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Note

Bacteriophage degradation of the capsular polysaccharide of *Klebsiella* K24 and determination of the position of the *O*-acetyl group

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The elucidation of the structure of the capsular polysaccharides from pathogenic bacteria has attracted the attention of research workers for many years. The main motivation for this work has been the possibility of utilising capsular polysaccharides (CPS) or derived structures as vaccines. In some cases non-carbohydrate substituents, and particularly *O*-acetyl groups, form part of the immunodominant structures of bacterial polysaccharides[1]. Thus, the location of these substituents is an important aspect of polyaccharide structural determination.

In a previous study[2], it was established that the repeating unit structure of *Klebsiella* K24 CPS is:

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

In addition, *O*-acetyl groups were found to be present and were tentatively assigned to one of the mannose units.

We now report the *O*-acetyl group to be located on C-6 of the 2-linked α-D-mannose residue. This was determined by ^{1}H- and ^{13}C-n.m.r. spectroscopic analysis of the intact native and *O*-deacetylated K24 capsular polysaccharide and of an oligosaccharide (**P1**), generated by a bacteriophage glycanase, in conjunction with chemical and methylation analyses of **P1**.

Klebsiella K24 CPS was depolymerised using a crude bacteriophage preparation. Dialysis of the depolymerised material and gel filtration of the dialysate yielded **P1** (7.5% yield) and a higher oligomer (45% yield). Reduction and preparation of the peracetylated aldononitrile derivatives[3] of **P1** showed glucose at the

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TABLE I

ANALYSIS OF REDUCTION PRODUCTS OF **P1**

Peracetylated derivative of	T^a	*Mole %*[b]	
		I	*II*
Mannononitrile	1.00	76.6	74.2
Glucononitrile	1.03		16.8
Mannitol	1.37		4.7[c]
Glucitol	1.40	23.4	4.4[c]

[a]Retention time relative to that of peracetylated mannononitrile, determined on a column programmed to hold for 2 min at 180°, then increase 5°/min to 220°. [b]I, **P1** alditol; II, product from **P1** after successive *O*-deacetylation, reduction, and carboxyl-reduction. [c]Recovery was poor, for unknown reasons.

reducing end, and characterised the ϕ24 glycanase as a β-glucosidase. Methylation of the reduced **P1** oligosaccharide (**P1** alditol) by the method of Prehm[4] confirmed (Table II) the presence of glucose at the reducing end. The ratio of 3,4,6-tri-*O*-methylmannose to 3,4-di-*O*-methylmannose shows that approximately 20% of the 2-linked α-D-mannose units carry *O*-acetyl groups at the C-6 position. The low level of 2,4,6-tri-*O*-methylmannose is a result of the 3-linked α-D-mannose being substituted by the D-glucuronic acid (glycosidic linkages of uronic acids being relatively stable to acid hydrolysis). It is of interest that some of the *O*-acetyl groups survived the reduction of **P1** with sodium borohydride. It has previously been noted in the literature that *O*-acetyl groups may remain intact during reduction under basic conditions[5,6].

Anomeric signals in the ^{1}H-n.m.r. spectra (Table III) of **P1** and its derivatives were assigned by comparison of spectral data with those previously published[2]. The spectra of K24 CPS and the *O*-deacetylated polymer are very similar. Signals integrating to the equivalent of five protons were seen in the anomeric regions of both

TABLE II

METHYLATION ANALYSIS OF **P1** ALDITOL BY THE PREHM METHOD

Partially methylated alditol acetates of[a]	T^b	*Mole %*[c]
1,2,4,5,6-Glc	0.75	24.28
2,3,4,6-Man	0.97	28.31
3,4,6-Man	1.34	33.04
2,4,6-Man	1.49	6.86
3,4-Man	1.70	7.48

[a]1,2,4,5,6-Glc = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylglucitol, etc. [b]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, determined on a column programmed to hold for 1 min at 180°, then increase at 2°/min to 250°. [c]Values corrected using e.c.r. factors given by Sweet *et al.* (ref. 13).

