Carbohydrate Research, 142 (1985) 237–251
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

COMPARATIVE STUDIES ON THE POLYSACCHARIDES OF *Cladonia alpestris* (REINDEER MOSS), *Cladonia confusa*, AND *Cladonia amaurocraea*†

MARCELO IACOMINI, CLAUDETTE L. SCHNEIDER,
Departamento de Bioquímica, Universidade Federal do Paraná, C.P. 19046, 80.000 Curitiba (Brazil)

AND PHILIP A. J. GORIN*
Plant Biotechnology Institute, National Research Council, Saskatoon, Saskatchewan, S7N OW9 (Canada)

(Received October 10th, 1984; accepted for publication, December 26th, 1984)

ABSTRACT

The chemical structures of polysaccharide components of three species of the lichen genus *Cladonia* were compared. *C. alpestris* and *C. confusa* are similar in overall growth appearance despite different habitats, and each contains traces of water-insoluble nigeran. The residual lichens gave almost pure D-galacto-D-mannans isolated via insoluble Cu complexes formed with Fehling solution. They were not identical but structurally related having (1→6)-linked α-D-mannopyranosyl main-chains substituted in different patterns by β-D-galacto- and α-D-mannopyranosyl groups. Supernatant solutions of the Fehling-solution precipitation contained high proportions of β-D-galactofuranosyl residues. The polysaccharide of *C. alpestris* contained consecutive (1→2)-linked α-D-mannopyranosyl units substituted in the 6-position by β-D-galactofuranose, whereas that of *C. confusa* was a D-galactan with both pyranosyl and furanosyl forms. The D-glucan component of *C. amaurocraea* was isolated together with D-galacto-D-mannan as insoluble Cu complexes. The former was isolated in good yield and proved to be water-insoluble pustulan. The galactomannan had the same overall structure as those of *C. alpestris* and *C. confusa*, but showed differences according to the ^{13}C-n.m.r. spectra.

INTRODUCTION

In 1955, Aspinall *et al.*[1] examined the chemical structures of polysaccharides of reindeer moss *(Cladonia alpestris)*. The lichen, which was collected in Norway, is widely distributed throughout northern regions of Europe, America, and Asia. Polysaccharides were isolated following successive extractions with 5 and 24% cold aqueous potassium hydroxide, and each fraction contained galactose, mannose, and glucose. The first fraction (2.2% yield) contained these components in a

†NRCC No. 23678.
*Present address: Departamento de Bioquímica, Universidade Federal do Paraná, C.P. 19046, 80.000 Curitiba, Brazil.

0008-6215/85/$ 03.30

13:34:52 ratio, whereas the second fraction (1.3% yield) had a 13:47:40 ratio, indicating some degree of heterogeneity. Consistent with this, the first preparation, on treatment with Fehling solution, gave an insoluble Cu complex whose component polysaccharide had a diminished glucose content (15:50:28 ratio). Methylation analysis of both preparations gave mixtures of *O*-methyl aldoses that were qualitatively similar, as indicated by paper chromatography. Some of these components were characterized by crystallization or formation of crystalline derivatives, indicating the presence of nonreducing end-groups of galacto-, manno-, and glucopyranose, and 2,6-di-*O*-substituted mannopyranosyl units. Interestingly, the isolation of a mono-*O*-methylmannose suggested the presence of highly branched tri-*O*-substituted mannopyranosyl residues. As this investigation had only been intended to be preliminary and because the techniques of polysaccharide analysis have improved during the past 30 years, the polysaccharides of *C. alpestris* have now been reexamined by use of a sample collected in the boreal region of Central Saskatchewan, Canada. Also, *Cladonia confusa* and *Cladonia amaurocraea* (Central Saskatchewan) were collected and their polysaccharides examined in order to carry out a comparative study within the same genus. *C. confusa* is of significance as it has a growth form similar to that of *C. alpestris*, but grows in a region having drastically different climatic conditions, namely in Ilha do Mel, Paraná, Southern Brazil.

EXPERIMENTAL

Lichens. — *C. alpestris* and *C. amaurocraea* grew in close proximity in a forest clearing close to the west shore of Mountain Lake, Stanley Mission, Saskatchewan, Canada. *C. confusa* was collected from Ilha do Mel, Paraná, Brazil. The exact locale was near a mangrove swamp in a flat, sandy part of Praia do Belo, which is a beach facing the mainland.

Aqueous methanol extraction of lichens and analysis of soluble polyols. — A sample of each lichen (1.0 g) was treated under reflux with 2:1 (v/v) benzene–ethanol (20 mL), a process which dissolved 20 mg of material. The residual lichen was then extracted with 80% aqueous methanol (20 mL) under reflux and, following evaporation and de-ionization, the extract was examined on a paper chromatogram in 2:1:1 (v/v) butanol–ethanol–water (spray: ammoniacal $AgNO_3$). The spots corresponded to arabinitol and a small proportion of mannitol. In the case of *C. confusa*, another spot having R_{GAL} 0.6 was also detected.

The extracts were converted into mixtures of polyol acetates by treatment with acetic anhydride–pyridine at 100°. The products were analyzed[2] in a g.l.c. column (120 × 0.4 cm i.d.) of 3% (w/w) ECNSS-M on Chromosorb W (80–100 mesh), from 130 to 180° (4°/min, then hold). The presence of arabinitol and mannitol was confirmed.

Quantitative analyses were carried out by extraction of samples (1.0 g) of lichens in the presence of allitol or xylitol (10.0 mg) as internal standards. Extracts

were acetylated and the products submitted to g.l.c.

Preparation of polysaccharides from C. alpestris *and* C. confusa. — Lichen samples (1.0 g), previously extracted with benzene–ethanol, were treated with water (50 mL) at 100° for 6 h. Following filtration, each filtrate was centrifuged rapidly while hot and then evaporated to 3 mL. The solutions were frozen and then allowed to thaw in a refrigerator overnight, resulting in the formation of insoluble polysaccharides. These were centrifuged off, washed twice with ice-cold water, and dried. Supernatant solutions were evaporated to 2 mL and soluble polysaccharides precipitated by addition of the solution to an excess of ethanol (10 mL).

