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Note

Bacteriophage degradation of *Klebsiella* K30 capsular polysaccharide. An NMR investigation of the 3,4-pyruvated galactose-containing repeating oligosaccharide

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Pyruvic acid, linked as a cyclic acetal to a pyranosyl residue, is a common component of bacterial polysaccharides[1]. It is most often found linked 4,6 to a pyranosyl residue. In such cases, the absolute configuration at the acetal carbon can be determined from the ^{1}H and ^{13}C chemical shifts of the methyl group[2]. For pyruvic acetals linked to O-3 and O-4, the difference in chemical shifts is insufficient to distinguish between the *R* and *S* configurations, although this may be achieved after reduction of the carboxyl group[2]. In a previous study[3] of the repeating unit of the polysaccharide of *E. coli* K47, we demonstrated that the configuration of the acetalic carbon of a 2-linked 3,4-pyruvated β-D-Gal*p* residue could be determined as *R* from the NOE between the pyruvic methyl group and H-2 of the Gal*p* in a NOESY experiment on the polysaccharide. The configuration of a pyruvic acetal 2,3-linked to an α-Gal unit of *Streptococcus pneumoniae* Type 4 polysaccharide[4] was similarly determined by NOE measurements. In *E. coli* K47 polysaccharide, the conformation of the pyruvic-substituted → 2)-β-D-Gal*p* unit was perturbed towards a skew half-chair as was evidenced by a lessening in the H-2,3 and H-3,4 dihedral angles. The present investigation was undertaken in order to establish whether 2D ROESY and NOESY experiments could be used to establish the configuration of the acetalic carbon of the terminal 3,4-pyruvated β-D-Gal*p* present in the pentasaccharide produced by bacteriophage degradation of the *Klebsiella* K30 capsular polysaccharide. In a previous study[2], the configuration was determined as *S* by 1D NMR analysis of the carboxyl-reduced polysaccharide.

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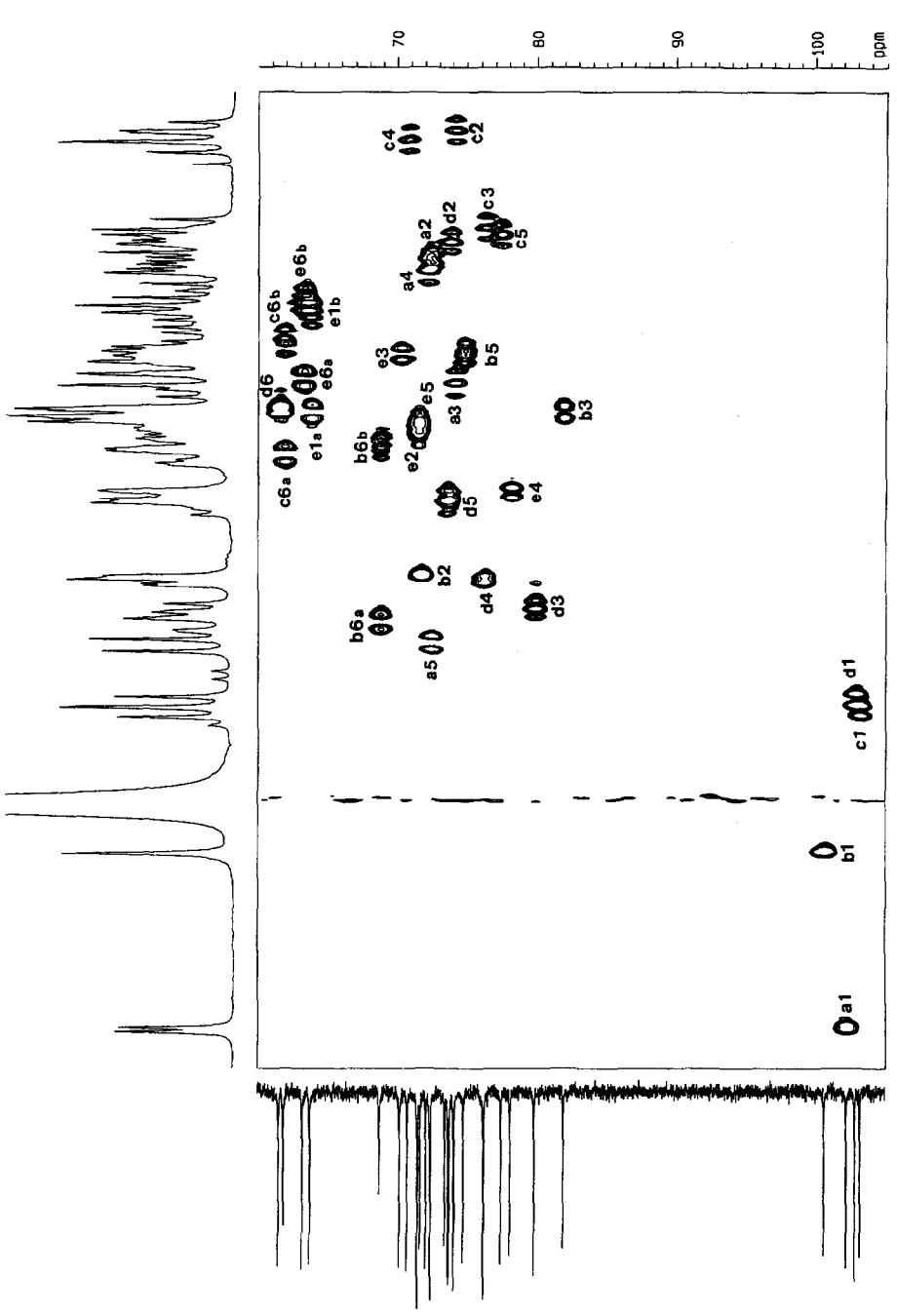


