

## A GLUCOMANNAN FROM THE TUBERS OF *ORCHIS MORIO*

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(Received 11 July 1973 Accepted 28 August 1973)

**Key Word Index**—*Orchis morio*, Orchidaceae, salep mannan, glucomannan, mucilage, reserve polysaccharide

**Abstract**—A glucomannan containing D-glucopyranosyl and D-mannopyranosyl residues in the molar ratio *ca* 1 : 3 : 3 has been extracted with water from the tubers of *Orchis morio*. The polysaccharide has a  $\overline{DP}_n$  of *ca* 665 and consists of a backbone of  $\beta(1 \rightarrow 4)$ -linked glucosyl and mannosyl residues with *ca* 7 branch points per molecule, probably at C3 positions of the hexose residues. The polysaccharide contains acetyl groups linked almost exclusively to the C2 or C3 positions of mannose residues. Structural studies showed that this water-extracted glucomannan is similar to the material of the mucilage globules which had been mechanically separated and isolated from the tissue of young tubers.

### INTRODUCTION

THE tubers of orchis species contain a water-soluble reserve polysaccharide which has been named salep mannan. Early workers<sup>1,2</sup> showed the mucilage to be a (1  $\rightarrow$  4) linked mannan of high  $\overline{DP}_n$  which is partially acetylated. Later studies<sup>3</sup> showed that the salep mannan from various species of orchids consists of a glucomannan containing  $\beta(1 \rightarrow 4)$ -linked D-glucopyranosyl and D-mannopyranosyl residues in the ratio *ca* 1 : 3 : 3. Methylation analysis indicated that the glucomannans isolated from some species could be branched<sup>3</sup> and it was also stated that acetyl groups were absent.<sup>3</sup> Structural studies, reported below, have been carried out on glucomannans isolated from the young tubers of *Orchis morio*. Related work on the biosynthesis of salep mannan has already been published<sup>4,5</sup>.

### RESULTS AND DISCUSSION

The plants were harvested during the months of June–August and the young tubers were immediately ground and heated in boiling EtOH to inactivate the enzymes present.<sup>2</sup> The crude polysaccharide obtained from the aqueous extract of the plant material was treated with  $\alpha$ - and  $\beta$ -amylase to degrade starch. The residual polysaccharide on acid hydrolysis gave glucose and mannose in the molar ratio 1 : 3 : 3 and precipitation with Fehling's solution did not significantly alter this ratio.

The mucilage in orchid tubers is formed in specialized very large cells which are finally completely filled out with the polysaccharide. Whole mucilage globules, free of starch and

<sup>1</sup> KLAGES, F. and NIEMANN, R. (1936) *Ann.* **523**, 224.

<sup>2</sup> HUSEMANN, E. (1940) *J. Prakt. Chem.* **155**, 241.

<sup>3</sup> DALOUL, M., PETEK, F. and COURTOIS, J. E. (1963) *Bull. Soc. Chim. Biol.* **45**, 1247; COURTOIS, J. E., DALOUL, M. and PETEK, F. (1963) *Bull. Soc. Chim. Biol.* **45**, 1255; PETEK, F., COURTOIS, J. E. and DALOUL, M. (1963) *Bull. Soc. Chim. Biol.* **45**, 1261.

<sup>4</sup> FRANZ, G. and MEIER, H. (1971) *Planta Med.* **19**, 326.

<sup>5</sup> FRANZ, G. (1973) *Phytochemistry* **12**, 2369.

other extraneous material were isolated. On acid hydrolysis they released glucose and mannose in the molar ratio 1:3:6.

The water-extracted glucomannan was methylated successively by the methods of Haworth<sup>6</sup> and Hakomori<sup>7</sup> and the product showed no hydroxyl absorption in its IR spectrum. A sample of the methylated glucomannan was methanolysed and examined by GLC. A second sample was hydrolysed<sup>8</sup> and the derived glycol acetates were examined by GLC. The hydrolysate was also examined by PC and TLC. The following sugars were identified: 2,3,4,6-tetra-*O*-methylhexose (glucose and mannose), 2,3,6-tri-*O*-methylmannose, 2,3,6-tri-*O*-methylglucose and di-*O*-methylhexose in the peak area ratio 10:59:18:5:0:5. The di-*O*-methylhexose is neither 2,3-di-*O*-methylmannose nor 2,3-di-*O*-methylglucose but is probably a mixture of the corresponding 2,6-di-*O*-methylhexoses. Only traces of tetra-*O*-methylglucose were detected.

