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The structure of *Escherichia coli* K26 antigen

Linda M. Beynon and Guy G. S. Dutton
Department of Chemistry, University of British Columbia, Vancouver, B.C., V6T 1Y6 (Canada)

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ABSTRACT

The structure of the capsular antigen of *E. coli* K26 has been found by a combination of chemical and spectroscopic techniques to be of the "5+1" type shown. An important step was the simultaneous separation and identification of a mixture of neutral and acidic oligosaccharides by g.l.c. c.i.-m.s.

→3)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*A-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→
4
↑
1
3,4-pyr-α-L-Rha*p*

INTRODUCTION

E. coli K26 has been classified as having an A type capsular (K) antigen which, since it lacks amino sugars, may be expected to resemble the K antigens of *Klebsiella*[1]. The structure of the K26 antigen now proposed confirms the predictions made by Heidelberger on the basis of serological cross reactions[2].

RESULTS AND DISCUSSION

Composition. — The purified capsular polysaccharide was found by gel-permeation chromatography to be monodisperse (M_r 1 × 10^7) and, after depyruvylation, had $[\alpha]_D$ −13° (water). Sugar analysis prior and subsequent to reduction of the uronic acid suggested a hexasaccharide repeating-unit composed of L-rhamnose, D-galactose, and D-glucose in the ratios 4:1:1. The configurations of the sugars were assigned by comparison of the retention times of their (−)-2-octyl glycoside acetates with those of standards[3].

The size of the repeating unit was confirmed by the ^{1}H-n.m.r. spectrum of the native polysaccharide, which showed six signals in the region for anomeric protons (Table I). These corresponded to three α linkages (δ 5.10, 3 H), one β linkage (δ 4.74), and a borderline signal (δ 4.91) of relative intensity two. This last signal was found by a single-frequency off-resonance decoupled experiment (SFORD) to result from one α and one β linkage. Two signals were visible at high field, namely, at δ 1.35 (corresponding to the four rhamnose residues) and 1.59 (from a 1-carboxyethylidene group which,

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TABLE I

N.m.r. data for *E. coli* K26 capsular polysaccharide and derived oligosaccharides

Compound	*^{1}H-N.m.r. data*				*^{13}C-N.m.r. data*	
	δ[a] (p.p.m.)	*$J_{1,2}$[b] (Hz)*	*Integral (no. of H)*	*Assignment[c]*	*P.p.m.[d]*	*Assignment[e]*
K26 polysaccharide →3)-α-Rha-(1→3)-β-Gal-(1→3)- [3,4-pyr-α-Rha-(1→4)]- β-GlcA-(1→3)-α-Rha-(1→3)-α-Rha-(1→	5.10	s	3	→3)-α-Rha-	104.16	→3)-β-Gal-
	4.91	b	2	→3)-β-Gal- α-Rha-	103.59	-3,4-β-GlcA-
	4.74	b	1	-3,4-β-GlcA-	103.11 102.99	→3)-α-Rha-
	1.59	s	2	CH_3 (pyr)		
	1.35	b	12	CH_3 (Rha)	102.04	-3,4-pyr-α-Rha-
					62.21	C-6 (hex)
					23.61	CH_3 (pyr)
					17.61	C-6 (Rha)
K26 polysaccharide (autohydrolysis; spectrum run at pH ~3)	5.06	s	3	→3)-α-Rha-	173.85	C=O (GlcA)
	4.87	s	1.4	→3)-β-Gal- α-Rha-	104.57	→3)-β-Gal-
	4.71	7	1	-3,4-β-GlcA-	103.67 103.65	→3)-β-GlcA- -3,4-β-GlcA-
	1.31	6	12	CH_3 (Rha)	103.04 103.00 102.97	→3)-α-Rha-
					99.94	α-Rha-
					61.93	C-6 (hex)
					17.47	CH_3 (pyr)
K26 polysaccharide (autohydrolysis; spectrum run at pH 7)	5.08	s	3	→3)-α-Rha-		
	4.88	8	1	→3)-β-Gal-		
	4.85	s	1	α-Rha-		
	4.80	8	1	-3,4-β-GlcA-		
	1.32	6	12	CH_3 (Rha)		

