ACETOLYSIS OF DEXTRAN NRRL B1397. PREPARATION OF TRISACCHARIDES CONTAINING (1→2)- AND (1→6)-α-D-GLUCOSE LINKAGES*

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ABSTRACT

Dextran NRRL B1397 was acetylated and then acetolyzed. Fractionation of the deacetylated products of the acetolysis gave, after charcoal, paper, and Dowex-1 (borate) chromatography, three trisaccharides. Partial hydrolysis and acetolysis, before and after reduction, and methylation showed the structure of these trisaccharides to be *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose, *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose, and *O*-α-D-glucopyranosyl-(1→2)-*O*-[α-D-glucopyranosyl-(1→6)]-D-glucose, respectively.

INTRODUCTION

Acetolysis of dextrans has been widely used to isolate oligosaccharides containing (1→2)-, (1→3)- and (1→4)-α-D linkages, since these linkages are more stable to acetolysis but less stable to hydrolysis than are the (1→6)-α-D linkage[1–10]. On the basis of periodate oxidation studies, Jeanes *et al.*[11] reported, that Dextran B1397 has 75% (1→6)- and 25% (1→4)-α-D-like linkages, but methylation studies[12] had shown the presence of (1→2)- and (1→3)-α-D linkages. Preliminary acetolysis studies[13] had resulted in the isolation of kojibiose [*O*-α-D-glucopyranosyl-(1→2)-D-glucose], and accordingly partial acetolysis was undertaken to isolate oligosaccharides, having a higher mol.wt. and containing (1→2)-α-D linkages.

RESULTS AND DISCUSSION

Acetolysis of Dextran NRRL B1397 gave three trisaccharides, *O*-α-kojibiosyl-(1→6)-D-glucose [*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-gluco-

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se] (**1**), *O*-α-isomaltosyl-(1→2)-D-glucose [*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose] (**2**), and *O*-α-D-glucopyranosyl(1→2)-*O*-[α-D-glucopyranosyl-(1→6)]-D-glucose (**3**), but *O*-α-kojibiosyl-(1→2)-glucose [*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose] could not be obtained (see Tables I and II). The structures were established by hydrolysis and acetolysis, before and after reduction (Table III), and by the methylation procedure (Table IV).

It has been postulated that the branches containing the α-D-(1→2) linkages were predominantly one D-glucose residue in length[1,2]. Thus, Trisaccharides **1** and **3** were probably liberated from the branch points by the acetolysis. However, the isolation of Trisaccharide **2**, although its yield was small, indicated that the structure of the dextran contains either isomaltosyl side-chains at the α-D-(1→2) branch points or *O*-α-D-glucosyl-(1→2)-D-glucose residues in the main chain, or both.

TABLE I

FRACTIONATION ON A DARCO G-60–CELITE COLUMN OF DEACETYLATED SUGARS FROM THE METHANOL–BENZENE ELUATE OBTAINED FROM ACETOLYZED DEXTRAN NRRL B1397

Fraction	*Tube No.*	*Ethanol conc.* (%)	*Compounds detected by paper chromatography*
1	21–29	1	G[a]
2	30–195	1	G, IM_2, nigerose, kojibiose[b]
3	196–250	5	IM_2, IM_3
4	251–380	5	IM_2, IM_3, oligosaccharides (IM_2–IM_3)[c]
5	381–475	7.5	IM_2, IM_3, oligosaccharides (IM_2–IM_3)[c] oligosaccharides (IM_3–IM_4)[c]
6	476–640	10	IM_3, IM_4[d], IM_5[d], oligosaccharides[e]
7	641–790	12.5	IM_4[d], IM_5[d], oligosaccharides[e]
8	791–	30	IM_6[d], oligosaccharides[e]

[a]The following abbreviations are used: G, D-glucose; IM_2, isomaltose; IM_3, isomaltotriose; IM_4, isomaltotetraose; IM_5, isomaltopentaose; IM_6, isomaltohexaose. [b]Kojibiose was separated by paper chromatography, crystalline octaacetates were prepared and were found to have the same properties as the known compounds. [c]These oligosaccharides were detected between spots of IM_2 and IM_3 or those of IM_3 and IM_4, respectively. [d]These spots were each corresponding to IM_4, IM_5 or IM_6, respectively, in their R_F values, but they were considered to be contaminated by other oligosaccharides. [e]These indicate various unidentified oligosaccharides.

