THE CONFIGURATION OF GLYCOSIDIC LINKAGES IN OLIGOSACCHARIDES

III. *O*-α-D-MANNOPYRANOSYL-(1→2)-*O*-α-D-MANNOPYRANOSYL-(1→2)-D-MANNOSE[1]

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ABSTRACT

A trisaccharide obtained after acetolysis of *Saccharomyces rouxii* mannan is shown to be *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose. Methylation and lead tetraacetate oxidation techniques define the positions of the glycosidic linkages. The configurations of the two linkages are determined through use of two novel degradative methods which yield 2-*O*-α-D-mannopyranosyl-D-mannose and 2-*O*-α-D-mannopyranosyl-glycerol, each containing one of the glycosidic linkages of the parent trisaccharide.

An earlier paper (7) described a mannan which was isolated from a fermenting culture of *Saccharomyces rouxii* (15). Two oligosaccharides were obtained after acetolysis of the polysaccharide, one of which was found to be 2-*O*-α-D-mannopyranosyl-D-mannose and the other an amorphous trisaccharide composed of D-mannose units. The latter is now shown to be *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-D-mannose (I).

Methylation of the trisaccharide and hydrolysis of the methylated product yielded two parts of crystalline 3,4,6-tri-*O*-methyl-D-mannose (2), and one part of 2,3,4,6-tetra-*O*-methyl-D-mannose, characterized as the corresponding D-mannonic acid phenylhydrazide (5). The positions of both glycosidic linkages of the trisaccharide are therefore 1,2-.

The compound consumed lead tetraacetate only slowly, and in the potassiumacetate-catalyzed oxidation the trisaccharide and the corresponding triitol each yielded one mole of formaldehyde, in agreement with the formulation of a 1,2-linkage at the reducing end (4). Further, the central D-mannose unit in the triitol consumed 1 mole of lead tetraacetate, as expected for a 1,2- (or 1,4-) linkage, but its rate of oxidation was slow relative to the oxidative attack at the end-units. It has been noted elsewhere (14) that in the glucose and xylose series a *trans*-2,3-diol adjacent to a 1,4-glycosidic linkage is not oxidized by lead tetraacetate. The current results demonstrate a resistance to oxidation also by a *trans*-3,4-diol in a mannose residue adjacent to a 1,2-α-mannosidic linkage.

To determine the configurations of the two glycosidic linkages it was proposed to chemically degrade the trisaccharide and obtain two disaccharides, each containing one of the glycosidic linkages of the parent compound. This approach has been successfully employed, for example, by Wolfrom *et al.* (16) who obtained maltitol and isomaltose by partial hydrolysis of reduced panose, and more recently by Kuhn *et al.* (11), for a trisaccharide from human milk. In the present study, selective degradation of the mannotriose was achieved by the use of novel methods which permitted preferential removal either of the non-reducing end-unit or of the reducing end-unit.

The non-reducing end-unit was removed in the following way. The mannotriose (I) was first reduced with sodium borohydride (17) to an amorphous mannotriitol (II)

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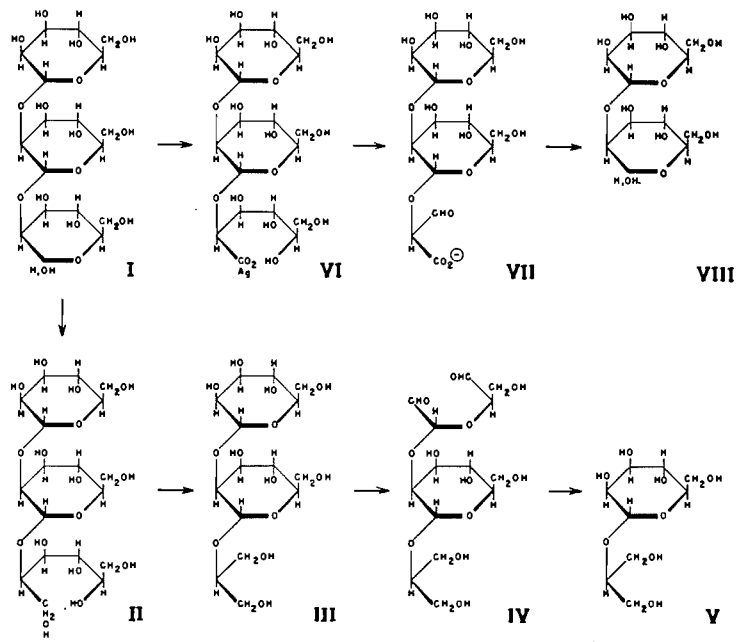
characterized as its duodecabenzoate. This was then treated with 3 moles of lead tetra-acetate, and the oxidation product reduced with sodium borohydride to give an amorphous mannobiosyl-glycerol (III), which yielded a solid nona-*p*-nitrobenzoate. These steps are analogous to the stepwise degradation of 2-*O*-α-D-mannopyranosyl-D-mannose (3). The mannobiosyl-glycerol (III) was then oxidized with two moles of lead tetra-acetate which, in agreement with the results noted above, preferentially attacked the terminal D-mannose unit and produced the dialdehyde (IV). Treatment of this product with phenylhydrazine acetate according to the method of Barry (1) afforded 2-*O*-α-D-mannopyranosyl-glycerol (V) (3) characterized as its hexa-*p*-nitrobenzoate derivative. The 1,2-glycosidic linkage between the central residue and the reducing end-unit was thus found to possess the α-configuration.

