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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* TYPE 33

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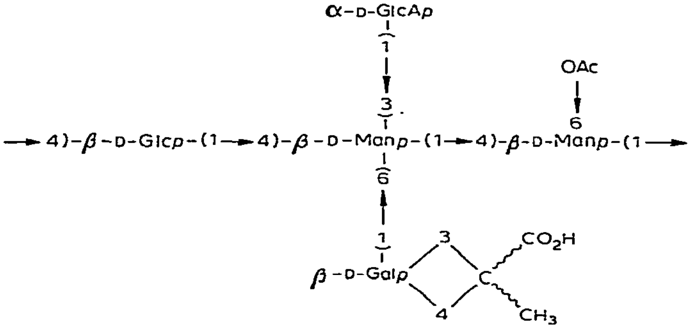
ABSTRACT

The structure of the capsular polysaccharide from *Klebsiella* type 33 has been investigated. Methylation analysis, various specific degradations, graded hydrolysis with acid, and n.m.r. spectroscopy were the principal methods used. It is concluded that the polysaccharide is composed of pentasaccharide repeating-units having the following structure.



The D-galactopyranosyl group, with pyruvic acid linked as a ketal to O-3 and O-4, was degraded on treatment of the fully methylated polysaccharide with strong base. It is proposed that methyl pyruvate is eliminated, in an E2 type of reaction.

INTRODUCTION

The capsular polysaccharide from *Klebsiella* type 33 (K 33) was investigated by Nimmich[1], who found that it contained mannose, galactose, glucose, and glucuronic acid. Several of the *Klebsiella* capsular polysaccharides contain these sugars[1,2], and structural studies of those from types 7[3], 13[4], 28[5], 59[6], and 62[7] have been reported. We now report structural studies of K 33.

RESULTS AND DISCUSSION

Analyses of hydrolysates of K 33 and of carboxyl-reduced[8] K 33 demonstrated that it is composed of mannose, galactose, glucose, and glucuronic acid in the molar proportions 2:1:1:1 and thus, most probably, is composed of pentasaccharide repeating-units. The sugars obtained from carboxyl-reduced K 33 were shown to belong to the D series by g.l.c. of their acetylated (+)-2-octyl glycosides[9].

The polysaccharide also contained pyruvic acid and *O*-acetyl groups. The pyruvic acid was identified by t.l.c. as its 2,4-dinitrophenylhydrazone. The ^1H- and ^{13}C-n.m.r. spectra were not well resolved, probably because of the poor solubility of K 33. From the intensities of the ^1H-n.m.r. signals at δ 1.60 (pyruvate CH_3) and δ 2.16 (*O*-acetyl CH_3), these components occur in approximately equimolecular proportions. A weak signal at δ 2.21 may indicate that most, but not all, of the *O*-acetyl occupies a unique position in the polysaccharide. The corresponding signals in the ^{13}C-n.m.r. spectrum appeared at 25.5 (pyruvate CH_3) and 21.3 p.p.m. (*O*-acetyl CH_3), and were of comparable intensities. Studies reported below further demonstrate that K 33 contains one mole of each of these components per pentasaccharide repeating-unit.

The polysaccharide had $[\alpha]_{578}$ +22°, suggesting that it contains both α- and β-glycosidic linkages. A well-resolved ^{13}C-n.m.r. spectrum was obtained from a material which had been partially depolymerised by mild hydrolysis with acid, during which all of the pyruvic acid and *O*-acetyl groups were also removed. In this spectrum, signals for the five anomeric carbon atoms appeared at 104.3, 103.5, 102.8, and 101.5 p.p.m. (two C). The signals of three of the C-6 atoms of the hexose residues appeared

