PERGAMON

Phytochemistry 51 (1999) 395–402

PHYTOCHEMISTRY

Novel structures in galactoglucomannans of the lichens *Cladonia substellata* and *Cladonia ibitipocae*: significance as chemotypes

Sandra M. Woranovicz[a], B. Mario Pinto[b], Philip A.J. Gorin[a], Marcello Iacomini[a,*]

[a]*Departamento de Bioquímica, Universidade Federal do Paraná, C.P. 19046, 81531-990, Curitiba, PR, Brazil*
[b]*Department of Chemistry, Simon Fraser University, Burnaby, BC, Canada V5A 1S6*

Received 27 May 1998; received in revised form 25 August 1998

Abstract

The galactoglucomannans of two species of the lichen genus *Cladonia*, *C. substellata* and *C. ibitipocae*, were compared. They were homogeneous on gel-filtration chromatography and structurally related, having (1 → 6)-linked α-D-mannopyranosyl main-chains, but were substituted in different patterns by α- and β-D-galacto-, β-D-gluco- and α-D-mannopyranosyl groups. The C-1 portions of their ^{13}C-NMR spectra are typical of the lichen species and indicate differences between the two polysaccharides. Partial acetolysis of the galactoglucomannan from *C. substellata* gave rise to oligosaccharides and three were identified, namely α-D-Man*p*-(1→3)-αβ-D-Gal*p*, α-D-Man*p*-(1→2)-αβ-D-Man*p* and α-D-Man*p*-(1→2)-[β-D-Glc*p*-(1→4)]-αβ-D-Man*p*, whereas only the latter two were obtained from that of *C ibitipocae*. Methylation and Smith degradation data confirmed these results. Whereas the mannobiose represents a common structure in lichen heteropolysaccharides, it is the first time that the other oligosaccharides have been isolated from those of lichens.

Keywords: Lichens; *Cladonia* spp; Galactoglucomannans, new structures; Chemotyping

1. Introduction

The use of structurally different mannose-containing polysaccharides for the classification and identification of yeasts (Gorin, & Spencer, 1970) led to the investigation of these polysaccharides isolated from ascomycetous lichens via Fehling precipitation. Their structure, as evidenced by chemical and ^{13}C-NMR studies (Gorin, Baron, & Iacomini, 1988; Gorin, Baron, Silva, Teixeira, & Iacomini, 1993; Teixeira, Iacomini, & Gorin, 1995) proved to be typical of the parent lichen and could thus be utilized in chemotyping studies.

As part of these investigations, we have previously investigated in detail members of the genus *Cladonia*, namely *C. alpestris*, *C. confusa* and *C. amaurocraea* (Iacomini, Schneider, & Gorin, 1985) and in less detail *C. substellata* (Teixeira et al., 1995). Recently (Woranovicz, Gorin, Marcelli, Torri, & Iacomini, 1997), mannose-containing polysaccharides were isolated from *C. signata*, *C. furcata*, *C. imperialis* and *C. clathrata* and as with those of other *Cladonia* species, they contained a main chain of (1 → 6)-linked α-D-Man*p* units either unsubstituted or substituted in various patterns by side chains of β-D-Gal*p*-(1→4)-, α-D-Gal*p*-(1→2)-, and α-D-Man*p*-(1→2)- groups, arranged as mono- or as disubstituents on the same main-chain residue (Iacomini et al., 1985; Gorin et al., 1988; Gorin et al., 1993). These, as in other systems, are the principal heteropolysaccharides in ascomycetous lichens.

We now analyze the galactoglucomannans of *C. substellata* and *C. ibitipocae* and describe some previously undescribed structures. Also of interest was the presence of glucosyl units, since although glucose is present in the vast majority of lichen mannose-containing polysaccharides prepared via Fehling precipitation

* Corresponding author. Fax: +55-41-2662042.

0031-9422/99/$ - see front matter
PII: S0031-9422(99)00005-9

Table 1
Analysis of partially *O*-methylated alditol acetates obtained from *O*-methylated galactoglucomannans[a]

O-Methylalditol acetate	*C. substellata* (% galactoglucomannan[a])	*C. ibitipocae* (% galactoglucomannan[a])
2,3,4,6-Me_4-Man	18	25
2,3,4,6-Me_4-Glc	9	2
2,3,5,6-Me_4-Gal	–	5
2,3,4,6-Me_4-Gal	11	19
3,4,6-Me_3-Man	3	–
2,3,6-Me_3-Man	7	–
2,4,6-Me_3-Gal	9	–
2,3,4-Me_3-Man	2	7
2,3,6-Me_3-Gal	4	–
2,3,6-Me_3-Glc	4	–
2,3-Me_2-Man	9	3
3,4-Me_2-Man	5	7
3-Me-Man	15	27

[a] Percentage of peak area relative to total peak area.

(Gorin et al., 1993), doubts still remain as to whether they are chemically linked or arise from a glucan contaminant. Thus, Lichenan (Karrer, & Joos, 1924) and pustulan (Iacomini, Gorin, Baron, Tulloch, & Mazurek, 1988), which are (1→3), (1→4)-linked and (1→6)-linked β-glucans, respectively are also precipitated. The presence of covalently linked glucosyl units has only been demonstrated in a galactoglucomannan of a *Sticta* sp. (Corradi da Silva, Iacomini, Jablonki, & Gorin, 1993) and a glucomannan of *Tornabenia intricata* (Teixeira, Iacomini, & Gorin, 1992).

