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A GEL-FORMING (1→3)-β-D-GLUCAN ISOLATED FROM *Cyttaria harioti* FISCHER

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ABSTRACT

An alkali-soluble glucan, $[\alpha]_D$ +11° (M potassium hydroxide) having a degree of polymerization of 220, has been isolated from the fruit bodies of the tree fungus *Cyttaria harioti* Fischer Periodate oxidation and methylation analysis show that it consists of a highly branched β-D-(1→3)-linked backbone Hydrolysis of the methylated polysaccharide yielded 2,3,4,6-tetra-*O*-methyl- (24 5 mol%), 2,4,6-tri-*O*-methyl- (39 4 mol%), 2,3,4-tri-*O*-methyl- (8 6 mol%), and 2,4-di-*O*-methyl-D-glucose (27 5 mol%) Periodate-oxidation results substantiate the methylation studies The general structural features of the glucan are discussed

INTRODUCTION

In previous papers[1–3], we have described structural features of a water-soluble heteropolysaccharide isolated from the fungus *Cyttaria harioti* Fischer These are the first reports on polysaccharides of the *Cyttariales* (Discomycetes)[4], the latter organisms are parasites of the tree *Nothofagus*, on which they produce characteristic tumors that infect the tree and finally cause its death The polysaccharide, $[\alpha]_D$ +95 8° (M potassium hydroxide), contained D-glucose, D-*arabino*-hexulosonic acid, and D-fructose in the molar ratio of 98 6 1. We now report on a gel-forming (1→3)-β-D-glucan obtained in good yield by alkaline extraction

RESULTS AND DISCUSSION

The residue from the cold-water extraction[1,2] of powdered, dried, fruit-bodies of *Cyttaria harioti* Fischer, was extracted with M sodium hydroxide After centrifugation, the viscous supernatant solution was neutralized by exhaustive dialysis, after which the glucan separated in the cellulose bags as a uniform gel It was dehydrated by freeze-drying (yield, 36 5% on the basis of dry fungus) and purified by

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redissolution in alkali and reprecipitation by neutralization with acetic acid until the specific rotation remained constant, $[\alpha]_D$ +11 0° (M potassium hydroxide)

Approximately 98% of the polysaccharide was precipitated from a dilute alkaline solution at an ethanol concentration of 45–48% This narrow range of concentration indicates homogeneity of the polysaccharide[5]. The low specific rotation, together with infrared absorption[6] at 890 cm^{-1} but not at about 840 cm^{-1} are characteristic of the β-configuration On acidic hydrolysis, the polysaccharide gave D-glucose as the sole product, characterized as *N*-*p*-nitrophenyl-D-glucopyranosyl amine[7] The degree of polymerization, determined by the method of Unrau and Smith[8], was 220 if the terminal group was considered to be a glucose residue linked through O-3.

On periodate oxidation, the polysaccharide consumed 0 61 moles of periodate per glucose residue with production of 0 31 moles/residue of formic acid, indicating the presence of 31% of (1→6)-linked D-glucose residues, together with terminal D-glucose Borohydride reduction of the polyaldehyde, followed by total acid hydrolysis afforded glucose (65 5 mol%) and glycerol (34 5 mol%), as determined by g l c.

Smith-type degradation[9] of the polyaldehyde produced only glycerol Non-hydrolyzed material was recovered in ~60% yield and remained unaltered when subjected to renewed periodate oxidation, thus indicating the presence of a (1→3)-linked, β-D-glucose backbone The fact that no glucosylglycerol was formed by mild, acid hydrolysis indicates the absence of alternate (1→3)- and (1→6)-linkages

The polysaccharide was methylated once by the Hakomori method[10] and the product thus obtained still showed the characteristic infrared absorption of free hydroxyl groups As remethylation by the same procedure caused degradation of the polysaccharide, fully methylated polysaccharide was obtained by succesive application of the Hakomori (once) and Purdie[11] (thrice) methods of methylation Hydrolysis was effected by the method recommended by Lindberg and coworkers[12] as causing minimal degradation. The sugars thus obtained were characterized by g l c of the alditol acetates and comparison with authentic samples as 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl-, 2,3,4-tri-*O*-methyl-, and 2,4-di-*O*-methyl-D-glucose The molar proportions observed (Table I) indicate a highly branched structure and are in total agreement with the periodate-oxidation data.

