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Heteropolysaccharides of the lichen *Evernia prunastri*

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Abstract

Extraction of *Evernia prunastri* with hot aqueous alkali solubilized heteropolysaccharide-containing material which was purified via Fehling precipitation. Further workup of this polysaccharide with Cetavlon gave two fractions having related but different structures. Each structure consisted of a 1 → 6-linked α-D-Man*p* main chain partially monosubstituted at O-2 with side chains of α-D-Gal*p* and partially disubstituted at O-2 and O-4 with α-D-Gal*p* and β-D-Gal*p*, respectively. The fractions differed in that one contained much more uronic acid than the other, with corresponding predominance of side chains containing α-D-Glc*p*A-(1 → 3)-D-Glc*p*, α-D-Gal*p*-(1 → 2)-D-Glc*p*, and β-D-Gal*f* units.

1. Introduction

Mannose-containing heteropolysaccharide are components of the predominant ascomycetous mycobionts of all lichens and their isolation and chemical structures have been recently reviewed [1,2]. These generally have a 1 → 6-linked α-D-Man*p* main chain substituted with various side chains, including α- and β-D-Gal*p*, β-D-Gal*f*, α- and β-D-Glc*p*, and α-D-Man*p* units, most of which are found in the mannose-containing polysaccharides of yeasts [3,4]. These lichen heteropolysaccharides are readily prepared via hot alkaline (or sometimes aqueous) extraction, followed by precipitation with Fehling's solution. Further purification can be achieved by fractionation using Cetavlon, and recently polysaccharides prepared from four lichen species were found to be precipitated at pH 7.0, as well as at pH

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8.5 in the presence of borate, due to the presence of acidic moieties [5]. The precipitation at pH 7.0 was expected in the case of one of the lichens examined, *Evernia prunastri* (L.) Ach., which has been reported to have a galactomannan containing galacturonic acid [6]. However, the presence of a much less acidic fraction precipitated at pH 8.5 was not anticipated. We now report an investigation of the two fractions, particularly because of this unexpected report of units of galacturonic acid, only glucuronic acid having been previously found in fungal heteropolysaccharides [4]. Nevertheless, since the heteropolysaccharide of the alga *Nostoc* contains galacturonic acid units [7] it is possible that these units could be present in a phycobiont component.

2. Results and discussion

E. prunastri was pre-extracted with refluxing 9:1 benzene–EtOH, followed by aq 80% MeOH, to remove nonpolar material and low-molecular weight carbohydrates, respectively. Extraction of the residue with aq KOH at 100°C, followed by neutralization with acetic acid, and treatment of the extract with excess EtOH gave a precipitate of crude polysaccharide (44% yield, based on the weight of lichen extracted). Fractionation of the crude material using Fehling's solution and decationization of the insoluble copper complex gave rise to a polysaccharide (12.5%

