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An unusual juxtaposition of polysaccharide components of *Collema leptosporum*

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Abstract

The lichenised ascomycete, *Collema leptosporum* Malme, was extracted with aqueous methanol to give traces of mannitol and 3-*O*-β-D-glucopyranosyl-D-mannitol (2.7% yield). The residue was consecutively extracted with hot water to give a complex uronic acid-containing polysaccharide, and then with hot aqueous alkali which provided a mixture of polysaccharides. This was fractionated with Cetavlon to give a branched galactomannan, which had the lowest content of galactose yet reported for such a lichen polysaccharide. It has a main chain of (1→6)-linked α-Man*p* units partly substituted at O-2,4 by non-reducing end-units of Man*p* and Gal*p*, shown by NMR spectroscopy to have α- and β-configurations, respectively. The other polysaccharide component was unexpectedly a branched (1→3), (1→6)-linked β-glucan, which is typical of a basidiomycete, whereas those of ascomycetes contain similar linkages but in linear glucans.

Keywords: Lichenised ascomycetes; *Collema leptosporum*; Galactomannan; Branched glucans

1. Introduction

Lichenised ascomycetes generally contain polysaccharides with branched heteromannan structures, such as galactomannans and glucomannans, which are typical of the lichen, having (1→6)-linked α-D-mannan main-chains with galactose-, glucose-, or mannose-containing side-chains. These are accompanied by linear glucans with a variety of glycosidic linkages such as (1→3), (1→4) and (1→6), which have α- and β-glycosidic configurations (Gorin, Baron, & Iacomini, 1988). In the only lichenised basidiomycete examined (*Cora pavonia*, now *Dictyonema glabratum*), the heteromannan has a (1→3)-linked α-mannan side-chain with xylosyl side-chains and a β-glucan that is branched (Iacomini, Zanin, Fontana, Hogge, & Gorin, 1987). These polysaccharide structures are similar to those of free asco- and basidiomycetes, respectively (Barreto-Bergter & Gorin, 1983). We now report an exception to this rule, in the case of the glucan of *Collema leptosporum*.

2. Materials and methods

2.1. Lichen

The lichen was found on the cortex of a palm tree growing on the campus of the Universidade Federal do Mato Grosso do Sul, Campo Grande, State of Mato Grosso do Sul, Brazil. A specimen was deposited in the herbarium of the Instituto de Botânica, São Paulo-SP, Brazil, as SP-263959, I. Divicenzi *leg*.; M. Fleiz e M. Marcelli *det*.

2.2. General methods

Specific rotations were obtained from 1% aqueous solutions of polysaccharides at 25°C. Paper chromatography (PC; Hough & Jones, 1962) was carried out using Whatman no. 1 filter paper (solvent: *n*-BuOH–pyridine–H_2O; 5:3:3) and sugars detected by the acetone–$AgNO_3$ dip (Trevelyan, Procter & Harrison, 1950) and *p*-anisidine hydrochloride spray methods (Hough, Jones, & Wadman, 1950). The homogeneity and molecular weight of polysaccharide preparations were determined by the application of samples of polysaccharide (2.0 mg) in H_2O to columns of Sepharose 4B (31 × 1.6 cm, i.d.), which was eluted with H_2O. The resulting fractions (5 ml) were tested for carbohydrates by the phenol–H_2SO_4 method of Dubois, Gilles, Rebers, and

