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Escherichia coli SEROTYPE K44: AN ACIDIC CAPSULAR POLYSACCHARIDE CONTAINING TWO 2-ACETAMIDO-2-DEOXYHEXOSES

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ABSTRACT

The structure of the capsular polysaccharide from *Escherichia coli* O8:K44 (A):H$^-$ (K44 antigen) has been established using the techniques of methylation, β-elimination, deamination, and Smith degradation. N.m.r. spectroscopy (^{13}C and ^{1}H) was used extensively to establish the nature of the anomeric linkages of the polysaccharide and of oligosaccharides derived through degradative procedures. The K antigen is comprised of repeating units of the linear tetrasaccharide shown. This acidic polysaccharide represents the first instance of an *E. coli* K antigen in this series (group A) that has been found to contain two different 2-acetamido-2-deoxyhexoses.

→4)-β-D-GlcpA-(1→3)-α-L-Rha*p*-(1→4)-α-D-Glc*p*NAc-(1→6)-β-D-Gal*p*NAc-(1→

INTRODUCTION

Capsular antigens of *E. coli* have been classified[1] into two main groups depending on their thermal stability. Many of those that are heat stable (A type) are said to be similar in composition and structure to the K antigens of *Klebsiella*, but there is a group of A-type capsular antigens that is distinctly different in that they contain amino sugars[2]. We now report the structure of the K antigen of *E. coli* serotype K44, the first member of this group to be examined in detail.

RESULTS AND DISCUSSION

Composition and n.m.r. spectra.—The polysaccharide from *E. coli* K44 was isolated and purified according to the procedure previously described[3]. The acidic polysaccharide obtained from two cetyltrimethylammonium bromide precipitations had $[\alpha]_D^{25}$ +50.4°.

Determination of the neutral and amino sugars as alditol acetates revealed the

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TABLE I

SUGAR ANALYSIS OF *E. coli* K44 POLYSACCHARIDE AND DERIVED PRODUCTS

Sugar or derived compound (as alditol acetate)	*Molar ratios*[a,b]						
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*
Rhamnose	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
Glucosamine	0.61	0.40		0.76		0.65	0.47
Galactosamine	0.48	0.28		0.35			
Glucose		0.95	0.90	0.68	0.77		
2,5-Anhydromannitol			0.54				
2,5-Anhydrotalitol			0.42				
Glycerol						0.15[c]	0.19[c]
Erythritol						0.67	

[a]Using a DB-17 capillary column to hold at 180° for 2 min, then increase 5°/min to 220°. [b]I, native acidic polysaccharide hydrolyzed with 2M HCl for 7 h at 95°; II, products of successive methanolysis, carboxyl-reduction, and hydrolysis; III, products of successive carboxyl-reduction, methanolysis, and deamination; IV, carboxyl-reduced polysaccharide; V, compound **1** (from deamination) after methanolysis, then carboxyl-reduction; VI, polyol from the periodate oxidation of the carboxyl-reduced polysaccharide; VII, disaccharide glycoside **2** from the Smith degradation. [c]Quantitation inaccurate because of the high volatility of the acetate.

presence of rhamnose, glucosamine, and galactosamine in the polysaccharide (Table I, column I). The formation of equimolar proportions of 2,5-anhydromannitol and 2,5-anhydrotalitol on deamination followed by reduction of some hydrolyzates confirmed the presence of glucosamine and galactosamine in the polysaccharide (Table I, column III). Both the carboxyl-reduced methyl glycosides[4], and the carbodiimide-reduced polysaccharide[5] on analysis as alditol acetates showed the presence of glucose (0.95 and 0.68 moles respectively, Table I, columns II and IV). Despite the low values for the amino sugars, these results, in conjunction with data cited later, suggest that *E. coli* K44 consists of a tetrasaccharide repeating unit of one residue each of galactosamine, glucosamine, glucuronic acid, and rhamnose.

