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STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE K58

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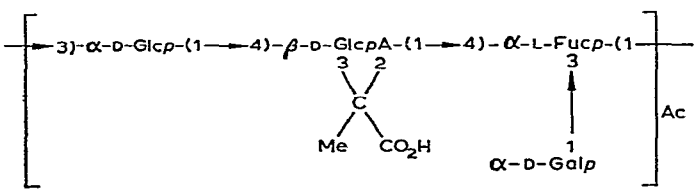
ABSTRACT

Klebsiella K58 capsular polysaccharide has been investigated by the techniques of methylation, Smith degradation–periodate oxidation, uronic acid degradation, and partial hydrolysis, in conjunction with ^{1}H-n.m.r. spectroscopy at 100 and 220 MHz, and ^{13}C-n.m.r. spectroscopy at 20 MHz. The structure has been found to consist of the tetrasaccharide repeating-unit shown, with one *O*-acetyl group per repeating unit. A uronic acid residue bearing a 1-carboxyethylidene group has previously been found, in this series, only in the polysaccharide from *Klebsiella* K1.



INTRODUCTION

The genus *Klebsiella* has been classified by Ørskov[1] into approximately 80 serotypes, based on their antigenic, capsular polysaccharides. Nimmich[2,3] qualitatively analyzed the polysaccharide from each strain and found that K58 contains glucose, galactose, fucose, glucuronic acid, and pyruvic acid. In addition, K58 was shown to contain one *O*-acetyl group per repeating unit. As part of our continuing investigation of the relationship between primary, chemical structure and immunological activity, we now report on the elucidation of the structure of the K58 polysaccharide.

RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — *Klebsiella* K58 bacteria were grown on an agar medium, and the capsular polysaccharide isolated was purified by one precipitation with Cetavlon. The product had $[\alpha]_D$ +19.0°.

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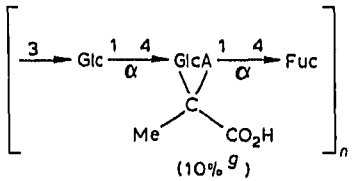
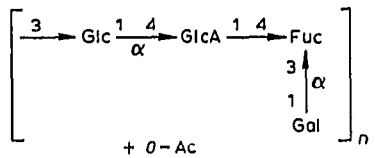
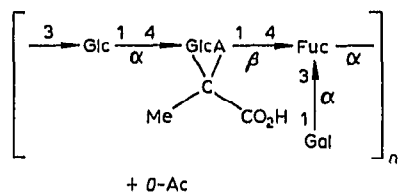
TABLE I

