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STRUCTURAL INVESTIGATION OF *Klebsiella* K-TYPE 4 CAPSULAR POLYSACCHARIDE

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ABSTRACT

The capsular polysaccharide from *Klebsiella* K4 contains the tetrasaccharide repeating-sequence →3)-α-D-Glc*p*-(1→2)-α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→3)-β-D-Glc*p*-(1→. P.m.r. spectroscopy and the measurement of optical rotation were used to establish the anomeric linkages in the polysaccharide and in the oligosaccharides derived by partial hydrolysis. The repeating unit also contains one *O*-acetyl group.

INTRODUCTION

Klebsiella K4 polysaccharide is made up of residues of D-glucuronic acid, D-glucose, and D-mannose, and constitutes one of four serologically different K-types having this sugar composition[1]. Known originally as *Klebsiella ozaenae* type D polysaccharide, this carbohydrate was the subject of a note by Joner[2], who performed a number of inhibition experiments to investigate the role of the constituent sugars in determining the antigenic specificity of the polysaccharide. Mild oxidation with periodic acid was found to modify some of the groupings responsible for the antigenic specificity, but no qualitative or quantitative differences in composition between the native and oxidized polysaccharides were detected.

More recently, studies on graded hydrolysis with acid[3] have been used to determine the size of the repeating unit in the K4 polysaccharide, and Heidelberger and Nimmich have examined the serological cross-reactivity of this strain with a number of Pneumococcal antisera[4].

RESULTS AND DISCUSSION

The acidic polysaccharide used in this investigation was produced by a strain isolated from a patient having a chronic lung-infection, and had the same characteristics as those previously reported. Electrophoresis on cellulose acetate, and ultracentrifugation, indicated that the polysaccharide was homogeneous and monodisperse. P.m.r. spectroscopy showed that one *O*-acetyl group was present per four sugar residues, and the chemical shifts of the signals in the anomeric region suggested that

TABLE I

METHYL ETHERS FROM THE HYDROLYZATES OF THE METHYLATED (A) AND REDUCED, METHYLATED (B) POLYSACCHARIDE FROM *Klebsiella* K4

Sugar[a]	*T*[b]	*Molar proportions (%)*	
		A[c]	*B*[d]
2,4,6-Man	1.19	30.7	26.1
2,4,6-Glc	1.34	69.3	52 6
3,4-Glc	2.13	—	21 3

[a]2,4,6-Man = 2,4,6-tri-*O*-methyl-D-mannose, *etc.* [b]Retention time of the corresponding (Me_3Si) alditol, relative to that of 2,3,4,6-tetra-*O*-methyl-D-galactose on a column of SE-52 at 140°. [c,d]Hydrolyzates examined after hydrolysis for 18 and 6 h, respectively.

TABLE II

ANALYSIS OF THE OLIGOSACCHARIDES FROM *Klebsiella* K4 CAPSULAR POLYSACCHARIDE

Oligomer	$[\alpha]_D$ *(degrees)*	R_{Glc} *(solvent A)*	*Mol. wt.*[a]	*Neutral sugars (molar proportions)*	*Methylated (Me_3Si) alditols (molar proportions)*
Neutral disaccharide **1**	+19	0.73	340	Glc	2,3,4,6-Glc (1.0) 2,4,6-Glc (1.0)
Aldobiouronic acid **2**	+27	0.74	360	Man	2,4,6-Man
Aldotriouronic acids **3**	+48	0 42	520	Glc (1 0) Man (0.7)	2,3,4,6-Glc (1.0) 2,4,6-Man (0.6) 2,4,6-Glc (0.8)
Aldotetraouronic acid **4**	+98	0.25	720	Glc (2.0) Man (0.8)	2,3,4,6-Glc (0.6) 2,4,6-Man (0.6) 2,4,6-Glc (1.0)

[a]Gel-permeation chromatography on Bio-Gel P-2, using D-glucose and an authentic biouronic acid as the calibrating solutes.

three of these sugars were linked by α-D-glycosidic bonds and one by a β-D bond; no bound pyruvic acetal groups were detected. The molar ratio of the neutral sugars in a hydrolyzate was found by g.l.c. analysis to be mannose (0.6) and glucose (2.0); this agrees with values reported previously[3]. Circular-dichroism measurements on the derived alditol acetates proved that the sugars were of the D configuration. Glucuronic acid was shown to have the same configuration, by procedures to be described.

