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Note

A D-galacto-D-mannan from *Melilotus officinalis* seeds*

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Seeds of *Melilotus officinalis* Lam. (*Leguminosae*), cultivated in India[1] (Ladakh and Nubra parts), are rich in mucilaginous matter and are used as a gruel to treat bowel complaints, especially infant diarrhoea. Anderson[2] determined the percentage of endosperm in the seeds. Tookey and Jones[3] determined their content of water-soluble gum. Reid and Meier[4] determined the neutral sugar constituents (1:1.19 Gal:Man) of the polysaccharide, but gave no details of its structure. The present communication deals with the isolation, purification, and structural analysis of the galactomannan of the seeds of *M. officinalis* Lam.

TABLE I

IDENTIFICATION OF THE NEUTRAL SUGARS FROM *M. officinalis* POLYSACCHARIDE

Component[a]	R_T[b] (OV-225)	m/z[c]	*Molar ratio*
D-Mannose	18.4	43, 73, 103, 115, 145, 157, 217, 289	1.09
D-Galactose	20.12	43, 73, 103, 115, 145, 157, 217, 289	1.00

[a]As alditol acetates. [b]Retention times of neutral sugars are for column of 3% of OV-225; the values were identical with those of standard sugars. [c]Characteristic fragments are reported in the literature[7].

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RESULTS AND DISCUSSION

Extraction of the powdered and defatted seeds of *M. officinalis* with water furnished a crude polysaccharide that was further purified *via* its copper complex[5]. The purified polysaccharide was water-soluble and neutral, and had $[\alpha]_D^{30}$ +70.1° (water). It appeared to be homogeneous by moving-boundary electrophoresis[6]. Hydrolysis of the polysaccharide produced galactose and mannose in the molar ratio of 1.00:1.09 (see Table I).

The polysaccharide was methylated, first by the Haworth method[8] and then by the Hakomori method[9]; this procedure was used because the unmethylated polysaccharide is insoluble in dimethyl sulfoxide. Hydrolysis[10] of the permethylated polysaccharide gave 2,3,4,6-tetra-*O*-methyl-D-galactose (11.4 mol), 2,3,6-tri-*O*-methyl-D-mannose (1.0 mol), and 2,3-di-*O*-methyl-D-mannose (11.2 mol) (see Table II). These results suggested a typical galactomannan structure in which, on average, 11 of every 12 (1→4)-linked mannosyl units in the main chain are substituted with a single galactosyl group as a side chain.

Upon oxidation until consumption of periodate ion ceased (8 days), the polysaccharide consumed 1.46 mol of sodium metaperiodate per mol, with the liberation of 465 mmol of formic acid per hexosyl unit. (A high concentration of periodate and a long reaction time are required for complete oxidation of a galactomannan[11,12] because the oxidation of mannosyl units adjacent to oxidized, 6-*O*-substituted mannosyl units is very slow owing to hemiacetal formation[13,14].) The periodate-oxidized polysaccharide was reduced with sodium borohydride, and the derived polyalcohol was hydrolyzed with acid[15] to give glycerol (1.00 mol) and erythritol (1.09 mol). None of the monomer units in the polysaccharide survived periodate oxidation.

Partial, acid-catalyzed hydrolysis of the purified polysaccharide gave four oligosaccharides (see Table III): **1**, *O*-β-D-mannopyranosyl-(1→4)-D-mannose; **2**, *O*-α-D-galactopyranosyl-(1→6)-D-mannose; **3**, *O*-α-D-galactopyranosyl-(1→6)-*O*-

TABLE II

IDENTIFICATION OF THE METHYLATED SUGARS OBTAINED FROM PERMETHYLATED *M. officinalis* POLYSACCHARIDE

Component[a]	R_T[b] (SE-30)	m/z[c]	*Molar ratio*
2,3,4,6-Me_4-D-Gal	2.9	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	11.4
2,3,6-Me_3-D-Man	3.7	43, 45, 87, 99, 101, 113, 117, 233	1.0
2,3-Me_2-D-Man	5.3	43, 101, 117, 261	11.2

[a]As alditol acetates. [b]Retention times of methylated sugars are for column of SE-30; the values are identical with those of standard sugars. [c]Characteristic fragments are reported in the literature[7].