2. Results and discussion

2.1. Galactoglucomannan from Cladonia substellata

The heteropolysaccharide obtained via Fehling precipitation contained galactose, mannose, and glucose in a 27:59:12 molar ratio. Chromatography on a column of Sepharose CL-4B indicated homogeneous material with M_r 1.8×10^6.

Methylation analysis showed a complex structure with formation of 12 *O*-methylalditol acetates (Table 1) in significant proportions, which indicated a structure with similarities to those of other heteropolysaccharides isolated from lichens of the genus *Cladonia* (Iacomini et al., 1985; Woranovicz et al., 1997) [this complexity was reflected in its ^{13}C-NMR spectrum, which contains many signals in the C-1 region (Fig. 1A)]. These corresponded, according to methylation data (Table 1), to nonreducing end-groups of Man*p* (18%), Gal*p* (11%) and Glc*p* (9%), whose total percentage corresponds to components of the Man*p* core, which contained 2-*O*- (3%), 4-*O*- (7%), 6-*O*- (2%), 4,6-di-*O*- (9%), 2,6-di-*O*- (5%) and 2,4,6-tri-*O*-substituted (15%) units. 3-*O*- (9%) and 4-*O*-substituted Gal*p* units (4%) were also present, along with those of 4-*O*-substituted Glc*p* (4%).

These structures and percentage values agreed with those obtained on Smith degradation of the heteropolysaccharide. It gave rise to a high content of glycerol (33%), but lower than in other species of the genus *Cladonia* (Iacomini et al., 1985; Woranovicz et al., 1997), this component arising mainly from nonreducing end-groups of Man*p*, Gal*p*, and Glc*p*, but also from 6-*O*- and 2,6-di-*O*-substituted Man*p* units. The high proportion of erythritol liberated in this case arose due to Man*p* and Glc*p* units substituted at O-4 (16%), whereas a lesser content of threitol (3%) was formed from similarly substituted Gal*p* residues. The detection of mannitol acetate (22%) was consistent with the presence of 2,4,6-tri-*O*-substituted Man*p* units. The high content of galactitol acetate (20%) was also consistent with periodate-resistant Gal*p* units substituted at O-3.

The ^{13}C-NMR spectrum of the galactoglucomannan (Fig. 1A), contained predominantly C-1 signals, that indicated a branched structure with nonreducing end-groups of β-D-Gal*p* (δ 104.7) linked (1→4)- and those of α-D-Man*p* (δ 103.5) linked (1→2) to α-D-Man*p* units, along with 6-*O*- (δ 100.5) and 2,6-di-*O*-substituted (δ 99.6) units of α-D-Man*p* from the core (Gorin, 1973).

Its specific rotation (+63°) would be expected from α-D-Man*p* units (+89°), diminished by the presence of those of β-D-Gal*p* (Haworth, Hirst, & Isherwood, 1937).

Partial acetolysis of the galactoglucomannan gave rise to a mixture of at least 6 acetylated products, according to its HPLC reversed phase profile. These were RP-1 of solvent (retention time, 1.3 min), RP-2 (1.7 min, 7% of total area), RP-3 (2.5 min, 28%), RP-4 (3.7 min, 34%), RP-5 (4.9 min, 6%), RP-6 (6.25 min, 13%) and RP-7 (8.0 min, 12%). The RP-4 com-

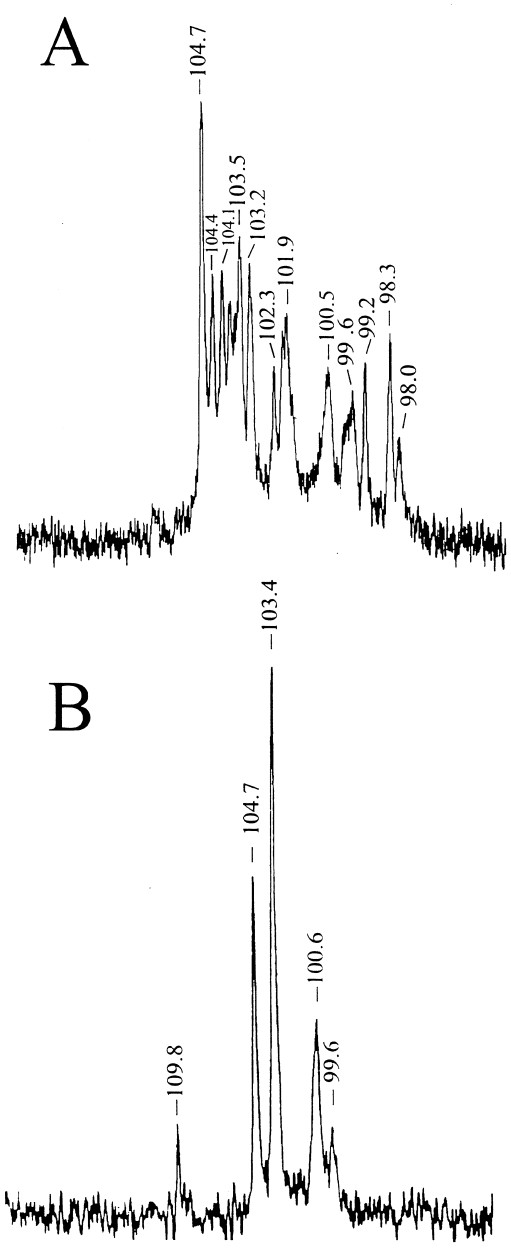


Fig. 1. C-1 region of ^{13}C-NMR spectra of galactoglucomannans of *C. substellata* (A) and *C. ibitipocae* (B).

ponent was isolated and its ^{1}H and H,H COSY NMR spectra suggested the presence of a mixture of acetylated α-D-Man*p*-(1→2)-αβ-D-Man and α-D-Man*p*-(1→3)-αβ-D-Gal.