K26 polysaccharide	5.08	s	3	→3)-α-Rha-	104.43	→3)-β-Gal-
(selective hydrolysis)	4.81	8	1	→3)-β-Gal-	103.85	-3,4-β-GlcA-
					103.81	→3)-β-GlcA-
	4.71	8	1	→3)-β-GlcA-	103.06	
	1.32	5–6	12	CH_3 (Rha)	103.01	→3)-α-Rha-
					102.93	
					101.44	-3,4-pyr-α-Rha-
					99.65	α-Rha-
					61.86	C-6 (hex)
					23.00	CH_3 (pyr)
					17.49	CH_3 (Rha)
K26 polysaccharide	5.08	s	3	→3)-α-Rha-		
(carbodiimide reduced)	4.97	s	1	α-Rha-		
	4.91	8	1	→3)-β-Gal-		
	4.74	8	1	-3,4-β-Glc-		
	1.32	s	13	CH_3 (Rha)		
K26 polysaccharide	5.08	s	3	→3)-α-Rha-		
(Smith-degraded)	4.81	8	1	→3)-β-Gal-		
	4.75	7	0.7	→3)-β-GlcA-		
	4.63		0.3			
	1.32	6	9	CH_3 (Rha)		
Oligosaccharide **1**	5.10	s	0.6	→3)-α-Rha-OH		
β-GlcA-(1→3)-Rha	4.85	s	0.4	→3)-β-Rha-OH		
	4.78					
	4.77	7–8	1	β-GlcA-		
Oligosaccharide **2**	5.10	1–2	0.7	→3)-α-Rha-OH	104.81	
β-GlcA-(1→3)-α-Rha-(1→3)-Rha	5.08	s	0.5	→3)-α-Rha-	104.03	β-GlcA-
	4.88	s	0.3	→3)-β-Rha-OH	102.92	→3)-α-Rha-
	4.75				94.78	→3)-α-Rha-OH
	4.70	7–8	1.5	β-GlcA-	94.20	→3)-β-Rha-OH
	1.32	6		CH_3 (Rha)	17.63	CH_3 (Rha)

[a] Chemical shift relative to internal acetone (δ 2.23 downfield from external sodium 4,4-dimethyl-4-silapentane-1-sulfonate). [b] Key: b = broad, unable to assign accurate coupling constant; s = singlet. [c] For example, →4)-β-GlcA refers to H-1 of a 4-linked β-glucuronic acid residue. The absence of a numeral prefix indicates a non-reducing terminal group. [d] Chemical shift relative to internal acetone (δ 31.07 downfield from external reference in *a*). [e] As for *c*, but for C-1.

from the integral, was judged to be present on two out of three repeating units).

When the native polysaccharide was subjected to autohydrolysis, the ^{1}H-n.m.r. spectrum of the degraded material showed the loss of the pyruvate signal at δ 1.59 (Table I). Altering the acidity of the solution from pH 3 to 7 caused the signal at δ 4.87 to be resolved into two at δ 4.88 ($J_{1,2}$ 8 Hz) and 4.85, and that at δ 4.71 to move downfield to δ 4.80. The signals in the region for anomeric protons were assigned by comparison of the spectra of the native polysaccharide and of oligosaccharides derived subsequently (see Table I).

The results of the analysis of the ^{13}C-n.m.r. spectrum of the native polysaccharide are shown in Table I. On removal of the pyruvate acetal, the signal at 102.04 p.p.m. moved upfield to 99.94 p.p.m. and disappeared when the lateral rhamnose residue was removed by selective hydrolysis. The signal at 62.21 p.p.m. demonstrates that D-galactose, the only hexose, is not linked through C-6. Incomplete removal of the lateral rhamnose residue resulted in twinning of the signal at 103.81 p.p.m.; hence, the resonance at 103.59 p.p.m. in the native polysaccharide was assigned to D-glucuronic acid, the branch point.