TABLE III

^{1}H-N.M.R. DATA FOR *Klebsiella* K24 POLYSACCHARIDE AND ITS DERIVED PRODUCTS

Compound	*Chemical shift*[a] (δ)	$J_{1,2}$ *(Hz)*	*No. of protons*	*Assignment*[b]
Native K24 CPS	5.43		2	-2,4GlcAα-, -Man(6-Ac)α-
	5.03		1	-3Manα-
	4.71		2	Manβ-, -3Glcβ-
	2.16		2.9	CH_3 of acetate
O-Deacetylated K24 CPS	5.45		2	-2,4GlcAα, -2Manα-
	5.04		1	-3Manα-
	4.71		1	Manβ-
	4.70	8-10	1	-3Glcβ-
P1	5.43		1	-2Man(6-Ac)α-, -2Manα-
	5.26 / 5.24	3-4	1.3	-4GlcAα-, -3GlcOHα
	5.05		1	-3Manα-
	4.70		1	Manβ-
	4.65	8-10	0.7	-3GlcOHβ
	2.16		1.40	CH_3 of acetate
O-Deacetylated **P1**	5.45		0.76	-2Manα-
	5.36		0.20	-3ManOHα
	5.29	3-4	1	-4GlcAα-
	5.25	3-4	0.22	-3GlcOHα
	5.07		1	-3Manα-
	4.72		0.22	-3ManOHβ
	4.70		1	Manβ-
	4.66	8-10	0.34	-3GlcOHβ
Alditol from *O*-deacetylation of **P1**	5.30	3-4	1	-4GlcAα-
	5.26		1	-2Manα-
	5.07 / 5.02		2	-3Manα-, unassigned
	4.70		1	Manβ-
P1 alditol	5.26		2	-2Manα-, -2Man(6-Ac)α-, -4GlcAα-
	5.07		1	-3Manα-
	4.70		1	Manβ-
	2.21		0.66	CH_3 of acetate

[a]Downfield from Me_4Si, measured from internal acetone at δ 2.23. Spectra were recorded at 90°.
[b]The numerical prefix indicates the position at which the sugar is substituted; α or β indicates the configuration of the glycosidic bond, or the anomer in the case of a reducing-terminal residue. Thus -2Manα- refers to the anomeric proton of a 2-substituted mannose residue in α-glycosidic linkage. The absence of a numerical prefix indicates a nonreducing terminal group.

spectra, consistent with the chemical evidence for a pentasaccharide repeating unit. The similarity of the spectra is evidence for the *O*-acetyl group being located at a C-6 position, for when an acetyl group is an *O*-substituent at a ring carbon, the ring

proton resonance is typically shifted downfield into the anomeric region[7], as noted in a recent study[8] of *E. coli* K32 CPS.

The ^{1}H-n.m.r. spectrum of **P1** differed from that of the native polyaccharide in that the resonance assigned to α-glucuronic acid was displaced upfield to 5.26 p.p.m. and the resonances of the α- and β-anomeric forms of glucose at the reducing end were present (δ 5.24 and δ 4.65 respectively). From the integral values it appeared that approximately half the repeating-unit oligomers were acetylated, indicating some loss of these groups during preparation. When **P1** was *O*-deacetylated by treatment with dilute alkali, about 50% of the reducing-end glucose was epimerised to mannose giving rise to one pair of α- and β-anomers for each reducing sugar. The resonances of these four forms were observed in the spectrum of *O*-deacetylated **P1**. The presence of reducing-end mannose was confirmed by the detection of mannitol hexaacetate in the mixture of peracetylated aldononitriles obtained from **P1** after *O*-deacetylation and reduction (Table I). Reduction of the *O*-deacetylated **P1**

