A MANNAN PRODUCED BY SACCHAROMYCES ROUXII[1]

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ABSTRACT

The main slime polysaccharide produced by *Saccharomyces rouxii* during the fermentation of D-glucose to D-arabitol is a mannan. Acetolysis of the polymer has afforded di- and tri-saccharides in good yield, but no higher oligosaccharides were produced. The disaccharide is shown by methylation and lead tetraacetate oxidation to be 2-*O*-α-D-mannopyranosyl-D-mannose, establishing the presence of a 1,2-α-linkage in the polysaccharide. On acid hydrolysis the methylated mannan gives mainly 2,3,4,6-tetra-*O*-methyl-D-mannose, 3,4,6-tri-*O*-methyl-D-mannose, and 3,4-di-*O*-methyl-D-mannose; lesser components found are 2,4,6-tri-*O*-methyl-D-mannose and 3-*O*-methyl-D-mannose. The methylation and acetolysis data together suggest an average structural unit consisting of a main chain of D-mannopyranose units containing alternate 1,2- and 1,6-linkages, to which single D-mannopyranose units are attached by 1,2-linkages as side chains; alternatively, 1,2-disaccharide units are attached as side chains by 1,2-linkages to a 1,6-linked main chain.

During the fermentation of D-glucose to D-arabitol by *Saccharomyces rouxii* (14) a slime is produced in the culture medium. The slime was isolated from an active culture filtrate by repeated precipitation with ethanol from water as a light brown powder containing approximately 15% protein. It yielded mainly mannose, on acid hydrolysis, although traces of pentose and another hexose were detected in its hydrolyzate on paper chromatograms. After acetylation, followed by deacetylation of the product, a polysaccharide was obtained which was virtually free of protein and contained only mannose. This purified material had $[\alpha]_D$ +58°, and in common with other mannans formed an insoluble copper complex.

Partial acetolysis of the polysaccharide gave rise to a mixture of mono-, di-, and tri-saccharide acetates which, after saponification, were chromatographed on charcoal (15) and then on cellulose (10). The yields of the di- and tri-saccharide were 10% and 25%, respectively. The disaccharide fraction was amorphous but appeared, from paper chromatographic examination, to be comprised of a single component. It yielded a crystalline octa-*O*-methyl ether, which on hydrolysis gave only crystalline 3,4,6-tri-*O*-methyl-D-mannose (1) and 2,3,4,6-tetra-*O*-methyl-D-mannose, characterized as the phenylhydrazide of the corresponding acid (5). The disaccharide, therefore, was 2-*O*-D-mannopyranosyl-D-mannose; as it has not been crystallized, its possible identity with the crystalline 1,2-mannobiose synthesized by Gakhokidze and Kutidze (6) is not determined. Sodium borohydride reduction of the compound gave crystalline 2-*O*-D-mannopyranosyl-D-mannitol, which, in agreement with the formulated structure, consumed 5 moles of lead tetraacetate per mole (12), producing 1 mole of formaldehyde per mole. The configuration of the glycosidic linkage was established by controlled lead tetraacetate oxidation of the

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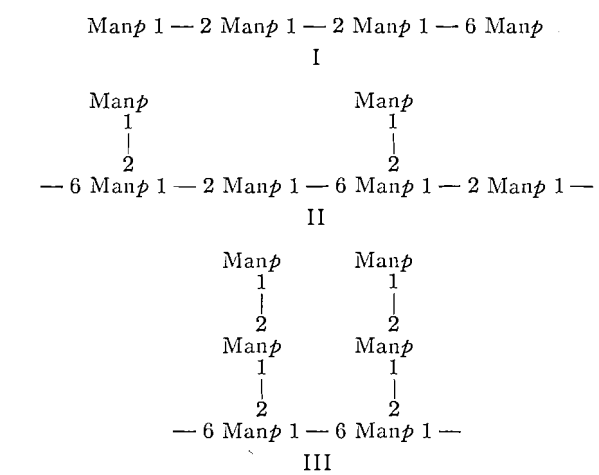
mannobiitol to give 2-*O*-D-mannopyranosyl-D-glyceraldehyde, which was then reduced to sirupy 2-*O*-D-mannopyranosyl-glycerol. The latter proved to be the α-anomer since it and its crystalline hexa-*p*-nitrobenzoate had more positive optical rotations, respectively, than the corresponding β-anomer and its derivative (3), respectively. Therefore the 1,2-linkage in the polysaccharide, as represented by the mannobiose, must possess the α-configuration.

