The Structure of a Galactomannan from *Anthyllis vulneraria* L.

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The seeds of *Anthyllis vulneraria* L. (Leguminosae) contain a galactomannan, $[\alpha]_D^{25}$ $+80°$, which by complete hydrolysis gave 1.32 mole of D-mannose per 1.00 mole of D-galactose. The only two disaccharides present in the partial acid hydrolysate of the galactomannan were 4-*O*-β-D-mannopyranosyl-D-mannose (mannobiose) and 6-*O*-α-D-galactopyranosyl-D-mannose. The hydrolysate also contained tri- and tetrasaccharides of the formula (mannose)$_n$-galactose. These results, together with the course of the periodate oxidation and the enzymic hydrolysis (by α-galactosidase) of the polysaccharide, are in good agreement with the general formula of galactomannans from Leguminosae (Fig. 1).

The reserve polysaccharides, galactomannans, from about twelve species of Leguminosae have been examined by several authors to determine their structure. Except for the variation of the galactose content, the galactomannans all appear to have the same structural features (Fig. 1), although, as mentioned earlier,[1] significant structural deviation from this pattern has been observed.[2,3]

In the majority of works on the subject the main methods used to solve the structure have been methylation and periodate oxidation. The results obtained were found to be in good agreement and thus contribute to support
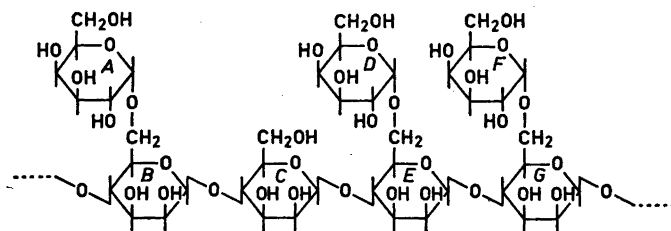

Fig. 1. Section of a galactomannan molecule.

the general character of the formula of Fig. 1, but it should be emphasized that they also fit into the pattern of quite different structures.

The methylation procedure demonstrates by the yield of tetramethyl-D-galactopyranose that all the galactose is present as terminal non-reducing D-galactopyranosyl groups. They are situated as single molecular side chains bound to the mannose backbone by their C-1 carbon atoms. The methylated mannose derivatives usually found, 2,3-dimethyl-D-mannose and 2,3,6-trimethyl-D-mannose do not indicate an unambiguous polysaccharide structure. The identification of these methylated sugars does not confirm whether they come from mannose in the pyranose or the furanose form, or whether the branching points are 1,4- or 1,6-linked. It is clear however, that the unbranched mannose units participate in a 1,4-linkage. From the comparatively slow hydrolysis rate of the galactomannans examined it may be inferred that the furanose form of mannose is less probable. The periodate oxidation data cannot clarify the lacking details, they merely confirm the methylation results.

The anomeric configurations of the mannose (β) and the galactose (α) residues in a leguminous galactomannan are postulated mainly by calculation from optical rotation data of the free polysaccharide and its methylated derivative.

Confirmation of this postulate and answer to the former mentioned uncertainties may, however, be obtained by means of the partial acid hydrolysis technique, which has been used to determine the structure of the galactomannans from comparatively few (3) leguminous species.[4-7]

The present paper represents a continuation of our work[1] on galactomannans from plants of agricultural interest and here we have chosen the technique of partial acid hydrolysis as the main procedure to clarify the structure of a galactomannan from *Anthyllis vulneraria* L.

The galactomannan obtained from *Anthyllis vulneraria* L. was a white water soluble powder, yield 1.8 %, $[\alpha]_D^{25} + 80°$. A hydrolysate of the homogeneous polysaccharide contained only galactose and mannose in the molecular proportion 1:1.32, which corresponds to a content of 43 % of galactose in the galactomannan molecule. This is in agreement with the high positive rotation and the amount of formic acid produced by the periodate oxidation. The liberated formic acid corresponds to 40 % of anhydro hexose as terminal groups, which probably are represented by the galactose molecules. The amount of formic acid formed would account for the consumption of 0.80 mole periodate per anhydro hexose unit. The uptake of periodate was found to be 1.2 mole which is taken as a proof that vicinal hydroxyl groups other than those of the terminal hexopyranosyl units are present in the polysaccharide molecule.