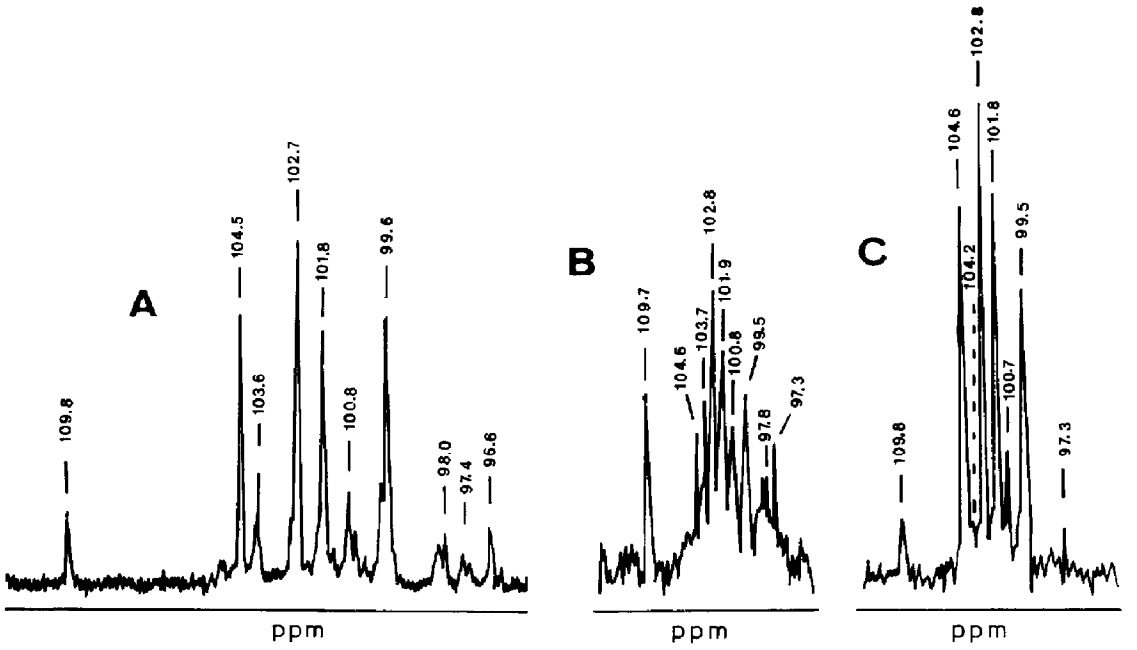


Fig. 1. C-1 portions of ^{13}C NMR spectra of heteropolysaccharides of *E. prunastri* obtained from the Fehling precipitate (A) and by further fractionation with Cetavlon at pH 7.0 (B) and at pH 8.5 in the presence of borate (C).

yield) [5] whose ^{13}C NMR spectrum (Fig. 1A) contained prominent C-1 signals at δ 99.6, 101.8 102.7, and 104.5, and smaller ones at δ 96.6, 97.4, 98.0, 100.8, 103.6, and 109.8, along with a minor carboxyl signal at δ 178.2.

The polysaccharide contained mannose, galactose, and glucose in a 53:42:5 molar ratio [5]. In order to determine the structures of the side chains and to assign the C-1 signals in the ^{13}C NMR spectrum, the polysaccharide was fragmented using partial acetolysis. The resulting mixture of oligosaccharides, which represented the side-chain structures, was chromatographed on a cellulose column. Successively eluted were mannose, glucose (tr.), galactose, and oligosaccharide fractions with R_{Lact} 1.46 (17%), 1.13 (2%), 0.35 (2%), 0.66 (9%), and 0.46 (7% yield) on paper chromatography (PC) in 1-butanol–pyridine–water. The isolated oligosaccharides were characterized as follows.

R_{Lact} 1.46 fraction.—This fraction contained equimolar amounts of galactose and mannose and its ^1H NMR spectrum corresponded to that of α-D-Gal*p*-(1 → 2)-α-D-Man*p* [8].

R_{Lact} 1.13 fraction.—Acid hydrolysis of the amorphous product provided galactose and glucose in a 1.1:1 molar ratio. Methylation in combination with GC–MS analysis gave acetates of 3,4,6-Me_3-glucitol and 2,3,4,6-Me_4-galactitol in a 3:4 molar ratio. Its ^{13}C NMR spectrum contained C-1′ signals at relatively high field (δ 98.0 and 99.3), indicating an α-glycosidic linkage and thus an α-galactopyranosyl-(1 → 2)-α,β-glucose structure. This agreed with its $[\alpha]_D$ of +132° [9].

