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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDE FROM *Klebsiella* TYPE 1

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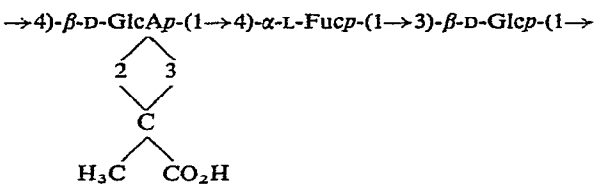
ABSTRACT

The structure of the capsular polysaccharide from *Klebsiella* type 1, which is composed of D-glucose, D-glucuronic acid, L-fucose, and pyruvic acid (1:1:1:1), has been investigated. Methylation analysis, n.m.r. spectroscopy, graded hydrolysis, and periodate-oxidation studies were the principal methods used. These studies demonstrated that the polysaccharide consists of the following trisaccharide repeating-unit:



INTRODUCTION

Preliminary studies on the structure of the capsular polysaccharide from *Klebsiella pneumoniae* type A, now described as *Klebsiella* type 1, were reported by Barker and his co-workers[1]. They found that the polysaccharide from strain 1265 was composed of D-glucose, D-glucuronic acid, and L-fucose. Methylation analysis and periodate-oxidation studies indicated that most of the sugars were linked through O-3. We now report further studies on the polysaccharide from a *K. pneumoniae* type 1 strain having the same chemical composition as that described by Barker *et al.*[1].

RESULTS AND DISCUSSION

In addition to D-glucose, D-glucuronic acid, and L-fucose, a hydrolysate of the capsular polysaccharide from *Klebsiella* type 1 (K-1) also contained pyruvic acid. The polysaccharide did not contain O-acetyl groups. The ratio of D-glucose to L-fucose in a hydrolysate of original K-1 was 1.3:1, and in a hydrolysate of carboxyl-reduced[2]

K-1, 2:1. The n.m.r. spectrum showed, *inter alia*, signals for methyl protons (δ 1.23, $J_{5,6}$ 6 Hz) from the L-fucose residues, for methyl protons (δ 1.55) from the pyruvic acid residues, and from 3 anomeric protons [δ 4.64, $J_{1,2}$ 7 Hz, δ 4.95, $J_{1,2}$ 7 Hz, and δ 5.28, $J_{1,2}$ 1 Hz] in the relative proportions 3:3:1:1:1. These figures indicate that K-1 is composed of trisaccharide repeating-units, containing one residue each of the three component sugars and one of pyruvic acid. The polysaccharide showed $[\alpha]_{578}^{22}$ −85°.

Attempted methylation of K-1 by the Hakomori procedure[3] was unsuccessful, as the polysaccharide did not dissolve completely in methyl sulfoxide. However, an acetylated sample could be methylated. The neutral sugars obtained on hydrolysis of this product were analysed, as their alditol acetates, by g.l.c.–m.s.[4] (Table I, column A). A second sample was carboxyl-reduced[2] using sodium borodeuteride, and subjected to methylation analysis (Table I, column B). The results demonstrate that the D-glucosyl residue is pyranosidic and linked through O-3, and that the L-fucosyl residue is either pyranosidic and linked through O-4, or furanosidic and linked through O-5. The result of the graded, acid hydrolysis of carboxyl-reduced K-1 indicates, however, that all sugar residues are pyranosidic.

TABLE I

METHYL ETHERS OBTAINED FROM THE HYDROLYSATE OF THE METHYLATED (*A*), REDUCED AND METHYLATED (B), REDUCED, GRADED ACID-HYDROLYSED, AND METHYLATED K-1 (*C*)

Sugars and location of methoxyl groups[a]	*T*[b]	*Mole %*		
		A	*B*	*C*
2,3,4-Fuc	0.67	5	1	2
2,3-Fuc	1.02	41	28	30
2,4,6-Glc	1.71	54	40	42
2,3,6*-Glc	1.90		3	26
6*-Glc	3.89		28	

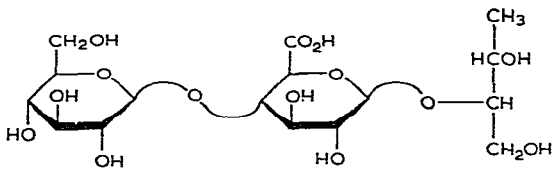
[a]The asterisk indicates the location of deuterium at C-6. [b]Retention time of the corresponding alditol acetate, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on a glass column wall-coated with SP-1000, at 220°.

