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STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* SEROTYPE 55

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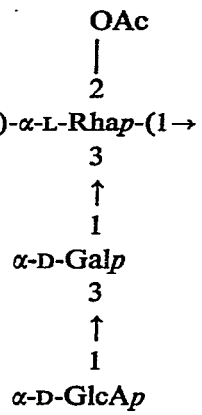
ABSTRACT

The structure of the capsular polysaccharide from *Klebsiella* type 55 has been investigated by using the techniques of methylation, Smith periodate oxidation, and partial, acid hydrolysis. The anomeric configurations of the glycosidic linkages were determined by performing ^{1}H-n.m.r. and ^{13}C-n.m.r. spectroscopy on the polysaccharide and derived poly- and oligo-saccharides obtained through degradative procedures. The position of the *O*-acetyl group was located by devising an improved method for its replacement by a methyl ether group. The structure was shown to consist of the following tetrasaccharide repeating unit.



INTRODUCTION

The approximately eighty serologically different strains of *Klebsiella* bacteria have been grouped[1,2] according to the qualitative composition of their respective, capsular polysaccharide. The capsular polysaccharides from *Klebsiella* types K12, 36, 45, 55, and 70 all consist of D-glucuronic acid, D-glucose, D-galactose, and L-rhamnose residues; however, each capsular polysaccharide has a unique structure

determining its serological specificity. Continuing our examination of the members of this group[3,4], we now report the structure of the capsular polysaccharide of serotype 55. Structures previously elucidated have been summarized[5], together with their immunochemical relationships. The prediction[5] that K55 capsular polysaccharide would have a nonreducing D-glucosyluronic acid end-group has been confirmed by chemical analysis.

RESULTS AND DISCUSSION

Composition and n.m.r. spectra. — *Klebsiella* K55 capsular polysaccharide, isolated by centrifugation and purified by Cetavlon precipitation, moved as one band during gel electrophoresis, and showed $[\alpha]_D$ +90°. Before acceptable spectroscopic analyses could be obtained, it was necessary to decrease the extreme viscosity of the native polysaccharide in aqueous solution by mild depolymerization with acid. The ^{13}C-n.m.r.[6] and ^{1}H-n.m.r. spectra clearly indicated a 1:1 ratio of *O*-acetyl group to ω-deoxy sugar, as well as signals from four anomeric atoms (carbon and hydrogen, respectively). Of the four anomeric signals, the ^{1}H-n.m.r. (p.m.r.) spectrum revealed that three arise from α-linked hexoses[7], and that the other must, because of the large coupling constant, be attributed to either a β-linked glucose or galactose residue[8]. The p.m.r. spectrum of the deacetylated polysaccharide showed that the anomeric signal at lowest field had shifted upfield, suggesting that this signal is from the sugar that bears the *O*-acetyl group. Assignment of the anomeric signals was achieved by examination of the p.m.r. spectra of poly- and oligo-saccharides derived from the capsular polysaccharide during the structural investigation (see Table I).

An acid hydrolyzate of the carboxyl-reduced polysaccharide contained rhamnose, galactose, and glucose in the relative proportions 0.98:1:2, analyzed as their alditol acetates by gas–liquid chromatography (g.l.c.). Rhamnose was confirmed to be of the L configuration, and glucose of the D configuration by circular dichroism (c.d.) measurements[10] on their alditol acetates. An acid hydrolyzate of the aldobiouronic acid isolated gave a positive reaction with D-Galactostat reagent, thus confirming the D configuration of the galactose.

Methylation analysis. — Methylation[11–17] of the acidic polysaccharide (see Table II, column I) supported the concept of a four-sugar repeating unit, and, also, indicated that rhamnose constitutes a branch point. The presence of 2,3,4-tri-*O*-methylglucose, in the absence of any di- or tetra-*O*-methylhexose, indicated that the D-glucuronic acid residue must be the terminal sugar of the side chain. The pyranosidic nature of all but the rhamnose residue is also demonstrated by the methylation results. The positions of methylation in the derived alditol acetates were determined by mass spectrometry (m.s.) and, in this investigation, individual stereoisomers were readily identified by their characteristic retention-times[16,18] and by co-chromatography with authentic standards.

Periodate oxidation. — Two, sequential, Smith periodate degradations[20,21] of the carboxyl-reduced polysaccharide established the presence of a two, rather than

TABLE I

P.M.R. DATA FOR *Klebsiella* K55 CAPSULAR POLYSACCHARIDE AND DERIVED POLY- AND OLIGO-SACCHARIDES

Compound	*Repeating unit of compound*	τ^a	$J_{1,2}$ *(Hz)*	*Integral ratios*	*Proton assignment*
Acidic polysaccharide with acetate	3 1 4 1 —Glc——Rha— β α 3 |α 1 Gal 3 |α 1 GlcA	4.47 4.86 5.25 7.81 8.65	2[b] b 8 s 6[b]	1 2 1 3 3	α-Rha α-Gal α-GlcA β-Glc CH_3 of acetate CH_3 of Rha
Acidic polysaccharide, deacetylated	3 1 4 1 —Glc——Rha— β α 3 |α 1 Gal 3 |α 1 GlcA	4.75 5.26 8.66	b 8 6[b]	3 1 3	α-Gal α-Rha α-GlcA β-Glc CH_3 of Rha
Carboxyl-reduced polysaccharide	3 1 4 1 —Glc——Rha— β α 3 |α 1 Gal 3 |α 1 Glc	4.73 4.79 4.91 5.25 8.67	3 1.5 3 8 6	1 1 1 1 3	α-Gal α-Rha α-Glc β-Glc CH_3 of Rha
P3	3 1 4 1 —Glc——Rha— β α 3 |α 1 Gal	4.74 4.77 5.27 8.66	3 2 7.5 6	1 1 1 3	α-Gal α-Rha β-Glc CH_3 of Rha
P2	3 1 4 1 —Glc——Rha— β α	4.82 5.26 8.67	1.5 7.5 6	1 1 3	α-Rha β-Glc CH_3 of Rha

