

- 1) This work was presented at the 87th Annual Meeting of the Pharmaceutical Society of Japan, Kyoto, April 1967.
- 2) Part V: T. Miyazaki and T. Yadomae, *Chem. Pharm. Bull.* (Tokyo), **16**, 1721 (1968).
- 3) Location: 600 Kashiwagi 4-chome, Shinjuku-ku, Tokyo.

When the polysaccharide was heated with 0.5N sulfuric acid for 5 hours and the product submitted to charcoal column chromatography, monosaccharide corresponding to about 50% of the total hexose and two main oligosaccharides were obtained. Results of partial hydrolysis by several steps are given in Chart 1 (details are given in the experimental part).

Oligosaccharide-I consisting of mannose was calculated as a disaccharide and oligosaccharide-II as a mannotrisaccharide from their end group assay. After methylation of these oligosaccharides with dimethyl sulfate-alkali and methyl iodide-silver oxide, followed by acid hydrolysis, the O-methyl-monosaccharides formed were identified by paper chromatography and paper electrophoresis, with reference to the previous results²⁾ of periodate oxidation. From oligosaccharide-I, 2,3,4,6-tetra-O-methyl- and 2,4,6-tri-O-methyl-D-mannose were detected and from oligosaccharide-II, 2,3,4,6-tetra-O-methyl-, 2,4,6-tri-O-methyl- and 3,4,6-tri-O-methyl-D-mannose were detected in the same ratio. Optical rotation: Oligosaccharide-I, $[\alpha]_D +11.1^\circ$ ($c=1.79$, H_2O); Oligosaccharide-II, $[\alpha]_D +16.6^\circ$ ($c=4.1$, H_2O), were lower than those of O- α -D-mannopyranosyl (1 \rightarrow 3)-D-mannose, $[\alpha]_D +50^\circ$,⁴⁾ and O- α -D-mannopyranosyl (1 \rightarrow 2)-O- α -D-mannopyranosyl(1 \rightarrow 2)-D-mannose, $[\alpha]_D +55^\circ$.⁵⁾ Therefore, presumably their linkages take β -configuration.

After complete methylation of the polysaccharide using the Haworth and the Purdie methods, followed by formic and sulfuric acid hydrolysis, the O-methyl-monosaccharides formed were examined by paper chromatography, thin-layer chromatography, and paper electrophoresis. Tetra-O-methyl-monosaccharide was identified with the authentic sample as 2,3,5,6-tetra-O-methyl-D-galactose. Although 2,4,6- and 3,4,6-tri-O-methyl-D-mannose and 2,3,6- and 2,4,6-tri-O-methyl-D-galactose were detected with a trace of di-O-methyl fraction, their componental ratio could not be determined because of the difficulty of their complete resolution.

The foregoing results showed that the intracellular polysaccharide has an acid-labile galactose portion corresponding to 32.2% of the total hexose. A part of galactose, 18.3% of the total hexose, and its oligosaccharides were rapidly released by treatment with 0.01N hydrochloric acid at 100° for 2 hours, and other part of galactose and trace of mannose were released after a prolonged heating under the same condition.

From the results of methylation, it is clear that the acid-labile galactose portion is in a furanose form and the non-reducing end of the polysaccharide is also a galactofuranose because of the sole detection of 2,3,5,6-tetra-O-methyl-D-galactose. Furthermore, the threitol-producible linkage described in the previous paper²⁾ is a 1 \rightarrow 5 linked galactofuranose by reason of not detecting 2,3,5-tri-O-methyl-D-galactose.

Thus, a large amount of galactose in the polysaccharide is present as a galactofuranosylglycan such as galactocarolose, a 1 \rightarrow 5 linked galactofuranosylglycan, which was isolated from *Penicillium charlesii*.⁶⁾ A small portion of galactose which is not acid-labile and is mainly 1 \rightarrow 3 linked pyranose, is attached to a mannan core in such a way as to form a linear structure.

The mannan core contains the units of mannose di- and trisaccharides. The structures of these units, based on the result of methylation and their optical rotations, are O- β -D-mannopyranosyl (1 \rightarrow 3)-D-mannose and O- β or α -D-mannopyranosyl (1 \rightarrow 2 or \rightarrow 3)-O- β or α -D-mannopyranosyl (1 \rightarrow 3 or \rightarrow 2)-D-mannose.

Therefore, the most probable structure of the polysaccharide would be:

D-Gal_f-(1 \rightarrow 5)-(D-Gal_f-(1 \rightarrow 5)-D-Gal_f)₄-D-Gal_p-(1 \rightarrow 3)-D-Gal_p-(1 \rightarrow 3)-D-Gal_p-(D-Man_p-(1 \rightarrow 2 and \rightarrow 3)-D-Man_p)₁₇-D-Man.

