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Klebsiella K43 capsular polysaccharide: primary structure and depolymerisation by a viral-borne endoglycanase

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ABSTRACT

The capsular polysaccharide of *Klebsiella* K43 has been studied by glycose analysis, methylation analysis, and NMR spectroscopy, and by bacteriophage depolymerisation of the native polysaccharide. Additional evidence for the structure of the repeating unit came from base-catalysed degradation of the methylated polysaccharide, and from NMR spectroscopic analysis of the lithium-degraded polysaccharide and of the oligosaccharide-alditol derived from the repeating unit oligosaccharide obtained from a bacteriophage degradation. The polysaccharide was shown to have the repeating unit:

$$\begin{array}{l} \rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1 \rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1 \rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1 \rightarrow \\ \qquad\qquad\quad 2 \\ \qquad\qquad\quad \uparrow \\ \qquad\qquad\quad 1 \\ \beta\text{-D-Man}p\text{-}(1 \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A} \end{array}$$

INTRODUCTION

The structure of the capsular antigen of *Klebsiella* K43 is the 74th *Klebsiella* K-antigen to be reported. There remain only the capsular antigens of serotypes K29, K42, and K65 to be elucidated. Interest in the capsular antigen of *Klebsiella* K43 stems from its serological cross-reaction in Pneumococcal XVIII antiserum[1] and its inclusion in an experimental 24 valent *Klebsiella* polysaccharide vaccine[2].

RESULTS AND DISCUSSION

Isolation, composition, and methylation analysis. – *Klebsiella* K43 bacteria were grown on sucrose-rich agar as described[3] and the capsular material was extracted with aqueous 1% phenol. The crude polysaccharide material, which was obtained by precipitation of the cell-free aqueous phenol extract into ethanol, was treated with cetyltrimethylammonium bromide to afford capsular material free of O-antigen. The purified capsular polysaccharide (**PS**) had $[\alpha]_D$ +29° (H_2O) and showed a broad distribution of molecular weights in GPC on Sephacryl S500 with a maximum (M_r) at 1.1×10^7.

TABLE I

Methylation analysis of *Klebsiella* K43 polysaccharide and derived products

Methylated sugar [a] (as alditol acetate)	Molar ratios [b]		
	1	2	3
1,2,4,5,6-Gal			0.46
2,3,4,6-Man	0.90		0.96
3,4,6-Man	1.00	0.78	2.00
2,4,6-Gal	0.99	1.00	
2,4,6-Man		0.96	
4,6-Man	0.86		
2,3-Glc	0.49		0.60

[a] 1,2,4,5,6-Gal = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol, etc. [b] 1, Methylated reduced **PS**; 2, base-degraded methylated **PS**; 3, methylated carboxyl-reduced **P1-ol.**

GLC of the acetylated aldononitriles[4], derived from the products of acid hydrolysates of **PS** with and without prior reduction of the uronic acid group, showed it to be composed of GlcA, Man, and Gal in the molar ratios 1:3:1. The residues were shown to be D by GLC of their acetylated (−)-2-octyl glycosides[5].

The ^{1}H NMR spectrum of **PS**, recorded at 80°C, contained H-1 resonances for α-residues at δ 5.29 (unresolved d), 5.27 ($J_{1,2} \sim 4$ Hz), and 5.20 (unresolved d), and for β-residues at δ 4.69 (unresolved d) and 4.59 ($J_{1,2}$ 8 Hz). The resonances at δ 5.29 and 5.20 were assigned to H-1 of two α-D-Man*p* residues and that at δ 4.69 to H-1 of a β-D-Man*p* residue. The ^{13}C NMR spectrum contained C-1 signals at 102.64, 101.92, 101.03, 100.58, and 96.02 ppm, a signal for C=O at 172.43 ppm, and signals for CH_2OH at 62.45, 62.04, 61.89, and 61.71 ppm. The latter chemical shifts indicated that none of the residues was 6-linked. These results suggest **PS** to have a pentasaccharide repeating unit.

PS was methylated by the Hakomori method[6] and the partially methylated alditol acetates, prepared from an acid hydrolysate of the methylated polysaccharide with reduction of the methoxycarbonyl function, were analysed by GLC–MS (Table I, column 1). The results indicated terminal Man, 2-linked Man, 2,3-linked Man, 3-linked Gal, and 4-linked GlcA.

