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Note

Isolation and identification of a linear (1→3)-linked β-D-glucan and other carbohydrate components of the lichen *Stereocaulon ramulosum* (SW.) Räusch

MADALENA BARON, PHILIP A.J. GORIN, AND MARCELLO IACOMINI
Departamento de Bioquímica, C.P. 19046, Universidade Federal do Paraná, 81.504 Curitiba-PR (Brazil)
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The lichen *Stereocaulon ramulosum* was analyzed for component carbohydrates and found to contain a linear (1→3)-linked β-D-glucopyranan, the first time that such a polysaccharide has been encountered in a lichen. Also isolated and characterized were an α-D-glucan resembling isolichenan, and several low molecular weight carbohydrates often found in lichens.

Native lichen was extracted by reflux in benzene–ethanol, followed by methanol containing 20% of water, the latter step furnishing a mixture of low molecular weight carbohydrates (3.3% yield). Paper chromatography, in conjunction with g.l.c. of derived acetates, showed the presence of arabinitol (1.9%), mannitol (0.3%), and *myo*-inositol (0.04%). Cellulose column chromatography of the extract provided 2-*O*-β-D-galactofuranosyl-D-arabinitol (umbilicin; 0.9%) and α,α-D-trehalose (0.08%).

Residual lichen was then extracted with hot water to yield soluble polysaccharides containing glucose, galactose, and mannose in a 18:1:1 molar ratio. The preparation was fractionated by addition of Fehling solution to an aqueous solution, which gave rise to an insoluble copper complex and a supernatant, from which a glucan (4.5%) was isolated. The complex was mainly derived from a galactomannan, which was not further investigated. The glucan proved to be homogeneous when chromatographed on columns of DEAE-cellulose and Sepharose 6B and had a specific rotation of +171° indicating an α-D configuration. Its ^{13}C-n.m.r. spectrum was identical to that of isolichenan[1] with relatively high-field C-1 signals at δ 101.8, 101.1, and 100.8, typical of an α-D configuration[2], and others at δ 81.6 and 81.9 (substituted O-3), and 79.1 (substituted O-4) (ref. 1). A ratio between (1→3)- and (1→4)-linkages of 1.6:1 was found by methylation analysis incorporating g.l.c.–m.s. examination of derived, partially *O*-methylated glucitol acetates[3]. This ratio

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was confirmed by periodate oxidation, in which 0.39 mol/mol of oxidant was consumed, and by a Smith degradation using strong hydrolytic conditions which provided a mixture of erythritol and glucose, quantitated by g.l.c. of derived alditol acetates.

Cellulose column chromatography of the product formed on Smith degradation using mild hydrolytic conditions provided erythritol, 2-*O*-α-D-glucopyranosyl-D-erythritol, and *O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→2)-D-erythritol in a molar ratio of 1:2.4:3.9. The formation of erythritol from a glucan containing preponderantly (1→3)-linkages showed that consecutive (1→4)-linkages are present, and that the distribution of the two linkage types, along the linear chain, is not completely even.

Lichen remaining from the above described extraction was treated with 2% aqueous potassium hydroxide at 100°, the resulting polysaccharide was dissolved in hot water, and the solution submitted to successive freezing and thawing. Insoluble material was isolated and purified by successive dissolution in aqueous alkali, neutralization, dialysis, and isolation of insoluble material, a process that was repeated two more times. The isolate (0.19% yield) was homogeneous when chromatographed on columns of Sepharose 4B-200 and Sepharose 6B, had $[\alpha]_D$ +3°, and gave a ^{13}C-n.m.r. spectrum similar to that of linear (1→3)-linked β-D-glucan with signals at δ 104.8 (C-1), 87.6 (*O*-substituted C-3), 77.5, 75.0, 69.9, and 62.5 (ref. 4). Methylation analysis confirmed this structure by the formation of 2,4,6-tri-*O*-methylglucitol acetate as the sole product identified by g.l.c. The sensitivity was such that the 2,3,4,6-tetra-*O*-methyl derivative would have been detected in amounts >0.5%, thus indicating a chain length of >200 units.

