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STRUCTURE OF THE 3-DEOXY-D-*manno*-OCTULOSONIC ACID-(KDO)-CONTAINING CAPSULAR POLYSACCHARIDE (K14 ANTIGEN) FROM *Escherichia coli* 06:K14:H31*

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ABSTRACT

The chemical structure of the K14-antigenic polysaccharide (K14 antigen) of *Escherichia coli* 06:K14:H31 was elucidated by determination of the composition, ^{1}H- and ^{13}C-n.m.r. spectroscopy, periodate oxidation, and study of the oligosaccharides obtained by partial hydrolysis. The polysaccharide consists of [*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1→5)-*O*-(3-deoxy-β-D-*manno*-octulopyranosylonic acid)-(2→6)] repeating units, ~60% of the octonic acid units being *O*-acetylated and ~10% *O*-propionylated at O-8. The sequence of acetylated and propionylated residues is not known. The serologically-specific part of the K14 antigen resides in the polysaccharide part.

INTRODUCTION

On the basis of chemical, physical, and genetic characteristics, the capsular polysaccharides of *Escherichia coli* may be divided into two distinct groups[1,2]. The polysaccharides of one group occur preponderantly together with the O-8 or O-9 cell-wall antigen (lipopolysaccharide), *i.e.*, preponderantly in *E. coli* O groups 8 and 9, have high-molecular weights (often several hundred thousand), and are genetically determined at chromosomal sites near *his* and *trp*[1–3]. The polysaccharides of the second group occur together with any of the many O-antigenic lipopolysaccharides, *i.e.*, in many *Escherichia coli* O groups, have molecular weights of ~30 000 or lower, and are genetically determined at a chromosomal site near *ser A*[1,2,4,5]. Whereas the former polysaccharides have hexuronic acids as negatively charged components, the latter contain less-common, charged components. These may be[2] phosphate, *N*-acetylneuraminic acid, or 3-deoxy-D-*manno*-octulosonic acid (KDO, **1**). It is only within the last few years that **1** was found to be a characteristic component not only of Gram-negative enterobacterial lipopolysaccharides[6,7], but also of certain capsular polysaccharides. This was first observed[8] in

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the chemical analysis of the capsular polysaccharide of *Neisseria meningitidis* serogroup 29-e, and then also observed[2] for the capsular polysaccharide of extra-intestinal *E. coli*. The K6, K13, K20, and K23 polysaccharides of *E. coli* contain **1** and ribose[9–14], the K12 antigen contains **1** and rhamnose[15], and the K15 antigen consists of **1** and 2-acetamido-2-deoxyglucose[14]. We have now found another capsular polysaccharide from *E. coli* 06:K14 that contains **1**. Its chemical structure is described herein.

RESULTS AND DISCUSSION

Isolation and characterization. — The capsular (K14) polysaccharide was isolated from liquid cultures of *Escherichia coli* 2701. As described previously[12,16], the bacteria and capsular polysaccharide were precipitated with hexadecyltrimethylammonium bromide (Cetavlon) and, from the precipitated complexes, the polysaccharide was extracted with calcium chloride. The crude polysaccharide preparation was purified[12] by precipitation with ethanol, followed by extraction with cold, buffered phenol at pH 6.5. This procedure yielded ~150 mg of purified K14 polysaccharide per L of culture. The final product contained ~0.5% of nucleic acid and <0.5% of protein.

The K14 polysaccharide is composed of 2-acetamido-2-deoxygalactose and 3-deoxy-D-*manno*-octulosonic acid (KDO, **1**). Quantitative determinations after hydrolysis showed that 2-acetamido-2-deoxygalactose and **1** were present in the K14 polysaccharide in equimolar proportions (*Anal.*: Found 1:0.86). Since the ^{1}H-n.m.r. spectrum (*vide infra*) indicated the presence of *O*-acetyl groups, the K14 polysaccharide was treated with ethanolic hydroxylamine according to Snyder and Stevens[17], and the hydroxamates formed were subjected to paper chromatography. Staining with ferric chloride revealed two spots, one having the mobility of acetohydroxamic acid (R_F 0.59), and a minor one having the same mobility as propionohydroxamic acid (R_F 0.73). G.l.c. analysis[18], with butyric acid as internal reference compound revealed that the K14 polysaccharide contained 0.6 *O*-acetyl and 0.1 *O*-propionyl group/disaccharide unit. Both groups could be removed at pH 11, in the cold, without alteration of the polysaccharide backbone.