Base-catalysed degradation[7] of **PS**.— Methylated **PS** was degraded with methylsulfinyl carbanion and the products were alkylated with methyl iodide. GLC–MS (Table I, column 2) of the partially methylated alditol acetates derived from an acid hydrolysate of the products showed complete loss of the terminal and 2,3-linked Man, and the concomitant formation of 3-linked Man. These results are consistent with the presence of the partial structure **1** in the repeating unit of **PS**.

D-Man-(1 → 4)-D-GlcA-(1 → 2)-D-Man-(1 →
3
↑

1

TABLE II

NMR data for the lithium-degraded *Klebsiella* K43 polysaccharide (**DP**) at 50°C

Residue	^1H or ^{13}C chemical shifts (ppm) [a]							
		1	2	3	4	5	6a	6b
a								
3)-α-Gal	^1H	5.34	3.95	4.04	4.22	4.06	3.78	3.78
	^{13}C	101.20	67.96	79.93	66.12	72.02	62.13	
b								
2)-α-Man	^1H	5.30	4.04	4.03	3.74	3.91		
	^{13}C	95.44	74.41	70.87	67.83	73.64		
c								
3)-α-Man	^1H	5.05	4.28	3.97	3.83	3.83		
	^{13}C	103.03	70.48	79.03	67.19	74.16		

[a] Relative to internal acetone at δ 2.23 and 31.07 ppm for ^1H and ^{13}C, respectively.

Thus, the main chain of **PS** comprises a 2-linked Man, a 3-linked Man, and a 3-linked Gal with the Man-(1 → 4)-GlcA side chain linked to O-2 of the 3-linked Man.

The sequence of the residues in the main chain of **PS** was established by NMR spectroscopy of the degraded polysaccharide (**DP**) produced on treating **PS** with lithium in ethylenediamine[8]. GLC of the acetylated aldononitriles derived from an acid hydrolysate of **DP** showed Gal and Man in the ratio 1:2.

The ^1H NMR spectrum of **DP**, recorded at 50°C, contained H-1 resonances for three α-linked residues at δ 5.34 ($J_{1,2}$ 4.0 Hz), 5.30 (unresolved d), and 5.05 ($J_{1,2}$ 1.4 Hz), and the ^{13}C NMR spectrum contained C-1 signals at 103.03, 101.20, and 95.44 ppm. The chemical shifts of most of the ^1H and ^{13}C resonances of **DP** (Table II) were established mainly from ^1H–^1H correlation (COSY[9], RELAY COSY[10], and HOHAHA[11]) and ^1H–^{13}C correlation (HETCOR[12]) experiments. The residues were labelled arbitrarily **a**–**c** in order of decreasing chemical shift of the H-1 resonances.

Residue **a** *[→ 3)-α-Gal].*—The chemical shifts for the H-1/2 resonances were established from the COSY spectrum, and the connectivities up to H-4 were noted in the HOHAHA contour map. The chemical shift for the H-5 resonance was determined from the NOE observed between H-4 and H-5 in a phase-sensitive NOESY[13] experiment. The H-6a and H-6b resonances were then identified from the COSY spectrum.

Residue **b** *[→ 2)-α-Man].*—The chemical shifts for the H-1/2 resonances were established from the COSY spectrum. The chemical shift for H-3 was established from the H-1 track in the RELAY COSY contour plot, while those for the H-4 and H-5 resonances were noted in the H-3 track.

Residue **c** *[→ 3)-α-Man].*—The chemical shifts for the H-1/2/3 resonances were traced in the COSY spectrum and that for H-4 in the H-3 track of the RELAY COSY contour map. The chemical shift for the H-5 resonance was

