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STRUCTURE OF THE AMINO ACID-CONTAINING CAPSULAR POLYSACCHARIDE (K54 ANTIGEN) FROM *Escherichia coli* O6:K54:H10

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ABSTRACT

The structure of the K54-antigenic polysaccharide (K54 antigen) of *Escherichia coli* O6:K54:H10 was elucidated by determination of the composition, ^{1}H- and ^{13}C-n.m.r. spectroscopy, periodate oxidation, and a study of the oligosaccharides obtained by partial hydrolysis with acid. The K54 polysaccharide consists of →3)-β-D-glucosyluronic acid-(1→3)-α-L-rhamnosyl-(1→ repeating-units. Of the glucuronic acid residues, ~85% are substituted in the ratio 9:1 with L-threonine and L-serine amidically linked to the carboxyl group. The K54 polysaccharide has a molecular weight of ~160,000, corresponding to ~380 repeating-units.

INTRODUCTION

There is a correlation between the pathogenicity of *Escherichia coli* and the nature of the surface polysaccharides[1,2], and the capsules play an important role in the pathogenicity of many of these bacteria. The capsules are composed of acidic polysaccharides (K antigens). They can be divided into polysaccharides of high molecular weight with low charge-density and polysaccharides of low molecular weight with high charge-density[3]. Invasive *E. coli* causing septicaemia or urinary tract infections mostly have capsules composed of the latter type of polysaccharide[1–3]. The acidic components of these capsular polysaccharides may be phosphate, *N*-acetylneuraminic acid, glucuronic acid, or 3-deoxy-D-*manno*-2-octulosonic acid (KDO). The K6, K13, K20, and K23 antigens contain KDO and ribose[4–7], the K12 antigen contains KDO and rhamnose[8], and the K14 antigen contains KDO and 2-acetamido-2-deoxygalactose[9]. The K1 antigen is a poly-2,8-α-*N*-acetylneuraminic acid[10,11] and the K2 antigen is a teichoic acid-like polymer[12], consisting of galactose and glycerol phosphate[13,14]. Galactose phosphate and fructose were recently found to be constituents of the K52 antigen[15]. The capsules of most invasive *E. coli* are expressed at 37° but not at room temperature (F. Ørskov, personal communication), and this is true for all capsular antigens mentioned above. However, there are some exceptions and one of these is the K54 antigen of uropathogenic *E. coli*, which is expressed by the bacteria at all temperatures. We now describe an investigation of the K54 antigen and propose a primary structure.

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RESULTS AND DISCUSSION

The capsular (K54) polysaccharide was precipitated[6,16] from liquid cultures of *E. coli* A12b (O6:K54:H10) together with the bacterial cells using hexadecyltrimethylammonium bromide (Cetavlon) and extracted from the precipitate with calcium chloride. After repeated precipitation from aqueous solution with ethanol, extraction with cold phenol at pH 6.5, and dialysis, the polysaccharide was isolated by lyophilisation in a yield of 80–100 mg/L of culture medium.

The polysaccharide comprised glucuronic acid and rhamnose in the molar ratio 0.9:1, together with threonine and serine in the molar proportions of 0.6 and 0.1, respectively, relative to glucuronic acid. No acetyl groups were detected.

The L configuration of the rhamnose was established by g.l.c. of the derived (+)-2-octyl 2,3,4-tri-*O*-acetylrhamnosides[17], which exhibited the same patterns of peaks (*T*, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, 1.61 and 2.44 at 150° on ECNSS-M; 3.85, 4.29, and 5.13 at 170° on 2.5% SE-30) as those obtained for the derivative prepared from L-rhamnose. The absolute configuration of threonine was determined as L by g.l.c. of its (+)-2-butyl ester[18].