Fig. 1. HMQC contour plot for K30 **P1-o1** showing the region δ 3.2–5.3 for f_2 and 60–105 ppm for f_1; **a1** connotes the cross-peak between H-1 and C-1 of residue **a**, etc. The 1D ^1H and ^{13}C spectra are projected along the f_2 and f_1 axes, respectively. (See Table I for identification of **a**-**e**).

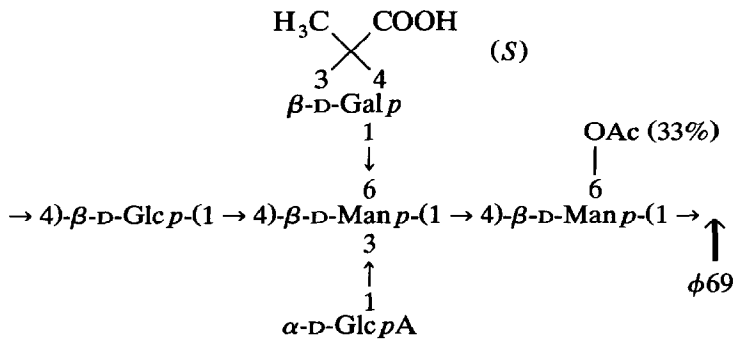
TABLE I

^1H and ^{13}C NMR data [a] for *Klebsiella* K30 oligosaccharide **P1-o1**

Residue		Proton or carbon								Pyruvic acetal		
		1a	1b	2	3	4	5	6a	6b	CH_3	C-2	COOH
a	H	5.214		3.565	3.831	3.589	4.388					
α-GlcA	^3J [b]	4.0		9.8	9.6	9.9						
	C	102.27		72.50	74.14	72.12	72.45	174.80				
b	H	4.836		4.245	3.898	4.086	3.766	4.344	3.971			
→ 3,4,6)-β-Man	^3J	nr [c]		2.6	9.8	9.8		2.1, 11.1	6.4			
	C	100.66		71.70	81.96	73.50	77.48	68.77				
c	H	4.533		3.282	3.496	3.305	3.510	3.987	3.743			
β-Glc	^3J	8.6		9.4	9.3	9.3		2.0, 12.5	7.6			
	C	103.25		74.17	76.24	70.81	77.48	61.88				
d	H	4.510		3.527	4.316	4.253	4.084	3.890		1.675		
β-Gal	^3J	8.6		7.5	5.6	2.0		nr				
3 4 \ / Pyr	C	102.88		73.82	79.86	76.21	73.73	61.53		23.74	107.23	175.20
e	H	3.895	3.682	3.948	3.769	4.068	3.913	3.821	3.652			
→ 4)-Man-ol	^3J	nr, 12.0	6.2		1.3	9.1		3.4, 12.1	6.0			
	C	63.77		71.49	70.24	78.13	71.53	63.22				

[a] Chemical shifts in ppm with acetone as internal reference, δ 2.23 and 31.07 ppm, respectively, for ^1H and ^{13}C. [b] ^1H–^1H coupling constants in Hz. [c] Not resolved.

The capsular polysaccharide of *Klebsiella* K30[5] has the structure **1**.