TABLE IV

^{13}C-N.M.R. DATA FOR *Klebsiella* K24 POLYSACCHARIDE AND DERIVED PRODUCTS

Compounds	*Chemical shift*[a] *(δ)*	*Assignment*[b]
Native K24 CPS	104.75	-3Glcβ-
	102.95	Manβ-
	100.86	-2,4GlcAα, -2Man(6-Ac)α-
	100.59	-3Manα-
O-Deacetylated K24 CPS	104.69	-3Glcβ-
	103.23	Manβ-
	100.86	-2,4GlcAα-
	100.63	-3Manα-
	100.22	-2Manα-
P1	103.10	Manβ-
	101.20, 101.05[c]	-4GlcAα-
	100.56	-3Manα-
	100.50, 100.43	-2Man(6-Ac)α-
	100.21, 100.12	-2Manα-
	96.85, 96.77	-3GlcOHβ
	93.09, 93.05	-3GlcOHα
O-Deacetylated **P1**	103.03, 102.90	Manβ-
	101.19, 101.16	-4GlcAα-
	100.71	-3Manα-
	100.24, 100.14	-2Manα-
	96.76	-3GlcOHβ
	93.31, 93.05	-3GlcOHα, -3ManOHα

[a]Downfield from Me_4Si, measured from internal acetone at δ 31.07. [b]As in Table III. [c]Two chemical shift values indicate that two resonances were resolved, but assigned to the same anomeric carbon.

and ^{1}H-n.m.r. analysis provided a spectrum in which the resonances previously assigned to the reducing-end residues were absent. Reduction of these residues also resulted in the anomeric resonance of the 2-linked α-mannose unit being shifted upfield to δ 5.26.

^{13}C-N.m.r. analysis before and after base treatment of the native K24 CPS showed that the *O*-deacetylation resulted in the loss of a signal at 64.14 p.p.m. and an increase in the intensity of a signal at 61.63 p.p.m., which is the region usually associated with hydroxymethyl carbon resonances. This confirms that the acetate group resides on C-6 of one of the residues. *O*-Deacetylation of K24 CPS also resulted in an upfield shift of an anomeric resonance from 100.86 to 100.22 p.p.m. (Table IV).

The resonance at 104.69 p.p.m. in the ^{13}C-n.m.r. spectrum of K24 CPS was absent from the spectrum of **P1**. This anomeric resonance must therefore be that of the β-glucose. In the ^{13}C-n.m.r. spectrum of **P1** two pairs of signals from the reducing end were present (96.85, 96.77 and 93.09, 93.05 p.p.m.). These signals represent the α- and β-forms of the reducing-end glucose, which are split since only half of the **P1** oligosaccharides still carry *O*-acetyl groups (from the ^{1}H-n.m.r. data). The reduced levels of acetate groups in the **P1** oligosaccharide were also reflected by two anomeric signals (each of which was split) at 100.21, 100.12 p.p.m. and 100.50, 100.43 p.p.m. These signals arose from the same residue in repeating units with and without acetate. The additional splitting was caused by the α and β forms of the neighbouring reducing end. As 2-linked α-mannose is located adjacent to the reducing-end glucose, this is further evidence that this mannose residue carries the *O*-acetyl group. A signal observed at 64.14 p.p.m. again indicated that the acetate is located at the C-6 position. This signal disappeared on *O*-deacetylation of **P1**.

Once the anomeric resonances of the β-glucose and 2-linked α-mannose were identified the remaining C-1 signals could be assigned from knowledge of the anomeric configurations of the residues involved and reference to published data[7].

The anomeric resonance of the 2-linked α-mannose in the ^{13}C-n.m.r. spectrum of *O*-deacetylated **P1** was also split due to the α–β mutarotation of the reducing end and appeared at 100.24, 100.14 p.p.m. An extra resonance at 93.31 p.p.m. was also seen, and assigned to reducing-end α-mannose which, as described previously, resulted from epimerisation of the reducing-end glucose during treatment of **P1** with base to effect *O*-deacetylation. The reducing β-anomeric resonance was not observed. It may have been under the anomeric resonance of the reducing-end glucose.