Methylation analyses[4,5]. — The results are presented in Table II and are consistent with a side chain of 3,4-*O*-(1-carboxyethylidene)rhamnose linked to O-4 of glucuronic acid, the branch point. A base-catalyzed uronic acid degradation[6] of the methylated native polysaccharide indicated the glucuronic acid to be linked to position 3 of a rhamnose residue. That the main chain consisted solely of 3-linked residues was confirmed by a Smith degradation[7] on depyruvylated material, which gave a polymer of high molecular weight.

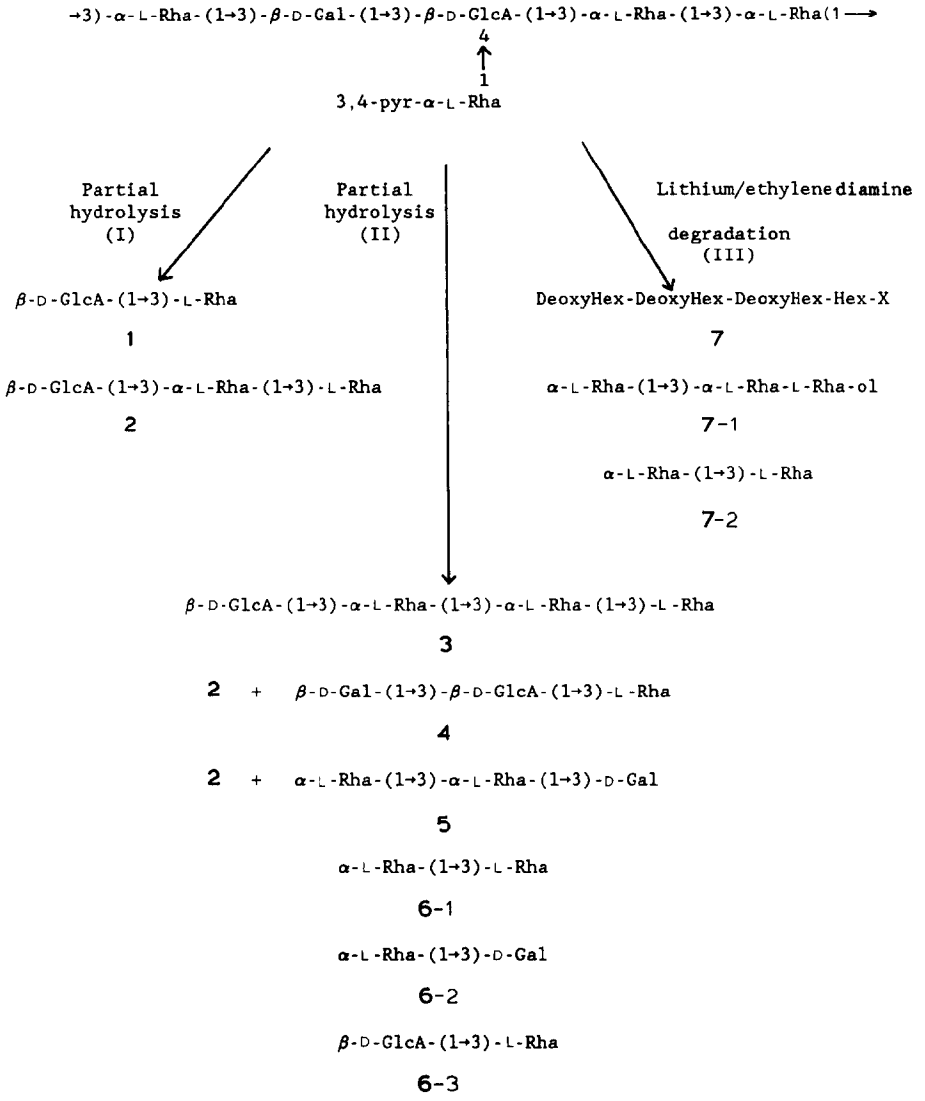
TABLE II

Methylation data for *E. coli* K26 polysaccharide and derived products

Methylated sugar[a] *(as alditol acetates)*	*Molar ratios*							
	I[b]	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*
1,2,4,5-Rha								0.29
2,3,4-Rha	0.23	0.62	0.98	1.71	0.53	0.21		
2,4-Rha	2.11	1.93	2.87	3.42	2.58	2.54	1.94	0.35
2,3,4,6-Gal					0.43			0.18
2-Rha	0.55							
2,3,4-Glc								1.00
2,4,6-Gal	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
2,6-Glc			0.65					
2,4-Glc					0.45		0.38	
2-Glc					0.53			

[a] 2,3,4,6-Gal = 1,5-di-O-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, *etc.* [b] I, native; II, depyruvylated polysaccharide; III, depyruvylated, uronic-ester-reduced, remethylated polysaccharide; IV, product from β-elimination; V, product from selective hydrolysis (I), uronic-ester-reduced; VI, product from selective hydrolysis (II); VII, Smith-degradation product; VIII, reduced oligosaccharide **2**, after reduction of uronic ester.