TABLE I

METHYLATION ANALYSES OF ORIGINAL AND MODIFIED *Klebsiella* TYPE 33 CAPSULAR POLYSACCHARIDES

Methylated sugar[a]	T[b]	*Mole %*[c]				
		A	B	C	D	E
2,3,4,6-Glc	1.00		21	21		31
2,3,4,6-Gal	1.14			23		
2,3-Et,6-Et-Man	1.55				20	
2,3,6-Man	1.79	24	22	21	32	19
2,3,6-Glc	1.94	24	23	19	31	21
2,6-Et-Man	2.61				4	19
2,6-Gal	2.77	26	17		2	3
2,3-Et-Man	2.86				8	
2-Man	4.85	26	17	16	2	8

[a] 2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.* [b] Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an SP-1000 column at 220°. [c] Polysaccharide: *A*, original; *B*, carboxyl-reduced; *C*, carboxyl-reduced, methylated, partially hydrolysed, and remethylated; *D*, methylated, uronic acid-degraded, and ethylated material; *E*, carboxyl-reduced material, methylated, base-treated, hydrolysed, and ethylated.

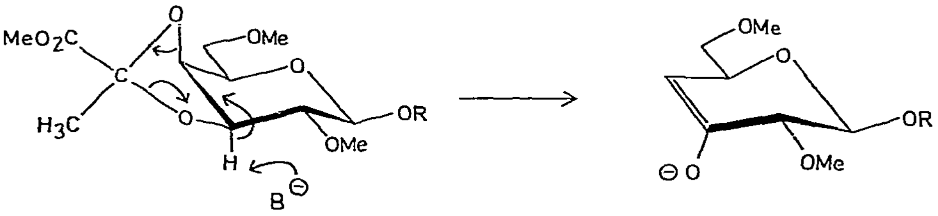
at ~62.3 p.p.m., indicating that the fourth C-6 is substituted and that its signal is therefore shifted downfield.

Methylation analysis of K 33 (Table I, column *A*) and of carboxyl-reduced K 33 (Table I, column *B*) supports the assumption of a pentasaccharide repeating-unit. These analyses further demonstrate that the D-glucuronic acid is terminal, and that the D-glucosyl and one of the D-mannosyl residues are linked through O-4.

In order to locate the pyruvic acid residue, K 33 was subjected to carboxyl-reduction, methylation, hydrolysis under mild conditions to remove the modified pyruvic acid residue, remethylation, hydrolysis, and analysis of the components (Table I, column *C*). The 2,6-di-*O*-methyl-D-galactose found in the analysis of the original material was completely replaced by 2,3,4,6-tetra-*O*-methyl-D-galactose, demonstrating that pyruvic acid is linked as a ketal to O-3 and O-4 of a terminal D-galactopyranosyl group. One of the D-mannosyl residues is consequently linked through O-3, O-4, and O-6. As no glycosidic linkages were cleaved during the mild hydrolysis with acid, it is concluded that they are all pyranosidic.

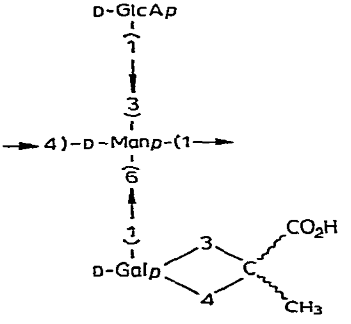
K 33 was subjected to a uronic acid degradation[10], *i.e.*, methylation by the Hakomori procedure, treatment with base, and mild hydrolysis with acid. By this treatment, the terminal D-glucopyranosyluronic acid group should be eliminated. Ethylation of the product and investigation of its hydrolysate (Table I, column D) should therefore reveal the position to which it was linked. Unexpectedly, however, the major portion of the pyruvylated D-galactopyranosyl group was simultaneously eliminated during this treatment. The formation of 3,6-di-*O*-ethyl-2-*O*-methyl-D-mannose demonstrates that the two terminals are linked to O-3 and O-6 of the double-branched D-mannopyranosyl residue.