On treatment with lead tetraacetate the mannobiose consumed only traces of oxidant during a reaction time of 30 min., and when oxidation was promoted by addition of potassium acetate (12) 1 mole of formaldehyde per mole was produced within the same period. These results, together with the observed rate of formic acid production and the quantity of reagent consumed, are in complete accord with those anticipated for a 1,2-hexose disaccharide (4, 13), and hence validate the earlier suggestion that lead tetraacetate oxidations may be useful for determining the position of linkage of 1,2-disaccharides as well as of other disaccharide types. Since all but 1,2-reducing disaccharides rapidly consume at least 1 to 2 moles of oxidant in the uncatalyzed reaction (4), the absence of significant lead tetraacetate uptake by the amorphous disaccharide constitutes further evidence of its essential homogeneity.

The mannan was methylated by three treatments with dimethyl sulphate – sodium hydroxide, and the product was then subjected to methanolysis followed by hydrolysis with 80% formic acid at 100° C. Paper chromatographic examination indicated that the hydrolyzate contained five component reducing *O*-methyl ethers. These were separated by chromatography on cellulose and found to be: (*a*) 2,3,4,6-tetra-*O*-methyl-D-mannose, a sirup characterized by conversion to crystalline 2,3,4,6-tetra-*O*-methyl-D-mannonic acid phenylhydrazide (5); (*b*) crystalline 3,4,6-tri-*O*-methyl-D-mannose (1); (*c*) 2,4,6-tri-*O*-methyl-D-mannose, a sirup which was characterized by the fact that on bromine oxidation followed by treatment with methanolic ammonia it gave 2,4,6-tri-*O*-methyl-D-mannonic acid amide (7) and that it was unoxidized by aqueous periodic acid; (*d*) crystalline 3,4-di-*O*-methyl-D-mannose, which was identified from the fact that on lead tetraacetate oxidation it gave mono-*O*-formyl-2,3-di-*O*-methyl-D-arabinose, which in turn was converted to crystalline 2,3-di-*O*-methyl-D-arabonic acid amide; and (*e*) 3-*O*-methyl-D-mannose, characterized since it rapidly consumed 1 mole per mole of lead tetraacetate (13) to yield a material giving a pink color with the *p*-anisidine hydrochloride spray (10) on a paper chromatogram. The ratio of compounds $a:b:c:d$ was approximately 10:7:2:10, and the mono-*O*-methyl ether comprised about 5% of the total. In addition to the 1,2-linkage, already established by isolation of the 1,2-mannobiose, the presence of the 1,6-linkage as a second major type is indicated by the methylation results, and the polysaccharide is shown to possess a highly branched structure. The isolation of 2,4,6-tri- and 3-mono-*O*-methyl-D-mannose in small amounts suggests that the polymer also contains a small proportion of 1,3- and 1,4-linkages but it is possible that these minor components originate through incomplete methylation.

A variety of possible repeating units may be devised using this methylation data. The results of partial acetolysis, however, assist considerably in elimi-

nating certain of these possibilities. As noted above, the acetolysis experiment furnished oligosaccharides having no more than three mannose residues. The trisaccharide fraction, although not yet completely characterized, is known from methylation results to contain only the 1,2-type of glycosidic linkage* making it improbable that the sequence of mannose residues linked 1,2- in the polysaccharide rarely, if ever, exceeds two. Since the yield of trisaccharide is high and because almost 90% of the linkage types encountered are 1,2- and 1,6-, in a ratio of close to 2: 1, then these linkages must frequently occur in a sequence such as represented by (I). To account for the tetra-*O*-methyl (end-groups) and di-*O*-methyl (branch-points) ethers in the proportion in which they are obtained, but not the minor component *O*-methyl ethers, structure (I) is enlarged to give (II) and (III). The latter two types of structural unit are



consistent with all of these data and, accordingly, are suggested as possible average representations for a major proportion of the mannan.