Partial hydrolyses. — Three groups of experiments were carried out. In the first, referred to as selective hydrolysis, conditions were chosen in an attempt to remove the lateral rhamnosyl unit. In the first attempt, only ~50% of the lateral rhamnose was removed, but the formation of 2,4-di-*O*-methylglucose (after reduction) demonstrated that the side chain was attached to the glucuronic acid at O-4 (Table II, column V). A second, more vigorous hydrolysis removed all the terminal rhamnose, but did not reveal any other terminal sugar (Table II, column VI).



Scheme 1. Oligosaccharides obtained from *E. Coli* K26 capsular polysaccharide.

TABLE III

G.l.c.–c.i.-m.s. of methylated oligosaccharides obtained from *E. coli* K26 polysaccharide

Oligosaccharide fraction	*Relative retention time[a] (area%)*	*Chemical-ionization mass-spectral ions* (m/z) *(Relative abundance)*							
		$(M+NH_4)^+$	$(M+H)^+$	ROH_2^{+b}	R^{+b}	G^{+b}	$(R-MeOH)^+$	$(G-MeOH)^+$	*Other fragment ions*
3 β-GlcA-(1→3)-α-Rha-(1→3)-α-Rha-(1→3)-Rha-ol	5.80 (34)	820 (18)		223 (25)	205 (9)	233 (9)	173 (35)	201 (100)	423 (25)
2 β-GlcA-(1→3)-α-Rha-(1→3)-Rha-ol	3.34 (14)	646 (18)		223 (10)	205 (2)	233 (13)		201 (100)	407, 424 (11) (20)
4 β-Gal-(1→3)-β-GlcA-(1→3)-Rha-ol	3.54 (4)	676 (39)		223 (4)	205 (7)	219 (7)		187 (23)	437, 405 (1) (1)
5 α-Rha-(1→3)-α-Rha-(1→3)-Gal-ol	3.02 (33)	632 (13)	615 (4)	253 (80)	235 (13)	189 (61)		157 (20)	380, 363, 205 (42) (32) (52)

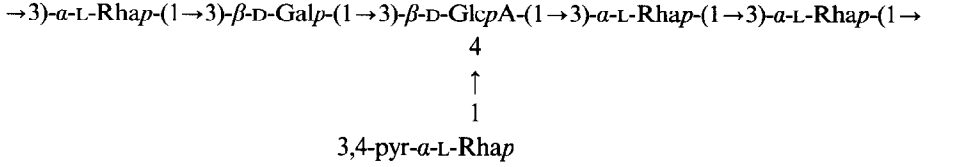
[a] DB-5, 210° for 2 min, then 6°.min^{-1} to 290°. Retention times relative to that of methylated sucrose (3.24 min). [b] G and R represent the terminal non-reducing sugar and reducing sugar (converted into its alditol), respectively.

