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Preliminary communication

Structural investigation on the carbohydrate backbone of the lipopolysaccharide from *Klebsiella pneumoniae* rough mutant R20/O1$^-$

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Klebsiella pneumoniae is an important opportunistic Gram-negative pathogen [1] causing serious infections like pneumonia and urinary tract infections, especially in immunocompromised patients, often leading to septicemia. Thus, *K. pneumoniae* infections are a major cause of mortality in hospital-acquired infections [2]. Lipopolysaccharide (LPS) and capsules have been identified as virulence determinants in *K. pneumoniae* [1,2]. The capsular and O-antigens have been investigated extensively and to date eight O-serogroups [2] and 77 K-antigens [3] are differentiated. The majority of O-antigens have been structurally characterised [4–17]; however, nothing is known about the structural features of the core and lipid A region of the LPS. We now report the structure of the decasaccharide bisphosphate **1**, representing the lipid A backbone and the major part of the core region of the LPS from *K. pneumoniae* rough strain R20/O1$^-$.

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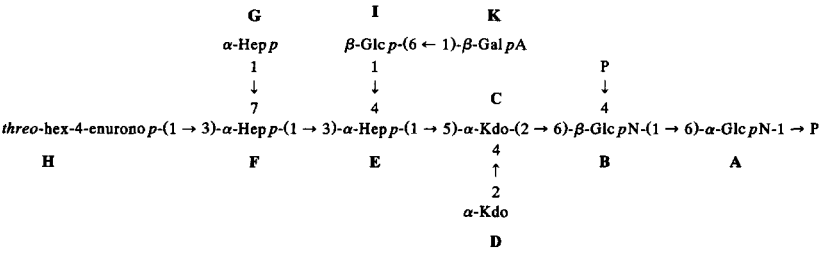

1

Klebsiella pneumoniae rough strain R20 was isolated as a spontaneous mutant from *K. pneumoniae* O1:K20 (strain 889/50, [3]). It was grown in Petri dishes (18 cm in diameter) on Nähragar I (Sifin, Berlin, FRG). The bacteria were killed (1% phenol), and washed successively with ethanol, acetone (twice), and ether. The LPS was extracted from dried bacteria by the phenol–chloroform–light petroleum method [18] in a yield of 3.6% of bacterial dry mass. Compositional analyses [19] indicated the presence of Glc, Hep, GlcN, 3-deoxy-D-*manno*-octulopyranosonic acid (Kdo), phosphate, and the fatty acids 3OH-C14:0, C14:0, and small amounts of 2OH-C14:0. Additionally, galacturonic acid (GalA) was identified by GLC (temperature programme: 110°C for 5 min, then 5°C min^{-1} to 250°C) and GLC–MS [20] after methanolysis of LPS (M methanolic HCl, 85°C, 4 h) and acetylation. The LPS (120 mg) was *O*-deacylated [21,22] (94 mg, 73.4% of the LPS), then *N*-deacylated [23] followed by neutralisation and extraction of the free fatty acids, and gel permeation chromatography on a column (2.5 × 50 cm) of TSK HW-40 (S) (Merck) in water. Two fractions were obtained, the major of which was lyophilised (28 mg, 23.3% of the LPS), then dissolved in water and separated by high-performance anion-exchange chromatography (HPAE) as described [24], but with the following modifications: flow rate, 4 mL min^{-1}; 30–70% M sodium acetate in 0.1 M NaOH increasing linearly over 70 min. Four fractions were detected, the major of which (eluting between 250–260 mM sodium acetate) was desalted by gel permeation chromatography and lyophilised (5 mg, 4.2% of the LPS). Its structure **1** was elucidated by 600-MHz ^{1}H, 150.9-MHz ^{13}C, and 145-MHz ^{31}P NMR spectroscopy (pD 9.4). The assigned chemical shifts, the couplings of the protons, and the NOE contacts as revealed by a NOESY spectrum are summarised in Tables 1–4. The anomeric region of the ^{1}H NMR spectrum contained eight signals (Table 1), three of which (residues **E–G**) could be assigned to heptose residues which possess the *manno* configuration, as revealed by their $^{3}J_{n,n+1}$ ^{1}H-coupling constants (Table 2). Five anomeric signals (residues **A**, **B**, **H–K**) were assigned to monosaccharides possessing an axial H-2. Three of these are β-linked ($J_{1,2}$ 7.5–8.5 Hz, residues **B**, **I**, **K**) and two are α-linked ($J_{1,2}$ 3.1–3.9 Hz, residues **A** and **H**). The signal at 5.489 ppm due to H-1 of **A** appeared as a double doublet indicating the esterification of O-1 by phosphate. Another signal at 5.678 ppm represented H-4 of a hex-4-enuronopyranosyl residue [25] which according to ^{1}H-chemical shifts and $^{3}J_{n,n+1}$-coupling constants possesses the *α-threo* configuration. In the region 1.70–2.15 ppm, the characteristic signals of H-3 of two α-linked Kdo residues (**C** and **D**) were present. The resonances attributed to these residues indicated an α-(2 → 4)-linked Kdo disaccharide [26], of which residue **C** was in addition substituted