In immunoelectrophoresis, the polysaccharide exhibited a precipitation line with anti-K14 antiserum, which indicated serological K14 specificity. Immunoelectrophoresis, gel-permeation chromatography, and analytical ultracentrifugation revealed, in the K14 polysaccharide preparations, a heterogeneity of the apparent molecular weight. The preparations appeared homogeneous after being heated to 100° at pH 5–6, or treated with a base, or in the presence of 0.2% of Triton X-100. These characteristics have been observed for several capsular polysaccharides from extra-intestinal *E. coli*, including strain 2701, and were shown to be due to micelle formation *via* a phosphoric acid-bound lipid substituent at the reducing end of the polysaccharide chains[19]. The results described earlier[19] are fully applicable to the K14 polysaccharide described herein. The optical rotation of the native polysac-

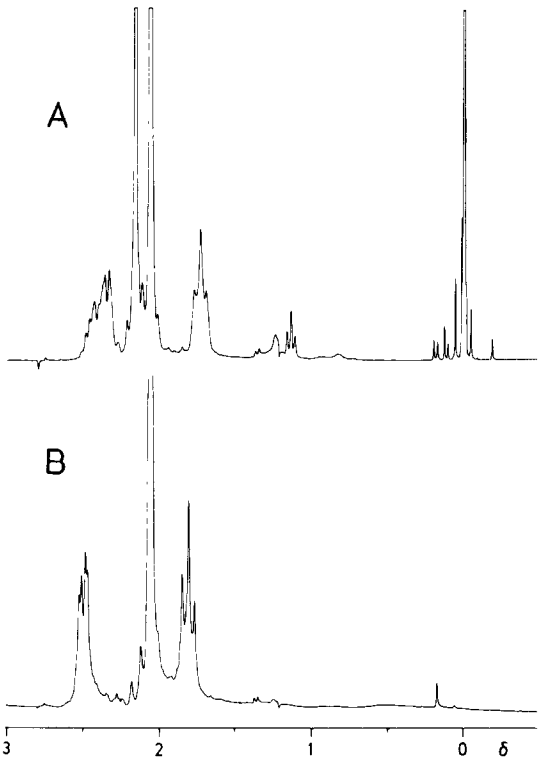


Fig. 1. ^{1}H-N.m.r. spectrum at 300 MHz (δ 0–3 region) of the K14 polysaccharide before (A) and after *O*-deacylation (B) for a solution in deuterium oxide at 70° with sodium 4,4-dimethyl-4-sila-(2,2,3,3-$^2H_4$)pentanoate as external reference.

charide was $[\alpha]_D^{25}$ 37.9° (*c* 0.14, water) and that of the polysaccharide without the lipid was $[\alpha]_D^{25}$ 33.3° (*c* 0.07, water).

Periodate oxidation. — Oxidation of the native and *O*-deacylated K14 polysaccharides with sodium metaperiodate resulted in the degradation of all 2-acetamido-2-deoxyygalactose residues, without apparent loss of **1**. Paper electrophoresis of hydrolyzates from both reaction products (pH 5.3, 42 V/cm), when stained with the thiobarbituric acid reagent, indicated the presence of **1** and a second thiobarbituric acid-reactive compound that had a relative mobility of M_1 1.14. The paper electropherogram of the *O*-deacylated and oxidized polysaccharide showed only the faster-moving, thiobarbituric acid-reactive compound. As described by Schmidt and Jann[15], reduction of the carbonyl group, followed by permethylation, hydrolysis, and mass spectrometry of both substances, showed that the faster-moving compound was 3-deoxy-D-*lyxo*-heptulosonic acid (**2**). In the oxidized K14 polysaccharide, compounds **1** and **2** were present in the molar ratio of ~1.5:1.

These results suggest that, in the K14 polysaccharide, the 2-acetamido-2-

deoxygalactosyl residues are linked at O-6, and that ~70% of the octulosylonic residues are *O*-acylated at O-7 or -8, and are thus protected against oxidative degradation to **2**. From this, it may be deduced that **1** is substituted by the 2-acetamido-2-deoxygalactosyl residue at O-4 or -5. The ratio of **1**:**2** found after periodate oxidation agrees well with the degree of *O*-acylation of the polysaccharide.