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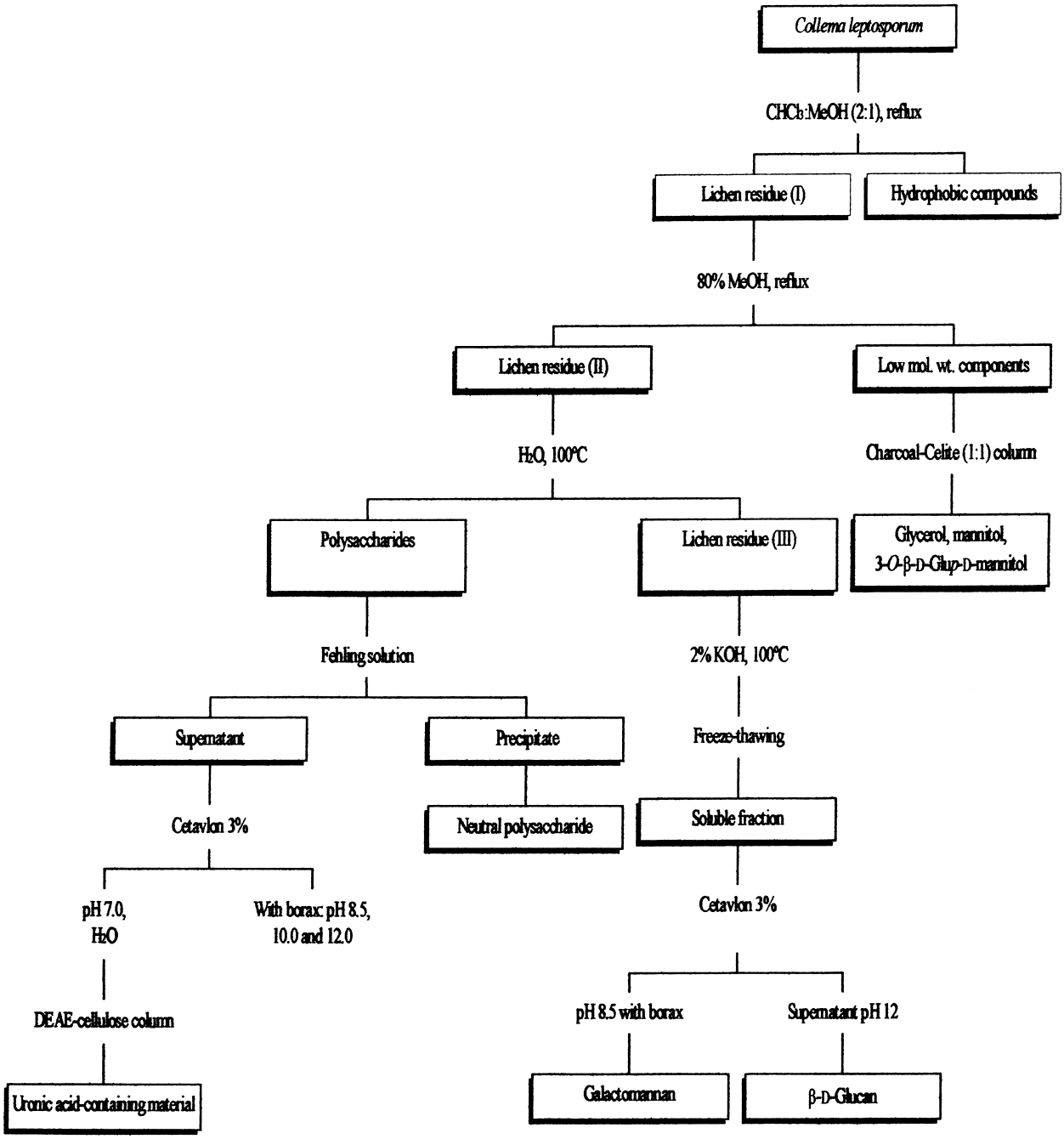


Scheme 1. Isolation of carbohydrate components from *C. leptosporum.*

Smith (1956) and molecular weights determined by comparison with Blue Dextran standards of 4.0×10^4, 7.8×10^4, 1.5×10^5, 5.0×10^5 and 2.0×10^6 Da.

Monosaccharide components of polysaccharides and oligosaccharides were identified and quantitated by the hydrolysis of a sample (2 mg) in 2 M TFA for 8 h at 100°C, followed by evaporation to residues, which were successively reduced with $NaBH_4$ and acetylated in Ac_2O–pyridine (1:1, v/v) at 25°C. The resulting mixtures of alditol acetates were examined by GC–MS, using a capillary column of OV-225 (30 m × 0.25 mm, i.d.); carrier gas, He; temperature programme, 50°C (40°C min^{-1}) → 220°C (constant temperature); the components being identified by their typical retention times and electron impact spectra, and quantitated by their peak area.

2.3. Sequential lichen extraction procedures

This was carried out according to Scheme 1. A manually cleaned, dried and ground sample (40 g) was defatted × 2 in $CHCl_3$–MeOH (300 ml; 2:1, v/v) for 2 h, filtered off and dried. The extract was evaporated to dryness and the

residual lichen then refluxed × 3 in MeOH–H_2O (300 ml; 4:1, v/v) for 3 h, filtered off and dried. The filtrate was evaporated to dryness (2.9% yield).

The residue (32 g) was treated with H_2O (500 ml) for 4 h at 100°C, and the extraction repeated thrice. The combined extracts were evaporated to 50 ml and the polysaccharide (9.8 g) precipitated by addition to EtOH (200 ml).