4) J.K.N. Jones and W.H. Nicholson, *J. Chem. Soc.*, 27, (1958).

5) P.A.J. Golrin and S.A. Perlin, *Can. J. Chem.*, 34, 1796 (1956).

6) W.N. Haworth, H. Ristrick and M. Stacey, *Biochem. J.*, 31, 640 (1937).

Experimental

Paper chromatograms were run by the ascending or descending method using the following solvent systems (*v/v*): (1) EtOAc-pyridine-H₂O=10:4:3, (2) EtOAc-pyridine-EtOH-H₂O=3:2:2:2, (3) 1-butanol-EtOH-H₂O=4:1:5, (4) 1-butanol-EtOH-H₂O=10:3:3, (5) EtOAc-AcOH-H₂O=9:2:2.

Gradual Hydrolysis of the Polysaccharide—1) Fragments produced by means of 0.01N HCl (for 2 hr), 0.1N and 2N H₂SO₄: The polysaccharide (21.9 mg) was hydrolysed with 0.01N HCl (2 ml) in a sealed tube in a boiling water bath for 2 hr. After neutralisation and filtration, the hydrolysate was concentrated to dryness and dried in a vacuum desiccator. The residual syrup was dissolved in precisely 0.5 ml of distilled water and 0.2 ml portion of the solution was separated by paper chromatography on a large sheet of filter paper using the solvent system (1). After air drying overnight, the section of the paper corresponding to galactose was quantitatively extracted with 10 ml of distilled water. The extract was filtered through a sintered glass filter and the amount of the sugar in 0.2 ml of the filtrate was estimated colorimetrically by the method of Dubois, *et al.*⁷⁾ Estimation of the total hexose was performed by the same procedure on the extract from an undeveloped paper. The content of liberated galactose in the total hexose was 18.3%.

The polysaccharide (102.0 mg) was hydrolysed under the same condition, the reaction mixture was neutralised with 0.01N NaOH, and dialysed in a Visking tubing against distilled water for 24 hr. The external solution was concentrated *in vacuo* to a syrup and examined by paper chromatography using the solvent system (2).

Three oligosaccharides were obtained (Fig. 1) and each component was hydrolysed with 2N H₂SO₄ in sealed tube at 100° for 3 hr. After neutralisation and filtration, the hydrolysate was concentrated *in vacuo* to a syrup which was examined by paper chromatography, using the solvent system (1). Each hydrolysate gave a spot corresponding to galactose.

Solution of the material in the Visking tube was concentrated *in vacuo* and EtOH was added to this concentrate. The precipitate that appeared was collected by centrifugation, washed with EtOH and ether, and dried *in vacuo*. Yield, 73.3 mg (71.8%).

This fragment was electrophoretically pure. A part of this fragment (5.7 mg) was hydrolysed in a sealed tube with 2N H₂SO₄ at 100° for 10 hr. After neutralisation and filtration, the hydrolysate was separated by paper chromatography using the solvent system (1) and componental sugars in the hydrolysate were estimated by the method of Dubois, *et al.* The molar ratio of galactose and mannose was 1:3.9.

Another part of the non-dialysable fragment was hydrolysed with 0.1N H₂SO₄ at 100° for 5 hr in a sealed tube. After neutralisation and filtration, the filtrate was dialysed in a Visking tubing against distilled water for 24 hr. The external solution was concentrated *in vacuo* to a syrup which was examined by paper chromatography. Monosaccharides in the dialysable fraction were galactose and mannose in the ratio of 2.3:1.

In addition to these monosaccharides, not sufficient amount of oligosaccharide to examine was detected on a paper chromatogram.

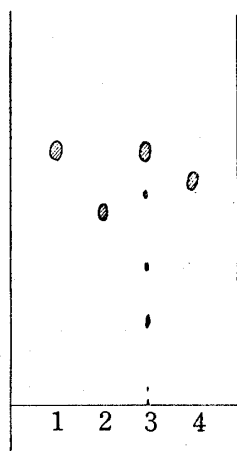


Fig. 1. Chromatogram of Fragment with 0.01N HCl for 2 hr

1: galactose 2: lactose
3: hydrolysate 4: maltose
sol. system (2) AgNO₃-NaOH

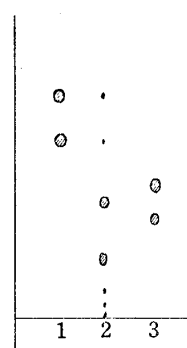


Fig. 2. Chromatogram of 5% EtOH Fraction

1: mannose, galactose
2: 5% EtOH fraction
3: maltose, lactose
sol. system (1) AgNO₃-NaOH

7) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

Solution of the material in the tube was concentrated to a syrup and EtOH was added to this concentrate. The precipitate that appeared was collected by centrifugation, washed with EtOH and ether, and dried *in vacuo*. Yield, 26.5 mg (48.5%).