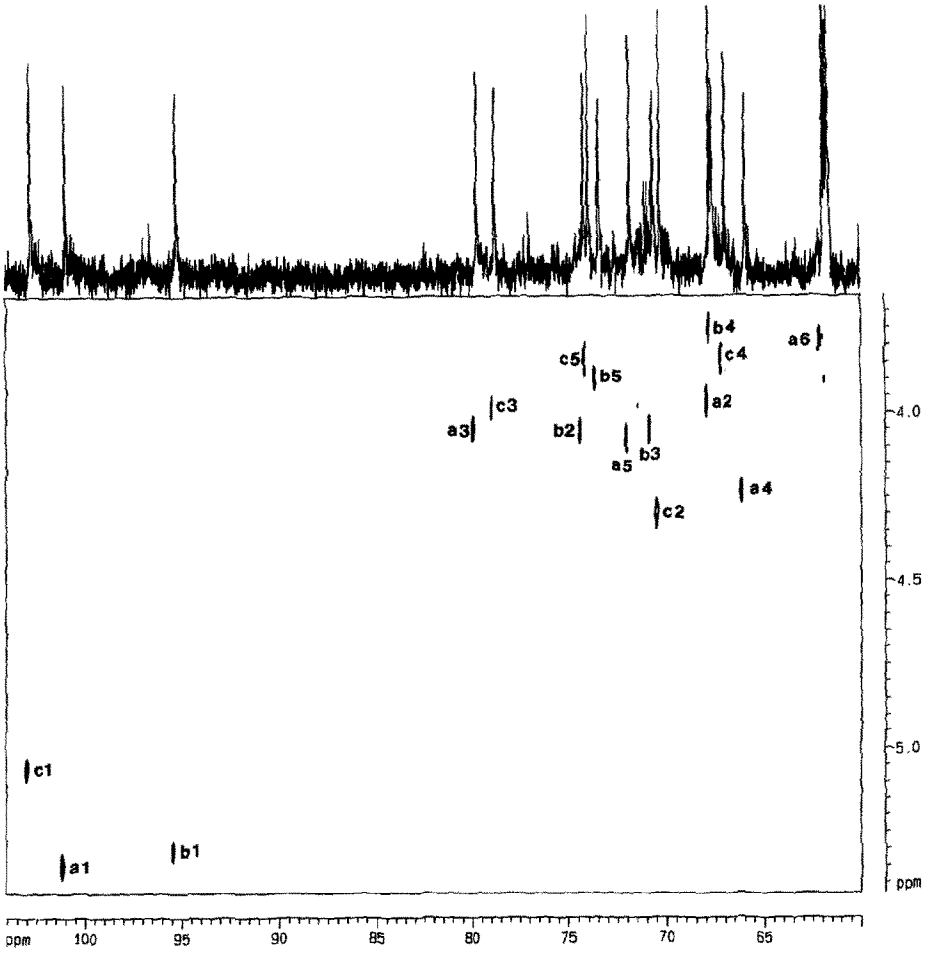

Fig. 1. ^{1}H–^{13}C correlation spectrum of **DP**. **a1** connotes the cross-peak between H-1 and C-1 of residue **a**, etc.

established from the NOE between H-3 and H-5 in the NOESY experiment. The ^{13}C resonances for residues **a**–**c** (Table II) were assigned by comparing the ^{1}H assignments with the ^{1}H–^{13}C correlation data obtained from the HETCOR experiment (Fig. 1). The identity of residues **a**–**c**, their linkage positions, and anomeric configurations followed from a comparison of the NMR data (Table II) with those for methyl glycosides[14,15].

The sequence of the residues in the repeating unit of **DP** was established from a phase-sensitive NOESY experiment[13]. The H-1 track of residue **a** showed an intense NOE cross-peak at $\delta \sim 3.96$. This cross-peak probably consists of an intramolecular NOE from H-1 to H-2 and an overlapping intermolecular NOE from H-1 of **a** to H-3 of **c**. H-1 of residue **c** showed the expected intramolecular

NOE to H-2 and an intermolecular NOE to H-2 of **b**. H-1 of residue **b** showed intermolecular NOEs to H-3 and H-4 of **a** and an intramolecular NOE to H-2. Although the cross-peaks of the intermolecular NOE to H-3 and that of the intramolecular NOE to H-2 were overlapped, both NOEs were expected, the former because Gal was shown to be 3-linked by the methylation results and the latter because residue **b** has the α configuration. The sequence **2** may thus be written for **DP**

$$\overset{\textbf{c}}{\rightarrow 3)\text{-}\alpha\text{-D-Man}p\text{-}(1} \rightarrow \overset{\textbf{b}}{2)\text{-}\alpha\text{-D-Man}p\text{-}(1} \rightarrow \overset{\textbf{a}}{3)\text{-}\alpha\text{-D-Gal}p\text{-}(1} \rightarrow$$

2

Since it has already been established that the Man-(1 → 4)-GlcA side chain is linked to O-2 of the 3-linked Man in the main chain, structure **3** may be written for the repeating unit of **PS**.

```
→ 3)-α-D-Manp-(1 → 2)-α-D-Manp-(1 → 3)-α-D-Galp-(1 →
         2
         ↑
         1
β-D-Manp-(1 → 4)-β-D-GlcpA
```

3

The structure of the repeating unit was confirmed by studying the bacteriophage depolymerisation of **PS**.