Remaining residue (20 g) was extracted with 2% aq. KOH (100 ml) for 2 h at 100°C and the process repeated twice. The combined solutions were neutralised with HOAc, and similarly evaporated to a small volume and precipitated with EtOH: yield 4.8 g (12%).

2.4. Characterisation of low molecular weight carbohydrate components

The aq. MeOH extract (5 mg) was acetylated using Ac_2O–pyridine and the product examined by GC–MS (for conditions, see above). Acetates of glycerol and mannitol were identified and quantified, according to their retention times and EI–MS spectra, along with peak areas in the presence of an internal standard of allitol.

The remaining extract was dissolved in H_2O and fractionated on a column of charcoal–Celite (1:1). Glycerol and mannitol were eluted with H_2O, while 30% aq. EtOH eluted 3-*O*-β-D-glucopyranosyl-D-mannitol (2.7%). Hydrolysis gave glucose and mannitol (PC and GC–MS). Its ^{13}C NMR spectrum was identical with that of authentic material (2) with signals at δ 62.6 (C-1′), 64.4 and 64.7 (C-1 and C-6), 71.0, 71.6, 72.0 (2 superimposed), 72.4, 74.9, 77.2, 79.4 (C-3) and 103.3 (C-1′).

2.5. Freeze-thawing of polysaccharide solutions

The polysaccharides obtained by aqueous and aqueous alkaline extractions were each dissolved in H_2O (120 ml), frozen and thawed at 4°C. The precipitates were centrifuged and the solutions retained for further fractionations.

2.6. Fehling precipitation of aqueous extract

The extract was dissolved in water (300 ml) and treated with Fehling solution (300 ml), and the mixture was kept at 4°C for 48 h (Gorin & Spencer, 1970). The precipitated copper complex was centrifuged, the sediment washed with 2% aq. KOH and methanol, and then suspended in H_2O containing mixed ion-exchange resins. The suspension was then filtered off, the filtrate evaporated to a small volume, and the polysaccharide precipitated with excess EtOH.

2.7. Cetavlon fractionation of polysaccharides

This was carried out on polysaccharides isolated via Fehling precipitation and alkaline extraction, according to a combined method of Scott (1965) and Duarte and Jones (1971). Precipitations were carried out successively at pH 7.0 and 8.5, and at pH 12.0, the last two in the presence of borate. The supernatant of the final precipitation was neutralised (HOAc), dialysed, and the polysaccharide isolated after EtOH precipitation.

2.8. Methylation analysis of polysaccharides

The polysaccharides (∽ 50 mg) were successively methylated by the methods of Haworth (1915), Ciucanu and Kerek (1984) and Kuhn, Trischmann, and Löw (1955). The fully methylated polysaccharides were refluxed in 3% MeOH–HCl, hydrolysed with 1 M H_2SO_4 at 100°C for 18 h, and the resulting mixtures of partially methylated aldoses reduced with $NaBH_4$ and acetylated in Ac_2O–pyridine. The partially methylated alditol acetate mixtures were examined by GC–MS with capillary columns of OV-225 and DB-210 (each 30 m × 0.25 mm, i.d.) under the same conditions as described above for alditol acetates.

2.9. Partial acid hydrolysis followed by enzymolysis of galactomannan

The galactomannan (17 mg) in 0.17 M H_2SO_4 (1 ml) was heated at 100°C for 10 h, dialysed to remove the acid and evaporated to a residual polysaccharide (7 mg). This in H_2O (0.5 ml) was treated with 1 drop of *exo*-α-mannosidase from jack bean (Sigma: EC 3.2.1.24; suspension in 3.0 M $(NH_4)_2SO_4$ and 0.1 mM $Zn(OAc)_2$, pH 7.5: 5.0 mg protein ml^{-1}; 19 units mg^{-1} protein: 1 unit hydrolysing 1.0 μmol of *p*-nitrophenyl-α-D-mannopyranoside at pH 4.5 and 25°C). After 6 h, the solution was dialysed, heated at 100°C for 15 min, dialysed again, filtered, lyophilised, and the residue examined by ^1H NMR spectroscopy. The process was repeated with 2 drops of enzyme for 18 h and the final product also examined by ^1H NMR spectroscopy.

2.10. ^{13}C NMR and ^1H NMR spectroscopy

^{13}C NMR, ^1H NMR, DEPT and HMQC spectra were obtained from D_2O solutions at 30°C with a Bruker 400 MHz DRX NMR spectrometer. Chemical shifts are in δ (ppm), relative to that of Me_4Si ($\delta = 0$).