The mucilage globules were methylated and examined in the way described for the water-extracted glucomannan. Hydrolysis of the fully methylated material gave 2,3,4,6-tetra-*O*-methylhexose (glucose and mannose), 2,3,6-tri-*O*-methylmannose, 2,3,6-tri-*O*-methylglucose and di-*O*-methylhexose in the peak area ratio 10:31:10:0:6.

The methylated water-extracted glucomannan and the methylated globules from the mucilage cells are quite similar. However, the percentage of non-reducing end groups (tetra-*O*-methylhexoses) and also that of the branch points (di-*O*-methylhexoses) is about twice as high in the glucomannan of the mucilage globules than in the water-extracted glucomannan. This difference could be explained by some depolymerization during the somewhat lengthy, but necessary treatment of the water-extracted glucomannan with the amylases. As it is difficult to estimate the values for the  $\overline{DP}_n$  from the methylation analysis (the difference in the quantities of the tetra-*O*-methylhexoses and di-*O*-methylhexoses being small) the water-extracted glucomannan was nitrated<sup>9</sup> and the molecular weight was determined by membrane osmometry. A value of *ca.* 665 was obtained. Using this value and the percentage of tetra-*O*-methylhexose the number of branch points in the water-extracted glucomannan is *ca.* 7 per molecule. In the native glucomannan (mucilage globules), whose  $\overline{DP}_n$  was not determined by osmometry, the number of branch points for a molecule of a certain  $\overline{DP}_n$  appears to be about twice that of the water-extracted glucomannan.

The water-extracted glucomannan on partial acid hydrolysis gave *inter alia* the following oligosaccharides: mannobiose, 4-*O*- $\beta$ -D-mannopyranosyl-D-glucose, 4-*O*- $\beta$ -D-glucopyranosyl-D-mannose, cellobiose, mannotriose, 4-*O*- $\beta$ -D-glucopyranosyl-D-mannobiose, 4-*O*- $\beta$ -D-mannobiosyl-D-glucose and probably mannotetraose. The isolation of these oligosaccharides shows that there are both contiguous glucosyl and mannosyl residues which are  $\beta(1 \rightarrow 4)$ -linked.

The acetyl content of the glucomannan is *ca.* 5.3% and on deacetylation the polysaccharide becomes water insoluble. Samples of the acetylated and deacetylated polysaccharides were oxidized with NaIO<sub>4</sub>, the oxidized polysaccharides were reduced with NaBH<sub>4</sub> and hydrolysed. The polysaccharide which had been deacetylated gave glycerol, erythritol, mannose and glucose in the molar ratio 10:35:4:0:2:0:2 whereas the acetylated material gave these compounds in the ratio 10:9:4:2:2:trace. In the acetylated sample a high proportion of the mannose residues but only a few glucose residues survived oxidation. The

<sup>6</sup> HAWORTH, W. N. (1915) *J. Chem. Soc.* **107**, 8.

<sup>7</sup> HAKOMORI, S. (1964) *J. Biochem. (Tokyo)* **55**, 205.

<sup>8</sup> BOUVENG, H. O., KJESLING, H., LINDBERG, B. and MCKAY, J. E. (1962) *Acta Chem. Scand.* **16**, 615.

<sup>9</sup> DAVIDSON, G. F. (1938) *J. Textile Inst.* **29**, T195.

small quantities of surviving hexose residues in the deacetylated sample probably arose from branching at C3 positions or from incomplete oxidation. The acetyl content and the proportion of mannose residues resistant to oxidation (17.5%) are in good agreement if one assumes that virtually all of the acetyl groups are linked to C2 or C3 positions of mannose residues. In this respect the glucomannan is similar to that isolated from *Pinus sylvestris*<sup>10</sup>. The branch points most probably occur at C3 positions of hexose residues. The work of Courtois *et al.*<sup>3</sup> on the glucomannans of *Loroglossum hircinum* and *Orchis intacta* (?) suggested that branching occurred at C6 positions.

