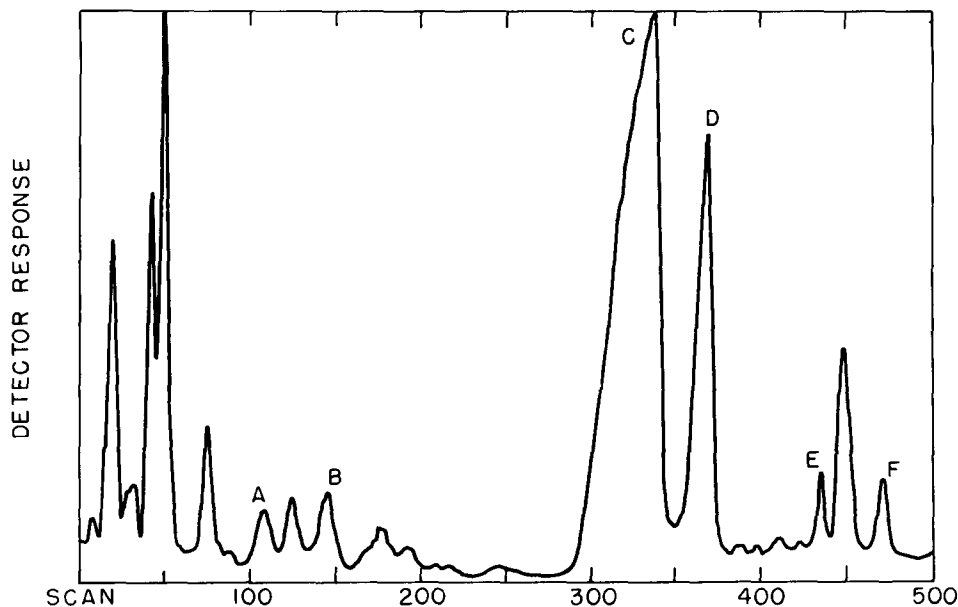


FIG. 1. Computer printout of the GLC spectrum of the GLC-MS identification of the alditol acetates prepared from the acid hydrolyzate of the methylated glucomannan. See text for description of conditions. Scan numbers represent the points at which MS scans of the effluent were taken. See Table 1 for the key to the sugars represented by peaks A, B, C, D, E, and F and also for retention times. See Table 2 for the  $m/e$  fragments resulting from the MS scans of peaks A, B, C, D, E, and F. MS scans showed that peaks other than A, B, C, D, E, and F were not carbohydrates but were from the solvent and acetylating reagents used in preparing the alditol acetates.

nation of methoxyl content. We carefully performed the experimental research described herein so as to reduce these limitations as much as possible.

The glucomannan was successively methylated by the Haworth (Wolfrom et al. 1961), Hakomori (Hakomori 1964; Sandford and Conrad 1966), and Purdie (Hirst and Percival 1965) methods. The product, in chloroform, was poured into 10 volumes of petroleum ether (b.p. 30–60 C) and the mixture refrigerated for 3 days. The white precipitate that formed was recovered by filtration and dried in a desiccator over phosphorus pentoxide, yielding a white powder.

The glucomannan proved difficult to methylate, as polysaccharides of this type usually do. However, after the several methylations described above, the methoxyl content by analysis was 45.3%, close to the theoretical value of 45.6% for a hexosan with an average of three hydroxyl groups per anhydrohexose unit. The glucomannan was thus considered fully methylated.

#### *Hydrolyzing the methylated glucomannan*

The methylated glucomannan (40 mg) was hydrolyzed with 10 ml of formic acid at 95 C for 3 h. The solution was cooled and the formic acid removed by evaporation under reduced pressure. A few drops of water were added and reevap-

TABLE 1. Retention times of methyl ethers obtained on methylation of the glucomannan from Douglas-fir inner bark.

Sugar	Peak <sup>3</sup>	Sugars, this study <sup>2</sup> (min)	Ratio of retention times <sup>1</sup>	
			This study	Harwood
1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methyl-D-glucitol	A <sup>4</sup>	5.00	1.00	1.00
1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methyl-D-galactitol	B	6.25	1.25	1.26
1,4,5-tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methyl-D-mannitol	C	11.30	2.26	2.27
1,4,5-tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methyl-D-glucitol	D	13.25	2.65	2.62
1,4,5,6-tetra- <i>O</i> -acetyl-2,3-di- <i>O</i> -methyl-D-mannitol	E	25.25	5.05	4.87
1,4,5,6-tetra- <i>O</i> -acetyl-2,3-di- <i>O</i> -methyl-D-glucitol	F	29.25	5.85	5.67

<sup>1</sup> Relative to retention times of the standard 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol for this study and those from Harwood (1973).

