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OCCURRENCE OF D-*ARABINO*-HEXULOSONIC ACID IN POLYSACCHARIDES OF *CYTTARIA* SPECIES

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Key Word Index—*Cyttaria darwinii*, *C. harioti*; Cyttariaceae; structural elucidation; ^{13}C NMR spectroscopy; fungal polysaccharides; D-*arabino*-hexulosonic acid.

Abstract—A heteropolysaccharide (CD_1) was isolated by water extraction from *Cyttaria darwinii*, a parasite of *Nothofagus* spp., in 32% yield. The only neutral sugar component was D-glucose (94%), the other component being D-*arabino*-hexulosonic acid, a sugar acid not normally found in polysaccharides. The linkages between the glucosyl groups were determined by methylation analysis and the anomeric configurations by chromium trioxide oxidation of the acetylated polysaccharide. ^{13}C NMR spectroscopy proved to be useful in determining structural similarities between CD_1 and CH_1, isolated from the related species *C. harioti*.

INTRODUCTION

Cyttariaceae (Discomycetes) is a family of fungi confined to the Southern hemisphere, where the different species grow as obligate parasites of *Nothofagus* on which they produce characteristic tumours that infect the tree and finally cause its death. The systematic study of Argentine Cyttariales was carried out by Gamundi [1] and investigations on the chemistry of these fungi were undertaken in our laboratory in an attempt to support the taxonomic studies in establishing relationships among the species. *Cyttaria harioti* Fischer has been extensively studied [2–10]. *Cyttaria darwinii* is a species taxonomically related with *C. harioti*. The first chemical studies led to the isolation of a water-soluble α-glucan characterized as pullulan [11]. A heteropolysaccharide composed of D-glucose and D-*arabino*-hexulosonic acid was also isolated from the aqueous extract. Since hexulosonic acids are not normally found in fungi, structural studies on this polysaccharide were undertaken.

RESULTS

The heteropolysaccharide CD_1 was isolated as the major component of the aqueous extract of *C. darwinii* (32% of the fungus dry wt). After purification it was eluted as a single peak from Sephacryl S-300 and an average M_r of 135 000 was estimated by comparison with the elution volumes of known dextrans. The optical rotation of purified CD_1 was $[\alpha]_D$ +57° (1 M KOH; *c* 0.5). Neutral sugars accounted for 91–92% of the material (phenol–sulphuric acid method). D-Glucose was the only neutral sugar component since similar results were obtained by the phenol–sulphuric acid and the D-glucose oxidase methods. It was further characterized by GC of the corresponding alditol acetate. Characterization of D-*arabino*-hexulosonic acid was effected by paper chromatography and detection with *p*-anisidine hydrochloride (characteristic pink colour) and by GC as its trimethylsilyl derivative (same R_t as an authentic sample).

Methylated CD_1 was hydrolysed with sulphuric acid and the partially methylated glucoses thus obtained were characterized by GC and GC-MS of the alditol acetates, and comparison with authentic samples (Table 1). Although 2,3,6-tri-*O*-methyl and 2,3,4-tri-*O*-methylglucose could not be properly resolved by GC, the fragmentation pattern observed by GC-MS suggested the presence of both methylated sugars in the poorly resolved signal.

After reduction and acetylation, CD_1 was oxidized with chromium trioxide [12] in the presence of *myo*-inositol as internal standard. After 2.5 hr, most of the glucosyl units (80%) had been oxidized, while D-*arabino*-hexulosonic acid remained unaltered.

The ^{13}C NMR spectrum of the heteropolysaccharide CD_1 (Fig. 1a) showed two main signals in the anomeric region, corresponding to β-(1→3)- and α-(1→6)-linked D-glucose units (δ 104.2 and 99.4, respectively). At higher fields, two signals were observed for *O*-substituted C-3 (δ 88.1 and 87.4). The signal at δ 67.4 was assigned to *O*-substituted C-6. The ^{13}C NMR spectrum of the heteropolysaccharide isolated from the aqueous extract of the related species *C. harioti* Fischer [3–5] was run under the same conditions (Fig. 1b).

After isolation of the polysaccharides through ethanol precipitation of the aqueous extract, the supernatant solution was evaporated to dryness and methanol added to the residue. Paper chromatography of the insoluble material showed the presence of D-*arabino*-hexulosonic acid, which was further confirmed by GC of the corresponding *O*-trimethylsilyl derivative and comparison with an authentic sample.