TABLE I *(Continued)*

P.M.R. DATA FOR *Klebsiella* K55 CAPSULAR POLYSACCHARIDE AND DERIVED POLY- AND OLIGO-SACCHARIDES

Compound	*Repeating unit of compound*	τ^a	$J_{1,2}$ *(Hz)*	*Integral ratios*	*Proton assignment*
1	1 4 Glc——Rha-OH β	4.89	1.5	0.7	α-Rha-OH
		5.14	1	0.3	β-Rha-OH
		5.29	7.5	1	β-Glc
		8.66	6	3	CH_3 of Rha
2	1 3 GlcA——Gal-OH α	4.67	3	0.5	α-Gal-OH
		4.83	3.5	0.8	α-GlcA
		4.91	3	0.2	α-GlcA
		5.33	7	0.5	β-Gal-OH
3	1 4 Glc——Rha-OH β 3 |α 1 Gal 3 |α 1 GlcA	4.74	3	1	α-Gal
		4.83	1.5	0.7	α-Rha-OH
		4.85	3.5	1	α-GlcA
		5.15	1	0.3	β-Rha-OH
		5.29	7.5		
		5.31	7.5	1	β-Glc
		8.66	6	3	CH_3 of Rha

[a]Shifts are given relative to internal acetone; τ 7.77 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.). Singlets designated s and b indicates that it was not possible to measure the coupling constant accurately.

TABLE II

METHYLATION ANALYSES OF K55 CAPSULAR POLYSACCHARIDE AND DERIVED POLY- AND OLIGO-SACCHARIDES

Methylated sugar[a] (as alditol acetate)	T[b]	*I[c] (Mole %[d])*	*II*	*III*	*IV*	*V*
2,3-Rha	0.95			49.1		
2,3,4,6-Glc	1.0					47
2,3,4,6-Gal	1.1		31.0[e]			
2-Rha	1.3	24.7	34.4			26
2,4,6-Glc	1.5	25.2	34.6	50.9		27
2,4,6-Gal	1.6	24.7			50	
2,3,4-Glc	1.7	25.4			50	

[a]2,3-Rha = 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-L-rhamnitol, etc. [b]Retention time relative to the alditol acetate derivative from 2,3,4,6-tetra-*O*-methyl-D-glucose on an OV-225 column programmed at 180° for 8 min and then at 2° min to 200°. [c]Column I, acidic capsular polysaccharide; II, P3; III, P2; IV, aldobiouronic acid; and V, aldotetraouronic acid. [d]Values are corrected by use of the effective carbon response factors given by Albersheim *et al.*[19]. [e]Part of this volatile ether was probably lost during processing.

a one or three, sugar-unit side-chain and, also, that the side chain is attached to O-3 of rhamnose rather than O-4. As only the terminal sugar unit of the carboxyl-reduced polysaccharide is susceptible to periodate oxidation, it was selectively removed by Smith degradation, to give a polysaccharide having a three-sugar repeating-unit, designated P3. Methylation analysis of P3 yielded essentially equimolar amounts of 2,3,4,6-tetra-*O*-methylgalactose, 2-*O*-methylrhamnose, and 2,4,6-tri-*O*-methylglucose (see Table II, column II) confirming (*a*) the three-sugar repeating-unit, and (*b*) that the terminal glucose residue (glucuronic acid in the acidic polysaccharide) had been attached to O-3 of galactose. The p.m.r. spectrum of P3, when compared with that of the original, carboxyl-reduced polysaccharide, revealed the absence of a signal corresponding to the anomeric proton of an α-linked hexose. Thus, the terminal glucose residue (glucuronic acid in the original) is α-linked. The decrease in optical rotation from +98° for the carboxyl-reduced polysaccharide to +30° for P3 is in agreement with its assignment.

In the second, periodate degradation, it was again only the terminal unit that was oxidized, and, hence, the galactose was selectively removed to give P2. The identification of equimolar amounts of 2,3-di-*O*-methylrhamnose and 2,4,6-tri-*O*-methylglucose, resulting from the methylation analysis of P2, established that the galactose unit had been attached to O-3 of rhamnose. The total structure consists, therefore, of a backbone of glucose and rhamnose units, with an aldobiouronic acid side-chain attached to O-3 of rhamnose. The p.m.r. spectrum of P2 indicated the presence of one less α-D-hexose in P2 than in P3. This signifies that the galactose residue in P3 was also α-D-linked: the decrease in optical rotation from +30° for P3 to −39° for P2 is again consistent with this argument. The β configuration can, then, be assigned to the in-chain D-glucose residue, and the remaining α-linkage assigned to the L-rhamnose residue.