This fraction was deacetylated and its normal phase HPLC profile showed 2 components, namely NP-1, the solvent peak (retention time, 1.7 min), NP-2 (6.6 min, 34%), and NP-3 (8.2 min, 66%). The NP-2 component was isolated and its ^{1}H-NMR spectrum showed 2 main H-1 signals, which corresponded to those of an authentic sample of α-D-Man*p*-(1→2)-α-D-Man*p* at δ 5.02 (H-1′; d, $J_{1,2}$ 1.2 Hz) and 5.36 (H-1; d, $J_{1,2}$ 1.6 Hz). Its HMQC spectrum showed C-1 signals corresponding to α-D-mannopyranose at δ 92.8 and 102.2 for the reducing and non-reducing ring, respectively.

The ring proton signals were traced via COSY crosspeaks starting from each anomeric signal and were confirmed by the TOCSY spectrum. The NOESY spectrum of the disaccharide showed strong inter-residue contacts between H-1′ and H-2, confirming the structure (**1**)

α-D-Man*p*-(1→2)-αβ-D-Man*p*

1

α-D-Man*p*-(1→3)-αβ-D-Gal*p*

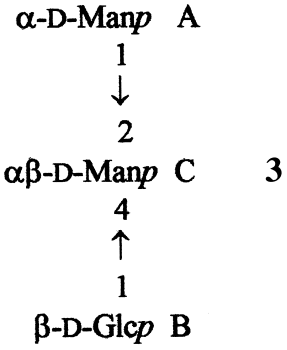
2

The NP-3 component had $[\alpha]_D$ +24° and gave rise to mannose and galactose on acid hydrolysis. It proved to be a disaccharide with 4 H-1 NMR signals corresponding to structure **2**. These were at δ 5.03 (d, $J_{1,2}$ 1.6 Hz; α-D-Man*p*-(1→3)-α-D-Gal*p*), 5.02 (d, $J_{1,2}$ 1.7 Hz; α-D-Man*p*-(1→3)-β-D-Gal*p*), 5.26 (d, $J_{1,2}$ 3.9 Hz; α-D-Man*p*-(1→3)-α-D-Gal*p*), and 4.60 (d, $J_{1,2}$ 7.9 Hz; α-D-Man*p*-(1→3)-β-D-Gal*p*). Its HMQC spectrum contained, for each C-1 nucleus, signals at δ 95.5, 95.8, 92.6 and 96.7 and its COSY, TOCSY, and NOESY spectra confirmed an α-D-Man*p*-(1→3)-αβ-D-Gal*p* structure (**2**). All signals were assigned, as were those of the acetylated derivative (see Section 4).

The ^{1}H-NMR spectrum of the non-purified acetylated sample (RP-4) contained signals for two H-1$_\alpha$s at δ 6.24 (d, $J_{1,2}$ 2.1 Hz) and δ 6.40 (d, $J_{1,2}$ 3.7 Hz) and a single H-1$_\beta$ at δ 5.64 (d, $J_{1,2}$ 8.01 Hz) and two H-1s at δ 4.94 (d, $J_{1,2}$ 1.8 Hz) and δ 5.02 (d, $J_{1,2}$ 1.6 Hz). COSY data obtained from this fraction at 25°C gave unambiguous assignments of H-1 signals, suggesting the presence of the two previously described disaccharides (see Section 4 for complete NMR data). The linkage for acetylated **2** was readily assigned from its COSY contour map, since the H-3 signal of β-Gal*p* did not appear at low field. In the acetylated Gal*p*, this resonance occurs at δ 5.08. Thus, as the H-2 signal of the acetylated Gal*p* unit in **2** gave crosspeaks with H-3 at δ 3.98, the linkage is 1→3.

The component of RP-6 was deacetylated providing material with $[\alpha]_D$ +3° and which gave mannose and glucose in a 2:1 molar ratio on acid hydrolysis. Its ^{1}H-NMR spectrum corresponded to a trisaccharide structure with a Man*p* reducing end, having three H-1 signals of approximately equal area at δ 4.55 (d, $J_{1,2}$ 8.0 Hz), 5.10 (d, $J_{1,2}$ 1.6 Hz), and 5.42 (d, $J_{1,2}$ 1.8 Hz). These signals were assigned to the corresponding trisaccharide with a β-Man*p* reducing residue. Assignments for all proton signals are presented in Table 2. They are based on the crosspeaks observed in the H,H COSY spectrum and the observed chemical shifts and coupling constants. It followed that there

were two units of α-Man*p* and one of β-Glc*p*. The sequence of these units was determined by a NOESY spectrum, which indicated that H-1 of the α-Man*p* nonreducing end (A) produced an inter-residue NOE at H-2 of the α-Man*p* reducing end (C) and the H-1 of β-Glc*p* nonreducing end (B) gave rise to enhancement at H-4 of C. This is consistent with the branched structure **3**.



In order to obtain ^{13}C signals, an HMQC analysis yielded the following chemical shifts for the C-1 residues: α-Man*p* reducing end, δ 92.7; α-Man*p* nonreducing end, δ 102.4; and β-Glc*p* nonreducing end, δ 102.9.

