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STRUCTURAL AND IMMUNOLOGICAL STUDIES OF THE *Escherichia coli* K7 (K56) CAPSULAR POLYSACCHARIDE

FAI-PO TSUI, ROBERT A. BOYKINS, AND WILLIAM EGAN*

Division of Biochemistry and Biophysics, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, Maryland 20205 (U.S.A.)

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ABSTRACT

The structure of the *Escherichia coli* K7 capsular polysaccharide has been investigated by a combination of chemical and spectroscopic methods. The structure of the repeating unit of the polymer was found to be →3)-β-D-ManNAcA-(1→4)-β-D-Glc-(1→; the O-6 atom of the D-glucosyl residue in the repeating unit is acetylated. The K7 polysaccharide is cross-reactive with the *Streptococcus pneumoniae* type 3 polysaccharide, the structure of which had previously been determined; our n.m.r. studies of the *S. pneumoniae* type 3 polysaccharide are in accord with this structure. The *E. coli* K7 and K56 capsular antigens have been shown by serology and ^{13}C-n.m.r. spectroscopy to be identical.

INTRODUCTION

Robbins *et al.*[1] reported that the capsular polysaccharide from *Escherichia coli* K7 strains is cross-reactive with that from *Streptococcus pneumoniae* type 3, a pneumococcal serotype often responsible for severe, invasive disease in children and adults[2]. The chemical structure of the type 3 capsule was determined by Hotchkiss and Goebel[3], and the repeating unit of the polymer was found to be →3)-β-D-GlcA-(1→4)-β-D-Glc-(1→. Although the composition of the *E. coli* K7 capsule is known (equimolar amounts of glucose and 2-amino-2-deoxymannuronic acid)[4,5], its structure is not. With a view to more firmly establishing the molecular basis for the cross-reactivity of these two polymers, we sought to determine the structure of the K7 polysaccharide, and we report our results herein.

RESULTS AND DISCUSSION

We have confirmed the composition of the K7 capsular polysaccharide reported by earlier workers[4,5]. Thus, subsequent to CMCT**-assisted, $NaBH_4$ reduction and

*To whom correspondence should be addressed.
**CMCT = sodium 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate.

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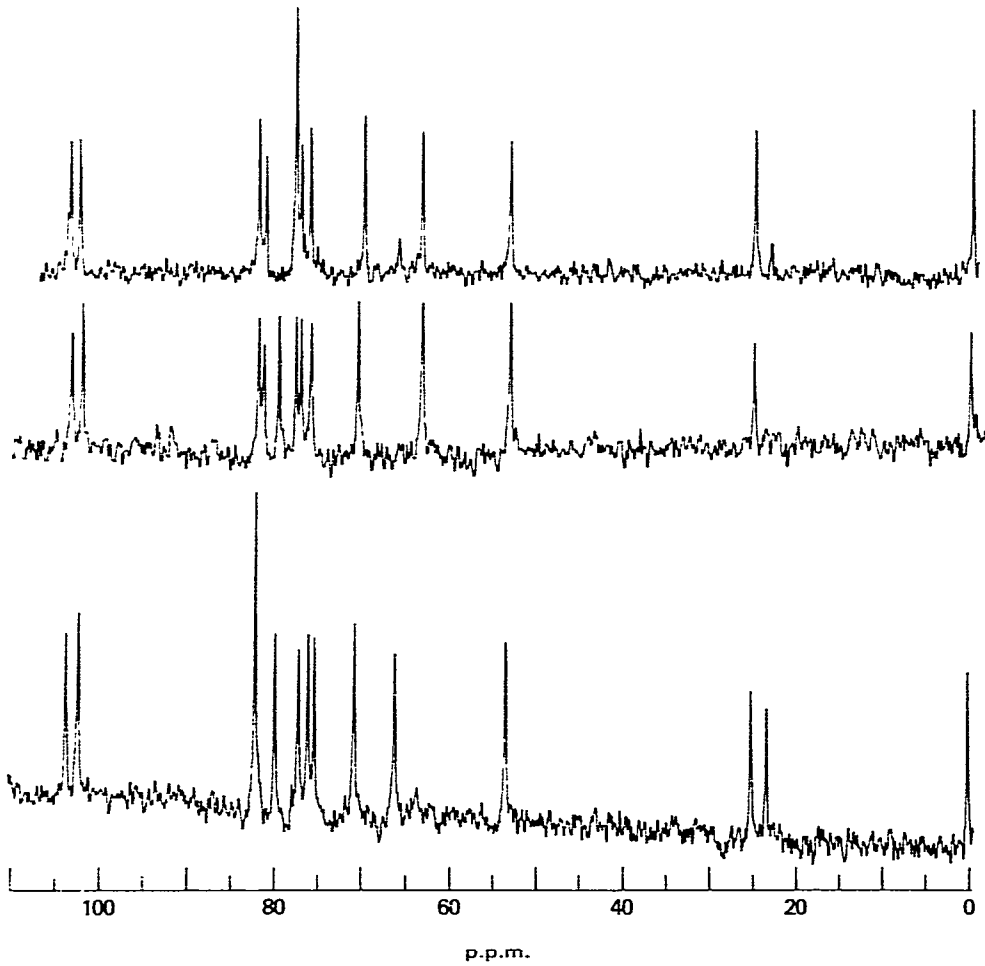