In many respects the slime mannan closely resembles the mannan isolated by Haworth and co-workers from baker's yeast by alkali extraction (7, 8). The acetate of the latter polysaccharide showed $[\alpha]_D$ +59° as compared with the present value of $[\alpha]_D$ +53°. Further, the two methylated mannans on hydrolysis have yielded the same *O*-methyl ethers in approximately the same proportions, with a few minor exceptions. The interpretations of the two sets of data, however, differ somewhat for, in contrast with (II) and (III), all three of the possible formulae advanced by the earlier workers (7) have chiefly 1,2-linkages in the main chain and 1,6-linkages in the side chain. Northcote and Horne (11) have produced evidence that the mannan constitutes part of the cell wall membrane rather than a storage material of the cell. The similarity in the fine structure of the two polysaccharides, therefore, raises the question as to whether the slime mannan is truly extracellular, or represents cell wall matter which is exuded by the active organism or released from spent cells through autolysis.

* *Unpublished data.*

EXPERIMENTAL

Paper chromatography was carried out on Whatman No. 1 filter paper using *n*-butanol–ethanol–water (40: 11: 19 v/v) as solvent and *p*-anisidine hydrochloride as spray reagent (10). Melting points are uncorrected. Evaporations were carried out under reduced pressure at 40° C. Optical rotations were measured at 27° C.

Preparation of Crude Mannan

A 30% D-glucose solution (5 gal.) containing corn steep liquor (1.1%) and urea (0.53%) was fermented for nine days at 30° C. by *Saccharomyces rouxii*. The cell-free culture medium was concentrated and added to an excess of ethanol and the slime thus obtained was twice reprecipitated by ethanol from water. The final product was isolated as a light brown powder (66 gm.; found: N, 2.62%) which gave on acid hydrolysis mainly mannose, detected on a paper chromatogram. Traces of another hexose and of a pentose were also shown to be present.

Purification of Mannan

The polysaccharide (5.4 gm.), obtained above, was pasted in formamide (50 ml.) and shaken for 18 hr. in a mixture of pyridine (50 ml.) and acetic anhydride (20 ml.). Most of the polysaccharide dissolved and the suspension was filtered off. The filtrate was added to ice water and the precipitate centrifuged off, washed twice with water, and dried. The acetylated material (6.2 gm.) had $[\alpha]_D$ +53° (*c*, 0.75, chloroform).

The acetate (0.89 gm.) was dissolved in chloroform (25 ml.) to which sodium methoxide (0.08 gm. of sodium in 75 ml. of methanol) was added. After one hour the solution was acidified with dilute hydrochloric acid and the precipitate which had formed was filtered off. The polysaccharide was precipitated once from water with an excess of ethanol, centrifuged off, washed with ethanol then ether, and dried. Yield 0.31 gm. The mannan had $[\alpha]_D$ +58° (*c*, 1.0, water) and gave an insoluble copper complex on mixing with Fehling's solution. Only mannose was detected on a paper chromatogram after acid hydrolysis of the product.

2-O-α-D-Mannopyranosyl-D-mannose

The crude polysaccharide (25 gm.) was dissolved by shaking in acetic anhydride (100 ml.) containing sulphuric acid (9 ml.), the temperature of the mixture being kept below 55° C. (2). After further treatment at 35° C. for two days the reaction mixture was filtered and poured into ice water. The mixture was then extracted with chloroform, which was washed with aqueous sodium bicarbonate, then water, dried (magnesium sulphate), filtered, and evaporated to give a solid crust. The latter was dissolved in methanol (200 ml.) to which sodium methoxide (sodium (0.5 gm.) in methanol (20 ml.)) was added. The precipitate which formed was filtered off (14.9 gm.) and the filtrate was evaporated to a solid which was deionized (Amberlites IR-120 and IR-4B) in aqueous solution and evaporated to a solid (3.4 gm.). Both the soluble and insoluble fractions gave spots on a paper chromatogram corre-

sponding to mono-, di-, and tri-saccharides only, and were combined. A sample of D-mannose was treated with acetic anhydride and sulphuric acid in the same way as the mannan; no reversion products were detectable in significant quantity on a paper chromatogram.

