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# STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE FROM *Klebsiella* SEROTYPE K-34 AND ITS CHARACTERIZATION BY N.M.R. SPECTROSCOPY

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ABSTRACT

The repeating unit of the capsular polysaccharide from *Klebsiella* type K-34 has been established by methylation, partial hydrolysis, and Smith degradation to consist of a hexasaccharide repeating-unit built up of four L-rhamnose, one D-glucose, and one D-galacturonic acid residues. The anomeric configurations of the linkages was determined by proton and $^{13}$C-n.m.r. spectroscopy at each step of the degradation procedures. Further evidence for the configurations of the glycosidic linkages involved the use of proton $T_1$ relaxation-times and oxidation by chromium trioxide. The data allowed assignment of the following structure for the repeating unit:

$$\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{A-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow$$

$$\begin{array}{l} 4 \\ \uparrow \\ 1 \\ \alpha\text{-L-Rha}p \end{array}$$

## INTRODUCTION

The capsular polysaccharide from *Klebsiella* K-34 belongs to a chemotype[1,2] that also includes serotype[3] K-48; it consists of D-galacturonic acid, L-rhamnose, and D-glucose residues. We report herein on the structure of the capsular polysaccharide of K-34, whose complexity arises mainly from the presence of four L-rhamnosyl residues among the six sugar residues of the repeating oligosaccharide. One of these rhamnose residues was expected to occupy a nonreducing, terminal position, as predicted by Heidelberger and Nimmich[4a,4b] by immunochemical cross-reaction studies. The chemical evidence presented in this communication confirm this prediction.

## RESULTS AND DISCUSSION

*Klebsiella* K-34 polysaccharide was isolated and purified as previously de-

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TABLE I

SUGAR ANALYSIS ON ORIGINAL AND MODIFIED *Klebsiella* K-34 POLYSACCHARIDE

| *Polysaccharide (or fragment thereof)* | *Sugars (as alditol acetates)*[a] | | | |
|---|---|---|---|---|
| | *Glc* | *Rha* | *Gal* | *Gal A* |
| A. Original K-34 | 1.00 | 3.70 | 0.15 | 1.00 |
| B, Carboxyl-reduced K-34 | 1.00 | 4.00 | 0.85 | |
| C, Acidic fraction after methanolysis and reduction | 1.00 | 3.00 | | 1.00 |
| D, Partially hydrolyzed K-34 | 1.00 | 2.90 | | 1.00 |
| E, Smith degradation of B | 1.00 | 0.9 | 0.85 | |
| F, Smith degradation of B + periodate | 0.25 | 0.3 | 1.00 | |

[a]In molar proportions.

scribed[5]. Acid hydrolysis of the original and carboxyl-reduced[6] polysaccharide showed the repeating unit to consist of L-rhamnose, D-glucose, and D-galacturonic acid in the molar proportions of 4 : 1 : 1 (Table I). Methanolysis of the acidic fraction of the hydrolyzate, followed by reduction with lithium aluminum hydride and subsequent hydrolysis revealed that the aldobiouronic acid was (D-galactosyluronic acid)L-rhamnose.

The $^{13}$C-n.m.r. spectrum of the original polysaccharide showed 29 distinct signals, including a carboxyl-group signal at $\delta$ 174 and a strong methyl-group signal at $\delta$ 17.4, corresponding to the four C-6 atoms of the rhamnose residues. In the anomeric region, five signals, at $\delta$ 104.6, 102.7, 100.8, 100.3 (double), and 98.4 (Table II) could be distinguished, one of them being double in intensity, in agreement with the chemical analysis. Further information was provided by the signal at $\delta$ 61.9, corresponding to a primary alcoholic group, indicating that the glucose residue was not linked through O-6. No characteristic signals for pyruvate or acetate groups could be detected in this spectrum nor in the $^{1}$H-n.m.r. spectrum. The latter showed seven signals in the region between $\delta$ 4.52 and 5.36, and a group of doublets centered at $\delta$ 1.32 corresponding to $CH_3$ of the rhamnose residues and integrating for 12 protons. The discrepancy between the observation of seven protons in the anomeric region and the $^{13}$C-n.m.r. and chemical analytical data was solved by selective heteronuclear $^{13}C\{^{1}H\}$ irradiation at the appropriate frequency for the protons concerned. Irradiation of the two protons resonation near $\delta$ 4.54 resulted in the coalescence of only one $^{13}$C signal in the anomeric region of the $^{13}$C n.m.r. spectrum. This result demonstrated, therefore, that one of these signals (most likely that at $\delta$ 4.52) was not an anomeric proton.