The tetrasaccharide repeating unit of the polysaccharide was more clearly demonstrated in the n.m.r. spectra (Fig. 1 and Table II). The ^{1}H-n.m.r. spectrum of the native polysaccharide (sodium salt) showed signals for four anomeric protons[6], one in an α-linkage at δ 4.95 ($J_{1,2}$ 3 Hz), two in β-linkages at δ 4.72 ($J_{1,2}$ 8 Hz), and δ 4.60 ($J_{1,2}$ 8 Hz), and an additional signal at δ 4.89. After passage through a column of cation-exchange resin, the polysaccharide (now in its acidic form) showed a distinct downfield shift of one of the anomeric signals, from δ 4.72 to δ 4.80. In the spectrum of the native polysaccharide, signals for methyl protons of rhamnose appeared at δ 1.34 (b, 3 H), and two signals attributed to methyl protons of *N*-acetyl groups were observed at δ 2.09 (s, 3 H), and δ 2.06 (s, 3 H), respectively.

The ^{13}C-n.m.r. spectrum of the native polysaccharide (Fig. 1) showed a total of 28 signals, and this, coupled with the presence of only 4 anomeric carbon reso-

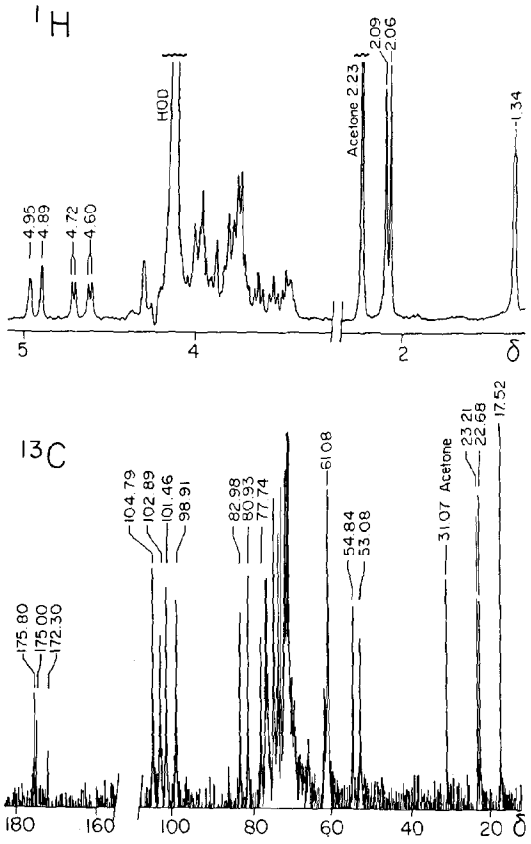

Fig.1. ^{1}H- And ^{13}C-n.m.r. spectra (400 MHz) of *E. coli* capsular polysaccharide.

nances[7], confirmed a tetrasaccharide repeating unit for the K44 polysaccharide. The three $C=O$ signals in the downfield region of the spectrum (between 170 and 180 p.p.m.) are consistent with the presence of two acetamido groups and one carboxyl group. The signals at 54.84 p.p.m. and 53.08 p.p.m. can be attributed to C-2 of glucosamine and galactosamine. In the upfield region of the spectrum three signals corresponding to the methyl carbons of the two *N*-acetyl groups and the rhamnose residue were observed.

A proton-coupled ^{13}C-n.m.r. spectrum of the native polysaccharide was obtained by the single frequency off-resonance decoupling (SFORD) technique. This spectrum showed signals at 104.2 p.p.m. ($^1J_{C\text{-}1,H\text{-}1}$ 160.9 Hz), 102.9 p.p.m. ($^1J_{C\text{-}1,H\text{-}1}$ 158.9 Hz), 101.3 p.p.m. ($^1J_{C\text{-}1,H\text{-}1}$ 167 Hz), and 98.9 p.p.m. ($^1J_{C\text{-}1,H\text{-}1}$ 171 Hz). The $^1J_{C\text{-}1,H\text{-}1}$ values reported for the α and β glycosidic linkages of 6-deoxyhexopyranoses and hexopyranoses are ~169 and ~160 Hz respectively[8,9]. Hence there are two α and two β linkages in the repeating unit of the K44 polysaccharide.