Remaining lichen was treated with 2% aqueous KOH (10 mL) at 100° for 1 h. The solutions were made neutral with acetic acid and filtered, and the filtrates evaporated to a small volume. Extracted polysaccharides were precipitated by addition to excess ethanol. They were dissolved in water (5 mL), Fehling solution (10 mL) was added, and the precipitates which formed were filtered off and washed successively with aqueous 2% KOH and methanol. The copper complexes were decomposed by shaking in an aqueous suspension of Amberlite IR-120 (H^+) cation exchange resin, which was filtered off, the filtrates were evaporated to 1 mL, and polysaccharides precipitated by addition of the solution to an excess of ethanol.

The supernatant solutions obtained after filtration of the Cu complexes were dialyzed against tap water and then de-ionized with a mixed-bed, ion-exchange resin. The solutions were evaporated to a small volume and added to an excess of ethanol to precipitate the polysaccharides.

Preparation of polysaccharides from C. amaurocraea. — A mixture of glucan and heteropolysaccharide was isolated from lichen by successive alkaline extraction and precipitation of Cu complexes, as just described. In the course of evaporation of the filtrate, obtained following treatment with Amberlite IR-120 cation-exchange resin, a precipitate formed. In order to facilitate isolation of this material, the evaporation was continued to a volume of 5 mL, and the mixture frozen and gradually thawed overnight in a refrigerator. Insoluble glucan was centrifuged off, and the resulting pellet washed twice with cold water and dried. The supernatant solutions were combined, evaporated to a small volume, and added to an excess of ethanol to give a precipitate consisting of D-galacto-D-mannan contaminated with a small proportion of D-glucan. Yields, specific rotations (in H_2O for water-soluble and in 2% aqueous NaOH for water-insoluble polysaccharides, *c* 0.2%), sedimentation coefficients (as 0.45% solutions in 0.5M aqueous NaOH), and sugar compositions are recorded in Results.

Sugar composition of polysaccharides. — The sample of polysaccharide was hydrolyzed with 2M trifluoroacetic acid for 18 h at 100° and the solution evaporated. The residue was converted into alditol hexaacetates by successive $NaBH_4$ reduction and acetylation with acetic anhydride–pyridine at 100°. Mixtures were analyzed by g.l.c. under conditions described earlier for alditol acetates derived from aqueous methanol extracts.

Methylation analysis of polysaccharides. — Samples were subjected to

methylation analysis under conditions previously outlined[3], *i.e.*, successive methylations by the methods of Haworth[4] and Kuhn *et al.*[5], and conversion of the methylated products into mixtures of partially *O*-methylated alditol acetates that were examined by g.l.c.–m.s.[6] using a capillary column coated with 3:1 or 1:3 OV-225–OV-17. The peaks were identified by e.i.–m.s. spectra[7] and retention times, and final identification was obtained by co-injection with standards.

1,2,4,5,6-Penta-*O*-acetyl-3-*O*-methylmannitol was identified as a product of methylation analyses carried out on galactomannans, a finding that is ambiguous because the 3-*O*-methyl is identical with the 4-*O*-methyl isomer. *O*-Methylhexose mixtures were therefore converted into acetylated methyl glycosides and the resulting methyl 2,4,6-tri-*O*-acetyl-3-*O*-methylmannoside identified by g.l.c.–m.s. The conditions used for g.l.c. differed from those described in a previous publication[3], where a conventional ECNSS-M column was used. This was replaced by a capillary column coated with DB-210; injections were made in the split mode at 50° and a rapid program (40°/min) to 225° (hold) was carried out. The carrier gas was helium (linear velocity, 22 cm/s). The retention times of the principal peaks were 16.3 min, for the 3-*O*- and 20.5 min for the 4-*O*-methyl isomer. A much improved resolution was observed over that of the conventional method.

Partial acetolysis of D-*galacto*-D-*mannans*. — The polysaccharides were partly acetolyzed according to the method of Lee and Ballou[8]. The products were examined on paper chromatograms in 2:1:1 (v/v) butanol–ethanol–water (spray, *p*-anisidine hydrochloride). Under these conditions and when a chromatogram was run for 3 days, 2-*O*-α-D-galactopyranosyl-D-mannose migrated a little faster than 2-*O*-α-D-mannopyranosyl-D-mannose, and the spots could be distinguished. The trisaccharide *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose is sufficiently mobile in this solvent to allow a reliable comparison with authentic material.

Smith degradation of polysaccharides. — Degradations were carried out using hydrolytic conditions, as previously described[3].

Degradation of D-*galacto*-D-*mannan to (1→6)-linked* α-D-*mannopyranan mainchains*. — Galactomannans (100 mg) were treated with 0.16M H_2SO_4 (10 mL) for 3 h at 100°. The hydrolyzates were made neutral ($BaCO_3$), filtered, concentrated to 0.5 mL, and the polysaccharide isolated by precipitation with an excess of ethanol.

Partially hydrolyzed polysaccharide (20 mg), dissolved in water (1.0 mL), was treated with a solution (0.2 mL) of jack-bean α-D-mannosidase (Sigma Chemical Co.) containing 0.68 unit of enzyme (1 unit hydrolyzes 1.0 μmol of *p*-nitrophenyl α-D-mannopyranoside/min at pH 4.5 and 25°). The product was heated at 100° for 5 min and de-ionized, the solution evaporated to a small volume, and the polysaccharide isolated by precipitation with an excess of ethanol.

^{13}C-N.m.r. spectroscopy. — The spectra were recorded under the conditions previously described[3]. The water-insoluble polysaccharides were examined as solutions in D_2O containing 2% of NaOD, whereas the water-soluble polysaccharides

were examined as D_2O solutions; all spectra were recorded at 70°. Chemical shifts (δ) are expressed relative to the resonance of Me_4Si obtained in a separate experiment.