Fig. 1. ^{13}C-N.m.r. spectra (75.47 MHz) of the 0–110-p.p.m. region of native *E. coli* K7 polysaccharide (bottom trace), and *O*-deacetylated K7 polysaccharide at pH 6.5 (middle trace) and pH 1.5 (top trace). [Samples (~30 mg) were dissolved in 1:19 D_2O–H_2O (~1.5 mL) containing 0.01M EDTA, and 1% of sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (TSP) as the internal reference-standard, and placed in 10-mm, n.m.r. tubes. Approximately 25,000 transients were collected for each spectrum. Spectra were obtained by using $\pi/2$ pulses (~25 μs), 8192 data points, a 20-kHz spectral-window and matched filter, and a pulse repetition-time of 2.5 s. Prior to Fourier transformation, the free-induction-decay signal was zero-filled and then exponentially multiplied, so as to result in an additional, 1-Hz line-broadening in the frequency-domain spectrum.]

acid-catalyzed hydrolysis, only two monosaccharides were detected in appreciable quantities, namely, D-glucose and 2-amino-2-deoxy-D-mannose. (The CMCT–$NaBH_4$ reduction is required prior to the hydrolysis step, because the 2-acetamido-2-deoxymannosyluronic residue is unstable to the reaction conditions; see ref. 6.) The identity and absolute configuration of the mannuronic acid residue was established as described[6,7]; the identity and absolute configuration of the glucose residue

TABLE I

^{13}C-N.M.R. SPECTRA OF NATIVE AND CHEMICALLY MODIFIED *Escherichia coli* K7 POLYSACCHARIDE[a,b]

Carbon atom	*K7 Polysaccharide*	*O-Deacetylated K7 polysaccharide*	*$NaBH_4$-reduced K7 polysaccharide*
-NH-*C*(O)CH_3	177.90	177.90 (177.9)	177.65
-O-*C*(O)CH_3	176.59	—	—
C-6	177.9	177.9 (174.42)	62.8
C-1′	103.20	102.86 (103.03)	103.0
C-1	101.77	101.77 (102.0)	102.25
C-3	81.70	81.74 (81.74)	81.43
C-4′	81.70	81.19 (80.94)	81.17
C-5	79.45	79.35 (77.51)	78.98
C-5′	75.00	77.44 (77.51)	77.20
C-3′	76.73	76.89 (76.92)	76.58
C-2	75.66	75.76 (75.89)	75.45
C-4	70.42	70.39 (69.74)	67.81
C-6′	65.70	63.14 (63.21)	62.93
C-2	53.05	53.11 (53.0)	53.10
-NHC(O)*C*H_3	24.88	25.07 (24.97)	24.90
-OC(O)*C*H_3	23.06	— —	—

[a]Primed carbon atoms refer to the D-glucosyl, and unprimed carbon atoms to the 2-acetamido-2-deoxy-D-mannosyluronic acid, residues. [b]Values in parentheses refer to a solution of pH ~1.1; otherwise, of pH 6.5.

was established by a combination of chromatographic and enzymic methods (see Experimental section).

