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STUDIES OF THE PRIMARY STRUCTURE OF THE CAPSULAR POLYSACCHARIDE FROM *Klebsiella* SEROTYPE K15

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ABSTRACT

The capsular polysaccharide of *Klebsiella* serotype K15 has been investigated mainly by methylation analysis, characterisation of the oligosaccharides obtained by partial acid hydrolysis, periodate oxidation, enzymic degradation, and ^{1}H- and ^{13}C-n.m.r. spectroscopy, and shown to have the hexasaccharide repeating-unit **1**. The glycan does not contain any pyruvic acetal or *O*-acetyl substituents.

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                β-D-Glcp-(1→4)-α-D-GlcpA
                                     1
                                     ↓
                                     2
→4)-β-D-Galp-(1→3)-β-D-Galp-(1→4)-β-D-Galp-(1→6)-β-D-Galp-(1→
```

1

INTRODUCTION

Serotype K15 belongs to one of the eighty-one serologically classified strains[1–3] of *Klebsiella* and to one of the 20 chemotypes, which also includes K8, K25, K27, and K78, the structures of which have been published[4–7]. We now report on the structure of the capsular polysaccharide of K15.

RESULTS AND DISCUSSION

The dry bacteria, isolated from the *Klebsiella* K15 strain, yielded 3.8% of capsular polysaccharide. The native polysaccharide was used for the spectral analysis, and the alkali-treated[8] material for methylation and other experiments.

Quantitative analysis[9,10] of the constituent sugars in the native polymer revealed (Table I) D-glucose, D-galactose, and D-glucuronic acid in the molar ratios ~1:4:1. The carboxyl-reduced[11] product yielded only D-glucose and D-galactose

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TABLE I

SUGAR COMPOSITION OF K15 CAPSULAR POLYSACCHARIDE

Polysaccharide	*Molar ratios of constituent sugars*[a]			*Equiv. wt.*[b]
	D-*Glc*	D-*Gal*	D-*GlcA*	
Native	1.00	3.60	1.10	1012 ±10
Alkali-treated[c]	1.00	3.80	1.05	1005 ±10
Carboxyl-reduced[d]	2.00	3.80	0.20	n.d.

[a]Hexoses determined by g.l.c. of the alditol acetates[9] and hexuronic acid by the carbazole–sulphuric acid method[10]. [b]Determined by conductometric titration of the acidic polysaccharide with 0.1M NaOH. [c]Polysaccharide was treated[8] for 4 h at 56° with 0.25M NaOH. [d]Method of Taylor and Conrad[11].

(1:2), the additional proportion of glucose being derived from glucuronic acid. The equivalent weight (1012 ±10) of the polymer accorded with the results of sugar composition. The optical rotations of the sugars isolated from the hydrolysate indicated that they were D.

There were six signals (Table II) in the anomeric region of the ^{13}C-n.m.r. spectrum of the K15 polysaccharide at 99.9, 104.0, 104.4, 105.1, 105.3, and 105.6 p.p.m. The signal at 176.5 p.p.m. was attributed to C-6 of the D-glucuronic acid

TABLE II

N.M.R. DATA FOR K15 CAPSULAR POLYSACCHARIDE AND THE OLIGOSACCHARIDES DERIVED THEREFROM

Compound[a]	*^{1}H Data*			*^{13}C Data*	
	δ[b] *(J in Hz)*	*Intensity*	*Assignment*	*P.p.m.*[c]	*Assignment*
α-GlcA-(1→2)-Gal-OH (I)	5.22 (3.0)	1.6	α-GlcA, α-Gal-OH		
	4.88 (7.0)	0.4	β-Gal-OH		
α-GlcA-(1→2)-β-Gal-(1→4)- -β-Gal-(1→6)-Gal-OH (II)	5.27 (3.0)	1.0	α-GlcA		
	5.18 (3.0)	0.5	α-Gal		
	4.75 (7.0)	1.0	β-Gal		
	4.65, 4.59	1.5	β-Gal, β-Gal		
β-Glc-(1→4)-α-GlcA-(1→2)→β-Gal- →4)-β-Gal-(1→3)→β-Gal- -(1→4)-β-Gal-(1→6)-β-Gal-(1→ (III)	5.06 (3.0)	1.0	α-GlcA	176.5	C-6 of GlcA
	4.88 (7.0)	1.0	β-Glc	105.6	β-Glc
	4.74 (7.5)	1.0	β-Gal	105.3	β-Gal
	4.71 (7.5)	1.0	β-Gal	105.1	β-Gal
	4.69 (7.0)	1.0	β-Gal	104.4	β-Gal
	4.52 (7.0)	1.0	β-Gal	104.0	β-Gal
				99.9	α-GlcA
				62.2, 61.6	C-6 of Glc and Gal

[a]I, aldobiouronic acid (H2); II, aldotetraouronic acid (H4); III, type-15 polysaccharide. [b]Chemical shift relative to that of acetone (δ 2.22). [c]Chemical shift relative to that of acetone (31.07 p.p.m.).