Partial hydrolysis of oligosaccharide osazones has been suggested as a possible means for preferential removal of reducing end-units (6). However, this method was not applicable to the mannotriose, which cannot form an osazone. An alternative method for the reducing end-unit was therefore developed. The trisaccharide was oxidized with bromine water in the presence of calcium benzoate (10) to the mannobiosyl-mannonic acid isolated as the silver salt (VI), which in turn was oxidized with 3 moles of lead tetra-acetate to give presumably a salt of *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-L-*glycero*triuronic acid (VII). Treatment of the latter with hot dilute acetic acid readily hydrolyzed off the tartronic semialdehyde residue, affording 2-*O*-α-D-mannopyranosyl-D-mannose (VIII), characterized by reduction with sodium borohydride to crystalline 2-*O*-α-D-mannopyranosyl-D-mannitol (7). It was evident, therefore, that the glycosidic linkage between the non-reducing end-unit and the central residue of the trisaccharide also possesses the α-configuration.

As previously noted (7), the disaccharide, 2-*O*-α-D-mannopyranosyl-D-mannose, represents 10% of the polysaccharide, and the present trisaccharide, 25%. The high



total yield obtained of these two compounds shows that the 1,2-glycosidic linkages in the polymer have predominantly, if not entirely, the α-configuration. The configuration of the 1,6-linkage, the other major type present, is yet to be determined.

EXPERIMENTAL

Evaporations were carried out under reduced pressure at 40° C. Optical rotations were measured at 27° C. All melting points are uncorrected. Paper chromatograms were developed using Whatman No. 1 filter paper, *n*-butanol–ethanol–water (40:11:19 v/v) as solvent, and *p*-anisidine hydrochloride (9) and ammoniacal silver nitrate (12) as spray reagents.

Positions of Linkage in the Trisaccharide

The mannotriose (298 mg.) ($[\alpha]_D$ +55°, *c*, 1.5, water) was methylated six times with aqueous sodium hydroxide – methyl sulphate. After destruction of excess methyl sulphate the aqueous solution was neutralized using acetic acid and then extracted continuously with chloroform. The extract was dried over magnesium sulphate, filtered, and evaporated to a sirup (301 mg.) which was distilled rapidly at 1 mm. pressure. The product (181 mg.) was heated at 100° C. for 18 hours in 90% formic acid (5 ml.). The hydrolyzate was then concentrated to a sirup which was dissolved in water and heated at 100° C. for 30 minutes, the procedure being repeated three times.

The mixture of *O*-methyl sugars, which on paper chromatographic examination was shown to contain no di-*O*-methyl mannose, was fractionated on a cellulose column (8). Benzene–ethanol–water (10:1: trace, v/v) eluted the tetra-*O*-methyl component (53 mg.), $[\alpha]_D$ +25° (*c*, 1.7, methanol). Oxidation of this sugar by bromine water, followed by lactonization and treatment of the lactone with an equivalent of phenylhydrazine in refluxing ethanol, yielded, after three recrystallizations from benzene, 2,3,4,6-tetra-*O*-methyl-D-mannonic acid phenylhydrazide. The substance had m.p. and mixed m.p. 180°–181° C. and gave an X-ray diffraction pattern identical with that of an authentic specimen. Calculated for $C_{16}H_{26}O_6N_2$: —OCH_3, 36.2%. Found: —OCH_3, 35.8%.