R_{Lact} 0.66 fraction.—This fraction had $[\alpha]_D$ +78°, and acid hydrolysis gave galactose and mannose in a 2:1 molar ratio. Its ^{13}C NMR spectrum contained C-1 signals at δ 94.1, 102.2, and 104.5, which were assigned to reducing Man*p* residues substituted at O-2 by α-D-Gal*p* [10] and at O-4 by β-D-Gal [11], respectively. Other signals were present at δ 78.2 (O-substituted C-4 of β-D-Gal*p*-(1 → 4)-α-D-Man*p*) [11] and 80.6 (O-substituted C-2 of α-D-Gal*p*-(1 → 2)-α-D-Man*p*) [10]. These data are consistent with the structure α-D-Gal*p*-(1 → 2)-[β-D-Gal*p*-(1 → 4)]-α-D-Man*p*, and agree with the ^1H NMR spectrum, which contained H-1 signals at δ 5.36, *J* 1.7 Hz (H-1); δ 5.16, *J* 3.5 Hz (α-D-Gal*p*); and δ 4.44, *J* 7.6 Hz (β-D-Gal*p*). Further evidence for the proposed structure was obtained by methylation analysis, which gave acetates of 2,3,4,6-Me_4-galactitol and 3,6-Me_2-mannitol in a 2.2:1 molar ratio.

Two other fractions (R_{Lact} 0.35 and 0.46) were obtained which contained uronic acid units as judged by the presence of of low-field ^{13}C NMR signals for C-6 at δ 174.2. However, the spectra were very complex, suggestive of two or more components in each fraction, which were therefore not further analyzed.

In order to isolate a pure aldobiouronic acid the Fehling-precipitated polysaccharide was partially hydrolyzed with acid, and the product was chromatographed on a cellulose column to give the desired fraction with R_{Lact} 0.46 (3% yield). Acid hydrolysis provided glucuronolactone and glucose, and the ^{13}C NMR spectrum contained signals consistent with the mixed α,β anomers of an aldobiouronic acid. A signal at δ 174.2 was assigned to $C_{\alpha\beta}6'$ while other signals could be attributed to the α and β anomers of α-D-Glc*p*A-(1 → 3)-D-Glc. The larger signals, from the β anomer, were at δ 100.4 (C-1′), 99.7 (C-1), and 84.6 (O-substituted C-3), and those

of the α isomer were at δ 100.7 (C-1'), 94.0 (C-1), and 82.3 (O-substituted C-3). In accordance with these data, methylation analysis gave rise to 2,4,6-Me_3-Glc, and hydrolysis of material obtained by lithium aluminum deuteride reduction of the per-*O*-methylated aldobiouronic acid provided 2,3,4-Me_3-Glc-6-$^2H_2$ and 2,4,6-Me_3-Glc (GC–MS of *O*-Me-alditol acetates).

The polysaccharide arising from the Fehling precipitate was further fractionated with Cetavlon and precipitates obtained at pH 7.0 and then at pH 8.5 in the presence of borate were regenerated to give Fractions A (4.5% yield) and B (5.2% yield). Each of their ^{13}C NMR spectra (Figs. 1B and 1C, respectively) contained signals with similar chemical shifts, but in different proportions. Also, the presence of only a very small carboxyl signal (Na salt) at δ 178.2 in the spectrum of Fraction B (complete spectrum) showed that an almost complete resolution of components with and without uronic acid units had been effected.

Fractions A and B were homogeneous on gel filtration using Sepharose 4B-200, with Fraction A having a higher molecular weight. Each fraction was electrophoretically homogeneous in borate buffer.

Fraction A had an equivalent weight by alkali titration of 1650 and $[\alpha]_D$ + 109°, and contained mannose, galactose, and glucose in a 49 : 42 : 9 molar ratio [5], uronic acid (5.1%), phosphorus (3.4%), and no protein. GC–MS examination of a trimethylsilylated methanolyzate established that the uronic acid was glucuronic acid. Methylation analysis showed the presence of fragments corresponding to nonreducing-end units of Glc*p* (6%), Gal*f* (5%) and Gal*p* (20%), and to 6-*O*- (30%), 2,6-di-*O*- (9%), 3,6-di-*O*- (5%), and 2,4,6-tri-*O*-substituted (5%) Man*p*, and 3-*O*-substituted Glc*p* units (9%) [1] (Table 1). Fully *O*-methylated polysaccharide was reduced with lithium aluminum deuteride, and the product was converted into a mixture of *O*-methylalditol acetates. GC–MS analysis showed the presence of fragments corresponding to nonreducing-end units of Gal*p* (32%), and to Man*p* units that were 6-*O*- (33%), 2,6-di-*O*- (10%), and 2,4,6-tri-*O*-substituted (9%). Also detected were acetylated 2,3,4-tri-*O*- (3%) and 2,4-di-*O*-methyl derivatives (3%) of a hexitol doubly labelled with ^2H at C-6 (MS). These alditol acetate peaks had GC retention times corresponding with those of derivatives of glucitol, but not galactitol or mannitol, thus indicating that Glc*p*A may be present as nonreducing and 3-*O*-substituted units.

