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STRUCTURAL DIVERSITY OF D-GALACTO-D-MANNAN COMPONENTS ISOLATED FROM LICHENS HAVING ASCOMYCETOUS MYCOSYMBIONTS†

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ABSTRACT

D-Galacto-D-mannan fractions were isolated from six common Canadian lichens having ascomycetous mycosymbionts, namely *Parmelia sulcata, Stereocaulon paschale, Peltigera aphthosa, Letharia vulpina, Actinogyra muehlenbergii,* and an *Usnea* sp. Their chemical structures were compared with each other and those of six species previously investigated; only *Parmelia sulcata* and *Cetraria islandica* (Iceland moss) contained galactomannans of closely related structures. The structural diversity depends on substituents on the (1→6)-linked α-D-mannopyranosyl main-chains. The side-chains occur as monosubstituents at O-2 or O-4 or as disubstituents at O-2,4. Frequently, the main-chain units are unsubstituted. Thus far, eight types of substitution have been recognized (**1–8**) in which β-D-Gal*p* is linked (1→4), α-D-Gal*p* and α-D-Man*p* (1→2), and β-D-Gal*f* (1→4).

INTRODUCTION

Lichens having ascomycetous mycosymbionts contain glucan(s) and heteropolysaccharide(s). Detailed studies have been carried out on the heteropolysaccharide of *Evernia prunastri*; its components are galactose, mannose, and glucuronic acid as nonreducing end-groups of galactopyranose (36%) and mannopyranose (11%), and unidentified tri-*O*-substituted mannopyranosyl residues[1]. D-Galacto-D-mannans were isolated from *Cetraria islandica* (Iceland moss) and *Ramalina usnea via* insoluble Cu complexes. Each contains (1→6)-linked α-D-mannopyranosyl main-chains, but the *C. islandica* polysaccharide is more highly branched, some of the main-chain units being di-*O*-substituted by α-D-Gal*p*-(1→2) and β-D-Gal*p*-(1→4) side-chains (**5**). The galactomannan of *R. usnea*

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contains β-D-Gal*p*-(1→4) side-chains only[2]. Galactomannans of *Cladonia alpestris* (reindeer moss)[3,4], *Cladonia confusa*, and *Cladonia amaurocraea*[4] have related but different structures. (Polysaccharides obtained from the supernatant solution of the Fehling-solution precipitates of *C. alpestris* and *C. confusa* contain high proportions of β-D-galactofuranosyl units[4]). Thus far, each galactomannan has a typically characteristic structure as demonstrated by the C-1 signals of the ^{13}C-n.m.r. spectra. In the present study, the extent of this chemical diversity was further investigated by examination of lichens collected mainly in the boreal region of Central Saskatchewan, Canada. These include *Parmelia sulcata* (Tayl.), *Stereocaulon paschale* (L.) Hoffm., *Peltigera aphthosa* (L.) Wild, *Actinogyra muehlenbergii* (a rock tripe), and an *Usnea* sp. collected from dead branches of trees. *Letharia vulpina* (L.) Hue was collected in the Rocky Mountains.

EXPERIMENTAL

Lichens. — *A. muehlenbergii* (a rock tripe) was found on the prominent rock formations situated on the east side of Mountain Lake, close to Stanley Mission, Saskatchewan, Canada. *P. sulcata* grew in close proximity. An *Usnea* sp. was collected from the dead branches of spruce trees growing on the west side of the same lake. The source of *P. aphthosa* was Meadow Lake Provincial Park, Saskatchewan, at a point 500 m north of the Waterhen River and 1 km east of Lac des Iles. *S. paschale* was found 50 km northwest of Meadow Lake on the roadside of Highway 55. *L. vulpina* is not common in Central Saskatchewan and was collected at the 2300-m level in Larch Valley, 4 km west of Moraine Lake in Banff National Park, Alberta. It grew on dead alpine larch (*Larix lyalli*).

Aqueous methanol extraction of lichens and identification of polyols in the extracts. — Samples of lichens were extracted successively with cold 2:1 (v/v) benzene–ethanol and with methanol containing 20% (v/v) of water under reflux. The products were examined by paper chromatography and by gas-liquid chromatography of the derived acetates, and proportions and contents of arabinitol and mannitol were determined. All procedures are identical to those described in a previous publication[4].

In the case of the extracts from *P. aphthosa* and *A. muehlenbergii*, several components moving slower than mannitol were detected on paper chromatograms. They were purified by cellulose column chromatography as follows. *P. aphthosa* (13.7 g), previously extracted with benzene–ethanol, was treated with 80% aqueous methanol under reflux, the extract evaporated to dryness, and the product deionized with mixed-bed resins. The resulting mixture (1.52 g) was fractionated on a cellulose column in 10:1 (v/v) acetone–water which gave arabinitol (0.28 g) and mannitol (0.45 g); 7:1 (v/v) acetone–water gave a disaccharide (R_{Gal} 0.6; 0.11 g) that crystallized and on recrystallization (water–methanol–ethanol) had m.p. 170° and $[\alpha]_D^{25}$ +66° (*c* 0.5, water). Its ^{13}C-n.m.r. spectrum corresponded to that of sucrose. Further elution gave 3-*O*-β-D-glucopyranosyl-D-mannitol (R_{Gal} 0.5; 0.37

g); m.p. 96–99° (water–methanol–ethanol) and $[\alpha]_D^{25}$ −2° (*c* 0.5, water). This material has been previously identified as a component of *P. aphthosa*[5].