The degradation of the D-galactopyranosyl group substituted with a pyruvic acid ketal parallels the degradation of methyl 2,3:4,6-di-*O*-benzylidene-α-D-mannoside on treatment with butyl-lithium[11,12]. The reaction, which may occur as depicted in **1**→**2**, is reminiscent of an E2 elimination. The antiperiplanar disposition of the groups to be eliminated is probably a prerequisite. The modified sugar residue is then eliminated either by the basic conditions or during the subsequent mild hydrolysis with acid. The reaction is almost complete with the strong base sodium methylsulfinylmethanide in dimethyl sulfoxide. It also occurs to a considerable extent under milder conditions, *i.e.*, with sodium methoxide in methanol. No degradation was observed when the original polysaccharide was treated with sodium methylsulfinylmethanide in dimethyl sulfoxide, which is the first step in the Hakomori methylation.



1 **2**

A sample of K 33 was carboxyl-reduced, methylated, treated with sodium methylsulfinylmethanide in dimethyl sulfoxide, hydrolysed with acid under mild conditions, and ethylated. Analysis of the hydrolysate of this product (Table I, column *E*) revealed that the degradation discussed above proceeds equally well when 1-methoxypropan-2-one is ketal-linked to O-3 and O-4 of the D-galactopyranosyl group. The results demonstrate that the terminal D-galactopyranosyl group is linked to O-6 of the branching D-mannopyranosyl residue and, consequently, that the terminal D-glucopyranosyluronic acid group is linked to O-3 of the same residue. The partial structure **3** is thereby established.



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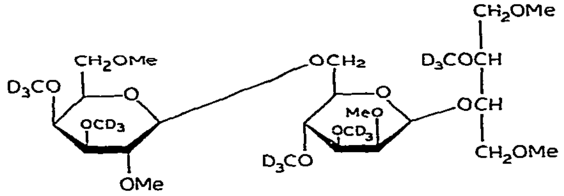


4

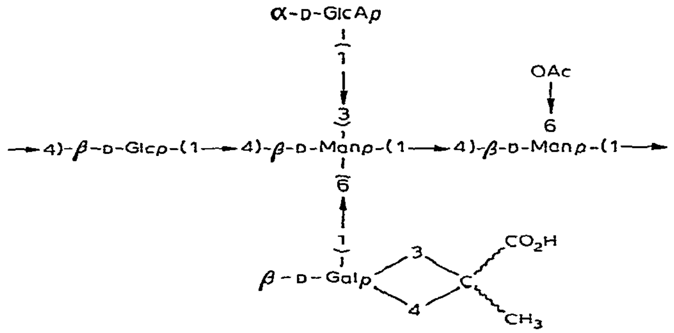
A modified Smith degradation of carboxyl-reduced K 33, involving periodate oxidation, borohydride reduction, methylation, hydrolysis under mild conditions, and remethylation, using trideuteriomethyl iodide, yielded the methylated trisaccharide **4**. It gave the expected mass spectrum[13], and 2,6-di-*O*-methyl-3,4-di-*O*-trideuteriomethyl-D-galactose and 2-*O*-methyl-3,4-di-*O*-trideuteriomethyl-D-mannose were obtained on hydrolysis. In the ^{1}H-n.m.r. spectrum, the anomeric protons appeared at δ 4.50 ($J_{1,2}$ ~1 Hz) and 4.33 ($J_{1,2}$ 7.5 Hz), respectively. Thus, the Smith degradation confirms the results of the base-degradation experiments and also demonstrates that both the D-galactosyl group and the branching D-mannosyl residue are β-linked.

Graded, acid hydrolysis of K 33 yielded acidic oligosaccharides which were separated by ion-exchange and gel-permeation chromatography. One component (**5**), $[\alpha]_{578}$ +45°, moved as a trisaccharide (R_{Maltose} 0.75) in p.c. On hydrolysis, it yielded D-mannose, and traces of D-glucose and D-galactose, as the neutral sugars. On hydrolysis of the trisaccharide alditol, D-mannitol and D-mannose were obtained. The trisaccharide was reduced with sodium borodeuteride and methylated. G.l.c. of this product gave a main peak at *T* 1.4, relative to fully methylated cellotri-itol. In g.l.c.–m.s., this component gave the spectrum expected for the methylated alditol of **5**, *e.g.*, the signals for the terminal components, *m/e* 233 (aA_1) and 236 (cA_1). Carboxyl-reduction of the methylated alditol of **5** with lithium aluminium deuteride, followed by acid hydrolysis, yielded 1,2,3,5,6-penta-*O*-methyl-D-mannitol-*1-d*, 2,3,4-tri-*O*-methyl-D-glucose-*6,6-d*$_2$ and 2,4,6-tri-*O*-methyl-D-mannose, thereby establishing the sequence of sugar residues and the substitution pattern in **5**. The ^{1}H-n.m.r.