TABLE I

HYDROLYSIS OF THE METHYLATED D-GLUCAN

Cleavage product (O-*methyl*-D-*glucose*)	*Structural feature indicated*	*Molar proportion*
2,3,4,6-Tetra-	Glc*p*-(1→	24 5%
2,4,6 Tri-	→3)-Glc*p*-(1→	39 4%
2,3,4-Tri	→6)-Glc*p*-(1→	8 6%
2,4-Di-	→6) →3)-Glc*p*-(1→	27 5%

From these results, the glucan consists of a (1→3)-linked β-D-glucose backbone, every second or third residue of which is substituted in the 6-position The (1→6)-linked β-D-glucose residues, present in low proportion, are in the side chains. The branches are short (one or two glucose residues) in accord with the methylation data and the physical properties of the glucan

Glucans containing β-(1→3) and β-(1→6) linkages are commonplace in fungal cell-walls and have been isolated *inter alia* from *Pullularia pullulans*, *Sclerotium glucanicum*, *Claviceps* species and *Plectoria occidentalis*[13] A similar structure has also been described for an extracellular glucan produced by the rot fungus *Stereum sanghinolentum*[14] Highly branched fungal and yeast glucans containing (1→6)-β-D-glucopyranosyl groups on a (1→3)-β-D-glucan backbone are known to be antitumor active[15] Interestingly the glucan described here is the major component of *Cyttaria harioti*

TABLE II

GAS–LIQUID CHROMATOGRAPHY OF *O*-METHYL-D-GLUCITOLS

Substitution mode of D-*glucitol*		*Retention time*[a,b]
O-*Methyl*	O-*Acetyl*	
2,3,4,6-	1,5	1 00
2,4,6-	1,3,5	1 71
2,3,4-	1,5,6	2 00
2,4-	1,3,5,6	3 73

[a]Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol [b]10% NPGS, T_{inj} 280, T_d 300°, T_c 200°. N_2 30 ml/min

EXPERIMENTAL

Material — Fruit bodies of *Cyttaria harioti* Fischer were collected in San Martin de los Andes (Neuquén, Argentina) in January, 1969

General — All evaporations were performed under diminished pressure at 40–50°. Paper chromatography was conducted by the descending method on Whatman No 1 paper with the following solvent systems (A) 5 2·2 butyl alcohol–ethanol–water, (B) 6.4·3 butyl alcohol–pyridine–water, (C) 18 3 1 4 ethyl acetate–acetic acid–formic acid–water, (D) 5 5 1 3 pyridine–ethyl acetate–acetic acid–water, (E) butanone–water azeotrope Detection was effected with (*a*) silver nitrate–sodium hydroxide[16]; (*b*) aniline hydrogen phthalate[17]; and (*c*) *p*-anisidine hydrochloride[18]. Melting points are uncorrected Optical rotations were recorded with a Perkin–Elmer 141 polarimeter and ir. spectra with a Perkin–Elmer Model 421 spectrophotometer Glc was effected with a Hewlett–Packard 5750 gas chromatograph equipped with stainless-steel columns (183 × 0 3 cm) packed with (a) 3% OV-17 on Chromosorb W (HP, 80–100 mesh) or (b) 10% NPGS on Chromosorb G, with nitrogen at a flow-rate of 30 ml/min The trimethylsilyl ethers were prepared with hexamethyldisilazane and

chlorotrimethylsilane in pyridine, as described by Sweeley and coworkers[19] Room temperature implies 20–25°