A. muehlenbergii (44 g) was subjected to the same extraction procedure giving a mixture of polyols (1.50 g). On elution with 10:1 (v/v) acetone–water, fractions corresponding to arabinitol (0.38 g) and mannitol (0.27 g) were obtained. With eluent having a 7:1 solvent ratio, 2-*O*-β-D-galactofuranosyl-D-arabinitol (R_{Gal} 0.9; 0.27 g) with $[\alpha]_D^{25}$ −62° (*c* 0.5, water) was obtained and identified by comparison of its ^{13}C-n.m.r. spectrum with that of authentic material[6]. Another fraction (R_{Gal} 0.6; 76 mg) was obtained which proved to be sucrose.

Preparation of galactomannans from P. sulcata, S. paschale, Usnea *sp., and* P. aphthosa. — Residual material remaining after benzene–ethanol extraction of lichen (10 g) was treated with 2% aqueous KOH (150 mL) for 1 h at 100°. The mixture was made neutral with acetic acid, centrifuged, and the supernatant solution combined with those obtained after washing of the pellet. Evaporation to a small volume, followed by the addition of the solution to an excess of ethanol, precipitated a polysaccharide. It was isolated and dissolved in water (20 mL), and Fehling solution (20 mL) added. The precipitated Cu complex was filtered off, washed successively with aqueous KOH and methanol, and then shaken with Amberlite IR-120 (H^+) cation-exchange resin. The suspension was filtered, the filtrate evaporated to a small volume, and the polysaccharide isolated by ethanol precipitation.

Preparation of galactomannan from A. muehlenbergii. — The alkaline extract of the lichen, obtained as just described, was dissolved in hot water (15 mL) with the aid of a homogenizer. The mixture was cooled and centrifuged. The supernatant solution was treated with Fehling solution as described earlier, and the insoluble complex isolated and decomposed with resin. On this treatment, the glucan precipitated and was removed with the resin. The filtrate was evaporated to 2 mL, the solution frozen, and gradually thawed overnight in a refrigerator. The insoluble polysaccharide was centrifuged off and the galactomannan isolated from the supernatant solution following precipitation with an excess of ethanol.

Isolation of galactomannan from L. vulpina. — A sample that had been obtained after benzene–ethanol extraction of lichen (3.0 g) was shaken in dimethyl sulfoxide (50 mL) for 45 h. The mixture was filtered through a sintered-glass funnel and washed with additional dimethyl sulfoxide (15 mL). The material was then treated with water (150 mL) for 6 h at 100° and then washed with water. These 2 steps removed β- and α-D-glucan, respectively[7]. Residual lichen was then subjected to alkaline extraction as described earlier to obtain a galactomannan-rich fraction.

Polysaccharide analysis. — The methods used for determination of sugar composition, methylation data, Smith-degradation products, partial-acetolysis products, structure of polysaccharide formed on partial acid hydrolysis and treatment with jack-bean α-D-mannosidase, and ^{13}C-n.m.r. spectra are described in a previous publication[4]. The solvents used in the determination of specific rotations were water for water-soluble polysaccharides and 2% aqueous NaOH for those that were water-insoluble. The concentrations were 0.2%.

RESULTS AND DISCUSSION

Components of lichens soluble in aqueous methanol. — Powdered lichens were first extracted with cold benzene–ethanol, a process that removed 2–3% of soluble material, and then with hot methanol–water to give extracts containing mannitol and arabinitol, according to p.c. and g.l.c.–m.s. of the derived acetates. The amounts present were, respectively: *A. muehlenbergii* (0.6, 1.2%), *P. aphthosa* (2.4, 2.8%), *Usnea* sp. (0.4, 4.0%), *L. vulpina* (0.4, 2.5%), *P. sulcata* (0.3, 2.5%), and *S. paschale* (0.8, 2.5%). In some of these extracts, other components were found. G.l.c.–m.s. of the derived acetates showed that *S. paschale* contained 0.3% of xylitol, and *Usnea* sp. and *L. vulpina* 0.5 and 0.2%, respectively, of an unidentified material having $R_{\text{allitol hexaacetate}}$ 0.93. Paper chromatography of aqueous extracts of *P. aphthosa* and *A. muehlenbergii* showed spots moving slower than mannitol. Components from *A. muehlenbergii* had R_{Gal} 0.9 and 0.6, and cellulose column chromatography provided 2-*O*-β-D-galactofuranosyl-D-arabinitol (umbilicin) (0.68% yield) and sucrose (0.19% yield). *P. aphthosa* extract yielded sucrose (R_{Gal} 0.6) in 0.77% yield and 3-*O*-β-D-glucopyranosyl-D-mannitol (R_{Gal} 0.5)

TABLE I

AMINO ACID COMPOSITION OF SIX LICHEN SPECIES[a]

Amino acid	P. sulcata	S. paschale	P. aphthosa	L. vulpina	A. muehlenbergii	Usnea *sp.*
Tryptophan	0.8	1.2	1.1	1.1	1 3	1.0
Lysine	3.6	3 7	4.3	4.7	5.3	3.4
Histidine	1.8	1.7	1.6	1.9	2.1	1.4
Ammonia	1.2	1.2	1.1	1.3	1.4	1.0
Arginine	3.2	2.5	3.1	3.4	6.1	2.7
Aspartic acid	7.8	7.8	7.8	8.6	7.5	7.3
Threonine	4.6	5.2	5.7	4.9	4.7	4.0
Serine	4.8	4.5	4.8	5.2	4.8	4.3
Glutamic acid	12.0	13.4	12.1	13.6	11.6	11.7
Proline	4.2	4.3	3.6	4.4	3.7	3.8
Glycine	4.6	4.6	3.5	4.7	4.2	4.1
Alanine	5.8	5.6	5.5	6.1	5.7	5.3
Half cystine	1.2	1.1	0.9	1.2	1.0	1.1
Valine	3.6	4.5	4.9	5.7	4.6	4.0
Methionine	1.6	1.6	0.8	1.4	1.7	1.7
Isoleucine	3.2	3.2	3.4	3.6	3 3	3.1
Leucine	5.8	5.1	4.8	6.4	5.9	5.5
Tyrosine	3.8	3.7	7.4	5.2	4.8	3.7
Phenylalanine	3.9	3.4	3.3	4.3	4.1	3 4
Total	77.5	78.3	79.7	87.7	83.8	72.5
Protein (%)	5.87	6.13	19.75	4.00	5.75	7.17
Nitrogen (%)	0.94	0.98	3.06	0.64	0.92	1.15

[a]Molar composition (%).

in 2.73% yield. The last-named compound had been isolated previously from this lichen[5].