spectrum of the original trisaccharide showed, *inter alia*, signals for anomeric protons at δ 5.21 (1 H, $J_{1,2}$ 3.7 Hz), 5.14 (0.5 H, $J_{1,2}$ 1.5 Hz), 4.86 (0.5 H, $J_{1,2}$ 1 Hz), and 4.71 (1 H, $J_{1,2}$ 1 Hz). The low-field signal is assigned to the D-glucopyranosyluronic acid group, which should be α-linked, and the high-field signal to the central D-mannopyranosyl residue, which should be β-linked. The two remaining signals are assigned to the α and β form, respectively, of the terminal D-mannopyranose residue. These results, in conjunction with previous results, therefore give the complete structure **6** for the repeating-unit of K 33, except for the anomeric nature of the two chain residues and the location of the *O*-acetyl group (which, however, are also included in the given structure).

α-D-GlcA*p*-(1→3)-β-D-Man*p*-(1→4)-D-Man*p*

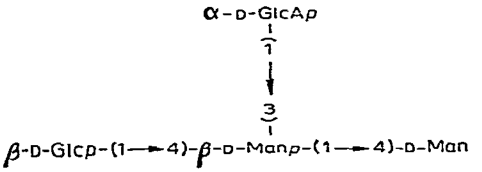
5



6

Another oligosaccharide from the graded hydrolysis with acid proved to be the tetrasaccharide **7**. Hydrolysis yielded D-glucose and D-mannose as the neutral sugars, and hydrolysis of the tetrasaccharide alditol demonstrated that part of the D-mannose was the reducing terminal. The tetrasaccharide **7** had $[\alpha]_{578}$ +35° and R_{Maltose} 0.49 in p.c. In addition to signals for anomeric protons corresponding to those observed for the trisaccharide, a signal at δ 4.52 (1 H, $J_{1,2}$ 7.5 Hz) was observed, demonstrating that the terminal D-glucopyranosyl group is β-linked. The fully methylated tetrasaccharide-alditol-*1-d* gave one peak in g.l.c., and m.s. of the material in this peak gave the expected spectrum, *i.e.*, A_1 fragments at *m/e* 219 and 233, from the terminal D-glucopyranosyl and D-glucopyranosyluronic ester groups, respectively. The methylated alditol was carboxyl-reduced, using lithium aluminium deuteride, and hydrolysed. Analysis of this product by acetylation and g.l.c., as devised by Bebault *et al.*[14], showed that it contained 2,3,4,6-tetra-*O*-methyl-D-glucose but no tetra-*O*-methyl-D-mannose. Analysis of the product, as the derived alditol acetates, gave equimolecular amounts of 1,2,3,5,6-penta-*O*-methyl-D-mannitol-*1-d*, 2,3,4,6-

tetra-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-glucose-6,6-d_2, and 2,6-di-*O*-methyl-D-mannose, in agreement with the postulated structure **7**.



7

The anomeric nature of the chain D-mannopyranosyl residue was not defined from the foregoing experiments. The carboxyl-reduced polysaccharide was therefore acetylated, and treated with chromium trioxide in acetic acid[15]. During this treatment, the β-hexopyranosyl residues should be oxidized to hex-5-ulosonic ester residues, but the α-hexopyranosyl residues should be resistant. Sugar analysis of the product gave ~1 mole of D-glucose which should derive from the D-glucopyranosyluronic acid group. This analysis indicates that the chain D-mannosyl residue is β-linked and confirms the assignments made from other evidence.