The tri-*O*-methyl fraction was eluted by benzene–ethanol–water (7:1: trace, v/v) and crystallized on evaporation of the solvent; yield, 97 mg. After recrystallization twice from ether the product (51 mg.) had m.p. 95°–99° C. undepressed on admixture with authentic 3,4,6-tri-*O*-methyl-D-mannose and $[\alpha]_D$ +20° → +7° (constant value; *c*, 1.15, water). The infrared spectrum of the product was identical with that of the known material. Calculated for $C_9H_{18}O_6$: C, 48.64%; H, 8.16%. Found: C, 48.92%; H, 8.11%. Only 18 mg. of the product could not be induced to crystallize.

A sample of the trisaccharide was treated with excess lead tetraacetate in acetic acid. The consumption of reagent, moles per mole, after 5, 31, and 55 minutes was 0.03, 0.27, and 0.42, respectively. In 90% acetic acid and in the presence of potassium acetate, lead tetraacetate oxidation (13) liberated 1.0 mole of formaldehyde per mole in 1 hour's reaction time.

O-α-D-*Mannopyranosyl*-(1→2)-*O*-α-D-*mannopyranosyl*-(1→2)-D-*mannitol*

The mannotriose (683 mg.) was treated with sodium borohydride (200 mg.) in water (20 ml.) for 18 hours. The solution was acidified with acetic acid, treated with Amberlite IR-120, and then concentrated to a solid. The latter was dissolved in methanol, which was evaporated off. This process was repeated three times to give the amorphous mannotriitol (674 mg.) which had $[\alpha]_D$ +49° (*c*, 0.8, water). On oxidation of the mannotriitol with

lead tetraacetate in 90% acetic acid containing potassium acetate the production of formic acid was constant in 40 minutes' reaction time at 2.3 moles per mole; the consumption of lead tetraacetate was 5.0 moles per mole. These values corresponded to oxidation of the two end-units, which would require 2.6 moles of formic acid (13) and 5.0 moles of lead tetraacetate per mole. During the next 1.5 hours the consumption of oxidant increased to 5.7 moles per mole, and a further 1 hour's reaction time raised the value to 5.8 moles per mole. This slow oxidation without further production of formic acid was interpreted as involving the central D-mannose unit. The yield of formaldehyde was 0.9 moles per mole (requires 1.0 mole per mole).

The mannotriitol (30 mg.) was benzoylated in a mixture of pyridine (0.20 ml.), benzoyl chloride (0.13 ml.), and chloroform (0.30 ml.). After 3 hours at room temperature the sugar had dissolved and the reaction mixture was added to chloroform, which was washed with 0.1 *N* sulphuric acid, aqueous sodium bicarbonate, and water successively, dried over magnesium sulphate, filtered, and evaporated to a white solid. The duodecabenzoate was purified by two precipitations from methanol. Yield 53 mg., m.p. 96°–98° C., and $[\alpha]_D$ −4° (*c*, 0.8, chloroform). Calculated for $C_{102}H_{81}O_{28}$: C, 69.36%; H, 4.65%. Found: C, 69.81%; H, 4.65%.

O-α-D-Mannopyranosyl-(1→2)-O-α-D-mannopyranosyl-(1→2)-glycerol

The mannotriitol (516 mg.) was dissolved in water (2 ml.), acetic acid (200 ml.) added, and the mixture treated with lead tetraacetate (1.35 g., 3.0 molar equivalents). After 18 hours lead was precipitated as lead oxalate by the addition of oxalic acid and the solution was filtered. The filtrate was evaporated to dryness and the residue was dissolved in water (50 ml.) containing sodium borohydride (500 mg.). After 1 hour the reaction mixture was worked up as described above. The amorphous mannobiosyl-glycerol thus formed (294 mg.) had $[\alpha]_D$ +49° (*c*, 1.4, water) and acid hydrolysis of the material gave mannose and glycerol which were detected on a paper chromatogram.

The glycerol derivative (45 mg.) was heated at 80° C. for 30 minutes in pyridine (1.7 ml.) containing *p*-nitrobenzoyl chloride (280 mg.). The precipitate which formed on addition of the reaction mixture to aqueous sodium bicarbonate was filtered off after 30 minutes and reprecipitated from acetone–ethanol. The amorphous white nona-*p*-nitrobenzoate (60 mg.) had m.p. 128°–131° C. and $[\alpha]_D$ −3° (*c*, 0.8, chloroform). Calculated for $C_{78}H_{55}O_{40}N_9$: C, 53.29%; H, 3.15%. Found: C, 53.03%; H, 3.20%.

The mannobiosyl-glycerol was oxidized with lead tetraacetate in acetic acid containing a trace of water. The reaction was very slow, only 0.16 and 0.90 mole per mole being consumed after 2 and 20 hours respectively.