The difference in the recovered yield of P3 (70%) compared to P2 (90%) may be explained by the difference in the pH at which the periodate oxidation was conducted. The periodate oxidation of P3, to afford P2, was accomplished in a sodium acetate buffer at pH 5, whereas the periodate oxidation of the carboxyl-reduced polysaccharide, to afford P3, was performed in an unbuffered solution. Presumably, in the latter case, the formic acid liberated effected the hydrolysis of some of the glycosidic bonds, and, hence, some oligomeric material was lost during dialysis; this could be expected to remove some of the attached side-chains, and thereby disrupt the repeating unit. However, this was not found to be the case, as total-sugar analysis of P3 revealed rhamnose, galactose, and glucose in the ratios of 1 : 1 : 1.03. As will be seen from the results of partial, acid hydrolysis, the most labile glycosidic bond in this polysaccharide is the α-L-linked rhamnose residue; therefore, glycosidic cleavage results in the loss of whole repeating units rather than side-chain fragments, and thus the integrity of the repeating unit is retained.

Partial hydrolysis. — Partial, acid hydrolysis of the acidic polysaccharide, followed by fractionation on an ion-exchange resin and then gel-filtration chromatography, provided oligomers that confirmed the structure previously assigned. The

neutral fraction contained only one disaccharide (R_{Glc} 1.2), and monosaccharides, mainly glucose and rhamnose. The neutral disaccharide was identified as 4-*O*-β-D-glucopyranosyl-L-rhamnose (**1**, scillabiose) by comparing values of optical rotation, and p.m.r. and ^{13}C-n.m.r. spectra, with those of an authentic[22,23] sample of the sugar, and by the melting point of the derived heptaacetate when mixed with an authentic standard[22]. This oligomer confirmed that D-glucose is attached to O-4 of L-rhamnose by a β-D-linkage.

A range of larger oligomers, as well as an aldobiouronic acid and at least one, or possibly two, aldotetraouronic acids, was isolated from the acidic fraction. The aldobiouronic acid **2**, $[\alpha]_D$ +137°, was shown by ^{13}C-n.m.r. and p.m.r. spectroscopy to contain no rhamnose, but the spectra revealed signals corresponding to one nonreducing α-D-hexose and two, fractional signals attributable to a reducing glucose or galactose unit (implied by the large coupling-constant of the β-D signal in the p.m.r. spectrum[8]). Methylation, lithium aluminum hydride reduction, hydrolysis, and derivatization as alditol acetates gave equimolar amounts of 2,4,6-tri-*O*-methylgalactose and 2,3,4-tri-*O*-methylglucose. The width of the 2,3,6-tri-*O*-methylgalactose peak during g.l.c., as well as a careful inspection of the mass spectrum, indicated that it was probably contaminated with some 2,5,6-tri-*O*-methylgalactose arising from the furanose form of the reducing sugar. The structure of the aldobiouronic acid **2** is thus established as 3-*O*-α-D-glucopyranosyluronic acid-D-galactose, in agreement with the proposal that the polysaccharide has a glucuronic acid unit linked to O-3 of galactose by an α-D-linkage. The occurrence of this aldobiouronic acid in a bacterial polysaccharide was first reported[24] in the case of *Klebsiella* strain 6412 (K-type 83), although it had been found[25] earlier as a component of the partial, acid hydrolyzate of ketha (*Feronia elephantum*) gum.

The other major, identifiable, acidic oligomers **3** were two compounds (R_{Glc} 0.20 and 0.37) having essentially identical properties in gel-permeation chromatography and, hence, inseparable by this method. The ^{13}C-n.m.r. and p.m.r. spectra of the mixture indicated that either the major component (R_{Glc} 0.20), or both components, contains one β-D-linked hexose and two α-D-linked sugars per reducing rhamnose unit. This suggests that at least the major component is an aldotetrauronic acid having the following structure.

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

The observations that all of the larger oligomers were acidic, and that rhamnose, rather than glucose, was found as the reducing sugar in the isolated oligomers, implied that by far the most labile glycosidic bond in this polysaccharide is the α-L-rhamnopyranosyl linkage. The assumption that mild, acid hydrolysis, to lower the degree of polymerization and facilitate handling, does not alter the structure of the repeating unit is justified if the only linkages cleaved are the α-L-rhamnosidic bonds. Additional evidence to support this supposition is the similarity between the total-sugar ratios of P3 and partially depolymerized P3. Partial, acid hydrolysis appears first to cleave the polysaccharide into aldotetraouronic acid units, which are then further broken down into an aldobiouronic acid and a neutral disaccharide.

TABLE III

SUGAR ANALYSES AFTER REPLACEMENT OF THE *O*-ACETYL GROUPS OF K55 CAPSULAR POLYSACCHARIDE BY *O*-METHYL GROUPS

Sugar[a]	T[b]	T[c]	*Mole %*[d]	
			I[e]	*II*[e]
2-Rha	27.8	10.9	11	16
Rha	31.9	12.6	13	8
Gal	62.7	26.6	28	27
Glc	65.7	28.3	48	49

[a]2-Rha = 1,3,4,5-tetra-*O*-acetyl-2-*O*-methyl-L-rhamnitol, etc. [b]Retention time (in min) on a column of 0.2% of polyethylene glycol succinate, 0.2% of polyethylene glycol adipate, and 0.4% of XF 1150, programmed from 125 to 200° at 1°/min. [c]Retention time (in min) on a column of 3% of SP2340 programmed from 165 to 230° at 2°/min. [d]Values are corrected by use of the effective carbon response factors given by Albersheim *et al.*[19]. [e]I = conventional, II = modified process; for details, see text.