In order to locate the *O*-acetyl groups, K 33 was treated with methyl vinyl ether in the presence of an acid catalyst, and the product was subjected to methylation analysis[16]. In this analysis, *O*-acetyl groups in the original material are replaced by *O*-methyl groups. D-Mannose, D-glucose, D-galactose, and 6-*O*-methyl-D-mannose were obtained in the molar proportions 1 :0.9 :0.6 : 1. The acetyl group is consequently linked to O-6 of the chain D-mannose residue. It has previously been demonstrated that uronic acid residues, which become fully protected during the reaction with methyl vinyl ether, are degraded during the treatment with strong base, which is the first step in the Hakomori methylation[17]. The present results also demonstrate that the D-galactosyl group, with pyruvic acid ketal-linked to O-3 and O-4, is also degraded during this treatment, although only partially.

As a result of the structural studies reported above, it is proposed that the capsular polysaccharide from *Klebsiella* type 33 is composed of pentasaccharide repeating-units having the structure **6**. The configuration of the pyruvic acid ketal has, however, not been determined. The structure contains a double-branched sugar residue. Another polysaccharide containing a similar structural feature is the *Klebsiella* type 38 capsular polysaccharide[18]. The present finding may indicate that such structures are more common than has been assumed.

EXPERIMENTAL

General methods. — General methods and methods for sugar and methylation analysis[19,20] and carboxyl-reduction[8] have been described previously.

For g.l.c., Perkin–Elmer 990 or Hewlett–Packard 5830A instruments equipped with flame-ionisation detectors were used. Separations were performed on glass columns (180 × 0.15 cm, with 100/120 mesh Gas Chrom Q as support material) containing *A*, 3% OV-225 (at 200° for alditol acetates); or *B*, 3% OV-1 (for oligosaccharide derivatives). Partially methylated alditol acetates and acetylated octyl glycosides were separated on an SP-1000 W.C.O.T. column (25 m × 0.25 mm) at 220°.

G.l.c.–m.s. was performed with a Varian MAT 311-SS 100 m.s.–computer system fitted with appropriate g.l.c. columns. Spectra were recorded at 70 eV, with an ionisation current at 1000 μA.

For n.m.r., a JEOL FX-100 instrument operated in the PFT-mode was used. The spectra were recorded for solutions in $CDCl_3$ or D_2O with internal or external tetramethylsilane, respectively, as standard. Optical rotations were recorded (10-cm micro-cell) with a Perkin–Elmer 241 instrument, and i.r. spectra were recorded with a Perkin–Elmer 257 instrument. Paper chromatography was performed on Whatman No. 1 paper in the solvent system ethyl acetate–acetic acid–water (3:1:1).

Isolation of the polysaccharide from Klebsiella *K 33* (*strain 6168*). — This was performed as previously described[1]. The polysaccharide had $[\alpha]_{578}^{23}$ +22° (*c* 0.6, water). In the i.r. (KBr), an absorption was observed at 1730 cm^{-1} (*O*-acetyl). The percentages of nitrogen (0.19%) and phosphorus (0.09%) in the material were insignificant. For some n.m.r. studies and oxidation experiments with chromium trioxide, native material was modified by treatment with hydrochloric acid at pH 2 for 6 h at 100°. The solution was neutralized, dialysed, and freeze-dried. Analyses of the recovered material revealed that pyruvic acid residues and *O*-acetyl groups had been cleaved, but that the polysaccharide backbone was essentially intact.

Degradation of methylated polysaccharide by treatment with strong base. — A sample (10 mg) of carefully dried, methylated K 33 was degraded by using sodium methylsulfinylmethanide in methyl sulfoxide, essentially as described earlier[10]. The recovered material was ethylated, dialysed, and hydrolysed, and the resulting sugars were analysed as their alditol acetates by g.l.c.–m.s. (Table I, column D).

An analogous degradation was performed starting from carboxyl-reduced, methylated K 33 (Table I, column *E*).