In immune electrophoresis, the K54 polysaccharide exhibited a precipitation line with Cetavlon[19] and also with the anti-K54 serum, but not with anti-O6 serum. This showed that the acidic polysaccharide exhibited serological K54 specificity. Immune electrophoresis, analytical ultracentrifugation, and gel-permeation chromatography revealed the K54 polysaccharide to be heterogeneous in terms of molecular weight. The preparations appeared to be homogeneous after heating to 100° at pH 5–6, incubation in 0.1M NaOH at 37°, or in the presence of Triton X-100. These characteristics have been described for some capsular polysaccharides of invasive *E. coli* and they apply fully to the K54 antigen.

The K54 polysaccharide had $[\alpha]_D^{25}$ −76° (*c* ~0.1, water) and an average molecular weight of 160,000 as determined by the method of Yphantis[20].

The amino acids could not be removed from the K54 polysaccharide by treatment with 0.1M sodium hydroxide for 2 h at room temperature, indicating that they were bound by amide linkages which was in accord with the n.m.r. data (see below). Incubation[21,22] of the polysaccharide in M sodium hydroxide for 6 h at 50° under nitrogen gave a product that was eluted with water in the void volume of Sephadex G-50 and contained glucuronic acid and rhamnose in the ratio 0.8:1. The average molecular weight of this amino acid-free polysaccharide was 160,000, as determined with the method of Yphantis[20], indicating that there was little or no degradation during the removal of the amino acids.

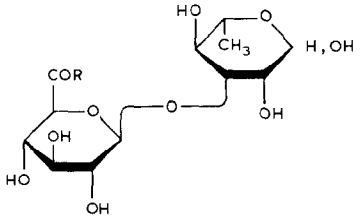
The K54 polysaccharide and the amino acid-free derivative were treated with periodate, and the products were reduced with borohydride. Subsequent analysis showed that the products had the same composition as the respective starting-materials, indicating that they contained (1→3) linkages.

The amino acid-free K54 polysaccharide was methylated by the Hakomori method[23,24] and then carboxyl-reduced with lithium aluminium deuteride. The

resulting polymer was hydrolysed and the products were converted into the alditol acetates. G.l.c. (170°, on ECNSS-M) revealed two components (T 1.00 and 5.08) which m.s. indicated to be 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl(6-$^2H_2$)glucitol. These results confirmed the presence of (1→3) linkages in the parent polysaccharide.

When the K54 polysaccharide was hydrolysed with 0.5M sulfuric acid at 100° for 2 h, preparative paper electrophoresis of the neutralised hydrolysate revealed two charged saccharides, **1** (M_{GlcA} 0.68) and **2** (M_{GlcA} 0.55), which were purified further by chromatography on Biogel P-2 (**1**, K_d 0.68; **2**, K_d 0.43). Both **1** and **2** contained equal amounts of glucuronic acid and rhamnose.

Oligosaccharide **1** (22% yield) had $[\alpha]_D^{25}$ −41° (*c* 0.06, water) and, on incubation with β-D-glucuronidase, gave glucuronic acid and rhamnose. Reduction of **1** with borodeuteride, followed by methylation[23,24], gave a product that was purified by chromatography on Sephadex LH-20. G.l.c. (200° on 2.5% of SE-30 on Chromosorb) of this product revealed one component (T 1.98) and c.i.-m.s. with ammonia as the reagent gas gave a peak for the quasi-molecular ion $(M + NH_4)^+$, with m/z 473, indicating a molecular weight of 455 and also that **1** was a disaccharide. The e.i. mass spectrum revealed the primary fragments, m/z 233 (→ 201 → 169) and 206 (→ 174 → 142). Fragment m/z 233 arose from the methylated, non-reducing glucosyluronic acid moiety, and the fragment m/z 206 arose from the deuterated, methylated rhamnitol. The fragmentation pattern of the latter indicated it to be substituted at C-3, further confirming the glucosyluronic acid-(1→3)-rhamnose structure.



1 R = OH

2a R = NH—CH(COOH)—CHOH(Me)

2b R = NH—CH(COOH)—CH_2OH

Oligosaccharide **2** (18% yield) had $[\alpha]_D^{25}$ −25° (*c* 0.02, water). Borodeuteride reduction of **2**, followed by methylation and purification on Sephadex LH-20, gave a product which contained three components in g.l.c. with T 16.46 (**3a**, major), 16.12 (**3b**), and 14.42 (**3c**).