Such a β-D-glucan structurally resembles those of callose, curdlan, pachyman, and a laminaran formerly called paramylum which are components of plants, bacteria, a fungus, and a green alga, respectively. Pachyman, isolated from *Poria cocos*, has a particularly high proportion of (1→3)-linkages since the molecule has a mol. wt. of ~370 000 with only a few 3,6-di-*O*-substituted branch points and one internal (1→6)-linkage[5-7]. Laminaran from the green alga, *Euglena gracilis*, is also (1→3)-linked and preponderantly linear since it consumes only 0.02 mol of periodate/mol[8]. This distinguishes it from the laminarans of *Laminaria* and other brown algae, which have d.p. values of 20–25 and preponderant (1→3)-linkages, with up to three units per molecule of 6-*O*- or 3,6-di-*O*-substituted units[9].

The isolation of the α-D-glucan from *St. ramulosum* in 4.5% yield showed that it likely arises from the mycobiont. Phycobionts generally constitute only a small proportion of the total lichen, and it is possible that it could be the source of the β-D-glucan, which was isolated in only 0.19% yield. However, this point rests on a future analysis of culture-grown myco- and phyco-bionts. The phycobiont(s) in our strain of *St. ramulosum* has not been identified. However, blue green algae (cyanobacteria) of *Scytonema* and *Stigonema* spp. occur in the cephalodia of this lichen[10]. In certain other *Stereocaulon* spp., the green alga *Trebouxia* is present in the thallus[11].

EXPERIMENTAL

Lichen. — *St. ramulosum*, which is common and widely distributed throughout Brazil, was collected in the coastal range, Serra do Mar, in the State of Paraná. It grew by the side of the Estrada Graciosa, 1 km from the Post of the Forestry Police in the direction of Morretes at an altitude of 900 m. Prior to extraction, it was cleaned to remove other plant material and dried.

^{13}C-N.m.r. spectroscopy. — This was carried out using previously described conditions[1].

Determination of monosaccharide composition of oligo- and poly-saccharides. — These compounds were hydrolyzed and the products converted into alditol acetates which were analyzed by g.l.c., as described by Sawardeker *et al.*[12].

Methylation analysis of polysaccharides. — This procedure was carried out, as previously described[1], by successive methylations by the methods of Haworth[13] and Kuhn *et al.*[14]. The per-*O*-methylated products were then converted into mixtures of *O*-methylalditol acetates, which were examined by g.l.c.-m.s.[15] using a capillary column coated with 3:1 OV-225–OV-17. Peaks were identified by their typical e.i.m.s. spectra[3] and typical retention times.

Identification and quantification of polyols in lichen. — The methodology was as previously described [16].

Aqueous methanol extraction of lichen and identification of soluble carbohydrates. — *St. ramulosum* (215 g) was extracted with refluxing 9:1 (v/v) benzene–ethanol (1.7 L), and then methanol containing 20% of water (1.7 L). The latter was evaporated, de-ionized, and the product (7.1 g; 3.3% yield) examined, as described previously[16], by paper chromatography and g.l.c. of derived acetates. The contents of component arabinitol, mannitol, and *myo*-inositol were determined.

A portion (0.70 g) of the methanolic extract was chromatographed on a cellulose column, which was eluted successively with acetone–water with v/v proportions of 7:1, 4:1, and 3:1. Isolated were arabinitol (397 mg), mannitol (71 mg), 2-*O*-β-D-galactofuranosyl-D-arabinitol (183 mg), α,α-trehalose (16 mg), and *myo*-inositol (8 mg).

2-*O*-β-D-Galactofuranosyl-D-arabinitol gave galactose and arabinitol on hydrolysis; ^{13}C-n.m.r. (D_2O; 70°): δ 108.2, 84.0, 82.2, 77.4, 71.8, 71.6, 71.1, 63.8, 63.6, and 62.2, corresponding to authentic material[17].

α,α-D-Trehalose gave glucose on hydrolysis; ^{13}C-n.m.r. (D_2O; 70°): δ 95.1, 74.3, 73.9, 72.8, 71.5, and 62.4, identical with data of authentic standard.