2.11. Controlled Smith degradation of glucan

This was carried out according to the method of Goldstein, Hay, Lewis, and Smith (1965) on glucan (5 mg). The product was not precipitated from H_2O (0.3 ml) with an excess EtOH–acetone (1:1 v/v) and PC (see Section 2.2) gave as oligosaccharides, a main spot with an R_f corresponding to that of 1-*O*-β-D-glucopyranosyl-glycerol and a minor one, which could arise from a glucobiosyl glycerol.

3. Results and discussion

C. leptosporum is a lichenised fungus, which grows on tree trunks in the savannah (*cerrado*) region of Brazil, and which becomes gelatinous on contact with water. It was

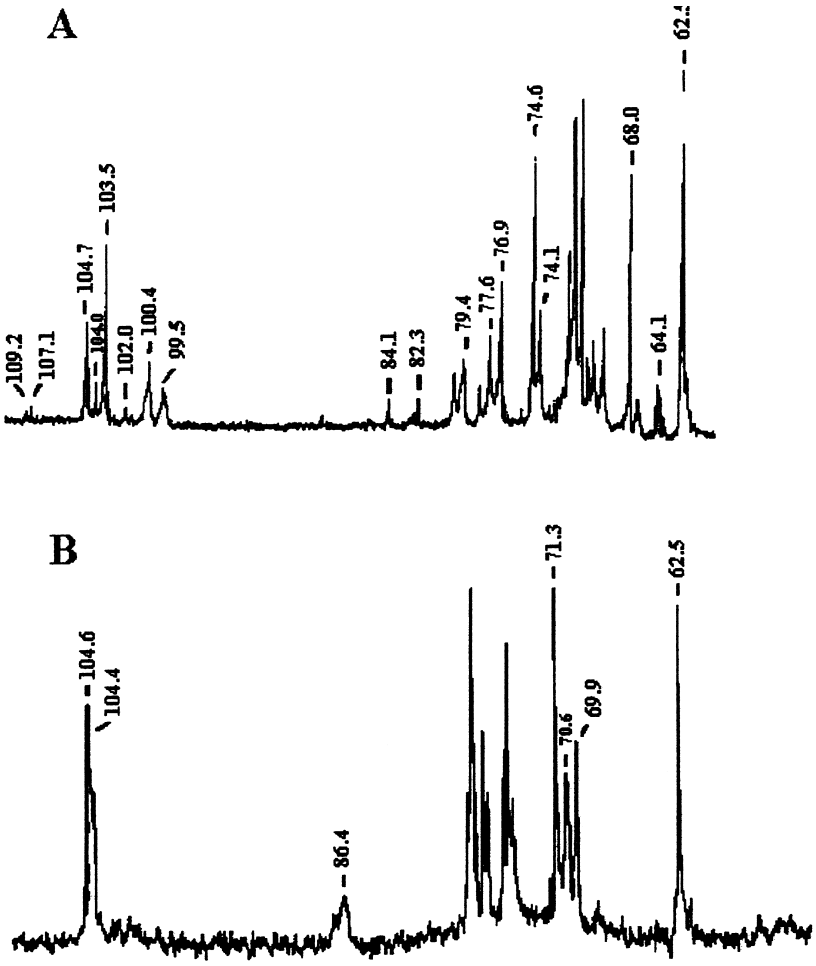


Fig. 1. ^{13}C NMR spectra of: (A) galactomannan; and (B) branched β-glucan.

defatted by an extraction with chloroform:methanol (2:1 v/v) (3.6% yield), followed by 80% aqueous methanol, to give glycerol (0.005%) and mannitol (0.24%), identified by GC–MS of their derived acetates, and 3-*O*-β-D-glucopyranosyl-D-mannitol (2.7%). This disaccharide has been found in *Peltigera aphthosa* (Lindberg, Silvander & Wachtmeister, 1963) and a *Sticta* sp. (Corradi da Silva, Iacomini, Jablonski & Gorin, 1993), and was now characterised by its glucose to mannitol ratio of 1:1 and its typical ^{13}C NMR spectrum.