C.i.-m.s. of the major component **3a** gave a quasi-molecular ion $(M + NH_4)^+$

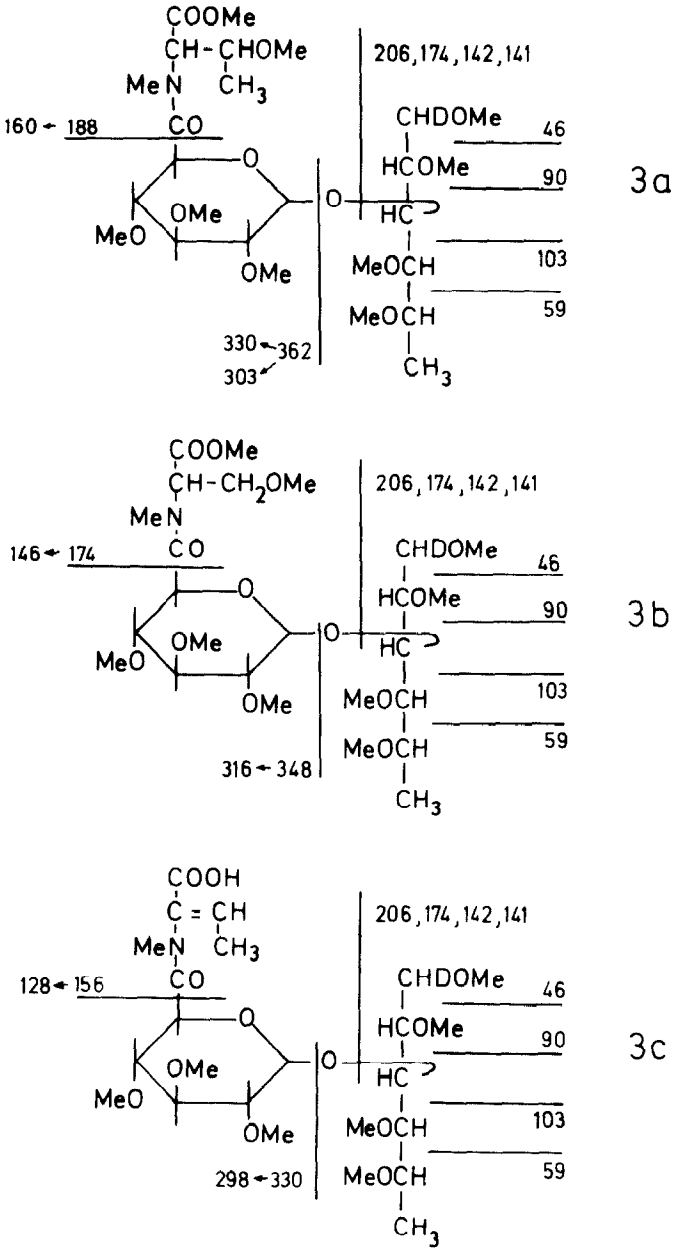


Fig. 1. Fragmentation patterns of **3a**, **3b**, and **3c**.

with *m/z* 602 indicating a molecular weight of 584, which was 129 mass units larger than the corresponding derivative of **1**. The fragmentation pattern reflected by the e.i. mass spectrum is shown in Fig. 1 and differed from that of the corresponding derivative of **1**. The formation of ions with *m/z* 188, 160, 362, and 330 showed that threonine was linked to the glucuronic acid residue, and the ions with *m/z* 156 (188