A chromium trioxide oxidation[10] conducted on the peracetylated, carbodi-

TABLE II

N.M.R. DATA FOR *E. coli* K44 POLYSACCHARIDE AND DERIVED PRODUCTS

Compound[a]	*^{1}H-N.m.r. data*				*^{13}C-N.m.r. data*	
	δ[b]	*$J_{1,2}$*[c] *(Hz)*	*Integral (no. of H)*	*Assignment*[d]	*δ*[e]	*Assignment*[f]
β-GlcA-(1→3)-Rha	4.68	b	1.0	GlcAβ1-		
(1)	5.17	b	0.9	-3Rhaα1-		
	1.34	b	3.0	CH_3(Rha)		
α-Rha-(1→4)-α-GlcNAc	4.96	b	1.0	-4GlcNAcα1-	101.60	Rhaα1-
(1→1)-glycerol	4.90	b	1.0	Rhaα1-	99.05	-4GlcNAcα1-
(2)	2.09	s	3.0	CH_3(*N*-Ac)	77.62	C-4 (GlcNAc)
	1.34	b	3.0	CH_3(Rha)	73.49	C-1 (glycerol)
					62.32	C-3 (glycerol)
					61.10	C-6 (GlcNAc)
					54.85	C-2 (GlcNAc)
						O ‖
					22.73	N-CCH_3
					17.48	C-6 (Rha)
→4)-β-GlcA-(1→3)-α-Rha-(1→4)-α-GlcNAc-(1→6)-β-GalNAc-(1→	4.95	3	1.0	-4GlcNAcα1-	175.80	*C*=O } *N*-acetyl of GlcNAc and GalNAc
	4.89	b	1.0	-3Rhaα1-	175.00	*C*=O }
[K44 polysaccharide (Na^+)]	4.72	8	1.0	-4GlcAβ1-	172.30	*C*=O (GlcA)
	4.60	8	1.0	-6GalNAcβ1-	104.77	-4GlcAβ1-
	2.09	s	3.0	CH_3(*N*-Ac)[g] (GlcNAc)	102.89	-6GalNAcβ1-

2.06	s	3.0	CH_3(*N*-Ac)[g] (GalNAc)	101.46	-3Rhaα1-
1.34	5	3.0	CH_3(Rha)	98.90	-4GlcNAcα1-
				82.98	C-4 (GlcA)
				80.93	C-3 (Rha)
				77.74	C-4 (GlcNAc)
				61.08	C-6 (GlcNAc)
				54.84	C-2 (GlcNAc)[g]
				53.08	C-2 (GalNAc)[g]
					O ‖
				23.21	N-CCH_3 (GalNAc)[g]
					O ‖
				22.68	N-CCH_3 (GlcNAc)[g]
				17.52	C-6 (Rha)

[a] For the origins of compounds **1** and **2** see text. [b] Chemical shift relative to external sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), referenced from internal acetone at δ 2.23. [c] Key: b = broad, unable to assign accurate coupling constant; s = singlet. [d] For example -3Rhaα1- refers to the anomeric proton of a 3-linked rhamnosyl residue in the α-anomeric configuration. The absence of a numerical prefix indicates a nonreducing terminal group. [e]Chemical shift relative to external DSS, referenced from internal acetone at 31.07 p.p.m. [f]As for *d*, but for ^{13}C nuclei. [g]These assignements are tentative and are based on the chemical shifts in compound **2**.

imide-reduced polysaccharide, followed by acid hydrolysis and passage of the acid hydrolyzate through a column of cation-exchange resin to remove the basic 2-amino-2-deoxysugars, left rhamnose as the only neutral sugar. Chromium trioxide in acetic acid has been shown[11] to selectively degrade peracetylated pyranose sugars in the β anomeric configuration. Hence this result indicates the presence of an α-rhamnosyl residue and a β-glucosyluronic residue in the K44 polysaccharide. Circular dichroism measurements[12] on the alditol acetates showed rhamnose to have the L configuration, whereas glucuronic acid, 2-acetamido-2-deoxygalactose and 2-acetamido-2-deoxyglucose have the D configuration. The c.d. spectra were compared with those of the corresponding authentic alditol acetates.