O-Acetylation at C-6 would be expected to influence the chemical shift values of the adjacent C-5 carbon (β-effect)[9] in addition to the effects it has on the resonances of C-6 and C-1, which have already been discussed. The resolution of the ^{13}C-n.m.r. spectra was good, particulary in the case of **P1** and *O*-deacetylated **P1**. Despite this, it was not possible to assign ring-carbon resonances with confidence because of their large number. The spectra of the oligosaccarides were further complicated by multiplicity caused by mutarotation. Thus, no β-effects of *O*-acetylation were identified. However, the combination of chemical and spectroscopic data does

allow the location of the acetate at C-6 of the 2-linked α-mannose to be made with confidence.

The results detailed here illustrate the use of bacteriophage-generated oligosaccharides in the analysis of polysaccharides carrying labile groups. Not only is n.m.r. analysis aided, but methylation analysis using the Prehm method to locate *O*-acetyl is more successful with oligosaccharides. Many polysaccharides, including K24 CPS, are not soluble in the reaction mixture[10].

EXPERIMENTAL

General methods. — ^{1}H-N.m.r. spectra were recorded with a Bruker WH-400 spectrometer at 90°. Samples (5–15 mg) were dissolved and lyophilised in D_2O twice and then redissolved in D_2O (0.5 mL) for analysis. Acetone was used as an internal standard (δ 2.23). ^{13}C-N.m.r. spectra were recorded on a Varian XL-300 spectrometer at ambient temperature. Samples (10–40 mg) were dissolved in D_2O (0.5 mL) for analysis and acetone was added as an internal standard (δ 32.07). Infrared spectra were recorded using a Perkin-Elmer model 457 spectrophotometer. Analytical g.l.c. separations were carried out with a Hewlett-Packard 5890 A gas chromatograph fitted with a Durabond DB17 capillary column. A Nermag R10-10 mass spectrometer fitted with a similar column was used for g.l.c.-m.s. analysis.

Propagation of φ24 and depolymerisation of K24 CPS. — Phage φ24 was propagated on its host in nutrient broth[11] to yield 1.5×10^{12} pfu/mL. The medium was dialysed and concentrated to 175 mL and added to the polysaccharide [1 g/100 mL in volatile buffer, 0.1M NH_4OAc and 0.05M $(NH_4)_2CO_3$, pH 7]. The mixture was incubated for 48 h at 37°, then concentrated to 100 mL and dialysed against distilled water (3 × 1 L). The dialysates were combined and freeze dried (yield 400 mg). The residue was applied to a BioGel P2 column (2.5 × 90 cm) to yield **P1** (30 mg) and a higher oligomer (180 mg) on elution with distilled water (acidified with one drop/L of formic acid).

Analysis of **P1**. — An aqueous solution of **P1** (2 mg) was reduced with $NaBH_4$ (3 h, room temperature) and the product after workup was hydrolysed in 2M trifluoroacetic acid (TFA) for 16 h at 100°, converted into the peracetylated aldononitriles[3], and analysed by g.l.c. (Table I).

Methylation of **P1** alditol (2 mg) was achieved using the method of Prehm[4]. The methylated product was hydrolysed (2M TFA, 16 h, 100°), reduced, converted into the permethylated alditol acetates, and analysed by g.l.c. (Table II).

P1 (~ 25 mg) was *O*-deacetylated by treatment with 0.1M NaOH, neutralised with dilute AcOH and evaporated to dryness under vacuum (40°). The *O*-deacetylated **P1** was reduced with $NaBH_4$, the alditol was treated with 0.3M methanolic HCl (reflux, 16 h), and then uronic-ester reduction[12] was effected using $NaBH_4$. The product was hydrolysed (2M TFA, 16 h), converted into the peracetylated aldononitriles, and analysed by g.l.c. (Table I, column II).

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