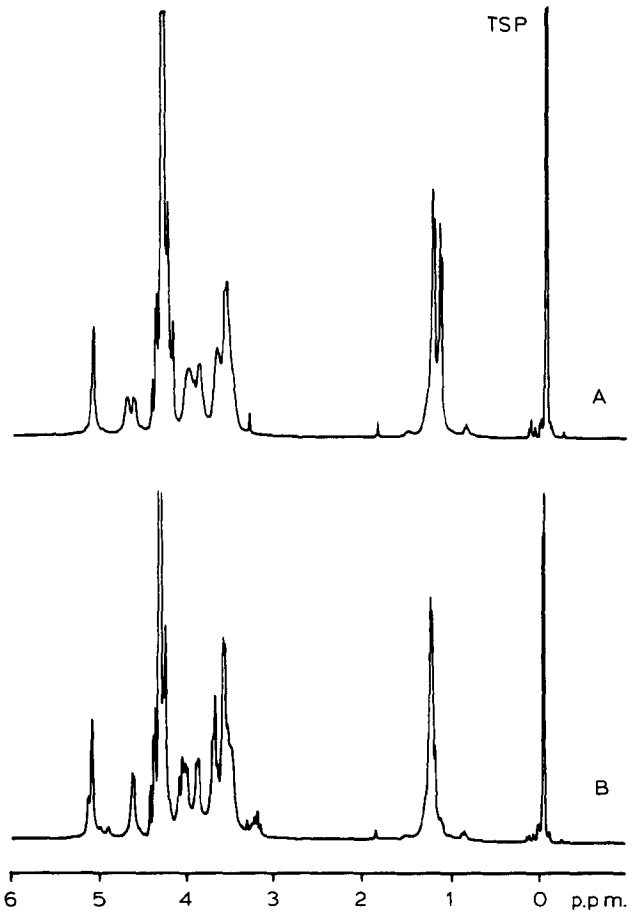


Fig. 2. ^{1}H-N.m.r. spectrum (300 MHz) of the native (A) and amino acid-free K54 polysaccharide (B) in solution in deuterium oxide at 70° with sodium 4,4-dimethyl-4-sila(2,2,3,3-$^2H_4$)pentanoate as external reference.

– MeOH) and 128 (160 – MeOH) were taken to indicate an amide linkage between the uronic acid and the threonine. The fragments derived from the rhamnitol moiety (*m/z* 46, 59, 90, and 103) showed that it was substituted at C-3. Thus, **3a** was identified as **1**, amidically linked to threonine.

Oligosaccharide **3b**, analysed as for **2a**, was found to be the serine analogue of **3a**. The fragmentation pattern is shown in Fig. 1.

The e.i. mass spectrum of **3c** revealed a fragmentation pattern (Fig. 1) which showed that **3c** was an artefact, derived from **3a** by loss of methanol or from **2a** by dehydration. The quasi-molecular ion $(M + NH_4)^+$ of **3c** had *m/z* 570, which was 32 mass units smaller than that of **3a**. The structures of the disaccharides as characterised by methylation analysis are **1, 2a,** and **2b**.

The ^{1}H-n.m.r. spectrum of the K54 polysaccharide (Fig. 2A) contained

TABLE I

TENTATIVE ASSIGNMENTS OF THE SIGNALS FROM THE ^{13}C-N.M.R. SPECTRA OF THE K54 POLYSACCHARIDE (PS), ITS AMINO ACID-FREE FORM (PSdaa), DISACCHARIDE **1**, AND REFERENCE COMPOUNDS

	T1[a]	*G6*	*G6a*	*G1*	*R1*	*G3*	*R3*	*G5*	*G2*	*G4*	*R4*	*R2*	*R5*	*Tβ*	*Tα*	*Tγ*	*R6*
K54 PS	177.5	176.8	171.5	104.9	102.1	83.4	82.2	77.7	75.1	72.4	71.7	71.2	70.1	69.3	61.1	20.5	18.0
				104.8	101.9	83.2	82.0	76.2	74.8	72.3	71.5						
K54 PSdaa		175.4		104.6	101.7	83.1	81.8	76.9	74.8	72.2	71.3	71.0	69.9				17.6
Me β-D-Glc*p*A-6(*N*)-L-Thr[21]	177.7		172.0	104.9		77.2		76.5	74.5	73.4				69.4	61.7	20.9	
1		176.3		104.2	94.6	76.7	83.0	76.3	74.1	72.5	72.0	71.1	69.0				17.6
					94.1		81.0				72.5	71.8					
Me α-Rha[30]					102.3		71.7				73.4	71.4	69.6				18.0
α-Rha[25]					94.7		70.9				73.1	71.2	68.9				17.6
β-Rha[25]					94.2		73.6				72.7	72.1	72.7				17.6