TABLE II

YIELDS AND PROPERTIES OF THE TRISACCHARIDES ISOLATED BY DOWEX-1 AND PAPER CHROMATOGRAPHY

Trisaccharide	*Yield*[a] (*mg*)	R_{IM_3}	$[\alpha]_D^{24}$ (*water*) (°)
1	65	1.15	+144
2	20	1.26	+138
3	150	1.10	+153

[a]From 26 g of dextran.

A mixture of α-kojibiosyl-(1→6)-D-glucose and *O*-α-D-glucopyranosyl-(1→2)-*O*-[α-D-glucopyranosyl-(1→6)]-D-glucose have been obtained by Lewis *et al.*[9]. In our study these two compounds could not be separated by charcoal and paper chromatography but by paper electrophoresis in borate solution. Because of the different chelating properties with borate, preparative separation was attempted with a column of Dowex-1 in the borate form and good separation of the two trisaccharides was obtained.

TABLE III

HYDROLYSIS AND ACETOLYSIS OF THE TRISACCHARIDES OBTAINED FROM DEXTRAN NRRL B1397, BEFORE AND AFTER REDUCTION

Trisaccharide	*Reduction*	D-*Glucose content*[a]	*Disaccharides detected in*	
			Hydrolyzates	*Acetolyzates*
1	Before	3.00	Isomaltose	Kojibiose
	After	2.06	Isomaltitol	Kojibiose, isomaltitol
2	Before	3.00	Isomaltose, kojibiose	Kojibiose
	After	1.87	Isomaltose, kojibiitol	Isomaltose, kojibiitol
3	Before	3.00	Isomaltose	Kojibiose
	After	2.09	Isomaltitol	Kojibiitol, isomaltitol

[a]Molar ratio.

TABLE IV

CLEAVAGE FRAGMENTS OF THE METHYLATED TRISACCHARIDES **1**, **2**, AND **3**

O-*Methyl*-D-*glucose*	*Molar ratios*[a]			*Linkage indicated*
	1	**2**	**3**	
2,3,4,6-Tetra-	1.0	1.0	1.0	[G] 1→
2,3,4-Tri-	1.0	1.0	0.06	→6 [G] 1→
3,4,6-Tri-	0.99	0,96		→2 [G] 1→
3,4-Di-	0.09	0.13	0.40	→2, →6 [G] 1→
4,6-Di-				→2, →3 [G] 1→

[a]Calculated from the area under the peak of the alditol acetate.

Since the dextran also contains α-D-(1→3) linkages, oligosaccharides having this linkage exist in the acetolyzate. Their isolation, however, was not performed because various oligosaccharides containing the α-D-(1→3) linkage have already been isolated by Bourne *et al.*[14] and Yamauchi and Matsuda[10].

The interaction of sugars with borate to form negatively charged complexes is well known[15] and it has been assumed that *cis* hydroxyl groups might play a role in the formation of the complex. The D-glucose-containing oligosaccharides examined can be divided into two groups on the basis of their behavior on Dowex-1 (borate) (Table V). All the compounds eluted with acid had M_G values higher than 0.5, with the exception of isomaltohexaose, and the hydroxyl groups at C-1, C-2, and C-4 of

TABLE V

SEPARATION OF D-GLUCOSE OLIGOSACCHARIDES ON A DOWEX-1 (BORATE) COLUMN[a]