Bacteriophage depolymerisation of **PS**.—A bacteriophage isolated from sewage and propagated on *Klebsiella* K43 bacteria as previously described[16] was used to depolymerise **PS**. The pentasaccharide repeating oligosaccharide (**P1**) isolated had a reducing galactose residue. Comparison of the ^{1}H NMR spectra of **PS** and **P1** clearly established that an α-linked residue in **PS** had been cleaved by the bacteriophage. These data establish the bacteriophage endoglycanase as an α-galactosidase. **P1** was reduced with sodium borodeuteride and the derived oligosaccharide-alditol was methylated. GLC and GLC–MS analysis of the partially methylated alditol acetates derived from a hydrolysate of the methylated product (Table I, column 3), after reduction of the methoxycarbonyl group, established the reducing end of **P1** as a 3-linked Gal and that this residue was linked to O-3 of the 2,3-linked Man in **PS**. **P1** was reduced with sodium borohydride and the derived oligosaccharide-alditol **P1-ol** was studied by ^{1}H and ^{13}C NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra are shown in Figs. 2 and 3, respectively.

NMR studies of **P1-ol**.—Complete assignment of the ^{1}H and ^{13}C resonances of the sugar residues and the alditol were made from ^{1}H–^{1}H correlation (COSY[9], RELAY COSY[10], and HOHAHA[11]) and ^{1}H–^{13}C correlation (HETCOR[12] and HMBC[17,18]) experiments.

The four sugar residues in **P1-ol** were labelled **a–d** in order of decreasing chemical shifts of their H-1 resonances and the alditol was labelled **e**. All of the ^{1}H

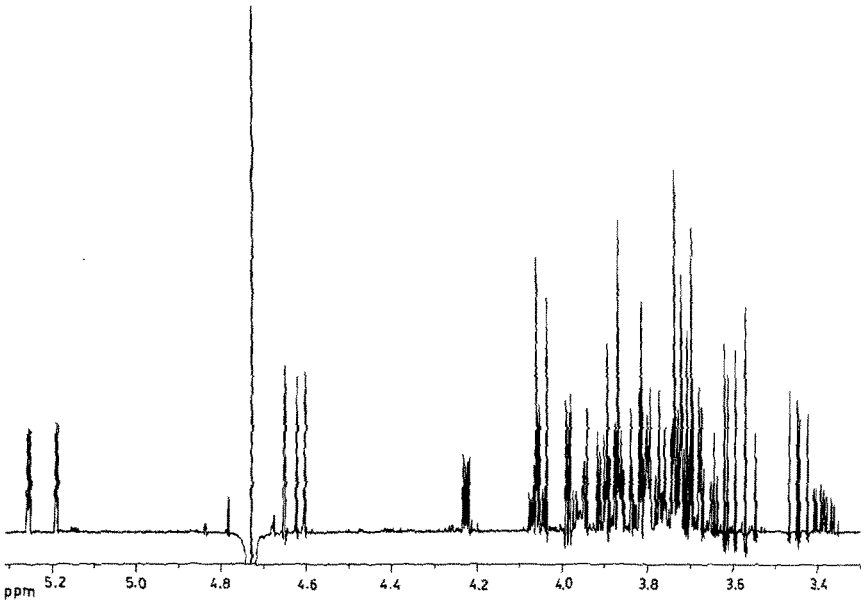

Fig. 2. Resolution-enhanced ^{1}H NMR spectrum of **P1-ol**.

resonances of residues **c** and **d**, H-1 to H-4 of residue **a**, and H-1 to H-5 of residue **b** could be assigned from the ^{1}H–^{1}H correlation spectra (Table III). These chemical shifts were compared with the ^{1}H–^{13}C correlation data obtained from the