The mixture (14.9 gm.) was partly fractionated on a charcoal column using ethanol–water mixtures (15) and then completely on a cellulose column (10). On the latter column *n*-butanol saturated with water eluted the monosaccharide portion, ethyl acetate – acetic acid – water (9:4:4 v/v) the disaccharide (1.7 gm.), and 70% ethanol the trisaccharide (4.3 gm.).

The disaccharide had $[\alpha]_D$ +40° (*c*, 2.3, water). Behavior of the compound on oxidation with lead tetraacetate (4, 13) eliminated the possibility that the linkage position was 1,3-, 1,4-, 1,5-, or 1,6-. The data obtained, however, was wholly consistent with a 1,2-linkage. Thus, in the uncatalyzed reaction only traces of oxidant were consumed during the first 0.5 hour's reaction time; all except 2-substituted compounds consume 1 to 2 moles per mole within the first 10 min. (4). Also, in the potassium acetate-catalyzed oxidation the compound produced 0.9 mole of formaldehyde per mole in 30 min.; the formic acid production at one, two, three, and five hours, respectively, was 1.5, 2.3, 2.9, and 3.4 moles per mole; and in five hours it produced 0.9 mole of formaldehyde per mole and consumed 6.3 moles of lead tetraacetate per mole. The corresponding data expected (based on the oxidation of 2-*O*-methyl-D-glucose and methyl α-D-mannopyranoside) was 1.0 mole of formaldehyde per mole; 1.8, 2.5, 2.9, and 3.5 moles of formic acid; 1.0 mole of formaldehyde per mole and 6.8 moles of lead tetraacetate per mole.

Methylation of 2-O-α-D-Mannopyranosyl-D-mannose

The disaccharide (0.290 gm.) was methylated four times with dimethyl sulphate – sodium hydroxide as described for the methylation of the mannan (see below) except that during the first treatment the dimethyl sulphate was added before the sodium hydroxide to minimize the possibility of degradation. The product (0.285 gm.) crystallized, was twice recrystallized from *n*-hexane, and had m.p. 43–46° C. and $[\alpha]_D$ +72° (*c*, 0.8, ethanol). Calculated for $C_{20}H_{38}O_{11}$: C, 52.85%; H, 8.43%; —OCH_3, 54.6%. Found: C, 53.25%; H, 8.53%; —OCH_3, 53.2%.

The methylated disaccharide (0.145 gm.) was hydrolyzed by being heated for 18 hr. at 100° C. in formic acid (5 ml.) containing water (0.5 ml.). The reaction mixture was then evaporated to a sirup, dissolved in water, and heated at 100° C. to destroy formate esters. The solution was evaporated to a sirup and the process was repeated twice. The mixture of *O*-methyl sugars was fractionated on a cellulose column. Benzene–ethanol–water (10:1: trace v/v) eluted the tetra-*O*-methyl sugar (0.066 gm.), and benzene–ethanol–water (5:1: trace v/v) the tri-*O*-methyl sugar (0.060 gm.). The tetra-*O*-methyl mannose had $[\alpha]_D$ +8° (*c*, 0.7, water) and was oxidized with bromine to its lactone, which was converted to 2,3,4,6-tetra-*O*-methyl-D-mannonic acid phenylhydrazide, m.p. 180–181° C. Calculated for $C_{16}H_{26}O_6N_2$: C, 56.12%; H, 7.65%. Found: C, 56.30%; H, 7.80%. The tri-*O*-methyl sugar crystallized

and was twice recrystallized from ether to give 3,4,6-tri-*O*-methyl-D-mannose with m.p. 99–101° C. and $[\alpha]_D$ +18° → +8° (constant, 18 hr.) (*c*, 0.7, water). Calculated for $C_9H_{18}O_6$: C, 48.64%; H, 8.16%. Found: C, 48.71%; H, 8.18%. The melting point of each of the above sugars was not depressed by admixture with known specimens, and the correct X-ray diffraction patterns and infrared absorption spectra were given.