The ^{13}C-n.m.r. spectrum of the K7 polysaccharide is shown in Fig. 1; *cf.* Table I. The resonances at 103.2 and 101.8 p.p.m. may be unambiguously assigned to the anomeric carbon atoms (C-1) of the two sugar residues; comparison of these chemical shifts with those for model compounds[6–8] showed that C-1 in each of the sugars is involved as a glycoside. The relative, integrated absorption-intensities of these resonances showed that the two sugars are present in equimolar proportions (±5%). The 53.05-p.p.m. resonance is assignable to C-2 of the 2-acetamido-2-deoxy-D-mannosyluronic acid residue[8]. From its resonance position[8], the signal at 65.7 p.p.m. was assigned to C-6 of the D-glucosyl residue; this assignment was confirmed by obtaining the spectrum under ^{1}H-coupled conditions and observing this resonance as a triplet (C-6 of D-glucose is the only carbon atom in the polysaccharide bearing two hydrogen atoms and, thus, the only one that could give a triplet under non-decoupling conditions). The ^{13}C chemical-shift for C-6 of D-glucose is ~2–3 p.p.m. to lower field than is usually observed[8]. Acetyl-methyl signals were observed at 23.06 p.p.m. (*O*-acetyl) and 24.88 p.p.m. (*N*-acetyl); the associated carbonyl-carbon resonances were observed at 176.59 and 177.90 p.p.m.

Treatment of the K7 polysaccharide with NH_4OH (pH 11.0; 20°) led to selective removal of the *O*-acetyl group[9] (and provided a chemical confirmation of its spectro-

scopically determined presence in the native polymer). Prior to the addition of NH_4OH, two acetyl signals of equal intensity were discernible in the ^{1}H-n.m.r. spectrum (300 MHz) at 2.02 and 2.11 p.p.m. With time, following the addition of NH_4OH, the resonance at 2.11 p.p.m. decreased in absorption intensity, and eventually disappeared; concomitantly, two signals, at 1.89 p.p.m. (major) and 1.95 p.p.m. (minor), arose. (The major signal derives from acetate ion and the minor one from acetamide, as determined by addition of authentic material.) The ^{13}C-n.m.r. spectrum of the polymer, following NH_4OH treatment and extensive dialysis, is shown in Fig. 1b. The resonances that had been present at 176.6 and 23.06 p.p.m. in the spectrum of the native polysaccharide were not observed in that of the NH_4OH-treated material. Additionally, the D-glucose C-6 resonance (at 65.70 p.p.m. for the native polymer) occurred at 63.14 p.p.m.; this change in the chemical shift of the C-6 signal furnished evidence for acetoxylation at this position[8–10]. Comparative integration of the *N*- and *O*-acetyl methyl group signals in both the ^{13}C- and the ^{1}H-n.m.r. spectra showed that, to within experimental error (±5%), each D-glucose residue was *O*-acetylated.