2.2. *Galactoglucomannan from* Cladonia ibitipocae

The polysaccharide contained galactose, mannose, and glucose in a 35:60:2 molar ratio; elution from a column of Sepharose CL-4B indicated a homogeneous preparation with M_r 2.0×10^6.

The polysaccharide was further analyzed using the protocol described above. Methylation analysis showed the presence of nonreducing end-units Man*p* (25%), Gal*p* (19%), Gal*f* (5%), and Glc*p* (2%) and 6-*O*- (7%), 4,6- (3%) and 2,6-di-*O*- (7%), and 2,4,6-tri-*O*-substituted Man*p* units (27%) of the core (Table 1). A Smith degradation of the galactoglucomannan gave rise to glycerol (44%) from nonreducing end-units and mannitol (44%), consistent with these data (for explanation, see above).

Its ^{13}C-NMR spectrum (Fig. 1B) showed C-1 signals at δ 99.6, 100.6, 103.4, and 104.7 that were also present in the spectrum of the *C. substellata* galactoglucomannan but was much less complex. A lower intensity, additional small signal at δ 109.8 was attributed to β-D-Gal*f* units, the chemical shift indicating a (1 → 6)-linkage to α-D-Man*p* units (Gorin, Barreto-Bergter, & Cruz, 1981). The specific rotation of $+52^\circ$ is consistent with a mixture of β-D-Gal*p* and α-D-Man*p* structures.

The galactoglucomannan was submitted to partial acetolysis and aliquots were examined by reverse-phase HPLC after 48, 96 and 144 h. The components had identical retention times for each reaction time, but the disaccharide yield was the greatest after 144 h. All peaks had retention times on HPLC and R_fs on TLC corresponding to those obtained from the *C. substellata* heteropolysaccharide. However, further examination showed that the component equivalent to RP-4 contained only acetylated α-D-Man*p*-(1 → 2)-αβ-D-Man*p* and not the acetylated α-D-Man*p*-(1 → 3)-αβ-D-Gal, as indicated by its ^1H-NMR (Ogawa, & Yamamoto, 1982), H,H COSY and NOESY spectra; the NOESY spectrum indicated a close contact between H-1′ and H-2 (see Section 4). The product was stable to the acetolysis conditions as its peak intensity did not diminish after 144 h.

An acetylated trisaccharide, which corresponded to RP-6, was isolated and its ^1H-NMR spectrum contained H-1 signals at δ 6.13 (d, $J_{1,2}$ 2.1 Hz), 4.92 (d,

Table 2
^1H-NMR data for trisaccharide α-D-mannopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-D-mannose and its acetylated derivative

Ring[a]	Chemical shifts (δ, PPM) and coupling constants (Hz)					
	H-1	H-2	H-3	H-4	H-5	H-6
Free oligo.[c]						
A	5.10, $J_{1,2}$ 1.6	4.13, $J_{2,3}$ 3.5	3.90, $J_{3,4}$ 9.7	3.68, $J_{4,5}$ 9.6	3.80, $J_{5,6}$ 4.9	3.92, $J_{6,6'}$ 12.6
B	4.55, $J_{1,2}$ 8.0	3.35, $J_{2,3}$ 8.8	3.55, $J_{3,4}$ 9.4	3.45, $J_{4,5}$ 9.8	3.88, $J_{5,6}$ 2.9	3.78, $J_{6,6'}$ 12.7
C	5.42, $J_{1,2}$ 1.8	4.05, $J_{2,3}$ 3.4	4.11, $J_{3,4}$ 8.7	3.95, $J_{4,5}$ 10.2	3.78, $J_{5,6}$ 4.9	3.98, $J_{6,6'}$ n.d.
Ac Deriv.[b,d]						
A	4.92, $J_{1,2}$ 1.8	5.37, $J_{2,3}$ 2.8	$J_{3,4}$ 5.29	5.32, $J_{4,5}$	4.13, $J_{5,6}$ 4.3 and 2.0	4.17–4.21, $J_{6,6'}$ 12.4
B	4.59, $J_{1,2}$ 8.1	4.95, $J_{2,3}$ 9.0	5.16, $J_{3,4}$ 9.5	5.11, $J_{4,5}$ 9.6	3.76, $J_{5,6}$ 3.1 and 1.8	4.08, 4.48, $J_{6,6'}$ 12.7
C	6.13, $J_{1,2}$ 2.1	3.99, $J_{2,3}$ 2.6	5.30, $J_{3,4}$	4.13, $J_{4,5}$ 9.4	3.91, $J_{5,6}$ 2.1 and 3.7	4.15, 4.45, $J_{6,6'}$ 12.4

[a] Rings A (nonreducing Man), B (nonreducing Glc), and C (reducing Man) correspond to those in structure **3**, solvent D_2O.
[b] Rings of acetylated derivative of the trisaccharide, solvent $CDCl_3$.
[c] For the free trisaccharide, there were interglycosidic NOEs between H-1 of A and H-2 of C, and H-1 of B and H-4 of C.
[d] For the acetylated trisaccharide, there were interglycosidic NOEs between H-1 of A and H-2 of C, and H-1 of B and H-4 of C.