TABLE III

DATA OBTAINED FOR THE ISOLATED OLIGOSACCHARIDES

Oligosaccharide	*D.p.*	R_{Gal}[a,b]	$[\alpha]_D^{26}$ *(degrees)*[a,c]	*Composition*	IO_4^- *consumed*[d]	HCO_2H liberated[d]	*Glycol composition*[e]
1	1.98	0.80	−7.9	Man	3.04	0.97	1:1.04 glycerol: erythritol
2	2.02	0.65	+122	1:1 Gal: Man	4.08	2.01	glycerol only
3	2.96	0.43	+36.8	1:2 Gal: Man	5.02	1.98	1:2.00 erythritol: glycerol
4	3.06	0.18	−21.8	Man	4.07	1.05	1:2.08 glycerol: erythritol

[a] The values are identical with those reported in the literature[16]. [b] Mobility relative to that of D-galactose in solvent B. [c] For a solution in water. [d] Mol/mol of the methyl glycoside of the oligosaccharide. [e] Released after periodate oxidation, reduction, and hydrolysis of the methyl glycoside of the oligosaccharide.

β-D-mannopyranosyl-(1→4)-D-mannose; and **4**, *O*-β-D-mannopyranosyl-(1→4)-*O*-β-D-mannopyranosyl-(1→4)-D-mannose.

The ^{1}H-n.m.r. spectrum of the polysaccharide (see Fig. 1) contained a signal at δ 4.8 ($J_{1,2}$ ~1.0 Hz) from the anomeric proton of β-D-mannopyranosyl units[17], and a doublet at δ 5.0 ($J_{1,2}$ ~3.0 Hz) from the anomeric proton of α-D-galactopyranosyl units[17].

All the carbon lines in the ^{13}C-n.m.r. spectrum of the polysaccharide (see Fig. 2) were resolved; their chemical shifts are recorded in Table IV. The spectrum indicated two types of anomeric carbon atom in the polymer: δ 101.8 and 103.0, for α-D-galactopyranosyl and β-D-mannopyranosyl units, respectively[17]. The resonances for C-5 and C-6 of β-D-mannopyranosyl units in the ^{13}C-n.m.r. spectrum also clearly differentiated and identified the unsubstituted (1→4)-linked β-D-mannopyranosyl units of the mannan backbone, as well as dominating the 6-*O*-substituted, (1→4)-linked β-D-mannopyranosyl units. The resonances in the ^{13}C-n.m.r. spectrum agree with those reported in the literature[17], and a D-galacto-D-mannan molar ratio of 1.0:1.1 was obtained from the relative peak areas of the two monomers.

The results of periodate oxidation, partial hydrolysis, and n.m.r. analysis supported the conclusion reached from methylation analysis of the polysaccharide.

