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Note

Characterization of a pullulan in *Cyttaria darwinii*

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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 tumors that infect the trees and finally cause their death. In previous papers, we have reported the isolation, and structural studies, of three polysaccharides[1–4] and a glycopeptide[5] from *Cyttaria harioti* Fischer, and a β-fructoglucan[6] from *Cyttaria johowii* which exhibits antitumor activity. We report herein the first chemical studies on *Cyttaria darwinii* which led to the isolation of a water-soluble α-glucan characterized as pullulan.

Stromata of *Cyttaria darwinii* were extracted with water at room temperature. Fractional precipitation with ethanol allowed the separation of two polysaccharide fractions, CD_1 having $[\alpha]_D$ +57° (potassium hydroxide) and CD_2, $[\alpha]_D$ +189° (water). Preliminary, hydrolytic analysis of CD_1 showed that this fraction was a heteropolysaccharide composed of D-glucose and D-*arabino*-hexulosonic acid; it is not further discussed herein.

The second fraction, CD_2, was purified by repeated redissolution in water and precipitation with ethanol until the optical rotation remained unchanged. The purified polysaccharide was precipitated in 91% yield from aqueous solution over a narrow range (38–40%) of ethanol concentration, and was eluted as a single peak from Sepharose 4B. By comparison with the elution volume of known dextrans, an average molecular weight of 140,000 was estimated (see Fig. 1). The high specific rotation, together with infrared absorption[7] at 850 cm^{-1} (but not at 890 cm^{-1}), are characteristic of the α-D configuration. On acid hydrolysis, the polysaccharide gave D-glucose as the sole product (p.c. and D-glucose oxidase method[8]), further characterized by g.l.c of the corresponding alditol acetate.

The polysaccharide was methylated by the Hakomori[9] method, and hydrolyzed as recommended by Lindberg and coworkers[10]. The partially methylated glucoses thus obtained were characterized by g.l.c. and g.l.c.–m.s. of the alditol

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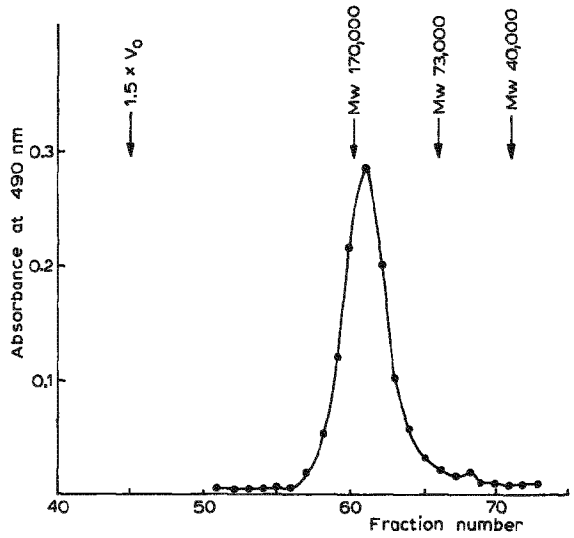

Fig. 1. Determination of the molecular weight of the pullulan from *Cyttaria darwinii* on a column of Sepharose 4B.

acetates, and comparison with authentic samples, as 2,3,4,6-tetra-*O*-methyl- (0.15%), 2,3,6-tri-*O*-methyl- (63.1%), and 2,3,4-tri-*O*-methyl-D-glucopyranose (36.7%). These results indicated a very linear structure having 4-*O*- and 6-*O*-substituted D-glucopyranosyl residues.

The acetylated α-D-glucan was partially acetolyzed by the method recommended by Kocourek and Ballou[11]. After deacetylation, p.c. and t.l.c. showed glucose, maltose, maltotriose, and only traces of higher oligosaccharides. The mixture was separated on a column of Bio Gel P-2, and maltose was further characterized, after reduction, by g.l.c. of the corresponding per-*O*-(trimethylsilyl) derivative. Methylation of the trisaccharide fraction, followed by acid hydrolysis, afforded 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-D-glucopyranose in 1:2 molar ratio, confirming its structure. The isolation of maltotriose, together with the fact that no significant amounts of higher oligosaccharides were obtained, indicates no more than two consecutive (1→4) linkages. Taking into account the higher lability of (1→6) linkages to acetolysis conditions[12], and the results of the methylation studies, a backbone of maltotriose units joined by (1→6) linkages could be postulated for the α-D-glucan from *Cyttaria darwinii*. This structure is similar to that of pullulans elaborated by various strains of *Pullularia pullulans*[13–15] and *Tremella mesenterica*[16], and to the α-D-glucan isolated from *Cyttaria harioti*[4].

This pullulan-type structure for the α-glucan from *Cyttaria darwinii* was confirmed by pullulanase treatment. Pullulans from *Pullularia pullulans* contain a minor sub-unit having three consecutive (1→4) linkages[17] which afford maltotetraose on enzymic hydrolysis. This structural feature was not observed in the pullulan-like α-glucans from *C. harioti*[4] and *C. darwinii*.