$J_{1,2}$ 1.8 Hz), and 4.59 ($J_{1,2}$ 8.1 Hz). Other chemical shifts and coupling constants (Table 2) were determined from H,H COSY and TOCSY spectra, in which H-4 signals corresponded to nonreducing and reducing α-Man*p* units. Thus, the H-3 signals of both units had similar δ values (5.29 and 5.30) and correlation of H-2 with H-5 of the reducing unit was clear. The COSY spectrum was then used to identify the H-4 signal of the reducing α-Man*p* unit from the H-5/H-4 correlation. It follows that the Man*p* unit has *O*-substitution at C-2 and C-4 because its H-2 and H-4 signals appear at high field (δ 3.99 and 4.13 respectively), as there are no acetyl groups in these positions. The linkages in the acetylated trisaccharide were confirmed by a NOESY spectrum, which showed main interglycosidic NOEs between H-1 of the Man*p* nonreducing and H-2 of the Man*p* reducing residue and also between H-1 of the Glc*p* nonreducing residue and H-4 of the Man*p* reducing residue.

3. Conclusions

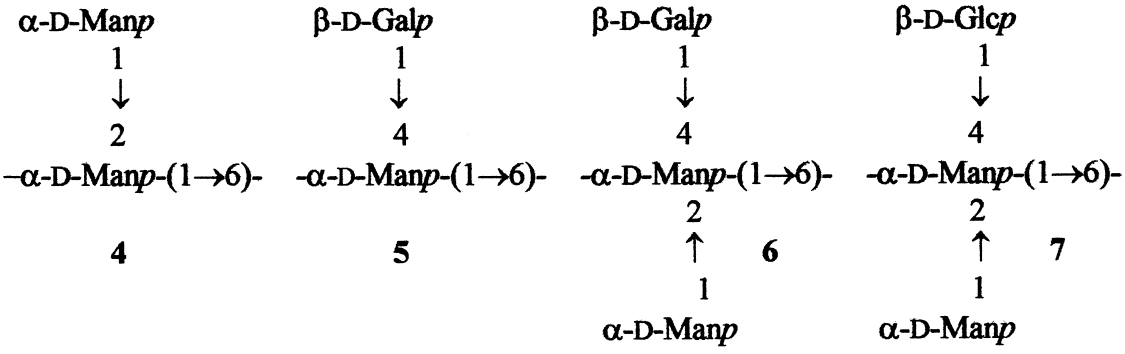
The data show that glucose is a component of the heteropolysaccharide isolated from each *Cladonia* species, although we do not yet know if this is a common factor in previously examined *Cladonia* species.

From the point of view of chemotyping, there are many similarities between the *C. substellata* and *C. ibitipocae* galactomannans although the former is much more complex, as evidenced by ^{13}C-NMR data. The glucogalactomannans are highly branched and contain, as main structural features, a (1 → 6)-linked α-D-Man*p* main-chain substituted predominantly at O-2 with α-D-Man*p* (**4**, C-1 signals at δ 103.4, 103.5), and at O-4 with β-D-Gal*p* (**5**; C-1 signals at δ 104.7). The possibility of α-D-Gal*p* units linked (1→2) to the main chain exists in the *C. substellata* galactoglucomannan, by virtue of the C-1 signal at δ 103.2. The presence of O-2,4 disubstituents in the galactoglucomannans of *C. substellata and C. ibitipocae* was shown by studies of methylation (15 and 27%, respectively) and Smith degradation (22 and 44%, respectively) (Table 1). In each case, these values are high compared with the glucose content of the galactoglucomannans (12 and 2%, respectively), which suggests the presence of respective O-2 and O-4 substituents of α-D-Man*p* and β-D-Gal*p* respectively (**6**), as well as those of α-D-Man*p* and β-D-Glc*p* (**7**) (it appears that β-Glc*p*-(1 → 4)-α-Man*p* linkages are much more stable to acetolysis than those of β-Gal*p*-(1 → 4)-α-Man*p*, as evidenced by the absence of a galactose-containing trisaccharide analogue). The methylation data and the complexity of the ^{13}C-NMR spectrum of the galactoglucomannan of *C. substellata* (Fig. 1A) suggest a much more complex structure than that present in *C. ibitipocae*. Also, the polysaccharide of the latter species does not contain a side chain with α-D-Man-(1 → 3)-D-Gal*p* units (**3**), although only it contains β-D-Gal*f* units, albeit in small amounts. However, each polysaccharide contains the trisaccharide structure **7**.

4. Experimental

4.1. General methods

Specific rotations were obtained with 1% aq. solutions using a Rudolph Research Autopol II automatic polarimeter, except in the case of the oligosaccharides where the concn. was 0.1%. Gas liquid chromatography-mass spectrometry (GC-MS) was performed with a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810 R-12 mass spectrometer, using He as carrier gas. Capillary columns of OV-225 (a) (30 m × 0.25 mm i.d.) and DB-225 (b) (15 m × 0.25 mm i.d.) were used. The former was held at 50°C during injection and then programmed at 40°C/min to 220°C (constant temperature). The injection temperature of the latter was 50°C with a program to 220°C (constant temperature). Columns (a) and (b) were used for quantitative analysis of alditol acetates and partially *O*-methylated alditol acetates (Sawardeker, Sloneker, & Jeanes, 1965). Descending paper chromatography (PC) (Hough, &



Jones, 1962) was carried out on Whatman No. 1 filter paper (solvent: *n*-BuOH–pyridine–H_2O, 5:3:3), sugars being detected by the acetone–$AgNO_3$ dip reagent (Trevelyan, Procter, & Harrison, 1950). Thin layer chromatography (TLC) was performed on aluminum plates precoated with Silica gel 60F-254 (solvents: EtOAc–MeOH–H_2O, 4:2:1 for polar samples and EtOAc–hexane, 1:1 for non polar samples), which were sprayed with a mixture of 1% ceric sulphate and 1.5% molybdic acid in 10% aq. H_2SO_4 and heated at 100°C. Acetylation of alditols was carried out with Ac_2O–pyridine (1:1) for 12 h at room temperature (Wolfrom, & Thompson, 1963a).