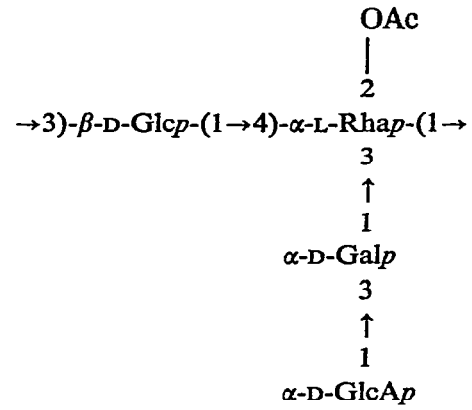
All of the evidence produced so far is consistent with the proposed structure, but the results do not exclude the possibility that the main chain is composed of blocks of D-glucose and of L-rhamnose units, rather than an alternating arrangement. The observed resistance of the β-D-glucosidic bond to acid hydrolysis, compared to that of the α-L-rhamnosidic bond, together with the complete absence of any neutral oligomers other than scillabiose (**1**) excludes a main chain consisting of blocks of D-glucose units. The presence of only the one neutral oligomer, 4-*O*-β-D-glucopyranosyl-L-rhamnose, in the partial, acid hydrolyzate of the acidic polysaccharide confirms the alternating pattern of the glucose and rhamnose units in the main chain.

Location of acetate group. — The only aspect of the K55 capsular polysaccharide structure still undetermined is the location of the *O*-acetyl group. This was shown, by using two somewhat different procedures, to be located at O-2 of L-rhamnose. Employing the method first used by De Belder and Normann[26], and later by Lindberg

et al.[18,27], the free hydroxyl groups in acidic K55 polysaccharide were protected by reaction with methyl vinyl ether, using *p*-toluenesulfonic acid as the catalyst. Purification by gel-permeation chromatography, followed by Hakomori methylation, lithium aluminum hydride reduction, and hydrolysis, provided 2-*O*-methylrhamnose, rhamnose, galactose, and glucose, identified and quantitated as their alditol acetates by g.l.c.–m.s. (see Table III, column I). As only the *O*-acetyl groups are replaced by methyl ether groups, the identification of 2-*O*-methylrhamnose indicated that the *O*-acetyl group is located on O-2 of rhamnose. The incomplete replacement of all of the acetate groups by methyl ether groups may be due to steric hindrance around O-2 of rhamnose. The sharp resolution of the *O*-acetyl signal in the p.m.r. spectrum is consistent with its location at a specific site in the polysaccharide, and the 1 : 1 integral of *O*-acetyl to rhamnose indicates that each rhamnose unit carried an *O*-acetyl group.

The main difficulties with the foregoing method arise from (*a*) the severe contamination of the fully acetalated polysaccharide with polymeric products of methyl vinyl ether, and (*b*) the subsequent difficulty of separating the fully acetalated polysaccharide from the *p*-toluenesulfonic acid and contaminating polymers without hydrolysis of the acid-labile acetal groups, particularly when the polysaccharide is itself acidic. Although it was reported[28] that methyl vinyl ether forms an ester with the carboxyl group, it is likely to be only stable enough to be purified under the most rigorously anhydrous, chromatographic conditions. In order to circumvent these difficulties, that is to prepare a polymer that could be readily handled and thoroughly purified on a larger scale, a procedure was devised in which, immediately after acetalation, all acidic groups were converted into their methyl esters by the addition of diazomethane. As the replacement of an *O*-acetyl group by a methyl ether involves attack by a nucleophile, rather than a base, the derivatized polymer was then treated with sodium methoxide in dimethyl sulfoxide, rather than dimsyl anion, to form sodium alkoxide functionalities at the oxygen atoms that originally bore acetyl groups. The addition of methyl iodide then rapidly led to etherification of those positions. The advantage of this procedure is that all of the steps can be performed in sequence in the same reaction-vessel, under a serum cap, by introducing reagents and removing by-products with hypodermic needles, thereby maintaining anhydrous conditions. The tagged polysaccharide can then be rigorously purified by column chromatography. β-Elimination may occur during the treatment with sodium methoxide; however, very little evidence of this was observed. An attempt to avoid the possibility of β-elimination by neutralizing the polymer, immediately after acetalation, with an equivalent of sodium hydride in dimethyl sulfoxide (instead of diazomethane) did not produce interpretable results. Analysis by this alternative procedure showed a greater proportion of 2-*O*-methylrhamnose (see Table III, Column II) than the previous method, thus supporting the attachment of the acetyl group to O-2 of rhamnose.

The aforementioned studies reveal that the capsular polysaccharide from *Klebsiella* type 55 is composed of tetrasaccharide repeating units having the following structure.



Of the *Klebsiella* capsular polysaccharides reported to date, that from *Klebsiella* strain 6412 (K-type 83) most closely resembles the structure presented here for the K55 antigen. In K83 polysaccharide[24], there is no acetate group, and a β-D-galactopyranosyl residue replaces the in-chain β-D-glucopyranosyl unit in K55; all other structural features are identical. The next closest analog is the polysaccharide from *Klebsiella* K20, which also has a terminal D-glucuronic acid unit[29].