Table 1
^{1}H NMR data [a] for **1**

Unit	Chemical shifts (δ)							
	H-1	H-2	H-3*ax* H-3*eq*	H-4	H-5	H-6a H-6b	H-7a H-7b	H-8a H-8b
A	5.489 [b]	2.996	3.730	3.510	4.103	4.290 3.732		
B	4.620	2.852	3.692	3.65–3.79 [c]		3.473 3.668		
C			1.900 2.117	4.131	4.235	3.703	3.832	3.871 3.669
D			1.763 2.144	4.066	4.032	3.626	4.004	3.958 3.726
E	5.262	4.044	4.126	4.326	4.192	4.062	3.794 3.960	
F	5.307	4.370	4.002	3.949	3.693	4.174	3.764 3.701	
G	4.896	3.994	3.851	3.857	3.645	4.002	3.666 3.729	
H	5.412	3.875	4.375	5.678				
I	4.568	3.282	3.495	3.431	3.593	3.901 4.186		
K	4.515	3.532	3.743	4.150	4.057			

[a] The spectrum was measured at 600 MHz in D_2O relative to acetone (δ 2.225). Assignments were made by a phase-sensitive double quantum-filtered(DQF) COSY experiment. Monosaccharide units **A**–**K** are as shown in the formula.
[b] $^3J_{H\text{-}1,P}$ 7.6 Hz.
[c] H-4 and H-5.

at position O-5. The relative configurations of GlcN, GalA, and Glc residues were identified by their ^{1}H-chemical shifts and $^3J_{n,n+1}$-coupling constants (Tables 1 and 2).

The ^{13}C NMR spectrum (Table 3) contained nine signals in the anomeric region, that at 100.75 ppm represented C-1 of residue **H** (determined by ^{1}H,^{13}C-COSY) and C-2 of residue **C** (determined by a DEPT experiment). The other signals confirmed the presence of two GlcN residues (**A** and **B**, characteristic resonances of C-2 at 56.01 and 56.80 ppm which were correlated to the high-field resonances of H-2 at 2.996 and 2.852 ppm, respectively), and of two Kdo residues [characteristic signals of C-3 at 35.13 (residue **C**) and 35.25 (residue **D**) ppm, and carboxyl resonances at 175.89 and 175.60 ppm]. Two signals of carboxyl groups were attributed to C-6 of GalA **K** and the *α-threo*-hex-4-enuronopyranosyl residue **H**. The other ^{13}C-chemical shifts of the latter were similar to those reported for the *β*-L-*threo*-hex-4-enuronopyranosyl residue obtained after deacylation of the LPS of *Vibrio cholerae* H11 [25]. Substitutions were identified by comparison of the data to those obtained for the unsubstituted monosaccharides [20,26,27]. Thus, residues **D**, **G**, **H**, and **K** are terminally linked. Both GlcN residues (**A**,**B**) are substituted at position O-6 (downfield shifts of ~ 9 and ~ 2.4 ppm, respectively), Kdo residue **C** at O-4 and O-5 (Δ ~ 4.3 and ~ 2.6 ppm, respectively), heptose **E** at O-3 and O-4 (Δ ~ 4.2 and ~ 6.4 ppm, respectively; *β*-shift of ~ 2.7 ppm