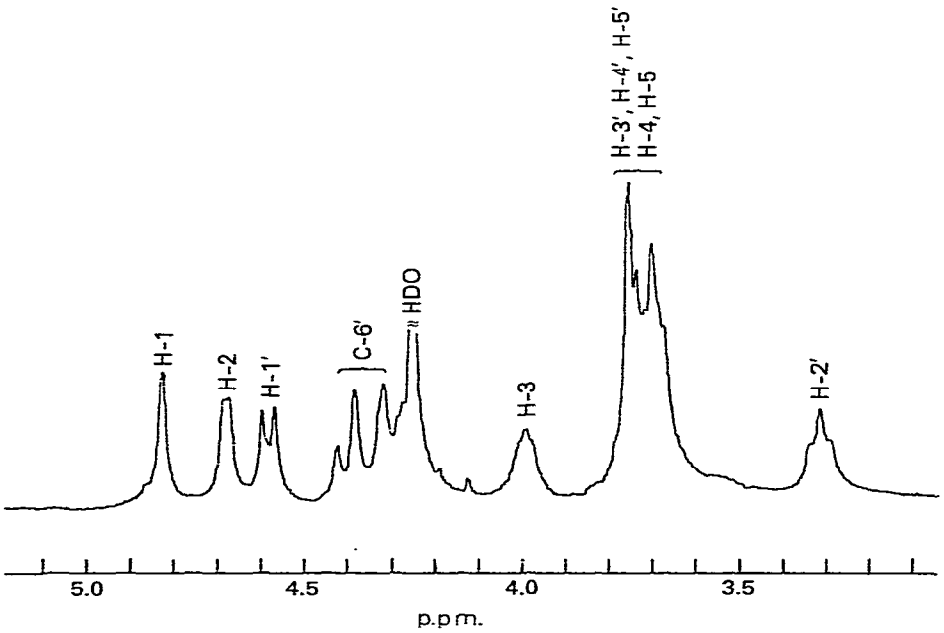


Fig. 2. ^{1}H-N.m.r. spectrum (300.15 MHz), in the ~3–5-p.p.m. range, of the native, *E. coli* K7 polysaccharide. [Following lyophilization from D_2O (99.7 atom% of D), the polysaccharide (5 mg) was dissolved in 0.5 mL of D_2O (99.98 atom% of D) to which a trace of TSP was added as a chemical-shift standard, and placed in a 5-mm, n.m.r. tube. Sixteen transients were collected; the spectrum was obtained by employing $\pi/2$ pulses (6 μs), 8192 data points, a 3-kHz spectral-window and matched filter, and a 2-s pulse-repetition rate. Prior to Fourier transformation, the free-induction-decay signal was zero-filled, and then exponentially multiplied, so as to result in an additional 0.25-Hz line-broadening in the frequency-domain spectrum. Spectral assignments were made on the basis of extensive, homonuclear-decoupling experiments (primed atoms refer to the D-glucosyl residue, and unprimed atoms, to the 2-acetamido-2-deoxy-D-mannosyluronic acid residue).]

The presence of a free carboxylic acid was established by titration of the carboxyl-carbonyl resonance in the ^{13}C-n.m.r. spectrum[6]. Thus, a change of ~3 p.p.m. in the chemical shift was found to accompany the lowering of the pH value of the solution from 6.5 to 1.5, *i.e.*, on passing through the pK_a value of the carboxylic acid (~3); this alteration in chemical shift with pH was reversible. In both the native and *O*-deacetylated polymers, a resonance at ~80 p.p.m. (at pH 7.0) was observed to titrate, and this was assigned to C-5 of the 2-acetamido-2-deoxy-D-mannosyluronic acid residue[6,7]. After reduction with CMCT–$NaBH_4$, the ^{13}C-n.m.r. spectrum of the polysaccharide was no longer pH-dependent (in the pH 1 to 7 region). The reduction also resulted in an additional, hydroxymethyl-group resonance, at 62.8 p.p.m., in the ^{13}C-n.m.r. spectrum.

The anomeric configuration of the D-glucosyl residue was determined by ^{1}H-n.m.r. spectroscopy; see Fig. 2. The observation of a doublet (*J* 7.7 Hz) at 4.58 p.p.m. (4.57 p.p.m. for the *O*-deacetylated material) established the β stereochemistry at C-1 of the D-glucosyl residue; additionally, no resonance was observed in the spectral region that is characteristic of the α-anomeric configuration (~5.1 p.p.m.)[11]. The value of the directly bonded, C-1–H-1 coupling constant, 164 Hz, confirmed the assignment of the β stereochemistry to the D-glucosyl residue[12].