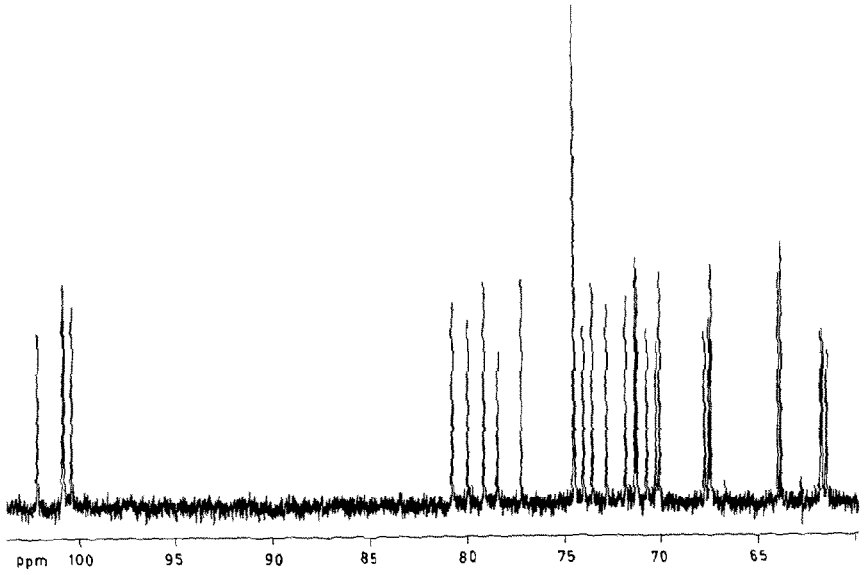

Fig. 3. ^{13}C NMR spectrum of **P1-ol**.

TABLE III

NMR data [a] for oligosaccharide **P1-ol**

Atom	Residue [b]				
	a 2)-α-Man	**b** 2)-α-Man	**c** β-Man	**d** 4)-β-GlcA	**e** 3)-Gal-ol
H-1a	5.256	5.192	4.651	4.611	3.726
H-1b					3.726
^3J [c]	1.9	1.8	0.7	7.9	
C-1	100.46	100.84	100.92	102.19	63.98
H-2	4.059	4.228	3.988	3.445	4.058
^3J	3.1	3.5	3.1	9.5	0.9
C-2	80.00	78.46	71.35	72.84	71.84
H-3	3.956	3.884	3.630	3.697	3.820
^3J	9.4		9.6		
C-3	70.74	70.29	73.57	74.53	79.16
H-4	3.740	3.692	3.572	3.839	3.890
^3J			9.6	9.8	
C-4	67.54	67.75	67.44	80.81	70.10
H-5	3.730	3.786	3.387	4.053	3.806
^3J			2.3, 6.6		
C-5	74.53	74.04	77.26	74.53	71.26
H-6a	3.800	3.785	3.734		3.709
H-6b	3.905	3.883	3.930		3.709
^3J			12.3		
C-6	61.72	61.47	61.78	173.52	63.83

[a] Chemical shifts with acetone as internal reference, δ 2.23 and 31.07 ppm, respectively, for ^1H and ^{13}C. [b] 2)-α-Man connotes a 2-linked α-mannopyranosyl residue, etc. [c] ^1H–^1H coupling constants in Hz.

HETCOR experiment, and permitted the unambiguous assignment of all of the ^{13}C resonances for residues **c** and **d**, C-1 to C-4 of residue **a**, and C-1 to C-5 of residue **b**. The assignment of the chemical shifts for C-5/H-5 and C-6/H-6a,6b for residue **a**, C-6/H-6a,6b for residue **b**, and all of the $^{13}C/^1H$ resonances for the alditol **e** was accomplished mainly from an HMBC[17,18] (heteronuclear multiple bond correlation) experiment which measures through-bond connectivity between C and H atoms two and three bonds distant. Thus, H-4 of **b** showed a correlation to a ^{13}C resonance at 61.47 ppm which was assigned to C-6 of this residue. The chemical shifts for H-6a and H-6b of **b** followed from the ^1H–^{13}C correlation data.