The partial hydrolysis and chromatographic separation on a carbon celite column resulted in isolation of four oligosaccharide fractions (fractions I, II, III, IV) and one fraction containing a mixture of mannose and galactose (fraction 0). The two monosaccharides were identified as D-mannose and D-galactose after further separation on a cellulose column. The oligosaccharides behaved as homogeneous fractions on high voltage electrophoresis in borate and germanate buffers. Fractions I and II were identified as the following compounds; I, 4-*O*-β-D-mannopyranosyl-D-mannose and II, 6-*O*-α-D-galacto-

pyranosyl-D-mannose. Fractions III and IV contained trisaccharides and tetrasaccharides representing one galactose residue (from side chain) combined with two or three mannose residues (from main chain): (mannose)$_n$-galactose. Fraction III is a trisaccharide or, as it emerges from the methylation data, presumably a mixture of two isomers, which would correspond to the molecule combination ABC and CDE, Fig. 1. ABC has been isolated earlier,[6] but CDE has not been described previously, at least not from this type of galactomannan. The analysis data obtained from fraction IV indicate a tetrasaccharide with one more molecule of mannose than fraction III. It may be one of three possible tetrasaccharides; ABCE, CDEG, or CEFG, or a mixture of any two or all three.

After the identification of fractions I and II, in addition to the rest of the data, it can be settled that the galactomannan from *Anthyllis vulneraria* L. possesses a structure in good agreement with the commonly accepted one for leguminous galactomannans shown in Fig. 1. Galactose molecules are connected to 76 % of the mannose residues of the backbone chain.

EXPERIMENTAL

Paper chromatograms were run by the descending method on Whatman No. 1 papers in the following solvent systems (v/v):

a. Butanol, pyridine, water, 10:4:3.
b. Ethyl acetate, pyridine, water, 10:4:3.
c. Propanol, water, 78:22.

Thin-layer chromatography was carried out on kieselgel G in the following systems (v/v):
d. Benzene, ethanol, 20:3.[15]
e. Butanone saturated with water.[16]

Aniline oxalate was used as spray reagent for the chromatograms.

High voltage electrophoresis (1500 V, 90 min) was carried out on glass fiber sheets (Whatman GF/C) in 0.1 M borate buffer, pH 10, and 0.05 M germanate buffer,[13] pH 10.7. The electropherograms were sprayed with $KMnO_4$.

Extraction and purification of the galactomannan. The milled seeds (100 g) were extracted with cold water (1500 ml) under continuous stirring for 2 h. The extract was centrifuged and the supernatant left for three days (with some toluene) to be clarified. After centrifugation the copper complex of the galactomannan was precipitated by adding Fehling's solution (350 ml) and purified further by ethanol precipitations.[8] The polysaccharide preparation was a white powder, yield 1.8 g; $[\alpha]_D^{25}$ +80° (*c* 1.0, water); N, nil; sulfated ash, 0.26 %; the aqueous solution was neutral and gave no coloration with iodine, showing that no starch was present. The homogeneity of the product was confirmed by electrophoresis in borate buffer and 2 N NaOH (120 mA, 18 h). The product moved towards the cathode the same distance as the galactomannan from *Lotus corniculatus* L.[1]

Identification and estimation of constituent sugars. Complete hydrolysis was achieved by heating the polysaccharide dissolved in N H_2SO_4 in a sealed tube for 15 h. The hydrolysate gave only mannose and galactose on examination by paper chromatography. The sugars were separated by solvent *a* and the molar proportion was determined by periodate oxidation.[9] As a result of replicate analysis the molecular proportion galactose/mannose was found to be 1.00/1.32 which corresponds to 43 % of anhydrogalactose in the polysaccharide. Mannose was characterized as D-mannose phenylhydrazone,[10] m.p. 195°, $[\alpha]_D^{20}$ +32° (*c* 0.5, pyridine), IR spectrum identical with that of a reference substance.

The monosaccharide fraction obtained from a partial acid hydrolysate by separation on the charcoal celite column, was transferred to a cellulose column and eluted with

butanol-ethanol (50:10), saturated with water. Chromatographically pure galactose was obtained and characterized as D-galactose by its optical rotation.