2-O-α-D-Mannopyranosyl-D-mannitol

2-*O*-α-D-Mannopyranosyl-D-mannose (0.235 gm.) was dissolved in water (20 ml.) containing sodium borohydride (0.10 gm.) and the solution was left for 18 hr. Acetic acid was added to destroy excess reagent and the solution then treated with Amberlite IR-120 and evaporated to dryness. Boric acid was removed by dissolving the sirup in successive portions of methanol and repeated evaporations of the solution. The product (0.204 gm.) crystallized and was recrystallized twice from methanol–ethanol. It had m.p. 136–137° C. and $[\alpha]_D$ +45° (*c*, 0.8, water). Calculated for $C_{12}H_{24}O_{11}$: C, 41.86%; H, 7.08%. Found: C, 41.66%; H, 7.09%. The alcohol was oxidized with lead tetraacetate in 90% acetic acid containing potassium acetate. After three hours (steady state) 5.2 moles of reagent per mole had been consumed by the carbohydrate and 1.0 mole of formaldehyde per mole liberated. (Calculated: 5.0 and 1.0 moles per mole, respectively).

Methylation of Mannan

The crude mannan (15.0 gm.) was dissolved in water (150 ml.) contained in a three-necked flask. To the vigorously stirred solution and under an atmosphere of nitrogen, sodium hydroxide (2.5 gm.) was added followed by dimethyl sulphate (2.3 ml.). The solution was kept at 0° C. for one hour and then treated with portions of 30% (w/v) sodium hydroxide (75 ml.), followed by dimethyl sulphate (70 ml.) three times. The reaction mixture was heated on a steam bath for two hours to destroy excess dimethyl sulphate, neutralized with acetic acid, and extracted twice with equal volumes of chloroform. The extract was dried (magnesium sulphate), filtered, and evaporated to a light brown solid (9.8 gm.); found: —OCH_3, 43.7%.

Separation of Component O-Methyl-D-mannoses After Hydrolysis of Methylated Mannan

The methylated polysaccharide (4.69 gm.) was refluxed in 2.5% methanolic hydrogen chloride for 18 hr. and the solution was neutralized (silver carbonate), filtered, and evaporated to a brown solid (4.57 gm.). The degraded material was dissolved in formic acid (40 ml.) containing water (10 ml.) and further hydrolyzed by heating of the solution at 100° C. for 48 hr. The solution was evaporated to a sirup which was dissolved in water (50 ml.); the mixture was then heated at 100° C. for three hours to hydrolyze formate esters, the solution being then evaporated to a sirup and the process being repeated four times. The sirupy mixture thus obtained (4.59 gm.) was examined on paper chromatograms and its components separated on a cellulose column as shown in Table I; 3.67 gm. of starting material was used in the separation.

TABLE I

SEPARATION OF *O*-METHYL-D-MANNOSES

Solvent used in column fractionation	Eluted sugar	Net yield, gm.
Benzene–ethanol–water 10: 1: trace v/v	2,3,4,6-Tetra-*O*-methyl-D-mannose	1.10
	3,4,6-Tri-*O*-methyl-D-mannose	0.68
	2,4,6-Tri-*O*-methyl-D-mannose	0.17
Benzene–ethanol–water 7: 1: trace v/v	3,4-Di-*O*-methyl-D-mannose	0.91
n-Butanol half-saturated with water	3-*O*-Methyl-D-mannose	0.11
Total (81% recovery)		2.97

*2,3,4,6-Tetra-O-methyl-*D*-mannose*

The sirup (0.148 gm.), which had $[\alpha]_D$ +23° (*c*, 4.8, methanol), was oxidized in bromine water. The reaction product was lactonized, and converted to 2,3,4,6-tetra-*O*-methyl-D-mannonic acid phenylhydrazide by the action of phenylhydrazine. Two recrystallizations from benzene gave a product (0.034 gm.) with m.p. 183–184° C. and $[\alpha]_D$ −28° (*c*, 0.8, chloroform). Calculated for $C_{16}H_{26}O_6N_2$: —OCH_3, 36.2%. Found: —OCH_3, 36.0%.