Isolation and purification of the glucan — Powdered, dried, fruit-bodies of *Cyttaria harioti* Fischer (100 g) were defatted with petroleum ether and soluble sugars were extracted with methanol Cold-water extraction of the residue, followed by ethanol precipitation afforded the heteropolysaccharide previously described[1–3] The water-insoluble material was stirred with M sodium hydroxide (2 liters) for 5 h at room temperature and centrifuged The extraction was repeated twice, and the combined supernatants were neutralized by dialysis against running water and then dialyzed against distilled water The glucan precipitated as a gel, which was dehydrated by freeze-drying, yield 36 5 g, $[\alpha]_D$ +9 2° (*c* 0 8, M potassium hydroxide)

For purification, the crude polysaccharide was redissolved in sodium hydroxide and reprecipitated by addition of acetic acid until the specific rotation remained constant (four times)

Properties of the polysaccharide — The purified polysaccharide (Found N, 0%), after having been dried at 60°/1 torr for 12 h over phosphorus pentaoxide, had $[\alpha]_D$ +11 0° (*c* 1 0, M potassium hydroxide), ν_{max}^{film} 3400 (s, broad), 2900 (m), and 890 cm^{-1} (w)

Portionwise addition of ethanol to a solution of the purified glucan (0 2% in 0 1M potassium hydroxide) resulted in no precipitation up to a concentration of 45% ethanol When the alcohol concentration was increased to 48%, the polysaccharide precipitated in 98% yield

The glucan swelled in water to form a rigid gel and its alkaline solutions were highly viscous.

Acid hydrolysis of the polysaccharide — The glucan (200 mg) was hydrolyzed with 0 5M sulfuric acid (20 ml) in a sealed tube for 16 h at 110° The hydrolyzate was neutralized with barium carbonate, filtered, and the filtrate decationized by stirring with Dowex-50 (H^+ form) resin. Paper chromatography (solvents A, B, C, D, detection reagents *a*, *b*, *c*) and g l.c [trimethylsilyl ethers, OV-17, T_{inj} (injection port) 250°, T_d (detector) 300°, T_c(column) 150°] showed glucose as the only component D-Glucose was further identified by preparation of *N*-(*p*-nitrophenyl)-D-glucopyranosylamine, m p and mixed m p 185°, in accord with reported values[7].

Determination of the degree of polymerization[8] — A suspension of the glucan (55 mg) in water (10 ml) was reduced by stirring with sodium borohydride (50 mg) for 48 h Excess borohydride was decomposed by dropwise addition of acetic acid, and the pH was adjusted to 7.0 by careful addition of sodium hydrogencarbonate Sodium metaperiodate (25 mg) was added and the mixture was stored in the dark at room temperature for 10 days, with occasional shaking The excess oxidant was decomposed, after acidification with 0 5M sulfuric acid, by addition of 0 5M sodium arsenite The mixture was dialyzed against an equal volume of distilled water for 48 h The formaldehyde produced was determined in the dialyzate by the chromotropic acid method[20]. The amount of formaldehyde corresponded to a degree of poly-

merization of 220, assuming that the terminal reducing group was linked through O-3 and would yield two molecules of formaldehyde

Periodate oxidation of the polysaccharide — The glucan (130 mg) was suspended in 0 1M sodium metaperiodate (50 ml) and water (50 ml) was added Oxidation was allowed to proceed at room temperature in the dark, with periodic shaking At intervals, the periodate uptake was determined by the arsenite method[21] and release of formic acid by titration with 10mM sodium hydroxide Extrapolating the data to zero time, the periodate consumption was 0 61 mole of periodate per glucose residue, with production of 0 31 mol/residue of formic acid After oxidation was complete, ethylene glycol was added and the mixture dialyzed The polyaldehyde was then reduced with sodium borohydride (150 mg) After 12 h, an additional amount (50 mg) of the reductant was added After a further 24 h, the excess of borohydride was decomposed by careful addition of acetic acid, the suspension was dialyzed, and the reduced product recovered by freeze-drying