Smith degradation of the polysaccharide[5,6,21,22]. — Carboxyl-reduced K 33 (18 mg) was dissolved in water (12 ml), and 0.2M sodium metaperiodate (3 ml) was added. The reaction mixture was kept in the dark at 5° for 120 h. Excess of periodate was reduced with ethylene glycol (1 ml), and the mixture was dialysed. The solution was concentrated to 30 ml, and sodium borohydride (100 mg) was added. After stirring overnight at room temperature, the excess of borohydride was destroyed by adding 50% acetic acid. The solution was dialysed, and concentrated to 2 ml, and the material was purified on a column (100 × 3 cm) of Sephadex G-25. The yield of polyalcohol was 7 mg. Sugar analysis of part (2 mg) of the material showed that the oxidation had gone to completion, D-mannose and D-galactose being obtained in the ratio 1:1. Another part (5 mg) of the polyalcohol was methylated, and recovered by dialysis. Part (10%) of this material was hydrolysed and analysed, giving 2-*O*-methyl-D-mannose and 2,6-di-*O*-methyl-D-galactose in the ratio 1:1. The remainder of the methylated material was treated with 90% formic acid (2 ml) at 40° for 1 h. The solution was evaporated to dryness, and methylated by using trideuteriomethyl iodide. Part (20%) of the recovered material was hydrolysed and analysed, giving 2,3,4,6-tetra-*O*-methyl-D-galactose (trideuteriomethylated at O-3 and O-4) and 2,3,4-tri-*O*-methyl-D-mannose (trideuteriomethylated at O-3 and O-4) in the ratio 1:1. The remainder of the material was purified by t.l.c.; R_F 0.32 (silica gel; chloroform–ethanol, 19:1). Permethylated β-D-Gal*p*-(1→6)-β-D-Man*p*-(1→2)-erythritol (4, ~1 mg) was obtained, showing $T_{\mathrm{Melibiitol}}$ 5.4 in g.l.c. (OV-1 column at 220°). The mass spectrum showed, *inter alia*, the following fragments (relative intensities in brackets, and some assignments[13] in square brackets): 91(100), 150(16) [cA_1], 158(6)[aA_3], 190(5)[aA_2], 213(5)[bcJ_1], 225(1)[aA_1], 293(1.5)[bcA_3], 325(1)[bcA_2], and 360(3.5)[bcA_1]. In the ^{1}H-n.m.r. spectrum ($CDCl_3$), the signals from the anomeric protons occurred at δ 4.50 ($J_{1,2}$ <1 Hz) and 4.33 ($J_{1,2}$ ~7.5 Hz).

Isolation and characterization of oligosaccharides obtained on graded hydrolysis with acid. — K 33 (300 mg) was hydrolysed in 0.13M sulfuric acid at 100° for 1.5 h. The hydrolysate was neutralized with barium carbonate and added to a column (30 × 1.5 cm) of Dowex-1 X8 resin. The neutral sugars were eluted with water, and the acidic oligosaccharides with acetic acid (6%). The acidic oligosaccharides were further fractionated on a column (60 × 1.5 cm) of Bio-Gel P-2, yielding two main components (5, 6.1 mg; and 7, 14.1 mg).