^{1}H-N.m.r. spectroscopy. — The ^{1}H-n.m.r. spectrum of the K14 polysaccharide exhibit a doublet at δ 4.7 (J 8.6 Hz) due to the anomeric proton of the 2-acetamido-2-deoxygalactose residue. In the region at δ 0–3 of the ^{1}H-n.m.r. spectra of the native and *O*-deacylated K-14 polysaccharides (see Fig. 1), a multiplet between δ 2.3 and 2.5 and a triplet were observed at δ 1.73 and are characteristic of H-3*eq* and H-3*ax* of the β anomer of **1**. The singlet signals at δ 2.14 and 2.05 arose from $OCOCH_3$ and $NHCOCH_3$, respectively, and have relative intensities of ~0.6:1 (not shown). The triplet at δ 1.13 was not detected in the capsular polysaccharides that we have studied previously. The chemical shift is characteristic of aliphatic CH_2, and the splitting indicated an adjacent methylene group. The detection of *O*-propionyl groups in the K14 polysaccharide described earlier suggests that the signal at δ 1.13 is due to propionyl groups in the polysaccharide. The signal of the respective methylene groups (δ 2.46, in a reference spectrum not shown) overlaps with that of H-3*eq* of **1**. In the spectrum of *O*-deacylated K14 polysaccharide (Fig. 1B), the signals at δ 1.13 and 2.14 were absent. Furthermore, instead of the poorly resolved multiplet that appeared in the spectrum of the native K14 polysaccharide (Fig. 1A) between δ 2.3 and 2.5 the spectrum of the *O*-deacylated polysaccharide exhibited a double doublet at δ 2.48 (Fig. 1B), characteristic of H-3*eq* of **1**. These results indicate that the K14 polysaccharide consists of 2-acetamido-2-deoxygalactosyl and 3-deoxy-β-D-*manno*-octulosonic acid residues, and that it is substituted with acetyl groups and, to a much lesser extent, with propionyl groups.

^{13}C-N.m.r. spectroscopy. — In the ^{13}C-n.m.r. spectra of the native, *O*-deacylated, and periodate-oxidized and borohydride-reduced K14 polysaccharides (see Table I), the signals[21] arising from CH_3CO (δ 21.8) and CH_3CO (δ 175.8) were absent in the spectra of the *O*-deacylated and periodate-oxidized polysaccharides. The latter compound was reduced with sodium borohydride after periodate oxidation, which removed the acyl groups. In the spectrum of the *O*-deacylated polysaccharide, the signal at δ 174.5 was attributed to C-1 of **1**, and that at δ 36.4 to C-3 of **1**. It could be demonstrated, by an off-resonance experiment[22] (not shown), that the signal at δ 103.8 was due to C-1 of the 2-acetamido-2-deoxygalactose unit (doublet through coupling with H-1), and that at δ 102.7 was due to C-2 of **1** (no splitting in the off-resonance mode). The signal at δ 103.8 was missing in the spectrum of the periodate-oxidized–sodium borohydride-reduced polysaccharide, in agreement with the opening of the D-galactose pyranose ring by periodate oxidation. The signal at δ ~54.2 was present in all three spectra and was due to C-2 of the 2-acetamido-2-deoxygalactose unit. Its chemical shift indicated the presence of the β-D anomer in the polysaccharide, which was confirmed by the ab-

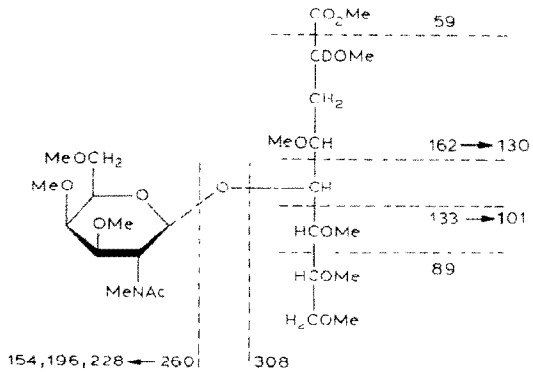
sence of a signal at δ 50–52 indicative of the α-D anomer[8]. As periodate oxidation showed that OH-7 or -8 was acylated, substitution of the octulosylonic residue by the 2-acetamido-2-deoxygalactosyl residue could be expected at either O-4 or -5. Substitution at O-4 would result in a distinct shift upfield (β) of ~3 p.p.m. of the signal arising from adjacent C-3 (see ref. 8), which was found to be δ 35.3 in the methyl β-D-glycoside of **1** (unpublished results); in the present spectra, the signal of C-3 was at δ 36.4. Therefore, it may be concluded that the 2-acetamido-2-deoxygalactosyl residue is linked to O-5 of the octulosylonic residue, as such a substitution would only slightly displace the signal of C-3.