Oxidation by periodate.[1] Results from a typical series of experiments are given in Table 1.

Table 1. Oxidation by periodate.

Time, h	Moles of periodate consumed per $C_6H_{10}O_5$	Acid, equivalents per $C_6H_{10}O_5$
1	0.67	—
4	0.82	—
24	1.02	0.400
48	1.10	0.395
72	1.14	0.401
120	1.19	

Hydrolysis by α- and β-galactosidase.[1] The anomeric configuration of galactose was the α-form as galactose was only liberated by α-galactosidase.

Methylation analysis. In the first[11] methylation step the oligosaccharide (0.25—1 mequiv. OH) was dissolved in 20 parts (w/w) of DMF (dimethylformamide) in a ground glass stoppered test tube. Silver oxide (2 equiv. per equiv. sugar) and methyl iodide (3 equiv. per equiv. sugar) were added, and the mixture shaken over night (16 h) at room temperature. After centrifugation, the decanted solution was evaporated to dryness under reduced pressure (70—100° in the water bath). The residue was dissolved in DMF (1.25 ml DMF/mequiv. sugar) and remethylated[12] by addition of 0.26 g/mequiv. sugar of a mixture consisting of BaO (25 parts) and $Ba(OH)_2 \cdot 8H_2O$ (1 part). Finally methyl iodide (0.25 ml/mequiv.) was added and the test tube shaken at room temperature for 20 h. After centrifugation and decantation the solution was diluted with chloroform (5 ml). This solution was shaken after addition of a few drops of sodium thiosulfate solution (0.1 N) and washed with water (5 × 2 ml). After drying with Na_2SO_4, the solution was evaporated under reduced pressure to dryness (40° in the water bath).

The fully methylated product was hydrolyzed by addition of 1 ml formic acid and kept at 100° with reflux for 1 hour. The acid was removed by evaporation under reduced pressure, and the residue rehydrolyzed in 1 N HCl for 3 h, neutralized with $BaCO_3$ and evaporated to dryness. This residue was dissolved in a small amount of chloroform and examined by thin-layer chromatography (Table 3).

Partial acid hydrolysis of the galactomannan

(a) *Hydrolysis.* After a long series of preliminary experiments the following procedure was adopted. A solution of the polysaccharide (2.5 %) in N HCl (100 ml) was heated at 80° for 4 h. The hydrolysate was neutralized by a column (2.4 × 12 cm) of Amberlite IR 4 B (OH⁻). The eluate (800 ml) was concentrated to about 10 ml (A). Paper chromatography (solvent a) revealed four reducing oligosaccharides (fractions I, II, III, IV) besides spots of galactose and mannose (fraction 0). Control experiments were performed using a mixture of 43 % of D-galactose and 57 % of D-mannose. This sugar mixture (2.5 %) was submitted to the same treatment as described for the polysaccharide. Paper chromatography of the concentrated solution (B) proved to give only negligible traces of oligosaccharide spots. When the sugar concentration was increased to 5 %, the amount of oligosaccharides formed by acid synthesis were more pronounced. It appears that the experimental conditions chosen for the partial hydrolysis would not produce synthetic oligosaccharides in more than negligible quantities.

(b) *Separation and identification of the fractions.* The concentrate (A) was transferred to a charcoal-celite column 1:1 (4 × 21 cm). Elution with water yielded a mixture of

Table 2. Paper chromatography (R_{Glc}) and electrophoresis (M_{Glc}) of the different fractions.

Fraction No.	R_{Glc} Solvent *a*	M_{Glc} borate buffer	M_{Glc} germanate buffer
I	0.56	0.55	0.69
II	0.43	0.52	1.14
III	0.19	0.39	0.69
IV	0.05	0.43	0.72

galactose and mannose (fraction 0) which was further separated into its two components on a cellulose column. Gradient elution with ethanol in water gave four apparently homogeneous fractions (I, II, III, IV) as shown by paper chromatography and high voltage electrophoresis in borate and germanate buffers [13] (Table 2).