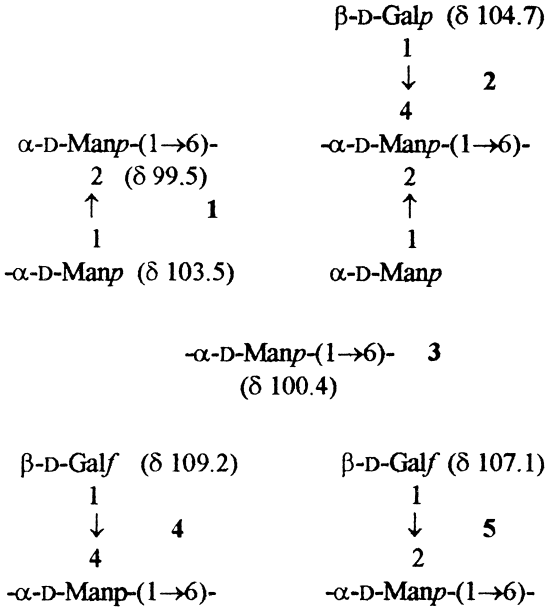
The residual material was extracted with hot water to give soluble polysaccharide (25% yield), which contained rhamnose, arabinose, xylose, mannose, galactose and glucose in a molar ratio of 0.2:1:27:24:22:26, as well as uronic acid and protein (1.2%). Fractionation with Fehling solution gave an insoluble complex, which was regenerated to give a neutral polysaccharide (0.2% yield based on the original lichen), which contained glucose and mannose in a molar ratio of 65:35. A lichen glucomannan has been previously characterised as a component only of *Tornabenia intricata* (Teixeira, Iacomini, McCune, & Gorin, 1992). The supernatant contained polysaccharide (16% yield based on lichen), which was further fractionated with Cetavlon resulting in an almost exclusive precipitation at pH 7.0, typical of an acidic structure. The supernatant, on adjustment to pH 12.0 in the presence of borate, gave rise to 0.7% of a high-glucose fraction.

Fractionation of the pH 7.0 precipitate was effected by DEAE–cellulose column chromatography. Elution with aqueous sodium chloride eluted a predominant uronic acid-containing material, which was not further examined.

Extraction of residual lichen with hot aqueous potassium hydroxide gave polysaccharide (12% yield based on original lichen) containing arabinose, xylose, mannose, galactose and glucose in a 1:16:32:14:37 molar ratio. The material was dissolved in water and the solution submitted to successive freezing and gentle thawing to give a soluble fraction (7.5% yield), having xylose, mannose, galactose and glucose in a 18:31:24:27 ratio. Fractionation with Cetavlon gave virtually no precipitate at pH 7.0, but at pH 8.5 in the presence of borate, a galactomannan was precipitated (1.9% yield), which had a hitherto unreported low proportion of galactose (18%). The pH was adjusted to 12.0 and a soluble fraction isolated (4.8% yield, Man–Gal–Glc ratio, 2:3:94). These two fractions were further examined.

The galactomannan, which had a molecular weight 1.4 ×

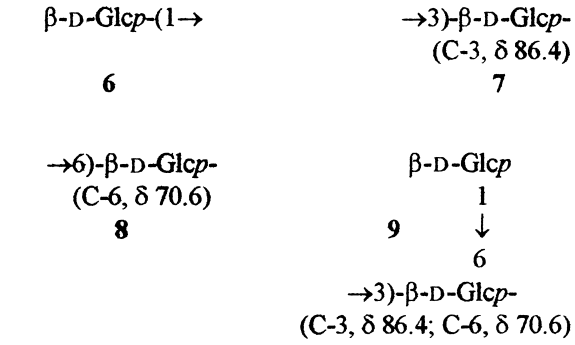
10^5 Da, was submitted to a methylation analysis, and a GC–MS examination of the resulting partly *O*-methylated alditol acetates showed the presence (in mol %) of non-reducing end-units of Man*p* (39%) and Gal*p* (19%) with Man*p* units that were 6-*O*- (12%), 2,6-di-*O*- (11%) and 2,4,6-tri-*O*-substituted (12%), together with a minute amount (0.3%) of non-reducing end-units of Gal*f* units. The $[\alpha]_D$ of the galactomannan was +40°, less than the value of +88° reported for an α-mannan (Haworth, Heath, & Peat, 1941), which suggested the presence of β-D-Gal*p* and/or β-D-Man*p* as well as predominant α-D-Man*p* units. The former possibility was indicated by the ^{13}C NMR spectrum of the galactomannan (Fig. 1(a)), which contained a C-1 signal at δ 104.7, typical of β-Gal*p* units linked (1→4) to a (1→6)-linked α-Man*p* main-chain (**2**; Gorin & Iacomini, 1984). Also, in its ^1H NMR spectrum (not shown) an H-1 doublet appeared at a high field of δ 4.617, typical of a *β*-pyranosyl structure, and its $J_{H\text{-}1,H\text{-}2}$ of 7.8 Hz was characteristic of β-Gal*p* rather than β-Man*p* units whose coupling would be much smaller. Correlation of ^1H and ^{13}C signals of the anomeric region was observed in the HMQC spectrum of the galactomannan (spectrum not shown). Other C-1 signals could be assigned, namely that at δ 103.5, which corresponded to α-Man*p* non-reducing end-units linked (1→2) to those of the same main-chain (**1**; δ 99.5), which were also 4-*O*-substituted by β-Gal*p* units (**2**; Gorin, 1973), while that at δ 100.4 arose from the unsubtituted α-Man*p* units of the main chain (**3**; Gorin, 1973). Very small low-field C-1 signals of β-Gal*f* at δ 109.2 and 107.1, corresponded to units linked (1→4) (**4**) and (1→2) (**5**) to Man*p* units (Gorin, Barreto-Bergter, & da Cruz, 1981).