Nonreducing-end units of Glc*p*A were indicated since they were oxidized by sodium periodate. Fraction A was subjected to successive oxidation with periodate, reduction with sodium borohydride, and strong acid hydrolysis. PC examination did not show the presence of glucuronolactone, and furthermore reduction of the hydrolyzate with sodium borodeuteride, followed by GC–MS examination of the derived acetates, gave a glucitol hexaacetate spectrum without a peak at m/z 141 that corresponded to doubly deuteriated C-6.

Partial acid hydrolysis of Fraction A provided ethanol-insoluble material (28% yield) lacking uronic acid. Methylation analysis showed the presence of nonreduc-

[1] Only *O*-methylated alditol acetates detected at levels of greater that 5 mol% are quoted here.

Table 1
GC–MS analysis of partly *O*-methylated alditol acetates obtained from *E. prunastri* polysaccharides

Alditol acetate [a]	Mole percent formed from *O*-methylated polymer derived from Cetavlon precipitates at the pH's shown		
	pH 7.0 (Fraction A)	pH 7.0 $LiAl^2H_4$-reduced	pH 8.5 (Fraction B)
2,3,4,6-Me_4-Man	1	3	1
2,3,4,6-Me_4-Glc	6	4	
2,3,5,6-Me_4-Gal	5		
2,3,4,6-Me_4-Gal	20	32	33
2,4,6-Me_3-Glc	9		1
2,4,6-Me_3-Gal	2		
2,3,6-Me_3-Gal	4		
2,3,4-Me_3-Man [b]	30	33	36
2,3,4-Me_3-Glc [b]		3	
2,6-Me_2-Gal	1		
4,6-Me_2-Glc	2		
2,3-Me_2-Man	1		3
3,4-Me_2-Man	9	10	17
2,4-Me_2-Man [b]	5	3	
2,4-Me_2-Glc [b]		3	
3-Me-Man	5	9	9

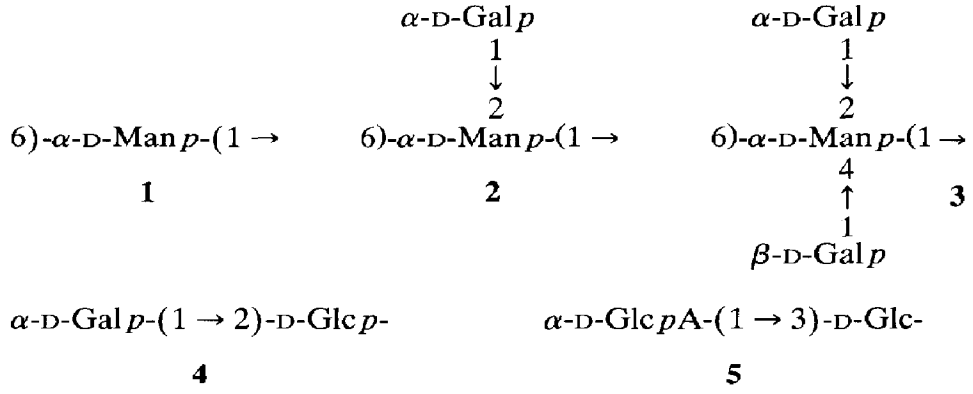
[a] Listed in order of their elution from a capillary column of OV-225.
[b] Retention times compared with that of 1,5-Ac_2-2,3,4,6-Me_4-glucitol: 2,3,4-Me_3-Man, 1.274; 2,3,4-Me_3-Glc-6-$^2H_2$, 1.288; 2,3,4-Me_3-Gal, 1.368; 2,4-Me_2-Man, 1.736; 2,4-Me_2-Glc-6-$^2H_2$, 1.698; and 2,4-Me_2-Gal, 1.807.