[a]T, threonine; G, glucuronic acid; R, rhamnose.

signals for the methyl protons of threonine (δ 1.15, $J_{\beta,\gamma}$ 6.2 Hz) and rhamnose (δ 1.23, $J_{5,6}$ 5.8 Hz). The anomeric region contained signals at δ 5.10 (H-1 of rhamnose[25]) and two poorly resolved doublets at δ 4.64 and 4.72; the signals at δ 1.15 and 4.72 were missing from the spectrum of the amino acid-free polysaccharide (Fig. 2B), reflecting the absence of threonine. The signal at δ 4.72 was probably due to H-1 of glucuronic acid substituted by threonine, whereas those at δ 4.64 for the native polysaccharide and at δ 4.63 for the amino acid-free form were due to H-1 of unsubstituted glucuronic acid[26,27]. There were no signals for acetyl and propionyl groups.

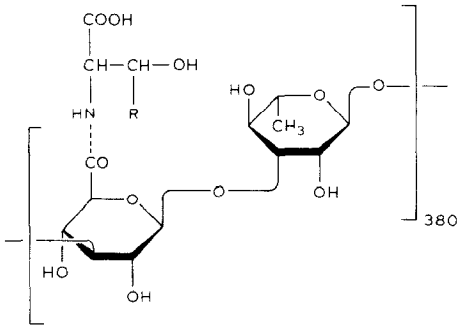
The ^{1}H-n.m.r. spectra of **1** and **2a** (not shown) were very similar to those shown in Figs. 2B and 2A, respectively. Since rhamnose is the reducing unit in **1** and **2a**, it gave two signals for H-1 at δ 4.81 (not given by the native polysaccharide) and 5.05 ($J_{1,2}$ 1.4 Hz) which was also given by the native and amino acid-free polysaccharide. The anomeric configuration of the rhamnose residue cannot be determined with certainty from the $J_{1,2}$ values[28,29], but a comparison of the chemical shifts of the signals for the anomeric protons of rhamnose in the polysaccharide and the disaccharides suggested an α linkage in the polymer.

The ^{13}C-n.m.r. data for the K54 polysaccharide and its amino acid-free form are shown in Table I. The presence of threonine was indicated[21] by the signals at δ 20.5, 61.1, 69.3, and 177.5. Due to incomplete substitution of the glucuronic acid by threonine, and to a much smaller degree with serine, most signals in the spectrum were split. This was also the case for the C-1 signal of rhamnose, which, in the polymer, seems to be close to the carboxyl group of glucuronic acid. The amino acid-free polysaccharide gave a simpler spectrum containing 12 signals which could be assigned with the aid of reference compounds[21,30] (Table I).

The anomeric configurations of the glucuronic acid and rhamnose moieties were determined by using a gated decoupling experiment[31–34] which gave $J_{1,2}$ values of 157.9 and 173.1 Hz, respectively.

The ^{13}C-n.m.r. spectra of **1** and **2a** (not shown) exhibited the same principal pattern of signals as those of the amino acid-free and native polysaccharide, respectively. Because of the reducing rhamnose moiety in **1** and **2a**, the α and β anomers gave rise to groups of signals, thus increasing the complexity of the spectra. The rhamnose C-1 signal of the amino acid-free polysaccharide appeared at lower field (δ 101.7) than the corresponding signal of the reducing rhamnose moiety in **1** (δ_α 94.6, δ_β 94.1). A similar α-shift was observed for the C-3 signal of glucuronic acid (δ 76.7 in **1** and δ 83.1 in the amino acid-free polysaccharide). The ^{13}C signals of **1** are included in Table I. The results of ^{13}C-n.m.r. spectroscopy show that the K54 polysaccharide contains (1→3)-linked β-glucosyluronic acid and α-rhamnosyl residues.