1

The structures of the *Klebsiella* K30 and K69[6] capsular polysaccharides differ only in the location of the pyruvic acetal which is linked 3,4 to Gal in K30 and 4,6 to Gal in K69. Studies have shown[6] that bacteriophage ϕ69 contains an endo-mannosidase which catalyses hydrolysis of the β-D-Man*p*-(1 → 4)-β-D-Glc*p* bond of the K69 polysaccharide. This bacteriophage also caused lysis of lawns of *Klebsiella* K30 bacteria and has now been used to depolymerise the K30 polysaccharide (see **1**). This cross-reaction shows that the position of the pyruvic acetal on the repeating unit is unimportant for bacteriophage enzyme binding and lysis. Such cross-reactivity of bacteriophages within the *Klebsiella* polysaccharide series is not unusual and has been reported before by Rieger-Hug and Stirm[7] who found 29 cross-reactions when examining the host capsule depolymerase activity of 50 *Klebsiella* bacteriophages. The K30 repeating unit **P1** and its dimer **P2** were isolated from the bacteriophage digest and purified by GPC. **P1** was reduced to its alditol **P1-o1**, which was then investigated by 2D NMR spectroscopy.

Complete assignment of the ^{1}H and ^{13}C resonances of the sugar residues and the alditol was made using COSY[8], HOHAHA[9], HMQC[10,11], and HMQC-TOCSY[12] experiments. Coupling constants were measured from the resolution-enhanced 1D ^{1}H spectrum. The sugar residues were labelled **a**–**d** in order of decreasing chemical shift of the H-1 resonances, and the alditol was labelled **e**. The resonances for most of the ^{1}H signals (Table I) for **a**–**d** could be assigned from the COSY contour plot and the rest were assigned from the HOHAHA spectrum. The chemical shifts for H-4/6 of **b** followed from the H-2 track and were corroborated in the H-6a track of the HOHAHA plot. The resonance for H-6 of **d** was assigned from the connectivities in the H-3 track. The ^{13}C assignments (Table I) followed from the HMQC experiment (Fig. 1), which also confirmed that H-6a and H-6b of **b** coincided, and permitted identification of the alditol (**e**) cross-peaks. The shift values for **e** were correlated using the HMQC-TOCSY spectrum while the assignments were made after identification of the most deshielded carbon

TABLE II

Inter- and intra-residue NOE contacts for K30 **P1-ol**

Residue	Proton	NOE contact to (ppm)
a	H-1	3.565 (**a**, H-2), 4.245 (**b**, H-2)
		3.898 (**b**, H-3)
b	H-1	4.245 (**b**, H-2), 4.068 (**e**, H-4)
		3.898 (**b**, H-3), 3.766 (**b**, H-5)
c	H-1	4.086 (**b**, H-4), 3.510 (**c**, H-5)
		3.496 (**c**, H-3)
d	H-1	3.971 (**b**, H-6b), 4.344 (**b**, H-6a)
d	H-2	1.675 (pyruvic CH_3)

signal (78.13 ppm) as that of the linkage carbon (C-4). The connectivity pattern of **e** could then be traced out in the expanded COSY contour plot. The HMQC-TOCSY spectrum also corroborated assignments made for **a–d**. Comparison of the NMR data for **a–e** with those for model compounds[13–15] identified the residues in **P1-ol** as indicated in Table I. The significant deshielding of C-3,4,6 of **b**, C-3,4 of **d**, and C-4 of **e** confirmed these as linkage positions.

Both ROESY[16] and NOESY[17] spectra were recorded for **P1-ol** and while the NOESY spectrum gave some information, by far the more informative was the ROESY spectrum. ROESY is well established as the 2D NOE experiment of choice for medium-sized molecules[18]. The combination of molecular rotational correlation time and spectrometer angular frequency gives rise sometimes to only very small NOEs; however, the ROESY experiment, which employs a spin-lock field, obviates this experimental difficulty and good 2D NOE spectra are obtained. It is important, however, to place the carrier frequency at the lower end of the spectrum in order to ensure that no complicating COSY or HOHAHA effects are seen[19].

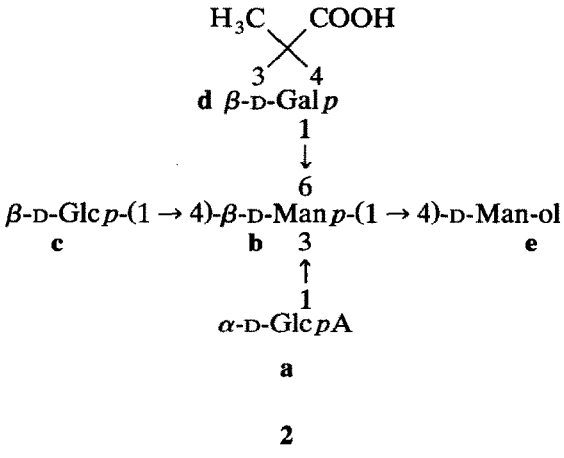
The sequence of sugar residues in **P1-ol** followed from the interresidue NOEs observed in the ROESY spectrum which also showed intraresidue NOEs consistent with the identities of the residues (Table II). In the case of the α-GlcA-(1 → 3)-β-Man linkage, besides the strong interresidue 1 → 3 NOE, a weaker interresidue NOE was observed between GlcA H-1 and Man H-2. This is in keeping with the close spatial arrangement (*gauche*) of Man H-2 and H-3. The HMBC[20]