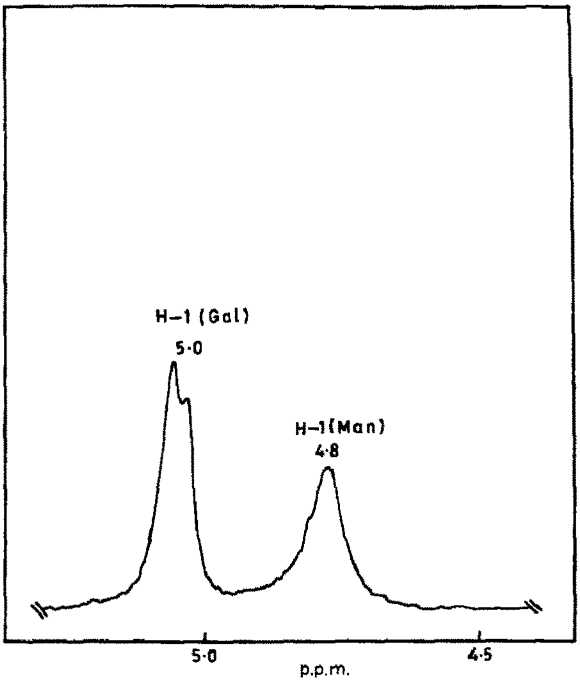


Fig. 1. ^{1}H-n.m.r. spectrum (100 MHz) of a solution of *M. officinalis* polysaccharide (10 mg/0.4 mL) in D_2O (at 90°).

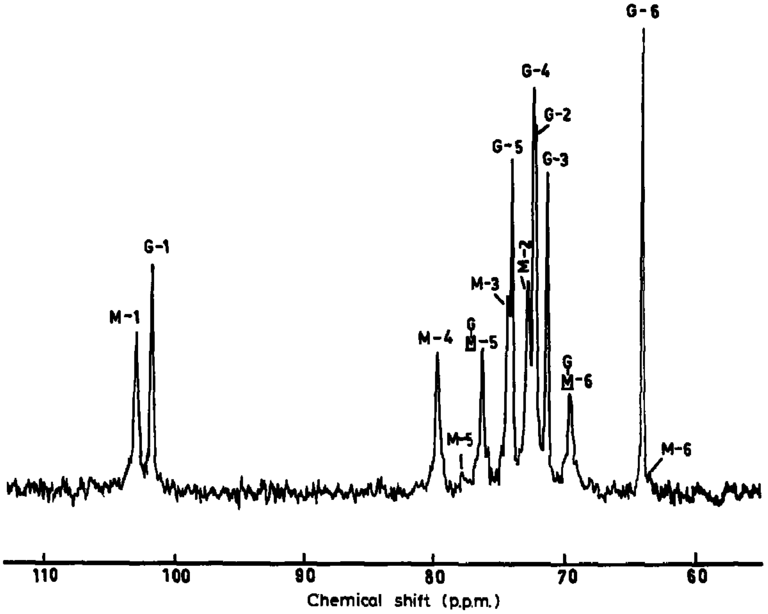


Fig. 2. ^{13}C-n.m.r. spectrum (25 MHz) of a solution of *M. officinalis* polysaccharide (32 mg/0.4 mL) in D_2O (at 90°).

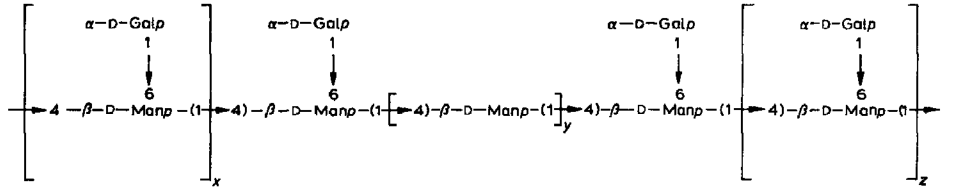
TABLE IV

ASSIGNMENTS OF PEAKS IN ^{13}C-N.M.R. SPECTRUM OF *M. officinalis* POLYSACCHARIDE[a]

Type of unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
α-D-Galactopyranosyl	101.8	72.3[b]	71.4	72.4[b]	74.1	64.1
β-D-Mannopyranosyl, unbranched at 0-6	103.0	72.9	74.4	79.3[c] 79.6[d]	78.0	63.6
β-D-Mannopyranosyl, branched at 0-6	103.0	72.9	74.4	79.6[c] 79.9[d]	76.3	69.6

[a] Shifts (p.p.m.) downfield from internal sodium 4,4-dimethylsilapentanoate-d_4. [b] These assignments may have to be interchanged. [c] When the preceding D-mannose residue is unbranched. [d] When the preceding D-mannose residue is branched. Apart from the major resonance at 79.9 ppm, the values of the chemical shift for C-4 of D-mannopyranose were taken from ref. 17.