ing-end units of Man*p* (4%) and Glc*p* (10%), and 6-*O*-substituted Man*p* residues (86%) corresponding to the main chain. The glycosidic linkages were α since the degraded polysaccharide had $[\alpha]_D + 87°$.

Fraction B had $[\alpha]_D + 102°$, contained mannose, galactose, and glucose in a 51 : 44 : 5 molar ratio [5], phosphorus (2.1%), uronic acid (1.1%), and virtually no protein. Methylation analysis indicated the presence of nonreducing-end units of Gal*p* (33%), and of 6-*O*- (36%), 2,6-di-*O*- (17%), and 2,4,6-tri-*O*-substituted (9%) units of Man*p*. Partial acid hydrolysis of Fraction B gave a polysaccharide ($[\alpha]_D + 78°$; yield 23%) containing mannose, galactose, and glucose in a 83 : 15 : 2 molar ratio. Methylation analysis demonstrated the presence of nonreducing-end units of Gal*p* (11%) and Man*p* (2%), and of 6-*O*- (63%), 4,6-di-*O*- (6%), and 2,6-di-*O*-substituted units (6%) of Man*p*, a result which is consistent with a predominance of $1 \rightarrow 6$-linked α-D-Man*p* units in the main chain.

According to methylation (Table 1) and ^{13}C NMR data (Fig. 1B), Fraction A contained structures in common with Fraction B (Fig. 1C), namely a main chain of $1 \rightarrow 6$-linked α-D-Man*p* units (**1**) partially monosubstituted with side chains of α-D-Gal*p* at O-2 (**2**; δ 102.8) [10] and having a lower proportion of disubstituted residues carrying α-D-Gal*p* (O-2) and β-D-Gal*p* (O-4) units (**3**; δ 104.6) [11]. However, structural differences can be observed between the two fractions. Fraction B has a higher content of nonreducing-end units of Glc*p* (6%) and β-D-Gal*f*

(5%; C-1, δ 109.7), and of Glc*p*A (C-6, δ 178.2), 3-*O*-substituted Glc*p*, and 3,6-di-*O*-substituted Man*p*, in addition to units responsible for a signal at δ 103.7, which were not nonreducing ends of Man*p* [12] (methylation data). Also the ^{13}C NMR spectrum of Fraction A (Fig. 1B) contains a more prominent signal at δ 100.8 than that of Fraction B (Fig. 1C) which arises from side chains containing α-D-Glc*p*A-(1 → 3)-D-Glc*p* groups (**5**). Furthermore, a small high-field C-1 signal at δ 97.8, which probably arises from α-D-Gal*p*-(1 → 2)-Glc*p*- (**4**) is larger in the



Fraction B ^{13}C NMR spectrum (Fig. 1C). The ^{1}H NMR spectrum of Fraction B is of interest since it contained contained small signals at δ 5.63 and 5.69, suggesting 1-phosphodiester linkages as present in the *O*-phosphonomannan of *Hansenula capsulata* [3]. The presence of a phosphodiester is also consistent with a C-1 signal [13] at δ 97.3 (Fig. 1C).