The first partial hydrolysis (I, see Scheme 1) produced four oligosaccharides (p.c.), but only two (**1** and **2**) were obtained in adequate quantities and in sufficient purity to allow analysis by ^{1}H-n.m.r. spectroscopy (Table I); **1** was shown to be β-D-GlcA-(1→3)-L-Rha, which had been characterized earlier[8], and **2** was shown to be β-D-GlcA-(1→3)-α-L-Rha-(1→3)-L-Rha by analysis of its carboxyl-reduced, methylated alditol (Table II, column VIII).

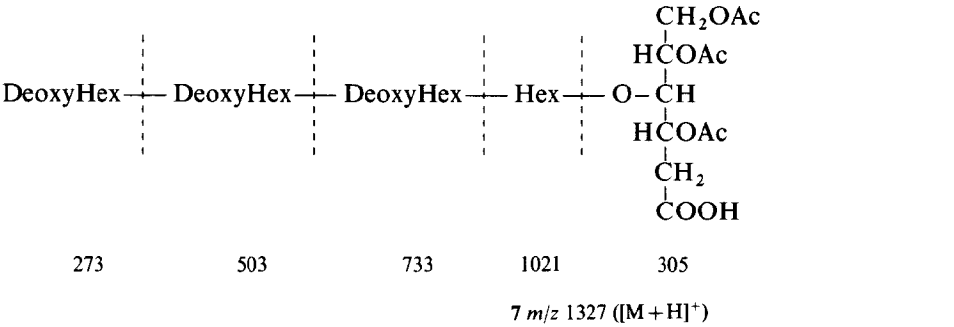
The saccharides **1** and **2** do not account for the fourth rhamnose residue nor the galactose. A second partial hydrolysis (II) was undertaken in an attempt to isolate an oligosaccharide which would enable the sequence of these residues to be established. Four fractions (**3**–**6**) (Scheme 1) were obtained by gel filtration on Bio-Gel P2, each was reduced and methylated, and the products were examined by g.l.c.–c.i.-m.s.

The main components of each fraction are presented in Tables III and IV, but each fraction contained minor components which were not examined further. From these results, it transpired that fractions **4** and **5** each contained **2**.

Despite the fact that p.c. of fraction **6** gave only a single discrete spot, analysis by g.l.c. of this fraction after methylation was revealing (Table IV). The analysis of mixtures of methylated oligosaccharides by g.l.c.–c.i.-m.s. has been reported[9] and the technique may be applied to mixtures of acidic and neutral oligosaccharides, thus avoiding the use of ion-exchange columns. G.l.c.–c.i.-m.s. of **3** and **4** (Table III) indicated the structure of the *E. coli* K26 antigen as shown.



Lithium–ethylenediamine degradation[10]. — This reaction was carried out in order to provide complementary evidence for the structure proposed. The product **7** was isolated by gel-permeation chromatography, and f.a.b.-m.s. of acetylated **7** indicated the following structure.



7 *m/z* 1327 ([M+H]$^+$)

TABLE IV

Relative retention times and ions obtained on g.l.c.–c.i.-m.s. of methylated disaccharides obtained from *E. coli* K26 capsular polysaccharide

Oligosaccharide fraction	Relative retention time[a]	Chemical-ionization mass-spectral ions (m/z) (*Relative abundance*)										
		$[M+NH_4]^+$	$[M+H]^+$	$[M+NH_4-MeOH]^+$	$[M+H-MeOH]^+$	ROH_2^{+b}	R^+	G^{+b}	G'^{+b}	$[R-MeOH]^+$	$[G'-\mathrm{MeOH}]^+$	$[G-MeOH]^+$
6-1 α-Rha-(1→3)-Rha	0.70	412 (17)		380 (18)	363 (35)				189 (100)			157 (95)
6-1′ α-Rha-(1→3)-Rha-ol	0.58		411 (8)		379 (51)	223 (78)	205 (16)		189 (100)	173 (9)		157 (45)
6-2 α-Rha-(1→3)-Gal	1.06	442 (12)		410 (10)	393 (38)			219 (93)	189 (94)		187 (68)	157 (86)
6-2′ α-Rha-(1→3)-Gal-ol	0.84		441 (19)		409 (10)	253 (93)	235 (72)		189 (100)	203 (2)		157 (60)
6-3 β-GlcA-(1→3)-Rha	1.21	456 (68)		424 (12)	407 (8)			189 (9)	233 (30)		157 (40)	201 (100)
6-3′ β-GlcA-(1→3)-Rha-ol	0.94	472 (26)	455 (10)		423 (20)	223 (69)	205 (13)		233 (30)	173 (28)		201 (100)