Other observations. — Although the primary structure of the capsular material from *Klebsiella* K55 has been determined, several unusual properties of this polysaccharide were observed during the investigation. Among these may be cited the viscosity of an aqueous solution, extremely high for a branched polysaccharide; the tendency of the acidic polysaccharide and of P2 to form insoluble gels; and, the ability of the acidic oligomers to aggregate, as judged by their behavior in gel-filtration. The high viscosity and tendency to gel formation may be related to the β-D-glucopyranosyl linkage to O-4 of a (deoxy)hexose, as this structure has a formal resemblance to that of cellulose. In the case of the intact polysaccharide, there is the additional possibility of the intramolecular formation of an ester between the uronic acid and, for example, O-4 of galactose; evidence for such esters has been reported[30].

In the p.m.r. spectra of the aldobiouronic acid and the aldotetraouronic acid, certain of the signals due to anomeric protons showed unexpected splitting. Thus, for the aldobiouronic acid, H-1 of the D-glucuronic acid gave two signals, at τ 4.83 and 4.91; although these may result from the influence of the reducing galactose residue, such an explanation is less likely to explain the splitting of H-1 in the β-D-glucopyranosyl unit of the aldotetraouronic acid, as linkage of the "aglycon" through O-4 is generally considered to be without such influence.

EXPERIMENTAL

General methods. — P.m.r. spectra were recorded with a Varian XL-100 instrument at ~90°, after samples had been dissolved in D_2O and exchanged. Acetone (τ 7.77) was used as the internal standard. ^{13}C-N.m.r. spectra were recorded

with a Varian CFT-20 instrument. Samples for ^{13}C-n.m.r. spectroscopy were dissolved in 50% aqueous D_2O, with 1,4-dioxane as the internal standard. Optical rotations were measured at 25 $\pm 1°$ with a Perkin–Elmer model 141 polarimeter by using a 10-cm cell. Circular dichroism spectra were recorded with a Jasco J20 automatic recording spectropolarimeter, and infrared spectra with a Perkin–Elmer 457 spectrophotometer. Descending, paper chromatography was conducted on Whatman No. 1 paper, with solvent systems *A* and *B*; (*A*) 4:1:1 ethyl acetate–pyridine–water, and (*B*) freshly prepared 2:1:1 1-butanol–acetic acid–water. Compounds were made visible by using silver nitrate in acetone[31]. Thin-layer chromatography (t.l.c.) and column chromatography were performed on silica gel G (from EM Reagents). Analytical, g.l.c. separations were performed with a Hewlett–Packard 5700 instrument fitted with dual flame-ionization detectors. An Infotronics CRS-100 electronic integrator was used to measure peak areas. Stainless-steel columns (1.8 m × 3 mm) were used with nitrogen as the carrier gas at a flow rate of 20 mL/min. Columns used were (*1*) 3% of OV-225 on Supelcoport (100–120 mesh); (*2*) 0.2% of polyethylene glycol succinate, 0.2% of polyethylene glycol adipate, and 0.4% of XF1150; (*3*) 3% of OV-1; and (*4*) 3% of SP2340; each of the last three on Gas Chrom Q (100–120 mesh). Preparative g.l.c. was performed in an F and M model 720 instrument having dual thermal-conductivity detectors. Columns (1.8 m × 6.3 mm) analogous to those used for analytical separations were used, except that (*5*) 3% of Silar 10C on Gas Chrom Q (100–120 mesh) was used instead of *2*. G.l.c.–m.s. was conducted with a Micromass 12 instrument fitted with a Watson–Biemann separator. Spectra were recorded at 70 eV, with an ionization current of 100 μA and an ion-source temperature of 200°. Gel-permeation chromatography was conducted on columns of Sephadex G-10 (170 × 2.5 cm) or Bio-Gel P-4 (minus 400 mesh; 120 × 2.5 cm). The columns were irrigated with water at a flow rate of ~7 mL/h. Fractions (1–2 mL) were collected, freeze-dried, and analyzed by paper chromatography. Melting points were obtained, for samples between glass slides, with a Fisher–Johns apparatus, and are uncorrected. Solutions were evaporated below 50° under diminished pressure.

Preparation and properties of Klebsiella *K55 capsular polysaccharide.* — A culture of *Klebsiella* K55 (3985/51) was obtained from Dr. I. Ørskov, Copenhagen, and was grown on a medium of NaCl (8 g), K_2HPO_4 (4 g), $MgSO_4 \cdot 7H_2O$ (1 g), $CaCO_3$ (2 g), sucrose (120 g), and Bacto yeast extract (8 g) in water (4 L) for 4 d. The cells were harvested, and diluted to twice their original volume (1.5 L) with water containing 1% of phenol; this suspension was then centrifuged, in batches, for 5 h at 35,000 r.p.m. in a Beckman L3-50 centrifuge with rotor type 35. The clear, supernatant liquors were separated, combined (~900 mL), and precipitated by pouring into 4:1 acetone–ethanol (2.5 L). The crude polysaccharide, isolated by decantation and centrifugation, was dissolved overnight in the minimal volume of water, and then precipitated with a 10% Cetavlon solution. The precipitate was isolated by centrifugation, washed with a small volume of cold water, and redissolved in the minimal amount of 4M NaCl overnight. The polysaccharide in the salt solution was precipitated by pouring it into 1 to 2 volumes of alcohol, with stirring. The Cetavlon-purified

polysaccharide was isolated by centrifugation, dissolved in water overnight, and then dialyzed overnight against running tap-water. Freeze-drying of this solution yielded 5 g of the partial sodium salt of the polysaccharide. The free-acid form of the polysaccharide was obtained by passing a solution of the partial sodium salt through a column of Amberlite IR-120 (H^+) resin, followed by dialysis and freeze-drying.