The 6-*O*-methyl-D-glucose obtained in the methylation analysis of carboxyl-reduced K-1 is dideuterated at C-6. Thus, it derives from the D-glucopyranosyluronic acid residue, which consequently is substituted at O-2, O-3, and O-4. As all sugars occur as residues of the main polysaccharide chain, the pyruvic acid should be linked to two of these positions.

On acid hydrolysis of carboxyl-reduced K-1 with 5mM sulfuric acid at 80° for 30 min, the pyruvic acid residues were removed, but the glycosidic linkages were left intact. Methylation analysis of this product (Table I, column C) confirmed previous results and demonstrated that the D-glucopyranosyluronic acid residues are linked through O-4. Consequently, the pyruvic acid residue in K-1 is linked to O-2 and O-3 of these residues. Pyruvic acid linked to vicinal *trans*-positions is unusual, but has been

observed, for example attached to the 3- and 4-positions of a L-rhamnopyranosyl residue in the *Klebsiella* type 72 capsular polysaccharide[5]. The *trans*-disposition of this residue accounts for its sensitivity to acid hydrolysis.

K-1 was subjected to a Smith degradation[6], that is periodate oxidation, borohydride reduction, and acid hydrolysis under mild conditions. The main reaction product, obtained after chromatography on Sephadex G-15, was the trisaccharide **1**, according to evidence presented below. Acid hydrolysis of the product yielded D-glucose and 1-deoxythreitol. Methylation, followed by carboxyl-reduction using lithium aluminium deuteride, and acid hydrolysis, yielded 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose dideuterated at C-6, and 1-deoxy-2,4-di-*O*-methyl-L-threitol. The optical rotation of the trisaccharide, $[\alpha]_{578}$ −75°, showed that both sugars were β-linked, and this was supported by n.m.r. evidence. The relative intensities of the signals from the two anomeric protons and the methyl protons was 1:1:3, which also agreed with the proposed structure.



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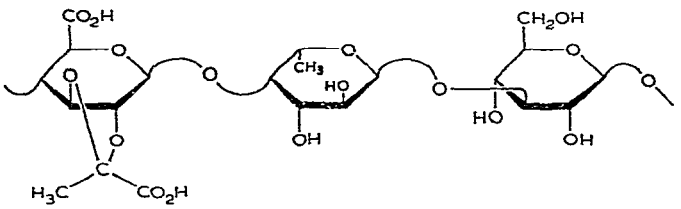
The strong, negative optical rotation of K-1 indicates that the D-glucopyranosyl and D-glucopyranosyluronic acid residues are β-linked and that the L-fucopyranosyl residue is α-linked. Confirmation of the configuration of the linkages was obtained through examination of products from partial, acid hydrolysis of the polymer. Neutral oligosaccharides were absent from partial, acid hydrolysates of K-1, but two oligosaccharides having fucose as the terminal reducing sugar were isolated in satisfactory yield. The chromatographically faster-moving fragment contained equal amounts of fucose and glucuronic acid, but no pyruvate. It was hydrolysed to D-glucuronic acid and L-fucose by β-glucuronidase. The second product was a trisaccharide containing equal amounts of the three component monosaccharides found in the polysaccharide. From it, D-glucose was removed by treatment with a β-D-glucosidase preparation; α-D-glucosidase had no effect. The partial, acid hydrolysates also contained lesser amounts of two other fragments, which were probably pyruvylated derivatives of the disaccharide and trisaccharide, respectively.

Partial, acid hydrolysis of the carboxyl-reduced K-1, in which reduction was effected with sodium borotritide, yielded three oligosaccharides. Two were disaccharides, one of which contained glucose and fucose, all the glucose being derived from glucuronic acid. It was hydrolysed by β-D-glucosidase but not α-D-glucosidase, indicating that the original polymer contained (β-D-glucosyluronic acid)fucose. The second disaccharide was indistinguishable, in all respects tested, from cellobiose.