Methylation analysis (see Table I) of the capsular polysaccharide established that D-glucopyranosyl and D-mannopyranosyl units were present in the ratio of 2:1,

and that all were linked through O-3; and methanolysis and g.l.c. of the methylated polysaccharide showed that the D-glucopyranosyluronic residues were also in-chain, being linked through O-2. These results were corroborated by g.l.c.–m.s. analysis of the hydrolyzate from ester-reduced, methylated polysaccharide, in which the appearance of 3,4-di-*O*-methyl-D-glucose is a consequence of the reduction of the ester group in the methylated, D-glucopyranuronic acid units linked through O-2 in the methylated polysaccharide chain.

The sequence of sugars in the linear polysaccharide was determined by partial hydrolysis with acid, and the isolation and identification of oligosaccharides **1**–**4**. These products were separated by gel-permeation chromatography, purified in some cases by preparative paper-chromatography, and examined by standard techniques (see Table II).

Neutral disaccharide **1** was identified as 3-*O*-β-D-glucopyranosyl-D-glucose on the basis of methylation analysis, the value of $[\alpha]_D$ (which was in agreement with that in the literature[5]), and paper-chromatographic comparison with an authentic specimen. Oligomer **2** was indistinguishable, by paper chromatography, o.r.d. measure-

β-D-Glc*p*-(1→3)-D-Glc
1

α-D-Glc*p*A-(1→3)-D-Man
2

ment, and p.m.r. spectroscopy, from the aldobiouronic acid 3-*O*-(α-D-glucopyranosyluronic acid)-D-mannose[6], and the methylated derivative, as well as the reduced, methylated derivative, gave the expected products on hydrolysis and methanolysis. The 2,3,4-tri-*O*-methylglucitol obtained from reduced, methylated **2** by hydrolysis and reduction with borohydride was isolated and *O*-demethylated, yielding glucitol; the D configuration of the glucose, and hence of the parent glucuronic acid, was proved by conversion of the glucitol into its hexaacetate and examination of the c.d. spectrum thereof.

Fraction **3** contained a mixture of triouronic acids **3a** (major component) and **3b**, as evidenced by the products of hydrolysis and methanolysis of permethylated **3**, the p.m.r. spectrum of **3**, and the presence in **3** of both D-mannose and D-glucose (reducing) end-groups. Both **3a** and **3b** are structurally related to the tetraouronic acid **4**, through their possessing one D-glucose unit less (removed from either terminal of **4**).

α-D-Glc*p*-(1→2)-α-D-Glc*p*A-(1→3)-D-Man
3a

α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→3)-D-Glc
3b

α-D-Glc*p*-(1→2)-α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→3)-D-Glc
4

Partial hydrolysis of **4** gave D-glucose, D-mannose, D-glucuronic acid, and **2**, and the reducing end-group was shown to be D-glucose. On hydrolysis and methanolysis, methylated **4** yielded products consistent with the structure shown, and the anomeric linkages between the sugar units were deduced from the p.m.r. spectrum of **4** in D_2O.

It follows from the foregoing evidence that the sequence of sugar residues formulated as **5** is present in the chains of K4 capsular polysaccharide, and, as there is no branching, this is the tetrasaccharide repeating-unit.

$$\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Glc}p\text{A-}(1\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow \quad \mathbf{5}$$

The *O*-acetyl group has not yet been located, but its presence, shown by the p.m.r. spectrum of the polysaccharide in D_2O, had also been inferred by Heidelberger and Nimmich[4], who noted an increase in the antibody precipitation with Anti-Pneumococcal type II after treatment of K4 polysaccharide with alkali.