RESULTS

Components of Cladonia *spp. soluble in aqueous methanol.* — Powdered lichen was shaken in 2:1 (v/v) benzene–ethanol, a process that extracted 2% of soluble material in each case. The insoluble residue was heated under reflux in methanol containing 20% of water to give rise to a soluble fraction containing hexitol and pentitol, according to paper chromatography. G.l.c.–m.s. of the derived acetates, carried out in the presence of allitol hexaacetate (internal standard), identified arabinitol and mannitol. The respective yields were as follows: *C. alpestris* (1.4, 0.3%), *C. amaurocraea* (1.5, 0.4%), and *C. confusa* (2.2, 0.4%). The extract of the last-named lichen also contained a component whose chromatographic mobility on paper corresponded to that of a disaccharide polyol. Values of protein content as Kjeldahl nitrogen and amino acid analysis data of benzene–ethanol extracted lichen are presented in Table I. The protein contents are low, as

TABLE I

AMINO ACID COMPOSITION OF LICHENS

Amino acid	*Molar composition (%)*		
	C. alpestris	C. confusa	C. amaurocraea
Tryptophan	1.0	1.2	1.2
Lysine	4.7	4.2	4.6
Histidine	2.0	1.5	1.7
Ammonia	1.4	1.7	1.5
Arginine	3.9	3.1	4.2
Aspartic acid	7.9	8.8	8.8
Threonine	4.3	4.0	4.4
Serine	4.4	4.5	4.3
Glutamic acid	13.4	12.6	14.1
Proline	3.5	4.1	3.9
Glycine	4.5	4.4	4.5
Alanine	5.5	5.5	5.6
Half cystine	1.2	1.2	1.2
Valine	4.4	4.6	4.3
Methionine	1.5	1.7	1.6
Isoleucine	3.2	3.1	3.3
Leucine	5.8	5.6	5.9
Tyrosine	3.9	3.7	3.7
Phenylalanine	4.1	3.8	3.9
Total	80.6	79.3	82.7
Protein (%)	2.69	2.25	2.69
Nitrogen (%)	0.43	0.36	0.43

compared with those of plants, being 2.25–2.69%. Whether these values show a seasonal variation is yet to be determined.

Polysaccharides of C. alpestris. — Powdered *C. alpestris*, previously extracted with benzene–ethanol, was extracted further with boiling water, a process that solubilized a D-glucan. The solution was concentrated to a small volume, which was frozen and then thawed, resulting in formation of an insoluble polysaccharide (0.12% yield). This contained glucose (91%), mannose (5%), and galactose (3%), and was homogeneous on ultracentrifugation which indicated a sedimentation coefficient of 2.1 *S*. The D-glucan had the α configuration since its ^{13}C-n.m.r. spectrum contained two C-1 signals at the typically high field[9] of δ 100.8 and 101.8. On methylation, followed by conversion into *O*-methylglucitol acetates, and analysis of the product by g.l.c.–m.s. using a capillary column coated with 1:3 OV-225–OV-17, 2,3,6- and 2,4,6-tri-*O*-methyl derivatives were detected. Their relative proportions indicated that (1→3)- and (1→4)-linked glucopyranosyl units were present in a 1:1 ratio. These were distributed regularly along a linear chain, as a Smith degradation incorporating mild hydrolytic conditions gave, on a paper chromatogram, product showing a single spot corresponding to 2-*O*-α-D-glucopyranosyl-D-erythritol.

The mother liquor of the extract that yielded the insoluble D-glucan (nigeran) contained a polysaccharide (2.0% yield) consisting of glucose (41%), mannose (32%), and galactose (26%), which suggests the presence of a galactomannan. In

TABLE II

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

O-Methylalditol[b]	*Polysaccharide of*				
	C. alpestris			C. confusa *(Fraction C[c])*	C. amaurocraea *(Fraction F)*
	Fraction A[c]	*Fraction B[d]*	*Fraction B[e]*		
2,3,4,6-Me_4-Man	29	4	6	28	17
2,3,5,6-Me_4-Gal	3	37		1	3
2,3,4,6-Me_4-Gal	13	2	4	23	19
3,4,6-Me_3-Man	5		55	5	17
2,4,6-Me_3-Glc		17	7	2	
2,3,6-Me_3-Man	7			4	1
2,3,6-Me_3-Gal	3	11		2	6
2,3,4-Me_3-Man	6	3	14	4	2
2,3,4-Me_3-Glc					16
2,3,6-Me_3-Glc	4	3	7	3	
2,3-Me_2-Man	2	1		4	7
3,4-Me_2-Man	16	16	7	10	2
3-Me-Man	12	4		14	9

[a]Proportion (%) of peak area relative to total peak areas. [b]Analyzed as peracetate. [c]Insoluble Cu complex. [d]Fehling soluble. [e]Acid degraded.