N.M.R. DATA FOR *Klebsiella* K58 CAPSULAR POLYSACCHARIDE AND THE DERIVED OLIGOSACCHARIDES

Compound[a]	^{1}*H-N.m.r. data*				^{13}*C-N.m.r. data*	
	δ[b]	$J_{1,2}$ *(Hz)*[c]	*Integral*	*Assignment*[d]	*(p.p.m.*[e]*)*	*Assignment*[f]
1 GlcA $\xrightarrow[\beta]{1\ \ 4}$ Fuc∼OH	5.26	3	0.5	α-Fuc∼OH	103.91	β-GlcA
	4.59	10	1.7	β-Fuc∼OH	97.07	β-Fuc∼OH
	4.54	6.5		β-GlcA	93.16	α-Fuc∼OH
	1.33	6.0	3	CH_3 of α-Fuc∼OH	23.7	CH_3 of 1-carboxyethylidene
	1.29	6.5		CH_3 of β-Fuc∼OH	16.3	CH_3 of fucose
	1.64	s	0.3	CH_3 of 1-carboxyethylidene		
2 Glc $\xrightarrow[\alpha]{1\ \ 3}$ GlcA $\xrightarrow[\beta]{1\ \ 4}$ Fuc∼OH	5.46	3.5	1	α-Glc	103.94	β-GlcA
	5.26	3	0.6	α-Fuc∼OH	99.5	α-Glc
	4.61	7	1.8	β-Fuc∼OH	97.16	β-Fuc∼OH
	4.57	7		β-GlcA	93.3	α-Fuc∼OH
	1.64	s	0.4	CH_3 of 1-carboxyethylidene	61.2	C-6 of Glc
	1.33	6	3	CH_3 of α-Fuc∼OH	23.7	CH_3 of 1-carboxyethylidene
	1.29	6		CH_3 of β-Fuc∼OH	16.25	CH_3 of fucose
3 Glc $\xrightarrow[\alpha]{1\ \ 3}$ GlcA $\xrightarrow[\beta]{1\ \ 4}$ Fuc∼OH, Gal $\xrightarrow[1]{3}\alpha$ Fuc∼OH	5.45	3.5	0.9	α-Glc	104.0	β-GlcA
	5.29	3	1.6	α-Fuc∼OH	99.7	α-Gal
				α-Gal	99.5	α-Glc
	4.60	8	1.7	β-Fuc∼OH	97.2	β-Fuc∼OH
	4.55	7		β-GlcA	93.3	α-Fuc∼OH
	1.64	s	0.4	CH_3 of 1-carboxyethylidene	61.07	C-6 of Glc
					62.59	C-6 of Gal
	1.33	6.5	3	CH_3 of α-Fuc∼OH	23.7	CH_3 of 1-carboxyethylidene
	1.29	6.5		CH_3 of β-Fuc∼OH	16.25	CH_3 of fucose

4	 (10%[g])	5.44	2	1	α-Glc	104.0	β-GlcA
		5.27	b	1	α-Fuc	100.3	α-Fuc
		4.57	8	1	β-GlcA	99.7	α-Glc
		1.64	s	1	CH_3 of 1-carboxyethylidene	61.3	C-6 of Glc
		1.33	6	3	CH_3 of fucose	23.5	CH_3 of 1-carboxyethylidene
						16.1	CH_3 of fucose
5		5.35	s	1	α-Glc		
		5.25	s	0.9	α-Fuc		
		5.16	s	1	α-Gal		
		4.63	8	1	β-GlcA		
		2.17	s	2	CH_3 of acetate		
		1.33	6	3	CH_3 of fucose		
6		5.45	2	1	α-Glc	104.5	β-GlcA[h]
		5.18 }	b	2	α-Fuc	101.2	α-Fuc
		5.13 }			α-Gal	99.5	α-Glc
		4.59	8	1	β-GlcA	97.5	α-Gal
		2.17	s	3	CH_3 of acetate	62.5	C-6 of Gal
		1.64	s	3	CH_3 of 1-carboxyethylidene	61.4	C-6 of Glc
		1.33	6	3	CH_3 of fucose	29.98	CH_3 of acetate
						23.4	CH_3 of 1-carboxyethylidene
						16.0	CH_3 of fucose

[a]For origin of compounds **1–5**, see text. [b]Chemical shift relative to internal acetone; δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.). [c]Key: b = broad, unable to assign accurate coupling-constant; s = singlet. [d]For example, α-Gal = proton on C-1 of α-linked D-Gal residue. [e]Chemical shift in p.p.m. downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. downfield from D.S.S. [f]As for footnote *d*, but for anomeric, ^{13}C nuclei. [g]The 10% of 1-carboxyethylidene remaining after Smith hydrolysis. [h]Spectrum recorded with a Brucker WP-80 instrument (20.1 MHz), using a 5-mm tube having a semi-micro volume, cylindrical cavity (6 × 4.2 mm).