Treatment of the original K-34 polysaccharide with 0.1M trifluoroacetic acid for 10 min at 100° afforded, after dialysis, a polymer from which one rhamnose residue had been removed (Table I), indicating that this rhamnose residue occupied

TABLE II

N.M.R. DATA FOR ORIGINAL AND MODIFIED *Klebsiella* CAPSULAR POLYSACCHARIDE

| *Compound* | *$^1$H-N.m.r. data* | | | | *$^{13}$C-N.m.r. data* | | | |
|---|---|---|---|---|---|---|---|---|
| | *$\delta^a$ (p.p.m.)* | *$^3J_{1,2}{}^b$ (Hz)* | *Integral* | *Assignment* | *$\delta^c$ (p.p.m.)* | *$^1J_{C-H}{}^d$ (Hz)* | *Integral$^e$* | *Assignment$^f$* |
| A B C D E<br>→3-α-Rha-(1→2)-α-Rha-(1→3)-β-Glc-(1→3)-α-GalA-(1→2)-α-Rha-(1→<br>4<br>↑<br>1<br>α-Rha F<br>Original K-34 polysaccharide | 5.36 | 3 | 1 | α-GalA D | 104.6 | 160 | 1 | C-1 of **C** |
| | 5.29 | n.o. | 1 | α-Rha F | 102.7 | 170 | 1 | C-1 of **A** |
| | 5.17 | n.o. | 1 | α-Rha } E B | 100.8 | 172 | 1 | C-1 of **F** |
| | 5.09 | n.o. | 1 | α-Rha } | 100.3 | 170 | 2 | C-1 of **D** and **B** (or **E**) |
| | 4.95 | n.o. | 1 | α-Rha A | 98.4 | 172 | 1 | C-1 of **B** (or **E**) |
| | 4.56 | n.o. | 1 | β-Glc C | 61.9 | | 1 | C-6 of **C** |
| | 4.52 | n.o. | 1 | H-4 or H-5 GalA | 17.5 | | 4 | C-6 of **A**,**B**,**E**, and **F** |
| | 1.32 | 6 | 12 | $CH_3$ of Rha | | | | |
| A B C D E<br>→3-α-Rha-(1→2)-α-Rha-(1→3)-β-Glc-(1→3)-α-GalA-(1→2)-α-Rha-(1→<br>K-34 polysaccharide after mild acid hydrolysis | 5.33 | 3 | 1 | α-GalA D | 104.6 | | 1 | C-1 of **C** |
| | 5.19 | n.o. | 1 | α-Rha } E, B | 102.7 | | 1 | C-1 of **A** |
| | 5.16 | n.o. | 1 | α-Rha } | 100.3 | | 2 | C-1 of **D** and **B** (or **E**) |
| | 4.99 | n.o. | 1 | α-Rha A | 98.4 | | 1 | C-1 of **B** (or **E**) |
| | 4.70 | n.o. | 1 | β-Glc C | 61.7 | | 1 | C-6 of **C** |
| | 1.30 | 6 | 9 | $CH_3$ of Rha | 17.5 | | 3 | C-6 of **AB** and **E** |
| β-Glc-α-Gal-glycerol<br>Smith oligosaccharide | 5.32 | 3 | 1 | α-Gal | | | | |
| | 4.73 | 7 | 1 | β-Glc | | | | |

[a]Chemical shift relative to internal acetone: 2.23 p.p.m. downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). [b]n.o., not observed. [c]Chemical shift relative to internal acetone: 31.07 p.p.m. from DSS. [d]Measured by gated-decoupling. [e]Recorded with a 5 $T_1$ delay between the pulses. [f]Assignments were made by selective, heteronuclear, double irradiation of the protons.

a terminal, nonreducing position. In the corresponding $^{13}$C-n.m.r. spectrum, the signal at $\delta$ 100.8 was missing and was therefore assigned to the pendant rhamnose residue on the chain. On the other hand, the $^{1}$H-n.m.r. spectrum of the partially hydrolyzed polysaccharide showed the disappearance of two signals. The first one (at $\delta$ 5.29) could be unambiguously assigned to the rhamnose residue that had been removed, because of the presence of a very small, residual peak at this chemical shift (Table II). The second missing signal had resonated at $\delta$ 4.52, indicating that it corresponded to a proton of the branch-point sugar residue, as is further demonstrated in the following discussion.