Considering all of the foregoing findings, a tentative structure (5) was assigned for the average repeating unit of *M. officinalis* galactomannan. The characteristic features of *M. officinalis* polysaccharide indicate that the backbone is composed of twelve (1→4)-linked β-D-mannopyranosyl units, to which are attached, by (1→6) linkages, on average, eleven α-D-galactopyranosyl single-unit side chains. It therefore possesses a classic, highly branched galactomannan structure.



$x + z = 9$, and $y = 1$

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EXPERIMENTAL

General methods. — Solutions were evaporated under diminished pressure at temperatures not exceeding 50°. Optical rotations were measured with a Perkin-Elmer model 141 polarimeter. Moving-boundary electrophoresis was carried out in an LKB electrophoresis apparatus. The i.r. spectrum was recorded with a Perkin-Elmer model 137-B infrared spectrophotometer. N.m.r. experiments were conducted in the Fourier-transform mode at 25 MHz for ^{13}C, and at 100 MHz for ^{1}H, with a JEOL FX100 spectrometer; spectra were recorded for solutions of the polymer in D_2O (32 mg/0.4 mL for ^{13}C- and 10 mg/0.4 mL for ^{1}H-n.m.r.) at 90°. Chemical shifts are expressed relative to internal sodium 4,4-dimethyl-4-silapentanoate-d_4.

Descending paper chromatography (p.c.) was conducted by using Whatman No. 1 paper (analytical), Whatman No. 3MM paper (preparative), and the following solvent systems (v/v): (*A*) 4:1:5 (upper layer) 1-butanol-ethanol-water[18], (*B*) 6:4:3 1-butanol-pyridine-water[16], and (*C*) butanone-water (azeotrope)[18]. The chromatograms were developed with (*a*) *p*-anisidine phosphate[19] and (*b*) periodate-benzidine[20]. G.l.c. of neutral sugars was performed on a Hitachi model 063 gas chromatograph equipped with a H_2 flame-ionization detector, using a column (0.3 × 200 cm, spiral glass) packed wih 3% of OV-225 on Gas Chrom Q (100-200 mesh), at 210° and a flow rate of N_2 of 30 mL/min. G.l.c.-m.s. of peracetylated alditol acetates was conducted with a JEOL model JGC-20K gas chromatograph and a JMS-D100 mass spectrometer under similar conditions. Partially methylated alditol acetates were analyzed by g.l.c.-m.s., using a Hewlett-Packard model 5985 apparatus equipped with a column (0.63 × 153 cm, stainless steel) packed with SE-30 and temperature-programmed from 140 to 230° at 4°/min; He at a flow rate of 40 mL/s was used as the carrier gas. Paper electrophoresis was conducted with a Laboratorium Felzerclsk Gyara apparatus (Budapest, Hungary), type DE-210, using Whatman No. 1 paper in sodium tetraborate buffer (0.05M, pH 9.2).

Isolation and purification of the polysaccharide. — *M. officinalis* seeds (200 g) were cleaved at low speed in a hand mill in order to break off the seed coat and separate the cotyledons. After their removal, the endosperm was finely powdered, dried, defatted, and extracted three times (12 h each) with water at 50°. The resulting viscous solution, after cooling, was filtered through muslin, centrifuged, and acidified (CH_3CO_2H). The crude polysaccharide was precipitated with ethanol, collected by centrifugation, and dried by solvent exchange with acetone, followed with absolute ethanol. The crude polysaccharide was further purified *via* its copper complex[5]. Finally, a solution of the purified polysaccharide was passed successively through columns of Dowex 50W-X8 (H^+) and Dowex 3 (OH^-) ion-exchange resins. Polysaccharide was precipitated from the final effluent with 3 volumes of ethanol, dehydriated by solvent exchange with acetone, followed by absolute ethanol, and finally dried in a vacuum desiccator at room temperature; yield 13.34 g. The product, $[\alpha]_D^{30}$ +70.1° (*c* 0.08, water), did not reduce Fehling solution, was water-soluble, neutral (pH 6.7), and free from halogens, methoxyl, nitrogen, pentose, sulfur, and uronic acid.