The size of the unit **5** is compatible with the results obtained by graded hydrolysis with acid[3]. It is also evident that periodate oxidation would alter the composition of K4 polysaccharide by cleavage of the constituent D-glucuronic acid units between C-3 and C-4, thus destroying one of the groupings responsible for the antigenic specificity of the polysaccharide. This would explain the marked diminution in the ability of the oxidized polysaccharide to precipitate antibody from homologous immune serum, noted by Joner[2].

Comparison of the molecular structure of *Klebsiella* K4 polysaccharide with those of K2 (ref. 7), K5 (ref. 8), and K24 (ref. 9) shows that it contains the same proportions of sugar units as K2 polysaccharide, but in a linear sequence. The bio-uronic acid linkage is common to K2 and K24, and is also in-chain in the K24 polysaccharide, as is the α-(1→3) linkage between mannose and glucose. No other inter-sugar linkages are common to the structure of K4 and any other polysaccharide in this group. The only other linear tetrasaccharide repeating-unit reported for a *Klebsiella* capsular polysaccharide occurs in that[10] of K6, but this carries an additional acidic function in the form of pyruvic acetal.

EXPERIMENTAL

General methods. — Paper chromatography (p.c.) was performed with Whatman No. 1 paper and the following solvent systems (all v/v): *A*, 2:1:1 1-butanol–acetic acid–water; *B*, 1-butanol–ethanol–water (4:1:5, upper phase); *C*, 8:2:1 ethyl acetate–pyridine–water; and *D*, butanone–water azeotrope. Components were detected by heating the papers for 5–10 min at 110° after spraying with *p*-anisidine hydrochloride in aqueous 1-butanol. Other general methods and methylation analyses were as previously described[6].

Properties of Klebsiella *K4 capsular polysaccharide.* — The capsular polysaccharide, grown and purified as previously reported[3], had $[\alpha]_D^{20}$ +90° (*c* 0.2), and contained N, 0.2%. $\overline{M}_w$ was 2.1 × 10^5 according to gel-permeation chromatography, and the equivalent weight ~710 (as acid, by titration) (compare ref. 3).

The p.m.r. spectrum of a 2% solution of the polysaccharide in D_2O showed a sharp singlet at δ 2.20 (acetate CH_3), and signals in the anomeric region at δ 5.40 (J 3 Hz), 5.24 (J 3 Hz), 5.18 (J 2 Hz), and 4.65 (J 7 Hz).

Hydrolysis of the polysaccharide; sugar analysis. — Hydrolysis of the polysaccharide in 2M trifluoroacetic acid for 18 h at 100°, followed by p.c. of the hydrolyzate (solvents *A* and *C*), showed the presence of glucose, mannose, glucuronic acid, and an aldobiouronic acid. The ratio of mannose to glucose was determined by g l.c. of the alditol acetates[11]. Circular-dichroism spectra were recorded for the crystalline acetates in acetonitrile, and a comparison thereof was made with those of authentic samples of the hexaacetates of D-mannitol and D-glucitol[12].

Methylation analysis. — A sample (0.25 g) of the capsular polysaccharide in the acid form was methylated once by Hakomori's[13] method and twice by that of Purdie and Irvine[14], to give an amorphous product (0.23 g), $[\alpha]_D^{20}$ +72° (*c* 1.2, chloroform), that showed no hydroxyl absorption in the i.r. spectrum. Methylated polysaccharide (0.15 g) was hydrolyzed with 2M trifluoroacetic acid for 18 h at 100°; p.c. of the hydrolyzate (solvents *B* and *D*) showed the presence of 2,4,6-tri-*O*-methylmannose and 2,4,6-tri-*O*-methylglucose. A portion of the hydrolyzate was reduced, the alditols were per(trimethylsilyl)ated, and the ethers analyzed by g.l.c., to give the proportions of sugars shown in Table I. These sugars were further characterized by mass spectrometry of the derived alditol acetates. A further sample of the methylated polysaccharide (5 mg) was heated with 10% methanolic hydrogen chloride for 18 h at 100°, the acid neutralized with silver carbonate, and the product analyzed by g.l.c., which revealed the methyl glycosides of 2,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylglucose, and methyl 3,4-di-*O*-methylglucuronate.