On the basis of literature values[19,20], which show that the chemical shifts for unlinked primary carbon atoms of alditols occur between 1–1.5 ppm to lower field than those of hexopyranoses, the unassigned sets of $^{13}C/^1H$ resonances at 63.98 ppm/δ 3.726 and 63.83 ppm/δ 3.709 were assigned to the C-1/H-1a,1b and C-6/H-6a,6b resonances of **e**. The remaining $^{13}C/^1H$ resonances for **e** were established by comparing the correlations established from the HMBC experiment (Fig. 4 and Table IV) with the ^1H–^{13}C correlation data from the HETCOR

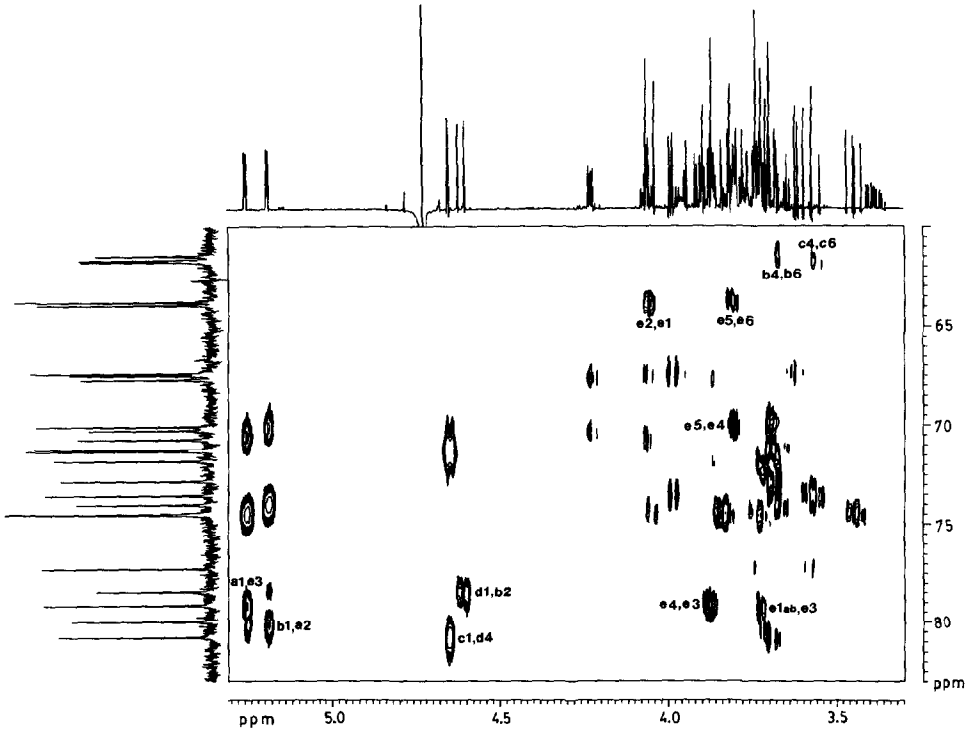

Fig. 4. HMBC contour map of **P1-ol** showing the region f_2 5.3–3.3 ppm and f_1 60–83 ppm. The ^{13}C NMR spectrum is projected along the f_1 axis and the ^1H spectrum is projected along the f_2 axis. **a1,e3** connotes H-1 of residue **a** and its correlation with C-3 of residue **e**, etc.

experiment. The correct sequence of the carbon atoms in **e** was established by assigning the most downfield ^{13}C resonance of **e** to the linkage carbon C-3.

The two remaining unassigned sets of $^1H/^{13}C$ resonances at 74.53 ppm/δ 3.730 and 61.72 ppm/δ 3.800,3.905 could now be assigned to C-5/H-5 and C-6/H-6a,6b of residue **a**, respectively. The $^1H-^1H$ coupling constants (Table III) for the sugar residues **a**–**d** were determined from the resolution-enhanced 1D spectrum (Fig. 2) of **P1-ol**.

Comparison of the NMR data for residues **a**–**e** with those for model compounds[14,15,19,20] identified the residues in **P1-ol** as indicated in Table III. The significant deshielding of C-2 of **a**, C-2 of **b**, and C-4 of **d** identified these as the linkage positions of the residues; these accord with the methylation results for **P1-ol**.