The foregoing results indicate that the K54 polysaccharide can be formulated as **4** in which the dotted line indicates incomplete substitution of the carboxyl group. The disaccharide β-D-glucosyluronic acid-(1→3)-L-rhamnose has also been isolated from the K26 polysaccharide of *E. coli* O8:K26:H$^-$ (K. Jann, unpublished).



4
R = Me (threonine, ~90 % of total substitution)
R = H (serine, ~10 % of total substitution)

Whereas *O*-acetylation is common with microbial polysaccharides, substitution with amino acids is not frequently encountered. *N*-Acetylserine is amide-linked to the amino group of 3-amino-3,6-dideoxyglucose in the cell-wall lipopolysaccharide of *E. coli* O114[35], and *N*-acetylalanine is linked likewise to the amino group of 2-amino-2-deoxyglucuronic acid in the cell-wall polysaccharide of *Staphylococcus aureus*[36]. In contrast, the *Shigella boydii* type 8 antigen[37] contains serine amide-linked through the amino group, the lipopolysaccharide of *Proteus mirabilis* strain 1959 contains amide-linked lysine[38], and the capsular polysaccharide of *Haemophilus influenza* type d contains amide-linked alanine, serine, and threonine[21,22]. The K54 polysaccharide also has this type of structure. Amino acids involved in ester linkages are found only in teichoic acids[39].

The K54 polysaccharide contains two amino acids (differing in size by one methylene group) as substituents competing for one substitution site. A similar situation was found in the *E. coli* K14 and K52 polysaccharides[9,15] where both acetyl and propionyl groups are present, but these groups have different positions in the K52 polysaccharide. Although substitution of microbial polysaccharides by non-carbohydrates is probably important in recognition processes (*e.g.*, antibodies or immune cells), the biological significance and the biosynthetic pathways are still unknown.

The K54 antigen belongs to a group of acidic polysaccharides which surround invasive *E. coli* cells as capsules. As was described for the K12 antigen of this group and demonstrated for others[40,41], the capsular polysaccharide described in this communication has its reducing end blocked by a phosphatidic acid[40] which allows the polysaccharide to form micelles and to associate with the bacterial cell-wall through hydrophobic interactions. The capsule expression, also with respect to temperature dependence, is currently being studied.

EXPERIMENTAL

Bacteria and cultivation. — *E. coli* A12b (O6:K54:H10) was obtained from

Drs. I. and F. Ørskov (Copenhagen) and grown to the late log phase (5–7 h) in a fermenter at 37° in 10-L batches, which contained per L: $K_2HPO_4 \cdot 3\,H_2O$ (9.7 g), KH_2PO_4 (2 g), sodium citrate · 5 H_2O (0.5 g), $MgCl_2 \cdot 7\,H_2O$ (0.1 g), casamino acids (20 g), and the dialysable part of yeast (100 mL from 500 g in 5 L of deionised water).

Isolation and purification of the capsular polysaccharide. — The acidic capsular polysaccharide and the bacterial cells were precipitated from the liquid culture by the addition of an equal volume of aqueous 2% hexadecyltrimethylammonium bromide (Cetavlon). All following operations were carried out at 4°. The polysaccharide was extracted from the precipitate with M calcium chloride, and purified by three cycles of precipitation from aqueous solution with ethanol (80% final concentration) followed by repeated extractions with cold phenol (80%, buffered to pH 6.5 with sodium acetate)[6,16]. The combined, final aqueous phases were centrifuged for 4 h at 105,000*g* and the supernatant solution was lyophilised. The residue was further purified by chromatography on Sephadex G-50.

Analytical methods. — Glucuronic acid was determined with the carbazole reagent[42], and rhamnose with the cysteine reagent[43] as well as by g.l.c. of the alditol acetate. Threonine and serine were determined, after hydrolysis of the polysaccharide with 4M hydrochloric acid for 18 h at 100°, with a Durrum D-500 amino acid analyser.