3. Conclusions

The chemical structures deduced for Fractions A and B differ widely from that proposed for the heteropolysaccharide prepared by Mićović et al. [6], which they suggested was a highly branched, acidic galactomannan with a main chain of 1 → 2- and 1 → 6-linked mannosyl units and side chains containing Man*p*, β-Gal*p*, and Gal*p*A units. The presence of 2,6-di-*O*-substituted mannofuranosyl units was inferred from a methylation analysis which gave rise to a fragment incorrectly identified as 3,5-di-*O*-methylmannose, and shown by others to be the 2,4-di-*O*-methyl isomer [14,15]. Mićović et al. also observed a high $[\alpha]_D$ of +120°, but did not consider the probability of α-D-Gal*p* units in addition to those of β-D-Gal*p*, identified by IR spectroscopy. Our present results show a markedly different composition contained in an acidic and a virtually neutral fraction incorporating structures **1** to **5**. The structures α-D-Gal*p*-(1 → 2)-D-Glc*p*- (**4**) and α-D-Glc*p*A-(1 → 3)-D-Glc*p*- (**5**) have not been previously found in lichen heteropolysaccharides.

The mannose-containing heteropolysaccharides of *E. prunastri* prepared via Fehling precipitation resemble those of *Parmotrema cetratum* [16], *P. sulcata* [17],

and *Cetraria islandica* [18] and have ^{13}C NMR spectra similar to those of *Parmotrema araucaria* and an *Usnea* sp. [2]. Such a chemotyping of this group suggests that their mycobionts are related.

4. Experimental

Lichen.—*Evernia prunastri* was collected in the Pacific coast region of the state of Oregon, USA.

Alkali-extracted polysaccharide and its fractionation.—The lichen (21.1 g) was extracted with refluxing 9:1 C_6H_6–EtOH (0.5 L) for 4 h, followed by refluxing 4:1 MeOH–H_2O (0.5 L) for 4 h. The residue (18.5 g) was isolated and treated twice with 2% aq KOH (0.5 L) at 100°C for 1 h, and the combined extract was neutralized (AcOH) and filtered. The filtrate was treated with 3 vol of EtOH, and the resulting precipitate was isolated (yield 9.25 g). The product was dissolved in H_2O (1 L), the solution was frozen, then thawed gradually, and the insoluble material present was centrifuged off. The supernatant was freeze dried, the residual polysaccharide (7.38 g) was dissolved in H_2O (250 mL), Fehling's solution (250 mL) was added, and the resulting precipitate was isolated and treated with an aqueous suspension of Amberlite IR 120 (H^+ form). The resin was filtered off, the filtrate was evaporated to 50 mL and added to EtOH (150 mL), and the resulting precipitate was isolated (yield 2.63 g). A portion (1.0 g) in H_2O (40 mL) was treated with 5% Cetavlon in H_2O (40 mL), the solution was adjusted to pH 7.0, and the precipitate was isolated. It was regenerated by the addition of 4 M NaCl, followed by precipitation with EtOH (160 mL), the process being repeated twice more. The final precipitate was then dialyzed and freeze-dried, giving Fraction A (0.36 g). The supernatant from the Cetavlon precipitation was adjusted to pH 8.5 with aq NaOH treated with 2% borax (80 mL), adjusted to pH 8.5 by addition of 3% boric acid. The resulting precipitate was acidified with aq AcOH, and polysaccharide was precipitated with 4 vol of EtOH, the procedure being repeated twice more. The polysaccharide was dissolved in H_2O (10 mL) and the solution was dialyzed and lyophilized. The yield of Fraction B was 0.42 g.

General methods.—Specific rotations were determined at 25°C using 0.2% aq solutions. Fractions A and B were analyzed for their contents of protein [19], phosphorus [20,21], carbohydrates [22], and uronic acid [23]. ^1H and ^{13}C NMR spectroscopic determinations were carried out with a 300 MHz Bruker spectrometer; chemical shifts are quoted in ppm downfield from the resonance of Me_4Si. The solvent was D_2O for oligosaccharides and D_2O containing 2% NaOH for polysaccharides. Measurements were made at 33°C, and ^{13}C chemical shifts were corrected (+0.6 ppm) to values that would be obtained at 70°C to permit comparison with shifts observed by the authors (P.A.J.G. and M.I) in earlier investigations on lichen polysaccharides.