Reduction of methylated K4 polysaccharide. — Methylated polysaccharide (14 mg) in oxolane (2 mL) was stirred with lithium aluminum hydride (100 mg) for 18 h. After the addition of aqueous ethanol, the solution was evaporated to dryness, and the residue was extracted with chloroform. The product showed no absorption at 1740 cm^{-1} (methyl ester CO) in the i.r. spectrum. This product (10 mg) was heated in 2M trifluoroacetic acid for 6 h at 100°, and the sugars present in the hydrolyzate were analyzed by p.c. (solvents *B* and *D*). Chromatograms showed the presence of two components, one of which gave a pink color, characteristic of the 2,4,6-tri-*O*-methyl sugars, when sprayed with *p*-anisidine, whereas the slower-moving component gave a brownish-yellow color, and had a mobility similar to that of 3,4-di-*O*-methyl-D-glucose. A portion of the hydrolyzate was reduced, the alditols were per(trimethylsilyl)ated, and the ethers analyzed by g.l.c., to give the proportions of sugars shown in Table I. A second portion was reduced, the alditols were acetylated, and the acetates analyzed by g.l.c.–m.s.

Partial hydrolysis of K4 polysaccharide with acid; isolation of oligosaccharides. — A sample of the acidic polysaccharide (1.0 g) in 0.01M trifluoroacetic acid (200 mL) was heated for 48 h at 100°, and the hydrolysis products separated by preparative gel-permeation chromatography on a column (112 × 4 cm) of Bio-Gel P-2, using water as the eluant. Some fractions were further purified by preparative paper-chromato-

graphy using Whatman No. 3MM paper and solvent *A*. Four components were isolated, the main characteristics of which are listed in Table II. The structures of these oligomers were determined by standard techniques, including partial hydrolysis with acid, methylation analysis, the use of p.m.r. spectroscopy, and the measurement of optical rotatory power. The proportions of neutral sugars were determined by g.l.c. of the derived alditol acetates after hydrolysis of the oligosaccharides with 2M trifluoroacetic acid for 8 h at 100°.

Compound **1** was identical in p.c. (solvents *A*, *B*, and *C*) to authentic 3-*O*-β-D-glucopyranosyl-D-glucose. Methanolysis of the fully methylated disaccharide (1 mg), followed by g.l.c. analysis, showed the methyl glycosides of 2,3,4,6-tetra-*O*-methylglucose and 2,4,6-tri-*O*-methylglucose. Methylated disaccharide (3 mg) was hydrolyzed with 2M trifluoroacetic acid for 4 h at 100°: p.c. of the hydrolyzate (solvents *B* and *D*) revealed the same methyl sugars.

Fully methylated aldobiouronic acid **2** was heated with 10% methanolic hydrogen chloride for 18 h at 100°. G.l.c analysis of the products showed the presence of methyl glycosides of methyl 2,3,4-tri-*O*-methylglucuronate and 2,4,6-tri-*O*-methylmannose. The identity of the 2,4,6-tri-*O*-methylmannose was confirmed by hydrolysis of the methylated aldobiouronic acid, and g.l.c. of the trimethylsilyl derivatives of the anomers of the tri-*O*-methyl sugar. Methylated **2** (10 mg) was treated with lithium aluminum hydride (100 mg) in oxolane (2 mL) for 18 h. After addition of aqueous ethanol, and evaporation of the mixture to dryness, the residue was extracted with chloroform (3 × 5 mL); the extracts were combined and evaporated, the residue was hydrolyzed with 2M trifluoroacetic acid for 4 h at 100°, and the resulting sugars were converted into their alditol acetates. Analysis by g.l.c. then showed products derived from 2,3,4-tri-*O*-methylglucose and 2,4,6-tri-*O*-methylmannose in approximately equal proportions. The glucose derivative was isolated by preparative g.l.c., and demethylated[15], to give glucitol, which was shown to have the D configuration by the c.d. spectrum of its hexaacetate. The p.m.r. spectrum of the aldobiouronic acid in D_2O gave anomeric signals at δ 5.22 (J 3.5 Hz), 5.12 (J 2 Hz), and 4.88 (J 2 Hz), consistent with the values given in the literature for 3-*O*-(α-D-glucopyranosyluronic acid)-D-mannose[16].