Preparation of α-D-glucan. — Residual lichen (189 g) was extracted with water (1.7 L) for 4 h at 100°, the mixture filtered when hot, and the process repeated twice. The combined filtrates were evaporated to a small volume, which was added to excess ethanol, and the precipitate which formed was isolated. This polysaccharide preparation (45 g) contained glucose, galactose, and mannose in a molar proportion of 18:1:1. It was dissolved in water (300 mL) with heating, and the solution frozen and thawed gradually. The insoluble material which formed was filtered off, this

process being repeated eight times. The combined precipitates contained galactose, glucose, mannose, xylose, fucose, and rhamnose in a molar ratio of 48:26:12:4:5:5 and was not investigated further. The soluble material still contained mannose (4%) and galactose (11%) as impurities and therefore an aqueous solution (150 mL) was further fractionated after treatment with Fehling solution (150 mL). The resulting copper complex was removed, and the supernatant made neutral with acetic acid, dialyzed, and de-ionized with resin. It was evaporated to a small volume and added to excess ethanol, and the precipitated polysaccharide isolated. The resulting α-D-glucan (9.6 g), $[\alpha]_D^{25}$ +171° (*c* 0.6, water) was homogeneous when chromatographed on columns of DEAE-cellulose (PO_4^{3+}, treated with increasing concentrations of phosphate buffer, pH 6.8) and Sepharose 6B (eluted with water).

Controlled Smith degradation of α-D-glucan. – The glucan (1.00 g) was oxidized in 0.05M $NaIO_4$ (1.0 L) for 96 h at 0–2° in the absence of light. 1,2-Ethanediol was then added, the solution dialyzed, and $NaBH_4$ added. After de-ionization, the pH was adjusted to 2, and the aqueous solution (50 mL) maintained for 40 min at 100°. Paper chromatography of the product in 1:5:3:3 (v/v) benzene–l-butanol–pyridine–water, showed three spots (reagent, $AgNO_3$–NaOH dip method) with mobilities corresponding to those of erythritol, glucosylerythritol, and glucobiosylerythritol formed on Smith degradation of isolichenan[18]. The mixture was chromatographed on a cellulose column and eluted successively with 9:1, 4:1, 3:1, and 2:1 (v/v) acetone–water. Isolated were erythritol (7.7 mg), 2-*O*-α-D-glucopyranosyl-D-erythritol (38.5 mg), and *O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→2)-D-erythritol (98.5 mg). Glucosylerythritol gave glucose and erythritol on hydrolysis; ^{13}C-n.m.r. (D_2O; 70°): δ 99.4, 80.2, many signals, 64.3, and 62.3. Glucobiosylerythritol also gave glucose and erythritol; ^{13}C-n.m.r. (D_2O; 70°): δ 100.9, 99.5, 81.2, 80.2, many signals, 64.3, 62.2, 62.1 and 61.4.

In the complete hydrolysis of polysaccharide oxidized with $NaIO_4$ and then reduced with $NaBH_4$, 0.5M H_2SO_4 for 7 h at 100° was used. The hydrolyzate was then reduced with $NaBH_4$, the product was treated with acetic anhydride–pyridine, and the resulting acetates examined by g.l.c. using a column of ECNSS[12] showed the presence of erythritol (38%) and glucitol (62%) derivatives.

Preparation of laminaran. — Remaining lichen (137 g) was treated with 2% aqueous KOH (1.5 L) for 2 h at 100°, the mixture filtered when hot, and the filtrate made neutral with acetic acid. It was concentrated to a small volume, added to excess ethanol, and the resulting precipitate isolated (26.2 g). It contained glucose, galactose, mannose, xylose, and rhamnose in a molar ratio of 47:28:24:0.3:1. The polysaccharide was shaken in water, and the mixture submitted to a process of repeated freezing and thawing, as described above. Insoluble material was collected, combined, and found to contain glucose, mannose, and galactose in a molar ratio of 65:13:21. After treatment with aqueous $NaBH_4$, this preparation was dissolved in 2% aqueous KOH (250 mL) for 20 min at 100°. The solution was made neutral with acetic acid, dialyzed against water, concentrated to 50 mL, and insoluble material centrifuged off. After two more identical treatments, the resulting laminaran

(0.41 g), $[\alpha]_D^{25}+3°$ (*c* 0.4, 1% aqueous NaOH), was homogeneous on column chromatography on Sepharose 4B-200 and Sepharose 6B (eluent, 0.1M NaOH). The mol. wt. was $<3 \cdot 10^6$; ^{13}C-n.m.r. (D_2O containing 1% NaOH; 70°): δ 104.8, 87.6, 77.5, 75.0, 69.9, and 62.5.

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