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Comparative studies of the polysaccharides from species of the genus *Ramalina*—lichenized fungi—of three distinct habitats

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Abstract

Several structurally different glucans (α- and β-) and galactomannans were characterized as components of four species of the genus *Ramalina*, namely *R. dendriscoides, R. fraxinea, R. gracilis* and *R. peruviana*. Freeze–thawing treatment of hot aqueous extracts furnished as precipitates (PW) linear α-D-glucans of the nigeran type, with regularly distributed (1→3)- and (1→4)-linkages in a 1:1 ratio. The supernatants (SW) contained α-D-glucans with (1→3)- and (1→4)-linkages in a molar ratio of 3:1. The lichen residues were then extracted with 2% aq. KOH, and the resulting extracts submitted to the freeze-thawing treatment, giving rise to precipitates (PK2) of a mixture of α-glucan (nigeran) and β-glucan, which were suspended in aqueous 0.5% NaOH at 50 °C, dissolving preferentially the β-glucan. These were linear with (1→3)-linkages (laminaran). The mother liquor of the KOH extractions (2% and 10% aq. KOH) was treated with Fehling's solution to give precipitates (galactomannans). The galactomannans are related, having (1→6)-linked α-D-mannopyranosyl main chains, substituted at *O*-4 and in a small proportion at *O*-2,4 by β-D-galactopyranosyl units. Despite the different habitats of these lichenized fungi, all species studied in this investigation have a similar pool of polysaccharides.

Keywords: Lichens; Lichenized fungi; *Ramalina dendriscoides*; *Ramalina fraxinea*; *Ramalina gracilis*; *Ramalina peruviana*; Polysaccharides; Galactomannans; α- and β-glucans

1. Introduction

Structurally different, species-specific, mannose-containing polysaccharides have been utilized for the classification and identification of yeasts (Gorin and Spencer, 1970). This led to investigations of polysaccharides isolated from different species of ascomycetous lichens (Teixeira et al., 1995). The best known polysaccharides of lichenized fungi are lichenan, isolichenan and galactomannan. Galactomannans have a range of different, but related, chemical structures depending on the species (Gorin et al., 1988), which are useful in chemotaxonomic studies (Teixeira et al., 1995; Gorin et al., 1993). Lichen homopolymers, namely glucans, have also been investigated as an approach to chemotaxonomy (Yokota et al., 1979) of different families and genera using ^{13}C NMR spectroscopy. Further studies were carried out on glucans of several ascomyceteous lichenized fungi (Gorin et al., 1993), which have a linear structure with an α- or β-glycosidic configuration. The α-D-glucans have (1→3) and (1→4)-linkages whose ratio showed considerable variation (1:1 to 4:1) (Baron et al., 1988; Woranovicz-Barreira et al., 1999a; Carbonero et al., 2001, 2002), controlling their solubility or insolubility in cold water. A soluble α-D-glucan with 3:1 ratio of (1→3) and (1→4)-linkages was isolated by Gorin and Iacomini (1984) and Stuelp et al. (1999) from the lichens *Ramalina usnea* and *R. celastri*, respectively. Moreover, these lichens also contain an insoluble α-D-glucan, having (1→3) and (1→4)-linkages in 1:1 ratio (Gorin and Iacomini, 1984; Stuelp et al., 1999). Linear (1→3)-linked β-D-glucans have been previously isolated from the lichens *Stereocaulon ramulosum* (Baron et al., 1988), *Ramalina celastri* (Stuelp et al., 1999) and *Cladonia* spp (Carbonero et al., 2001).