4.2. Preparation of galactoglucomannans

Lichen samples (50 g), previously extracted successively with $CHCl_3$–MeOH (2:1 v/v; 250 ml) (Machado et al., 1994) and 80% aq. MeOH (250 ml), were treated with 2% KOH at 100°C for 2 h (Iacomini et al., 1985). The solns. were neutralized with AcOH, filtered, and extrd. polysaccharides pptd. by addition to excess EtOH. They were isolated, dried, dissolved in hot H_2O, the solns. frozen, and then thawed at 4°C (Machado et al., 1994). The insol. material which formed was centrifuged off, and the freezing and thawing process repeated on the supernatants until ppts. no longer appeared. The combined supernatants were then treated with Fehling soln. (Jones, & Stoodley, 1965) and resulting ppts. of Cu complexes isolated by centrifugation and washed successively with aq. 2% KOH and MeOH. These were dissociated by shaking in an aq. suspn. of Dowex 50×8 (H^+ form) ion exchange resin, which was filtered off, the filtrates evaporated to small vol., and the polysaccharides solns. freeze-dried (yields: *C. substellata*, 2.7%; *C. ibitipocae*, 8.6%).

4.3. Homogeneity and M_r determinations on glucogalactomannans.

Samples (2.0 mg) were chromatographed on a column of Sepharose CL-4B (61×0.85 cm, i.d.) using H_2O as eluant. The resulting fractions (2 ml) were tested for carbohydrates (Dubois, Gilles, Rebers, & Smith, 1956) and the M_r values of each determined by comparison of the retention times of their single peaks with those of a battery of dextrans with M_r's 0.27×10^6, 0.50×10^6, and 2.0×10^6.

4.4. Monosaccharide composition analyses

Neutral monosaccharides, liberated by hydrolysis of polysaccharides and oligosaccharides with M TFA (1.5 ml) for 8 h at 100°C, were analyzed as their alditol acetates by GC-MS (see Section 4.1) by successive $NaBH_4$ redn. (Wolfrom, & Thompson, 1963b) and acetylation with Ac_2O–pyridine (1:1 v/v) at 25°C for 18 h.

4.5. Per-O-methylation of galactomannans

The polysacharides (~50 mg) were partially *O*-methylated using Me_2SO_4–30% aq. NaOH (w/v) (Haworth, 1915) and the process completed with NaOH–Me_2SO–MeI (Ciucanu, & Kerek, 1984) until the products did not have OH absorption in the IR at 3400 cm^{-1}. The methylated polysaccharides were treated with refluxing 3% MeOH–HCl for 3 h, then 2 M H_2SO_4 at 100°C for 18 h, and the resulting mixtures of *O*-methyl aldoses reduced with $NaBH_4$ and then acetylated. The partially *O*-methylated alditol acetates were analyzed by capillary GC-MS, as described in Section 4.1, and the resulting partially *O*-methylated alditol acetates identified by their typical electron impact breakdown profiles and retention times (Jansson, Kenne, Liedgren, Lindberg, & Lönngren, 1976).

4.6. Smith degradation analyses

Galactoglucomannans (50 mg) were oxidized to polyaldehydes with aq. 0.05 M $NaIO_4$ (50 ml) for 72 h at 25°C in the dark (Abdel-Akher, Hamilton, Montgomery, & Smith, 1952; Hay, Lewis, & Smith, 1962; Iacomini et al., 1981), the excess of oxidant then destroyed by addition of ethylene glycol (2.0 ml), and the solutions dialyzed against tap water for 24 h. The solns. were treated with $NaBH_4$ for 12 h at 25°C, excess reducing agent decomposed by addition of AcOH, the solns. dialyzed and then freeze-dried. A portion of the resulting polyalcohol was successively hydrolysed, reduced with $NaBH_4$, acetylated, and the resulting polyol mixture analyzed by GC-MS.

4.7. Partial acetolysis and HPLC fractionation of resulting oligosaccharides

Conventional acetolysis of the galactomannans (200 mg) was performed (Lee, & Ballou, 1965) for 6 days at 20°C. The production of oligosaccharides was monitored by TLC, using the nonpolar solvent as mobile phase (see Section 4.1). The mixtures were dissolved in CH_3CN (HPLC grade) and filtered through a 0.22 μm filter pore membrane. Separation of oligosaccharides was carried out by HPLC: Waters™ and equipped with Waters™ 600 Controller, using a Delta-PAK™ C^{18}-Waters, 100×8 mm column. It was eluted with a 3:2 (v/v) mixt. of H_2O and CH_3CN, flow rate 3.0 ml/min, the eluates being monitored with a differential refractometer. In cases where a single peak contained 2 disaccharides and another where a mixture of trisaccharide and unknown impurity were obtained, deacetylation

was carried out and the mixtures were rechromatographed on a normal phase column, Altech Carbohydrate 10 μm-Waters (C), 300 × 4.1 mm (solv: CH_3CN–H_2O, 7:3 (v/v); flow rate, 2.2 ml/min).