*3,4,6-Tri-O-methyl-*D*-mannose*

The material crystallized spontaneously and was recrystallized twice from ether. It had m.p. 101–103° C. and $[\alpha]_D$ +17° → +8° (constant, 18 hr.) (*c*, 0.9, water) and gave an X-ray diffraction pattern identical with that of an authentic specimen. Calculated for $C_9H_{18}O_6$: C, 48.64%; H, 8.16%. Found: C, 48.64%; H, 8.09%.

*2,4,6-Tri-O-methyl-*D*-mannose*

The sugar had $[\alpha]_D$ +12° (*c*, 1.2, water) and did not consume periodic acid. Calculated for $C_9H_{18}O_6$: —OCH_3, 41.9%; found: —OCH_3, 41.1%. The material was also characterized by conversion to 2,4,6-tri-*O*-methyl-D-mannonic acid amide, m.p. 142–143° C. after two recrystallizations from ethanol–ether–*n*-hexane. Haworth and co-workers (7) report m.p. 145° C.

*3,4-Di-O-methyl-*D*-mannose*

The sugar crystallized and was twice recrystallized from ethyl acetate to give a product with m.p. 70–73° C., which differs from other melting points of this material quoted in the literature (7, 8). Therefore, it (0.086 gm.) was left overnight with lead tetraacetate (0.18 gm.) in acetic acid (15 ml.) containing water (0.3 ml.). Excess lead was then precipitated by the addition of oxalic acid in acetic acid and the solution was filtered. The filtrate was evaporated to a sirup which gave a red-brown spot which moved at a distance of 1.2 compared with 2,3-di-*O*-methyl-L-arabinose on a paper chromatogram. This material (0.088 gm.), presumably 5-*O*-formyl-2,3-di-*O*-methyl-D-arabinose, was dissolved in water (10 ml.) containing bromine (0.2 ml.). After five days excess bromine was aerated off, the solution neutralized (silver carbonate), filtered, treated with Amberlite IR-120, and evaporated to a sirup. The product was heated at 100° C. for three hours to promote lactonization

and then dissolved in excess methanolic ammonia. The resulting sirup, obtained on evaporation of the solution, crystallized and was recrystallized three times from ethanol–*n*-hexane. Yield 0.033 gm. It had m.p. 156–158° C. and $[\alpha]_D$ −17° (*c*, 0.75, water), and gave an X-ray powder diagram identical with that of 2,3-di-*O*-methyl-L-arabonic acid amide (9). Calculated for $C_7H_{15}O_5N$: C, 43.51%; H, 7.83%. Found: C, 43.55%; H, 7.81%.

3-O-Methyl-D-mannose

The sirup had $[\alpha]_D$ +15° (*c*, 1.2, 50% ethanol), moved at a rate similar to that of 3-*O*-methyl-D-glucose, and gave a brown spot on a paper chromatogram. Calculated for $C_7H_{14}O_6$: $-OCH_3$, 16.0%; found: $-OCH_3$, 15.0%. A portion was oxidized with lead tetraacetate in acetic acid; 1 mole of reagent per mole was consumed immediately, very little more being taken up during the next 30 min. The reaction mixture was then evaporated to a sirup which was dissolved in water and treated with Amberlites IR-120 and IR-4B. The solution was filtered and evaporated to a sirup which on a paper chromatogram gave a pink spot corresponding to a pentose which moved 1.2 times the distance of rhamnose.

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