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Table 1 Methylation studies of polysaccharide CD_1

R_t (min)	*O*-Methyl	%	Primary fragments (*m/z*)	Structural feature
4 07	2,3,4,6-	13 4	45, 117, 161, 205	Glc*p*-(1-
6.95	2,4,6-	23.9	45, 117, 161, 233	-3)-Glc*p*-(1-
7 88	2,3,6-	36.6	45, 117, 233	-4)-Glc*p*-(1-
7 88	2,3,4-		117, 161, 189, 233	-6)-Glc*p*-(1-
14 59	2,4-	26 0	117, 189	-3,6)-Glc*p*-(1-

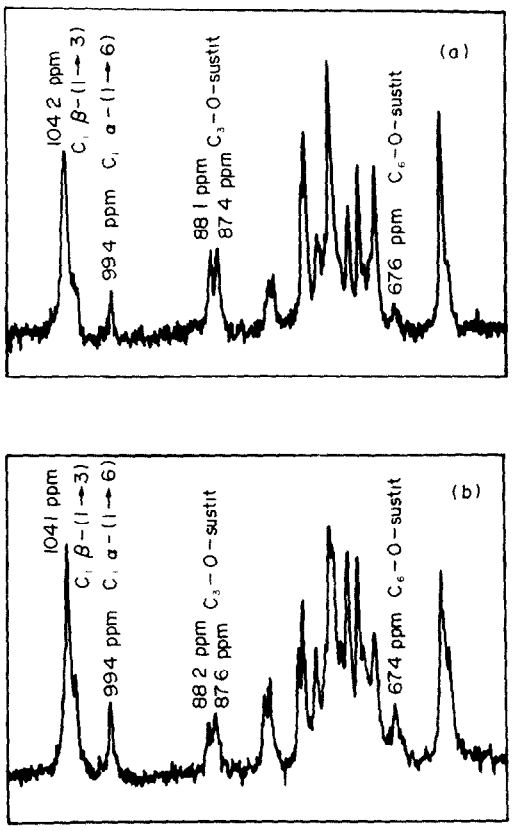

Fig. 1 ^{13}C NMR spectra of CD_1 from *C. darwinii* (a) and CH_1 from *C harioti* (b).

DISCUSSION

The presence of D-*arabino*-hexulosonic acid has not been frequently reported in natural products and its characterization in aqueous extracts of *C. harioti* and *C. johowii* [13] and as a component of the water soluble heteropolysaccharide (CH_1) from *C. harioti* [3–5] suggested that its occurrence could be considered as a characteristic feature of *Cyttaria* species. Taking into account the close taxonomical relationship between *C. harioti* and *C darwinii* [1] studies were undertaken on the latter species.

Preliminary composition analysis of CD_1 from *C. darwinii* showed that it was composed of D-glucose (94%) and D-*arabino*-hexulosonic acid, suggesting that it could be structurally related to CH_1 isolated from *C harioti*. Fructose, a minor sugar of CH_1, could not be detected in acid hydrolysates of CD_1. In the mother liquors from the precipitation of CD_1 and CD_2, D-*arabino*-hexulosonic acid was chromatographically detected, indicating its occurrence as a free component in *C. darwinii* stromata. The possibility of its release by autohydrolysis of the polysaccharide CD_1 could not be excluded.

Methylation analysis (Table 1) indicated the presence of the same type of linkages in CD_1 as in CH_1, although (1→4)-linkages appear to be in a higher proportion in CD_1. The characterization of tetra and di-*O*-methyl glucopyranoses is indicative of a branched structure. The difference in proportions could suggest the presence of D-*arabino*-hexulosonic acid as non-reducing terminal units.

In order to determine the anomeric configuration of the glucosyl residues, acetylated CD_1 was oxidized with chromium trioxide. Only 20% of the glucosyl residues remained unoxidized after 2 5 hr, suggesting a preponderance of β-linkages (β:α ratio, 4:1) as could be anticipated in view of the optical rotation. D-*arabino*-Hexulosonic acid residues were recovered unaltered after oxidation, but this result could not be related with the linkage configuration since the behaviour of methyl glycosides of ulosonic acids towards chromium trioxide oxidation has not been described.

The results obtained from the chemical studies of CD_1 suggested a close structural relationship with CH_1, which was further confirmed by ^{13}C NMR spectroscopy, both spectra are almost superimposable (Fig. 1). In the anomeric region, the β-configuration for the (1→3)-linkages and the α-configuration for the (1→6)-linkages could be unambiguously assigned. The (1→4)-linkages should also bear a β-configuration since no signal attributable to α-(1→4)-linked D-glucose units at *ca* δ100.6 [14] could be observed. The configuration of the linkages in CH_1 has been determined through characterization of the corresponding disaccharides, i.e, laminaribiose, *iso*-maltose and cellobiose [4]. We have shown that direct comparison of the ^{13}C NMR spectra of polysaccharides can be used to establish common structural features and can be used to relate species

EXPERIMENTAL

Material. Mature stromata of *C. darwinii* were collected in Parque Nacional 'Los Glaciares' (Santa Cruz, Argentina) in summer (January), under normal physiological conditions.