## EXPERIMENTAL

**General methods.** PC was on Schleicher and Schuell No. 2043b paper and TLC on Kieselgel G (Merck) using (a) EtOAc–pyridine–H<sub>2</sub>O (8:2:1), (b) EtOAc–pyridine–H<sub>2</sub>O (2:1:2), (c) EtOAc–HOAc–H<sub>2</sub>O (9:2:2) containing 0.55% phenylboronic acid, (d) MeCOEt–H<sub>2</sub>O–NH<sub>3</sub> (100:50:3), and (e) *n*-BuOH–EtOH–H<sub>2</sub>O–NH<sub>3</sub> (4:1:5:trace). Chromatographic detection reagents were alkaline AgNO<sub>3</sub>, naphth-1-ol–conc. H<sub>2</sub>SO<sub>4</sub> or NaIO<sub>4</sub>–benzidine. A Perkin–Elmer F30 chromatograph was used for GLC with glass columns (2 m × 2 mm i.d.) containing (a) 3% ECNSS-M on Gas Chrom Q (100–120 mesh) and (b) 10% *m*-bis(*m*-phenoxyphenoxy)benzene on AW DMCS Chromosorb W (100–120 mesh). Hydrolyses were carried out with 0.5 M H<sub>2</sub>SO<sub>4</sub> at 120° for 1 hr in an autoclave and the hydrolysates were neutralized with BaCO<sub>3</sub>. The sugars in hydrolysates were estimated by GLC (column a) of their glycol acetates.

**Isolation and  $\overline{DP}_n$  determination of the glucomannan.** The plants (*Orchis morio* L.) were harvested during the months of June–August. The young tubers were immediately separated, rinsed with H<sub>2</sub>O and homogenized at 4°. The plant tissue was then heated in boiling EtOH for 1 hr to inactivate enzymes<sup>2</sup> and subsequently extracted with H<sub>2</sub>O for 15 hr. The polysaccharide was precipitated by the addition of EtOH (4 vol.), dissolved in H<sub>2</sub>O and incubated with a mixture of  $\alpha$ -amylase (bacterial, Fluka) and  $\beta$ -amylase (from barley, Fluka) for 4 days (pH 5.8, 35°) in order to degrade contaminating starch. The low MW degradation products were removed by dialysis; neither mannose nor any mannose containing oligosaccharide was detected in the diffusible material. The purified polysaccharide had  $[\alpha]_D^{25} -36^\circ$  (c 0.9, H<sub>2</sub>O), OAc 5.3% and on acid hydrolysis gave glucose and mannose in the molar ratio 1:3.3. A sample (200 mg) of the glucomannan was nitrated<sup>9</sup> (N, 12.4%) and the  $\overline{DP}_n$  was determined to be ca. 665 by membrane osmometry using a Sartorius SM 11539 membrane and butyl acetate as solvent.

**Isolation of the entire content of the mucilage cells.** Freshly harvested (August) young tubers were cut into slices ca. 5 mm thick and the exposed tissue was moistened with 70% EtOH. By scraping over the surface with a razor blade large mucilage globules could be removed from the tissue. This material was immediately suspended in 70% EtOH and the suspension was subjected to mild ultrasonic treatment at 0° for 5 min to remove adhering starch granules and other cell debris from the globules of mucilage. Floating cell debris was removed by decantation and the starch granules were removed by filtration through a sieve (mesh size 0.3 mm) which exclusively retained the mucilage globules. The above procedure was repeated until microscopic examination revealed them to be free of extraneous material. The mucilage globules, which readily dissolved in H<sub>2</sub>O had  $[\alpha]_D^{25} -42^\circ$  (c 0.5, H<sub>2</sub>O) and on acid hydrolysis gave glucose and mannose in the molar ratio 1:3.6.