Protein composition of lichens. — Based on Kjeldahl, protein-nitrogen values, *P. aphthosa* contained much more protein (19.75%) than the other 5 lichens (4.00–7.17%). As can be seen from Table I, the molar content (in %) of each amino acid is similar, except for high values of arginine (6.1%) in *A. muehlenbergii* and tyrosine (7.4%) in *P. aphthosa*. The proportion of methionine (0.8%) in *P. aphthosa* is one half of that present in the other lichens.

Extraction and characterization of lichen heteropolysaccharides. — The residual material remaining after benzene–ethanol and aqueous methanol extraction of each of the six lichens was subjected to further extraction and fractionation procedures in order to isolate the component galactomannans. These procedures depended on the solubility properties of the galactomannan and its Cu complex and the accompanying glucan(s). The same procedure was used to isolate component heteropolysaccharides of *P. aphthosa, P. sulcata, S. paschale,* and *Usnea* sp.

Isolation, from P. aphthosa, P. sulcata, S. paschale, *and* Usnea *sp. of heteropolysaccharides and preparation of the (1→6)-linked α-*D*-mannopyranan main-chains.* As can be seen from the results reported in Table II, treatment of the lichens with hot water gave little soluble polysaccharide (2–4%), as compared with hot aqueous KOH extraction where 12–34% of extract containing mannose, galactose, and glucose was obtained. Further fractionation of the extract was achieved *via* precipitation of Cu complexes formed with Fehling solution, giving a polysaccharide (2–5% yield) rich in mannose and galactose. The supernatant solutions of the experiments with *P. aphthosa* and *P. sulcata* provided polysaccharides having 83 and 77% of glucose, respectively. These fractions have not yet been further examined.

The polysaccharide isolated *via* the Cu complex was partially hydrolyzed with 0.16M H_2SO_4 for 18 h at 100° to give the polysaccharide core consisting principally of mannose (Table II). (1→6)-Linked α-D-mannopyranans were obtained in the case of *P. sulcata* (Fig. 1E; typical C-1 signal[8] at δ 100.8) and *Usnea* sp. (Fig. 2E; typical H-1 signal[9] at δ 5.39). Products of partial hydrolysis obained from *S. paschale* and *P. aphthosa* galactomannans were branched since the ^{13}C-n.m.r. spectra contained C-1 signals (Figs. 2B and 1B, respectively) corresponding to non-reducing end groups (δ 103.7), and α-D-mannopyranosyl residues[8] substituted at O-6 (δ 100.9) and *O*-2,6 (δ 99.7). Treatment of the polysaccharides with jack-bean α-D-mannosidase removed side chains to give α-D-mannopyranans that were (1→6)-linked according to the main C-1 signal (δ 100.9; Figs. 2C and 1C, respectively).

Chemical structures of D-*galacto*-D-*mannan from* P. aphthosa. Preliminary studies of the galactomannan from *P. aphthosa* showed it to resemble that of *R. usnea*. The ^{13}C-n.m.r. spectra of both polysaccharides contained prominent C-1 signals at δ 104.8 and 101.9 (see Fig. 1A) and other similarities were observed (Table II) in the proportions of mannose, galactose, and glucose (58:39:2), and

TABLE II

SOME PROPERTIES OF POLYSACCHARIDE FRACTIONS OBTAINED FROM SIX LICHEN SPECIES

Lichen	*Yield of hot-water extraction (%)*	*Yield of hot KOH extraction (%)*	*Polysaccharide of Cu complex, obtained from KOH extract (A)*				*Polysaccharide of mother liquor of Cu complex (B)*		*Polysaccharide obtained on partial hydrolysis of B*		
			Man–Gal–Glc	*Yield (%)*	$[\alpha]_D$[a] *(degrees)*	S[b]	*Man–Gal–Glc*	*Yield (%)*	*Man–Gal–Glc*	*Yield (%)*	$[\alpha]_D$[a] *(degrees)*
L. vulpina	17	21	66:24:9	0.1		4.5	41:28:31	21[c]	92:1:7	15	+73
P. aphthosa	4	15	58:39:2	5.2	+60	3.3	43:38:18	1.8	88:0:12	20	+76
S. paschale	2	14	63:31:5	4.3	+63	3.8	9:8:83	2.5	93:0:7	13	+60
P. sulcata	2	12	49:40:10	2.5	+95	3.3	13:10:77	3.1	80:14:6	21	+90
Usnea sp.	4	34	43:42:14	2.0	+112	5.1	23:25:52	22	82:3:15	9	+66
A. muehlenbergii		9	15:7:78	4.7		6.7		[d]			
			62:30:8	0.6	+30	3.65			95:3.2	6	+64

[a]For a solution in water (*c* 0.2) at 25°. [b]For a 0.45% solution in 50mM aqueous solution hydroxide; all peaks showed polydispersity. [c]Obtained by potassium hydroxide extraction, after successive extractions with cold dimethyl sulfoxide and hot water; little insoluble Cu complex was formed. [d]Very low yield.