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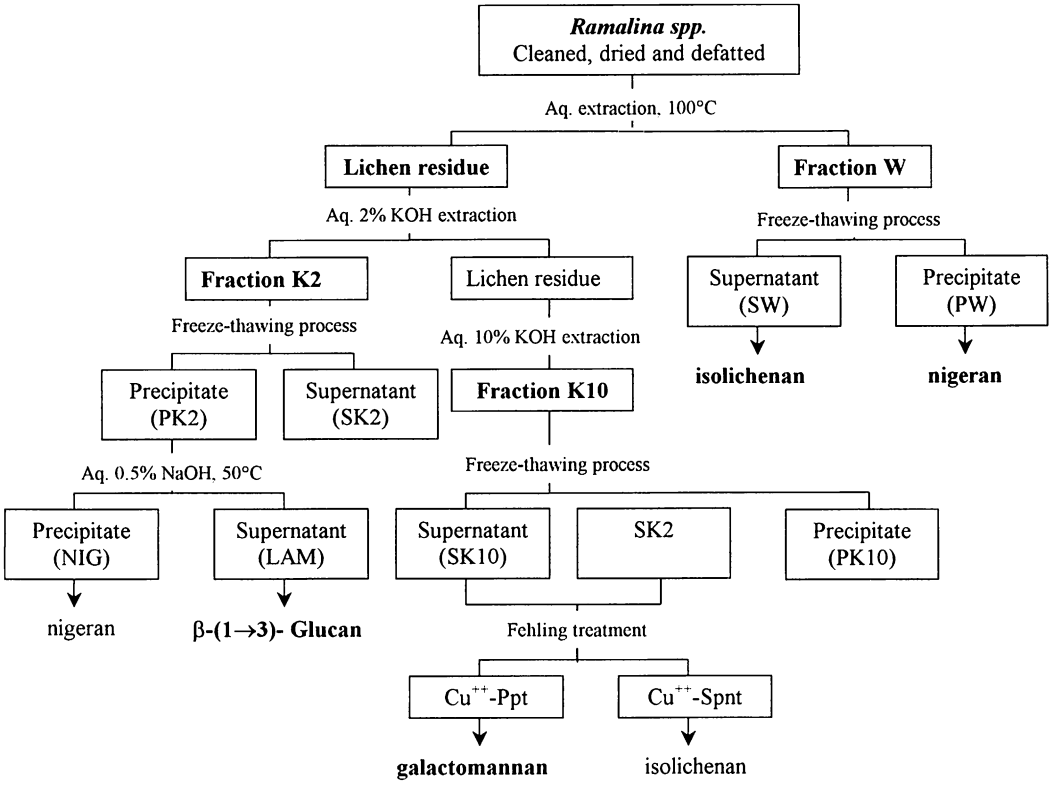
The lichenized fungi of the genus *Ramalina* are characterized by their fruticose thallus, either with soralia or apothecia and in most of the Brazilian species the habit types are commonly caespitose (e.g. *R. gracilis*), rarely decumbent, and pendulous (e.g. *R. usnea*). Pseudocyphellae are found in all Brazilian species of *Ramalina* with a solid thallus (Kashiwadani and Kalb, 1993).

The main goal of this investigation is an extensive study of the chemistry of polysaccharides from four *Ramalina* spp. collected in three different habitats (alpine, coastal sandy ecosystem—"Restinga" and subtropical forest) and a further objective is to compare the chemical structures of these polysaccharides with those of other lichens, from a chemotyping point of view.

Table 1
Yields of fractions extracted with hot water, 2% and 10% aq. KOH

Fraction	Yield (g%)[a]			
	R. dendriscoides (RD)	*R. gracilis* (RG)	*R. fraxinea* (RF)	*R. peruviana* (RP)
MeOH:$CHCl_3$ extract	7.1	10.2	2.9	11.0
Aqueous extract (W)	2.1	12.0	10.2	12.5
Supernatant (SW)	1.5	7.8	9.2	10.2
Precipitate (PW)	0.5	1.3	0.9	0.8
2% KOH extract (K2)	30.0	27.2	30.2	22.1
Supernatant (SK2)	17.7	23.0	28.2	18.2
Precipitate (PK2)	2.6	4.2	2.0	1.5
10% KOH extract (K10)	nd[c]	5.6	10.1	9.4
Supernatant (SK10)	nd[c]	4.6	9.9	9.1
Precipitate (PK10)	nd[c]	0.9	0.2	0.3
Fehling supernatant (Cu^{++}-Spnt)[b]	6.4	10.4	19.0	8.3
Fehling precipitate (Cu^{++}-Ppt)[b]	5.4	10.5	8.8	12.5

[a] Yield based on dry weight of lichen.
[b] Fraction SK2 and SK10 were combined and treated with Fehling solution.
[c] Not determined.



Scheme 1. Extraction and purification of polysaccharides of *Ramalina* spp.

2. Results and discussion

Variable amount of lipids and other low-molecular-weight material (Table 1), were extracted with $CHCl_3$–MeOH, the alpine *R. fraxinea* having the lowest yield (2.9%). Then, defatted lichen thalli were extracted with hot water at 100 °C (Scheme 1) and the extracted polysaccharides (fraction W) were precipitated with ethanol, dissolved in water and freeze-dried. The yields varied from 2.1 to 12.5%, with *R. dendriscoides* having the

Table 2
Monosaccharide composition of polysaccharides obtained from *Ramalina* species

Lichenized fungi	Hot aqueous extract (mol%)[a]		2% aq. KOH extract (mol%)[a]		Fehling treatment (mol%)[a]	
	Precipitate (PW)	Supernatant (SW)	Precipitate (PK2)	Supernatant (SK2)	Precipitate (Cu^{++}-Ppt)	Supernatant (Cu^{++}-Spnt)
R. dendriscoides	14:19:67	43:22:35	5:4:91	24:32:44	57:33:10	17:21:62
R. gracilis	9:5:86	13:10:77	8:8:84	27:23:50	53:37:10	6:8:86
R. fraxinea	8:8:74	14:10:76	23:20:57	15:10:75	55:39:6	3:1:96
R. peruviana	5:6:89	0:0:100	11:9:80	39:33:28	51:44:5	12:10:78

[a] Mannose:galactose:glucose ratios, determined by GC–MS.