This fragment was electrophoretically pure, and components of this fragment were mannose and a trace of galactose, its molar ratio being 28.3:1.

2) Fragments produced by means of 0.01N HCl (for 8 hr): The polysaccharide (14.3 mg) was hydrolysed with 0.01N HCl for 8 hr by the procedure described above.

The amount of galactose and mannose in 0.3 ml of the filtrate was estimated by the same method as in 1). Its results indicated that the content of galactose and mannose in the total hexose was respectively 32.2% and 1.9%. When the hydrolysate was separated by paper chromatography using the solvent system (1), oligosaccharides of low molecular size were not detected.

The original point of the paper chromatogram, which was used to determine monosaccharides, was extracted with distilled water, the extract was concentrated *in vacuo*, and the syrup was hydrolysed with 2N H₂SO₄ at 100° for 15 hr in a sealed tube. The hydrolysate was estimated by the method described above. The molar ratio of galactose to mannose was 1:7.0.

3) Fragments produced by means of 0.5N H₂SO₄: The polysaccharide (crude, 3.9 g) was dissolved in 0.5N H₂SO₄, heated in a boiling water bath for 5 hr. After neutralisation and filtration, the hydrolysate was concentrated *in vacuo* to about 30 ml and passed through a column of charcoal (5 × 55 cm). Elution was effected stepwise with water, 5, 15, 30 and 50% EtOH.

Each fraction was concentrated *in vacuo* to a small volume and then examined by paper chromatography. The water-fraction was only monosaccharides such as galactose and mannose, the 5% EtOH fraction was considerable amount of oligosaccharides of low molecular size and a small amount of monosaccharide. Fractions of 15, 30 and 50% EtOH were oligosaccharides of higher molecular size.

The fraction of 5% EtOH was separated by paper chromatography using the solvent system (1) (Fig. 2).

The fast moving component was named oligosaccharide-I and the slower moving one oligosaccharide-II. These saccharides were eluted with water from the appropriate sections of the chromatogram.

Characterisation of Oligosaccharide-I—Oligosaccharide-I was chromatographically pure and had $[\alpha]_D +11.1^\circ$ ($c=1.79$, H₂O).

Determination of its reducing power by the method of alkaline hypoiodite⁸⁾ gave the ratio of that of mannose to oligosaccharide-I as 1:0.516.

After hydrolysis of this by means of 2N H₂SO₄ in a sealed tube in a boiling water bath for 8 hr, paper chromatographic analysis of the hydrolysate showed it to be composed of mannose alone.

The disaccharide (50.7 mg) was methylated with Me₂SO₄ (3 ml) and 30% NaOH (9 ml), followed by second methylation using Me₂SO₄ (2.5 ml) and 30% NaOH (7.5 ml). The product, which was extracted with CHCl₃, was further methylated by the Purdie method⁹⁾ to give a syrup. The methylated oligosaccharide-I showed absence of hydroxyl group in its IR spectrum.

The methylated oligosaccharide was hydrolysed with 90% formic acid for 1 hr, 100°, and after cooling, the hydrolysate was concentrated *in vacuo*. To the concentrate, 5 ml of 0.5N H₂SO₄ was added, the mixture was heated for 5 hr at 100°. After neutralisation and filtration, the filtrate was concentrated to a small volume, and the residue was examined by paper chromatography using the solvent systems (3), (4) and (5), and by paper electrophoresis. Two spots corresponding to 2,3,4,6-tetra-O-methyl-D-mannose and 2,4,6-tri-O-methyl-D-mannose were detected on the paper chromatogram with *p*-anisidine.

The section of paper chromatogram corresponding to tri-O-methyl-D-mannose was extracted with CHCl₃ and the extract was examined by paper electrophoresis using 0.026 M borate buffer (pH 10.0). The *Mg.* value¹⁰⁾ of the extracted sugar was 0.

Characterisation of Oligosaccharide-II—Oligosaccharide-II was chromatographically pure and had $[\alpha]_D +16.6^\circ$ ($c=4.1$, H₂O).

Hydrolysis with 2N H₂SO₄ for 8 hr and paper chromatographic analysis of the hydrolysate showed a trisaccharide to be composed of mannose only.