Paper chromatography of an acid hydrolyzate of the polysaccharide showed the presence of glucose, galactose, fucose, and glucuronic acid. Carboxyl-reduced, K58 polysaccharide was hydrolyzed, and the presence of glucose, galactose, and fucose in the ratios of 2:1:1 was determined by gas–liquid chromatography (g.l.c.) of their alditol acetates. Fucose was shown to be of the L configuration, and glucose of the D configuration, by circular dichroism (c.d.) measurements on the derived alditol acetates[4]. Galactose was shown to be of the D configuration by the positive reaction of D-galactostat reagent with an acid hydrolyzate of the polysaccharide.

The 220-MHz, ^{1}H-n.m.r. spectrum of the polysaccharide showed sharp singlets at δ 2.17 and 1.64, and a doublet at δ 1.33, in the approximate ratios of 1:1:1. These were assigned to methyl groups of *O*-acetyl, 1-carboxyethylidene, and fucose, respectively[5–7]. Four discernible signals were observed in the anomeric region, at δ 5.45 (1 H, $J_{1,2}$ 2 Hz), 5.18–5.13 (2 H, broad), and 4.59 (1 H, $J_{1,2}$ 8 Hz). The ^{13}C-n.m.r. spectrum of the polysaccharide[8,9] showed signals of equal intensity at 16.0 (fucose CH_3), 23.4 (1-carboxyethylidene CH_3), and 29.98 p.p.m. (acetate CH_3). In the anomeric region, four signals in the ratios of 1:1:1:1 at 104.5, 101.2, 99.5, and 97.5 p.p.m. were observed (see Table I).

The signals at 61.4 and 62.5 p.p.m. were assigned to the C-6 atoms of hexoses. Both the ^{1}H- and ^{13}C-n.m.r. data indicate the presence of one β-linked and three α-linked residues. Assignments were made after n.m.r.-spectral investigation of oligosaccharides isolated after partial hydrolysis and periodate oxidation (see later).

Methylation of original polysaccharide. — Methylation[10,11] of K58 polysaccharide, followed by reduction of the uronic ester, hydrolysis, derivatization as the alditol acetates, and g.l.c.–m.s. analysis[12,13] indicated that K58 polysaccharide is composed of tetrasaccharide repeating-units. The sugar residues are in the pyranoid form, with fucose constituting a branch point (see Table II). Analysis of a re-methylated sample of the reduced product showed the formation of 6-*O*-methylglucose, thus establishing that the uronic acid is glucuronic acid. Mild hydrolysis of this re-methylated polysaccharide (to remove the 1-carboxyethylidene group), followed by methylation, showed, on g.l.c.–m.s. analysis, the formation of 2,3,6-tri-*O*-methylglucose, indicating that the 1-carboxyethylidene group is linked at O-2 and O-3 of the glucuronic acid residue; this was confirmed by methylation analysis of a sample of autohydrolyzed, native polysaccharide (see later).

Base-catalyzed degradation[14]. — To determine the location of the uronic acid, the methylated polysaccharide was subjected to base-catalyzed degradation, and the product was then directly ethylated[15]. The isolation of an oligosaccharide indicated that the uronic acid is in the backbone. On hydrolysis, and derivatization for g.l.c.–m.s., the compounds shown in Table II were obtained, indicating that the glucuronic acid is attached to O-4 of fucose. Loss of some glucose suggested that it is linked to the glucuronic acid in the backbone.

Periodate oxidation. — The native polysaccharide consumed 1.8 mol of periodate per mol of repeating unit[16] in 10 h, yielding, after reduction with sodium borohydride, and Smith hydrolysis[17], a polymeric product (**4**). On reduction of the uronic acid,

