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THE STRUCTURAL REPEATING-UNIT OF THE CAPSULAR POLYSACCHARIDE FROM *Klebsiella* SEROTYPE K48*

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ABSTRACT

Investigation of the structure of the capsular polysaccharide from *Klebsiella* K48, using methylation analysis, periodate oxidation, Smith degradation, and ^{1}H- and ^{13}C-n.m.r. spectroscopy, indicated the repeating unit to be the pentasaccharide

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow$$
$$\begin{array}{c} 2 \\ \uparrow \\ 1 \\ \alpha\text{-D-Gal}p\text{A} \end{array}$$

INTRODUCTION

Earlier studies by Heidelberger and Nimmich[1], who investigated the cross-reactions of K polysaccharides from *Klebsiella* and certain capsular polysaccharides from *Pneumococcus*, established correlations between chemical structure and serological specificity. Thus, *Klebsiella* type 48 should have rhamnosyl residues either hindered by a substituent or linked otherwise than (1→3), and a common feature with *Klebsiella* type 34 in their glucose residues. Since glucose is 3-linked[2] in the main chain of K34, it is expected to occupy a similar position in K48. These predicted structural features are now demonstrated to be true.

RESULTS AND DISCUSSION

The capsular polysaccharide, isolated and purified as described for other strains[2], gave L-rhamnose and D-glucose in the molar ratio 1.0:1.0 upon acid hydrolysis (Table I). The equivalent weight of the polysaccharide was estimated by titration with sodium hydroxide to be ~820. The carboxyl-reduced[3] polymer contained nearly equal proportions of L-rhamnose and D-glucose, together with D-

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TABLE I

SUGAR ANALYSIS ON THE ORIGINAL AND MODIFIED *Klebsiella* K48

	Molar ratios				
	Rha	*Glc*	*Gal*	*GalA*	*Erythritol*
Original polysaccharide	1.03	1.00		1.00	
Carboxyl-reduced	1.8	2.00	0.8		
Periodate-oxidised	0.95	1.00			
Oligosaccharide **1**	1.00	1.15			0.85

galactose which was thus derived from D-galacturonic acid. The sugar composition of the repeating unit of polysaccharide K48 was thus a pentasaccharide with L-rhamnose, D-glucose, and D-galacturonic acid in the molar ratios 2:2:1.

This carbohydrate analysis was in agreement with the ^{1}H-n.m.r. spectrum of a solution of the polymer in D_2O which showed five signals for anomeric protons at δ 5.25 (1 H), 4.95–5.05 (2 H), and 4.75 (1 H). A 6-proton doublet at δ 1.33 confirmed the presence of two rhamnosyl residues in the repeating unit. Similarly, the ^{13}C-n.m.r. spectrum showed five signals for anomeric carbons at δ 105.3, 99.3, 98.9, 98.5, and 98.3. The presence of a galactosyluronic acid residue was indicated by the resonance of its C-6 at δ 173.1 and that of the rhamnosyl residues by two overlapping signals at δ 17.3. The two signals at δ 62.5 and 61.4 for CH_2OH belong to the two glucosyl residues which, thus, are not 6-linked (Table II). No ^{1}H or ^{13}C signals corresponding to acyl or carboxyethylidene substituents were observed.

Methylation analysis of the K48 polysaccharide and g.l.c.-m.s. of the resulting alditol acetates gave the results shown in Table III. Derivatives corresponding to 3,4-di-*O*-methylrhamnose, 4-*O*-methylrhamnose, 2,4,6-tri-*O*-methylglucose, and 2,3,6-tri-*O*-methylglucose were found in the ratios 0.9:0.5:0.9:1.0. The low proportion of 4-*O*-methylrhamnose suggested that one of the rhamnosyl residues was involved in the aldobiouronic acid unit and therefore was only partially released upon acid hydrolysis. This rhamnose is also a branch point for a side chain. The borohydride-reduced carbodi-imide[3] derivative was also subjected to methylation analysis (Table III). The formation of 2,3,4,6-tetra-*O*-methylgalactose showed that galacturonic acid occupies a terminal non-reducing position in the side chain. Treatment of the methylated K48 polysaccharide with an excess of base[4] under the conditions for β-elimination resulted only in a relative increase of the release of 4-*O*-methylrhamnose. This result confirmed the terminal position of galacturonic acid in the side chain and its direct attachment to the rhamnosyl residue. When base-catalysed β-elimination was followed by re-methylation, the analysis (Table III, column V) showed again that galacturonic acid was in the side chain as a single sugar and that it was 2-linked to the rhamnosyl branch-point, as demonstrated by the formation of 2,4-di-*O*-methylrhamnose.