The oligosaccharide (48.0 mg) was methylated as described above and IR spectrum of the methylated oligosaccharide-II showed no hydroxyl group. The methylated material was hydrolysed by the same procedure as that of oligosaccharide-I and the residual syrup was examined by paper chromatography. In solvent systems (3), (4) and (5), *p*-anisidine revealed two spots corresponding to 2,3,4,6-tetra-O-methyl-D-mannose and tri-O-methyl-D-mannose. The section of the paper corresponding to tri-O-methyl-D-mannose was extracted with CHCl₃, the extract was examined by paper electrophoresis using the same condition as described above, and *p*-anisidine revealed two spots *Mg.* 0 and *Mg.* 0.33. The *Mg.* values of authentic 2,4,6-tri-O-methyl-D-mannose and 3,4,6-tri-O-methyl-D-mannose were 0 and 0.33, respectively.

8) M. Tomoda, *Yakugaku Zasshi*, **80**, 1696 (1960).

9) T. Purdie and J.C. Irvine, *J. Chem. Soc.*, **83**, 1021 (1903).

10) *Mg.* value represents the true movement relative to glucose and 2,3,4,6-tetra-O-methyl-D-glucose.

Methylation of the Polysaccharide—The polysaccharide (1.6 g) was dissolved in H_2O (20 ml), Me_2SO_4 (15 ml) and 30% NaOH (45 ml) were added to this mixture dropwise with vigorous stirring and water cooling, at such a rate that the mixture remained slightly alkaline. After 4 hr, the reaction mixture was allowed to warm to room temperature, and Me_2SO_4 (50 ml) and 30% NaOH (150 ml) were added dropwise during 6 hr, with stirring continued overnight. The mixture was heated on a boiling water bath for 1 hr to destroy the excess of Me_2SO_4 and the ice-cooled reaction mixture was extracted repeatedly with CHCl_3 . The product thus obtained was methylated again under the above condition. The methylated product was further methylated by refluxing with CH_3I (30 ml) and Ag_2O (3 g) which was added in small portions during 3 hr and refluxing was continued for further 24 hr. The same treatment was repeated four times. The final product was extracted with CHCl_3 from the reaction mixture. The CHCl_3 extract was washed with H_2O , dried over anhyd. Na_2SO_4 , and the solvent was distilled off. To the CHCl_3 solution of the final product, 0.5 volume of hexane was added, and after removal of a small quantity of the precipitate by centrifugation, excess of hexane was added to the supernatant. The precipitate was collected by centrifugation and dried *in vacuo* to a pale yellow powder. The content of $-\text{OCH}_3$ in the methylated polysaccharide was 42.8% (Zeisel).

Examination of the Methylated Monosaccharides—The methylated polysaccharide (138 mg) was hydrolysed by heating with 90% HCOOH (10 ml) for 10 hr at 100° and after removal of HCOOH , followed by heating with 1N H_2SO_4 (10 ml) for further 4 hr. The hydrolysate was neutralised with BaCO_3 , BaSO_4 was filtered off, and the filtrate was passed through a column of Amberlite IR-120 (H). The eluate on concentration, gave the methylated monosaccharides as a syrup. The syrup was examined by paper and thin-layer chromatographies, and also by paper electrophoresis. The syrup was separated on a sheet of filter paper Toyo Roshi No. 50 (40×40 cm) impregnated with the solvent system (5). The three bands on the paper chromatogram corresponded to the tetra-, tri-, and a small amount of di-O-methylhexose fractions.

Tetra-O-methylhexose Fraction: The fraction gave a single brown spot by *p*-anisidine on the paper and thin-layer chromatography, and its *R_f* value is identical with that of an authentic sample of 2,3,5,6-tetra-O-methyl-D-galactose, and by paper electrophoresis using 0.026 M borate buffer showed a single spot, *Mg.* 0.

Tri-O-methylhexose Fraction: Paper chromatography of the tri-O-methylhexose fraction in the solvent systems (4) and (5) disclosed at least two components. The fraction was then fractionated by thin-layer chromatography using the solvent system of acetone: $\text{C}_6\text{H}_6 = 1:1^{10}$ into fraction-I (faster component) and fraction-II (slower component).

Fraction-I was examined by paper electrophoresis and *p*-anisidine revealed one spot, *Mg.* 0 but multiple paper chromatography of the fraction with the solvent systems (3) and (4), showed two spots corresponding to 2,3,6-tri-O-methyl- and 2,4,6-tri-O-methyl-D-galactose and no 2,3,5-tri-O-methyl-D-galactose (brown spot with *p*-anisidine).

Fraction-II, on paper chromatography, showed component with *R_f* value corresponding to that of 2,4,6- and 3,4,6-tri-O-methyl-D-mannose, and its examination by paper electrophoresis, revealed two spots, *Mg.* 0 and 0.33.

The component of *Mg.* 0 was identical with that of 2,4,6-tri-O-methyl-D-mannose and that of *Mg.* 0.33 was identical with that of 3,4,6-tri-O-methyl-D-mannose.

11) R. Tschsche and G. Balle, *Tetrahedron*, **19**, 2323 (1963).