Elution with water		*Elution with acid*	
Compound	$M_G \times 100$[b]	*Compound*	$M_G \times 100$[b]
Kojibiose	32[c]	Glucose	100
	26[e]		
Maltose	32[c]	Nigerose	69[c]
	35[e]		70[e]
Cellobiose	23[c]	Isomaltose	69[c]
			70[e]
α-Isomaltosyl-(1→2)-D-glucose	20[e]	Gentiobiose	75[c]
O-α-D-Glucopyranosyl-(1→2)-*O*-[α-D-glucopyranosyl-(1→6)]-D-glucose	24[e]	Isomaltotriose	67[e]
α,α-Trehalose	19[c]	Isomaltotetraose	55[e]
Methyl α-D-glucopyranoside	10[d]	Isomaltopentaose	51[e]
		Isomaltohexaose	37[e]
		α-Kojibiosyl-(1→6)-D-glucose	64[e]

[a]The column (1 × 5 cm) was prepared with Dowex-1 (X-2, BO_4^{3-}, 100–200 mesh). Individual known sugars (10 mg) were applied to the column, eluted with water (40 ml), and then with 50mM acetic acid (40 ml). A mixture of kojibiose (10 mg), glucose (10 mg), and isomaltose (10 mg) was also examined, and kojibiose was well separated from the other two sugars. [b]Electrophoretic mobility compared to that of D-glucose. [c]Data from Ref. 29. [d]Data from Ref. 30. [e]Data from present work.

the reducing D-glucose residue were not substituted. On the other hand, the compounds eluted with water had M_G values lower than 0.35 and any one of the hydroxyl groups at C-1, C-2, or C-4 of the reducing D-glucose moiety was substituted. Thus, it appears that at least three hydroxyl groups at C-1, C-2, and C-4 of the reducing D-glucose residue are essential for the formation of a borate complex under the conditions used. This requirement may be related to a change from a glucopyranose to a glucofuranose structure at the reducing end. In a furanose ring having the α-D configuration, the hydroxyl groups at C-1 and C-2 show almost the structure of a *cis* glycol, which may produce a more stable borate complex. Methylation of the sugar-borate complex would solve this problem. Although isomaltohexaose has three free hydroxyl groups at C-1, C-2, and C-4 of the reducing D-glucose residue, its M_G value was 0.37. This low value may be due to the higher-molecular weight of the oligosaccharide. The compounds tested were clearly separated into two fractions. (Table V). Retreatment of the eluates was necessary when trailing occurred or when

the column was overloaded. A good separation was obtained with the use of 5 mg of material elutable with acid to 1 ml of resin. Column chromatography in Dowex-1 (borate) had previously been reported to be a powerful tool for separation of various pentoses, hexoses, oligosaccharides, and sugar alcohols[16–19]. As minimal amounts of borate reagent were used in the present study, removal of the borate ion from the sugar was very easily performed. It will be also of interest to determine whether this technique is effective for the separation of various oligosaccharides containing sugars other than D-glucose.

EXPERIMENTAL

Dextrans. — Dextrans NRRL B1397, NRRL B1299, N4, and N4-clinical were generous gifts of Meito Sangyo Co., Ltd., Nagoya (Japan), prepared from *Leuconostoc mesenteroides* NRRL B1397, NRRL B1299, and N4 strains, respectively. Strains of *L. mesenteroides* NRRL B1397 and B1299 were kindly provided by Dr. A. R. Jeanes, Peoria, Illinois.