<sup>2</sup> From GLC spectrum obtained on Hewlett-Packard 5750B gas chromatograph. See text for conditions.

<sup>3</sup> Designations A, B, C, D, E, and F refer to peaks in Fig. 1.

<sup>4</sup> This peak may also contain 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol.

orated to remove traces of formic acid. The resulting syrup was hydrolyzed with 5.0 ml of 0.5 N sulfuric acid for 2.5 h at 95 C. The hydrolyzate was neutralized with barium carbonate, and the solids were removed by centrifugation. The centrifugate was deionized with Amberlite IR 120 (H<sup>+</sup>) ion exchange resin and concentrated to a syrup on a rotary evaporator. The syrup was dissolved in a few drops of water and subjected to paper chromatography (solvents B, C, and D). We identified the compounds 2,3,6-tri-*O*-methyl-D-mannopyranose and 2,3,6-tri-*O*-methyl-D-glucopyranose by their movement relative to authentic 2,3,4,6-tetra-*O*-methyl-D-glucopyranose ( $R_G = 0.55$  and  $0.66$ , respectively, in solvent D). However, the method was not sensitive enough to detect trace sugars such as those that constituted end-groups.

The partially methylated sugars in the hydrolyzate were converted to their alditol acetates according to the method described by Borchardt and Piper (1970), except that acetic anhydride in pyridine (1:1 v/v), rather than acetic anhydride in sulfuric acid, was used in the acetylation. We tentatively identified the resulting partially methylated alditol acetates with GLC by comparing their retention times with those of authentic 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (Harwood 1973) (Table 1), which was prepared by reducing and acetylating authentic 2,3,4,6-tetra-*O*-methyl-D-glucopyranose. The partially methylated alditol acetates were positively identified by GLC-MS (Bjorndal et al. 1967) (Fig. 1, Table 2). However, Bjorndal et al. (1967) did not differentiate between alditol acetates with the same methylation pattern. For example, although 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-mannitol could not be differentiated from 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol on the basis of their MS fragmentation patterns, their retention times on GLC were different (Table 1). Therefore, by combining GLC and GLC-MS, we could identify with certainty the partially methylated monosaccharides.

#### ELUCIDATING THE STRUCTURE OF THE GLUCOMANNAN

The specific rotation of the glucomannan was  $-35.9^\circ$ , indicative of a  $\beta$ -glycosidic linkage (Wolfrom et al. 1961; Timell 1965). This value was close to the average rotation of  $-38^\circ \pm 2^\circ$  reported by Timell (1965) for many of the  $\beta$ -linked glucomannans isolated from gymnosperm woods. Therefore, we concluded that most of the linkages in the glucomannan were of the  $\beta$  configuration.

TABLE 2. Prominent MS peaks (mass/electrical charge, or *m/e*) of the alditol acetates derived from a methylated glucomannan of Douglas-fir inner bark.<sup>1</sup>

Sugar	Peak <sup>2</sup>	<i>m/e</i>											
		43	45	71	73	75	85	87	88	89	99	101	102
1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methyl-D-glucitol	A <sup>3</sup>	100.0	40.5	13.4				34.7	10.0	25.0		92.0	10.2
1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methyl-D-galactitol	B	98.3	51.2	15.8	14.3			25.5				100.0	12.3
1,4,5-tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methyl-D-mannitol	C	91.0	13.2					24.8			22.9	44.0	
1,4,5-tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methyl-D-glucitol	D	80.1	19.8				11.4	24.6			29.6	37.4	
1,4,5,6-tetra- <i>O</i> -acetyl-2,3-di- <i>O</i> -methyl-D-mannitol	E	60.4									14.1	32.6	
1,4,5,6-tetra- <i>O</i> -acetyl-2,3-di- <i>O</i> -methyl-D-glucitol	F	83.5					15.1	12.3			17.4	25.2	
		<i>m/e</i>											
		103	113	117	127	129	131	145	161	162	205	233	261
1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methyl-D-glucitol	A	15.2		84.3		49.4		42.5	82.7	10.8	35.4		
1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methyl-D-galactitol	B		11.8	100.0		69.6		94.1	69.8		42.5		
1,4,5-tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methyl-D-mannitol	C		46.6	100.0		14.7	11.3		13.3			37.2	
1,4,5-tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methyl-D-glucitol	D		46.9	100.0		19.8	13.6		11.0			47.9	
1,4,5,6-tetra- <i>O</i> -acetyl-2,3-di- <i>O</i> -methyl-D-mannitol	E			100.0	21.1								14.0
1,4,5,6-tetra- <i>O</i> -acetyl-2,3-di- <i>O</i> -methyl-D-glucitol	F			100.0	22.1								21.6

<sup>1</sup> Values in the table represent percentages of the base peak.<sup>2</sup> Designations A, B, C, D, E, and F refer to peaks in Fig. 1.<sup>3</sup> This peak may also contain 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol.