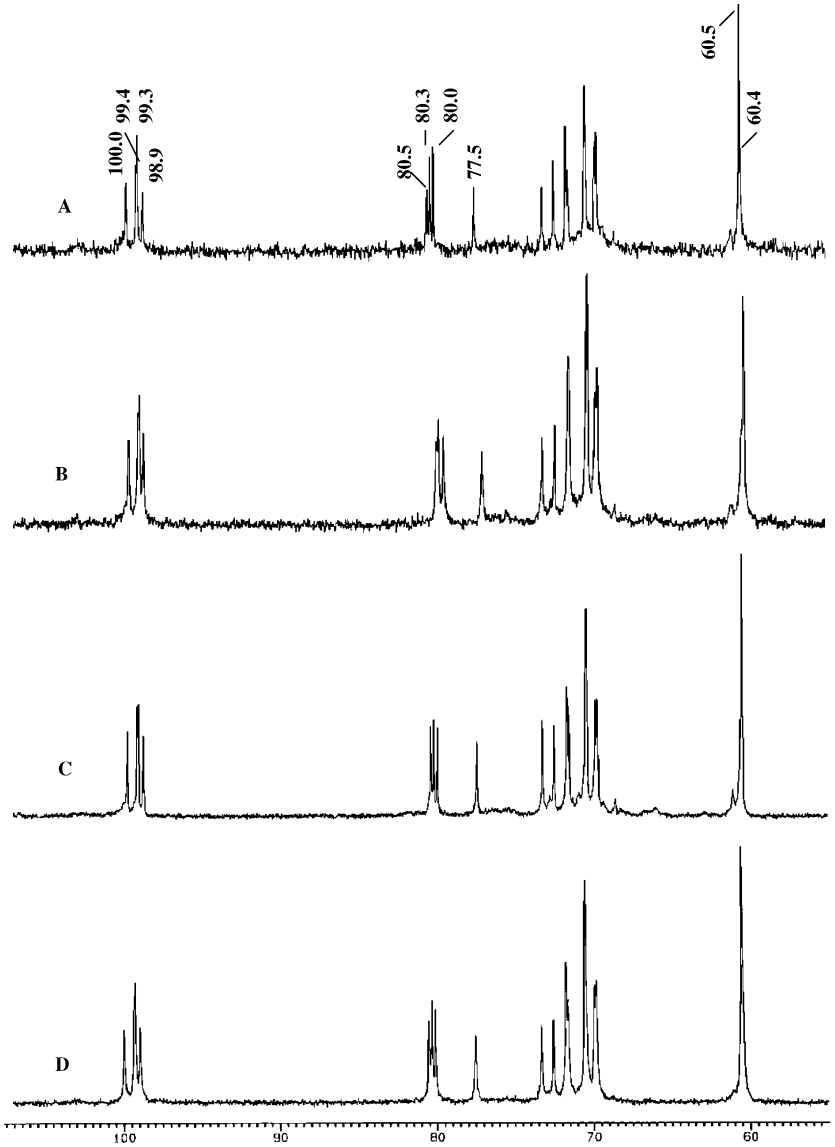


Fig. 1. ^{13}C NMR spectra of soluble (1→3)-, (1→4)-linked α-glucans (3:1 ratio) in D_2O, at 50 °C, (chemical shifts are expressed as δ ppm) obtained from fraction SWF of *R. dendriscoides* (A), and fractions SW of *R. gracilis* (B), *R. fraxinea* (C) and *R. peruviana* (D).

lowest polysaccharide content (2.1%, see Table 1). The aqueous extracts were then frozen and gently thawed, resulting in precipitates (PW) and supernatants (SW) composed mainly of glucose (Table 2), which the exception of the SW fraction of *R. dendriscoides* with contained a mixture of polysaccharides. Separation of the polysaccharides present in fraction SW was carried out via precipitation with Fehling's solution, resulting in a Fehling's supernatant (SWF) containing 90% glucose. These soluble D-glucans (fractions SW and SWF), obtained as supernatants of the freeze–thawing treatment of the four lichens studied herein, contained (1→3)- and (1→4)-linkages in a 3:1 ratio (isolichenan type), as demonstrated by their ^{13}C NMR spectra (Fig. 1), which showed four anomeric signals at δ 100.0, δ 99.4, δ 99.3 and δ 98.9, indicating an α-configuration (Stuelp et al., 1999); and others at δ 80.5, δ 80.3 and 80.0 (*O*-substituted C-3s); δ 77.5 (*O*-substituted C-4), and δ 60.5 and δ 60.4 (C-6). Methylation analysis provided a mixture of partially *O*-methylated alditol acetates, which was examined by capillary GC–MS, which indicated acetates of 2,4,6- and 2,3,6-tri-*O*-methylglucitol in a molar ratio of 2.9:1.0, corresponding to the (1→3)- and (1→4)-linkages, respectively.

Steric exclusion chromatography of *R. peruviana* α-D-glucan (isolichenan), which contained 100% glucose (Table 2) showed that it has a M_r of 103 kD, *dn*/*dc* 0.115 and was eluted as a single peak.