The galactomannan was partially hydrolysed (Gorin, Spencer, & Eveleigh, 1969) in order to preferentially remove β-D-Gal*p* side-chains to give a polysaccharide core containing mannose and galactose in a 19:1 molar ratio. Its ^1H NMR spectrum contained 6 H-1 signals, and these were correlated in an HMQC experiment to 3 C-1 signals typical of a structure containing mainly **1** and smaller amounts of **3**, with α-Man*p* units substituted at O-6 (δ 100.7; 5.032 and 5.044), 46% combined area), O-2,6 (δ 99.5; 5.184, 5.210 and 5.220), and those of non-reducing ends linked (1→2) to α-Man*p* residues (δ 103.5; 5.182, 5.210 and 5.251) (Gorin & Spencer, 1970; Gorin, 1973). These data were confirmed by the methylation analysis and the formation of corresponding amounts of acetates of 2,3,4,6-Me_4-, 2,3,4-Me_3- and 3,4-Me_2-mannitol.

Treatment of the acid-degraded galactomannan with an α-D-mannosidase (Gorin et al., 1969) from jack bean, which is specific for (1→2)-, (1→3)- and (1→6)-linked α-Man*p* oligosaccharides (Lu, 1967), until no more degradation took place. Exposed α-Man*p* side-chain units were removed to give a polysaccharide whose ^1H NMR spectrum (not shown) contained H-1 signals in a 2:1 ratio at δ 5.032 and a doublet at δ 5.185 and 5.217. The former singlet arose from 6-*O*-linked α-mannopyranosyl units of the main chain in the galactomannan, and suggests that this is the predominant structure of the main chain, any incomplete enzymolysis resulting due to the presence of galactosyl units in the side chains of the acid-degraded polysaccharide.

The glucan-rich fraction not precipitated at pH 12.0 in the presence of borate had a molecular weight 5.7×10^4 Da, and $[\alpha]_D$ 0°. This rotation corresponds to a β-glucosidic configuration, which was confirmed by its ^{13}C NMR spectrum, which contained typical low-field C-1 signals with approximately equal areas at δ 104.4 and 104.6 (Hall & Johnson, 1969; Iacomini et al., 1987).



Methylation analysis showed that non-reducing end- (**6**, 21%), 3-*O*- (**7**, 28%), 6-*O*- (**8**, 27%) and 3,6-di-*O*-substituted (**9**, 23%) Glc*p* units were present. These data correspond to ^{13}C at δ 86.4 (*O*-substituted C-3), 70.6 (*O*-substituted C-6, inverted in DEPT spectrum) and 62.5 (free C-6) (Saitô, Ohki, Takasuka, & Sasaki, 1977). A controlled Smith degradation of the glucan did not give rise to a residual polysaccharide, which would arise from a (1→3)-linked β-D-glucopyranosyl main-chain. Instead, 1-*O*-β-D-glucopyranosyl-glycerol was formed with a trace of

a possible glucobiosyl-glycerol, thus showing that the main chain contains periodate-sensitive (1→6)-linkages.

The presence of this branched glucan in a lichenised ascomycete was surprising, since ascomycetes and lichenised ascomycetes contain linear glucans (Barreto-Bergter & Gorin, 1983). The only glucan yet examined of a lichenised basidiomycete *Cora pavonia* (now named *Dictyonema glabratum*), contains a (1→3), (1→6) branched β-glucan (Iacomini et al., 1987), typical of basidiomycetes (Barreto-Bergter & Gorin, 1983).

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