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Polysaccharides Elaborated by *Pullularia pullulans*

Part II *. The Partial Acid Hydrolysis of the Neutral Glucan Synthesised from Sucrose Solutions

HANS O. BOUVENG, HANS KIESSLING, BENGT LINDBERG and JAMES McKAY**

Pappperstekniska och Träkemiska avdelningarna, Svenska Träforskningsinstitutet, Stockholm Ö, Sweden

Examination of the products of partial acid hydrolysis of the neutral glucan synthesised from sucrose has given evidence for the presence of a regular sequence of α-(1→4) and α-(1→6) glucosidic linkages in the structure [→6)-α-G-(1→4)-α-G-(1→4)-α-G-(1→]$_n$ where n is *ca.* 90. From the hydrolysate isomaltose, *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose (panose), and *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→6)-D-glucopyranose (isopanose) have been isolated and characterised.

The isolation and characterisation of a neutral glucan from cultures of *Pullularia pullulans*, grown on sucrose as carbon source, have already been reported [1]. The polysaccharide, containing α-D-glucopyranose units linked in an unbranched chain through positions 4 and 6 in the ratio 2:1, was concluded to be closely akin to the "restpullulan" of Wallenfels *et al.*[2] but possibly to differ in fine structure. The possibility of a different fine structure depended on the isolation of a trisaccharide, tentatively identified as isomaltotriose, from a partial acetolysate of the glucan. The complete enzymatic conversion of restpullulan to maltotriose [2] precludes the formation of such a fragment on chemical degradation.

Partial acid hydrolysis was selected as a means of settling the issue since this would favour strongly the formation of fragments retaining (1→6)-linkages. From the data of Wolfrom *et al.*[3], and assuming a random distribution of linkages, conditions of hydrolysis were deduced which would permit high yields of trisaccharides containing (1→6)-linkages to be obtained. Examination of the products of hydrolysis showed that the polysaccharide was broken down to give isomaltose as the major disaccharide and *O*-α-D-glucopyranosyl-

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** Present address: Dept. of Chemistry, University of Edinburgh, Edinburgh, Scotland.

(1→6)-*O*-α-D-glucopyranosyl-(1→4)-α-glucopyranose (panose) and *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→6)-D-glucopyranose, for convenience referred to as isopanose, as the principal trisaccharides. Small amounts of maltose and maltotriose were also formed but isomaltotriose, if present at all, was so to no significant extent.

Isomaltose was characterised as the crystalline β-octaacetate, panose as the crystalline sugar and as the derived panitol dodecaacetate. The two trisaccharides could be distinguished in the first instance by their paper-chromatographic and electrophoretic mobilities. Both sugars yielded glucose, maltose and isomaltose on partial acid hydrolysis, while panitol and isopanitol yielded glucose and isomaltose, and glucose and maltose, respectively, as the only reducing sugars. On oxidation with periodate panose showed distinct overoxidation and after 30 h had consumed 8.5 moles of periodate and liberated 3.3 moles of formic acid per mole. Isopanose showed no apparent overoxidation, the periodate consumption and formic acid release being 7.5 moles/mole and 5.0 moles/mole, respectively. The trisaccharides were converted to the corresponding crystalline alditol dodecaacetates which differred markedly in melting-point and specific rotation but had almost identical infra-red spectra, differences occurring only in the region 950—750 cm^{-1} in which the absorptions characteristic of α-(1→4) and α-(1→6) glucosidic linkages occur. The properties of the trisaccharides and their derivatives are compared in Table 1. Applying Hudson's rule to the known optical rotations of panitol dodecaacetate, maltose and isomaltose octaacetates, and maltitol and isomaltitol nonaacetates, the specific rotation of isopanitol dodecaacetate was calculated to be + 87°.

Maltose and maltotriose were identified by paper chromatography but isomaltotriose could not be detected on chromatograms with any certainty. It is clear, of course, that the possible presence of a small amount of isomaltotriose among the hydrolysis products cannot be excluded. These observations are in agreement with the presence of the repeating unit [→6)-α-G-(1→4)-α-G-(1→4)-α-G-(1→] proposed by Wallenfels *et al.* for restpullullan on the basis of the more clear-cut enzymatic degradation. Estimation of DP by the measurement of the osmotic pressures of solutions of the polysaccharide methyl ether gave a value of *ca.* 280, corresponding to some 90 repeating units per molecule.

Table 1. Properties of panose, isopanose and the derived alditol dodecaacetates.

	R_G[a]	M_G[b]	$[\alpha]_D$ (equil.)	m.p.	DP	Alditol acetate m.p.	$[\alpha]_D$
Panose	0.54	0.24	+ 148°	218—220°	3.05	148.5—150.5°	+ 118°
Isopanose	0.50	0.59	+ 152°	—	2.96	69—72°	+ 88°

[a] R_F compared to that of glucose in isoamyl alcohol, pyridine, water, 7:7:6.
[b] Electrophoretic mobility compared to that of glucose in borate buffer, pH 10.

EXPERIMENTAL

Paper chromatograms were run on Whatman No. 1 papers in the following solvents:
A. Ethyl acetate, pyridine, water, 10:4:3.
B. Isoamyl alcohol, pyridine, water, 7:7:6 (2 descents).