4.8. NMR spectroscopy

1D ^{1}H-NMR spectra, 2D H,H COSY (Zähringer et al., 1995), NOESY (Macura, & Ernst, 1980), TOCSY (McNicholas, Batley, & Redmond, 1987) and ^{13}C–^{1}H inverse detected (Friebolin, 1993) were recorded using Bruker WM-400 and -600 MHz NMR spectrometers at 25°C. $CDCl_3$ was the solvent for derivatized compounds and D_2O for unsubstituted sugars. Chemical shifts are in PPM downfield from Me_4Si ($\delta = 0$) for spectra in $CDCl_3$ and downfield from DSS for spectra in D_2O. Chemical shifts and coupling constants were obtained from a first-order analysis of the spectra. ^{13}C-NMR spectra were recorded under previously described conditions (Gorin, & Iacomini, 1984). Chemical shifts (δ) of polysaccharides are expressed relative to the resonance of Me_4Si ($\delta = 0$) obtained in a separate experiment, and to internal DSS in the case of free oligosaccharides, and to internal Me_4Si for acetylated oligosaccharides.

4.9. ^{1}H-NMR data for 2-O-α-D-mannopyranosyl-αβ-D-mannose (in D_2O)

H-1:δ 5.36, $J_{1,2}$ 1.2 Hz; H-2: δ 3.93, $J_{2,3}$ 3.5 Hz; H-3: δ 3.67, $J_{3,4}$ 9.6 Hz; H-4: δ 3.78, $J_{4,5}$ 10.4 Hz; H-5: δ 3.73, $J_{5,6's}$ n.d.; H-6′s: δ 3.74, $J_{5,6}$ 5.0 Hz, $J_{6,6'}$ 11.5 Hz.

H-1′: δ 5.01, $J_{1,2}$ 1.7 Hz; H-2′: δ 4.04, $J_{2,3}$ 3.3 Hz; H-3′: δ 3.83, $J_{3,4}$ 9.5 Hz; H-4′ δ 3.61, $J_{4,5}$ 9.7 Hz; H-5′: δ_3.75, $J_{5,6's}$ 4.1 and 2.0 Hz; H-6′s: δ 3.85, $J_{6,6'}$ 12.6 Hz. N.d = not detected. Main NOEs between H-1′ and H-2, and H-1 and H-2.

4.10. ^{1}H-NMR data for 3-O-α-D-mannopyranosyl-αβ-D-galactose (in D_2O)

α-Gal*p*, H-1α: δ 5.26, $J_{1,2}$ 3.9 Hz; H-2α: δ 3.87, $J_{2,3}$ 10.4 Hz; H-3α: δ 3.94; $J_{3,4}$ 3.1 Hz; H-4α: δ 4.22, $J_{4,5}$ 0.7 Hz; H-5α: δ 4.05 Hz, $J_{5,6}$ n.d.; H-6α′s: δ 3.85–3.89, *J*s n.d.

β-Gal*p*, H-1β: δ 4.60, $J_{1,2}$ 7.9 Hz; H-2β: δ 3.53, $J_{2,3}$ 10.0 Hz; H-3β: δ 3.74, $J_{3,4}$ 3.6 Hz, H-4β: δ 4.16, $J_{4,5}$ n.d.; H-5β: δ 3.65–3.76, $J_{5,6}$ 7.7 Hz; C-6β′s: δ 3.66–3.76/3.70, $J_{6,6'}$ 12.8 Hz.

α-Man*p*, H-1′ linked to α-Gal*p*: δ 5.03, $J_{1,2}$ 1.7 Hz; H-1′ linked to β-Gal*p*: δ 5.02, $J_{1,2}$ 1.8 Hz; H-2′: δ 3.98, $J_{2,3}$ 3.5 Hz; H-3′: δ 3.67, $J_{3,4}$ 9.8 Hz; H-4′: δ 4.05, $J_{4,5}$ 9.55 Hz; H-5′: δ 3.65–3.75, $J_{5,6}$ 2.5 Hz; H-6′s: δ 3.83–3.91, *J* n.d.

The main interglycosidic NOE was between H-1′ and H-3.

4.11. ^{1}H-NMR data of 1,2,4,6-tetra-O-acetyl-3-O-(2′,3′,4′,6-tetra-O-acetyl-α-D-mannopyranosyl)-αβ-D-galactose (in $CDCl_3$)

H-1α: δ 6.40, $J_{1,2}$ 3.7 Hz; H-2α: δ 5.23, $J_{2,3}$ 10.2 Hz; H-3α: δ 4.23, $J_{3,4}$ 3.4 Hz; H-4α: δ 5.48, $J_{4,5}$ 1.1 Hz; H-5α: δ 4.22–4.26, $J_{5,6}$ n.d.; H-6α′s: δ 4.24, $J_{6,6'}$ 12.6 Hz.

H-1β: δ 5.64, $J_{1,2}$ 8.3 Hz; H-2β: δ 5.31, $J_{2,3}$ 10.1 Hz; H-3β: δ 3.98, $J_{3,4}$ 3.4 Hz; H-4β: δ 5.42, $J_{4,5}$ 1.0 Hz; H-5β: δ 4.11, $J_{5,6}$ 7.6 Hz, Hβ-6′s: δ 4.16–4.24, $J_{6,6'}$ 11.9 Hz.

H-1′ linked to α-Gal*p*: δ 5.04, $J_{1,2}$ 1.6 Hz; H-1′ linked to β-Gal*p*: δ 5.05, $J_{1,2}$ 1.4 Hz; H-2′: δ 5.10, $J_{2,3}$ 3.2 Hz: H-3′: δ 5.15, $J_{3,4}$ 10.2 Hz; H-4′: δ 5.24, $J_{4,5}$ 9.9 Hz; H-5′: δ 3.93, $J_{5,6}$ 2.5 and 5.1 Hz; H-6′s: δ 4.18, $J_{6,6'}$ 12.5 Hz.