Reduction and hydrolysis liberated radioactive glucitol and unlabelled glucose in equal quantities. Thus, the disaccharide is derived from the sequence ...β-D-Glc-(1→4)-D-GlcA... in K-1. The third fragment, obtained in lesser yield, was a trisaccharide containing glucose and fucose in a 2:1 molar ratio, with fucose as the terminal reducing sugar.

From the combined evidence presented above, it is concluded that K-1 is composed of trisaccharide repeating-units having the structure **2**. The only structural feature not determined is the configuration at the acetalic carbon atom in the pyruvic acid residue.



2

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure, at bath temperatures not exceeding 40°. G.l.c. was conducted with a Perkin–Elmer 990 instrument, equipped with a glass column containing 3% of ECNSS-M on Gas Chrom Q at 200°, for the alditol acetates, and a Hewlett-Packard 5830 A instrument with a glass capillary column (25 m × 0.25 mm) wall-coated with SP-1000, for partially methylated alditol acetates. N.m.r. spectra were recorded on a Varian XL-100 spectrometer using sodium 1,1,2,2,3,3-hexadeuterio-4,4-dimethyl-4-silapentane-1-sulfonate as reference. Optical rotations were determined on a Perkin–Elmer 141 polarimeter. Mass spectra were recorded on a Perkin–Elmer 270 and a Varian MAT-311-SS100 combined gas chromatograph–mass spectrometer fitted with columns containing OV-225.

β-D-Glucosidase (EC 3.2.1.21) and β-D-glucuronidase (EC 3.2.1.31) were purchased from British Drug Houses, Poole, England, and α-D-glucosidase (EC 3.2.1.20) from Boehringer Corporation, Mannheim, W. Germany. Oligosaccharides were incubated with sufficient enzyme to hydrolyse equimolar amounts of cellobiose, *p*-nitrophenyl β-D-glucosiduronic acid and maltose, respectively, bacterial growth being inhibited with toluene. D-Glucose released was determined with the D-glucose oxidase reagent, and L-fucose by the cysteine–sulfuric acid procedure.

Preparation of Klebsiella *K-1 polysaccharide.* — The *Klebsiella* A-1 strain used in earlier studies[7] was grown on trays of nitrogen-deficient solid media. The culture was removed, suspended in 0.9% (w/v) saline containing 1% (v/v) of formalin, and stirred in a blender for 5–10 min. The bacteria were removed by centrifugation and

the polysaccharide was recovered from the supernatant fluid by precipitation with several volumes of cold (−40°) acetone. Purification was by reprecipitation after pronase digestion. The product was dried *in vacuo*.

Methylation of K-1. — Acetic anhydride (1.5 ml) and pyridine (1.5 ml) were added to a solution of K-1 (10 mg) in formamide (10 ml). The reaction mixture was kept for 18 h at room temperature, dialysed, and freeze-dried. The product and methyl sulfoxide (4 ml) were kept in a sealed bottle at room temperature in an ultrasonic bath for 30 min. 2M Methylsulfinyl anion in methyl sulfoxide (4 ml) was added, and the mixture was agitated in an ultrasonic bath for 30 min and then kept for 18 h at room temperature. Methyl iodide (4 ml) was added with external cooling and the reaction mixture was agitated ultrasonically for 30 min, poured into water, dialysed, and concentrated to dryness. The residue was treated with 90% formic acid (2 ml) for 1 h at 100°, the solution was concentrated, and the residue was hydrolysed with 0.25M sulfuric acid (2 ml) for 16 h at 100°. The sugars were converted into their alditol acetates, and analysed by g.l.c.–m.s.[4] (Table I, column A). The identifications of the sugars, from their mass spectra, were unambiguous and will not be discussed.

Carboxyl-reduction of K-1. — K-1 (32 mg) was carboxyl-reduced, as described by Taylor and Conrad[2] using 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) as the activating agent and sodium borodeuteride as the reducing agent. The product was purified by dialysis and freeze-dried, yielding modified polysaccharide (30 mg). Part of this material (4 mg) was hydrolysed with 0.25M sulfuric acid, and the sugars were analysed by g.l.c.–m.s. as their alditol acetates (Fuc, 34%; Glc, 66%). Another part (4 mg) was methylated, hydrolysed, and analysed by g.l.c.–m.s. as described before (Table I, column B).

