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STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDES FROM *Klebsiella* TYPES 8 AND 82, A REINVESTIGATION

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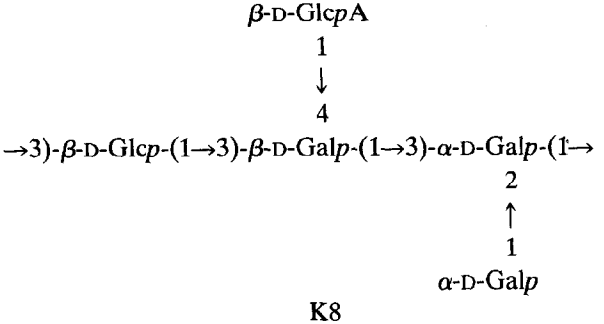
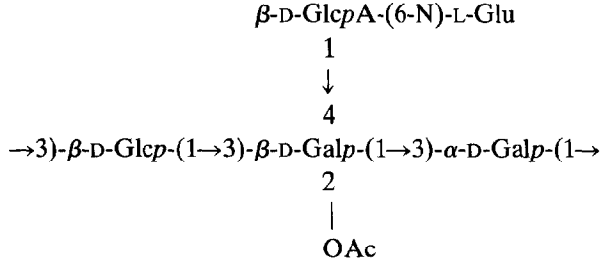
ABSTRACT

The structures of the capsular polysaccharides elaborated by *Klebsiella* types 8 (K8) and 82 (K82) have been reinvestigated. N.m.r. spectroscopy of the original and chemically modified polysaccharides was the principal method used. It is concluded that the polysaccharides are composed of repeating units having the following structures.



K8



K82

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The presence of L-glutamic acid, linked as an amide to the carboxyl group of a uronic acid, has not been observed hitherto in bacterial polysaccharides.

INTRODUCTION

During the development of a computer program for structural analysis of polysaccharides using their ^{13}C-n.m.r. spectra[1], suitable unbranched polysaccharides having a regular structure were needed for the testing of the program. One of these was the Smith-degradation product of carboxyl-reduced *Klebsiella* K82[2], and another was obtained by Smith degradation of carboxyl-reduced *Klebsiella* K8[3]. The observation that their ^{13}C-n.m.r. spectra were identical was not in agreement with the published structures[2,3], and prompted a reinvestigation of the structures of the parent polysaccharides.

The previously published structure for K82 differs from that in the Abstract in that the non-carbohydrate constituents were omitted and the anomeric configurations of the two D-galactopyranosyl residues were reversed. For K8, the terminal D-galactopyranosyl group was omitted.

RESULTS AND DISCUSSION

K82. — The ^{13}C-n.m.r. spectrum of the Smith-degraded K82 (K82S) showed 18 signals, in agreement with a trisaccharide repeating-unit. Methylation analysis of K82S showed the presence of 3-linked D-glucose and 3-linked D-galactose in the ratio 1:2 (ref. 2). The ^{1}H-n.m.r. spectra of K82S (Table I), original K82 (Table II), and *O*-deacetylated K82 (Table III) were determined and signals were assigned to different protons by means of COSY and RELAYED COSY spectra. The two galactopyranosyl residues in K82S were identified from the chemical shifts of H-3 and H-4, which occur at lower fields than the corresponding shifts for a glucopyranosyl residue. The sequence of the sugar residues (α-Gal, β-Gal, and β-Glc) was determined by means of a 2D-n.O.e. spectrum. The H-1 in α-Gal gave cross-peaks with H-2 in the same residue and with H-3 in β-Glc. The H-1 in β-Glc gave cross-peaks with H-3 in the same residue and with H-3 in β-Gal. Finally, H-1 in β-Gal showed n.O.e. contacts to H-3 and, tentatively, to H-5 in the same residue (at δ 3.74) and to H-3 in α-Gal. From the inter-residue contacts, it is concluded that K82S is composed of trisaccharide repeating-units having structure **1**.