Fraction I, mannobiose, 4-O-β-D-mannopyranosyl-D-mannose. It was obtained as a white powder; yield 67 mg; degree of polymerisation (DP) 2; $[\alpha]_D^{20}$ −5.6° (*c* 1.43, water); paper chromatographic and electrophoretic mobilities are given in Table 2. Fraction I gave exclusively mannose by hydrolysis. By paper chromatography (solvents *a, b, c*) it was undistinguishable from an authentic sample of mannobiose. It proved to be 1,4-linked by the test of formaldehyde formation after oxidation by periodate of the corresponding sugar alcohol.[14] Methylation [11,12] and thin-layer chromatographic separation [15] (Table 3) of the hydrolysed product gave 2,3,6-trimethyl-D-mannose and 2,3,4,6-tetramethyl-D-mannose, which confirms the identification of fraction I as mannobiose.

Fraction II, 6-O-α-D-galactopyranosyl-D-mannose. This was obtained as a white powder; yield 34 mg; DP 2; $[\alpha]_D^{23}$ +120° (*c* 1.0, water); paper chromatographic and electrophoretic mobilities are given in Table 2. Fraction II gave galactose and mannose in equimolecular amounts by complete hydrolysis. The disaccharide was attacked by α-galactosidase and not by β-galactosidase. It proved to be 1,6-linked when submitted to the periodate oxidation of the corresponding sugar alcohol.[14] Examination of the methylated product (Table 3) gave 2,3,4-trimethyl-D-mannose and 2,3,4,6-tetramethyl-D-galactose which confirms the identification of fraction II as 6-O-α-D-galactopyranosyl-D-mannose.

Fraction III. This fraction is probably a mixture of ABC and CDE (Fig. 1). It was obtained as a white powder; yield 135 mg; DP 3; $[\alpha]_D^{25}$ +39° (*c* 2, water) [ABC, $[\alpha]_D^{25}$ +93.3 → +98.4° (*c* 1.22, water)].[6] The fraction appeared to be homogeneous by paper chromatography and electrophoresis (Table 2). It gave galactose and mannose in the proportion 1/1.9 after total hydrolysis. It was attacked by α-galactosidase only and gave galactose, unattacked III, and a component which moved identical with fraction I compared by paper chromatography. Partial acid hydrolysis followed by examination

Table 3. R_F values of methyl derivatives obtained from the fractions I, II, and III. Thin-layer chromatography.

Methyl derivatives	Fraction I Solvent *d*	Fraction II Solvent *d*	Fraction III Solvent *d*	Fraction III Solvent *e*
2,3-Me$_2$Man *a*			0.11	0.43
2,3,6-Me$_3$Man	0.22		0.22	0.62
2,3,4-Me$_3$Man		0.23	0.23	0.65
2,3,4,6-Me$_4$Man	0.35		0.35	0.71
2,3,4,6-Me$_4$Gal		0.31	0.31	0.80

a 2,3-Dimethyl-D-mannose, *etc.*

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by paper chromatography with reference substances revealed galactose, mannose, unhydrolyzed III, and two substances with the same mobilities as fractions I and II. Methylation and two-dimensional thin-layer chromatographic separation (solvents *d, e*) of the hydrolyzed methylated product resulted in the following components, undistinguishable from authentic specimens (Table 3); 2,3-dimethyl-D-mannose, 2,3,6-trimethyl-D-mannose, 2,3,4-trimethyl-D-mannose, 2,3,4,6-tetramethyl-D-mannose, and 2,3,4,6-tetramethyl-D-galactose. This mixture is obtainable if the fraction consists of a mixture of the molecule combinations ABC and CDE (Fig. 1).

Fraction IV was a reducing tetrasaccharide with one more molecule of mannose than fraction III. It may be one of the three possible tetrasaccharides ABCE, CDEG, and CEFG (Fig. 1), or a mixture of any two or all three. The fraction was obtained as a white powder; yield 73 mg; $[\alpha]_D^{20}$ +37° (*c* 1.0, water). It appeared to be homogeneous as shown by paper chromatography and electrophoresis (Table 2). The reducing ability before and after total hydrolysis was 1:4.5 compared with glucose. Total hydrolysis revealed galactose and mannose in the proportion 1:3.17. The anomeric galactose configuration was the α-form as judged by the enzymic hydrolysis. Examination by paper chromatography of the partial acid hydrolysate revealed the following components: galactose, mannose, fractions I, II, III, and unhydrolyzed IV.

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