Paper electrophoresis was carried out on Whatman No. 3 and 3 MM papers in 0.1 M borate buffer pH 10 at 0.8 kV.

Sugar determinations were made by the hypoiodite method; periodate and formic acid were determined iodometrically.

Partial hydrolysis of the glucan. The polysaccharide (12 g) was dissolved in 500 ml 0.05 N sulphuric acid and the solution was heated at 100° for 5 h. The cooled solution was neutralised with barium carbonate and the filtrate deionised with Dowex 50 and Dowex 3 resins. Evaporation of the solution yielded a syrup (12.8 g). The syrup was placed on a column (9.5 × 25 cm) of carbon-Celite and elution carried out with 5 l batches of aqueous ethanol of increasing ethanolic concentration to give seven fractions.

Examination of the fractions. Fraction I (1.28 g), eluted with water, showed glucose along with a trace of isomaltose on paper chromatograms.

Fraction II (2.23 g), eluted with 5 % ethanol, contained glucose and isomaltose. Fractionation of a portion (0.56 g) on a column (2.5 × 140 cm) of Sephadex G-25 [4] gave pure isomaltose (100 mg) with $[\alpha]_D$ + 110° (*c*, 1.0 in water). Acetylation with acetic anhydride and sodium acetate at 120° yielded a crystalline acetate, m.p. and mixed m.p. 146 – 148°, $[\alpha]_D^{22}$ + 97° (*c*, 0.85 in chloroform) and with infra-red spectrum identical to that of authentic isomaltose β-octaacetate.

Fraction III (1.28 g), eluted with 10 % ethanol, was found by examination on paper chromatograms in solvent B to consist mainly of isomaltose, with minor amounts of glucose, maltose, a trisaccharide with R_G 0.50 and possibly a trace of isomaltotriose (R_G 0.40, corresponding to isomaltotriose in a dextran hydrolysate).

Fraction IV (2.45 g), eluted with 15 % ethanol, had a main component with R_G 0.50 along with minor amounts of glucose, maltose, isomaltose and higher saccharides. No isomaltotriose could be detected. Fractionation on the Sephadex column of a portion (0.49 g) yielded a trisaccharide fraction (158 mg) which could be resolved by paper electrophoresis into components A and B with M_G 0.24 and 0.59, respectively. Both yielded glucose, maltose and isomaltose on partial hydrolysis (3.5 h at 100° in 0.05 N sulphuric acid). Partial hydrolysis of the derived alditols gave glucose and isomaltose, in the case of A, and glucose and maltose, in the case of B, as the only reducing sugars. The remainder of the fraction was refractionated by carbon-Celite column chromatography using borate buffer containing an ethanol gradient of 0 – 25 % to give trisaccharide A (0.59 g) and trisaccharide B (0.41 g). The minor components included a small amount of maltotriose.

Trisaccharide A had DP 3.05 (reducing power), was identical in chromatographic and electrophoretic mobility to panose (R_G 0.54, M_G 0.24) and had, on crystallisation from aqueous methanol, m.p. and mixed m.p. 218 – 220° and $[\alpha]_D^{22}$ + 160° → + 148° (*c*, 3.1 in water). The infra-red spectrum was identical to that of an authentic sample of panose. The sugar suffered marked overoxidation by periodate, consuming 8.5 moles periodate per mole and yielding 3.3 moles formic acid per mole after 30 h. Acetylation of the derived alditol [5] yielded a crystalline acetate, m.p. 148.5 – 150.5° and $[\alpha]_D^{22}$ + 118° (*c*, 1.12 in chloroform).

Trisaccharide B had R_G 0.50, M_G 0.59, $[\alpha]_D^{22}$ + 152° (*c*, 1.26 in water) and DP 2.96. Oxidation with periodate was complete after 30 h, 7.5 moles periodate per mole being consumed and 5.0 moles formic acid per mole being liberated. Acetylation of the derived alditol yielded a crystalline acetate, m.p. 69 – 72° and $[\alpha]_D^{22}$ + 88° (*c*, 0.43 in chloroform). (Found: C 50.0; H 6.11. Calc. for $C_{42}H_{58}O_{28}$: C 49.9; H 5.75). Comparison of the infra-red spectra of the two alditol dodecaacetates (KBr discs) showed the following differences in absorption frequency:

Panitol dodecacetate:	940,	891,	772,	765 cm⁻¹.
Isopanitol dodecacetate	948,	896,		760 cm⁻¹.

All other absorptions were coincident.

Fractions V (1.88 g), *VI* (1.49 g) and *VII* (1.44 g), eluted with 20 %, 25 %, and 50 % ethanol, respectively, were combined and the hydrolysis procedure was repeated. The sugars obtained were identified on paper chromatograms as glucose, isomaltose, trisaccha-

rides A and B (compound spot) and a fourth component slower than isomaltotriose, presumably a tetrasaccharide.

Determination of molecular weight. Osmotic pressure measurements were made on solutions of the methyl ether of the polysaccharide in butyl acetate at concentrations of 1.370 %, 0.822 % and 0.662 %. The derived molecular weight was 57 140, *i.e.* DP 280.

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