→3)-β-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→

1

The ^{13}C-n.m.r. spectrum of K82 contained, *inter alia*, four signals for anomeric carbons at δ 100.2, 102.9, 103.2, and 105.3. One of these derives from a D-glucopyranosyluronic acid group, linked to O-4 of one of the D-galactopyranosyl

TABLE I

^{1}H-N.M.R. DATA (50°) FOR SMITH-DEGRADED K82

Atom	*α-Gal*	*β-Glc*	*β-Gal*
H-1	5.39 (3.3)[a]	4.71 (7.9)	4.67 (7.9)
H-2	4.03	3.49	3.78
H-3	4.05	3.69	3.83
H-4	4.26	n.a.[b]	4.17

[a]$J_{1,2}$ values in brackets. [b]Not assigned.

TABLE II

CHEMICAL SHIFTS OF SELECTED SIGNALS IN THE ^{1}H-N.M.R. SPECTRA (50°) OF NATIVE K82

Atom	*α-Gal*	*β-GlcA*	*β-Gal*	*β-Glc*
H-1	5.31	4.96	4.85	4.57
H-2	3.90	3.45	5.17	3.42
H-3	3.99	n.a.[a]	4.12	n.a.
H-4	4.22	n.a.	4.50	n.a.

[a]n.a., Not assigned.

TABLE III

CHEMICAL SHIFTS OF SELECTED SIGNALS IN THE ^{1}H-N.M.R. SPECTRA (85°) OF DEACETYLATED K82

Atom	*α-Gal*	*β-GlcA*	*β-Gal*	*β-Glc*
H-1	5.39	4.96	4.73	4.73
H-2	4.07	3.44	3.91	3.53

residues[2]. The appearance of an anomeric proton signal at δ 4.96, $J_{1,2}$ 7.5 Hz, in the ^{1}H-n.m.r. spectrum demonstrates that this group is β-linked. The considerable difference between the chemical shifts for H-4 of α-Gal and β-Gal in K82 and in K82S (Tables I and II) demonstrates that the β-D-glucopyranosyluronic acid group is linked to O-4 of the latter residue. The repeating unit, except for non-carbohydrate substituents, consequently has structure **2**.

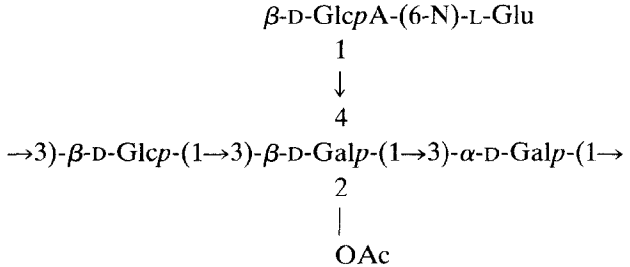
β-D-Glc*p*A
1
↓
4
→3)-β-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→

2

A signal in the ^{13}C-n.m.r. spectrum of K82 at δ 21.4 could be assigned to the methyl carbon of an *O*-acetyl group and a corresponding signal appeared at δ 2.14 in the ^{1}H-n.m.r. spectrum, and both disappeared on treatment of K82 with dilute aqueous alkali at room temperature. Comparison of the ^{1}H-n.m.r. spectra for the original (Table II) and *O*-deacetylated K82 (Table III) demonstrates that the acetyl group is linked to O-2 in β-Gal, as the corresponding methine proton is shifted from δ 5.17 to 3.91 on *O*-deacetylation. A shift of this magnitude was also observed for methyl 2-*O*-acetyl-β-D-galactopyranoside[4]. *O*-Acetyl groups often migrate and may thus occupy two or more different positions in a sugar residue. This possibility is excluded here, as O-3 in the same residue is substituted.