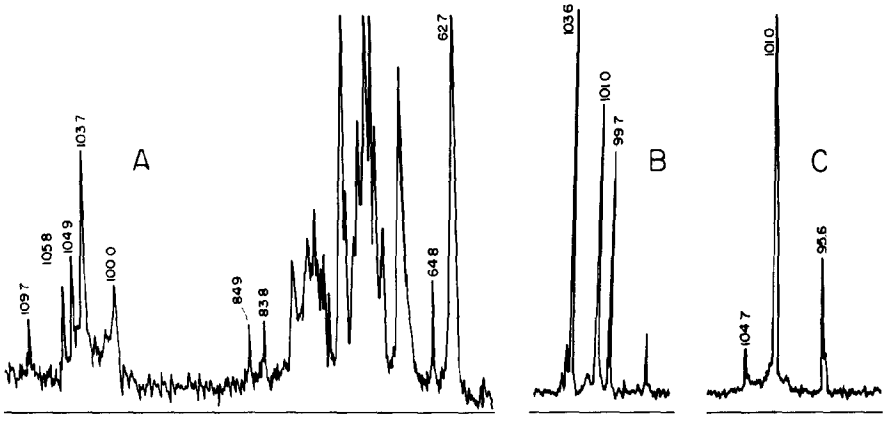
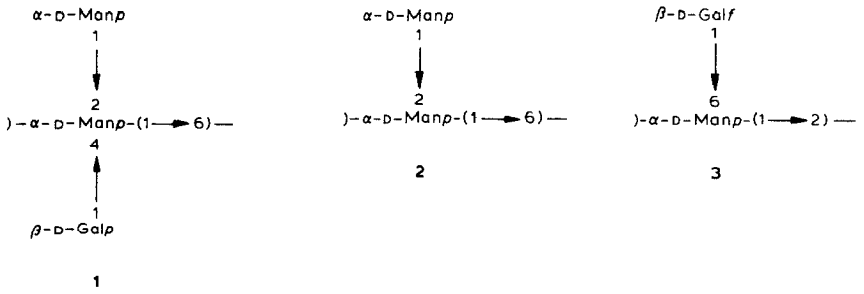


Fig. 1. ^{13}C-N.m.r. spectra (δ) of: (A) *C. alpestris* D-galacto-D-mannan isolated *via* its water-insoluble Cu complex, (B) partial hydrolysis product, and (C) material formed on further treatment with jack-bean α-D-mannosidase.

an attempt to isolate the galactomannan component(s) in a higher yield, the hot-water-insoluble lichen material was subjected to a more vigorous extraction procedure. Treatment with 2% aqueous KOH at 100°, followed by neutralization (AcOH), and ethanol precipitation, gave a polysaccharide (9.6% yield) containing mannose (49%), galactose (28%), and glucose (23%). By following the method of Aspinall *et al.*[1], an insoluble Cu complex was obtained by the action of Fehling solution. This complex gave Fraction A (33% yield, based on KOH extract) containing mannose (66%), galactose (24%), and little glucose (9%). It had $[\alpha]_D^{25}$ +52° and a sedimentation coefficient of 6.4 *S*. On methylation analysis, Fraction A gave at least eleven *O*-methylalditol acetates (Table II), indicating a complex structure very different from that of the D-galacto-D-mannan of *Cetraria islandica*[3]. This complexity was reflected in the ^{13}C-n.m.r. spectrum which contained eight signals in the C-1 region, each of which representing at least one different structure (Fig. 1A). According to the methylation analysis, Fraction A is highly branched with non-reducing end-groups of mannopyranose (29%), galactopyranose (13%), and galactofuranose (3%). Their total proportion corresponds to that of the mannan core which contains 4,6-di- (2%), 2,6-di- (16%), and 2,4,6-tri-substituted (12%) units of D-mannopyranose, each serving as branch points. Since the characterization of 3-*O*-methylmannitol acetate, which arose in the methylation analysis, did not distinguish between the formation of 3-*O*- and 4-*O*-methylmannose intermediates, the mixture of partially *O*-methylated aldoses was converted into the methyl glycoside acetates. G.l.c. analysis[3] showed that only the 3-*O*-methyl derivative was present. The only information obtainable on the D-glucose-containing component was that it contained 4-substituted glucopyranosyl units (Table II). Information on the structure of the mannan core was obtained by partial acid hydrolysis of Fraction A, which gave rise to an ethanol-insoluble moiety (21% yield). It contained mannose (87%), glucose (9%), and galactose (4%), and had $[\alpha]_D^{25}$ +69°. Its ^{13}C-n.m.r. spectrum

(Fig. 1B) indicated a branched structure with nonreducing end-groups (δ 103.6), and 6-substituted (δ 101.0) and 2,6-disubstituted (δ 99.7) units of α-D-mannopyranose. Such shifts are typical for C-1 atoms occurring in similar structures of yeast mannans and derived oligosaccharides[10]. Treatment of the degraded polysaccharide with jack-bean α-D-mannosidase provided a polymer whose ^{13}C-n.m.r. spectrum contained a C-1 signal typical[10] of a (1→6)-linked α-D-mannopyranan at δ 101.0. Other signals, which represent the α and β forms of the reducing end of the low-molecular-weight molecule, were observed (Fig. 1C) in the region of δ 95.6.

On the basis of these data and the formation of (1→2)-linked mannobiose and mannotriose on partial acetolysis, it is not possible to propose a complete structure for the galactomannan of Fraction A. However, the main chain consists of (1→6)-linked α-D-mannopyranosyl residues substituted at O-2 by α-D-mannopyranosyl groups, but the mode of linkage of the nonreducing end-groups of mannopyranose and galactopyranose to the main chain remain to be determined completely. As the specific rotation of Fraction A (+52°) is close to that of an acid-degraded polysaccharide from which the D-galactosyl units had been removed (+69°), these units exist preponderantly in the β-D configuration. It seems likely that they are linked (1→4) to the main chain and perhaps to other mannopyranosyl units. This linkage probably occurs on the main-chain of D-mannopyranosyl residues that are also substituted at O-2 by an α-D-mannopyranosyl group (**1**). Monosubstitution occurs as in structure **2**. These structures are similar to those occurring in the *C. islandica* D-galacto-D-mannan[3]. However, the α-D-Gal*p*-(1→2)-α-D-Man*p* structure is absent, as shown by a partial acetolysis experiment[8].