Table 2
Observed first-order $J_{n,n+1}$ values (Hz) for **1**, determined by DQF ^{1}H,^{1}H-COSY

Unit [a]	$J_{1,2}$	$J_{2,3}$	$J_{3ax,4}$ $J_{3eq,4}$ $J_{3ax,3eq}$	$J_{4,5}$	$J_{5,6a}$ $J_{5,6b}$	$J_{6a,6b}$ $J_{6,7a}$ $J_{6,7b}$	$J_{7a,7b}$ $J_{7,8a}$ $J_{7,8b}$	$J_{8a,8b}$
A	3.3	10.3	8.5	11.4	3 8.2	11.8		
B	8.5	10.3	7.3	m	4 6.3	11.8		
C			5.4 11.9 13.2	3	3	9.3	6.1	11.8
D			5.2 11.0 13.5	3	3	7.7		
E	1.5	3	9.3	10.0		 5 7.6	12.0	
F	ND [b]	3	9.5	9.3		 4 8.5	11.8	
G	1.4	3	10.1				11.4	
H	3	7.4	3					
I	7.5	10.2	8.6	10.8	3 6.7	12.0		
K	7.6	10.3	3	2				

[a] Monosaccharide units **A–K** are as shown in the formula.
[b] ND, Not determined.

Table 3
^{13}C NMR data [a] for **1**

Unit	Chemical shifts (ppm)							
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
A	94.33	56.01 [b]	72.61	70.64	72.78 [c]	70.38		
B	102.16 [d]	56.80	75.08	73.95 [b]	75.15	63.67		
C	175.89 [m]	100.75 [e]	35.13	71.20 [f]	69.82	72.78 [c]	70.44	64.28
D	175.60 [m]	NA [l]	35.25	66.95	67.39	72.85	71.32 [g]	63.78 [h]
E	99.94	71.32 [g]	75.86	73.31	69.20 [i]	69.76 [k]	64.65	
F	102.73	69.47	81.32	66.07	72.78	69.20 [i]	71.78	
G	102.16 [d]	70.78	71.32 [g]	66.95	72.38	69.76 [k]	63.78 [h]	
H	100.75 [e]	71.14	66.49	107.47	146.34	170.40		
I	102.47	74.67	76.05	70.74	76.79	69.36		
K	103.39	71.24	73.38	71.20 [f]	76.39	175.28 [m]		

[a] The spectrum was measured at 150 MHz in D_2O relative to dioxane (67.40 ppm). Monosaccharide units **A–K** are as shown in the formula.
[b] $^3J_{C\text{-}2,P}$ 7 Hz. $^2J_{C\text{-}4',P}$ 5 Hz.
[c–k] Non-resolved.
[l] NA, Not assigned.
[m] Interchangeable.

Table 4
NOE signals of **1**, observed in the NOESY spectrum [a], which were important for the structural determination

Unit	NOE signal [b]		
	From	Intraunit	Interunit
A	**A1**	**A2** (m)	
B	**B1**	**B3** (m), **B5** (s)	**A6a** (w)
C	**C3***ax*	**C4** (w), **C6** (w), **C3***eq* (s)	**E5** (w), **D6** (m)
	C3*eq*	**C4** (m), **C6** (w), **C3***ax* (s)	**D6** (s)
	C5	**C4** (m), **C6** (m), **C7** (w)	**E2** (w), **E7b** (w)
D	**D3***eq*	**D4** (w), **D3***ax* (s)	**E5** (w)
E	**E1**	**E2** (m)	**C5** (m), **C7** (s)
	E2	**E3** (w)	**C5** (w), **C7** (w), **C6** (w)
F	**F1**	**F2** (m)	**E2** (w), **E3** (s), **I2** (w)
	F2	**F3** (w)	**I6a** (w), **I6b** (w)
G	**G1**	**G2** (w), **G3** (w)	**F6** (w), **F7a** (m), **F7b** (m)
H	**H1**	**H2** (m)	**F2** (w), **F3** (s), **F4** (m)
I	**I1**	**I3** (m), **I5** (s)	**E4** (s), **E6** (s)
K	**K1**	**K3** (w), **K5** (w)	**E4** (w), **I6a** (m), **I6b** (w)