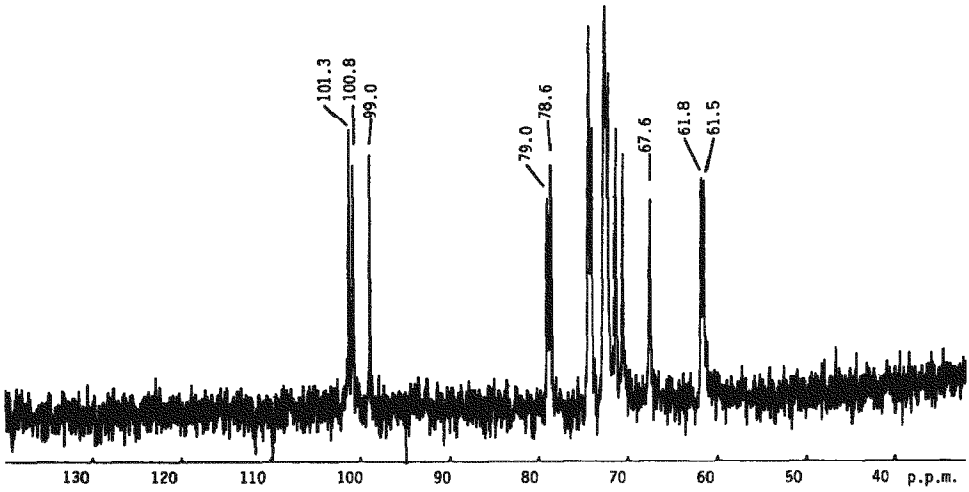

Fig. 2. ^{13}C-N.m.r. spectrum of the pullulan from *Cyttaria darwinii*.

The anomeric region of the ^{13}C-n.m.r. spectrum (see Fig. 2) showed 3 signals corresponding to α-(1→4) (101.3 and 100.8 p.p.m.) and α-(1→6) linkages (99.0 p.p.m.)[18], whose relative area was 2:1, in accordance with the repeating unit postulated. The splitting of the resonances for C-4 (79.0 and 78.6 p.p.m.) and C-6 (61.8 and 61.5 p.p.m.) of the (1→4)-linked units is due to the sensitivity of this position to the nature of the linkage to C-1. The C-6 resonance for *O*-6-substituted glucose residues appeared as a sole signal at 67.6 p.p.m.

Pullulan has been so far described as an extracellular polysaccharide of only a few fungi. In *C. harioti*, pullulan accounted for 1.5% of the dry weight, and the possibility of this polysaccharide's being elaborated by an internal yeast was not excluded. Gamundi[19] observed that yeasts of the Sacharomyces type could inhabit the stromata, but these yeasts have not been reported as producing pullulans. The isolation of a pullulan-like α-D-glucan from *Cyttaria darwinii* in 12% yield confirms the intracellular occurrence of this polymer, at least in two species of *Cyttaria*.

EXPERIMENTAL

Material. — *Cyttaria darwinii* was collected in Parque Nacional "Los Glaciares" (Santa Cruz, Argentina) in January, 1985.

General. — All evaporations were performed at 40° under diminished pressure. Small volumes of aqueous solutions were lyophilized. Optical rotations were recorded with a Perkin–Elmer 141 polarimeter, and i.r. spectra with a Perkin–Elmer 710 B infrared spectrophotometer.

Paper chromatography was conducted by the descending method on Whatman No. 1 paper, with the following solvent systems: (*A*) 6:4:3 1-butanol–pyridine–water, (*B*) 5:2:2 1-butanol–ethanol–water, and (*C*) 4:3:4 1-butanol–

pyridine–water. Detection was effected with (*a*) silver nitrate–sodium hydroxide[20] and (*b*) *p*-anisidine hydrochloride[21]. T.l.c. was carried out on BC-Alufolien Keiselgel 60 (Merck) plates in (*D*) 7:1:2 1-propanol–ethanol–water and (*E*) 600:150:9:4.5 acetone–benzene–water–NH_3; spots were detected with 5% sulfuric acid in ethanol.

Total carbohydrate was determined by the phenol–sulfuric acid method[22], and D-glucose in the hydrolyzate was estimated by the D-glucose oxidase–peroxidase procedure[8].

G.l.c. was performed with a Hewlett–Packard 5830 gas chromatograph equipped with a flame-ionization detector, on glass columns packed with: (I) 3% ECNSS-M on Gas Chrom Q (0.2 × 180 cm); (II) 10% NPGS on Chromosorb W AW-DMCS (60-80) (0.2 × 120 cm); and (III) 2% OV-101 on Chromosorb W AW DMCS (60–80) (0.2 × 120 cm). G.l.c.–m.s. was performed with a 8% NPGS column in a Varian 1440 chromatograph coupled to a Varian-MAT CH-7 A mass spectrometer controlled by 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 10 min at 60°.

The ^{13}C-n.m.r. spectrum was recorded at 50 MHz with a Bruker spectrometer operated in the F.t. mode for a solution in 3:1 H_2O–D_2O (90 mg/4 mL) in a 12-mm tube at 27°. The spectral width was 10 kHz, the pulse angle 45°, the acquisition time 0.49 s, and the number of transients ~50,000. 1,4-Dioxane was used as the external standard (67.4 p.p.m. downfield from the signal of Me_4Si).