The mother liquor, obtained after the precipitation step with Fehling solution, gave Fraction B (12% yield, based on total KOH extract). It contained a considerably higher proportion of galactose (49%), along with mannose (27%) and glucose (24%). The optical rotation value, $[\alpha]_D^{25}$ −31°, was much lower than that of Fraction A. Fraction B has a structure different from that of Fraction A, the former having a high proportion of D-galactofuranosyl units (methylation analysis; Table II) which are nonreducing end-groups (37%) or residues substituted at O-5 (11%). Fraction B gave low-field C-1 signals at δ 107.4–111.3 (Fig. 2A), indicative of the β-D configuration[11]. These units contribute to the low specific rotation of

−31°. However, α-D-galactopyranosyl units are also present since 2-*O*-α-D-galactopyranosyl-D-mannose was detected (paper chromatogram) following partial acetolysis. Partial acid hydrolysis of Fraction B removed galactosyl units to form a product (11% yield, $[\alpha]_D^{25}$ +45°), containing mannose (67%), galactose (4%), and glucose (29%). Its ^{13}C-n.m.r. spectrum (Fig. 2B) contained a C-1 region with a large signal at δ 102.2 corresponding to α-D-mannopyranosyl units substituted at O-2 and smaller signals of nonreducing end-groups (δ 103.8), and di-substituted residues[10] at O-2,6 (δ 99.9). These assignments were confirmed by methylation analysis (Table II), which indicated the presence of nonreducing (6%), 2- (55%) and 6-substituted (14%), and 2,6-di-substituted (7%) residues of mannopyranose, and glucopyranose units substituted at O-3 (7%) and O-4 (7%). Further degrada-

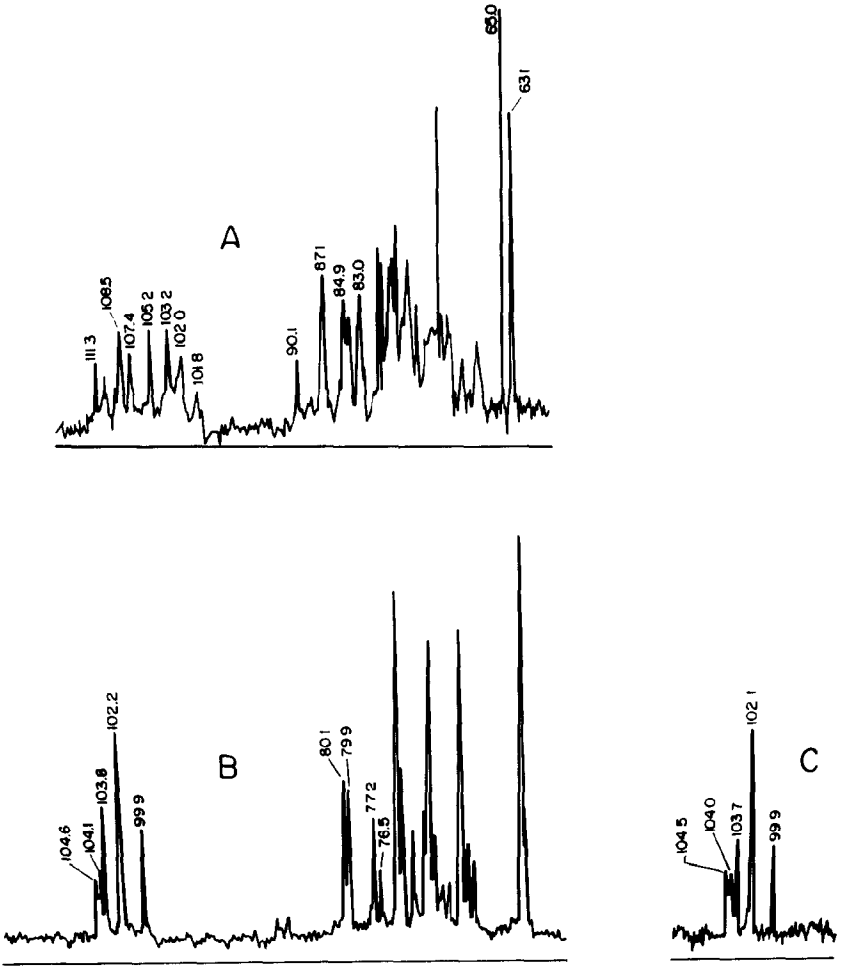


Fig. 2. ^{13}C-N.m.r. spectra (δ) of: (A) *C. alpestris* polysaccharide isolated from mother liquor of Fehling-solution precipitation, (B) partial hydrolysis product, and (C) material formed on further treatment with jack-bean α-D-mannosidase.

tion by jack-bean α-D-mannosidase apparently removed side chains since the ^{13}C-n.m.r. spectrum of the product (Fig. 2C) indicated an even higher proportion of (1→2)-linked α-D-mannopyranosyl units, which correspond probably to a main chain. These data, and the presence of 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methylmannitol and the absence of the 3,4,6-tri-*O*-methyl derivative in the methylation analysis carried out on Fraction B, show a substantial proportion of (1→2)-linked α-D-mannopyranosyl units substituted at O-6 by β-D-galactofuranosyl residues (**3**). Fraction B also contains much glucose (24%) which does not arise from the water insoluble α-D-glucan. A methylation analysis carried out on Fraction B showed that these structures were present as 17 and 3%, respectively, of the total polysaccharide. A β-D-configuration was indicated by the ^{13}C-n.m.r. spectrum of