2-O-α-D-Mannopyranosyl-glycerol

Mannobiosyl-glycerol was oxidized with lead tetraacetate under the conditions mentioned immediately above except that the proportion of water in the reaction mixture was increased.

The sugar (248 mg.) was dissolved in water (1 ml.) and made up to 50 ml. with acetic acid to which lead tetraacetate (560 mg.; 2.1 molar equivalents) was added. After 18 hours all of the reagent had been consumed and 10% oxalic acid in acetic acid (1.3 ml.) was added. The precipitate which formed was filtered off and the filtrate evaporated to dryness. The residue was dissolved in water (25 ml.) containing phenylhydrazine (1 ml.) and acetic acid (1 ml.) and the solution heated at 100° C. for 30 minutes. The mixture was evaporated to a sirup, which was dissolved in aqueous ammonia. The solution was

extracted twice with chloroform, treated with Amberlites IR-120 and IR-4B, and evaporated to a sirup (122 mg.). The material was fractionated on a cellulose column using *n*-butanol saturated with water as the mobile phase. 2-*O*-α-D-Mannopyranosyl-glycerol (42 mg.) was isolated as a sirup and identified by its infrared absorption spectrum and its $[\alpha]_D$, which was +59° (*c*, 1.4, water) (3).

The mannosyl-glycerol (28 mg.) was converted to its hexa-*p*-nitrobenzoate derivative by the method described above. The product (60 mg.), which was precipitated twice from ethanol–acetone, had m.p. 108°–110° C., $[\alpha]_D$ −71° (*c*, 0.7, chloroform) and an infrared absorption spectrum identical with that of the α- but not the β-anomer. Calculated for $C_{51}H_{36}O_{26}N_6$: C, 53.31%; H, 3.15%. Found: C, 53.17%; H, 3.12%.

2-O-α-D-Mannopyranosyl-D-mannose

The mannotriose (373 mg.) was oxidized with bromine (0.04 ml.) in water (25 ml.) containing calcium benzoate (300 mg.). After 3 days the solution did not reduce Fehling's solution and bromine was aerated off. The solution was treated with Amberlite IR-120, extracted three times with chloroform, and then neutralized with silver carbonate. The insoluble material was filtered off and the filtrate evaporated to a small volume. Addition of an excess of ethanol precipitated a white powder, presumably the silver salt of the trisaccharide aldonic acid, which was isolated. Yield 251 mg.

A sample of the product was oxidized with lead tetraacetate in 99% acetic acid. The observed rate of oxidation was as follows:

Time, min.	0	4	13	30	55	125	185
Reagent uptake, moles per mole	0	1.7	2.6	3.2	3.5	4.0	4.2

This relatively rapid uptake of slightly over 3 moles per mole of reagent, which was followed by a slower oxidation, corresponded to the formation of a good proportion of the expected *O*-α-D-mannopyranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-L-*glycero*-triuronic acid.

Therefore the silver salt (230 mg.) was dissolved in water (2 ml.) to which acetic acid (200 ml.) was added, and the mixture treated with lead tetraacetate (500 mg.; 3 molar equivalents). After 18 hours the solution was evaporated to a solid mass, which was dissolved in water, and 10% oxalic acid in acetic acid (1.1 ml.) added. The solution was filtered and water was added until the volume was 40 ml. Acetic acid (4 ml.) was added and the solution was heated at 100° C. for 1 hour. It was then shown to contain a material which gave a spot on a paper chromatogram corresponding to 2-*O*-α-D-mannopyranosyl-D-mannose.

The solution was evaporated to dryness and the residue was reduced with sodium borohydride (200 mg.) in water (20 ml.). After 18 hours the reaction mixture was worked up as described above and the product was purified by chromatography on a cellulose column using ethyl acetate – acetic acid – water (9:2:2, v/v). Yield 107 mg. Four recrystallizations from ethanol–methanol gave a product with $[\alpha]_D$ +36° (*c*, 1.0, water) and m.p. 136°–137° C., undepressed on admixture with 2-*O*-α-D-mannopyranosyl-D-mannitol. The crystals gave an infrared absorption spectrum and an X-ray diffraction pattern identical with those of the known compound. Calculated for $C_{12}H_{24}O_{11}$: C, 42.10%; H, 6.48%. Found: C, 41.94%; H, 6.52%.

An attempt to degrade the hydroxy-malondialdehyde derivative, prepared by careful lead tetraacetate oxidation of the reducing end-unit of the trisaccharide, using the conditions as for the corresponding *glycero*triuronic acid derivative (VII), was unsuccessful.

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