[a] Spectra were recorded at 600 MHz at 300 K; the mixing time was 200 ms. Monosaccharide units **A**–**K** are as shown in the formula.
[b] w, Weak; m, medium; s, strong.

for the C-5 resonance), heptose **F** at O-3 and O-7 [Δ ~ 9.7 and ~ 8 ppm, respectively; β-shifts of the resonances of C-2 (~ 1.3 ppm) and C-4 (~ 0.8 ppm)], and Glc **I** at O-6 (Δ ~ 7.9 ppm).

In the ^{31}P NMR spectrum, two signals at 2.84 and 4.29 ppm were found which were assigned to phosphate residues at C-1 of GlcN **A** and C-4 of GlcN **B**, respectively [28].

The intramolecular NOE contacts (Table 4) obtained from a NOESY spectrum confirmed the α- and β-*gluco* configuration of residues **A** and **B**, the β-*gluco* configuration of **I**, and the α- and β-*galacto* configuration of residues **H** and **K**. The sequence of the residues was established by analysis of the interresidue NOE contacts. Proton **H1** gave NOE signals to protons **F2**, 3, and 4, **F1** to **E2** and 3, and **E1** to **C5**, 7, and 8a, thus establishing the tetrasaccharide unit **H-F-E-C**. This unit is substituted at residue **F** by residue **G** (NOE, **G1** to **F7a,b** and 6), at **E** by residue **I** (**I1** to **E4** and 6), and at **C** by **D** in α-(2 → 4) linkage which was elucidated by the characteristic NOE contact **D6** to **C3***ax,eq* [26]. Furthermore, residue **I** is substituted by **K** (**K1** to **I6a,b**), and **A** by **B** (**B1** to **A6a**). Consequently, although not indicated by NOE contacts, **C** must be linked to **B**. In summary, the NOE signals and the data from the ^{1}H, ^{13}C, and ^{31}P NMR spectra determine the structure of the isolated decasaccharide as **1**.

The presence of the *threo*-hex-4-enuronopyranosyl residue **H** indicates a substituent at O-4 of the GalA residue linked to Hep **F**, which has been eliminated by treatment with hot alkali. The nature of the eliminated substituent is not known. However, the isolated decasaccharide **1** already reveals characteristic features of the core region of *K. pneumoniae* strain R20. First of all, the lipid A backbone consists of β-Glc*p*N-(1 → 6)-α-Glc*p*N 1,4′-bisphosphate which represents the structural principle of lipid A of all enterobacterial and many other LPSs [29]. Since this structure is highly conserved, it is

probable that it is generally present in *Klebsiella* lipid A. The structural element characteristic of enterobacterial core regions [30], α-Hep-(1 → 7)-α-Hep-(1 → 3)-α-Hep-(1 → 5)[α-Kdo-(2 → 4)]-α-Kdo which is substituted at O-3 of the second Hep by a hexose residue, is also present in the decasaccharide. However, the substitution at O-3 by GalA was previously found only in the core region of *Proteus mirabilis* R110/1959, whereas the other enterobacterial core oligosaccharides possess a Glc residue at that position. A major difference of the core region of *K. pneumoniae* strain R20 compared to the other core regions of *Enterobacteriaceae* investigated so far is the lack of phosphate. Negative charges are provided only by Kdo and GalA residues. Furthermore, the substitution at O-4 of the first Hep is only present in *P. mirabilis* R110/1959 (a Glc disaccharide) and *Yersinia enterocolitica* 75S (Glc).

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