The α-D-glucans obtained as a precipitate of freeze–thawing process (fractions PW) also have (1→3) and (1→4)-linkages, but in a ~1:1 ratio (nigeran type), being insoluble in cold water and isolated by centrifugation. On methylation analysis of the *R. peruviana* nigeran, it gave rise to 2,4,6- and 2,3,6-tri-*O*-methylglucitol acetates in a molar ratio of 1.2:1. When subjected to gel permeation chromatography on Sepharose CL-6B, it gave a single peak with M_r ~94 kD. ^{13}C NMR spectroscopy of all four lichens (Fig. 2; solvent DMSO-d_6) showed high-field C-1 signals at δ 100.2 and 99.3, showing an α-configuration, with other signals at δ 82.5 and 78.9 which corresponded to *O*-substituted C-3 and C-4, respectively, and δ 60.5 and δ 60.1 to unsubstituted C-6, similar to those of a previously described nigeran from *R. celastri* (Stuelp et al., 1999), *Cladonia* spp. (Woranovicz-Barreira, 1999a) and *Cladina* spp. (Carbonero et al., 2002).

The lichen residues obtained after aqueous extraction of *Ramalina* spp, were then submitted to 2 and 10% aq. KOH extraction at 100 °C and the resulting polysaccharides were obtained in 22.1–30% (fractions K2) and 5.6–9.4% (fractions K10) yield (Table 1). Following freeze-thawing treatment, each fraction (supernatant and

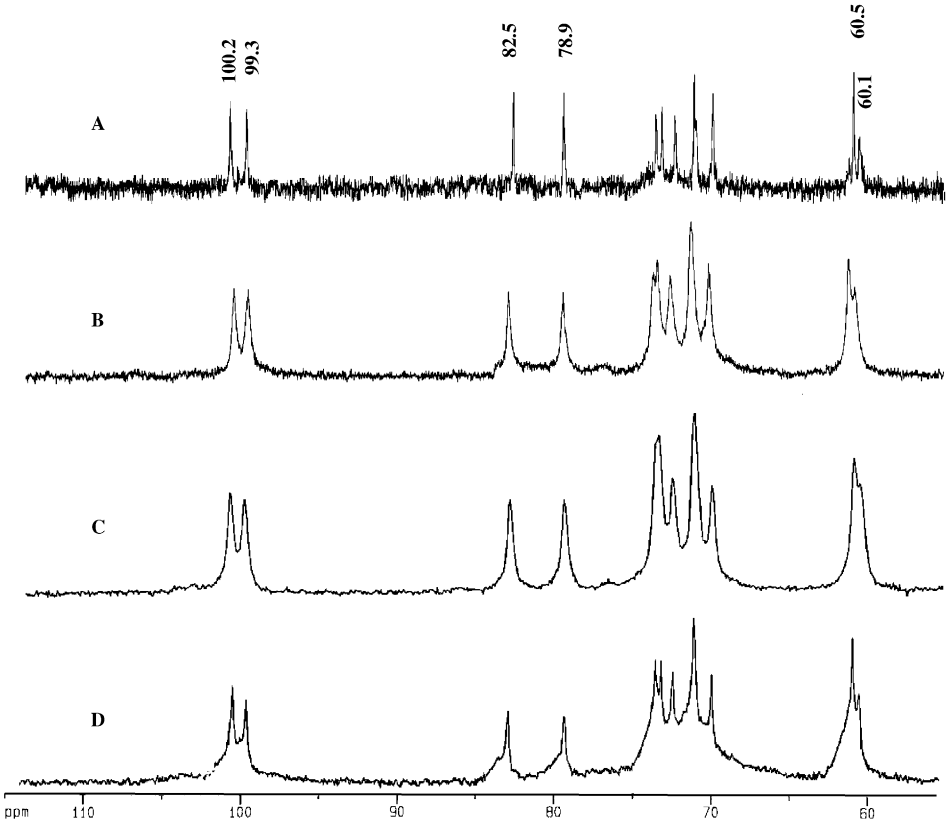


Fig. 2. ^{13}C NMR spectra of insoluble (1→3)-, (1→4)-linked α-glucans (1:1 ratio, fractions PW) in DMSO-d_6, at 50 °C, (chemical shifts are expressed as δ ppm) obtained from *R. dendriscoides* (A), *R. gracilis* (B), *R. fraxinea* (C) and *R. peruviana* (D).

precipitated) was isolated, depending on the species, in varying yields (see Table 1), mannose-, galactose-, and glucose-containing components being present in different molar ratios (Table 2). The water-insoluble PK2 fractions contained a mixture of (1→3),(1→4)-linked α-D-glucan and (1→3)-linked β-D-glucan, according to the ^{13}C NMR spectra (Fig. 3 A). The glucan mixtures of each species were then suspended in 0.5% aq. NaOH at 50 °C, which dissolved the β- (fraction LAM), but not the α-D-glucan (fraction NIG). These NIG fractions had a similar structure as that found in an aqueous extract (nigeran), by comparison of their ^{13}C NMR spectra. The soluble β-glucans (laminaran type) gave rise to ^{13}C NMR spectra (Fig. 3) with signals at δ 103.1 (C-1), δ 86.3 (*O*-substituted C-3), δ 76.5 (C-5), δ 73.1 (C-2), δ 68.6 (C-4) and 61.0 (C-6), identical to those of authentic (1→3)-linked β-D-glucan (Baron et al., 1988; Stuelp et al., 1999; Carbonero et al., 2001, 2002). The low-field signal of C-1 at δ 103.1 is consistent with a β-configuration.