The assignment of the position of *O*-acylation (at O-7 *vs.* O-8 of **1**) was similarly attempted. Signals at δ ~66.1 and ~71.5 have been assigned[15] to C-8 and -7 of **1**, respectively, and substitution at C-7 has been shown[8] to shift the corresponding signal to δ ~77–79. Comparison of the spectra showed that, upon *O*-deacylation, the signal at δ 65.2 increased in intensity, and the signals at δ 67.2 and 68.6 disappeared. The increase of intensity of the signal at δ 65.2 indicated that this signal was exhibited by C-8 of the octulosylonic residue. The signal at δ 67.2, which disappeared after *O*-deacylation, was attributed to H_2COAc-8 of the octulosylonic residue. The signal at δ 68.6 was difficult to assign. It may be caused by a β-shift of the C-7 signal due to *O*-acylation. Since the signal at δ 70.4 increased after *O*-deacylation, it was assigned to C-7. Similar α- and β-shifts have been discussed for the *O*-acetylation of OH-2, -3, or -6 of D-galactose[23]. No corresponding changes could be observed in the region of the C-7 signal. Therefore, it is tentatively proposed that **1**, in the K14 polysaccharide, is acylated at O-8. Signals arising from the methyl and methylene carbon atoms of the *O*-propionyl substituent (δ 8.9 and 28.3; spectrum not shown; *cf.* ref. 21) were not apparent in the spectrum of the native polysaccharide. This may be due to the low degree of substitution. Comparison of the spectra exhibited by the *O*-deacylated and by the periodate oxidized and reduced polysaccharide (Table I) shows that not only the signal of C-1 of the 2-acetamido-2-deoxygalactosyl residue is missing, but that several signals are shifted after oxidation and reduction of the K14 polysaccharide. Comparison with the signals[8,15,20] of **1** indicated that unchanged signals, except those of C-2 of the 2-acetamido-2-deoxygalactosyl residue and of $NHCOCH_3$, seem to be due to the carbon atoms of **1**. This is in agreement with the results of the periodate oxidation and with substitutions at O-6 of the 2-acetamido-2-deoxygalactosyl and at O-5 of the octulosylonic residue.

Structure of oligosaccharide obtained by hydrolysis. — The K14 polysaccharide was hydrolyzed with dilute sulfuric acid, and the hydrolyzate was subjected to preparative paper electrophoresis. Staining of guide strips indicated the presence of 2-acetamido-2-deoxygalactose, **1**, and a compound migrating toward the anode with an electrophoretic mobility of M_1 0.55. The last-named compound was eluted with water, chromatographed on Bio-Gel P-2 with water (K_d 0.3), and the solution lyophilized. The resulting material, which was obtained in 30% yield, consisted of equimolar amounts of 2-acetamido-2-deoxygalactose and **1**. The oc-

tulosonic residue could be detected, albeit in low yield, in a direct thiobarbituric acid assay, without prior hydrolysis. A substituent at O-5 but not at O-4 of **1** is known to be eliminated during a direct reaction with thiobarbituric acid and, accordingly, an O-5 substituted but not an O-4 substituted **1** exhibits the characteristic color during the thiobarbituric acid reaction[20]. Therefore, it is concluded that mild acid hydrolysis of the K14 polysaccharide liberated the disaccharide *O*-(2-acetamido-2-deoxy-β-D-galactosyl)-(1→5)-3-deoxy-D-*manno*-octulosonic acid (**3**).