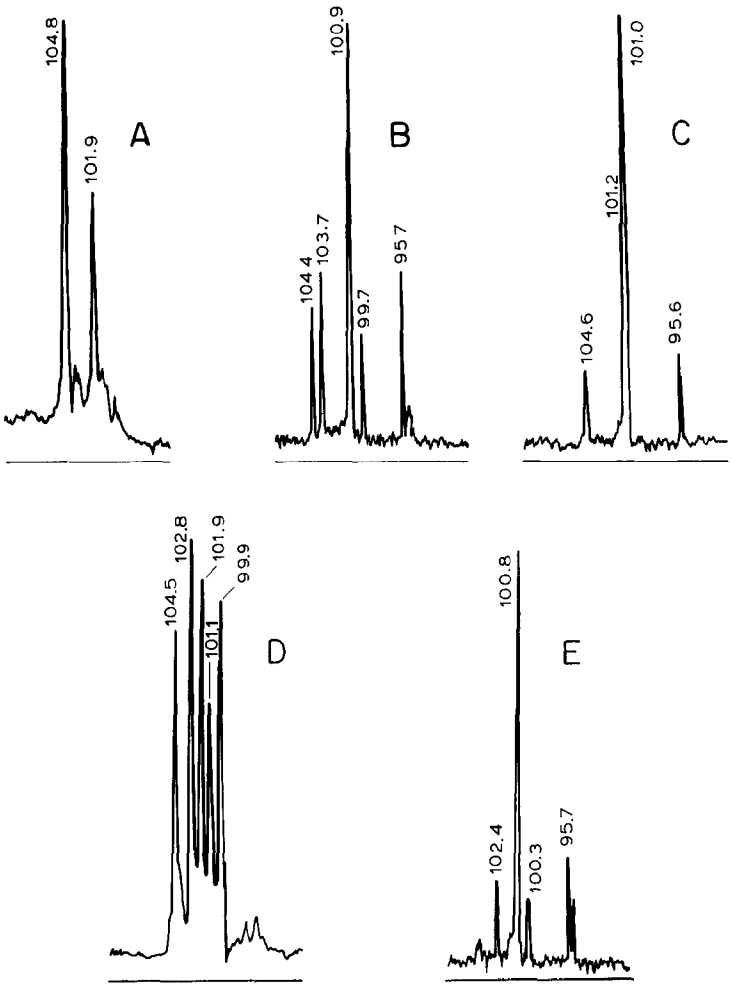


Fig. 1. C-1 portions of ^{13}C-n.m.r. spectra (δ) of polysaccharides of *P. aphthosa* and *P. sulcata*: (A) Polysaccharide of *P. aphthosa* obtained from its insoluble Cu complex; (B) polysaccharide obtained from A by partial hydrolysis; (C) product arising from the action of jack-bean α-D-mannosidase on partially hydrolyzed polysaccharide; (D) polysaccharide of *P. sulcata* obtained from its insoluble Cu complex; and (E) polysaccharide obtained from D by partial hydrolysis.

optical rotation ($[\alpha]_D$ +60°); *R. usnea* has corresponding sugar ratios of 53:43:3 and $[\alpha]_D$ +63°. However, methylation analysis (Table III) revealed nonreducing galactopyranosyl (42%) and mannopyranosyl (8%) end-groups, and mannopyranosyl residues substituted at O-6 (11%), O-4,6 (24%), and O-2,4,6 (5%), with respective proportions for the *R. usnea* galactomannan of 40, 2, 19, 37, and 0%. Galactofuranosyl units (3%) were also present in the latter polysaccharide.

The occurrence of disubstituted units in the (1→6)-linked D-mannopyranan main-chain is a feature not present in the *R. usnea* D-galacto-D-mannan (**1**). This