The supernatants of freeze–thawing treatment (SK2 and SK10) were combined and treated with Fehling's solution, and the resulting precipitates (as Cu^{2+}-complexes) removed by centrifugation. Polysaccharides present in the Fehling's supernatants (Cu^{++}-Spnt) contained mainly glucose and had structures similar (as seen by ^{13}C NMR comparison) to isolichenan. Examination of the monosaccharide composition of Fehling's-precipitated polymers (Cu^{++}-Ppt) showed mainly mannose and galactose (Table 2), indicating the presence of galactomannans. In terms of the purity of the galactomannans prepared via Fehling precipitation, their ^{13}C NMR spectra showed similarities and only small differences in their mannose, galactose, to glucose ratios (Table 2). According to their ^{13}C NMR spectra (Fig. 4), the C-1 signal at low field of δ 103.1 correspond to non-reducing end of β-D-Gal*p* linked (1→4) to α-D-Man*p* of the main-chain. The C-1 signal at δ 100.1 arose from (1→6)-linked α-D-Man*p* units of the main chain substituted at *O*-4 by

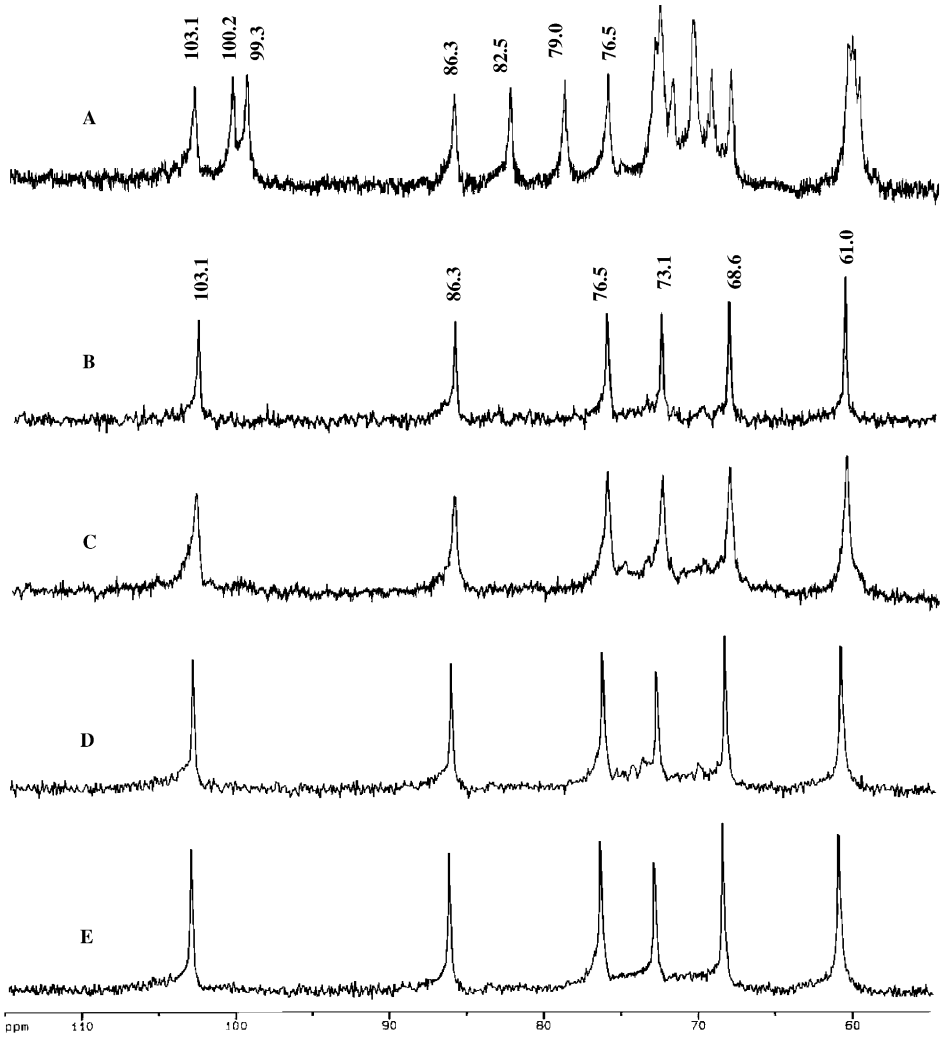


Fig. 3. ^{13}C NMR spectra of PK2 fractions, in DMSO-d_6, at 50 °C, (chemical shifts are expressed as δ ppm): (A) mixed nigeran and laminaran from PK2 of *R. peruviana*, (B) purified (1→3)-linked β-glucan (fraction LAM) obtained from *R. dendriscoides*, (C) from *R. gracilis*, (D) *R. fraxinea* and (E) *R. peruviana*.