TABLE II

METHYLATION ANALYSIS OF NATIVE, AND DEGRADED, *Klebsiella K58* CAPSULAR POLYSACCHARIDE

Methylated sugars[a] *(as alditol acetates)*	T[b]		*Mole %*[c]					
	Column A[d] *(OV-225)*	*Column B*[e] *(ECNSS-M)*	*I*[f]	*II*	*III*	*IV*	*V*	*VI*
2,3,4-Fuc	0.80	—				} 4		
2,3-Fuc	0.82	0.92					30	
4-*O*-Et,2-Fuc	—	0.9						38
2,4-Fuc	0.89	—	2	2	2	2		
2,3,4,6-Gal	1.00	1.00	28	28	29	23		43
2-Fuc	1.14	1.23	22	23	25	19	4	
2,4,6-Glc	1.31	1.45	25	25	25	26	36	
2,3,6-Glc	1.39	—		3	19			
2,3-Glc	2.05	—	3			21		
6-Glc	2.17	—		19				
Glc	2.78	3.4	20			5	30	19

[a]2,3,4-Fuc = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-fucitol, etc. [b]Retention time relative to that of the alditol acetate derivative of 2,3,4,6-Gal. [c]Values corrected by using effective carbon-response factors[22], and adjusting to the nearest integer. [d]Program: 180° for 4 min, and then 2° per min to 200°. [e]Program: 160° for 4 min, and then 4° per min to 200°. [f]I, original polysaccharide, methylated, and uronic ester reduced; II, as in I, but re-methylated; III, as in II, and then hydrolyzed to remove 1-carboxyethylidene group, and re-methylated; IV, autohydrolyzed polysaccharide, methylated, and uronic ester reduced; V, Smith-degradation product, methylated, and uronic ester reduced; VI, product of uronic acid degradation, and ethylation.

followed by total hydrolysis, and derivatization, g.l.c. showed the presence of glucose and fucose in the ratio of 2 : 1. N.m.r. spectroscopy (^1H and ^{13}C) of the Smith-degradation product **4** showed the presence of one β-linked and two α-linked sugars, indicating that the oxidized (terminal) galactosyl group is α-linked (see Table I).

Methylation of a portion of the Smith-degradation product, followed by reduction of the uronic ester, and derivatization for g.l.c.–m.s., gave the results shown in Table II, indicating that the side-chain galactose is linked to O-3 of fucose.

Autohydrolysis. — A sample of K58 polysaccharide in the free-acid form was autohydrolyzed for 3 h at 95° and then dialyzed. ^1H-N.m.r. spectroscopy of the product (**5**) showed the absence of 1-carboxyethylidene groups. Methylation of **5**, followed by reduction of the uronic ester and derivatization for g.l.c.–m.s., gave the results shown in Table II, indicating that the 1-carboxyethylidene group is attached to O-2 and O-3 of the glucuronic acid residue.

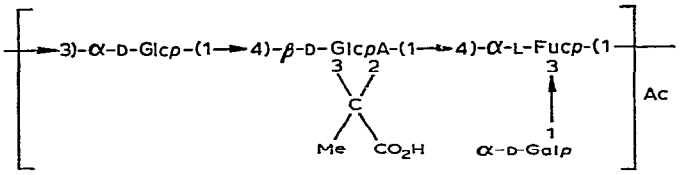
Partial hydrolysis. — A sample of K58 polysaccharide was hydrolyzed with 0.5M H_2SO_4 for 30 min at 95°, yielding a mixture of oligosaccharides. Preparative paper-chromatography gave an aldobiouronic acid (**1**), an aldotriouronic acid (**2**), and an aldotetraouronic acid (**3**). The ^1H- and ^{13}C-n.m.r. data (see Table I) indicated that the glucuronic acid is β-linked, and the glucose is α-linked, and confirmed

that the side-chain galactose is α-linked. Comparison of the spectral data for **2** and **4** indicated that the fucose is α-linked.

Location of the O*-acetyl group.* — The ^{1}H-n.m.r. spectrum of the native polysaccharide showed a sharp acetate peak at δ 2.17 suggesting that the group appears in a discrete position in each repeating unit. In order to locate the *O*-acetyl group, K58 polysaccharide was treated with methyl vinyl ether in the presence of an acid catalyst, and the product was subjected to methylation analysis[18]. However, many methylated sugars appeared in the g.l.c.–mass spectrum, and comparison of data from analysis of the native polysaccharide and of a deacetylated sample did not give interpretable results.