Five signals in the ^{13}C-n.m.r. spectrum of *O*-deacetylated K82, at δ 177.9, 175.3, 52.6, 30.8, and 26.6 (pD 2), were not accounted for by the tetrasaccharide repeating-unit (**2**). Signals in the ^{1}H-n.m.r. spectrum, at δ ~1.9 (1 H), ~2.1 (1 H), ~2.2 (2 H), and ~4.2 (~1 H), were also attributed to a non-carbohydrate substituent in K82. These chemical shifts correspond to those expected for glutamic acid. The fact that it was not split off on treatment with dilute alkali at room temperature indicates that it was linked as an amide to the carboxyl group of the β-D-glucopyranosyluronic acid residue. The diester obtained on solvolysis of K82 with acidified (+)-2-butanol was also indistinguishable, in g.l.c.–m.s., from the diester obtained from L-glutamic acid but differed, in g.l.c., from that prepared from D-glutamic acid. The ^{13}C-n.m.r. spectrum of *O*-deacetylated K82 was determined at pD 2 (see above) and at pD 12, where the signals given by the substituent appeared at δ 182.9, 179.0, 55.9, 34.8, and 29.3. The considerable shifts of both carboxyl signals demonstrate that the substituent is L-glutamic acid, with two free carboxyl groups, and not L-glutamine.

From the results of these studies, it is concluded that K82 is composed of tetrasaccharide repeating-units having structure **3**. Amide-linked amino acids have been found in other bacterial polysaccharides, but L-glutamic acid, linked in this manner, has not been observed before.



3

K8. — Previous results, showing that glucose, galactose, and glucuronic acid

are components of K8, were confirmed, and the D configuration of the sugars was demonstrated, using the method devised by Gerwig *et al.*[5]. N.m.r. studies, discussed in more detail below, indicated that K8 is composed of pentasaccharide repeating-units and does not contain *O*-acetyl groups or other non-carbohydrate components.

Methylation analysis of K8, without and with carboxyl-reduction of the methylated product, gave the sugars listed in Table IV, columns *A* and *B*. The results confirm that K8 is composed of pentasaccharide repeating-units containing terminal glucuronic acid, terminal galactose, glucose linked through O-3, galactose linked through O-2 and O-3, and galactose linked through O-3 and O-4. As mentioned above, Smith degradation of carboxyl-reduced K8 yielded a polymer, K8S, composed of trisaccharide repeating-units with the structure **1**. Uronic acid degradation[6] of K8, by treatment of the methylated product with base, methylation (using trideuteriomethyl iodide), and hydrolysis, yielded the sugars listed in Table IV, column *C*. The results demonstrate that the uronic acid is linked to O-4 of one of the galactose residues.

In the ^{1}H-n.m.r. spectrum of K8 (Table V), signals were assigned by a COSY experiment. The coupling constants for the signals given by the anomeric protons

TABLE IV

METHYLATION ANALYSIS OF NATIVE AND MODIFIED K8[a]

Sugar[b]	T[c]	*Mole %*		
		A	B	C
2,3,4,6-Gal	1.18	19	14	38
2,4,6-Glc	1.86	28	26	25
2,4,6-Gal[d]	2.03			22
2,3,4-Glc	2.26		17	
2,6-Gal	3.14	26	21	1
4,6-Gal	3.23	27	22	14

[a]Key: *A*, methylated polysaccharide; *B*, methylated and carboxyl-reduced polysaccharide; *C*, uronic acid-degraded polysaccharide. [b]2,3,4,6-Gal = 2,3,4,6-tetra-*O*-methyl-D-galactose, *etc.* [c]Retention time of the corresponding alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a DB-225 capillary column at 200°. [d]Trideuteriomethyl at O-4.

TABLE V

CHEMICAL SHIFTS OF SELECTED SIGNALS IN THE ^{1}H-N.M.R. SPECTRA (40°) OF NATIVE K8

Atom	*α-Gal I*	*α-Gal II*	*β-GlcA*	*β-Gal*	*β-Glc*
H-1	5.63	5.26	4.91	4.72	4.70
H-2	4.20	3.87	3.39	3.85	3.54
H-3	4.29	3.98	3.54	3.88	3.72

[$J_{1,2}$ ~4, 3.6, 7.8, and 7.5 (2 H)] indicate that two of the sugar residues are α-linked and the others β-linked. Signals for anomeric carbons were observed at δ 95.1, 96.7, 102.5, 104.8, and 105.0. As the linear backbone is the same in K8 and K82, one of the terminal sugars should be α-linked and the others β-linked.