Oligosaccharides. — Isomaltose [*O*-α-D-glucopyranosyl-(1→6)-D-glucose] and isomaltosyl oligosaccharides up to the hexamer were prepared from Dextran T40 (Pharmacia, Sweden) as described by Schlossman and Kabat[20], and showed $[\alpha]_D$ +113°, +141°, +150°, +158°, and +160°, respectively (lit.[20]: +111°, +137°, +141°, +151°, and +158°, respectively). Kojibiose was prepared by partial acetolysis of Dextran NRRL B1299, which contained α-D-glucosyl-(1→2)-D-glucose linkages[2,6,7]; the α anomer was obtained by crystallization from aqueous ethanol, m.p. 192–193°, $[\alpha]_D$ +140° (Takiura and Koizumi[21] reported m.p. 193–194°, Bailey *et al.*[22] $[\alpha]_D$ +135°). The α-octaacetate was obtained as described[6], m.p. 166–167° after recrystallization from 95% ethanol (Yamauchi and Aso[23] reported m.p. 166°, Torii *et al.*[6] 166–168°). Nigerose [*O*-α-D-glucopyranosyl-(1→3)-D-glucose] was obtained by partial acetolysis of Dextrans N4 and N4-clinical, which contained (1→6)- and (1→3)-α-D linkages[24], and it was purified by repeated paper chromatography and, finally, by charcoal treatment; it was compared with authentic nigerose generously supplied by Dr. K. Matsuda, Sendai (Japan). Sophorose [*O*-β-D-glucopyranosyl-(1→2)-D-glucose] was a gift from Dr. I. J. Goldstein, Ann Arbor, Michigan. Maltose [*O*-α-D-glucopyranosyl-(1→4)-D-glucose], cellobiose [*O*-β-D-glucopyranosyl-(1→4)-D-glucose], gentiobiose [*O*-β-D-glucopyranosyl-(1→6)-D-glucose], and laminaribiose [*O*-β-D-glucopyranosyl-(1→3)-D-glucose] were commercial products.

Acetyl derivatives of oligosaccharides of the isomaltose series were prepared as follows: each oligosaccharide (about 100 mg) was placed in a mixture of acetic anhydride (0.52 ml) and pyridine (0.78 ml) for 3 days at 0°. The reaction mixture was poured into ice–water and kept in a refrigerator. The precipitate was filtered off, washed with water, and dried; because it showed only one spot, the precipitate was used, without further purification, as reference substance for t.l.c.

Reduced oligosaccharides were prepared as follows: each sample (about 10 mg) was dissolved in water (2 ml) and treated with 1% sodium borohydride (1 ml) overnight

at 0°. The mixture was neutralized with Amberlite IR-120 (H^+) and concentrated to dryness. To remove the borate ions, dry methanol was added and the solution evaporated. After repeating this treatment several times, the final residue was dissolved in water and the solution lyophilized. Since each reduced oligosaccharide thus obtained showed only one spot, they were used, without further purification, as references for paper chromatography.

Chromatography. — Toyo filter paper No. 52 for chromatography was used, after being washed with water by the descending method for 3 days, with propyl alcohol–ethanol–water [7:1:2, v/v (Solvent A) and 6:1:3, v/v (Solvent B)] and ethyl acetate–pyridine–water–acetic acid [5:5:3:1, v/v (Solvent C)] and multiple ascending runs (usually 3 times). Color development was obtained with the silver nitrate–sodium hydroxide reagent[25].

Thin-layer chromatography was performed on Silica Gel G (E. Merck) plates in 2:23 (v/v) methanol–benzene. For detection of the spots, the plates were sprayed with dilute sulfuric acid and heated on a hot plate.

Paper electrophoresis was carried out in a Spinco Model R, Series D at 15 v/cm with 0.05M sodium borate buffer at pH ~ 8. Since borate ions interfered to some extent with the silver nitrate–sodium hydroxide reaction, the paper was dried in air after the electrophoretic run, dipped in methanol acidified with acetic acid (about 1% v/v) for 1 min, and then dried. This treatment improved the color development.

Sugar determination. — The phenol–sulfuric acid method was used[26].

Acetolysis of acetylated Dextran NRRL B1397. — Lyophilized dextran (19 g) was mixed with acetic anhydride (120 ml) and pyridine (180 ml) and heated for 4 h at 100° with stirring. When the dextran was almost completely solubilized, the mixture was poured into ice–water, kept overnight in a refrigerator, the precipitate filtered off, washed with water, and dried *in vacuo* in the presence of phosphoric anhydride and sodium hydroxide (29.3 g, 89%). Acetic anhydride–acetic acid–conc. sulfuric acid (24:16:3, v/v, 258 ml), was added to the precipitate and the solution was stirred for 30 min at 40°, then poured into ice–water, neutralized with solid sodium carbonate (about 300 g), and kept overnight in the refrigerator. Then, the precipitate was filtered off, washed with water, and dried (33 g). Extraction of the filtrate with chloroform did not give any significant amount of additional product. The dried sample was powdered in a mortar with methanol (200 ml) and filtered off.