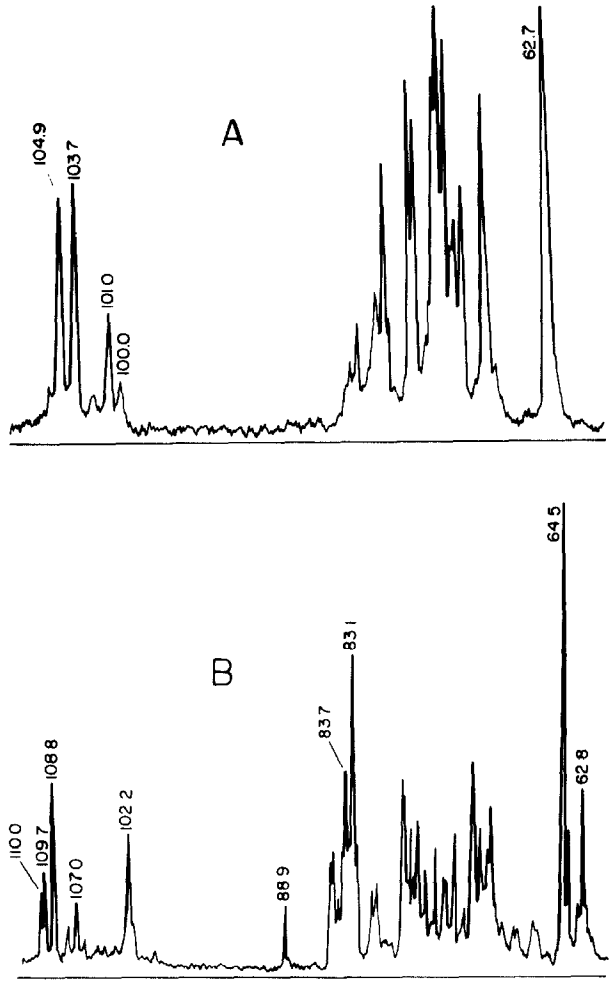


Fig. 3. ^{13}C-N.m.r. spectra (δ) of: (A) *C. confusa* polysaccharide isolated *via* water-insoluble Cu complex, and (B) material isolated from the mother liquor of Fehling-solution precipitation.

the acid-degraded material since the C-1 signals possibly attributable to the glucan are at a typically low field[9] (δ 104.1 and 104.6; Fig. 2B).

Polysaccharides of C. confusa. — As with *C. alpestris*, a water-insoluble α-D-glucan could be obtained, from the material washed with benzene–ethanol, by freezing hot-water extracts (0.3% yield). The resulting glucan had $[\alpha]_D^{25}$ +189°, and gave a ^{13}C-n.m.r. spectrum similar to that of the α-D-glucan of *C. alpestris*. Also, 3- and 4-substituted residues were present in a 53:47 ratio (methylation analysis) and were distributed regularly along a linear chain, as evidenced by the formation of 2-*O*-α-D-glucopyranosyl-D-erythritol on Smith degradation. The glucan has a structure like that of mycodextran.

Following a KOH extraction procedure similar to that used with *C. alpestris*, a polysaccharide was obtained, in 2.5% yield, containing glucose (12%), galactose (30%), and mannose (58%). The polysaccharide obtained *via* its water-insoluble Cu complex (1.6% yield) had $[\alpha]_D^{25}$ +54° and 3.4 *S*, and contained galactose (33%), mannose (63%), and glucose (4%). It was called Fraction C. Partial acetolysis gave α-D-(1→2)-linked mannobiose and mannotriose.

The ^{13}C-n.m.r. spectrum of Fraction C (Fig. 3A) showed that its structure was different from that of Fraction A, the corresponding component of *C. alpestris* (see spectrum of Fig. 1A); although the spectra contained common signals at δ 104.9, 103.7, and 100.0, their respective proportions were different. Methylation analysis (Table II) demonstrated the presence of nonreducing D-galactopyranosyl (23%) and D-mannopyranosyl (28%) end-groups and other principal components, namely D-mannopyranosyl substituted at O-2,6 (10%) and at 2,4,6 (14%) (characterized by the acetates of the methyl mannosides). Partial hydrolysis of Fraction C gave a polysaccharide ($[\alpha]_D^{25}$ +77°; yield 26%) containing mannose (88%), galactose (10%), and glucose (2%), whose ^{13}C-n.m.r. spectrum was similar to that of the corresponding material obtained from Fraction A of *C. alpestris* (see Fig. 1B). It showed the presence of nonreducing α-D-mannopyranosyl end-groups, and α-D-mannopyranosyl residues substituted at O-6 and 2,6-O. Further degradation of the polysaccharide by jack-bean α-D-mannosidase gave a material whose ^{13}C-n.m.r. spectrum corresponded to that of a low-molecular-weight, (1→6)-linked α-D-mannopyranan[10]. Thus, the polysaccharide contains a main chain substituted (**1** and **2**) as that present in the D-galacto-D-mannan of *C. alpestris*.

The polysaccharide obtained from the mother liquor of the Fehling-precipitation step (Fraction D) was isolated in only 0.15% yield, had $[\alpha]_D^{25}$ −47°, and contained galactose (86%), mannose (4%), and glucose (10%). Structural investigations were hindered by the lack of availability of Fraction D in more than milligram quantities. However, it is clearly different from the corresponding Fraction B isolated from *C. alpestris*, as shown by the high galactose content (86%) and unusual ^{13}C-n.m.r. spectrum (Fig. 3B) with two principal C-1 signals at δ 108.8 and 102.2. The 10% of glucose present seems, according to the methylation data (Table III), to arise from a linear polymer containing 3- (5%) and 4-substituted (6%) residues, the remainder consisting of a galactan. The latter product contains a high

TABLE III

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

O-Methylalditol	*Proportion of peak obtained from methylated polysaccharide (%)*
2,3,5,6-Me_4-Gal	30
2,3,4,6-Me_4-Gal	12
2,4,6-Me_3-Glc	5
2,3,6-Me_3-Gal	19
2,3,6-Me_3-Glc	6
2,4(3,5)-Me_2-Gal	30

[a]Fehling insoluble, Fraction D.

proportion of β-D-galactofuranosyl units since C-1 signals are present at the low field of δ 107.0–111.0. The methylation data are somewhat ambiguous, indicating 5- (19%) and 2,6-di-substituted D-galactofuranosyl (30%), or 4- (19%) and 3,6-di-substituted (30%) D-galactopyranosyl residues, respectively (Table III). However, D-galactopyranosyl nonreducing end-groups (12%) are definitely present. Residues of D-galactopyranose gave rise to the C-1 signal at δ 102.2, which is of an intermediate shift not sufficiently characteristic to distinguish between α- and β-D configuration. However, the specific rotation of Fraction D (−47°) is only marginally more positive than that of a pure β-D-galactofuranan[12] (−84°). This suggests a small rotational contribution, typical of β-D-galactopyranosyl units. Structures corresponding to those of Fraction D are likely present in Fraction B of *C. alpestris*, since the latter fraction gave rise to ^{13}C-n.m.r. signals at δ 102.0 and 108.5 (Fig. 2A).