The residue giving a signal for its anomeric proton at δ 5.63 was identified as the branching α-D-galactopyranosyl residue linked through O-2 and O-3 (α-GalI) from the low-field chemical shifts for H-2 and H-3. The signal for the corresponding anomeric proton in K8S appears at δ 5.39 and the deshielding of H-1 in K8 is due to the substituent in the 2-position.

The other α-linked sugar, giving a signal for the anomeric proton at δ 5.26, could be either terminal galactose or glucuronic acid. That it is the galactose (GalII) is evident from the chemical shifts for H-2 and H-3, which are similar to those expected for an α-D-galactopyranosyl group but at lower fields than expected for an α-D-glucopyranosyluronic acid group.

The sugar residues giving signals for anomeric protons at δ 4.91, 4.72, and 4.70 were identified as β-GlcA, β-Gal, and β-Glc, respectively, by comparison with the spectra of K82S and *O*-deacetylated K82.

With the identification of the structural element **4**, the structure of the pentasaccharide repeating-unit (**5**) of K8 is also defined.

α-D-Gal*p*-(1→2)-α-D-Gal*p*-(1→
3
↑

4

→3)-β-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→
4 2
↑ ↑
1 1
β-D-Glc*p*A α-D-Gal*p*

5

Except for the α-D-galactopyranosyl group, it has the same carbohydrate backbone as K82, which explains the weak immunological cross-reaction between K8 and K82 (ref. 10).

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at <40° (bath) or at room temperature by flushing with air. For g.l.c., a

Hewlett–Packard 5890 instrument fitted with a flame-ionisation detector was used. Separations were performed on a DB-225 (Durabond) capillary column or on a HP high-performance SE-54 capillary column. G.l.c.–m.s. was performed on a Hewlett–Packard 5970 instrument. Hydrolyses were performed with 2M trifluoroacetic acid for 16 h at 100°. Methylation analyses and work-up were performed essentially as previously described[7,8]. Absolute configurations of the sugars from K8 were determined according to the procedure of Gerwig *et al.*[5]. Smith degradation of K8 was performed essentially as described for K82 (ref. 2). Carboxyl-reduction and uronic acid degradation of methylated K8 were carried out essentially as described previously[9].

N.m.r. spectroscopy. — N.m.r. spectra of solutions in deuterium oxide were recorded with a JEOL GX-400 instrument, using sodium 3-trimethylsilylpropanoate-d_4, δ 0.00 (^{1}H), or 1,4-dioxane, δ 67.4 (^{13}C), as internal references. ^{13}C-N.m.r. measurements for native K82 were made at 70°, and for *O*-deacetylated K82 at 25°. The ^{13}C-n.m.r. spectrum of native K8 was measured at 25° with acetone, δ 31.0, as internal reference. Coupling constants of K8 were measured at 85°. All other measurements were made at 85° unless otherwise stated. Standard pulse sequences were used for the two-dimensional n.m.r. spectroscopy. For the NOESY spectrum, a mixing time of 250 ms was used.

Characterisation of the amino acid component of K82. — K82 (1 mg) was hydrolysed in 4M hydrochloric acid for 16 h at 100° and the hydrolysate was concentrated to dryness. The dry sample was suspended in 150 μL of (+)-2-butanol to which 30 μL of acetyl chloride was added. The suspension was kept for 16 h at 80° and then concentrated, and the residue was acetylated and analysed by g.l.c.–m.s. on an SE-54 capillary column, using a temperature programme, 190° for 3 min, 190→250°, 3°/min. Comparison was made with the corresponding derivatives of authentic L- and D-glutamic acid.

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