Two additional resonances were observed in the low-field portion of the ^{1}H-n.m.r. spectrum of the K-7 polysaccharide, at 4.67 and 4.82 p.p.m.; these respectively correspond to H-2 and H-1 of the D-mannosyluronic acid residue (^{1}H-n.m.r.-spectral assignments for the K7 polysaccharide were made on the basis of extensive, homonuclear, double-irradiation experiments). Sequential, low-power, continuous-wave ^{1}H-irradiation of the signals at 4.82, 4.67, and 4.58 p.p.m. resulted in singlet resonances in the ^{13}C-n.m.r. spectrum for the resonances at 101.77, 53.05, and 103.20 p.p.m., respectively. These decoupling experiments confirmed the results of the homonuclear-decoupling experiments already mentioned, and, moreover, allowed assignment of the ^{13}C-n.m.r. signals at 103.2 and 101.77 p.p.m. to the anomeric carbon atoms of the D-glucosyl and D-mannosyluronic acid residues, respectively.

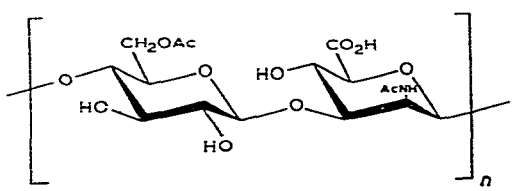
The anomeric stereochemistry of the D-mannosyluronic acid residue was determined primarily by ^{13}C-n.m.r. spectroscopy. The directly bonded, ^{13}C-1–^{1}H-1 coupling constant was 164 Hz, indicating β stereochemistry for this residue[12]. The resonance position for C-5, which is sensitive to anomeric stereochemistry, and occurs at 74.8 p.p.m. for the α and 79.1 p.p.m. for the β anomer[6] of 2-acetamido-2-deoxy-D-mannopyranose, was supportive of this assignment, as it occurred at 79.0 p.p.m. for the $NaBH_4$-reduced polysaccharide (the C-5 resonance position for the native polymer occurred at ~79 p.p.m. at pH 1.5, and ~77.0 p.p.m. at pH 6.5). The ^{1}H-n.m.r. chemical shift of the H-1 resonance was 4.82 p.p.m., corresponding to β stereochemistry for this residue (see ref. 6, and relevant citations therein).

The D-glucosyl residues of the polymer were oxidized by periodate, indicating substitution at either C-2 or C-4 (C-6 is acetoxylated). Acid-catalyzed hydrolysis, following periodate oxidation and $NaBH_4$ reduction, led to the formation of erythritol, identified as its peracetate by gas–liquid chromatographic comparison with authentic

material. The g.l.c. data thus provided evidence for C-4 substitution. The absence of a resonance signal in the 83–86-p.p.m. region of the ^{13}C-n.m.r. spectrum is consistent with the absence of either an O-3- or an O-2-linked D-glucosyl residue[8,10].

The remaining site of substitution on the 2-acetamido-2-deoxy-D-mannosyluronic acid residue was determined by ^{13}C-n.m.r. spectroscopy; the resonance at ~52.5 p.p.m., observed for the original polysaccharide, as well as for the chemically transformed polysaccharides (see Table I), is considerably shielded relative to the C-2 resonance for the (1→4)-linked 2-acetamido-2-deoxy-β-D-mannopyranosyl residues found in the *H. influenzae* type e polymer[6], and indicates alkoxylation at C-3 (alkoxylation generally causes an ~5–10-p.p.m., downfield shift of the signal of the contiguous carbon atom, and an ~0–3-p.p.m., upfield shift of those of adjacent carbon atoms[10]). Consistent with substitution at O-3 in this residue, the $NaBH_4$-reduced polymer was resistant to *N*-deacetylation by hydrazine (hydrazine-induced *N*-deacetylation is, however, readily accomplished for the afore-mentioned, O-4-linked material[6]).