The oligosaccharide 5 (4 mg) was transformed into its alditol by reduction with sodium borodeuteride (20 mg) in water (4 ml). After conventional work-up, part (20%) of the alditol was hydrolysed and divided into two parts. One part was acetylated, and g.l.c.–m.s. showed that it contained mannitol-*1-d* acetate. The other part was reduced with sodium borohydride and acetylated. G.l.c.–m.s. showed mannitol acetate (~40% deuterium at C-1) and trace amounts of galactitol and glucitol acetates. The remainder (80%) of the oligosaccharide alditol was methylated. The product showed $T_{\mathrm{Cellotriitol}}$ 1.4 (OV-1 column at 235°). The mass spectrum showed, *inter alia*, the following signals: *m/e* 88(25), 201(100)[aA_2], 233(14)[aA_1], and 236(8)[cA_1]. Part (20%) of the methylated material was hydrolysed and analysed, giving 1,2,3,5,6-penta-*O*-methyl-D-mannitol, 2,4,6-tri-*O*-methyl-D-mannose, and 2,3,4,6-tetra-*O*-methyl-D-glucose/D-mannose in the proportions 1:0.9:trace. The remainder of the methylated material was carboxyl-reduced by using lithium aluminium deuteride in ether, and after conventional work-up, the material was hydrolysed and analysed; 1,2,3,5,6-penta-*O*-methyl-D-mannitol, 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-mannose, and 2,3,4-tri-*O*-methyl-D-glucose-*6,6-d*$_2$ were obtained in the proportions 0.5:trace:1:1. The oligosaccharide 5 had $[\alpha]_{578}^{23}$ +45° (*c* 0.3, water) and R_{Maltose} 0.75 (p.c.; ethyl acetate–acetic acid–water, 3:1:1). In the ^{1}H-n.m.r. spectrum (D_2O, 85°), the signals from the anomeric protons appeared at δ 5.21 (1 H, $J_{1,2}$ 3.7 Hz), 5.14 (0.5 H, $J_{1,2}$ 1.5 Hz), 4.86 (0.5 H, $J_{1,2}$ 1 Hz), and 4.71 (1 H, $J_{1,2}$ 1 Hz).

The oligosaccharide 7 was subjected to the same characterization procedure as 5. It had $[\alpha]_{578}^{23}$ +35° (*c* 0.9, water) and R_{Maltose} 0.49 (p.c.; ethyl acetate–acetic acid–water, 3:1:1). In the ^{1}H-n.m.r. spectrum (D_2O, 85°), the signals from the anomeric protons appeared at δ 5.20 (1.5 H, $J_{1,2}$ <2 Hz), 4.91 (0.5 H, $J_{1,2}$ <2 Hz), 4.77 (1 H, $J_{1,2}$ <2 Hz), and 4.52 (1 H, $J_{1,2}$ 7.5 Hz). A portion of 7 was reduced with sodium borodeuteride. Hydrolysis and acetylation then gave mannitol-*1-d* acetate. Hydrolysis, reduction, and acetylation gave mannitol and glucitol acetates in the ratio 1.6:1. Analysis by g.l.c.–m.s. of reduced (sodium borodeuteride) and methylated 7 gave a single peak, $T_{\text{Cellotriitol}}$ 8.0 (OV-1 column at 260°). The mass spectrum showed, *inter alia*, the following fragments: *m/e* 88(97), 187(100)[a^1A_2], 201(83)[aA_2], 219(13)[a^1A_1], 233(11)[aA_1], and 236(25)[cA_1]. The methylated and carboxyl-reduced material was hydrolysed. Part of the hydrolysate was acetylated and analysed; 1-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-α,β-D-glucose was the only tetramethyl ether detected. The remainder of the material was analysed as alditol acetates; 1,2,3,5,6-penta-*O*-methyl-D-mannitol-*1-d*, 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-glucose-*6-d*$_2$, and 2,6-di-*O*-methyl-D-mannose were obtained in the proportions 0.8:1:0.8:1.

Chromium trioxide oxidation[15]. — Depyruvylated and carboxyl-reduced K 33 (20 mg) was dissolved in formamide (5 ml), and treated with acetic anhydride (1 ml) and pyridine (1 ml) overnight at room temperature. The acetylated material (23 mg) was recovered by dialysis and freeze-drying, and dissolved in acetic acid (1 ml); *myo*-inositol hexa-acetate (2 mg) was added as the internal standard. Part (67%) of the acetic acid solution was treated with chromium trioxide (60 mg) on an ultrasonic bath at 50° for 2 h. The material was recovered by partition between chloroform and water. The remaining portion (33%) was used as reference. Sugar analysis of the reference material showed D-glucose, D-galactose, and D-mannose in the molar proportions 2.3:1:2.3; for the oxidized material, these figures were 1.1:0:0.2.

Location of O-*acetyl groups*. — This was performed essentially as described earlier[6,16].

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