The structure of the remainder of the pentasaccharide repeating-unit could be

TABLE II

N.M.R. DATA FOR THE K48 CAPSULAR POLYSACCHARIDE AND THE OLIGOSACCHARIDE **1**

Compound	*^1H*			*^{13}C*	
	δ^a ($J_{1,2}$ *in Hz*)	Intensity	Assignment	δ^a	*Assignment*
→3)-β-Glc-(1→3)-α-Rha-(1→4)-α-Glc-(1→2)-α-Rha-(1→ 2 ↑ 1 α-GalA	5.25 (1)	1	2,3-αRha	173.1	C-6 of GalA
	5.10 (3)	1	α-GalA	105.3	β-Glc
	4.95–5.05 (n.o.)[b]	2	4-α-Glc	99.3	C-1
			2-α-Rha	98.9	
	4.75 (7)	1	3-β-Glc	98.5	
	4.70 n.o.	1	H-5 of GalA	98.3	
	1.33 (6)	6	CH_3 of Rha	98.3	
β-Glc-(1→3)-α-Rha-(1→2)-erythritol (**1**)				62.5	C-6 of Glc
	4.95 (1)	1	3-α-Rha	61.4	
	4.68 (7)	1	β-Glc	17.3	CH_3 of Rha

[a]In p.p.m. relative to the signal of internal acetone [CH_3 at 2.23 (^1H) and 31.07 p.p.m. (^{13}C)]. [b]Not observed.

deduced from the results of periodate oxidation and Smith degradation. Sugar analysis after periodate oxidation of the carboxyl-reduced polysaccharide yielded the expected 1:1 ratio of rhamnose and glucose (Table I). The absence of galactose amongst the products of hydrolysis indicated that the oxidation was complete. Borohydride reduction of the oxidised material followed by hydrolysis overnight at room temperature in 0.5M trifluoroacetic acid and preparative p.c. yielded an oligosaccharide **1** (R_{Glc} 0.5, 8:2:1 ethyl acetate–pyridine–water). Total acid hydrolysis and analysis of the derived alditol acetates showed that **1** contained erythritol, rhamnose, and glucose in the ratios 0.85:1.00:1.15. Only rhamnose survived a second Smith-degradation. Methylation of **1** followed by hydrolysis gave (g.l.c–m.s.) 2,3,4,6-tetra-*O*-methylglucose and 2,4-di-*O*-methylrhamnose in approximately equal proportion. Thus, it was concluded that **1** was Glc-(1→3)-Rha-(1→2)-erythritol, with the erythritol coming from the 4-linked glucose residue.

The ^{1}H-n.m.r. spectrum of **1** contained signals at δ 4.95 (s, 1 H) and 4.68 (d, 1 H) and a signal at δ 1.30 (d, 3 H) (Table II). Therefore, the 3-linked D-glucose residue is β. This conclusion allowed assignment of the signal at δ 4.75 ($J_{1,2}$ 7 Hz) in the spectrum of the polymer (Table II). Similarly, the signal (1 H, $J_{1,2} \sim 1$ Hz) at δ 4.95 can be ascribed to the 3-linked α-L-rhamnose. This signal is shifted downfield to δ 5.25 in the polymer when the corresponding α-L-rhamnose residue is branched at O-2 with the galacturonic acid. The peak appearing at δ 5.10 (d, $J_{1,2}$ 3 Hz) corresponds to H-1 of α-D-galacturonic acid.

The unresolved signal at δ 4.95–5.05 indicates that the 4-linked D-glucose and the 2-linked L-rhamnose are both α. A signal at δ 4.70 is also visible in the spectrum of the polymer but not in that of the periodate-oxidised polysaccharide. This signal may be assigned to the deshielded H-5 of D-galacturonic acid[8]. Its coupling constant could not be observed since the signal was partly overlapped by the H-1 doublet of

TABLE III

METHYLATION ANALYSES OF K48 CAPSULAR POLYSACCHARIDE AND DERIVATIVES

Methylated sugars[a] *(as alditol acetates)*	T[b]	*Molar ratios*[d]				
		Ic	*II*	*III*	*IV*	*V*
3,4-Rha	0.87	0.95	0.85	0.85	1.05	0.95
2,4-Rha	0.98					0.65
2,3,4,6-Gal	1.25		0.75			
4-Rha	1.77	0.55	0.80	0.80	1.00	0.20
2,4,6-Glc	2.06	0.90	0.90	0.90	1.00	1.00
2,3,6-Glc	2.69	1.00	1.00	1.00	1.00	1.00
2,3,4-Gal	3.84				0.90	

[a]3,4-Rha = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhamnitol, *etc.* [b]Retention times on 3% of ECNSS-M at 150° relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [c]I, K48 polysaccharide; II, carboxyl-reduced K48; III, β-eliminated K48; IV, K48 reduced with $LiAlH_4$ after methylation; V, β-eliminated K48 then methylated. [d]Calculated by effective carbon response factors according to Sweet *et al.*[9]. Average values from triplicate experiments.