Analysis of the reducing end-groups by the Somogyi method (Hodge and Hofreiter 1962) showed an average molecular weight of  $1.58 \times 10^4$ . On the basis of a molecular weight of 162 for an anhydrohexose unit, each molecule had an average of 97.5 anhydrohexose units. However, 7.7% of these units were galactose residues which were clearly present as nonreducing end-groups (Table 2). Therefore, the main chain of the polysaccharide had a degree of polymerization (DP) of 90 repeating anhydrohexose units.

The intrinsic viscosity of the glucomannan in cadoxen was 0.45 dl/g. Its DP by this method was determined from the relationship  $DP_n = K[\eta]$  (Browning 1967), where  $DP_n$  = number average degree of polymerization,  $[\eta]$  = intrinsic viscosity in dl/g, and  $K$  = a constant. Meier (1962) found a  $K$  value of 200 for glucomannans in cadoxen. Meier's (1962) glucomannans also contained galactose side chains, so use of the value of 200 was appropriate. Thus, the number average DP of the present glucomannan was 92. The DPs of 92 (from viscosity measurements) and 90 (by reducing end-group analysis), determined by these two quite independent methods, are amazingly close values.

On the basis of the previously described data, we propose a structure for the glucomannan (Fig. 2). The repeating unit, which occurs 10 times, consists of a polymer backbone of six anhydro-D-mannopyranose units and three anhydro-D-glucopyranose units. Their sequence may be random. Each repeating unit contains one D-galactopyranose unit as a side chain attached to position 6 of an anhydro-D-mannopyranose unit. However, 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol was also identified from the methylation studies (Table 1), so the D-galactopyranose side chain is sometimes attached to position 6 of an anhydro-D-glucopyranose unit. Although we have no data to indicate whether the D-galactopyranose units are connected by  $\alpha$ - or  $\beta$ -linkages, Fig. 1 shows  $\alpha$ -linkages because these are known to be present in other glucomannans (Timell 1965).

The backbone has a reducing and nonreducing end-group. Figure 2 depicts the nonreducing end-group as a glucopyranose unit. However, the GLC system used does not separate 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol (Harwood 1973). Therefore, the nonreducing end-group in some instances may be an anhydro-D-mannopyranose unit. The data also do not exclude the possibilities that some of the D-glucopyranose and D-mannopyranose units could be nonreducing side chains and that some D-galactopyranose units could exist as the nonreducing end of the backbone. However, these fine structural details would not be expected to influence glucomannan properties in recovery processes. Although variations in the fine structure of the polysaccharide are possible, the structure proposed (Fig. 2) is consistent with the data. In the native state in the bark, some acetyl groups may be attached to the glucomannan, but these would have been lost by hydrolysis when the polysaccharide was isolated.

#### CONCLUSIONS

The glucomannan isolated from the inner bark of Douglas-fir seems structurally and physically similar to the glucomannans known to be contained in the wood of all gymnosperms. Timell (1965) describes these as an alkali-soluble family of polysaccharides consisting of a framework of (1  $\rightarrow$  4)-linked  $\beta$ -D-glucopyranose and  $\beta$ -D-mannopyranose residues distributed at random. Some of the D-glucose



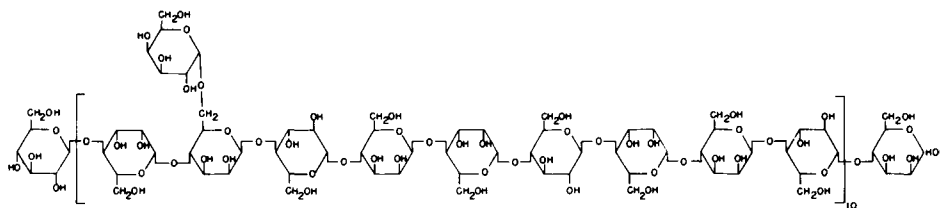


FIG. 2. Proposed structure for the glucomannan isolated from the holocellulose fraction of Douglas-fir inner bark.

and D-mannose residues carry side chains of  $\alpha$ -D-galactopyranose residues. The specific rotation in alkali of these glucomannans is usually about  $-38^\circ \pm 2^\circ$ , and their DPs are 90 to 100. The analytical results and physical properties of the glucomannan reported here certainly place it within this family.

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