Methylation analysis. — *E. coli* K44 polysaccharide was methylated according to the Hakomori procedure[13,14]. Hydrolysis of the methylated polysaccharide was carried out using two different acid conditions (Table III, columns I and II). The derivatives detected by g.l.c. and identified by g.l.c.-m.s. were 2,4-di-*O*-methylrhamnose, 2-deoxy-2-(*N*-methylacetamido)-3,6-di-*O*-methylglucose (hereafter referred to as 2,3,6-trimethylglucosamine) and 2-deoxy-2-*N*-methylacetamido)-3,4-di-*O*-methylgalactose (hereafter referred to as 2,3,4-trimetylgalactosamine). Methylation analysis of the carboxyl-reduced[5] polysaccharide showed, in addition, the presence of 2,3,6-tri-*O*-methylglucose (Table III, column III). These results demonstrated that the glucosamine and glucuronic acid are 4-linked, while the rhamnose is 3-linked and the galactosamine is 6-linked. There were no branch points or terminal

TABLE III

METHYLATION ANALYSES OF *E. coli* K44 POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugar or derived compound[a] *(as alditol acetate)*	*Mole percent*[b,c]						
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*
2,4-Rha	7.4	60.6	35.8	56.4		43.2	
2,3,4-Rha					48.5		65.3
2,3,4-Glc				43.5			
2,3,6-Glc			24.3				
3,6-GlcN(Me)Ac	52.9	23.9	27.2		45.6	26.7	34.6
3,4-GalN(Me)Ac	39.6	15.7	12.5		5.8		
1,4-Erythritol						27.0	
1-Glycerol						4.1[d]	

[a] 2,4-Rha = 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol; 3,6-GlcN(Me)Ac = 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol; etc. [b]Using a DB-17 capillary column programmed to hold at 180° for 1 min, and then increase 2°/min to 250°. [c]I, native polysaccharide, methylated components obtained by acetolysis; II, native polysaccharide, methylated products obtained by acid hydrolysis; III, carboxyl-reduced polysaccharide; IV, compound **1** (from deamination), carboxyl reduced after methylation; V, product from β-elimination and remethylation; VI, polyol from periodate oxidation of carboxyl-reduced polysaccharide; VII, disaccharide glycoside (**2**) from the Smith degradation. [d]Because of the high volatility of the acetate, quantitation was inaccurate.

sugars, which implied a linear tetrasaccharide repeating unit.

Deamination of the polysaccharide. — The polysaccharide was *N*-deacetylated[15] and the removal of the *N*-acetyl substituent was verified by ^{1}H-n.m.r. spectroscopy. The resulting polymer was deaminated and the product was separated by gel-permeation chromatography. Two fractions were obtained, the slower-moving one (eluted in the region corresponding to monosaccharides) in low yield. This fraction failed to give satisfactory n.m.r. and g.l.c. data. Sugar and methylation analyses (Tables I, column V, and III, column IV) conducted on the second fraction showed that it contained the aldobiuronic acid β-D-GlcA-(1→3)-L-Rha (**1**). The presence of 2,3,4-tri-*O*-methylglucose, on methylation followed by reduction of the uronate ester, proved that the glucuronic acid exists in the pyranoid configuration. In the ^{1}H-n.m.r. spectrum (Table II) the signal at δ 4.68 (broad, 1.0 H) was attributed to the β-glucuronic residue, whereas the signal at δ 5.17 (broad, 0.9 H) was attributed to the rhamnosyl reducing end.

Base-catalyzed β-elimination[16]. — The permethylated native polysaccharide was treated with sodium methylsulfinylmethanide for 18 h, and the product was methylated. G.l.c. analysis (Table III, column V) of the sugars as partially methylated alditol acetates showed 2,3,6-trimethylglucosamine and small quantities of 2,3,4-trimethylgalactosamine. The lower amount of the latter is probably due to it being the reducing sugar after liberation from the glucuronic acid by β-elimination, and undergoing degradation in the basic medium. A similar observation was noted for the Klebsiella K26 (ref. 17) and K79 (ref. 18) polysaccharides. In both cases, the reducing sugars obtained after a base-catalyzed β-elimination were 6-linked. The 2,4-di-*O*-methylrhamnose from the methylation of the native polysaccharide (Table III, column I and II) was replaced by 2,3,4-tri-*O*-methylrhamnose, indicating that the glucuronic acid was linked to O-3 of the rhamnose residue. These results permit the assignment of the following tentative partial sequence for the K44 repeating unit:

→6)-GalNAc-(1→4)-GlcA-(1→3)-Rha-(1→

Smith degradation. — Periodate oxidation[19] of the carbodiimide-reduced polysaccharide was followed by reduction and the polyol was isolated after dialysis against distilled water. The ^{1}H-n.m.r. spectrum of the polyol showed two prominent anomeric signals at δ 4.96 (1.0 H) and δ 4.89 (1.0 H), with greatly diminished signals at δ 4.72 (0.2 H), and δ 4.60 (0.1 H). Sugar (Table I, column VI) and methylation (Table III, column VI) analyses conducted on the polyol showed that the 3-linked rhamnose and 4-linked 2-acetamido-2-deoxyglucose survived the periodate oxidation, whereas the 6-linked 2-acetamido-2-deoxygalactose and 4-linked glucose (from reduction of glucuronic acid) were degraded. This result is in agreement with the evidence from the methylation analyses of the carboxyl-reduced K44 polysaccharide (Table III, column III). The presence of glycerol, the product of the

periodate-oxidized 6-linked GalNAc residue, confirmed that the amino sugars are adjacent to each other in the native polysaccharide.

To ensure complete glycol cleavage a second periodate oxidation was conducted on the polyol, and the product was worked up as just described. The polyol was then subjected to a Smith degradation[20], whereby the acyclic acetal linkages were cleaved in preference to the glycosidic (or cyclic acetal) linkages. Dialysis of the Smith-degraded product, followed by separation of the concentrated dialyzate using gel-permeation chromatography, gave a compound which by sugar (Table I, column VII) and methylation (Table III, column VII) analyses was shown to be the disaccharide glycoside **2**.

α-Rha-(1→4)-α-GlcNAc-(1→1)-glycerol

2

The assignments for the anomeric signals of **2**, presented in Table II, were made by comparison with the n.m.r. data for compound **3**, which was obtained by the periodate oxidation of compound **2**, followed by reduction. The ^{1}H-n.m.r. spectrum of **3** showed only one anomeric signal, at δ 4.97 ($J_{1,2}$ 3 Hz, 1 H), and a methyl resonance at δ 2.06 (s, 3 H), which is attributed to the *N*-acetyl group of the 2-acetamido-2-deoxyglucose residue (Table II). The degradation of the terminal rhamnosyl residue, as indicated by the absence of the signals at δ 4.90 and δ 1.34, is consistent with the methylation data for compound **2**. The Smith degradation of compound **3** followed by methylation analysis gave 2,3,4,6-tetramethylglucosamine.

CONCLUSION

These experiments demonstrate that the structure of the capsular polysaccharide from *E. coli* K44 is based on the linear tetrasaccharide repeating unit shown in the Abstract. Independent evidence for this structure has been obtained by studying the bacteriophage-induced degradation products of the K44 polysaccharide[21].

EXPERIMENTAL

General methods. — The instrumentation used has been described previously[18]. Whatman No. 1 paper was used for chromatography with solvent 1, 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, solvent 2, 8:2:1 ethyl acetate–pyridine–water, and solvent 3, 4:1:5 1-butanol–ethanol–water (upper phase). Analytical g.l.c. was carried out on an HP 5890A instrument using a fused silica capillary column coated with DB-17. For peracetylated alditols the temperature was held at 180° for 2 min and then raised at 5° /min to 220°; for partly methylated alditol acetates the program was 180° for 1 min, then increase at 2°/min to 250°. Preparative g.l.c. was performed on an F&M 720 instrument equipped with a column of 3% SP-2340 on

Supelcoport 100-120 mesh, temperature programmed from 190° to 240° at 4/min.

Preparation of E. coli *K44 capsular polysaccharide.*. — A culture of *E. coli* K44, obtained from Dr. Ida Ørskov (WHO International Escherichia Center, Copenhagen) was propagated on Mueller-Hinton broth and agar. The K44 polysaccharide (~0.3 g per liter of medium) prepared as previously described[3] and purified by two precipitations with cetyltrimethylammonium bromide, had $[\alpha]_D$ +50°.

Sugar analyses of the polysaccharide. — A sample (7 mg) of K44 polysaccharide was hydrolyzed with 2M HCl for 7 h on a steam bath (~95°). After removal of the acid by coevaporation with water (4 x 5 mL), paper chromatography of the hydrolyzate in solvents 1 and 2 showed the presence of rhamnose, glucosamine, galactosamine, and a slow-moving component (probably the aldobiuronic acid).