The disaccharide **3** was reduced with sodium borodeuteride and then methylated with dimethyl sulfoxide–sodium hydride–methyl iodide as described[24]. The purified, permethylated derivative was analyzed by g.l.c. (250°, isotherm, and 2.5% SE-30 on Chromosorb as stationary phase). Only one peak was detected, which indicated that the epimers formed by reduction of the carbonyl group of the octulosonic residue were not resolved under these conditions. The product was subjected to direct m.s. analysis. In the mass spectrum (see Fig. 2 and Scheme 1),



Scheme 1 Pattern of m.s. fragmentation of sodium borodeuteride-reduced, and permethylated disaccharide **3**

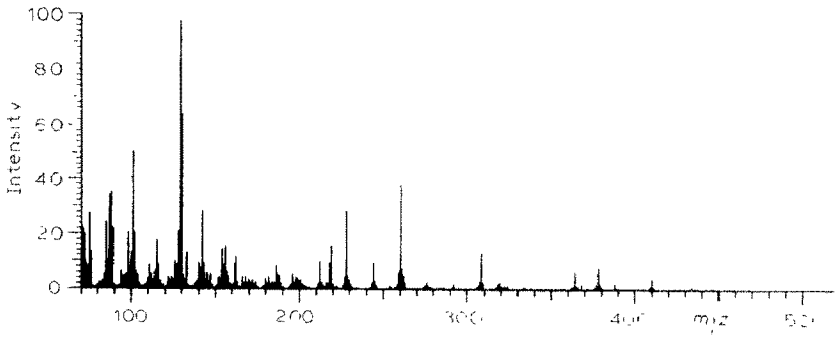
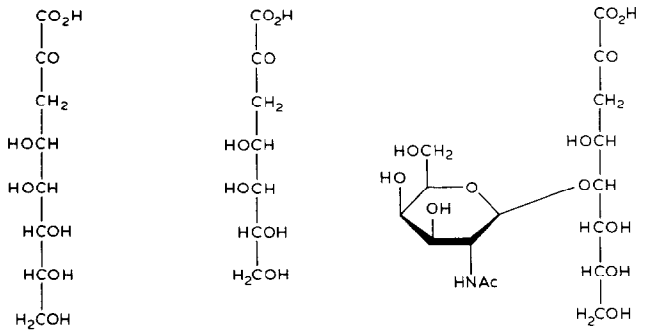
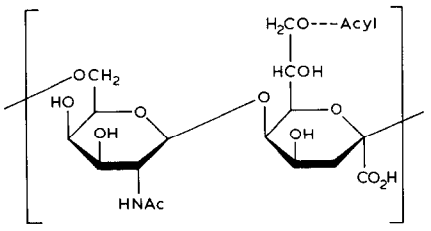


Fig. 2. Electron-impact ionization mass spectrum of the deuteroreduced and methylated derivative of **3** under standard conditions (70 eV, direct inlet)

the peak at *m/z* 260 was due to the permethylated 2-acetamido-2-deoxygalactosyl residue minus O-1, and the peak at *m/z* 308 to the permethylated and esterified octonic residue minus O-5. The presence of peaks at *m/z* 162, and 130 derived from it, as well as the absence of a peak at *m/z* 177 (or 145 derived from it) showed that the octulosonic residue is substituted at O-5 and not at O-4. This is in agreement with the results of the ^{13}C-n.m.r. spectroscopy, and also with the observation that, in the nonmethylated **3**, the octulosonic residue can be detected without prior hydrolysis (*vide supra*). In the mass spectrum obtained by chemical ionization in the presence of ammonia (not shown), a peak at *m/z* 585 was obtained. If a proton transfer[25] is considered, this quasimolecular ion agrees with a molecular weight of the deuteroreduced, permethylated **3** of 584.

On the basis of these results structure *O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1→5)-*O*-(3-deoxy-β-D-*manno*-octulopyranosylonic acid)-(2→6) (**4**) is proposed for the repeating unit of the K14 polysaccharide of *E. coli*. About 60% of the octulosonic residues are substituted with *O*-acetyl and ~10% with *O*-propionyl groups. Since acetylation and propionylation sites could not be differentiated, both substituents are described as *O*-acyl, and the incompleteness of acylation is indicated with a dotted line. It is not known whether the *O*-acylation is regularly distributed or concentrated in certain regions of the polysaccharide chain. The latter substitution pattern would result in domains of different hydrophobicity. The native polysaccharide is linked at its reducing end (octulosylonic residue) to a





lipid residue *via* a monophosphodiester group[19], which has been observed for all capsular polysaccharides of extraintestinal *E. coli* hitherto studied[19,20]. This hydrophobic component may play a role in the association of the polysaccharides with the bacterial cell to form a capsule.