CONCLUSION

It thus follows that the repeating unit of the *Klebsiella* K58 capsular polysaccharide has the following structure.



In this series, a uronic acid residue bearing a 1-carboxyethylidene group has previously been found[19] only in the polysaccharide from *Klebsiella* K1.

EXPERIMENTAL

General methods. — Evaporations were conducted under diminished pressure at bath temperatures not exceeding 40°. The equipment for m.s., n.m.r. spectroscopy, g.l.c., and g.l.c.–m.s. was the same as that used in the investigation of *Klebsiella* K23 polysaccharide[20]. Paper electrophoresis was performed in a Savant high-voltage (5 kV) system (model LT-48A), with kerosene as the coolant; the buffer used was 5:2:743 (v/v) pyridine–acetic acid–water, pH 5.3, and strips (77 × 20 cm) of Whatman No. 1 paper were used for all of these experiments, with application of 25–50 mA for 1.5 h. For descending paper-chromatography, the following solvent-systems (v/v) were used: (*1*) freshly prepared 2:1:1 1-butanol–acetic acid–water, (*2*) 8:2:1 ethyl acetate–pyridine–water, and (*3*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water. Sugars and oligosaccharides were detected with an alkaline silver nitrate reagent[21]. Analytical g.l.c. separations were performed in stainless-steel columns (1.8 m × 3 mm), with a carrier-gas flow-rate of 20 mL/min. Columns used were (*A*) 3% of OV-225 on Gas-Chrom Q (100–120 mesh); (*B*) 5% of ECNSS-M on the same support; and (*C*) 3% of SP-2340 on Supelcoport (100–120 mesh). Analogous columns (1.8 m × 6.3 mm) were used for preparative-g.l.c. separations.

Preparation and properties. — A culture of *Klebsiella* K58 (636/52) was obtained from Dr. I. Ørskov (Copenhagen). The polysaccharide, isolated as previously described[20], showed $[\alpha]_D$ +19.0° (*c* 1, water).

Analysis of constituent sugars. — Methanolysis of a sample (20 mg) of K58 polysaccharide was conducted with 3% methanolic hydrogen chloride, and subsequent reduction with sodium borohydride in anhydrous methanol reduced the uronic ester. Hydrolysis with 2M trifluoroacetic acid overnight at 95°, followed by reduction ($NaBH_4$), and acetylation, gave galactitol hexaacetate, glucitol hexaacetate, and fucitol pentaacetate in the ratios of 1:2:1 (column *C*, programmed at 180° for 8 min, and then at 4°/min to 240°). Circular dichroism (c.d.) of the last two components, isolated by preparative g.l.c., showed positive and negative c.d. curves, respectively, confirming that the glucose has the D configuration, and the fucose, the L configuration. Galactose was shown to be of the D configuration by the positive action of D-galactostat (Worthington Biochemical Co.) on the hydrolysis product of the polysaccharide.

Methylation analyses. — Methylation of K58 polysaccharide under the Hakomori[10] conditions, followed by a Purdie[11] treatment, yielded a product that showed no hydroxyl absorption in its i.r. spectrum. This material was reduced overnight with sodium borohydride in 1:1 (v/v) oxolane–ethanol. A portion of the product was hydrolyzed with 2M trifluoroacetic acid for 16 h at 95°, and the mixture was reduced with sodium borohydride, and the product acetylated. G.l.c.–m.s. gave the results shown in Table II.

Another portion of the reduced, uronic ester material was remethylated under the Purdie conditions for 2 days, and derivatized for g.l.c.–m.s., giving the compounds shown in Table II.

A portion (10 mg) of the remethylated material was hydrolyzed with 90% formic acid for 30 min at 95° to remove the 1-carboxyethylidene group. Methylation under the Purdie conditions for 2 days, and derivatization, gave the g.l.c.–m.s. results shown in Table II.