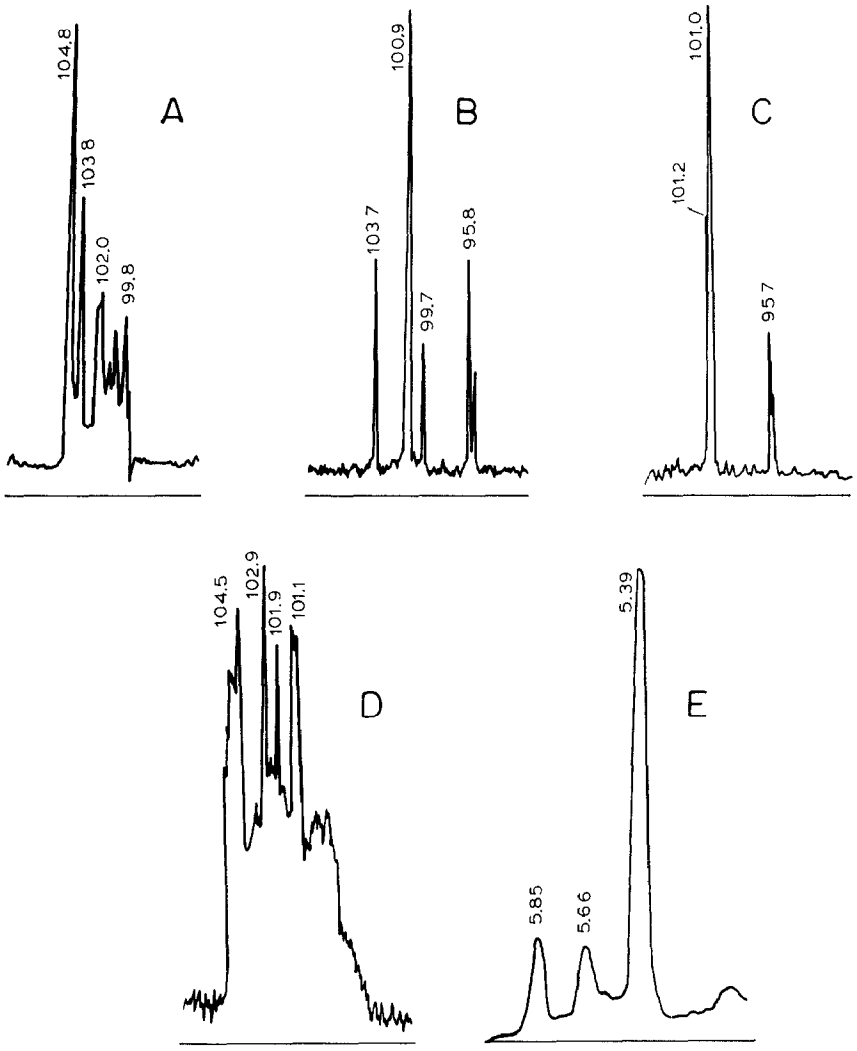


Fig. 2. C-1 portions of ^{13}C- (A–D) and ^{1}H- (E) n.m.r. spectra (δ) of polysaccharide obtained from *S. paschale* and *Usnea* sp.: (A) Polysaccharide of *S. paschale* obtained from its insoluble Cu complex; (B) polysaccharide obtained from A by partial hydrolysis; (C) high-molecular-weight material obtained by partial hydrolysis with jack-bean α-D-mannosidase; (D) polysaccharide of *Usnea* sp. prepared from its insoluble Cu complex; and (E) polysaccharide obtained from D by partial hydrolysis.

was reflected in the formation of 2-*O*-α-D-mannopyranosyl-D-mannose following partial acetolysis, and 1-*O*-α-D-mannopyranosyl-L-glycerol on Smith degradation of the galactomannan. These fragments arose from structure **2** (20%). The major structure **3** (48%) is present along with some unsubstituted main-chain residues (**4**; 6%) not linked, as in structure **2**, to disubstituted residues.

Chemical structure of D-*galacto*-D-*mannan from* P. sulcata. Examination of the C-1 region of the ^{13}C-n.m.r. spectrum of the *P. sulcata* heteropolymer (Fig. 1D)

TABLE III

G.L.C. ANALYSIS OF PARTIALLY *O*-METHYLATED ALDITOL ACETATES OBTAINED FROM METHYLATED POLYSACCHARIDES[a]

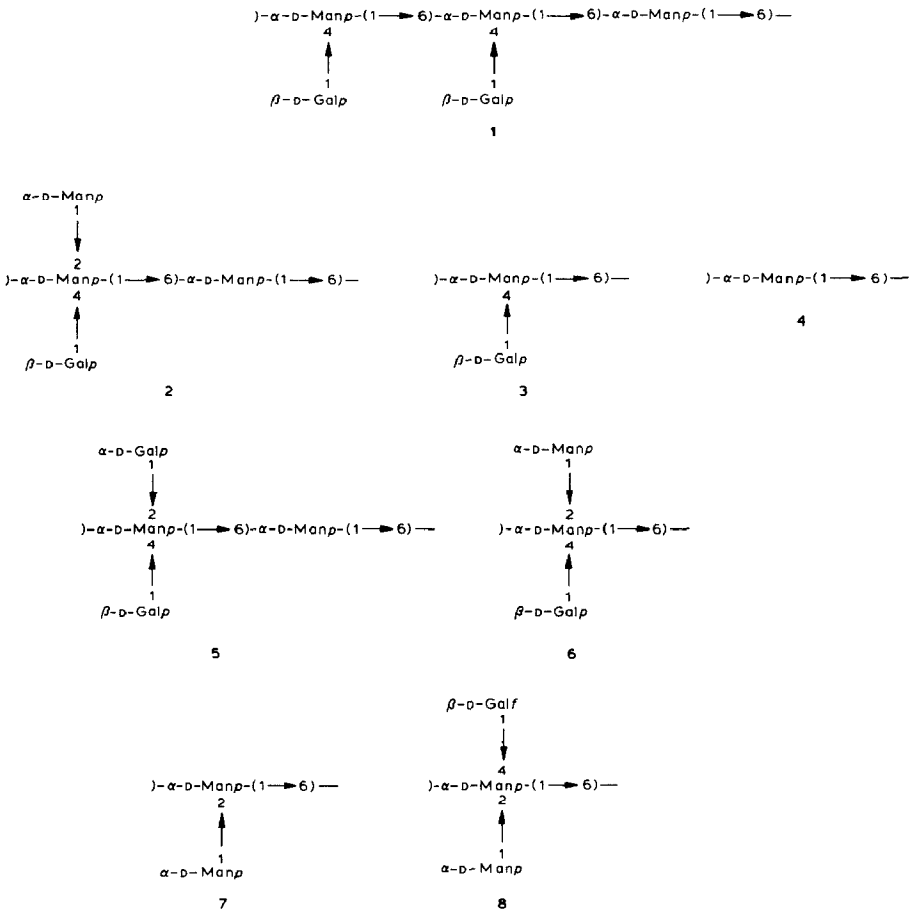
Alditol acetate	P. sulcata	S. paschale	P. aphthosa	L. vulpina	A. muehlenbergii	Usnea *sp.*
2,3,4,6-Me_4-Man	4	20	8	11	33(43)	13
2,3,4,6-Me_4-Glc	4					
2,3,5,6-Me_4-Gal	1			2	18	1
2,3,4,6-Me_4-Gal	34	35	42	21	2(2)	15
3,4,6-Me_3-Man		5			8(4)	
2,4,6-Me_3-Glc	6	3	4	20		21
2,3,6-Me_3-Gal				5		3
2,3,4-Me_3-Man	24		11	12	7(10)	16
2,3,6-Me_3-Glc	2		3	3		1
2,3-Me_2-Man	3	15	24	9	3	9
3,4-Me_2-Man	6	7	2	3	15(39)	5
3-Me-Man[c]	16	15	5	14	15(2)	16