residue[12] and those at 61.6 and 62.2 p.p.m. were assigned[13] to C-6 of the hexose residues. The ^{1}H-n.m.r. spectrum contained signals at δ 5.06, 4.88, 4.74, 4.71, 4.69, and 4.52 for six anomeric protons (Table II). The absence of signals at δ 1.5 and 2.2 indicated that pyruvic acetal[14] and *O*-acetyl[15] groups were absent from the polysaccharide.

Methylation analysis[16,17] of the K15 polysaccharide (Table III) yielded 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol, and 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylgalactitol in the molar ratios ~1:2:1:1. When the glucuronic acid in the methylated product was reduced–dideuterated[18] before hydrolysis, an additional 1 mol. equiv. of 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylglucitol (*m/z* 263; *cf.* *m/z* 261 for the undeuterated compound) was obtained.

The data in Tables I–III indicated that the K15 polysaccharide consists of hexasaccharide repeating-units made up of D-glucose, 4-substituted D-glucuronic acid, 6-substituted D-galactose, 2,3-disubstituted D-galactose, and two 4-substituted D-galactose residues.

The oligosaccharides obtained by partial acid hydrolysis[20] are listed in Table IV. Aldobio- (H2), aldotrio- (H3), and aldotetrao-uronic acid (H4) were isolated in considerable amounts. The oligosaccharides were subjected to methylation analysis[16,17]. When the methylated aldotetraouronic acid was carboxyl-reduced with calcium borodeuteride[18] before hydrolysis, an additional peak for 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl (6,6-$^2H_2$)glucitol was obtained (Table III, column VI) and identified by the mass-spectral peaks at *m/z* 191 and 235 (*cf.* *m/z* 189 and 233 for the undeuterated compound).

TABLE III

METHYLATION ANALYSIS DATA FOR NATIVE AND DEGRADED K15 CAPSULAR POLYSACCHARIDE

Methylated sugars[a] *(as alditol acetates)*	*Molar ratios*[b]						
	I[c]	*II*	*III*	*IV*	*V*	*VI*	*VII*
2,3,4,6-Glc	1.00	1.00	—	—	—	—	0.30
2,3,6-Gal	2.10	1.90	—	0.80	1.00	1.10	2.00
2,3,4-Gal	0.90	1.00	—	—	1.10	1.00	1.00
2,3,4-Glc[d]	—	—	—	—	—	0.80	—
3,4,6-Gal	—	—	1.00	1.00	1.00	1.00	—
4,6-Gal	0.80	0.80	—	—	—	—	0.80
2,3-Glc[d]	—	0.80	—	—	—	—	—

[a]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, *etc.* [b]Determined from peak areas in g.l.c.[17] at 170°, identified by retention time realtive to those of 2,3,4,6-Glc (*T* 1.00) and 2,3-Glc (*T* 5.39). [c]I, Methylated type-15 polysaccharide; II, methylated and borodeuteride-reduced type-15 polysaccharide; III, methylated aldobiouronic acid (H2); IV, methylated aldotriouronic acid (H3); V, methylated aldotetraouronic acid (H4); VI, methylated and carboxyl-reduced [$Ca(BD_4)_2$] aldotetraouronic acid (H4); VII, base-degraded[19] type-15 polysaccharide. [d]Dideuterated product, identified from the mass-spectral data.