[a] DB-17, 210° for 1 min, then 4°.min^{-1} → 240°. Retention times relative to that (5.98 min) of methylated sucrose. [b] G and G′ represent the non-reducing and reducing sugars, respectively, in samples. The alditols are represented by R.

The signal at *m/z* 1327, possibly for $(M + H)^+$ with weaker signals at *m/z* 1344 ($[M + NH_4]^+$) and 1349 ($[M + Na]^+$), suggested the presence of a chemically degraded product attached to the hexose residue[11,12]. G.l.c.–c.i.-m.s. indicated methylated **7** to contain mainly a rhamnose trisaccharide-alditol (**7**-1) with smaller amounts of a rhamnobiose (**7**-2) and the corresponding alditol (**7**-3). Thus, it is apparent that **7**-2 and **7**-2′ are identical to **6**-1 and **6**-1′ (partial hydrolysis II, Table IV). The higher-molecular-weight oligosaccharide **7** was not observed. These results suggest that some peeling[13] had occurred during the degradation, which is to be expected with a polymer containing all (1→3) linkages.

The structure of the *E. coli* K26 antigen, established as shown above, confirms the predictions of Heidelberger[2] who anticipated, on the basis of serological cross reactions, that one rhamnose residue would be present as a terminal unit in a side chain, glucuronic acid would be the branch point, and that at least one 3-linked rhamnose residue would be present. On the basis of the ^{1}H-n.m.r. spectrum, it is apparent that the pyruvate substitution is not completely stoichiometric.

EXPERIMENTAL

General procedures and instrumentation have been described[14]. Analytical and preparative p.c. were performed using *1*, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); *2*, ethyl acetate–pyridine–water (8:2:1); or *3*, 1-butanol–acetic acid–water (4:1:5). Chromatograms were developed using alkaline silver nitrate. Gel-permeation chromatography was performed on a column (95 × 3 cm) of Bio-Gel P2 by elution with acidified distilled water (1 drop of HCOOH per L).

Mono- and oligo-saccharides were reduced with aqueous sodium borohydride at room temperature for 3 h. Alditols were acetylated with acetic anhydride–pyridine (1:1) for 30 min at 100°. G.l.c. was performed on capillary columns with the following programs: alditol acetates (DB-17), 180° for 2 min then 5°.min^{-1} to 220°; partially methylated alditols (DB-17), 180° for 1 min then 2°.min^{-1} to 250°, methylated oligosaccharides (DB-5), 210° for 2 min then 6°.min^{-1} to 290°, and methylated higher oligosaccharides (DB-1), 240° for 1 min then 290° at 7°.min^{-1}. Retention times are expressed relative to that of methylated sucrose. Molar ratios are corrected by use of the effective carbon-response factors[15].

The ^{1}H- (95°) and ^{13}C-n.m.r. (~20°) spectra were recorded with Bruker WH-400 and Varian XL-300 instruments, respectively, with acetone as the internal standard (δ 2.23 for ^{1}H and 31.07 p.p.m. for ^{13}C, each value being measured against external sodium 4,4-dimethyl-4-silapentane-1-sulfonate).