A sample (11 mg) of the K44 polysaccharide was methanolyzed[4] with 3% HCl in methanol (16 h, 95°) and the acid was neutralized with $PbCO_3$. Reduction with $NaBH_4$ in anhydrous methanol (3 h, r.t.) was followed by the addition of 50% acetic acid and concentration. Portions of methanol (3 x 5 mL) were evaporated from the product, and the residue was hydrolyzed with 2M HCl (6 h, 95°). A portion of the hydrolyzate was then reduced (aqueous $NaBH_4$, 30 min, r.t.) and converted into alditol acetates. For g.l.c. data see Table I, column II.

The other portion was deaminated[22] (1 h, r.t.) with 33% acetic acid (1 mL) and 5% aqueous $NaNO_2$ (1 mL). This was followed by dilution with water (5 mL), and the solution was freeze dried. The product was reduced with aqueous $NaBD_4$ (2 h, r.t.), acetylated using 1:1 pyridine-Ac_2O (30 min, 95°), and analyzed by g.l.c.

Carbodiimide-mediated reduction of K44 polysaccharide. — Following the published procedure[5], K44 polysaccharide (250 mg) was reduced using 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (1 g) and aqueous $NaBH_4$ (3M, 150 mL). Neutral polysaccharide was obtained (ca. 95% yield) after two treatments. A sample (10 mg) of this neutral polysaccharide was hydrolyzed (2M HCl, 7 h, 95°) and converted into its alditol acetates by the procedures just described. The sugar composition is shown in Table I, column IV and the methylation data in Table III, column III.

Chromic acid oxidation[10]. — To a solution of the carbodiimide-reduced K44 polysaccharide (15 mg) in formamide (5 mL) were added Ac_2O (4 mL) and pyridine (4 mL), and the mixture was stirred for 2 d at room temperature. Following dialysis (mol. wt. cutoff 3,500) against running tap water, the retentate was freeze dried, and the residue was subjected to a second acetylation and workup. The peracetylated polysaccharide (checked by i.r. spectroscopy for absence of hydroxyl absorbance) was dissolved in glacial AcOH (3.5 mL), CrO_3 (100 mg) was added, and the mixture was stirred for 24 h at room temperature. The product, isolated by partition using $CHCl_3$-H_2O, was desalted by passage through a column of Sephadex LH-20, with acetone as the eluant. The degraded peracetylated product was hydrolyzed (2M HCl, 7 h, 95°) and reduced with $NaBH_4$ (30 min, r.t.). After treatment of the reaction mixture with Amberlite IR 120 (H^+) resin, the neutral residue was acetylated with 1:1 pyridine-Ac_2O.

Methylation analysis. — A sample of the native K44 polysaccharide (40 mg) in the free acid form [obtained by passing an aqueous solution of the isolated salt through a column of Amberlite IR 120 (H^+) resin] was methylated by the Hakomori procedure[13,14]. This involved dissolving the polysacharide in dry Me_2SO (7 mL) and treating it with sodium methylsulfinylmethanide (2M, 4 mL) for 2 h under a nitrogen atmosphere. Methyl iodide (10 mL) was added to the cooled mixture, which was stirred until a clear pale-yellow solution was obtained (~2 h). The product, recovered after dialysis (mol. wt. cutoff 3,500) against running tap water, was extracted into CH_2Cl_2 and the solution was passed through a column of Sephadex LH-20 (elution with 1:1 MeOH–$CHCl_3$). An i.r. spectrophotometric analysis of the purified product (37 mg) recovered from the eluate showed complete methylation, *i.e.* no hydroxyl absorption. The same procedure was performed on a sample (10 mg) of the carbodiimide-reduced polysaccharide.

One part (15 mg) of the methylated polysaccharide was acetolyzed[23] using 0.9M H_2SO_4 in 95% AcOH for 15 h at 80°, followed by addition of H_2O (2 mL) and further heating (2 h). The other part (12 mg) was hydrolyzed using 2M HCl for 7 h at 95°. The permethylated carboxyl-reduced polysaccharide was hydrolyzed using 2M HCl for 7 h at 95°. The extent of hydrolysis in each case was monitored by paper chromatography in solvent 3. The conversion of the sugars into partially methylated alditol acetates was achieved by the same procedure described for unmethylated sugars.