Total acid hydrolysis of the polyalcohol — The polyalcohol (10 mg) was hydrolyzed with 0 5M sulfuric acid (2 ml) in a sealed tube at 110° Paper chromatography (solvents A, B, reagents *a*, *b*) of the neutralized and decationized hydrolyzate showed only glucose and glycerol No erythritol could be detected G l c of the hydrolyzate (trimethylsilyl ethers) on OV-17 [T_{in} 250°, T_d 300°, T_c 3 min at 80° and then programmed from 80 to 150° (4°/min)] showed glucose (retention time 25 min 18 sec) and glycerol (retention time 6 min 24 sec), identified by comparison with authentic samples The molar proportion was calculated by comparison of the relative peak-area values with that of a standard mixture of the authentic components (glucose glycerol 1 9 1)

Smith degradation of the polyalcohol[9] — The polyalcohol (30 mg) was hydrolyzed with 50mM sulfuric acid for 24 h at room temperature The insoluble, degraded polysaccharide was recovered by centrifugation in ~60% yield The supernatant was neutralized with barium carbonate, filtered, and decationized Paper chromatography (solvents A, B, reagents *a*, *b*) and g l c (trimethylsilyl ethers, conditions already described) showed glycerol as the only component No glucosyl-glycerol could be detected

The insoluble, degraded polysaccharide was subjected to total acid hydrolysis as described before Paper chromatography (solvents A, B, reagents *a*, *b*) and g l c (trimethylsilyl ethers) showed glucose It was then subjected to periodate oxidation during 12 days in the dark The excess of oxidant was decomposed and the mixture dialyzed The resultant polyaldehyde was reduced with sodium borohydride as already described, and the polyalcohol was recovered after dialysis by freeze-drying Total acid hydrolysis with 0 5M sulfuric acid gave only glucose, as detected by paper chromatography and g l c

Methylation of the polysaccharide — The polysaccharide was methylated by the Hakomori method[10] The methylsulfinyl sodium reagent was prepared according to the method of Sandford and Conrad[22] Dried glucan (100 mg) was added to 10 ml of dry dimethyl sulfoxide Nitrogen was passed continuously. The suspension was

stirred at 60° until all the polysaccharide had dissolved and, after cooling to room temperature, methylsulfinyl sodium solution (~10 ml) was added The sample was stirred under nitrogen for 6 h An excess of methyl iodide was then added, and the mixture was stirred overnight The mixture was dialyzed against water and freeze-dried The product was remethylated thrice by Purdie's method[11], with methyl iodide and freshly prepared silver oxide, added in small portions The solids were removed by filtration and the filtrate evaporated to a syrup. The methylated polysaccharide was dissolved in benzene, filtered, and freeze-dried, yield, 40 mg It showed no hydroxyl group in the i r spectrum and had OMe 41 1%

Acid hydrolysis of the methylated polysaccharide — The methylated polysaccharide (30 mg) was soaked in 72% sulfuric acid (1 ml) with external cooling The solution was kept for 2 h at room temperature, the acid was diluted to 12%, and the container was sealed and heated for 4 h at 110° The solution was neutralized with barium carbonate and decationized with Dowex-50 (H^+ form) resin Paper chromatography (solvent E, reagent *c*) showed four main spots having colors and R_F values identical to those given by 2,3,4,6-tetra-*O*-methyl- (R_F 0 78, pink), 2,3,4-tri-*O*-methyl- (R_F 0 59, yellow), 2,4,6-tri-*O*-methyl- (R_F 0 48, pink), and 2,4-di-*O*-methyl-glucose (R_F 0 20, reddish brown)

The sugars in the hydrolyzate were reduced with sodium borohydride overnight The solution was acidified to decompose the excess of borohydride, decationized with Dowex-50 (H^+ form) resin, and evaporated Methanol was evaporated several times from the residue to remove boric acid To the dry residue were added pyridine (1 ml) and acetic anhydride (1 ml), and the sealed container was heated for 1 h at 70° After 24 h at room temperature, the solvents were removed and the final residue, containing the alditol acetate derivatives, was dissolved in a few drops of dichloromethane and analyzed by g l c Authentic samples of 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl-, 2,3,4-tri-*O*-methyl-, and 2,4-di-*O*-methyl-D-glucopyranose were reduced and acetylated as already described

G l c was performed on 10% NPGS (T_{inj} 280°, T_d 300°, T_c 200°) The retention times, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, and the molar ratios obtained, are shown in Table II

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