The sequence of the residues **a**–**e** in **P1-ol** was established from the HMBC spectrum (Fig. 4). Strong inter-residue correlations were observed (Table IV) between H-1 of **c** and C-4 of **d**, H-1 of **d** and C-2 of **b**, H-1 of **b** and C-2 of **a**, and

TABLE IV

Two- and three-bond $^{1}H-^{13}C$ correlations for **P1-ol**

Residue	Proton	Correlation to
a		
2)-α-Man	H-1	79.16 (**e**, C-3), 80.00 (**a**, C-2)
		70.74 (**a**, C-3), 74.53 (**a**, C-5)
b		
2)-α-Man	H-1	80.00 (**a**, C-2), 78.46(**b**, C-2)
		70.29 (**b**, C-3), 74.04(**b**, C-5)
	H-4	61.47 (**b**, C-6)
c		
β-Man	H-1	80.81 (**d**, C-4), 71.35(**c**, C-2)
	H-4	61.78 (**c**, C-6)
d		
4)-β-GlcA	H-1	78.46 (**b**, C-2)
	H-4, H-5	173.52 (**d**, C-6)
e		
3)-Gal-ol	H-1a, H-1b	79.16 (**e**, C-3), 71.84 (**e**, C-2)
	H-2	63.98 (**e**, C-1)
	H-4	79.16 (**e**, C-3)
	H-5	63.83 (**e**, C-6), 70.10 (**e**, C-4)

H-1 of **a** and C-3 of **e**. These data established structure **4** for **P1-ol**.

$$\beta\text{-D-Man}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-D-Gal-ol}$$

(**c** **d** **b** **a** **e**)

4

The study of the products of the bacteriophage degradation of **PS** thus confirms structure **3** as the repeating unit of **PS**.

EXPERIMENTAL

General methods.—GPC was performed on columns of Biogel P-4 (2.6 × 88 cm), Sephacryl S200 (1.6 × 88 cm), and Sephacryl S500 (2.6 × 88 cm), using 0.1 M sodium acetate buffer (pH 5.00) as eluent and a Waters R401 differential refractometer. Analytical GLC was performed with a Hewlett–Packard 5890A gas chromatograph fitted with flame-ionisation detectors, a 3392A recording integrator, and He as carrier gas. A J&W Scientific fused-silica DB-225 bonded-phase capillary column (30 m × 0.25 mm, 0.25-μm film) was used for separating partially methylated alditol acetates (210°C), acetylated aldononitriles (230°C), and acetylated (−)-2-octyl glycosides (220°C). The identity of each derivative was confirmed by GLC–MS on a Hewlett–Packard 5988A instrument, using the appropriate column, and with an ionisation energy of 70 eV and an ion-source temperature of 200°C.

Hydrolysis of samples with trifluoroacetic acid, carboxyl reduction of methyl esters resulting from methanolyses, determination of the absolute configuration of the sugars, preparation of acetylated aldononitriles and partially methylated alditol acetates, methylation analysis of the polysaccharide and derived products, and base-catalysed degradation of the methylated polysaccharide were carried out as previously described[21].

Preparation of Klebsiella *K43 polysaccharide* (**PS**).—An authentic culture of *Klebsiella* K43 (culture No. 2482) was obtained from Dr. I. Ørskov (Copenhagen) and the bacteria were grown on sucrose-rich agar at 37°C. The harvested slime was treated with an equal volume of aq 2% phenol and the mixture was stirred for 16 h at 4°C. The cells were separated by ultracentrifugation and the polysaccharides were precipitated in ethanol. **PS** was separated from the mixture and purified via complexation with cetyltrimethylammonium bromide.

Degradation of **PS** *with lithium in ethylenediamine.*—**PS** in dry ethylenediamine was treated with lithium as described[8]. The degraded polysaccharide (**DP**) was isolated by GPC on Sephacryl S200. **DP** was hydrolysed with 4 M CF_3CO_2H, and the products were converted into peracetylated aldononitriles and examined by GLC.

Bacteriophage-mediated depolymerisation of **PS**.—A bacteriophage which could be propagated on *Klebsiella* K43 bacteria was isolated from Grahamstown sewage and was used to depolymerise **PS**. The bacteriophage titre was increased by tube and flask lyses in nutrient broth until 5.3×10^{12} plaque-forming units were obtained. **PS** was dissolved in the dialysed bacteriophage solution which was incubated at 37°C for 3 days, then concentrated, and dialysed (mol wt cut-off, 3500) against frequently changed distilled water. The dialysates were combined and the solution was treated several times with Amberlite IR-120 (H^+) resin prior to GPC on Biogel P-4, to afford **P1** (14 mg), **P2** (8.5 mg), and **P3** (50 mg).

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying solutions in D_2O and were then dissolved in 99.99% D_2O (0.45 mL) containing a trace of acetone as internal reference (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). 1D and 2D NMR experiments were recorded with a Bruker AMX-400 spectrometer equipped with an X32 computer using UXNMR software release 920801.

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