Homogeneity tests on heteropolysaccharides.—Samples of heteropolysaccharide (10 mg) were applied to columns of Sepharose 4B-200 and eluted with 0.5 M NaCl,

and the eluate was monitored using phenol–H_2SO_4 [22]. The polysaccharides were dyed with Procion Blue and examined electrophoretically, with cellulose acetate as support and 0.05 M borax–NaCl, pH 9.0, as buffer [24].

Partial acetolysis of heteropolysaccharide.—This was carried out using the Fehling-precipitated polysaccharide (500 mg), according to the method of Lee and Ballou [25]. The product was *O*-deacetylated and the resulting mixture examined by PC on Whatman no. 1 paper using 5:3:3 *i*-BuOH–pyridine–H_2O as the developing solvent. The product was chromatographed on a cellulose column using the following acetone–H_2O mixtures as eluants: 10:1 for monosaccharides, 7:1 for disaccharides, and 4:1 and 3:1 for higher oligosaccharides.

Partial acid hydrolysis of heteropolysaccharide to form aldobiouronic acid.—Polysaccharide (0.40 g) obtained via Fehling precipitation was treated with 2 M CF_3CO_2H (2 mL) at 100°C for 6 h, the solution was concentrated to dryness, and the resulting mixture was chromatographed on a cellulose column.

Partial acid hydrolysis of Fractions A and B to form polysaccharide cores.—Polysaccharides (20 mg) were each treated with 0.16 M H_2SO_4 for 18 h at 100°C [26], and the solutions were neutralized ($BaCO_3$), filtered, and concentrated to small volumes. The precipitates which formed on the addition of 4 vol of EtOH were washed with MeOH and dried.

Acid hydrolysis of polyol obtained via periodate oxidation and borohydride reduction.—Polysaccharide (50 mg) obtained via Fehling precipitation was oxidized in H_2O (2 mL) containing $NaIO_4$ (200 mg) for 72 h. Ethylene glycol (0.10 mL) was added and the solution was dialyzed and lyophilized. The product was reduced with NaB^2H_4, the excess reagent was destroyed with AcOH, and the solution was dialyzed, then lyophilized. The resulting polyol was hydrolyzed with 4 M CF_3CO_2H for 3 h at 100°C and the product examined by PC, which showed glycerol, mannose, galactose, and glucose, but no glucuronolactone. GC–MS of derived alditol acetates showed the aldoses to be present in a molar ratio of 72:13:10:5.

Methylation analysis of oligo- and poly-saccharides, and GC–MS.—To oligosaccharide (3 mg) in H_2O (1 mL) was added Me_2SO_4 (0.1 mL) with stirring, and the pH of the solution was immediately monitored and maintained at 7–10 by the addition of 1% NaOH. After 1 h the usual Haworth procedure using 30% NaOH–Me_2SO_4 [27] was adopted, and this was followed by a Kuhn methylation [28].

The polysaccharides were methylated directly with 30% NaOH–Me_2SO_4 and then DMF–MeI–Ag_2O. One half of the fully *O*-methylated polysaccharide, obtained via precipitation of the alkali-extracted lichen polysaccharide with Cetavlon at pH 7.0, was dissolved in dry THF (1 mL) and $LiAl^2H_4$ (5 mg) was added. After 18 h the reagent was destroyed with EtOAc followed by dilute H_2SO_4, and the mixture was partitioned between H_2O and EtOAc. The EtOAc layer was washed thrice with H_2O and evaporated to dryness.

Each fully *O*-methylated product was treated with refluxing MeOH–3% HCl for 3 h and then with M H_2SO_4 at 100°C for 18 h, and the products were converted to mixtures of partially *O*-methylated alditol acetates. These were

examined by GC–MS on a capillary column of OV-225 (30 m × 0.25 mm i.d.), held at 50°C during injection, then programmed at 40°C/min to 220°C, then held.

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