Deamination of K44 polysaccharide. — *N*-Deacetylation of the polysaccharide (18 mg) was performed[15] by dissolving it with stirring in a mixture of H_2O (0.5 mL), Me_2SO (2.5 mL), NaOH (200 mg), and a drop of thiophenol. The reaction mixture was kept at 80°for 15 h . The excess base was neutralized with 2M HCl and the solution was dialyzed (mol. wt. cutoff 3,500) against running tap water overnight. The retentate was concentrated and centrifuged, and the supernatant was freeze-dried.

The resulting polymer, dissolved in H_2O (0.5 mg), was deaminated as described above. The product, after reduction with $NaBD_4$ (50 mg, 2 h), was treated with Amberlite IR 120 (H^+) resin to destroy the excess $NaBD_4$ and decationize the mixture. The solution obtained on filtration was concentrated, and methanol (4 portions) was evaporated from the residue. The product was fractionated on a column (2.5 cm x 25 cm) of Bio-Gel P-2 using 5:2:500 pyridine-acetic acid-water as eluant.

Base-catalyzed β-elimination[16]. — A dried sample (20 mg) of methylated K44 polysaccharide was dissolved in 9:1 Me_2SO–2,2-dimethoxypropane (10 mL) containing a trace of *p*-toluenesulfonic acid. Sodium methylsulfinylmethanide (2M, 5 mL) was added and the mixture was stirred for 18 h at room temperature.The degraded polymer was remethylated by adding CH_3I (10 mL) to the cooled solution and further stirring for 1 h. The methylated, degraded product was isolated by partition after the addition of CH_2Cl_2–H_2O and further purified by passing the CH_2Cl_2 solution through a column of Sephadex LH-20 (elution with 1:1 MeOH–$CHCl_3$.

The product was hydrolyzed (2M HCl, 7 h, 95°) and the sugars released were analyzed (Table III, column V).

Periodate oxidation[19] *and Smith degradation* [20] *of carboiimide-reduced* K44 *polysaccharide.* — A sample (190 mg) of the carbodiimide-reduced polysaccharide was dissolved in H_2O (120 mL) and $NaIO_4$ (1.42 g) was added. The reaction mixture was let stand in the dark for 4 d at room temperature. Ethylene glycol (0.1 mL) was added to destroy the excess periodate (2 h) and the reaction mixture was dialyzed (mol. wt. cutoff 3,500) against distilled water. The concentrated retentate was reduced with aqueous $NaBH_4$ (1 h, r.t.) and excess $NaBH_4$ was destroyed with 50% AcOH. Dialysis (mol. wt. cutoff 3,500) of the reaction mixture against distilled H_2O followed by lyophilization of the retentate yielded the polyol (127 mg) . The composition of the polyol is shown in Tables I and III and the n.m.r. data are presented in Table II. A second periodate oxidation was conducted on the polyol, and the product worked up as just described.

Smith degradation of the polyol using 0.5M trifluoroacetic acid (2 d, r.t.) was followed by dialysis (mol. wt. cutoff 3,500) against distilled H_2O. Acid was removed from the dialyzate by alternate concentration and additions of water, then evaporation to dryness. A solution of the residue was deionized by passage through separate columns of Amberlite IR 120 (H^+) resin and Amberlite IR 45 (OH^-) resin. Separation of the Smith-degraded product using a Bio-Gel P-2 column and water as the eluant gave compound **2** (77 mg). Sugar (Table I, column VII), n.m.r. (Table II) and methylation (Table III, column VII) analyses showed that compound **2** is a neutral disaccharide glycoside. Compound **2** (27 mg) was subjected to a periodate oxidation (400 mg of $NaIO_4$ in 25 mL H_2O) for 1 d at room temperature in the dark. After destruction of the excess $NaIO_4$ with ethylene glycol (0.1 mL), the reaction mixture was concentrated to ~10 mL and reduced with $NaBH_4$ (30 min, r.t.). Sequential treatment with 50% AcOH, concentration, addition and evaporation of MeOH (4 x 5 mL), dissolution in water, and deionization gave compound **3**. The ^{1}H-n.m.r. spectrum showed one anomeric signal at δ 4.97 ($J_{1,2}$ 3 Hz, 1 H), and in the high field region a methyl resonance at δ 2.06 (s, 3 H). Smith degradation of the compound was accomplished in 0.5M trifluoroacetic acid overnight at room temperature, followed by concentration and the addition and evaporation of H_2O (4 x 5 mL). A methylation analysis conducted on the residue yielded only 2,3,4,6-tetramethylglucosamine.

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