Preparation and properties of the K26 capsular polysaccharide. — A culture of *E. coli* K26 bacteria, obtained from Dr. I. Ørskov (Copenhagen), was grown on Mueller–Hinton agar (containing 0.5% of NaCl) for 6 days at 37°. The acidic capsular polysaccharide was isolated and purified by precipitation with cetyltrimethylammonium bromide (CTAB) as described[14]. The purified polysaccharide was shown to be homogeneous by gel chromatography on Sepharose 4B and its molecular mass was estimated as

1×10^7 Da. The native and depyruvylated polysaccharides were examined by ^{1}H- and ^{13}C-n.m.r. spectroscopy, and the principal signals and their assignments are recorded in Table I.

Methylation analysis. — The K26 polysaccharide (40 mg) was converted into its free acid form and methylated by the Hakomori procedure[4,5]. The product showed no i.r. absorption for hydroxyl groups. The methylated polysaccharide was hydrolyzed with 2M trifluoroacetic acid (18 h, 95°), and the resulting partially methylated sugars were analyzed as their alditol acetate derivatives by g.l.c.–m.s. (Table II, column I). The polysaccharide (17 mg) was depyruvylated by passage of an aqueous solution through a column of Amberlite IR-120 (H^+) resin (X4) and the product was methylated. A part of the product was hydrolyzed and analyzed as before (Table II, column II). The remainder (10 mg) was reduced with lithium aluminium hydride in oxolane (18 h, 25°), remethylated, and hydrolyzed (2M trifluoroacetic acid, 4 h, 95°). The g.l.c. results for the partially methylated alditol acetates are given in Table II, column III.

β-Elimination[6]. — To a solution of methylated K26 polysaccharide (30 mg) in methyl sulfoxide and 2,3-dimethoxypropane (19:1, 20 mL) was added a trace of *p*-toluenesulfonic acid. Sodium methylsulfinylmethanide (2M, 10 mL) was added and the solution was stirred, under nitrogen, overnight at room temperature. Methyl iodide (3 mL) was added to the cooled mixture which was stirred for 3 h, then partitioned between chloroform and water. The chloroform-soluble material was purified by passage through a column of Sephadex LH 20, then hydrolyzed (2M trifluoroacetic acid, 4 h, 95°), and the products were analyzed as the partially methylated alditol acetates by g.l.c.–m.s. (Table II, column IV).

Selective hydrolysis. — A solution of the K26 polysaccharide (580 mg) in 0.1M trifluoroacetic acid was heated for 15 min on a steam bath. The excess of acid was removed by repeated co-evaporation with water, and an aqueous solution of the residue was dialyzed overnight, then lyophilized. A portion (5 mg) of the residue (230 mg) was methylated, reduced, and hydrolyzed. G.l.c.–m.s. of the products as the partially methylated alditol acetates showed that 50% of the terminal rhamnosyl units had been removed (Table II, column V). A second selective hydrolysis on K26 polysaccharide (80 mg), using trifluoroacetic acid (10 mL, 0.1M, 30 min, 95°), gave, after isolation of the product on Bio-Gel P2, a product which had lost at least 80% of the terminal rhamnose (Table II, column VI). This result and the presence of a small ^{13}C resonance at 23.00 p.p.m. allowed a residual signal at 101.44 p.p.m. to be assigned to rhamnose with its pyruvate substituent still attached, and that at 99.65 p.p.m. to the terminal rhamnose without a pyruvate substituent.

Periodate oxidation and Smith degradation[7]. — A solution of depyruvylated K26 polysaccharide (15 mg) in 0.1M sodium metaperiodate was kept in the dark at room temperature. After 72 h, ethylene glycol (0.5 mL) was added, the polyaldehyde was reduced with sodium borohydride, the excess of $NaBH_4$ was neutralized with acetic acid (50%), and the mixture was dialyzed against tap water and then lyophilized. The polyol was hydrolyzed with 0.5M trifluoroacetic acid (16 h, 25°), the hydrolysate was desalted by passage through a column of Sephadex G10, and a portion (5 mg) was methylated,

reduced, and hydrolyzed (2M trifluoroacetic acid, 18 h, 100°). The partially methylated sugars were analyzed as their alditol acetates by g.l.c.–m.s. (Table II, column VII).