[a]Proportion (%) of each peak relative to total peak area. Prior to methylation, each polysaccharide was isolated *via* the insoluble Cu complex formed with Fehling solution, except that of *L. vulpina*. [b]In parentheses, methylation data of partly hydrolyzed (50mM sulfuric acid, 100°, 3 h) *A. muehlenbergii* galactomannan. [c]Derived from 3-*O*-methylmannose, identified by g.l.c. examination of the derived methyl glycoside acetates[2].

showed it to be identical to that of *Cetraria islandica,* except for the presence of an additional signal at δ 101.1. The ratios of mannose, galactose, and glucose were 49:40:10, resembling that (49:45:6) of *C. islandica*[2]. A marked similarity was also observed on methylation analysis, which showed as principal components nonreducing galactopyranosyl end-groups (34%) and mannopyranosyl units substituted at O-6 (24%) and O-2,4,6 (16%) (Table III). The corresponding values for the *C. islandica* polysaccharide[2] were 36, 19, and 15%. Partial acetolysis of the *P. sulcata* polysaccharide gave 2-*O*-α-D-mannopyranosyl-D-mannose and 2-*O*-α-D-galactopyranosyl-D-mannose. Formation of the latter disaccharide is in agreement with the high optical rotation, $[\alpha]_D^{25}$ +95° (Table II), like that of *C. islandica* (+88°). The formation, on Smith degradation, of 1-*O*-α-D-mannopyranosyl-L-glycerol and the methylation data show that 64% of the *P. sulcata* heteropolymer contains structure **5**, like that of the *C. islandica* D-galacto-D-mannan.

Despite the similarity of the *P. sulcata* polysaccharide to that of *C. islandica, P. sulcata* does not contain significant quantities of D-glucan (lichenan) extractable with hot water and insoluble in cold water[10].

Chemical structure of the D-*galacto*-D-*mannan from* S. paschale. The galactomannan of *S. paschale* contains mannose, galactose, and glucose in a 63:31:5 ratio. The C-1 region of its ^{13}C-n.m.r. spectrum (Fig. 2A) differs from that of other lichen D-galacto-D-mannans previously examined[2,4]. The methylation data (Table III) show that the main structures are nonreducing mannopyranosyl (20%) and galactopyranosyl (35%) end-groups, and mannopyranosyl residues substituted



at O-4,6 (15%) and O-2,4,6 (15%). Partial acetolysis of the galactomannan gave 2-*O*-α-D-mannopyranosyl-D-mannose. These data show that the structure of the galactomannan differs from those of other lichen counterparts previously investigated, having disubstituted units **6**, but lacking adjacent unsubstituted units as in **2**. Structure **3** is also present.

Chemical structure of the galactomannan of Usnea *sp.* The galactomannan of *Usnea* sp. contains mannose (43%), galactose (42%), and a high proportion of glucose (14%). Its specific rotation, $[\alpha]_D^{25}$ +112°, was greater than the highest one previously observed, for the galactomannans of *P. sulcata* and *C. islandica*, namely +95° and +88°, respectively. This was in accord with the formation, by partial acetolysis of the galactomannan of *Usnea* sp. of 2-*O*-α-D-galactopyranosyl-D-mannose, indicating that highly dextrorotary α-D-galactopyranosyl groups were present (as in *C. islandica* and *P. sulcata* galactomannans). Nonreducing mannopyranosyl (13%) and galactopyranosyl (15%) end-groups were present, along with 6- (16%), 4,6-di- (9%), 2,6-di- (5%), and 2,4,6-tri- substituted (16%) manno-

pyranosyl residues (see methylation data, Table III). Although it is not possible to suggest a repeating structure for this polysaccharide because of its complexity (see ^{13}C-n.m.r. spectrum, Fig. 2D), the formation, on Smith degradation, of 1-*O*-α-D-mannopyranosyl-L-glycerol suggested a 2,4-disubstituted main-chain unit (1→6)-linked to an unsubstituted unit. This and other data indicated that the galactomannan is structurally related to those of other lichens.

The formation of 2,4,6- (11%) and 2,3,6-tri-*O*-methylglucitol triacetate (1%) in the methylation analysis (Table III) suggested the presence of a preponderantly (1→3)-linked linear glucan which coprecipitated with the galactomannan on treatment with Fehling solution. This glucan differs from the material extracted, in 12% yield, from lichen with cold dimethyl sulfoxide which contained 3- and 4-substituted β-D-glucopyranosyl units in a 1:3 ratio[7]. The latter glucan could be distinguished from the galactomannan by its sedimentation coefficient of 1.8 *S*, lower than that of 5.1 *S* of the galactomannan (Table II).

Isolation and chemical structure of heteropolysaccharide of L. vulpina. The method used earlier for the isolation of galactomannans, namely, the treatment of the potassium hydroxide extract with Fehling solution to form an insoluble Cu complex was not successful for *L. vulpina*, since little precipitate was formed. Instead, a different isolation method was devised. A glucan having β-D-glucopyranosyl residues substituted at O-3 and O-4 in a 1:3 ratio[7] and 4.5 *S* was extracted with cold dimethyl sulfoxide in 12% yield. Further extraction of the residue with hot water, followed by cooling, provided a precipitate of an α-D-glucan[7] containing (1→3) and (1→4) linkages in a 1.2:1 ratio and having 9.1 *S* (4% yield). The remaining lichen was treated with 2% aqueous potassium hydroxide at 100° to give, in 21% yield, a polysaccharide containing mannose, galactose, and glucose in a 41:28:31 ratio and having 4.5 *S*. Partial acid hydrolysis gave a polysaccharide containing 92% of mannose whose ^{13}C-n.m.r. spectrum (C-1 portion) showed a main signal at δ 100.9 (Fig. 3B) corresponding to a (1→6)-linked α-D-mannopyranan.