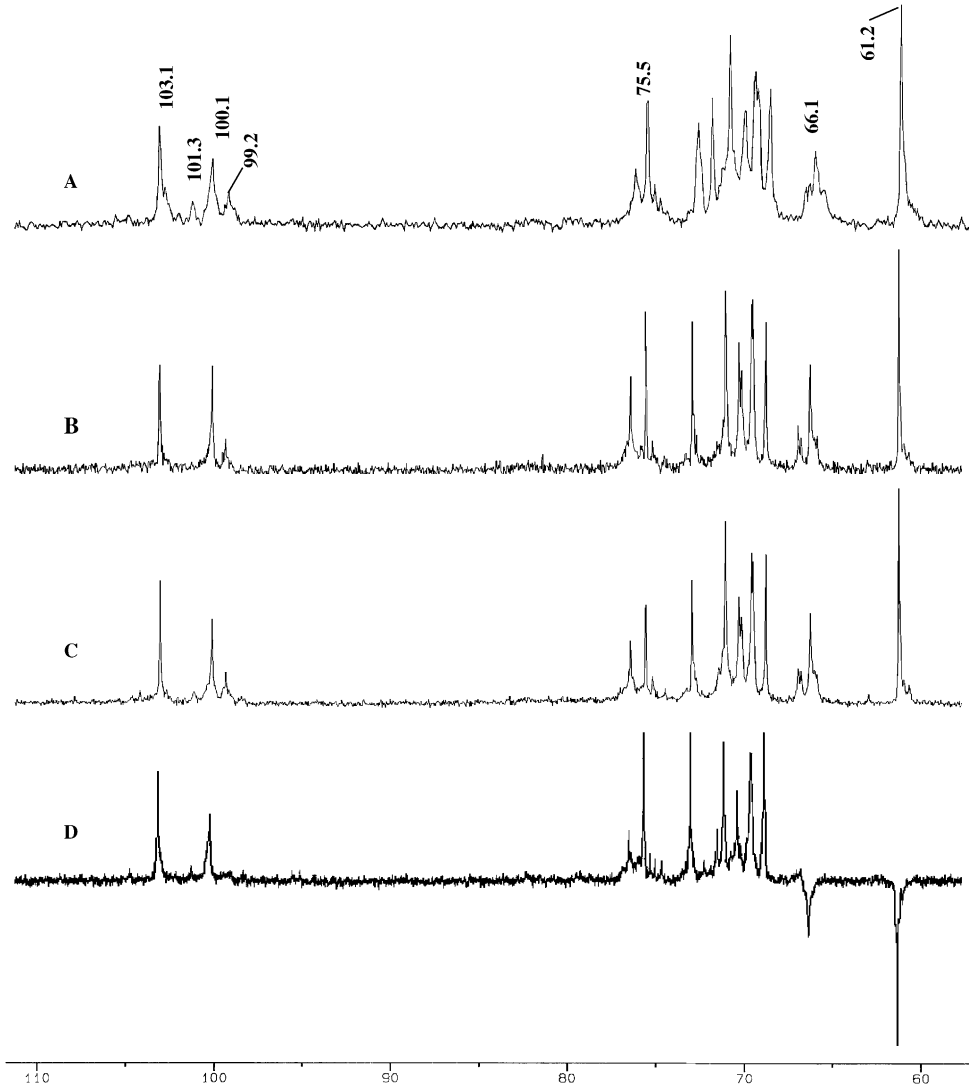


Fig. 4. ^{13}C NMR spectra of galactomannans (Cu^{++}-Ppt) in D_2O, at 50 °C, (chemical shifts are expressed as δ ppm) obtained from *R. dendriscoides* (A), *R. gracilis* (B), *R. fraxinea* (C). In (D) the DEPT experiment of galactomannan from *R. peruviana* is shown.

β-D-Gal*p*. In addition, two small signals appeared at δ 101.3 and δ 99.2, which correspond respectively to C-1 of a (1→6)-linked α-D-mannopyranosyl main-chain and 2,4,6-tri-*O*-substituted α-D-mannopyranosyl residues (Gorin, 1981; Gorin and Iacomini, 1984, 1985). The 6-*O*-substituition of α-D-Man*p* residues was also observed on DEPT examination (Distortionless Enhancement by Polarization Transfer, Fig. 4D), which provided an inverted signal at δ 66.1 corresponding to substituted CH_2OH.

Methylation analysis of the galactomannans indicated non-reducing end units of Gal*p* (36.0–39.7%) and Man*p* (2.0–3.0%), as well 6-*O*- (19.0–26.0%), 4,6-di-*O*- (30.0–32.7%) and 2,4,6-tri-*O*-substituted Man*p* residues (5.0–9.0%), according to the species (Table 3). The methylation data are consistent with the presence of a (1→6)-linked α-D-mannopyranosyl backbone mainly substituted at *O*-4 and *O*-2,4 by galactopyranosyl groups.