The substitution pattern for the D-glucosyl and D-mannosyluronic acid residues was supported by methylation analysis of the $NaBH_4$-reduced K7 polysaccharide, using g.l.c.–mass spectrometry to identify the alditols produced. The $NaBH_4$-reduced K7 polysaccharide was methylated according to a procedure described by Björndal *et al.*[13] (which also results in methylation of the acetamido group). The permethylated polymer was hydrolyzed, the sugars reduced with deuterated sodium borohydride, and the alditols peracetylated. G.l.c. analysis of the mixture of products revealed two major components, which were identified as 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol-*1-d* and 1,3,5-tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-mannitol-*1-d* by their mass-spectral fragmentation-patterns[13]. The fragmentation pattern for the glucitol derivative was identical to that published by Jansson *et al.*[14], with the exception that, in our spectrum, fragment-ions involving C-1 occurred at *m/z* values one unit higher than those shown by them; this is a result of the use, by us, of deuterated sodium borohydride in forming the alditols. The mannitol derivative featured fragment-ions that were consistent with a 1,3 substitution pattern[13,15]. These fragment-ions, and their intensities in parentheses, relative to the base ion (*m/z* 43), included: 45 (33), 74 (10), 75 (27), 100 (10), 101 (10), 117 (56), 129 (17), 142 (5), 159 (28), 161 (3), and 231 (2). A small, molecular-ion peak was seen at *m/z* 392 (0.5). The methylation analysis also shows that each of the sugars is present in the pyranoid form.



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The structure of the K7 polymer, as deduced from the results of the chemical and spectroscopic experiments presented, is as shown in formula **1**.

The *E. coli* K56 capsular polysaccharide was also reported, by Robbins *et al.*[1], to cross-react with the *S. pneumoniae* type 3 polysaccharide. To complete this study of cross-reacting antigens, we sought to determine the structure of the K56 capsular polysaccharide. An *E. coli* K56 strain was kindly given to us by Drs. Ida and Frits Ørskov of the WHO International Escherichia and Klebsiella Centre (Copenhagen, Denmark); these investigators indicated (personal communication) that K7 and K56 are, most probably, identical. The ^{13}C-n.m.r. spectrum of the capsular polysaccharide isolated from the K56 organism was identical in all respects to that derived from K7 organisms. The results of double-immunodiffusion studies were also consistent with the K7 and K56 polysaccharides's being identical; that is, the polysaccharides gave lines of identity when diffused against either K7 or K56 antiserum.

The ^{13}C- and ^{1}H-n.m.r. spectra of the pneumococcal type 3 capsular polysaccharide were consistent in all regards (composition, linkages, and anomeric stereochemistry) with the structure originally determined by Hotchkiss and Goebel[3] (see the Introduction).

The *S. pneumoniae* type 3 and *E. coli* K7 polymers differ primarily at the C-2 atom of their respective uronic acid residues, the substituent thereon being a hydroxyl group for the glucosyluronic residue in the pneumococcal type 3 polymer and an acetamido group for the 2-deoxymannosyluronic residue in the *E. coli* K7 polymer (the remaining difference is that O-6 of the glucosyl residue is *O*-acetylated in the K7 polymer). Apparently, these distinctions are not sufficient to preclude a cross-reaction. The K7 polymer did not cross-react with either the *H. influenzae* type e or the type d polysaccharide, both of which contain 2-acetamido-2-deoxymannosyluronic acid residues in the repeating unit[6,7].

EXPERIMENTAL

Materials. — D-Glucose, 2-acetamido-2-deoxy-D-mannose, and D- and L-arabinose were obtained from Calbiochem (San Diego, CA). Erythritol was obtained from Supelco, Inc. (Bellefonte, PA). D-Glucose oxidase was obtained from Worthington Biochemical Corp. (Freehold, NJ). Deuterated sodium borohydride (98 atom% of ^{2}H) and *d*-2-octanol were obtained from Aldrich Chemical Co. (Metuchen, NJ). Dialysis tubing (molecular-weight cut-off of ~3500) was obtained from Spectrum Medical Industries (Los Angeles, CA). All additional, analytical reagents were of the highest purity available.