Polysaccharides of C. amaurocraea. — An α-D-glucan, analogous to that obtained from *C. alpestris* by hot-water extraction and precipitation in the cold, was not present in *C. amaurocraea*. Instead, the hot-water-extracted polysaccharide (1% yield) contained mannose (20%), galactose (20%), and glucose (61%). However, it was found that the lichen contained a higher proportion of D-glucan, since hot aqueous KOH extraction gave, in 11% yield, a polysaccharide having mannose (36%), galactose (23%), and glucose (40%). On treatment with Fehling solution, 86% of the total carbohydrate precipitated as the Cu complex. This was isolated, shaken in aqueous suspension with Amberlite IR-120 (H^+) cation-exchange resin until dissolution, and the filtrate concentrated by evaporation resulting in precipitation of a polysaccharide. In order to obtain this material in better yield, the partly concentrated solution was frozen and thawed, and the insoluble material isolated (Fraction E; yield 29%, based on Fehling solution-precipitated polysaccharide). This proved to be a glucan contaminated with only traces of mannose and galactose (total 4%). It had $[\alpha]_D^{25}$ −35° corresponding to a β-D-glucan, and gave a single peak (3.0 *S*) on ultracentrifugation. Its ^{13}C-n.m.r. spectrum was identical to that of

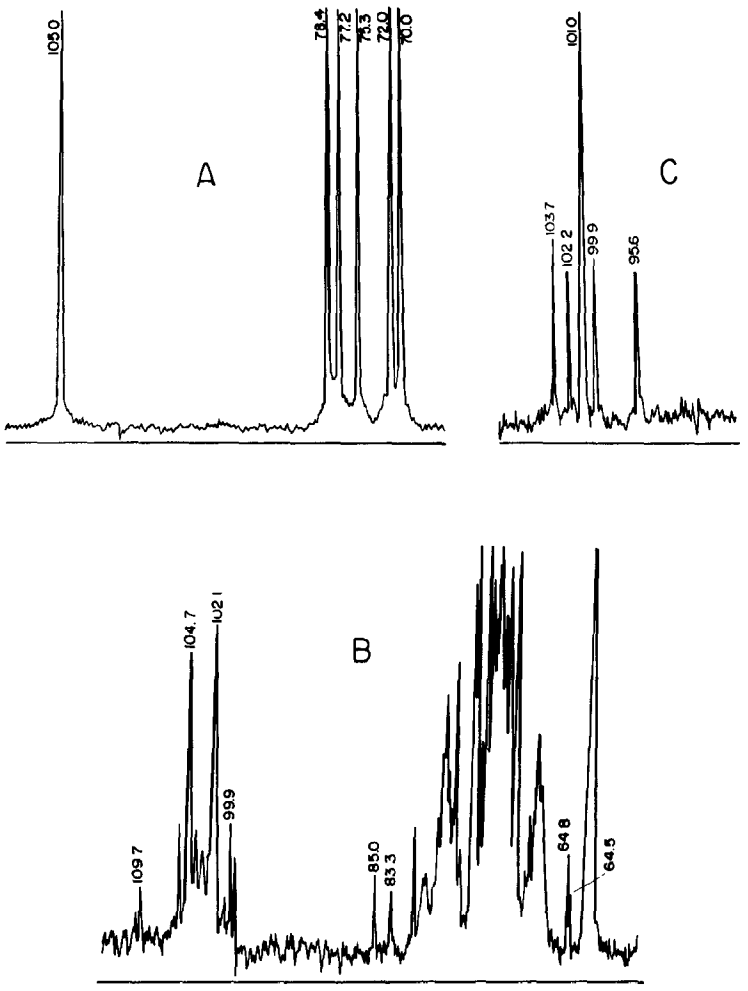


Fig. 4. ^{13}C-N.m.r. spectra (δ) of: (A) water-insoluble glucan and (B) water-soluble polysaccharide of *C. amaurocraea* isolated *via* insoluble Cu complexes, and (C) product formed on partial hydrolysis of the latter polysaccharide.

pustulan (Fig. 4A), a (1→6)-linked β-D-glucopyranan that is found, substituted with a small proportion of acetyl groups, in *Gyrophora esculenta* Miyoshi, *Lasallia papulosa* (Ach.) Llano[13], and unacetylated in *Umbilicaria pustulata*[14].

The water-soluble material, obtained from the Fehling solution-precipitated polysaccharide in 71% yield (Fraction F), contained mannose (50%), galactose (29%), and glucose (20%), had $[\alpha]_D^{25}$ +46°, and a sedimentation coefficient of 6.6 *S* different from that of the glucan component. The ^{13}C-n.m.r. spectrum showed two principal C-1 signals at δ 104.7 and 102.1 (Fig. 4B). Data obtained on methylation analysis (Table II) showed that Fraction F contained 16% of pustulan. The rest consisted of a galactomannan having, as principal structural components, non-

reducing mannopyranosyl (17%) and galactopyranosyl end-groups (19%), and 2- (17%), 4,6-di- (7%), 2,4,6-tri-substituted (9%) residues of mannopyranose. Thus, Fraction F contains a branched D-galacto-D-mannan whose main chain was shown to be a (1→6)-linked α-D-mannopyranan as follows. Partial acid hydrolysis gave, in 9% yield, a polysaccharide ($[\alpha]_D^{25}$ +76°) containing mannose (80%), galactose (6%), and glucose (14%). Its ^{13}C-n.m.r. spectrum contained C-1 signals at δ 103.7, 102.2, 101.0, and 99.9 (Fig. 4C), corresponding respectively to nonreducing α-D-mannopyranosyl end-groups, and 2-, 6-, and 2,6-di-substituted units of α-D-mannopyranose. The degraded polymer was treated with jack-bean α-D-mannosidase to form a polysaccharide whose ^{13}C-n.m.r. spectrum corresponded to that of a (1→6)-linked α-D-mannopyranan.