β-D-glucose. In the ^{13}C-n.m.r. spectrum of the polymer, only the signal at δ 105.3 has a chemical shift typical of a β-D-hexose residue and can therefore be assigned to the β-D-glucose. Thus, the other residues of the D-hexose series, namely, the second D-glucose and the D-galacturonic acid residue with C-1 signals at $\delta < 100$, are α. This assignment of the anomeric configurations accords with the positive $[\alpha]_D$ value of the polysaccharide.

From the above data, it follows that the repeating unit of the capsular polysaccharide from *Klebsiella* serotype K48 is the pentasaccharide shown in the Abstract.

The structural features predicted[1] for *Klebsiella* K48 polysaccharide on the basis of its cross-reactivity with *anti-Pneumococcus* I serum are fully verified. The prediction that rhamnosyl residues should be either hindered by a substituent or linked other than (1→3) is confirmed, with one L-rhamnosyl residue as a branch point and the other 2-linked. The prediction of a 3-linked D-glucopyranosyl residue present in the main chain, as in *Klebsiella* K34[2] is also confirmed. This structural similarity is enhanced by the presence of D-glucose in the two polysaccharides and by the same partial sequence

→2)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→.

EXPERIMENTAL

General methods. — G.l.c. was performed on glass columns (180 × 0.15 cm) containing 3% of SP-2340 on Supelcoport (100–120 mesh) at 185°. For g.l.c.-m.s. of partially methylated alditol acetates, an OV-225 and an SP 2340 S.C.O.T. column (25 m × 0.25 mm from Chromopack) were used at 180° programmed at 2°/min to hold at 220°. Mass spectra were recorded on an A.E.I. MS-30 spectrometer. The ^{1}H- and ^{13}C-n.m.r. spectra were recorded at 90° for solutions in D_2O (internal acetone) with a CAMECA 250 spectrometer.

Isolation of the polysaccharide and sugar analysis. — A culture of *Klebsiella* serotype K48 was obtained from Dr. I. Ørskov (WHO International Escherichia Center, Copenhagen). The capsular polysaccharide, collected and purified as described[5] for other strains, had $[\alpha]_D$ +38° (*c* 0.25, water). Carbohydrate analysis involved hydrolysis either with aqueous 72% sulfuric acid initially at room temperature and then after dilution to 0.5M for 6 h at 100°, or with M trifluoroacetic acid for 3 h at 100°. The products were converted into the alditol acetates and analysed by g.l.c. The equivalent weight was determined by titration with 0.01M sodium hydroxide.

Methylation analysis. — Methylations were carried out by the Hakomori procedure[6] using potassium methylsulphinylmethanide[7]. The methylated products were hydrolysed with formic acid (aqueous 90%, 1 h, 100°) and then with trifluoroacetic acid (2M, 3 h, 100°).

Uronic acid degradation. — Methylated K48 polysaccharide was degraded by

using potassium methylsulfinylmethanide in methyl sulfoxide overnight at room temperature under nitrogen. The mixture was neutralised with aqueous 50% acetic acid and dialysed, and the product was hydrolysed and analysed by g.l.c.-m.s.

Periodate oxidation and Smith degradation. — A solution of the carboxyl-reduced polysaccharide (50 mg) in water (20 mL) was oxidised with 0.1M sodium metaperiodate (20 mL) for 96 h at room temperature in the dark. The oxidised polymer was reduced conventionally with $NaBH_4$. After dialysis, the product was hydrolysed overnight at room temperature in 0.5M trifluoroacetic acid, and the hydrolysate was concentrated to dryness. P.c. (8:2:1 ethyl acetate–pyridine–water) gave a spot of R_{Glc} 0.50, which was eluted with water. After preparative p.c. in the same solvent, a part of the oligomer was hydrolysed in 2M trifluoroacetic acid (3 h, 100°), and another part was methylated.

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