Partial hydrolysis. — (*a*) A solution of the selectively hydrolyzed polymer in M trifluoroacetic acid was heated for 1 h (95°). After removal of the excess of acid by co-evaporation with water, the hydrolysate was dialyzed (mol. wt. cut-off 3500) against distilled water and then lyophilized, and the residue was subjected to a second hydrolysis (M trifluoroacetic acid, 1 h, 95°), followed by dialysis. The diffusates were combined and lyophilized, and the oligosaccharides were isolated by preparative p.c. (solvent *1*). Two acidic oligosaccharides (**1** and **2**) were obtained in sufficient purity and yield to merit further analysis. On the basis of its ^{1}H-n.m.r. spectrum, **1** (5 mg) was deduced to be the aldobiouronic acid previously isolated and fully characterized (methylation gave 2,4-di-*O*-methylrhamnose)[8]. An aqueous solution of **2** (5 mg) was reduced with excess of $NaBH_4$ (3 h, 25°), and the product was methylated, carboxyl-reduced, and analyzed (Table II, column VIII).

(*b*) A solution of K26 polysaccharide (1.6 g) in H_2SO_4 (200 mL, 0.025M) was heated on a steam bath for 1.5 h, then neutralized with lead carbonate, and centrifuged. The supernatant solution was dialyzed against distilled water, and the diffusate was concentrated, deionized, and analyzed by p.c. (solvents *1* and *2*). Rhamnose appeared to be the only component of the diffusate. The retentate was lyophilized and subjected to a second mild acid hydrolysis (0.5M trifluoroacetic acid, 1.5 h, 95°). The excess of acid was removed under diminished pressure and the final traces were neutralized with sodium hydrogencarbonate. The hydrolyzate was desalted on a column (86 × 3 cm) of Sephadex G10 and subjected to gel-permeation chromatography to give four fractions **3**–**6**.

Each of the fractions **3**–**5** was reduced and methylated, and the products were examined by g.l.c.–c.i.-m.s. (Table III). Fraction **6**, a minor fraction, appeared to be pure by p.c. (solvent *3*, R_{GAL} 0.49) and, following methylation, was analyzed by g.l.c.–c.i.-m.s. before and after reduction to the alditol (Table IV).

Lithium–ethylenediamine-mediated degradation[10]. — Dry K26 polysaccharide (130 mg) was suspended in ethylenediamine (7 mL), with stirring, for 30 min. Three pieces of lithium wire (3 x 3 mm), washed in hexane, were added at intervals of 15 min. The reaction began almost immediately and a deep blue color was maintained for 1 h. The reaction was terminated by the addition of anhydrous methanol (1–2 mL) to the cooled mixture, and the excess of ethylenediamine and methanol was removed under vacuum, over H_2SO_4 and NaOH, for 3 h. Glacial HOAc was added to the residue, with cooling, to destroy the lithium methoxide, an aqueous solution (4 mL) of the product was desalted on a column of Sephadex G10, and the fractions containing sugar (phenol–sulfuric acid[16]) were combined and eluted from a column of Bio-Gel P2 to give **7**. Acetylated oligosaccharide **7** was analyzed by f.a.b.-m.s. and methylated **7** by g.l.c.–c.i.-m.s.

Determination of absolute configuration. — A solution of carbodiimide-reduced K26 polysaccharide (16.0 mg) in 2M trifluoroacetic acid was kept overnight at 95°. The resulting monosaccharides were separated by preparative p.c. (solvent *2*). Each sugar (2

mg) was treated with refluxing (−)-2-octanol containing one drop of trifluoroacetic acid (95°, 18 h). Each mixture was concentrated to dryness under vacuum (50°) and the residue was acetylated (1:1 acetic acid–pyridine, 30 min, 95°). The acetylated octyl glycosides were analyzed by g.l.c.–m.s. (DB-17 at 180° for 2 min, then 5°.min^{-1} to 220°) and their absolute configurations were determined by comparison with authentic standards.

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