TABLE IV

OLIGOSACCHARIDES OBTAINED BY PARTIAL ACID HYDROLYSIS OF K15 CAPSULAR POLYSACCHARIDE

	Oligosaccharides[a]		
	H2	*H3*	*H4*
Yield (%)	8.4	5.2	18.3
Approximate molar ratio[b] of D-Gal and D-GlcA	0.75:1	1.8:1	2.6:1
Reducing-end sugar[c]	Gal	Gal	Gal
Ratio of reducing/non-reducing hexoses[d]	—	1:0.9	1:1.7
M_{GlcA} (paper electrophoresis[e])	0.66	0.46	0.36

[a]Obtained by partial acid hydrolysis of the polysaccharide with 0.5M H_2SO_4 at 100°. H2 (aldobiouronic acid), 90 min; H3 (aldotriouronic acid), 60 min; H4 (aldotetraouronic acid), 45 min. [b]Gal determined by g.l.c. of the alditol acetate[9] and GlcA by the carbazole–H_2SO_4 method[10]. [c]Identified[22] by g.l.c. of the alditol acetate after reduction with $NaBH_4$, hydrolysis, and conversion of the other constituents into acetylated aldononitriles. [d]Ratio of acetylated alditol/aldononitriles (GlcA derivative was not recorded in g.l.c.). [e]At pH 5.3 in pyridine–glacial acetic acid–water (10:4:86)[20].

From the results in Tables III and IV, the oligosaccharides H2, H3, and H4 were identified as D-GlcA-(1→2)-D-Gal, D-GlcA-(1→2)-D-Gal-(1→4)-D-Gal, and D-GlcA-(1→2)-D-Gal-(1→4)-D-Gal-(1→6)-D-Gal, respectively.

From the structure of H4 and the methylation analysis data (Table III), it can be deduced that the D-Gal linked to D-GlcA forms the branch point. However, there are several alternative positions for the fourth D-Gal in the repeating unit.

The results of base-catalysed degradation[19] of the methylated polymer are shown in Table III (column VII). After β-elimination, only the yield of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol was considerably diminished. Thus, D-Glc is linked to position 4 of D-GlcA and, since D-Glc occupies the terminal position, D-Glc-(1→4)-D-GlcA is the side chain. The structures of H2–H4 indicate that D-GlcA of the branch is linked directly to position 2 of D-Gal. Thus, the side chain in the hexasaccharide repeating-unit (**1**) consists of D-Glc-(1→4)-D-GlcA-(1→, and the chain comprises →3)-D-Gal-(1→4)-D-Gal-(1→6)-D-Gal-(1→4)-D-Gal-(1→, the 3-substituted D-Gal carrying the side chain at position 2.

The anomeric configurations of the glycosidic linkages can be assigned on the basis of the ^{1}H- and ^{13}C-n.m.r. data (Table II). The ^{1}H-n.m.r. spectrum of the polymer revealed six anomeric protons, of which only one (δ 5.06, J 3 Hz) indicated an α linkage and which was assigned to D-GlcA since the aldobiouronic acid (H2) had a similar signal for H-1 (δ 5.22, J 3 Hz). Hence, the signal at 99.9 p.p.m. in the ^{13}C-n.m.r. spectrum of the polymer was assigned to C-1 of α-D-GlcA. That the D-GlcA was α was further supported by the fact that no glucuronic acid was liberated when the aldobiouronic acid was treated with β-D-glucuronidase[21].

The structure of the capsular polysaccharide from *Klebsiella* serotype K15 is represented by the hexasaccharide repeating-unit **1**.

EXPERIMENTAL

Isolation of polysaccharide. — A culture of *Klebsiella* serotype K15, obtained from Dr. I. Ørskov (WHO International *Escherichia* Center, Copenhagen), was grown in nutrient agar medium in $D_{1.5}$ agar plates for 48 h at 37°, and then for another 48 h at room temperature. The capsular polysaccharide was isolated from the dry bacteria by the phenol–water–Cetavlon method[20]. From 100 agar plates, 22.5 g of dry bacteria were obtained, from which 0.85 g (3.8%) of capsular polysaccharide was isolated.

The native polysaccharide was mildly treated with alkali[8]. Carboxyl-reduction was effected by the method of Taylor and Conrad[11]. The equiv. wt. of the polysaccharide was determined by conductometric titration.

Sugar analysis. — The polysaccharide (1%) was hydrolysed with 0.5M H_2SO_4 (20 h, 100°), and the monosaccharides were identified by p.c. (Whatman No. 1 paper), using *A*, ethyl acetate–pyridine–water (4:1:1); and *B*, ethyl acetate–glacial acetic acid–formic acid–water (18:3:1:4).

Sugars were quantified by g.l.c. of the alditol acetates[9], using a Chemito Gas Chromatograph-3800 fitted with an ECNSS-M column. Uronic acid was determined colorimetrically[10] in the unhydrolysed product.

Methylation analysis. — Methylation was carried out by the Hakomori[16] method according to Hellerqvist *et al.*[17]. A portion of the methylated product was carboxyl-reduced–dideuterated[18] before hydrolysis. The hydrolysed products were analysed by g.l.c. and mass spectrometry. Mass spectrometry was performed with a Finningan combined g.l.c.–m.s. instrument.