Homogeneity of the polysaccharide. — The polysaccharide (1% solution) was analyzed by moving-boundary electrophoresis in 0.2M Tris·HCl buffer (pH 8.5) for 5 h at 12 V and 5 mA. Only one sharp symmetrical peak, both in the descending and the ascending arm, was recorded with a Schlieren optical system[6].

Hydrolysis of the polysaccharide. — Complete hydrolysis of the purified polysaccharide (1.8 g), as determined by the point of maximum reducing power (iodometric titration[21]), was accomplished with 2M trifluoroacetic acid (140 mL) for 10 h at 100°. The suspension was filtered, and the acid was removed by evaporation to dryness under a stream of N_2. The residue was heated with dry methanol at gentle reflux temperature; the resulting suspension was filtered, and the filtrate evaporated to a syrup. Resolution of the sugars was accomplished on a cellulose column using solvent *A*. D-Galactose and D-mannose were characterized by their melting points, specific optical rotations, and properties of characteristic derivatives (data not reported); all values were identical with those reported in the literature[22]. Another portion of the polysaccharide (0.2 g) was hydrolyzed with 2M trifluoroacetic acid (16 mL) in a sealed tube under similar conditions. A part of the resultant syrup (20 mg) was converted[23] into alditol acetates of the component sugars and these were analyzed by g.l.c. and g.l.c.-m.s. The results are given in Table I.

Methylation of the polysaccharide. — The polysaccharide (2 g) was subjected to four Haworth methylation[8], followed by a Hakomori methylation[9], to yield the fully methylated polysaccharide (1.42 g), which did not show any hydroxyl absorption in the infrared spectrum. The fully methylated polymer (1.2 g) was hydrolyzed according to the method of Croon *et al.*[10]. The hydrolyzate was diluted with water (50 mL), the acid neutralized with $BaCO_3$, the suspension filtered, and the filtrate evaporated *in vacuo*. The residue was then successively extracted with methanol and chloroform, and the combined extracts were evaporated to a light-yellow syrup. A part of the syrup was subjected to cellulose-column chromatography using solvent *C*. After p.c. examination using solvent *C*, the appropriate fractions were combined. Final resolution of the fractions into their individual components was achieved by preparative p.c. using solvent *C*. The three methylated sugars so obtained were characterized by their R_F values in solvent *C*, R_{TG} values using solvent *A* and spray reagent *a*, specific rotations, and melting points of their characteristic derivatives (data not reported); all values were identical with those reported in the literature[15,18].

The other part of the syrup (25 mg) was reduced in water with $NaBH_4$ (40 mg) for 2 h. After neutralization of the base with Dowex 50W-X8 (H^+) resin, the filtrate was evaporated to dryness, and boric acid was removed by repeated addition and evaporation of methanol. Then, the products were acetylated[23] with 1:1 acetic anhydride-pyridine for 20 min at 100°. After evaporation of the solution, the residue was dissolved in 1:1 chloroform-methanol and analyzed by g.l.c. and g.l.c.-m.s. The results of integration are given in Table II.