Structural analysis by methylation of the original and the carboxyl-reduced polysaccharide gave the results shown in Table III. The glucose residue is linked through O-3, galacturonic acid carries linkages on O-3 and O-4 and constitutes therefore the branch point of the repeating unit. Of the four rhamnose residues, two are linked through O-2, one through O-3, and the fourth occupies a terminal, non-reducing position. The existence of a rhamnosyl group as the terminal sugar of the side chain thus confirms the indication obtained by partial acid hydrolysis and the prediction from immunological cross-reactions[4a]. This prediction is now also verified in the case of *Klebsiella* serotype[4b] K-53.

The methylated polysaccharide was subjected to a base-catalyzed $\beta$-elimination[7]. This degradation, followed by mild acid hydrolysis caused loss of 2,3,4-tri-*O*-methyl-L-rhamnose, which was the only sugar residue removed under the $\beta$-elimination conditions (Table III). This result proves that rhamnose was the only constituent sugar residue of the side chain and that it was attached to O-4 of the galacturonic acid. During the degradation, an oligomeric material was obtained before mild acid treatment. The cleavage of the glycosidic linkage of the unsaturated uronic acid derivative has already been mentioned[8], and arisen from use of an excess of strong base for the $\beta$-elimination reaction. This cleavage was even more quantitative when methylsulfinyl potassium (a stronger base) was used instead of the usual sodium salt.

TABLE III

METHYLATION ANALYSIS OF ORIGINAL AND MODIFIED *Klebsiella* K-34 POLYSACCHARIDE

| *Methylated sugars*[a] | *Original* | *Carboxyl-reduced* | *Methylated and reduced* | *After β-elimination* |
|---|---|---|---|---|
| 2,3,4-$Me_3$-Rha | 0.75 | 0.70 | 0.75 | |
| 2,4-$Me_2$-Rha | 0.96 | 0.71 | 0.90 | 1.00 |
| 3,4-$Me_2$-Rha | 1.76 | 1.52 | 1.80 | 1.85 |
| 2,4,6-$Me_3$-Glc | 1.00 | 1.00 | 1.00 | 1.00 |
| 2,6-$Me_2$-Gal | | 0.63 | | |
| 2-Me-Gal | | | 0.30 | |

[a]In mole percent as alditol acetate derivative. 2,3,4-$Me_3$-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, etc.

As the rhamnose residue of the side-chain is directly linked to O-4 of the galacturonic acid branch-point, it was then possible to justify the presence of the seventh signal in the anomeric region of the $^4$H-n.m.r. spectrum (at $\delta$ 4.52), and its disappearance after partial acid hydrolysis. It has been established in the gluco-pyranobiose series[9] that the proton attached to the carbon atom involved in the interglycosidic linkage is shifted downfield, and that the two protons on the adjacent carbon atoms of the ring also undergo a slight downfield shift. By removal of the rhamnose residue linked to O-4 of galacturonic acid, the signal originally at $\delta$ 4.52 was moved upfield by at least 0.2 p.p.m. It is nevertheless difficult to assign this signal to H-4, as in the (1→4)-linked galacturonic acid series[10] it has been shown that H-5 vicinal to the linked O-4 undergoes a greater downfield shift that H-4.

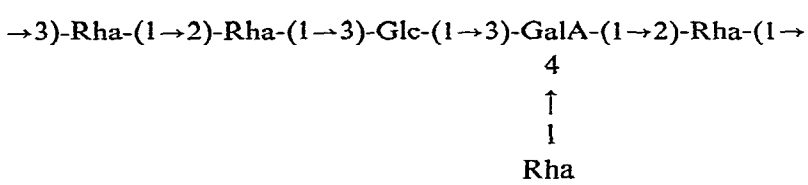
Smith degradation of the carboxyl-reduced K-34 polysaccharide gave an oligosaccharide (**1**) consisting of glucose, galactose, and glycerol, together with a rhamnosyl-glycerol (**2**). A second periodate oxidation on the oligosaccharide, followed by hydrolysis and g.l.c. analysis of the alditol acetates showed galactitol as the only reduced sugar surviving this second oxidation. This result demonstrates that glucose occupied the terminal, nonreducing position in the oligomer. Furthermore, the presence of glycerol in the oligomeric products from the Smith degradation arises from two rhamnose residues linked through O-2.