The chain of the K14 polysaccharide of *E. coli* has the same sugar constituents in the same sequence as that of the capsular polysaccharide of *Neisseria meningitidis* serogroup 29-e. However, in contrast to the K14 polysaccharide, the *N. meningitidis* 29-e polysaccharide contains (2→3)-linked 2-acetamido-2-deoxy-α-D-galactosyranosyl and (1→7)-linked 3-deoxy-β-D-*manno*-octulopyranosylonic acid residues[8]. The difference in structure between these capsular polysaccharides is reflected by the absence of a serological cross-reaction (unpublished observation).

The K14 polysaccharide belongs to the group of capsular polysaccharides from *E. coli* that contains **1** as a major constituent[2,8-12,15], a characteristic which they share with the capsular polysaccharide[8] of *N. meningitidis* serogroup 29-e. In all these polysaccharides, **1** is linked in the chain either at O-5 (*E. coli* K12, K14, and K15) or at O-7 (*E. coli* K6, K13, K20, K23, and *Neisseria meningitidis* 29-e); respective substitutions at the alternative positions O-4 and O-8 of **1** not being found. In most of these polysaccharides, **1** is *O*-acetylated either at O-7 or -8 for a (1→5) linkage, or at O-4 or -5 for a (1→7) linkage. Thus, the members of this group of polysaccharides are closely related and of interest not only with respect to structural correlations and biosynthetic patterns, but also with respect to immunological specificity. It is interesting to note that most of the aforementioned *E. coli* strains, including *E. coli* 2701, may cause urinary-tract infections in which the capsular polysaccharides play a role[27-29].

EXPERIMENTAL

Bacteria and cultivation. — *E. coli* 2701 (O6:K14:H31) was obtained from Dr. Bertil Kaijser (Göteborg). They were grown to the late logarithmic phase (6–8 h) in a fermenter in 10-L batches, which contained per L: $K_2HPO \cdot 3H_2O$ (9.7 g), KH_2PO_4 (2 g), sodium citrate · $5H_2O$ (0.5 g), $MgCl_2 \cdot 7H_2O$ (0.1 g), casamino acids (20 g), D-glucose (2 g), and the dialyzable part of yeast (100 ml, from 500 g in 5 L of de-ionized 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 0.2% hexadecyltrimethyl ammonium bromide (Cetavlon, equal vol.). All following operations were carried out at 4°. The polysaccharide was extracted from the precipitate with M calcium chloride. It was purified by three cycles of precipitation with ethanol (80% final concentration), followed by repeated extractions with cold phenol, buffered to pH 6.5 with sodium acetate[13,16]. The combined, final aqueous phases were centrifuged for 4 h at 105 000*g*, and the supernat-

TABLE I

^{13}C-N M R DATA FOR K14 POLYSACCHARIDE, AND *O*-DEACYLATED AND PERIODATE-OXIDIZED-BOROHYDRIDE-REDUCED DERIVATIVES[a]

Polysaccharide		
K14	*K14* O-*deacylated*	*K14* IO_4^--oxidized–$^3H_4^-$-reduced
176.1	176 1	175.8
175.8		
174.6	174.5	174.2
103 8	103.8	
103.1		102 8
102.7	102.7	102.4
		77.5
75.3	75.0	75.0
74.8	74.7	
72.5	72.5	72.3
70 4	70.4	70.6
69.3	69.4	69.4
69.0	69.1	
68.6		
67.2		
65.2	65.2	65.3
64.9	65.0	65.0
		63.0
		62.4
54.2	54.2	54.4
36.4	36.4	36.9
23.9	23 9	23.4
21.8		

[a]The chemical shifts were measured against external sodium 4,4-dimethyl-4-sila-(2,2,3,3-$^2H_4$) pentanoate and corrected (subtraction of 1.31 p.p.m.) by measuring dioxane (δ 67.4, based on tetramethylsilane) so that they may be directly compared with those observed using a tetramethylsilane reference.

ant solution was lyophilized. The residue was further purified by chromatography on Sephadex G-50.