According to the ^{13}C-n.m.r. spectrum (Fig. 3A) of the heteropolymer, its chemical structure is complex. Methylation data (Table III) indicated nonreducing mannopyranosyl (11%) and galactopyranosyl (21%) end-groups and mannopyranosyl residues substituted at O-6 (12%), O-4,6 (9%), and O-2,4,6 (14%). Glucopyranosyl residues substituted at O-3 (20%) and O-4 (3%) were also present, apparently arising from an impurity. These limited data are not inconsistent with the types of linkages described earlier as components of lichen galactomannans.

Isolation and chemical structure of D-*galacto*-D-*mannan of* A. muehlenbergii. On extraction with hot potassium hydroxide, *A. muehlenbergii* gave a polysaccharide in 9% yield. The product was largely insoluble in water and only water-soluble material was treated with Fehling solution. The precipitate gave polysaccharides (4.7% yield) containing mannose, galactose, and glucose in 15:7:78 ratio, and being composed of a glucan having 6.7 *S* and a galactomannan having 3.65 *S*. The glucan component could also be extracted from the lichen with cold dimethyl sulfoxide and was characterized as a water-insoluble, (1→6)-linked β-D-

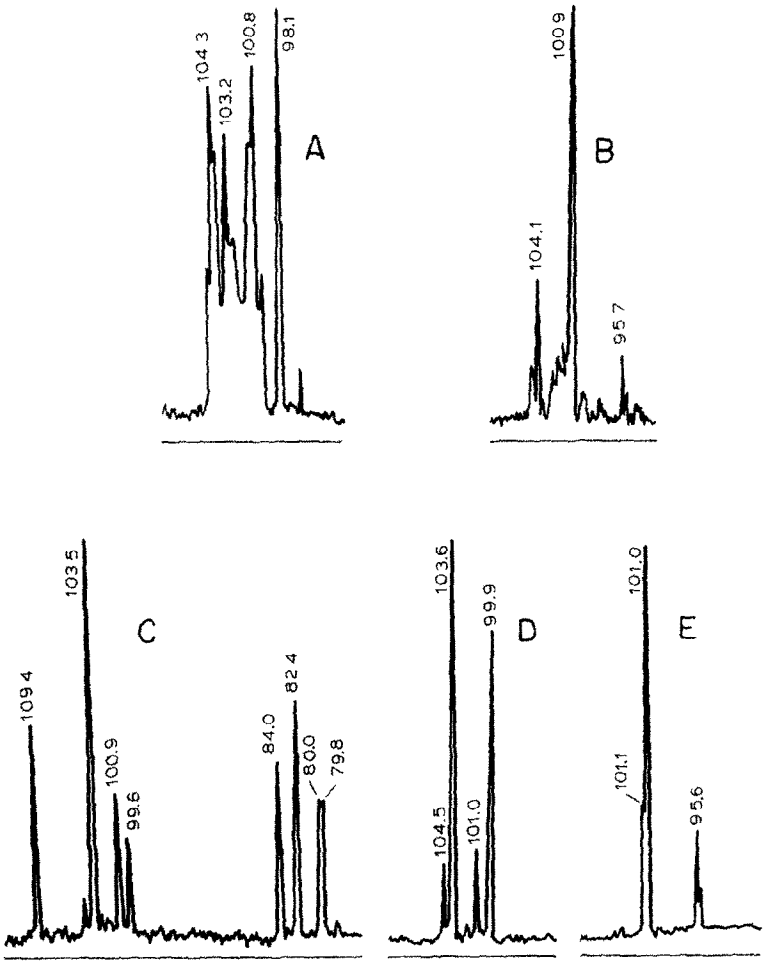


Fig. 3. ^{13}C-n.m.r. spectra (δ) (C-1 portions) of polysaccharides obtained from *L. vulpina* and *A. muehlenbergii*: (A) Hot, aqueous potassium hydroxide extract of *L. vulpina* carried out after successive cold dimethyl sulfoxide and hot-water extractions; (B) polysaccharide obtained from A by partial hydrolysis; (C) water-soluble fraction obtained from Cu complex of *A. muehlenbergii* polysaccharides (including other low-field signals); (D) product arising from mild acid hydrolysis (50mM sulfuric acid, 100°) of C; and (E) high-molecular-weight material prepared from acid-degraded polysaccharide by the action of jack-bean α-D-mannosidase.

glucopyranan (pustulan) in a separate experiment[7].

The component heteropolysaccharide was isolated by solubilisation in water (0.6% yield). It contained mannose, galactose, and glucose in a 31:15:4 ratio and its ^{13}C-n.m.r. spectrum (Fig. 3C) showed a low-field C-1 signal at δ 109.4 typical of β-D-galactofuranosyl groups[11]. These groups, which were present in an unusually high proportion, caused the relatively low specific rotation, $[\alpha]_D^{25}$ of the polysaccharide as a (1→5)-linked β-D-galactofuranan[12] has $[\alpha]_D$ −84°. By successive partial

hydrolysis and enzymolysis with jack bean α-D-mannosidase, the main chain of the heteropolymer was shown to be a (1→6)-linked α-D-mannopyranan (see Fig. 3E). However, the partial hydrolysis step was carried out under conditions (50mM sulfuric acid, 100°, 3 h) milder than those used to remove galactopyranosyl units. Such conditions hydrolyzed only the galactofuranosyl residues, leaving intact the mannan core, which was of further help in elucidating the structure of the heteropolymer.