The sequence of oligomer **1** was further confirmed by methylation analysis, which gave 2,3,4,6-tetra-*O*-methylglucose and 2,4,6-tri-*O*-methylgalactose. The mass spectrum of the permethylated oligomer gave the expected peaks[11] at *m/z* 103, 159, 187, 219, 307, 391, and 423, in agreement with the following structures:

Glc-(1→3)-Gal-(1→2)-glycerol (**1**)

and Rha-(1→2)-glycerol (**2**)

These results allowed assignment of the sequence of the repeating hexasaccharide unit as:




Examination of oligomer **1** by $^1$H-n.m.r. spectroscopy showed two anomeric signals at $\delta$ 5.32 ($^3J$ ~3 Hz) and 4.73 ($^3J$ ~7 Hz), obviously indicating the $\alpha$ and $\beta$ configuration, respectively. As after oxidation[12] by chromium trioxide of the acetylated polysaccharide and of the periodate-oxidized and acetylated K-34 polysaccharide, galactose resisted oxidation under conditions where glucose was removed (Table IV), it may be concluded that D-glucose had the $\beta$ configuration and D-galacturonic acid the $\alpha$.

TABLE IV

OXIDATION ON *Klebsiella* K-34 AND MODIFIED K-34 POLYSACCHARIDES BY CHROMIUM TRIOXIDE

| *Polysaccharide* | *Reaction time (min)* | *Sugar analysis*[a] | | |
|---|---|---|---|---|
| | | *Glc* | *Gal* | *Rha* |
| Carboxyl-reduced K-34 | 0 | 1.00 | 1.00 | 4.00 |
| | 15 | 0.13 | 1.15 | 3.77 |
| | 30 | 0.00 | 0.81 | 2.15 |
| Carboxyl-reduced and Periodate-oxidized K-34 | 0 | 1.00 | 0.94 | 0.90 |
| | 15 | 0.05 | 0.87 | 0.85 |
| | 30 | 0.00 | 0.37 | 0.35 |

[a]In mola: proportions.

At this stage, the problem of assigning configurations for the four rhamnose residues remained. Empirical evidence sets a limit in $^1$H-n.m.r. spectroscopy at about $\delta$ 4.95 between the $\alpha$ and $\beta$ configurations. As amongst the anomeric signals, the K-34 polysaccharide exhibits a rhamnosyl signal at 4.95, the corresponding configuration is difficult to ascertain. Additional confusion could arise from the low-field shifts induced by the substitution[13,14] at O-2 of two of the rhamnose residues. In $^1$H-n.m.r. spectroscopy, sugars having the *manno* configuration (such as rhamnose) show characteristic chemical shifts for their anomeric protons, but do not exhibit the usual clear-cut coupling-constant values that exist for *trans*-diaxial or equatorial–axial relationships. The methyl glycosides of L-rhamnose display very similar coupling values for the $\alpha$ and $\beta$ anomers (1.8 and 1.1 Hz, respectively).

Furthermore, the chemical shifts in the $^{13}$C-n.m.r. spectra in this series of sugars do not provide a clear answer for the anomeric configurations. Only the values of the $^1J_{C\text{-}1,H\text{-}1}$ coupling constants are clearly distinct and characteristic[15]. In the proton spectrum of the K-34 polysaccharide, the four anomeric signals of the rhamnose residues resonated at $\delta$ 5.29, 5.17, 5.09, and 4.95, corresponding to at least 3 $\alpha$-linked rhamnose residues. In order to establish the correspondence between the protons and $^{13}$C signals of the anomeric regions, selective, heteronuclear spin-decoupling ($^{13}C\{^1H\}$) experiments were performed (Table II). The $^1J_{C\text{-}1,H\text{-}1}$ couplings were determined by gated decoupling and showed a single value of 160 Hz, corresponding to the $\beta$ configuration of the D-glucose residue ($\delta$ 104.6). Because of the correspondence between the proton signal at $\delta$ 4.95 and the carbon resonance at $\delta$ 102.7, exhibiting a coupling constant of 170 Hz, it may be ascertained that the fourth rhamnose residue is $\alpha$-linked.