Analytical methods. All evapns were performed below 40° under red. pres. Small vols of aq. solns were lyophilized PC was performed by the descending method on Whatman No 1 paper with the following solvent systems (*a*) *n*-BuOH–pyridine–H_2O

(6·4·3), (*b*) *n*-BuOH–EtOH–H_2O (5:2:2) Detection was effected with (i) $AgNO_3$–NaOH [15] and (ii) *p*-anisidine HCl [16] GC was performed with a Hewlett–Packard 5830 GC equipped with a FID on glass columns packed with (a) 3% ECNSS-M on Gas Chrom Q (0.2 × 180 cm), (b) 3% OV-17 on Chromosorb W AW DMCS (60–80) (0 2 × 180 cm). GC-MS was performed with a 8% NPGS column in a Varian 1440 chromatograph coupled to a Varian MAT 166 data system. The trimethylsilyl ethers were prepared by dissolving the dried samples in pyridine and heating with Tri-Sil (Pierce) for 15 min at 60°.

Total carbohydrate was determined by the phenol–sulphuric acid method [17] and D-glucose in the hydrolysates was estimated by the D-glucose oxidase–peroxidase procedure [18].

^{13}C NMR spectra were recorded at 25.2 MHz. Samples were dissolved in 3·1 H_2O–D_2O (50 mg/0.4 ml) at room temp. 1,4-Dioxane was used as the ext. standard (67.4 ppm downfield from the signal of TMS).

Isolation, purification and properties of the heteropolysaccharide. Powdered, dried stromata of *Cyttaria darwinii* (14.2 g) were extracted with H_2O (0.7 l) in a Waring blender at room temp. and then centrifuged. The extraction was repeated twice; EtOH (to 55%) was added, and the insoluble product (5.8 g; 41%) was collected by centrifugation and dried by solvent exchange.

Portionwise addition of EtOH to a soln of the crude product (0.3 g) in H_2O (150 ml) resulted in pptn at 37–38% EtOH. Centrifugation at 2500 r.p.m. for 20 min gave the heteropolysaccharide CD_1 (32% of the fungus dry wt). After concentration, lyophilization of the initially cloudy supernatant liquor afforded polysaccharide CD_2, characterized as pullulan [11].

Heteropolysaccharide CD_1 was purified by repeated redissolution in H_2O and reprecipitation with EtOH until the specific rotation remained constant. The purified polysaccharide was subjected to gel permeation on a column of Sephacryl S-300 (1.5 × 92 cm) in Pi buffer, pH 7.2, 0.1% SDS. Blue dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used for the determination of the void vol. The average M_r was estimated by using dextrans of known M_rs (Sigma)

Acid hydrolysis. Acid hydrolysis of CD_1 was performed by the following procedure· (a) heating the sample with 90% HCOOH for 3 hr at 100°; (b) diluting the acid soln with an equal vol. of water and heating for a further 3 hr at 100°; (c) evapg the sample to a syrup; (d) rehydrolysing it with 0.5 M H_2SO_4 for 24 hr at 100°. The hydrolysate was neutralized with $BaCO_3$ and decationized with Dowex 50 (H^+ resin). It was analysed for total carbohydrate and D-glucose content. PC was performed by the descending method with solvents *a* and *b*; detection was effected with (i) and (ii) Sugar composition was estimated by GC under the following conditions. (a) as alditol acetates [19], using column *a* (Ti 200°, Td 210°, Tc 190°; flow 24 ml of N_2/min); (b) as trimethylsilylethers using column *b* (Ti 290°, Td 310°, Tc programmed from 120 to 200°, rate 4°/min; flow, 30 ml of N_2/min).

Methylation analysis. The polysaccharide (15 mg) was methylated by the method of ref. [20]. The fully methylated product was hydrolysed with 72% H_2SO_4 (3 ml) for 2 hr at 25° and then for 4 hr under reflux with 12% H_2SO_4 The partially methylated glucoses were analysed by GC as alditol acetates [19], using column *a*, Tc 155°, flow 29 ml of N_2/min). Further characterization was achieved by GC-MS (column *a*, Ti 250°, Tc 225°, flow 25 ml of He/min)

Chromium trioxide oxidation. CD_1 (5 mg) was reduced with $NaBH_4$ (5 mg, 25°, 24 hr). After dialysis and lyophilization, it was dissolved in formamide (1 ml) and Ac_2O (1 ml) and pyridine (1 ml) were added. After being stirred for 15 hr at room temp, the soln was evapd and the residue partitioned between H_2O and $CHCl_3$. The organic layer was concd and reacetylated in the same way The acetylated product was dissolved in HOAc (4.5 ml), CrO_3 (300 mg) was added and the mixture was kept at 50° for 2.5 hr. It was diluted with water to stop the oxidation, and extracted with $CHCl_3$. The dried extract was deacetylated with NaOMe, hydrolysed with 0.5 M H_2SO_4 (8 hr, 100°) and analysed by PC (solvents *a* and *b*; reagents i and ii).

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