The ^{13}C-n.m.r. spectrum of the mannan core showed two main C-1 signals at δ 103.6 and 99.9 (Fig. 3D) corresponding to structure **7** with nonreducing α-D-mannopyranosyl end-groups and 2,6-disubstituted α-D-mannopyranosyl residues. A minor signal at δ 101.1 is attributable to unsubstituted, (1→6)-linked main-chain units. In agreement with these findings, methylation analysis (Table III) indicated nonreducing mannopyranosyl end-groups (43%), and mannopyranosyl residues linked at O-6 (10%) and O-2,6 (39%). Since the original heteropolymer contained 15% of 2,4,6-trisubstituted mannopyranosyl residues, it follows that the residues of the chain are substituted with β-D-galactofuranosyl groups (**8**) which were subsequently removed by partial hydrolysis. As shown by the formation of 1-*O*-α-D-mannopyranosyl-L-glycerol on Smith degradation, some of the polysaccharide components represented by structure **8** are linked glycosidically to unsubstituted main-chain units. Thus, the *A. muehlenbergii* galactomannan has a unique chemical structure. Other lichens contain polysaccharides having galactofuranosyl groups[4], but these are present in small proportions in fractions not precipitated by the Fehling solution.

CONCLUSIONS

Recently, it was found that the ascomycetous lichens *C. islandica* (Iceland moss) and *R. usnea* contain D-galacto-D-mannans which can be conveniently purified *via* insoluble Cu complexes formed with Fehling solution[2]. The structures are related, having main-chains of (1→6)-linked α-D-mannopyranosyl residues which are partly substituted with structurally different side-chains. The *C. islandica* polysaccharide is more highly branched with α-D-Gal*p*-(1→2) and β-D-Gal*p*-(1→4) groups linked to the same α-D-mannopyranosyl residues (see structure **5**), whereas that of *R. usnea* contains β-D-Gal*p*-(1→4)-linked groups, as in structure **1**. In early studies, a heteropolymer consisting of galactose, mannose, and glucuronic acid and having a possibly related structure was isolated from *E. prunastri*[1]. The presence of D-galacto-D-mannans in *C. alpestris* (reindeer moss) was suggested by the work of Aspinall *et al.*[3] and, recently, the D-galacto-D-mannan isolated *via* its insoluble Cu complex has been shown to be related to, but structurally different from, those previously investigated. Structurally different D-galacto-D-mannans were also isolated from *Cladonia confusa* whose growth form is similar to that of reindeer moss, and from *Cladonia amaurocraea*[4]. In the course of collaborative studies carried out between the National Research Council of Canada, Saskatoon

(Canada) and The Department of Biochemistry, Federal University of Paraná, Curitiba (Brazil), we have found that ^{13}C-n.m.r. spectroscopy is an excellent investigative tool. The C-1 region of the spectra serves both as an aid in structural determination and as a fingerprint for comparative purposes.

In the present study, as in previous ones[2,4], purification of the D-galacto-D-mannans of *P. aphthosa, P. sulcata, S. paschale,* and *Usnea* sp. was achieved with hot aqueous KOH extraction, followed by fractionation *via* insoluble Cu complexes formed on treatment with Fehling solution. The product obtained in this way from *A. muehlenbergii,* however, contained only a small proportion of galactomannan, as it was contaminated with an excess of glucan whose Cu complex was also insoluble. Fortunately, the glucan component (pustulan) was water insoluble, a property that was utilized in its separation from the water-soluble galactomannan. By contrast, in the case of *L. vulpina,* the Cu complex of the galactomannan was water soluble, and it was necessary to remove the glucan components with successive cold dimethyl sulfoxide and hot-water treatments[7] prior to its preparation *via* hot, aqueous potassium hydroxide extraction.

The experiments presently carried out on six species of ascomycetous lichens confirmed the structural diversity of the component D-galacto-D-mannans. Only in one instance was a structural resemblance between two polysaccharides uncovered, namely between the galactomannans of *P. sulcata* (Fig. 1D) and *C. islandica*[2]. In this case, the ^{13}C-n.m.r. spectra were almost identical, and the similarity was confirmed by methylation analysis, partial acetolysis, Smith degradation, etc. (The overall polysaccharide composition of *P. sulcata* was different, as lichenan could not be separated as a precipitate on cooling of a hot-water extract.) In other cases where resemblances in the spectra were observed, namely between the galactomannans of *P. aphthosa* (Fig. 1A) and those of *R. usnea*[2] and *C. amaurocraea*[4], structural differences became apparent on further investigation.

The structures of the galactomannans and linear α and β-D-glucan components discussed herein differ from those of basidiomycetous *Cora pavonia.* The latter are typical of basidiomycetes, one being a branched heteropolymer having a (1→3)-linked α-D-mannopyranan main chain and β-D-xylopyranosyl side-chains, and the other a branched β-D-glucopyranan having 3-, 6-, and 3,6-disubstituted residues[13].

The ^{13}C-n.m.r. spectra of galactomannans of *Usnea* sp., *S. paschale, L. vulpina,* and *A. muehlenbergii* proved to be different from each other (Figs. 2D, 2A, 3A, 3C, respectively), and from the spectra obtained in previous studies[2,4]. These and other galactomannans currently isolated from *P. aphthosa* contain main chains of (1→6)-linked α-D-mannopyranosyl residues and differences in structure arise from the degree and sequence in which these residues are unsubstituted, or substituted at O-4 or O-2,4 by various side-chains. The better characterized structures are components of galactomannans of the following lichens: *P. aphthosa* (**2**) (20%), **3** (48%), and **4** (6%); *P. sulcata* (**5**); *S. paschale* (**6** and **3**); and *A. muehlenbergii* (**7** and **8**). Structure **8** is of particular interest since it contains β-D-

galactofuranosyl residues, a feature that has not been previously recognized as a major component of lichen galactomannans.

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