Acknowledgements

The authors would like to thank Mrs. Marcelline Tracey and Mr. César Augusto Tischer for the measurement of NMR spectra, and Dr. Blair D. Johnston for helpful discussions. For financial assistance, thanks are also due to the Brazilian PRONEX-CARBOIDRATOS, PADCT II/SBIO, and CNPq, and the Canadian NSERC.

References

Abdel-Akher, M., Hamilton, J. K., Montgomery, R., & Smith, F. (1952). *Journal of the American Chemical Society*, *74*, 4970.

Ciucanu, I., & Kerek, F. (1984). *Carbohydrate Research*, *31*, 209.

Corradi da Silva, M. A. L., Iacomini, M., Jablonski, E., & Gorin, P. A. J. (1993). *Phytochemistry*, *33*, 547.

Dubois, M., Gilles, K. A., Rebers, P. A., & Smith, F. (1956). *Anal. Chem.*, *28*, 350.

Friebolin, H. (1993). In *Basic One- and Two Dimensional NMR Spectroscopy* (2nd. ed.) (p. 368). Weinheim, Germany: VCH Verlagsgesselschaft.

Gorin, P. A. J. (1973). *Canadian Journal of Chemistry,*, *51*, 2375.

Gorin, P. A. J., & Iacomini, M. (1984). *Carbohydrate Research*, *128*, 119.

Gorin, P. A. J., & Spencer, J. F. T. (1970). *Advances in Applied Microbiology*, *13*, 25.

Gorin, P. A. J., Barreto-Bergter, E. M., & Cruz, F. S. (1981). *Carbohydrate Research*, *88*, 177.

Gorin, P. A. J., Baron, M., & Iacomini, M. (1988). In M. Galun, *Handbook of Lichenology*, *Vol. III* ((p. 9). Boca Raton, FL: CRC Press.

Gorin, P. A. J., Baron, M., Silva, M. L. C., Teixeira, A. Z. A., & Iacomini, M. (1993). *Ciência e Cultura*, *45*, 27.

Haworth, W. N. (1915). *Journal of the Chemical Society*, *107*, 8.

Haworth, W.N., Hirst, E.L., & Isherwood, F.A. (1937). *Journal of the Chemical Society*, 782.

Hay, G. W., Lewis, B. A., & Smith, F. (1962). *Methods in Carbohydrate Chemistry*, *5*, 357.

Hough, L., & Jones, J. K. N. (1962). *Methods in Carbohydrate Chemistry*, *1*, 21.

Iacomini, M., Duarte, G. R., Duarte, E. R., Duarte, H. S., Fontana, J. D., & Duarte, J. H. (1981). *Agricultural and Biological Chemistry*, *45*, 1373.

Iacomini, M., Schneider, C. L., & Gorin, P. A. J. (1985). *Carbohydrate Research*, *142*, 237.

Iacomini, M., Gorin, P. A. J., Baron, M., Tulloch, A. P., & Mazurek, M. (1988). *Carbohydrate Research*, *176*, 117.

Jansson, P.-E., Kenne, L., Liedgren, H., Lindberg, B., & Lönngren, J. (1976). *Journal of Chemical Communications, 1976. University of Stockholm*, *8*, 1.

Jones, J. K. N., & Stoodley, M. (1965). *Methods in Carbohydrate Chemistry*, *5*, 36.

Karrer, P., & Joos, B. (1924). *Hoppe Seyler's Zeitschrift für Physiolische Chemie*, *141*, 311.

Lee, Y.-C., & Ballou, C. E. (1965). *Biochemistry*, *4*, 257.

Machado, M. J., Gorin, P. A. J., Torri, G., & Iacomini, M. (1994). *Brazilian Journal of Medical and Biological Research*, *27*, 523.

Macura, S., & Ernst, R. R. (1980). *Molecular Physics*, *41*, 95.

McNicholas, P. A., Batley, M., & Redmond, J. W. (1987). *Carbohydrate Research*, *165*, 17.

Ogawa, T., & Yamamoto, H. (1982). *Carbohydrate Research*, *104*, 271.

Sawardeker, J. S., Sloneker, J. H., & Jeanes, A. (1965). *Analytical Chemistry*, *37*, 1602.

Teixeira, A. Z. A., Iacomini, M., & Gorin, P. A. J. (1992). *Phytochemistry*, *31*, 3467.

Teixeira, A. Z. A., Iacomini, M., & Gorin, P. A. J. (1995). *Carbohydrate Research*, *266*, 309.

Trevelyan, W. E., Procter, D. P., & Harrison, J. S. (1950). *Nature*, *166*, 444.

Wolfrom, M. L., & Thompson, A. (1963a). *Methods in Carbohydrate Chemistry*, *2*, 211.

Wolfrom, M. L., & Thompson, A. (1963b). *Methods in Carbohydate Chemistry*, *2*, 65.

Woranovicz, S. M., Gorin, P. A. J., Marcelli, M. P., Torri, G., & Iacomini, M. (1997). *The Lichenologist*, *29*, 471.

Zähringer, U., Knirel, Y. A., Lindner, B., Helbig, J. H., Sonesson, A., Marre, R., & Reitschel, E. T. (1995). In J. Levin, C. R. Alving, R. S. Munford, & H. Redl, Progress in Clinical and Biological Research, (vol 392) (p. 113). New York: Wiley and Liss.