Periodate (Smith) degradation of the polysaccharide. — The polysaccharide (1 g) was dissolved in water (250 mL), and 0.5м sodium metaperiodate (27 mL) was added. Oxidation was allowed to proceed at 5° in the dark. Aliquots were removed at intervals for estimation of periodate[24] and formic acid[25], the concentrations of which became constant after 8 days. The excess of periodate was removed with 1,2-ethanediol; the solution was dialyzed and then reduced[26] with $NaBH_4$ (900 mg) overnight at room temperature. After reduction, the solution was made neutral with 50% acetic acid, de-ionized, and evaporated to dryness. The last traces of boric acid were removed from the residue by repeated addition and evaporation of methanol. The residue was hydrolyzed with 0.5м sulfuric acid in a sealed tube for 10 h at 100°. The hydrolyzate was made neutral with $BaCO_3$, de-ionized, and evaporated to a syrup. P.c. using solvent *A* and spray reagent *b* revealed the presence of glycerol and erythritol (characterized by cochromatography with standard glycols); no sugars were detected. Glycerol and erythritol were quantified by the chromotropic acid method[27].

Partical hydrolysis of the polysaccharide. — The polysaccharide (2.5 g) was hydrolyzed with 0.05м sulfuric acid (35 mL) for 9 h at 100°. The hydrolyzate was made neutral with $BaCO_3$, the suspension filtered, and the filtrate de-ionized, and evaporated to a syrup. P.c. of the syrup using solvent *B* with spray reagent *a* revealed four oligosaccharides, in addition to galactose and mannose. The syrup was added to a column of 1:1 charcoal-Celite; components were successively eluted with water and 0.5, 5, and 10% ethanol. Fractions containing oligosaccharides were further separated by preparative p.c. in solvent *B*, to give oligosaccharides **1**-**4** in pure form. Homogeneity of the oligomers was established by paper electrophoresis. The d.p. of the four oligosaccharides was determined by the anthrone-sulfuric acid method[28].

The oligosaccharides were hydrolyzed with м trifluoroacetic acid for 5-6 h at 100°. After the usual treatment, the component sugars were identified by p.c. in solvent *A* with spray reagent *a*, and quantified by the phenol-sulfuric acid method[29]. The oligosaccharides were converted into methyl glycosides by treatment with 2% dry methanolic HCl at 0° under strictly anhydrous conditions, followed by p.c. to check completeness of the reaction, conditions that have been shown to yield methyl glycopyranosides of oligosaccharides, including those containing mannose at the reducing end[30-32]. Periodate oxidation of each oligosaccharide glycoside was conducted with 0.1M $NaIO_4$ solution in the dark at 5°. When the uptake became constant, periodate consumption[24] and formic acid liberation[25] were determined (mol/mol of methyl glycoside derivative). The periodate-oxidized product was reduced with $NaBH_4$, and the product hydrolyzed with M trifluoroacetic acid for 6 h at 100°. After the usual treatment, p.c. of the released glycols, using solvent *A* and spray reagent *b*, revealed the presence of glycerol and erythritol, as characterized by cochromatography with standard glycols. The glycols were quantitatively estimated by the chromotropic acid method[27].

The linkages of trisaccharides **3** and **4** were determined by methylation analysis using the method of Hakomori[9], followed by that of Purdie and Irvine[33], for methylation, and the method of Croon *et al.*[10] for hydrolysis of the methylation product. After the usual treatment, the mixture of methylated sugars obtained from **3** was separated by preparative p.c. in solvent *A*, to give three methylated sugars that were identified as 2,3,4,6-tetra-*O*-methyl-galactose, and 2,3,4-tri- and 2,3,6-tri-*O*-methylmannose by their migration rates (R_{TG} 0.89, 0.85, and 0.81) in p.c. and by cochromatography with authentic samples using solvent *A* and spray reagent *a*. Following identical treatment, the methylated sugars obtained from **4** were identified as 2,3,4,6-tetra-*O*-methylmannose and 2,3,6-tri-*O*-methylmannose by their migration rates (R_{TG} 0.96 and 0.81). The d.p., migration rate (R_{GAL}), optical rotation, sugar composition, periodate consumption, formic acid liberation, and Smith degradation products of each oligosaccharide are tabulated in Table III.

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