Uronic acid degradation. — The methylated product was degraded[19] by using methylsulphinylmethanide, the product was isolated by partition between chloroform and water and then hydrolysed, and the derived alditol acetates were analysed by g.l.c. and m.s.

Isolation of oligosaccharides. — The polysaccharide was partially hydrolysed with 0.5M H_2SO_4 at 100° (90 min for H2, 60 min for H3, and 45 min for H4), and the oligosaccharides were isolated by high-voltage electrophoresis[20] and analysed by the usual methods.

The reducing end-group of the oligosaccharides was determined by the method of Morrison[22]. The derived acetylated alditol (from the reducing end-group) and the monomers of acetylated aldononitriles were analysed by g.l.c. on OV-17.

N.m.r. spectroscopy. — ^{1}H- (90 MHz) and ^{13}C-n.m.r. (75.47 MHz) spectra for solutions in D_2O were obtained at 70° with a Bruker-HFX spectrometer (standards: ^{13}C, sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate; ^{1}H, acetone).

Enzymic degradation[21]. — A solution of the aldobiouronic acid (1 mg) in phosphate buffer (pH 6.8, 2 mL) was incubated with β-D-glucuronidase (1 μL) from *Escherichia coli* (10^5 U, Sigma) at 37° for 24 h. No glucuronic acid was liberated.

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REFERENCES

1 W. NIMMICH, *Z. Allg. Mikrobiol.*, 15 (1975) 127–129.
2 B. LINDBERG AND W. NIMMICH, *Carbohydr. Res.*, 48 (1976) 81–84.
3 I. ØRSKOV AND M. A. FIFE-ASBURY, *Int. J. Syst. Bacteriol.*, 27 (1977) 386–387.
4 I. W. SUTHERLAND, *Biochemistry*, 9 (1970) 2180–2185.
5 H. NIEMANN, B. KWIATKOWSKI, U. WESTPHAL, AND S. STIRM, *J. Bacteriol.*, 130 (1977) 366–374.
6 S. C. CHURMS, E. H. MERRIFIELD, AND A. M. STEPHEN, *Carbohydr. Res.*, 81 (1980) 49–58.
7 A. K. CHAKRABORTY, U. DABROWSKI, H. GEYER, R. GEYER, AND S. STIRM, *Carbohydr. Res.*, 103 (1982) 101–105.
8 A. K. CHAKRABORTY, H. FRIEBOLIN, H. NIEMANN, AND S. STIRM, *Carbohydr. Res.*, 59 (1977) 525–530.
9 J. S. SAWARDEKER, J. H. SLONEKER, AND A. JEANES, *Anal. Chem.*, 12 (1965) 1602–1604.
10 T. BITTER AND M. H. MUIR, *Anal. Biochem.*, 4 (1962) 330–334.
11 R. L. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.
12 J.-P. JOSELEAU, F. MICHON, AND M. VIGNON, *Carbohydr. Res.*, 101 (1982) 175–182.
13 G. G. S. DUTTON AND T. E. FOLKMAN, *Carbohydr. Res.*, 80 (1980) 147–161.
14 Y. M. CHOY, G. G. S. DUTTON, A. M. STEPHEN, AND M. T. YANG, *Anal. Lett.*, 5 (1972) 675–681.
15 G. M. BEBAULT, Y. M. CHOY, G. G. S. DUTTON, N. FUNNEL, A. M. STEPHEN, AND M. T. YANG, *J. Bacteriol.*, 113 (1973) 1345–1347.
16 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–207.
17 C. G. HELLERQVIST, B. LINDBERG, S. SVENSSON, T. HOLME, AND A. A. LINDBERG, *Carbohydr. Res.*, 8 (1968) 43–55.
18 A. K. CHAKRABORTY, *Makromol. Chem.*, 183 (1982) 2881–2887.
19 B. LINDBERG AND J. LÖNNGREN, *Methods Carbohydr. Chem.*, 7 (1976) 142–148.
20 H. NIEMANN, N. FRANK, AND S. STIRM, *Carbohydr. Res.*, 59 (1977) 165–177.
21 H. THUROW, Y. M. CHOY, N. FRANK, H. NIEMANN, AND S. STIRM, *Carbohydr. Res.*, 41 (1975) 241–255.
22 I. M. MORRISON, *J. Chromatogr.*, 108 (1975) 361–364.