Table 3
Analysis of partially *O*-methylated alditol acetates obtained from methylated galactomannans isolated from lichens of the genus *Ramalina*

O-Methylaldose[b]	Source of galactomannan analysed[a]			
	R. dendriscoides	*R. gracilis*	*R. fraxinea*	*R. peruviana*
2,3,4,6-Me_4-Man	2.3	3.0	2.8	2.0
2,3,4,6-Me_4-Gal	39.7	38.5	37.2	36.0
2,3,4-Me_3-Man	19.0	20.8	21.0	26.0
2,3-Me_2-Man	30.0	32.7	32.0	30.0
3-Me-Man	9.0	5.0	7.0	6.0

[a] Percentage of peak area relative to total peak areas.
[b] Analysed as derived *O*-methylalditol acetates.

According to these results (see Table 3), the galactomannans showed the same degree of *O*-4 substitution (~30.0%), and only small differences were observed in

O-2,4 substitution, with the *R. gracilis* galactomannan having the least of the di-*O*-substitution of the main chain.

It is possible to conclude that all *Ramalina* spp. have closely related polysaccharide structures, independent of their habitat. *R. gracilis* is adapted to grow in very hot, humid and salty areas. This Brazilian "Restinga" is a conglomerate of coastal sandy ecosystems with floristically and physiognomically distinct communities, according to Falkenberg (1999). He observed that these plant communities colonize sediments of very diverse origins (marine, fluvial, lagoonal, eolian, or a combination of these), forming a complex vegetation that occupies a narrow belt along the coast, including such distinct regions as beaches, dunes and associated depressions, sand ridges, terraces and plains. In great contrast, *R. fraxinea* is able to survive, exposed to lower and sometimes extremely low temperatures in the Alps, over a long winter period. Despite these different habitats, no modifications on the qualitative content of polysaccharides were observed.

The determination of the contents of polysaccharides could be utilized as an alternative method for chemotyping, it could also be used as a marker to aid lichen taxonomy and as a powerful phylogenetic characteristic when allied with DNA analysis (ITS rDNA, 18S rDNA). ^{13}C NMR spectroscopy of polysaccharides has been also suggested as a tool to identify lichen species (Yokota et al., 1979; Teixeira et al., 1995; Woranovicz et al., 1997).

The nigeran and laminaran found in *Ramalina* spp. have been previously found in several lichen genera (Baron et al., 1988; Woranovicz-Barreira et al., 1999a; Carbonero et al., 2002). However, the heteropolysaccharides (galactomannans) of *Ramalina* spp., when compared with galactomannans of other lichens (Gorin and Iacomini, 1985), are chemotypes which could have a significant influence in aiding the taxonomical classification of this genus and those of related genera.

Strong support that polysaccharides can be used for chemotyping of lichens was demonstrated by Woranovicz et al. (1999), Woranovicz-Barreira et al. (1999a,b) and Carbonero et al. (2001, 2002) for lichenized fungi of the genus *Cladonia* and *Cladina*. In these studies, α- and β-D-glucans, galactoglucomannans and galactomannoglucans were suggested as markers for lichen indentification in the *Cladonia* genus, and were also found in the three *Cladina* spp. examined. These results, along with the DNA studies of Ahti and Depriest (2001) suggested that *Cladina* must return and be classified within the genus *Cladonia*.

Another facet of the applicability of polysaccharides in chemotaxonomy was shown by Domenech et al. (1996) using polysaccharides to generate antibodies that can be used to distinguish related species of fungi.

3. Conclusion

Studies involving the chemistry of polysaccharides from lichens of genus *Ramalina* showed that all species studied herein, as well as *R. usnea* (Gorin and Iacomini, 1984) and as *R. celastri* (Miceno et al., 1991; Stuelp et al., 1999) have structurally similar components (isolichenans, nigerans, laminarans and galactomannans), demonstrating the importance of chemotyping in taxonominal classification, as already demonstrated by Woranovicz et al. (1999), Woranovicz-Barreira et al. (1999 a,b) and Carbonero et al. (2001, 2002) for *Cladonia* and *Cladina* genus, respectively.

4. Experimental

4.1. General experimental procedures

GC–MS was performed using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap model 800 mass spectrometer, with He as carrier gas. A capillary column (30 m×0.25 mm i.d.) of DB-225, held at 50 °C during injection and then programmed at 40 °C min^{-1} to 220 °C (constant temperature) was used for quantitative analysis of alditol acetates and partially *O*-methylated alditol acetates. Acetylation of alditols was carried out with Ac_2O–pyridine (1:1, v/v) at 100 °C for 1 h.

^1H and ^{13}C NMR spectra were obtained using a Bruker DRX 400 spectrometer incorporating Fourier transform. Analyses were performed at 50 °C, the water soluble samples were dissolved in D_2O and the water insoluble being dissolved in DMSO-d_6. Chemical shifts are expressed as δ ppm, relative to the resonances of CH_3 groups of acetone internal standard (^1H at δ 2.224; ^{13}C, δ 30.2), or DMSO-d_6 (^1H at δ 2.60; ^{13}C, δ 39.7).