Deacetylation of K55 polysaccharide was achieved by treatment with 0.1M NaOH for 2.5 h at 40°, followed by dialysis, desalting with Amberlite IR-120(H^+) resin, dialysis, and freeze-drying. The p.m.r. spectrum of the deacetylated polysaccharide indicated the complete absence of an *O*-acetyl signal. Deacetylated, partially depolymerized K55 polysaccharide had $[\alpha]_D$ +90° (*c* 1.2, water).

Spectroscopic analyses were performed on K55 polysaccharide, and on derived polysaccharides that had been partially depolymerized by hydrolysis in 0.1M trifluoroacetic acid (100 mg/50 mL) for 0.5 h at 95°, and then isolated by dialysis and freeze drying (92% recovery).

The p.m.r. spectrum of partially depolymerized K55 polysaccharide in D_2O at 90° showed a sharp singlet at τ 7.81 due to the CH_3 of the *O*-acetyl group, which integrated with the doublet due to the CH_3 of rhamnose at τ 8.65 in the ratio of 1:1. In the anomeric region, four discernible signals were observed at τ 4.47, 4.86 (two protons), and 5.25 (see Table I). The p.m.r. spectrum of deacetylated, partially depolymerized, K55 polysaccharide revealed three anomeric signals at τ 4.75 and one at τ 5.26, as well as a three-proton doublet at τ 8.66 arising from the CH_3 of rhamnose (see Table I).

The ^{13}C-n.m.r. spectrum of K55 polysaccharide confirmed the presence of rhamnose (C-6 at 18.0 p.p.m.) and the *O*-acetyl group (CH_3 at 21.2 p.p.m.). Two signals attributable to C-6 of hexoses (62.0 p.p.m.) and two signals due to linkage-position carbon atoms (85.6 p.p.m.) were also observed. In the anomeric region, four signals could be distinguished at 103.1, 99.6, 95.9, and 93.4 p.p.m. (see Table I).

Methylation analysis of the polysaccharide. — *Klebsiella* K55 capsular polysaccharide (25 mg, deacetylated) in dimethyl sulfoxide (4 mL) was methylated[11,12] by treatment with dimsyl anion (4 mL) for 6 h, and then overnight with methyl iodide (2 mL). After removal of the excess of the reagents by dialysis, the methylated polysaccharide was isolated by freeze-drying. Subsequent Purdie[13] treatment with silver oxide and methyl iodide yielded a methylated polysaccharide that showed no hydroxyl group absorption in the infrared spectrum. This methylated polysaccharide, $[\alpha]_D$ +74° (*c* 1.2, chloroform), was reduced overnight with lithium aluminum hydride in refluxing tetrahydrofuran (absence of carbonyl absorption in the infrared spectrum), and then purified by chromatography on a column of silica gel, with elution first with 1:9 ethyl acetate–chloroform, and then with 1:9 ethanol–chloroform. After hydrolysis of the methylated, reduced polysaccharide with 2M trifluoroacetic acid for 12 h at 95°, followed by reduction of the hydrolyzate with sodium borohydride, and then acetylation of the products with acetic anhydride in pyridine, equimolar amounts were obtained of the alditol acetates of 2-*O*-methylrhamnose, 2,4,6-tri-*O*-methylglucose, 2,4,6-tri-*O*-methylgalactose, and 2,3,4-tri-*O*-methylglucose, identified by comparative

g.l.c. and mass-spectrometric analyses with authentic standards (see Table II, column I).

Carboxyl reduction of the polysaccharide, and sugar analysis of the carboxyl-reduced polysaccharide. — A portion of the acidic capsular polysaccharide was carboxyl-reduced[9]; two treatments were performed, to ensure complete reduction; yield 75%. The resulting, neutral polysaccharide had $[\alpha]_D$ +98° (*c* 1.3, water). The p.m.r. spectrum in D_2O at 90° showed a doublet at τ 8.67 due to the CH_3 of rhamnose, and four signals in the anomeric region at τ 4.73, 4.79, 4.91, and 5.25 (see Table I). The ^{13}C-n.m.r. spectrum showed a signal from C-6 of rhamnose at 17.9 p.p.m., and four signals in the anomeric region at 93.2, 96.3, 101.7, and 103.2 p.p.m., as well as three signals attributable to C-6 of hexose sugars and two signals assigned to linkage-position carbon atoms[6]. After hydrolysis of a sample of the carboxyl-reduced polysaccharide with 2M trifluoroacetic acid for 8 h at 95°, derivatization of the liberated monosaccharides as their alditol acetates gave L-rhamnitol pentaacetate, galactitol hexacetate (m.p. 168°), and D-glucitol hexaacetate (m.p. 99°) in the ratios of 0.98 : 1 : 2 (column *2*, programmed from 125 to 200° at 1°/min, or column *5*, programmed from 180 to 230° at 2°/min). Circular dichroism measurements[10] of the collected rhamnitol pentaacetate indicated, by comparison with authentic standards, that the rhamnose was in the L configuration; ($\Delta\varepsilon_{213}^{MeCN}$ −1.3). Similarly, the glucitol hexaacetate was shown to have the D configuration; ($\Delta\varepsilon_{215}^{MeCN}$ +0.19).