Analytical methods. — 2-Acetamido-2-deoxygalactose was determined, after hydrolysis with 4M hydrochloric acid for 18 h at 100° with a Durrum D-500 amino acid analyzer, and **1** with the thiobarbituric acid assay[30] after optimal hydrolysis (0.1M hydrochloric acid for 30 min at 100°). *O*-Acetyl and *O*-propionyl groups were detected by descending paper chromatography of the hydroxamate derivative[17] on Whatman paper No. 1 with the upper phase of 4:1:5 (v/v) 1-butanol–ethanol–water and made visible with 10% aqueous ferric chloride. These acyl substituents were quantitatively determined by g.l.c. on Porapak QS according to Fromme and Beilharz[18].

Optical rotations were measured with a Perkin–Elmer Polarimeter 141. G.l.c. analyses were performed with a Varian Aerograph Series 1400 instrument, equipped with an autolinear temperature programmer and a Hewlett–Packard

3380A integrator. ^{1}H- and ^{13}C-N.m.r. spectra were recorded with a Bruker WM 300 spectrometer in the FT mode at 70° with sodium 4,4-dimethyl-4-sila-(2,2,3,3-$^2H_4$)pentanoate as external standard. Mass spectrometry was performed with a Finnigan MAT 44S mass spectrometer.

O-Deacylation. — The K14 polysaccharide (100 mg) was dissolved in dilute aqueous ammonium hydroxide (5 mL) of pH 10.5, and the solution was kept for 16 h at 4°, and then lyophilized. The residue was chromatographed on Sephadex G-50.

Periodate oxidation. — Native or *O*-deacylated K14 polysaccharide (100 mg) was dissolved in 50mM sodium metaperiodate (20 mL), and the solutions were kept for 100 h at 4°. After addition of 1,2-ethanediol (0.5 mL) each mixture was kept for 4 h at room temperature, and then dialyzed against de-ionized water. To the retentate was added sodium borohydride (150 mg) in water (5 mL), and the mixture was maintained for 24 h at 4°. It was then treated with Dowex 50 (H^+), evaporated *in vacuo*, and repeatedly methanol was added and evaporated. The final product was chromatographed on Sephadex G-50.

Isolation of **3**. — Native K14 polysaccharide (150 mg) was hydrolyzed with 50mM sulfuric acid for 30 min at 100°. The neutral and concentrated hydrolyzate was subjected to paper electrophoresis (pH 5.3, 42 V/cm, 90 min) on Schleicher and Schüll paper No. 2043b. The material having an electrophoretic mobility M_1 0.55 (detected on guide strips by staining with alkaline silver nitrate) was eluted from the paper with water, the solution lyophilized, and the residue chromatographed on Bio-Gel P-2 with water as eluent. Lyophilization of the fraction eluted at K_d 0.30 gave the purified disaccharide (45 mg) which exhibited a single spot on paper electrophoresis under the aforementioned conditions.

Methylation of **3**. — The disaccharide **3** (10 mg) was first reduced with sodium borodeuteride in deuterium oxide as described for reduction with sodium borohydride. After removal of borate ions with methanol, the residue was dried in the presence of phosphorus pentaoxide for 3 days at 40° *in vacuo*. The reduced disaccharide was dissolved in dimethyl sulfoxide (0.5 mL) and methylated in a glass vial closed with a rubber cap as described[24]; briefly the dimethylsulfinyl reagent (0.75 mL) was injected with a dry syringe and, after treatment for 2 h at 37° in a sonication bath, the mixture was frozen and methyl iodide (0.75 mL) was injected. After 3 h in the sonication bath at 37°, the mixture was poured into water (3 mL) and the solution extracted with chloroform (3 × 2 mL). The combined chloroform extracts were washed twice with water (1 mL) and processed as described[24]. The material eluted (after 10 mL) with 1:2 (v/v) chloroform–ethanol from a column (22 × 1 cm) of LH-20 was detected by spotting on silica gel plates and heating with 10% sulfuric acid in ethanol. The solution was evaporated *in vacuo* and the residual permethylated **3** was kept until further use under nitrogen at −25°.

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