Aldotriouronic acid mixture **3** (2 mg) in 2M trifluoroacetic acid was heated for 8 h at 100°. P.c. of the hydrolyzate (solvents *A* and *C*) revealed glucose, mannose, glucuronic acid, and the aldobiouronic acid **2**. A sample of **3** (10 mg) was dissolved in water, and reduced with sodium borohydride during 16 h. Acidification with Amberlite IR-120 (H^+) resin, followed by removal of the borate by distillation with methanol, yielded a product that was hydrolyzed with 2M trifluoroacetic acid for 5 h at 100°. To the hydrolyzate was added M sodium hydroxide (3 mL), and the solution was boiled for 4 h under oxygen. After de-ionization with Amberlite IR-45 (OH^-) and IR-120 (H^+) resins, the solution was evaporated, the residue acetylated, and the acetate analyzed by g.l.c., giving a molar ratio of 1.0:1.8 for glucose and mannose. Hydrolysis of methylated **3** with 2M trifluoroacetic acid for 8 h at 100°, followed by p.c. of the hydrolyzate (solvents *B* and *D*), showed 2,3,4,6-tetra-*O*-methylglucose,

2,4,6-tri-*O*-methylmannose, and 2,4,6-tri-*O*-methylglucose. G.l.c. analysis of these sugars as the per(trimethylsilyl)ated, derived alditols gave the proportions shown in Table II. Methanolysis and g.l.c. analysis of methylated **3** (2 mg) gave the methyl glycosides of methyl 2,3,4-tri-*O*-methylglucuronate, methyl 3,4-di-*O*-methylglucuronate, 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylglucose, and 2,4,6-tri-*O*-methylmannose. The p.m.r. spectrum of **3** in D_2O showed signals in the anomeric region at δ 5.40 (J 3 Hz), 5.24 (J 3 Hz), 5.16 (J 2 Hz), 4.88 (J 2 Hz), and 4.65 (J 7 Hz), consistent with the presence of a mixture of **3a** and **3b**.

The aldotetraouronic acid **4** (5 mg) was reduced with sodium borohydride, the product hydrolyzed with 2M trifluoroacetic acid for 6 h at 100°, and the material treated with hot, M sodium hydroxide for 4 h under oxygen, to yield glucitol (identified, as its hexaacetate, by g.l.c.). Hydrolysis of **4** with 2M trifluoroacetic acid for 8 h at 100°, followed by p.c. of the hydrolyzate (solvents *A* and *C*), showed the presence of glucose, mannose, glucuronic acid, and **2**. Methylated **4** (5 mg) was hydrolyzed with 2M trifluoroacetic acid for 18 h at 100°, and the sugars released were identified, by p.c. (solvents *B* and *D*) and by g.l.c. of the per(trimethylsilyl)ated, derived alditols, as 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylglucose, and 2,4,6-tri-*O*-methylmannose. Methanolysis of methylated **4**, and g.l.c. of the products, showed the presence of the methyl glycosides of methyl 3,4-di-*O*-methylglucuronate, in addition to those of the aforementioned, neutral sugars. The p.m.r. spectrum of **4** in D_2O gave signals in the anomeric region at δ 5.40 (J 3 Hz), 5.32 (J 3 Hz), 5.22 (J 2 Hz), and 4.68 (J 7 Hz).

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