Taking into account the effect of substitution at O-2 or at O-3 on the chemical shift of anomeric carbon atoms, it could be deduced that the signals at 100.3 and 98.4 p.p.m. arise from the two 2-substituted rhamnose residues, and the signal at 102.7 p.p.m. from the 3-substituted rhamnose. As the correspondence between the proton

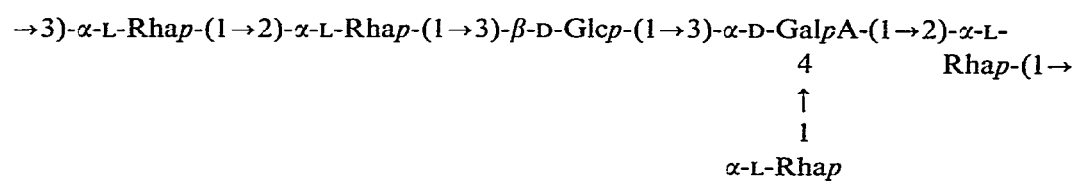
resonances and $^{13}C$ signals had been established by selective, heteronuclear spin-decoupling ($^{13}C\{^1H\}$), it was then possible to assign the signals at $\delta$ 5.17 and 5.09 to the residues linked through O-2, and the signal at $\delta$ 4.95 to the residue linked through O-3.

Additional evidence for the $\alpha$ configuration of the rhamnose residue linked through O-3 was obtained by measuring the $T_1$ values of the anomeric protons, and comparing them to those measured for model compounds[16a]. Under our experimental conditions, rhamnose residue linked through O-3 showed a $T_1$ value of 0.82 sec, characteristic of the $\alpha$ configuration, as compared with 0.53 sec for a $\beta$-glucopyranosyl group. These values compare well with those found by Josephson and Bundle for $\alpha$-rhamnosyl oligosaccharides[16b].

Further indication of the anomeric configurations of the glycosidic linkages is provided by using Hudson's rules[17] of isorotation. Comparison of the measured specific rotation of the K-34 polysaccharide, $[\alpha]_D$ $-8°$ (*c* 3.75, water), agreed well with the value calculated for one $\beta$-D-glucose, one $\alpha$-D-galacturonic acid and four $\alpha$-L-rhamnose residues in the repeating unit.

In Table IV it may be noted that the $\alpha$-L-rhamnose residues are more susceptible to oxidation by chromium trioxide than the $\alpha$-D-galactose residue[18]. Some caution has to be taken in interpreting the results of this method for determination of anomeric configurations, especially as several L-rhamnose residues are present in the polysaccharide under study[19].

From all of the foregoing evidence, the hexasaccharide repeating-unit of the serotype K-34 polysaccharide from *Klebsiella* is demonstrated to have the following structure:




EXPERIMENTAL

*General methods.* — Analytical paper chromatography was performed on Whatman No. 1 paper, and Whatman No. 3 MM paper was used for preparative purposes; the following solvent systems (v/v) were used: (*A*) 8:2:1 ethyl acetate–pyridine–water; (*B*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; (*C*) 10:4:3 ethyl acetate–pyridine-water; and (*D*) 2:1:1 1-butanol–acetic acid–water. Chromatograms were developed with silver nitrate. G.l.c. analyses were performed with a Packard–Becker 417 instrument fitted with dual flame-ionization detectors. Peak areas were measured with a Hewlett–Packard 3380 A digital integrator. Glass columns (3.175 mm o.d.) were used, with a carrier-gas flowrate of 60 mL/min. Columns were (*A*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 180°

(for alditol acetates) or 150° (for partially methylated alditol acetates), (*B*) 3% of OV-225 on Chromosorb WAW-DMCS (100–210 mesh) at 150° (for partially methylated alditol acetates), (*C*) 3% of SP-2340 on Chromosorb WAW-DMCS (100–210 mesh), and (*D*) 2% of XE-60 on the same support (for permethylated oligosaccharide derivatives). G.l.c.–m.s. was performed on a Girdel 3000 chromatograph coupled to an AEI MS-30 mass spectrometer. Spectra were recorded at 70 eV with an ionization current of 100 μA and an ion-source temperature of 100°.