TABLE III

Three-bond interresidue ^1H–^{13}C correlations (HMBC)

Proton	Long-range connectivity (ppm)
H-1 **a**	81.96 (**b**, C-3)
H-1 **b**	78.13 (**e**, C-4)
H-1 **c**	73.50 (**b**, C-4)
H-1 **d**	68.77 (**b**, C-6)
H-3 **d**	107.23 (pyruvic acetal, C-2)

experiment provided heteronuclear interresidue connectivities and also proof of the location of the pyruvic acetal (Table III). These data establish the structure **2** for **P1-o1**.



2

The NOE observed between the CH_3 of the pyruvic acetal and H-2 of residue **d** in the ROESY and the NOESY spectra fixed the orientation of the methyl group as endo in the bicyclic system. Application of the Cahn et al.[21] rules established the configuration of the acetalic carbon as *S*.

The values, 7.5 and 5.6 Hz, for $J_{2,3}$ and $J_{3,4}$, respectively, of the pyruvated β-D-Gal*p* unit suggest that the H-2,3 and H-3,4 dihedral angles have lessened and that the $^4C_1$ conformation is distorted towards a skew half-chair by the 5-membered ring of the pyruvic acetal; $J_{2,3}$ is smaller than the expected 8–10 Hz and $J_{3,4}$ is larger than the expected 3–4 Hz for a conventional chair conformation. The extent of the distortion appears less than that for the 3,4-pyruvated Gal*p* in *E. coli* K47[3] ($J_{2,3} = J_{3,4} = 6.1$ Hz), possibly because the latter is 2-linked and not terminal as in K30.

This study demonstrates the ability of high-field NMR analysis to describe fine structure and shows that the absolute configuration of 3,4-linked pyruvic acetals can be determined on the natural acetal and so reduction to the corresponding hydroxyisopropylidene derivative is not always necessary.

EXPERIMENTAL

Klebsiella bacteria were obtained from Dr. I. Ørskov (Copenhagen) and grown on sucrose-rich nutrient agar. The acidic capsular polysaccharide (**PS**) was separated from the cells by ultracentrifugation and purified by precipitation with cetyltrimethylammonium bromide[22]. Bacteriophage ϕ69, isolated from Grahamstown sewage water, was used to depolymerise **PS** (500 mg) using methods described elsewhere[6]. GPC of the products of the digest on Bio-Gel P4 (0.1 M

pyridinium acetate, pH 5.0) afforded equal amounts of **P1** and **P2** oligosaccharides. **P1** was reduced to **P1-o1** with $NaBH_4$ (1 h, water).

The ^1H and ^{13}C NMR spectra were recorded at a probe temperature of 30°C with a Bruker AMX-400 spectrometer. **P1-o1** (10 mg) was deuterium-exchanged by freeze-drying from D_2O and then dissolved in 99.99% D_2O (0.5 mL) containing a trace of acetone as internal standard (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). The parameters used for 2D experiments were as follows: COSY [512 × 2048 data matrix, zero-filled to 1024 data points in f_1; 68 scans per t_1 value; recycle delay, 1 s; spectral width, 1602 Hz; unshifted sine-bell filtering in t_1 and t_2]. HOHAHA [256 × 2048 data matrix; otherwise as for COSY; shifted sine-squared filtering in t_1 and t_2; mixing time, 84 ms]. NOESY [256 × 2048 data matrix, zero-filled to 1024 points in f_1; 40 scans per t_1 value; recycle delay, 4 s; shifted sine-squared filter; spectral width, 1767 Hz]. ROESY [512 × 2048 data matrix, zero-filled to 1024 data points in f_1; 88 scans per t_1 value; recycle delay, 1.5 s; spectral width, 3623 Hz; shifted sine-squared filter in t_1 and t_2; the carrier frequency was placed at the lower end of the spectrum to minimise COSY and HOHAHA peaks[19]]. HMQC [256 × 4096 data matrix, zero-filled to 1024 points in f_1; 84 scans per t_1 value; recycle delay, 1 s; spectral width, 1602 Hz in t_2 and 14022 Hz in t_1; shifted sine-squared filter]. HMQC-TOCSY [as for HMQC but with 48 scans per t_1 value; mixing time, 24 ms]. HMBC [512 × 4096 data matrix, zero-filled to 1024 data points in f_1; 56 scans per t_1 value; spectral width, 1602 Hz in t_2 and 20733 Hz in t_1; fixed delays of 3.45 ms and 60 ms; recycle delay, 1 s; shifted sine-squared filter].

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