Graded, acid hydrolysis of carboxyl-reduced K-1. — Carboxyl-reduced K-1 (22 mg) was treated with 5mM sulfuric acid for 30 min at 80°, and the solution was dialysed and freeze-dried. Part (3 mg) of the residue (15 mg) was methylated, hydrolysed, and analysed by g.l.c.–m.s. (Table I, column C). The n.m.r. spectrum, which showed no signal at δ 1.5, demonstrating that all the pyruvic acid residues had been removed, contained, *inter alia*, four doublets at δ 1.28 (3 H, $J_{5,6}$ 6 Hz), 4.45 (1 H, $J_{1,2}$ 6 Hz), 4.50 (1 H, $J_{1,2}$ 7 Hz), and 5.29 (1 H, $J_{1,2}$ 1 Hz).

Smith degradation of K-1. — The polysaccharide (50 mg) in 0.04M sodium metaperiodate (25 ml), buffered to pH 3.9 with sodium acetate, was kept in the dark for 120 h at 4°. Excess of periodate was reduced with ethane-1,2-diol (1 ml), and the solution was dialysed overnight, reduced with sodium borohydride (300 mg), neutralized with 50% aqueous acetic acid, dialysed, and freeze-dried, yielding the polyalcohol (40 mg). Sugar analysis by g.l.c.[8]–m.s.[9] of the alditol acetates showed 1-deoxythreitol, 15%; fucose, 15%; and glucose, 71% (response on the flame-ionization detector). The polyalcohol was treated with 0.25M sulfuric acid for 60 h at 25°, and the neutralised (barium carbonate) solution was filtered and concentrated to dryness. The reaction product was fractionated on a column (2.6 × 100 cm) of Sephadex G-15 with water as irrigant. The fractionation was followed by differential refractometry, and an oligomeric product (2.3 mg) having $[\alpha]_{578}^{24}$ −75° (*c* 0.1, water)

was eluted in the di–trisaccharide region. The n.m.r. spectrum showed, *inter alia*, signals at δ 1.20 (3 H, $J_{5,6}$ 6 Hz), 4.52, (1 H, $J_{1,2}$ 7 Hz), and 4.60 (1 H, J 7 Hz). Part of this material was used for sugar analysis, showing 1-deoxythreitol and glucose. The rest of the material, in methyl sulfoxide (1 ml), was treated first with 2M sodium methylsulfinyl anion in methyl sulfoxide (1 ml) and then with methyl iodide (1 ml). The methylated product was reduced with lithium aluminium deuteride in dichloromethane–ether (1:2) and then hydrolysed, and the sugars were analysed as their alditol acetates by g.l.c.–m.s. The analysis showed 3-*O*-acetyl-1-deoxy-2,4-di-*O*-methyl-L-threitol, 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol (deuterated at C-6), and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

N.m.r. spectroscopy of K-1. — A solution of K-1 (20 mg) in D_2O (1 ml) was freeze-dried, the procedure was repeated in order to exchange all -OH for -OD, and the product was then dissolved in D_2O (0.5 ml) containing traces of the reference. The n.m.r. spectrum showed, *inter alia*, signals at δ 1.23 (d, 3 H, $J_{5,6}$ 6 Hz), 1.55 (s, 3 H), 4.64 (d, 1 H, $J_{1,2}$ 7 Hz), 4.95 (d, 1 H, J 7 Hz), and 5.28 (d, 1 H, $J_{1,2}$ 1 Hz).

Preparation of oligosaccharides by partial, acid hydrolysis of K-1. — The polysaccharide was converted into the acid form by passage through an Amberlite IR-120 (H^+) resin. A 1% aqueous solution of the acid polysaccharide was allowed to autohydrolyse in a sealed tube at 100° for 16 h. Tritiated, carboxyl-reduced polymer was prepared from the acid form as described earlier[10]. The product was hydrolysed with 0.5M H_2SO_4 at 100° for 30 min, and the acid was neutralised with aqueous $Ba(OH)_2$. Oligosaccharides were separated by the preparative paper electrophoresis and chromatography systems listed[10].

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