4.2. Lichenized fungi

The selected subtropical species of the family Ramalinaceae were collected in South Brazil, namely in Paraná and Santa Catarina States. *Ramalina dendriscoides*, *R. peruviana* were collected growing on trunks of trees in the vicinity of Curitiba, Paraná State, at an altitude of 900 m, during May of 2001 and June of 2000, respectively. The thalli of *R. gracilis* were found growing on twigs of shrubs, in a salty and moist coastal area, exposed to light (in Brazil known as "Restinga"), in Santa Catarina Island, Campeche Beach, Santa Catarina State, at an elevation of about 3 m, during November of 2000. The alpine lichen *R. fraxinea* was collected in the Alps, Italy, Udine, Passo Pura near Ampezzo, 12°44′37″O, 46°25′29″ (June/2000), at an elevation of 1400 m. Voucher specimens are deposited in the UPCB (Herbarium of the Federal University of Paraná), registration number 46.289 (*R. dendriscoides*),

46.286 (*R. gracilis*), 46.290 (*R. fraxinea*) and 46.287 (*R. peruviana*).

4.3. Extraction and purification of polysaccharides

R. dendriscoides (31 g), *R. fraxinea* (13.8 g), *R. gracilis* (53 g) and *R. peruviana* (66 g) were first extracted with 1:1 (v/v) $CHCl_3$–MeOH at 60 °C for 2 h (×2, 1 l each), to remove low molecular mass material. The residues were submitted to sequential extraction (Scheme 1) with hot water at 100 °C for 3 h (×2, 1 l each), 2% aq. KOH at 100 °C for 3 h (×2, 1 l each) and 10% aq. KOH at 100 °C for 3 h (×2, 1 l each). The resulting extracts were neutralised (HOAc), added to EtOH (3 vol.) and the resulting polysaccharide precipitates, dissolved in water and dialysed, giving rise to fractions W, K2 and K10, respectively. The solutions were frozen and then allowed to thaw, and the resulting insoluble materials (fractions PW, PK2 and PK10) centrifuged off. The SW fractions of *R. fraxinea*, *R. gracilis* and *R. peruviana* contained a pure α-glucan, while that of *R. dendriscoides* showed a mixture of polysaccharides. These were separated by Fehling treatment, the α-glucan being isolated from the Fehling supernatant (SWF). The supernatants SK2 and SK10 were combined and then treated with Fehling solution and the precipitated material (Cu^{++}-Ppt, galactomannan) centrifuged off. The precipitates of Cu^{2+}-complexes and supernatants (Cu^{++}-Spnt) were neutralised with HOAc, dialysed against tap water, deionised with mixed ion exchange resins and then freeze-dried. The PK2 fractions contained a mixture of glucans, which were then suspended in 0.5% aq. NaOH at 50 °C, which dissolved the β- (fraction LAM), but not the α-D-glucans (fraction NIG). Both fractions were neutralised with acetic acid and dialysed.

4.4. Monosaccharide composition of the polysaccharides

Monosaccharide components of the polysaccharides and their ratios were determined by hydrolysis with 2 M TFA for 8 h at 100 °C, followed by conversion to alditol acetates (GC–MS examination) by successive $NaBH_4$ reduction and acetylation with Ac_2O–pyridine.

4.5. Determination of homogeneity of polysaccharides and their molecular weight

The homogeneity and molecular weight (M_r) of water-soluble polysaccharides were determined by steric exclusion chromatography (SEC), using multidetection equipment in which a differential refractometer (Waters) and a multiangle laser light scattering apparatus (MALLS; Dawn DSP-F, Wyatt Technology) were adapted on-line. The eluent was 0.1 M $NaNO_3$, containing 0.5 g/l NaN_3. The polysaccharide solution was filtered through a membrane, with pores of 0.2 μm diameter (Millipore). The water insoluble glucans (4.0 mg) were dissolved in 1 M NaOH (1.0 ml) and the solution applied to a column of Sepharose CL-6B (42×1.2 cm i.d.). Then it was eluted with 0.2 M NaOH and the resulting fractions of 2 ml were tested for carbohydrates (Dubois et al., 1956). The column was calibrated for molecular mass using dextrans with M_rs of 81.6×10^3, 26.6×10^4, 5.0×10^5 and 2.0×10^6.

4.6. *Per*-O-*methylation of polysaccharides*

The sample were partially *O*-methylated using Me_2SO_4-aqueous NaOH (Haworth, 1915) and the process completed with NaOH–Me_2SO–MeI (Ciucanu and Kerek, 1984). The per-methylated polysaccharides were converted into partially *O*-methylated alditol acetates by sucessive treatments with 3% MeOH-HCl for 2 h at 100 °C, 0.5 M H_2SO_4 for 14 h at 100 °C, reduction with $NaBH_4$ and acetylation with Ac_2O-pyridine. The products were examined by capillary GC–MS, as described in Section 4.1, and identified by their typical electron impact breakdown profiles and retention times (Jansson et al., 1976).

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