Isolation, purification, and properties of the glucan. — Powdered, dried, stromata of *Cyttaria darwinii* (14.2 g) were extracted with water (0.7 L) in a Waring Blendor at room temperature, and then centrifuged. The extraction was repeated twice; ethanol (to 55%) was added to the combined supernatant solutions, and the insoluble product (5.8 g; 41%) collected by centrifugation and dried by solvent exchange. Portionwise addition of ethanol to a solution of the crude product (0.3 g) in water (150 mL) resulted in precipitation at 37–38% of ethanol. Centrifugation at 2,500 r.p.m. for 20 min gave fraction CD_1 [28.7%; $[\alpha]_D^{25}$ +57° (*c* 0.5; M potassium hydroxide)]. After concentration, lyophilization of the (initially cloudy) supernatant liquor afforded fraction CD_2 (12.3%).

Fraction CD_2 was purified by repeated redissolution in water, reprecipitation with ethanol (to 40%), and centrifugation at 10,000 r.p.m. (15 min) until the specific rotation remained constant. The purified polysaccharide (total carbohydrate, 97–98%) had $[\alpha]_D^{25}$ +189° (*c* 0.95; water); ν_{max}^{film} 3300 (s, broad), 2850 (m), and 850 cm^{-1} (w). Polysaccharide CD_2 was subjected to gel permeation on a column of Sepharose 4B (1.5 × 92 cm) in phosphate buffer, pH 7.2, 0.1% sodium dodecyl sulfate. Blue Dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used for determination of the void volume. The average molecular weight was estimated by using dextrans of known molecular weights (Sigma).

Acid hydrolysis of the glucan. — A solution of CD_2 (5 mg) in 0.5M H_2SO_4 (1 mL) was heated in a sealed tube for 16 h at 100°. The hydrolyzate was made neutral

with $BaCO_3$, filtered, and the filtrate decationized by stirring with Dowex 50-W (H^+) resin. Paper chromatography (solvent *A*; reagents *a* and *b*) showed glucose as the only component, and the ratio of total carbohydrate:glucose was 1:1. The monosaccharide was further characterized by g.l.c. (alditol acetate[23]; column I, T_i 200°, T_d 210°, T_c 190°; flow 25 mL of N_2/min).

Methylation analysis of the glucan. — The polysaccharide (34 mg) was methylated by the Hakomori method[9]. The fully methylated product was hydrolyzed with 72% H_2SO_4 (3 mL) for 2 h at 25° and then for 4 h under reflux with 12% H_2SO_4. The partially methylated glucoses were analyzed by t.l.c. (solvent *E*), and g.l.c. as alditol acetates[23] (column II, T_i 280°, T_d 225°, T_c 205°; flow 29 mL of N_2/min). 2,3,4,6-Tetra-*O*-methyl-(T_R 7.11); 2,3,6-tri-*O*-methyl-(T_R 13.35), and 2,3,4-tri-*O*-methylglucose (T_R 14.07) were characterized by comparison with authentic samples. Further characterization was achieved by g.l.c.–m.s. (column II, T_i 250°; T_c 225°; flow 25 mL of He/min).

Partial acetolysis of the glucan. — Glucan CD_2 (200 mg) was first acetylated and the product then acetolyzed according to the procedure recommended by Kocourek and Ballou[11]. A solution of the mixture of acetates in methanol (10 mL) was deacetylated overnight at room temperature with 0.1M sodium methoxide (5 mL). The resulting precipitate was dissolved by the addition of water, and the solution was passed through Dowex 50-W (H^+) resin, and concentrated to a syrup. P.c. (solvents *B* and *C*) and t.l.c. (solvent *D*) showed glucose, maltose, maltotriose, and traces of larger oligosaccharides. This mixture was fractionated on a column of Bio Gel P-2 (1.5 × 100 cm) which had previously been calibrated with malto-oligosaccharides.

The disaccharide fraction was reduced with $NaBH_4$, and the product per(trimethylsilyl)ated, and analyzed by g.l.c. (column III; T_i 300°, T_d 300°, T_c programmed from 200 to 280°, 4°/min; flow 26 mL of N_2/min; T_R 5.85 min). The trisaccharide fraction was purified by preparative t.l.c. (solvent *D*), and methylated by the Hakomori method[9]. The methylated glucotriose was hydrolyzed, and the products analyzed by g.l.c. employing the conditions previously described for the glucan.

Enzymic hydrolysis of the glucan. — The polysaccharide (11 mg) was incubated at 37° with pullulanase in 2.5 mL of 0.01M sodium citrate–citric acid buffer (pH 5.0). T.l.c. (solvent *D*) and p.c. (solvents *B* and *C*) of aliquots taken at 6 and 20 h showed a sole spot which was identified as that of maltotriose, R_{Glc} 0.58 (solvent *D*); 0.32 (solvent *B*); 0.75 (solvent *C*). Aliquots taken at 48 h also showed traces of glucose and maltose.

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