*Isolation of the polysaccharide from Klebsiella K-34.* — A culture of *Klebsiella* K-34 was obtained from Dr. I. Ørskov, Copenhagen, and was grown on Standard I Nutrient Agar Merck, No. 7881, for 4 days at 30°. The cells and mucus were harvested, and diluted with water containing 1% of phenol. The suspension was centrifuged for 1 h at 29,000 r.p.m. in a Beckman Spinco L 50 ultracentrifuge equipped with a titanium R30 rotor. The clear supernatant solution was concentrated to ~200 mL and poured into ethanol (1 L), and the crude polysaccharide was redissolved in water, precipitated with 3% Cetavlon, redissolved in 2M sodium chloride (100 mL), and reprecipitated by ethanol (600 mL). The purified polysaccharide was then dissolved in distilled water (200 mL), deionized with Amberlite IR-120 ($H^+$) resin, dialyzed and freeze-dried, to yield ~800 mg of the polysaccharide, $[\alpha]_D$ −8° (*c* 3.7, water); equivalent weight by sodium hydroxide titration: ~823.

*Hydrolysis of the native polysaccharide, and sugar analysis.* — The polysaccharide (10 mg) in 2M trifluoroacetic acid was totally hydrolyzed during 4 h at 100°. Reduction and subsequent acetylation yielded rhamnitol and glucitol acetates in the ratios expressed in Table I. The same hydrolysis procedure was applied with the carboxyl-reduced[6] polysaccharide, and gave galactose in addition to glucose and rhamnose (Table I).

*Partial acid hydrolysis.* — The rate of hydrolysis of the original K-34 polysaccharide was monitored by paper chromatography (solvent systems *A*, *B*, and *C*) after different times of reaction in the presence of various concentrations of trifluoroacetic acid (0.01, 0.05, 0.1, and 0.5M). Treatment of the polysaccharide with 0.1M acid for 16 min at 100° afforded non-dialyzable material containing rhamnose, glucose, and galacturonic acid in the respective molar proportions of 3 : 1 : 1. $^1$H-n.m.r. and $^{13}$C-n.m.r. examination showed that the cleavage reaction had taken place to at least 90%. This result allowed the assignment of the small remaining signal to H-1 of an α-L-rhamnose residue.

*Methylation analysis.* — The K-34 polysaccharide was dissolved in dimethyl sulfoxide and methylated by one Hakomori[20] treatment followed by two consecutive Purdie methylations[21] to yield a product that showed no hydroxyl absorption in the i.r. spectrum. A part of the methylated polysaccharide was hydrolyzed with formic acid (90%, 1 h at 100°) and then with trifluoroacetic acid (2M, 3 h at 100°). The resulting, partially methylated sugars were analyzed as their acetate derivatives by g.l.c.–m.s.[22] (Table III). A second portion of the methylated K-34 polysaccharide was reduced with lithium aluminium hydride (4 h, reflux); after isolation, the product was hydrolyzed and analyzed as before (Table III).

Similar conditions for methylation analysis were applied to the carboxyl-reduced K-34 polysaccharide.

*Uronic acid degradation*[23]. — Permethylated K-34 polysaccharide (55 mg) was dissolved in 19:1 dimethyl sulfoxide–2,2-dimethoxypropane and *p*-toluenesulfonic acid (5 mg) was added. Methylsulfinyl sodium in dimethyl sulfoxide (2M, 5 mL) was added, and the mixture was kept overnight under nitrogen at room temperature. The mixture was made neutral with 50% acetic acid, diluted with water, and then dialyzed against distilled water. Analysis of the dialyzable and of the non-dialyzable products gave similar results (Table III). When methylsulfinyl potassium was used instead of the sodium salt, all of the *β*-eliminated product was found in the dialyzate.