**Methylation of the water-extracted glucomannan and the mucilage globules.** A sample (100 mg) of the water-extracted glucomannan was methylated successively by the methods of Haworth<sup>6</sup> and Hakomori.<sup>7</sup> The product was extracted with petrol, and the residue (100 mg, yield 82%) which was soluble in CHCl<sub>3</sub> showed no hydroxyl absorption in its IR spectrum (CCl<sub>4</sub> soln). A sample of the methylated polysaccharide was heated with 4% MeOH–HCl in a sealed tube at 100° for 12 hr. The methanolysate was directly examined by GLC (column b) and the following sugars were identified as the methyl glycosides by comparison with authentic compounds: 2,3,4,6-tetra-*O*-methylmannose, 2,3,6-tri-*O*-methylmannose, 2,3,4,6-tetra-*O*-methylglucose, 2,3,6-tri-*O*-methylglucose and di-*O*-methylhexose. A sample of the methylated polysaccharide was hydrolysed,<sup>8</sup> reduced with NaBH<sub>4</sub> and acetylated with Ac<sub>2</sub>O–pyridine (1:1). The following sugars were identified by GLC (column a) as their derived glycol peracetates by comparison with authentic compounds: 2,3,4,6-tetra-*O*-methylmannose and 2,3,4,6-tetra-*O*-methylglucose (unresolved), 2,3,6-tri-*O*-methylmannose, 2,3,6-tri-*O*-methylglucose and a mixture of 2,6-di-*O*-methylhexoses (?) (glucose and mannose). Another sample of the hydrolysate was examined by TLC and PC (irrigants D and E) and the identities of the above sugars were further confirmed. The above methylation procedure was repeated using a sample (ca. 50 mg) of mucilage globules to yield a product (36 mg, yield 60%) which showed no hydroxyl absorption in its IR spectrum. GLC examination (column a) of the glycol acetates in a hydrolysate of the methylated material showed it to be similar to the hydrolysates of the methylated glucomannan.

**Partial acid hydrolysis of the water-extracted glucomannan.** A sample (3 g) of the glucomannan was dispersed in 2% H<sub>2</sub>SO<sub>4</sub> (600 ml) and heated for 5 hr at ca. 100°. The cooled hydrolysate was neutralized (BaCO<sub>3</sub>), deionized

<sup>10</sup> MEIER, H. (1960) *Acta Chem. Scand.* **14**, 749.

(Dowex 50 H<sup>+</sup>) and reduced in vol. The hydrolysate was fractionated on a column of activated charcoal and celite (1:1) using a H<sub>2</sub>O-EtOH gradient. The fractions were further purified by PC (irrigant B) to give glucose, mannose and 8 oligosaccharides. These oligosaccharides were identified by direct comparison with authentic compounds and by their  $R_G$  values.<sup>10</sup> The oligosaccharides were further examined by comparison of the hydrolysates of the oligosaccharides and the corresponding reduced oligosaccharides (PC, irrigants A and C, GLC, column a).

*Periodate oxidation of the glucomannan*<sup>11</sup> A sample of the polysaccharide was treated with 10% NaOH overnight and the deacetylated polysaccharide was recovered. Samples (50 mg) of the glucomannan and the deacetylated material were oxidized with 0.05 M NaIO<sub>4</sub> at room temp. in the dark. After 10 days the consumption of periodate, determined spectrophotometrically,<sup>12</sup> became almost constant. Values of 0.8 and 1.05 mol of periodate per hexose residue were obtained for the acetylated and deacetylated samples respectively. The periodate was removed by dialysis and the oxopolysaccharides were reduced with NaBH<sub>4</sub>. The products were hydrolysed and PC (irrigant A) showed the presence of glycerol, erythritol, glucose and mannose. The hydrolysates were reduced with NaBH<sub>4</sub> and acetylated with Ac<sub>2</sub>O/NaOAc. The glycitol acetate mixtures were examined by GLC (column a) and the following compounds were identified by comparison with authentic samples: glycerol triacetate, erythritol tetraacetate, glucitol hexaacetate and mannitol hexaacetate.

*Acknowledgements*—Thanks are expressed to the Royal Society for a European Fellowship to A.J.B. and to the Swiss National Research Foundation for financial support. We wish to acknowledge the technical assistance of Fräulein F. Busar.

<sup>11</sup> MEIER, H. (1961) *Acta Chem. Scand.* **15**, 1318.

<sup>12</sup> ASPINALL, G. O. and FERRIER, R. J. (1957) *Chem. Ind.* 1216.