Sequential, Smith periodate degradations, and methylation analyses of the degraded polysaccharides. — Carboxyl-reduced polysaccharide (400 mg) was subjected to periodate oxidation with 0.05M sodium periodate (50 mL) for 12 h at 5° in the dark. The excess of periodate was decomposed with ethylene glycol, and the mixture was dialyzed overnight. The material remaining was reduced with sodium borohydride, de-ionized with Amberlite IR-120 (H^+) resin, dialyzed, and freeze-dried. The polyol recovered was hydrolyzed with 0.5M trifluoroacetic acid (50 mL) for 8 h at 25°, dialyzed, and freeze-dried; yield 207 mg of P3. G.l.c. analysis of the alditol acetates from the total, acid hydrolyzate (2M trifluoroacetic acid for 8 h at 95°) of P3 and of partially depolymerized P3 disclosed that the peracetates of rhamnitol, galactitol, and glucitol were present in the ratios of 1 : 1 : 1.1 and 0.99 : 1 : 1.1, respectively (column *2* programmed from 125 to 200° at 1°/min). Partially depolymerized P3 had $[\alpha]_D$ +30° (*c* 1.5, water). The p.m.r. spectrum in D_2O at 90° showed a doublet at τ 8.66 due to the CH_3 of rhamnose, and three signals in the anomeric region, at τ 4.74, 4.77, and 5.27 (see Table I). The ^{13}C-n.m.r. spectrum showed a signal at 17.8 p.p.m. due to C-6 of rhamnose, and three signals in the anomeric region, at 94.3, 101.5, and 103.0 p.p.m., as well as two signals due to C-6 of hexoses and one signal due to a linkage-position carbon atom. A portion of degraded polysaccharide P3 was methylated, the ether purified and hydrolyzed, the product reduced, and the alditols acetylated by procedures analogous to those used for the native polysaccharide. The alditol acetates corresponding to 2,3,4,6-tetra-*O*-methylgalactose, 2-*O*-methylrhamnose, and 2,4,6-tri-*O*-methylglucose, identified by g.l.c. and m.s. analyses, were obtained in comparable amounts (see Table II, column II).

Degraded polysaccharide P3 (100 mg) was dissolved in sodium acetate buffer

(pH 5, 25 mL), and oxidized with sodium metaperiodate (270 mg) for 24 h at 5° in the dark. The polyol was prepared by procedures analogous to those previously described. Smith hydrolysis with 0.5M trifluoroacetic acid (50 mL) for 16 h at 25°, followed by dialysis and then freeze-drying of the recovered polyol, gave degraded polysaccharide P2; yield 60 mg. Partially depolymerized P2 showed $[\alpha]_D$ −39° (*c* 0.4, water). The p.m.r. spectrum in D_2O at 90° contained a doublet at τ 8.67 representing the CH_3 of rhamnose, and anomeric signals at τ 4.82 and 5.26 (see Table I). The ^{13}C-n.m.r. spectrum contained a signal at 17.8 p.p.m. resulting from C-6 of rhamnose, and two signals in the anomeric region, at 101.6 and 103.8 p.p.m., as well as one signal from C-6 of a hexose and two signals from linkage-position carbon atoms. Methylation analysis of degraded polysaccharide P2, employing procedures analogous to those used for P3, produced comparable amounts of 2,3-di-*O*-methylrhamnose and 2,4,6-tri-*O*-methyl-glucose as their alditol acetates (see Table II, column III). G.l.c. and m.s. comparisons with authentic standards were used for identification.

Partial, acid hydrolysis of the polysaccharide; isolation and characterization of the oligosaccharides. — Acidic polysaccharide K55 (0.2 g) was hydrolyzed in 0.05M trifluoroacetic acid (100 mL) for 16 h at 95°. After removal of the acid by evaporation with several portions of water, the hydrolyzate was made neutral (NaOH), and dialyzed against water (1 L). Residual polysaccharide (10 mg) remained in the dialysis sac. After concentration, the dialyzate was fractionated on a column (4 mL) of Dowex 1-X2 (formate). The column was first eluted with water (200 mL), yielding monosaccharides and a neutral oligomer (shown by paper chromatography to have R_{Glc} 1.2, solvent *A*), and then with 10% formic acid (100 mL), yielding the acidic oligosaccharides.

Gel chromatography on Sephadex G-10 of the neutral components provided pure 4-*O*-β-D-glucopyranosyl-L-rhamnose (**1**, 27 mg). Neutral disaccharide **1** (R_{Glc} 1.2, solvent *A*) had $[\alpha]_D$ −23° (*c* 1.4, water)[22]. The p.m.r. spectrum (D_2O, 90°) showed a doublet ($J_{5,6}$ 6 Hz) at τ 8.66 characteristic of the CH_3 of rhamnose, and anomeric signals at τ 4.89 (0.7 H, $J_{1,2}$ 1.5 Hz), 5.14 (s, 0.3 H), and 5.29 (1 H, $J_{1,2}$ 7.5 Hz). The ^{13}C-n.m.r. spectrum showed a signal at 18.2 p.p.m. from C-6 of rhamnose, and three signals from anomeric carbon atoms at 94.6, 95.9, and 104.4 p.p.m., in addition to signals at 82.0 and 82.5 p.p.m. attributable to linkage-carbon atoms. The n.m.r. spectra were exactly superposable with those of authentic scillabiose[22,23]. A portion of **1** was acetylated (acetic anhydride–pyridine), to yield a crystalline heptaacetate, m.p. 139–140°. The mixed melting point with an authentic specimen was undepressed[22].