Uronic acid degradation[14]. — A solution of carefully dried, methylated polysaccharide (100 mg) and *p*-toluenesulfonic acid (a trace) in 19:1 dimethyl sulfoxide–2,2-dimethoxypropane (20 mL) was prepared in a serum vial which was flushed with nitrogen and sealed with a rubber cap, and the solution was stirred for 3 h. Sodium methylsulfinylmethanide (2M) in dimethyl sulfoxide (10 mL) was then added with the aid of a syringe, and the solution was stirred overnight at room temperature. After cooling to 10°, ethyl iodide (3 mL) was added slowly, using a syringe[15].

Following addition of water, the ethylated, degraded product was isolated by partition between chloroform and the aqueous solution. Hydrolysis of the product isolated was performed with 2M trifluoroacetic acid, and g.l.c.–m.s. analysis of the alditol acetate derivatives yielded peaks corresponding to 4-*O*-ethyl-2-*O*-methylfucose, 2,3,4,6-tetra-*O*-methylgalactose, and 2,4,6-tri-*O*-methylglucose (see Table II).

Periodate oxidation. — *Klebsiella* K58 capsular polysaccharide (200 mg) was dissolved in water (25 mL), to which a solution (25 mL) of 0.1M sodium metaperiodate

was then added. The solution was stirred in the dark at 3°, and the periodate consumption was monitored by the Fleury–Lange method[16]; after 10 h, consumption had reached 1.8 molecules per repeating unit. Ethylene glycol (10 mL) was then added, and, after being stirred for a further 30 min, the mixture was dialyzed overnight against running tap-water, and the product was reduced with sodium borohydride. The polyol was isolated by dialysis and lyophilization. Smith hydrolysis overnight at room temperature with 0.5M trifluoroacetic acid gave a polymeric product. A portion (5 mg) of this material was hydrolyzed with 2M trifluoroacetic acid overnight at 95°; paper chromatography (solvent *2*) then showed the presence of glucose and fucose, and the absence of galactose. Analysis of the constituent sugars of the Smith-degradation product was performed as for the native polysaccharide. G.l.c. analysis showed the presence of glucose and fucose in the ratio of 2:1. The ^{1}H- and ^{13}C-n.m.r.-spectral data are given in Table I.

Methylation analysis, as for the native polysaccharide, of a portion (20 mg) of the Smith product gave the results shown in Table II.

Autohydrolysis. — A sample of *Klebsiella* K58 polysaccharide was changed to the free-acid form with Amberlite 1R-120 (H^+) ion-exchange resin, and the solution was lyophilized. A solution of a portion (100 mg) of the material in water (5 mL) had pH 3.0; it was introduced into a length of standard, cellulose, dialysis tubing which had been sealed at one end, mixed with water (100 mL), and autohydrolyzed for 3 h at 95° in the sealed tube. Loss of the 1-carboxyethylidene group was found by ^{1}H-n.m.r. spectroscopy to be complete. Methylation analysis of the product, as described earlier, gave the results shown in Table III.

Partial hydrolysis. — A sample (300 mg) of the native polysaccharide was hydrolyzed with 0.5M sulfuric acid for 30 min at 95°, and the products were separated by preparative paper-chromatography (solvent *3*). Compound **1** (35 mg), $[\alpha]_D$ −20.0° (*c* 1, water), R_{Glc} 0.63, was shown to be the aldobiouronic acid by ^{1}H- and ^{13}C-n.m.r. spectroscopy. Compound **2** (30 mg), $[\alpha]_D$ +16.0° (*c* 1, water), R_{Glc} 0.32, and compound **3** (10 mg), $[\alpha]_D$ +38.0° (*c* 0.5, water), R_{Glc} 0.12, were similarly shown to be the aldotriouronic and the aldotetraouronic acid, respectively (see Table I).

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