The methanol extract was concentrated to about 10 ml and placed on a column (5 × 10 cm) of silica gel (Merck) prepared in 2:23 (v/v) methanol–benzene. Elution was carried out first with 2:23 methanol–benzene (about 150 ml) and then with methanol (about 150 ml). Each eluate was evaporated to dryness, and the residue was examined by t.l.c. with the acetyl derivatives of the isomaltose oligosaccharides as reference substances. The methanol–benzene eluate contained materials of low-molecular weight, whereas the methanol eluate contained only higher-molecular-weight compounds.

The methanol-insoluble materials were further acetolyzed and the products again fractionated as just described. Acetolysis and fractionation were repeated 7

times to give 13.2 g of residue eluted with methanol–benzene. This residue was deacetylated by treatment with sodium methylate in methanol[6].

Charcoal chromatography. — A solution of the deacetylated material (12.5 g) in water (30 ml) was poured on a column (4 × 37 cm) of Darco G-60–Celite (240 g, 2:1) prepared in 1% ethanol. The material adsorbed on the column was eluted with 1, 5, 7.5, 10, 12.5, and 30%, respectively, of ethanol, at 25°. The flow rate was approximately 40 ml/h. Aliquots of 20 ml were collected. Every fifth tube was analyzed for sugar content by the phenol–sulfuric acid method and for oligosaccharides by paper chromatography with Solvent A or B. The combined fractions and their major components, detected by paper chromatography, are shown in Table I.

Fractionation of trisaccharides. — Fractions 4 and 5 were combined and further fractionated by repeated charcoal and paper chromatographies (Solvent A or B). A trisaccharide fraction thus obtained gave only one spot on paper, but it was shown by paper electrophoresis to be a mixture of two components, as reported by Lewis *et al.*[9].

Preparative chromatography of the two components was carried out on a column prepared by the method of Zill *et al.*[16]. The procedure was tested on known oligosaccharides (see Table V). The trisaccharide mixture (60 mg) was applied to the column (1 × 8 cm) of Dowex-1 (X-2, BO_4^{3-}, 100–200 mesh) and fractions of 4 ml were eluted (1 ml/min). From each tube an aliquot of 100 μl was taken and the sugar content was determined. Elution was first performed with water until a negative reaction was observed, and then with 50mM acetic acid. The fraction eluted with water (M_G 0.22) was separated into two components (Trisaccharides **2** and **3**) by repeated paper chromatography (Solvent A). The fraction eluted with acetic acid contained Trisaccharide **1** (M_G 0.64). The properties and yields of the trisaccharides are reported in Table II.

From the trisaccharide region of the paper chromatograms, five oligosaccharides were obtained after elution and repeated chromatography on paper, charcoal, and Dowex-1. Since Trisaccharides **1**, **2**, and **3** were shown to contain an α-D-(1→2) linkage, they were extensively studied.

Sugar determination of Trisaccharides **1**, **2**, *and* **3** *before and after reduction.* — A sample (2.5 mg) dissolved in water (1 ml) was reduced with sodium borohydride. The final residue was dissolved in water (1 ml). Aliquots (20 μl) of this sample and of an identical solution of unreduced sugar were determined with phenol–sulfuric acid. The ratios of the glucose contents obtained before and after reduction are given in Table III, indicating that each oligosaccharide studied is a trisaccharide. Isomaltose (IM_2), isomaltotriose (IM_3), and isomaltotetraose (IM_4) were also treated in the same way as the controls; ratios before and after reduction were 2.00:1.03, 3.00:2.05, and 4.00:3.08, respectively.

Partial hydrolysis and acetolysis of Trisaccharides **1**, **2**, *and* **3** *before and after reduction.* — Each trisaccharide solution (100 μl) was hydrolyzed in 0.1M sulfuric acid (0.5 ml) for 1 h at 100° in a sealed tube and the sulfuric acid was removed by Amberlite IRA-400 (OH^-). The solution was evaporated under reduced pressure and the residue analyzed by paper chromatography with Solvents A and C. In all experi-

ments D-glucose (or D-glucitol, or both) and unreacted trisaccharides were detected. The disaccharides found in the hydrolyzates are shown in Table III.