Gel chromatography (Bio-Gel P-4) of the neutralized, acidic components provided an aldobiouronic acid (**2**, 66 mg), and two components, inseparable by that technique, considered to be aldotetraouronic acids (**3**, 57 mg).

The aldobiouronic acid (**2**; R_{Glc} 0.58, solvent *B*) had $[\alpha]_D$ +137° (*c* 0.95, water). The p.m.r. spectrum (D_2O, 90°) showed anomeric signals at τ 4.67 (0.5 H, $J_{1,2}$ 3 Hz), 4.91 (0.2 H, $J_{1,2}$ 3 Hz), 4.83 (0.8 H, $J_{1,2}$ 3.5 Hz), and 5.33 (0.5 H, $J_{1,2}$ 7 Hz). The ^{13}C-n.m.r. spectrum showed three signals, from anomeric carbon atoms,

at 93.1, 95.9, and 97.2 p.p.m. Hakomori methylation of **2** yielded the permethylated aldobiouronic acid, which, in t.l.c., had R_F 0.5 (ethyl acetate). After reduction of this compound with lithium aluminum hydride in refluxing tetrahydrofuran, and hydrolysis of the product, followed by sodium borohydride reduction of the hydrolyzate, and acetylation, yielded equimolar amounts of 2,4,6-tri-*O*-methylgalactose and 2,3,4-tri-*O*-methylglucose as their alditol acetates (see Table II, column IV). Identification was made by comparative g.l.c. and mass-spectrometric analyses with authentic standards.

An acid hydrolyzate of the aldobiouronic acid produced a positive reaction with both D-Galactostat and D-Glucostat reagents.

The aldotetraouronic acids (**3**; R_{Glc} 0.20 and 0.37, solvent *B*) had $[\alpha]_D$ +132° (*c* 0.85, water). The p.m.r. spectrum (D_2O, 90°) showed a doublet ($J_{5,6}$ 6 Hz) at τ 8.66, characteristic of the CH_3 of rhamnose, and anomeric signals at 4.74 (1 H, $J_{1,2}$ 3 Hz), 4.83 (s, 0.7 H), 4.85 (1 H, $J_{1,2}$ 3.5 Hz), 5.15 (0.3 H, $J_{1,2}$ 1 Hz), and 5.31 (5.29) (1 H, $J_{1,2}$ 7.5 Hz). The ^{13}C-n.m.r. spectrum showed a split signal at 17.8 p.p.m. resulting from C-6 of rhamnose, and five signals from anomeric carbon atoms at 93.2, 93.8, 94.5, 95.5, and 103.2 p.p.m.

Location of O-*acetyl group.* — Both experiments to locate the acetate group were conducted on Cetavlon-purified, acidic (i.e., desalted), K55 polysaccharide that had been slightly depolymerized by autohydrolysis (100 mg/10 mL for 5 h at 95°), and then isolated by dialysis and freeze-drying. The p.m.r. spectrum still indicated the presence of one acetate group per repeating unit.

Procedure 1. The acetalation (of a 20-mg sample of K55) and fractionation were performed essentially as described by Lindberg *et al.*[18,27]. The acetalated polysaccharide was methylated, carboxyl-reduced (lithium aluminum hydride in refluxing tetrahydrofuran), and hydrolyzed (2M trifluoroacetic acid, 20 mL) for 8 h at 95°, and the resulting sugars were analyzed as their alditol acetates by g.l.c.–m.s.[14,15,17] (see Table III, column I).

Procedure 2. This entire reaction was conducted under a serum cap, with use of hypodermic needles for the introduction and removal of reagents. To a solution of K55 polysaccharide (20 mg, dried overnight by gentle heating under vacuum) in dimethyl sulfoxide (4 mL) were added *p*-toluenesulfonic acid (5 mg) and methyl vinyl ether (condensed at −35°, 2 mL), and then the mixture was stirred for 4 h at 15°. The excess of methyl vinyl ether was removed under vacuum, and diazomethane in anhydrous ether was added, with stirring, until a permanent, yellow color persisted. The stirring was continued for 20 min, and then all of the volatile compounds were removed under diminished pressure. Sodium methoxide (5 mg, anhydrous) in dimethyl sulfoxide (4 mL) was added, and the mixture was stirred for 40 min. Methyl iodide (1 mL) was added to the cooled mixture, and it was kept for 2 h. The resulting mixture was dialyzed overnight against running tap-water, and the derivatized polysaccharide was isolated by freeze-drying. After carboxyl-reduction with lithium aluminum hydride in refluxing tetrahydrofuran, the partially acetalated, partially methylated, neutral polysaccharide was fractionated on a column of silica gel, with

elution first with 45:4:1 chloroform–ethyl acetate–ethanol (to remove, mainly, polymeric contaminants formed from the methyl vinyl ether) and then with 9:1 chloroform–ethanol, to elute the derivatized polysaccharide (detected by t.l.c. and by the Molisch test). A polysaccharide fraction, R_F 0.14 (19:1 chloroform–ethanol), was analyzed by preparing the alditol acetates of its hydrolyzate, and identifying them by g.l.c.–m.s.[14,15,17] (see Table III, column II).

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