DISCUSSION

Each of the three species of *Cladonia*, chosen for the present study, contained small proportions of protein (2.3–2.7%), arabinitol (1.4–2.2%), and mannitol (0.3–0.4%), and thus derives little nutritive value from these components.

As in other lichens, D-glucan and D-galacto-D-mannan are present, and structural variation is possible from species to species. Two species having almost indistinguishable growth forms, *C. alpestris* (Central Saskatchewan) and *C. confusa* (Southern Brazil), gave a water-insoluble nigeran in 0.1–0.3% yield. However, their aqueous KOH-extracted galactomannan components, isolated *via* the insoluble Cu complexes formed with Fehling solution (3.2 and 1.6% yield, respectively), were different. Analysis based on monosaccharide composition, partial hydrolysis, specific rotation, methylation data, and partial acetolysis showed that each galactomannan contained a main chain of (1→6)-linked α-D-mannopyranosyl units. Some of these were substituted at O-2 by α-D-mannopyranosyl groups (**2**) or at O-4 by β-D-galactopyranosyl groups, and in some cases these gave rise to 2,4-di-substitution (**1**). More structural differences were found when the C-1 regions of the ^{13}C-n.m.r. spectra (Figs. 1A and 2A) were compared. In turn, these differed, from those of the D-galacto-D-mannans of *C. islandica* and *Ramalina usnea* previously investigated[3]. This variation from species to species will be further examined[15].

Structurally different polysaccharide components of *C. alpestris* and *C. confusa* were isolated in low yield (1.1 and 0.75%, respectively) from the supernatant solutions of the Fehling-solution precipitations. That of *C. alpestris* contained galactose, mannose, and some glucose, and a higher proportion of (1→2)-linked α-D-mannopyranosyl units, apparently in the form of a main-chain substituted at O-6 by β-D-galactofuranosyl units (**3**). Its ^{13}C-n.m.r. spectrum (Fig. 2A) had a complex C-1 region, differing from that of the D-galactan of *C. confusa*, which showed two main signals at δ 102.2 and 108.8 (Fig. 3B). However, these signals are common to both spectra. Although the D-galactan was isolated in a quantity too small to allow a full structural determination, it was found to contain both furanosyl and pyranosyl units.

C. amaurocraea contained two polysaccharides extractable with hot aqueous alkali and giving insoluble Cu complexes with Fehling solution. Virtually no polysaccharide was present in the mother liquor. Regeneration of the complexes gave water-insoluble pustulan, a (1→6)-linked β-D-glucopyranan previously found to be a component of other lichens, and a water-soluble D-galacto-D-mannan somewhat contaminated with pustulan. The galactomannan has an overall structure similar to those described earlier, but its ^{13}C-n.m.r. spectrum (Fig. 4B) showed a C-1 region differing from all those previously determined with the possible exception of that of the galactomannan of *R. usnea*[3].

ACKNOWLEDGMENTS

The authors thank Dr. Osório, Museo Nacional de Historia Natural, Montevideo (Uruguay) and Prof. W. Sheard, Dept. of Biology, University of Saskatchewan, Saskatoon (Canada) for identification of *Cladonia* spp., and Mr. L. Hogge and Mr. Mazurek, Plant Biotechnology Institute, National Research Council, Saskatoon, for g.l.c.–m.s. and ^{13}C-n.m.r. determinations, respectively.

REFERENCES

1 G. O. ASPINALL, E. L. HIRST, AND W. WARBURTON, *J. Chem. Soc.*, (1955) 651–655.
2 J. S. SAWARDEKER, J. H. SLONEKER, AND A. JEANES, *Anal. Chem.*, 43 (1965) 1602–1604.
3 P. A. J. GORIN AND M. IACOMINI, *Carbohydr. Res.*, 128 (1984) 119–132.
4 W. N. HAWORTH, *J. Chem. Soc.*, (1915) 8–16.
5 R. KUHN, H. TRISCHMANN, AND I. LÖW, *Angew. Chem.*, 67 (1955) 32–33.
6 P. A. J. GORIN, E. M. GIBLIN, G. P. SLATER, AND L. HOGGE, *Carbohydr. Res.*, 106 (1982) 235–238.
7 P. J. GAREGG, P.-E. JANSSON, B. LINDBERG, F. LINDH, J. LÖNNGREN, I. KVARNSTRÖM, AND W. NIMMICH, *Carbohydr. Res.*, 78 (1980) 127–132.
8 Y.-C. LEE AND C. E. BALLOU, *Biochemistry*, 4 (1965) 257–264.
9 L. D. HALL AND L. F. JOHNSON, *J. Chem. Soc., Chem. Commun.*, (1969) 509–510.
10 P. A. J. GORIN, *Can. J. Chem.*, 51 (1973) 2375–2383.
11 T. KOHAMA, M. FUJIMOTO, A. KUNIAKA, AND H. YOSHINO, *Agric. Biol. Chem.*, 38 (1974) 127–134.
12 W. N. HAWORTH, H. RAISTRICK, AND M. STACEY, *Biochem. J.*, 29 (1935) 612–621.
13 Y. NISHIKAWA, T. TAKEDA, S. SHIBATA, AND F. FUKUOKA, *Chem. Pharm. Bull.*, 17 (1969) 1910–1916.
14 B. DRAKE, *Biochem. Z.*, 313 (1943) 388–399.
15 P. A. J. GORIN AND M. IACOMINI, *Carbohydr. Res.*, 144 (1985) 253–267.