The trisaccharide solutions (150 μl of each) were evaporated to dryness, treated with the acetolyzing reagent (100 μl) for 2 to 3 days at room temperature and then heated at 50° for 30 min. Each reaction mixture was poured onto ice, neutralized with solid sodium carbonate (about 120 mg), and extracted three times with chloroform (2 ml). The combined extracts were washed with water and evaporated to dryness. The residue was dissolved in methanol and treated with M sodium methoxide (30 μl) overnight at 4°. Water was added to the mixture to dissolve the precipitates, and the solution was evaporated to dryness after being treated with Amberlite IR-120 (H^+). The residue was dissolved in a small volume of water and examined by paper chromatography. The disaccharides shown in Table III were detected on the chromatogram in addition to D-glucose or D-glucitol (or both) and unreacted trisaccharides.

All trisaccharides contained both α-D-(1→2) and (1→6) linkages. On hydrolysis and acetolysis, reduced **1** gave only isomaltitol, which indicates that **1** contains an α-D-(1→6) linkage at the reducing end. On the other hand, reduced **2** gave only kojibiitol, which indicates an α-D-(1→2) linkage at the reducing end of **2**. Trisaccharide **3** liberated both isomaltitol and kojibiitol on acetolysis after reduction, thus showing the presence of two D-glucose residues bound to O-2 and O-6 of the reducing D-glucose residue. Consequently, it is most probable that **1**, **2**, and **3** are α-kojibiosyl-(1→6)-D-glucose, α-isomaltosyl-(1→2)-D-glucose, and *O*-α-D-glucopyranosyl-(1→2)-*O*[-α-D-glucopyranosyl-(1→6)]-D-glucose, respectively. In these experiments nigerose, maltose, sophorose, cellobiose, gentiobiose, laminaribiose, and their reduced forms were absent as determined by paper chromatography (Solvent A, B, and C).

Structure determination of **1**, **2**, *and* **3** *by methylation.* — A dried sample (5 mg) of each trisaccharide was dissolved in dimethyl sulfoxide (2 ml) in a nitrogen atmosphere and alkoxylated with freshly prepared methylsulfinyl carbanion (0.5 ml), the reaction mixture being stirred for 3 h at 25° in the presence of nitrogen[27]. The alkoxylated material was then methylated by the addition of methyl iodide at 20°, after which the reaction mixture was diluted with water, and the methylated product was extracted with chloroform (5 × 5 ml). The combined extracts were washed several times with water, dried with magnesium sulfate, and evaporated to a syrup. Complete substitution of the hydroxyl groups by methoxyl groups was confirmed by i.r. spectroscopy.

The fully methylated product (5–6 mg) was methanolyzed by heating with 5% methanolic hydrogen chloride at reflux for 16 h. After neutralization with silver carbonate, the mixture of methyl glucosides was analyzed by g.l.c. in a Hitachi K35 Gas Chromatograph, equipped with a flame ionization detector and a stainless steel column (1-m length) packed with 15% butyl succinate polyester on Neosorb NC; nitrogen was used as a carrier gas at a flow rate of 60 ml/min at 175°.

To estimate the molar ratio of the *O*-methyl-D-glucose[28], the methanolyzate was hydrolyzed with 0.25M sulfuric acid (1 ml) for 15 h at 100°. After neutralization with barium carbonate, the hydrolyzate (5 ml) was reduced with sodium boro-

hydride at room temperature; the excess of the reagent was decomposed with Amberlite IR-120, and the boric acid was removed by repeated evaporation with methanol. For g.l.c. analysis the alditols were acetylated with pyridine (1 ml) and acetic anhydride (1 ml) for 30 min at 100°. The solution was evaporated and the acetates dissolved in a small volume of chloroform and analyzed by g.l.c. on 3% ECNSS-M on a Gas Chrom Q (2-m length) column, at a flow rate of nitrogen of 60 ml/min at 180°. The results of the methylation procedure are reported in Table IV.

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