*Periodate oxidation of the polysaccharide, and Smith degradation.* — Polysaccharide K-34 (300 mg) was dissolved in distilled water (50 mL), and sodium metaperiodate (0.1M, 50 mL) was added. The solution was kept in the dark at 5°. After 72 h, ethylene glycol (2 mL) was added. The material recovered by dialysis was reduced with sodium borohydride (300 mg). The resultant polyol was hydrolyzed with trifluoroacetic acid (2M, 3 h at 100°) and the products were converted into their alditol acetates.

*Smith degradation of the carboxyl-reduced K-34 polysaccharide.* — The carboxyl-reduced polysaccharide (200 mg), in 0.05M sodium metaperiodate, was kept in the dark for 72 h at 5°. The reaction was stopped by addition of ethylene glycol (2 mL) and the mixture was dialyzed for 24 h against running tap-water. The product was reduced with sodium borohydride and the resulting polyol isolated by dialysis and lyophilization.

A part of the polyol was hydrolyzed with 2M trifluoroacetic acid (overnight, at room temperature). After evaporation of the acid, the residue was reduced with sodium borohydride and examined by paper chromatography (solvents *A* and *C*). Two main compounds were observed, **1** ($R_{Glc}$ 2.80, solvent *A*) and **2** ($R_{Glc}$ 0.45 solvent *A*), which were isolated by preparative paper-chromatography on a Whatman No. 3 paper. Hydrolysis of compounds **1** and **2** with 2M trifluoroacetic acid showed respectively the presence of glycerol and rhamnose, and of glycerol, galactose, and glucose. Permethylation of **2** and subsequent hydrolysis gave 2,3,4,6-tetra-*O*-methyl-glucose and 2,4,6-tri-*O*-methyl galactose in equimolar proportion.

A second periodate oxidation was performed on compound **2**. Hydrolysis with 2M trifluoroacetic acid gave only galactose.

*Oxidation by chromium trioxide.* — The polysaccharide (10 mg) was dissolved in formamide (1.5 mL), and pyridine (1.5 mL) and acetic anhydride (1.5 mL) were added[24]. The mixture was stirred for 16 h, and then dissolved in chloroform and washed with water. Evaporation gave a residue that was reacetylated in pyridine–acetic anhydride (4 mL) for 1 h at 100°, in the presence of *myo*-inositol (2 mg) as the internal standard. Chromium trioxide (300 mg) in acetic anhydride was added, the mixture was kept at 50°, and aliquots (1 mL) were removed at intervals. The material was recovered by partition between chloroform and water and the sugars were analyzed conventionally as their alditol acetate derivatives.

The same experiment was performed on the periodate oxidized K-34 polysaccharide under the same conditions. The results are summarized in Table IV.

*N.m.r. spectroscopy.* — The $^1H$ and $^{13}C$-n.m.r. spectra were recorded with a CAMECA 250 or a Bruker spectrometer, in $D_2O$ solution. The $^1H$-n.m.r. spectra were recorded at 363K with 5-mm (o.d.) tubes.

The $^{13}C$-n.m.r. spectra were recorded in 8- or 10-mm tubes (~50 mg in 1.5 mL of $D_2O$) at 343K. Chemical shifts ($\delta$ values) are given in p.p.m. relative to internal acetone, $\delta = 2.23$ in $^1H$ and 31.07 in $^{13}C$, downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate. The measurements of proton $T_1$-values were performed with 1% (w/v) solutions in 99.96% $D_2O$ at 363K. The inversion–recovery method was used for $T_1$ determination [Two-pulse sequence (T...180...$t$...90)$n$], with 10–15 $t$ values. The peak heights of the different anomeric proton signals were measured as a function of delay times ($t$), and these data were then used for a computer program.

Normal $^{13}C$ spectra were recorded with complete proton-decoupling at 62.80 MHz with a spectrometer equipped with Fourier transform (spectral windows of 200 p.p.m. and digitalization into 12,000 data points); pulse width 10 $\mu$sec (~70°), and interval between the pulses 0.6 sec (corresponding to the acquisition time).

Coupling constants were determined with a gated, $^1H$-decoupler sequence to retain nuclear Overhauser enhancements (interval between the pulses: 1.6 sec; decoupling time: 1.0 sec). The selective, heteronuclear, double-irradiation spectra were obtained by application of a continuous wave of fixed frequency and with a weak field of ~0.1 gauss.

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