Isolation of polysaccharide. — The capsular polysaccharide from *E. coli* K7 was isolated as described[9] for strain WHO 252 (courtesy of Drs. Frits and Ida Ørskov, International Escherichia and Klebsiella Centre, State Serum Institute, Copenhagen, Denmark) and Bureau of Biologics strain 69–9846 RLM K7 (isolated at the Bureau of Biologics by Dr. Richard L. Myerowitz); ^{13}C-n.m.r. spectra showed that these two strains were identical, and therefore, structural studies were performed with strain

WHO 252. The polysaccharide from *E. coli* strain H17b (from Drs. Frits and Ida Ørskov), previously named 02:K56 and now named 02-K7:H7, was obtained as already described.

Polysaccharide sugar-analysis. — Hydrolysis of the native, and sodium borohydride-reduced (*vide infra*), K7 capsular polysaccharide was conducted as described, using methanesulfonic acid[9,16]. Amino sugars were analyzed with a Beckman 120-B amino acid analyzer. Neutral sugars were analyzed either underivatized, in an automated sugar-analyzer[16], or derivatized (as described[9]) in a Varian Associates 3700 gas chromatograph equipped with a column of 3% of OV-225. Identification of sugars was accomplished by comparison with authentic samples. The absolute configuration of the 2-amino-2-deoxymannose residue was determined as described[6]; the absolute configuration of the glucose was established enzymically, its disappearance being noted following the addition of D-glucose oxidase[16] (EC 1.1.3.4).

Reduction of the K7 polysaccharide with sodium borohydride. — The polysaccharide was reduced according to the method of Taylor and Conrad[17]; CMCT was used to facilitate the reduction (*cf.*, ref. 6). The yield of reduced polymer was ~80% of the theoretical, based on the amounts of glucose and 2-amino-2-deoxymannose released on acid-catalyzed hydrolysis.

Periodate oxidation of the polysaccharide. — The polysaccharide (25 mg) was treated in the dark, at 4°, with 0.09M $NaIO_4$ solution (3 mL). After ~12 h, the reaction was quenched by the addition of ethylene glycol (50 μL), and the products were reduced with sodium borohydride (60 mg) during 4 h at 15°. After dialysis (3 × 500 mL) and lyophilization, the residue was subjected to neutral-sugar, ion-exchange chromatographic, and n.m.r.-spectroscopic analyses. The glucose residue was found to be oxidized (>95%). Comparable results were obtained with the *O*-deacetylated polymer.

Product study of periodate oxidation of the polysaccharide. — The carbodiimide-assisted, sodium borohydride-reduced polysaccharide was oxidized with sodium metaperiodate, and the products were reduced with sodium borohydride as described in the previous paragraph. This reaction mixture was then hydrolyzed with 2M HCl (400 μL) for 1 h at 115° in an evacuated, pyrolysis tube. After drying in a stream of nitrogen gas, the residue was peracetylated for 1 h at 50° with 1:1 (v/v) acetic anhydride–pyridine (2 mL). After evaporation of the excess of acetic anhydride and pyridine in a stream of nitrogen gas, the residue was extracted with chloroform, and the chloroform-soluble material was analyzed by g.l.c. in a Varian Associates model 3700 chromatograph equipped with a column (1.83 m × 2 mm) on Supelcosorb (100–120 mesh). Erythritol and residual, unoxidized glucose were detected, and identified, by comparison with authentic material.

Permethylation analysis of the sodium borohydride-reduced polysaccharide. — The polysaccharide was permethylated, and the product hydrolyzed, according to described methods[13]. The mass spectrometry was performed in an LKB 2091 Gas Chromatography–mass spectrometer as described[18].

N-Deacetylation, O-deacetylation, and ninhydrin oxidation of the K7 polysaccharide. — *N*-Deacetylation with hydrazine, *O*-deacetylation with ammonium hydroxide, and oxidation by ninhydrin were conducted as described[6,7].

Additional, analytical instrumentation. — ^{13}C-N.m.r. spectra were recorded either with a JEOL FX-100 or a Bruker WM-300 spectrometer, and ^{1}H-n.m.r. spectra, with a Bruker WM-300 spectrometer.

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