To determine the absolute configuration of threonine, the K54 polysaccharide (50 mg) was hydrolysed with M sodium hydroxide (4 mL) for 6 h at 50°. After neutralisation with Dowex 50 (H^+) resin, the mixture was dialysed against deionised water and the diffusate was concentrated to dryness. To the residue were added (+)-2-butanol (1 mL) and a drop of trifluoroacetic acid, and the mixture was heated (15 h, 100°). After removal of excess of reagent *in vacuo,* the residue was treated for 30 min at 100° with acetic anhydride (0.5 mL) and pyridine (0.7 mL). The reagents were removed by co-distillation with toluene under reduced pressure and the product was analysed by g.l.c. on a 25-m Carbowax capillary column (140°).

For the determination of the absolute configuration of rhamnose, the polysaccharide (10 mg) was hydrolysed with 0.5M sulfuric acid (1 mL) for 2 h at 100°. The hydrolysate was neutralised and the rhamnose component was isolated by high-voltage paper electrophoresis (Schleicher and Schüll paper, 2043b, 42 V/cm, pH 5.3, 90 min). The sample was heated with (+)-2-octanol (1 mL) and one drop of trifluoroacetic acid (15 h, 100°). The reagents were removed by co-distillation with water under reduced pressure (55°) and the residue was acetylated as described above. The product was analysed by g.l.c. on ECNSS-M (150°) and on 2.5% SE 30 (170°).

Optical rotations were measured with a Perkin–Elmer 141 polarimeter. G.l.c. was performed with a Varian Aerograph Series 1400 instrument, equipped with an autolinear temperature programmer and a Hewlett–Packard 3380 integrator. N.m.r. spectra were recorded with a Bruker WM 300 spectrometer in the F.t. mode at 70° [external sodium 4,4-dimethyl-4-sila(2,2,3,3-$^2H_4$)pentanoate]. The ^{13}C values

were corrected (−1.31 p.p.m.), using 1,4-dioxane (δ 67.4 based on Me_4Si), and the ^1H values by −0.07 p.p.m. (1,4-dioxane signal at δ 3.7). C.i. and e.i. mass spectra were obtained using a Finnigan 1020 mass spectrometer.

Removal of the amino acid substituents. — A solution of the K54 polysaccharide (100 mg) in M sodium hydroxide (4 mL) was kept under nitrogen at 50° for 6 h, neutralised with Dowex 50 (H^+) resin, dialysed against deionised water, and lyophilised. The residue was dissolved in a minimum amount of water and eluted from a column of Sephadex G-50 with water. The material eluted in the void volume was isolated by lyophilisation.

Periodate oxidation and methylation. — The procedures have been described in detail[6,8,9,13].

Carboxyl-reduction of the methylated amino acid-free polysaccharide. — To a solution of the methylated derivative (from 10 mg of amino acid-free K54 polysaccharide) in ether and chloroform (10 mL, 3:1) was added lithium aluminium deuteride (60 mg), and the mixture was boiled under reflux for 4 h. Water was then added dropwise with cooling in an ice-bath, the precipitate was dissolved in M sulfuric acid, the aqueous phase was extracted with chloroform (4 × 2 mL), and the combined organic phases were concentrated to dryness. The residue was hydrolysed (1 h, 100°) with aqueous 90% formic acid (1 mL) and, after evaporation of the acid, with 0.125M sulfuric acid (1 mL, 3 h, 100°). The mixture was neutralised, reduced and acetylated as described[23,24], and then subjected to g.l.c. and m.s.

Isolation of **1** *and* **2**. — The K54 polysaccharide (100 mg) was hydrolysed (2 h, 100°) in 0.5M sulfuric acid (3 mL). The hydrolysate was neutralised with barium hydroxide, concentrated *in vacuo* to ~0.5 mL, and subjected to high-voltage paper electrophoresis on Schleicher and Schüll paper No 2043b (42 V/cm, pH 5.3, 90 min). Guide strips stained with alkaline silver nitrate were used to locate **1** and **2** which were eluted from the appropriate areas with water. They were subsequently chromatographed on Biogel P-2 and isolated by lyophilisation.

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