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Structural studies of the capsular polysaccharide (S-21) from *Klebsiella pneumoniae* ATCC 31314

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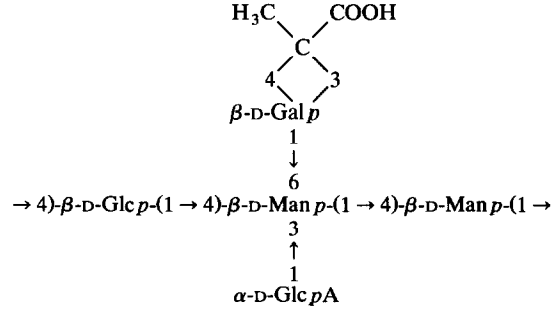
ABSTRACT

The structure of the polysaccharide (S-21) elaborated by *Klebsiella pneumoniae* ATCC 31314 has been investigated. NMR spectroscopy, sugar and methylation analysis, uronic acid degradation, and partial hydrolysis to oligosacccharides were the main methods used. In order to obtain good NMR spectra, the polymer was subjected to non-specific degradation by treatment with fuming hydrochoric acid. It is concluded that S-21 is composed of pentasaccharide repeating units with the following structure.



Approximately 0.7 equivalent of *O*-acetyl group, distributed over at least three positions, was also present but not located. The carbohydrate backbone in S-21 is identical to that of Klebsiella K30 and K33 capsular polysaccharides.

INTRODUCTION

The search for bacterial polysaccharides with potential industrial application has resulted, *inter alia*, in xanthan from *Xanthomonas campestris*. A related polysaccharide from a *Pseudomonas* species was investigated and found to have the same carbohydrate backbone but with almost stoichiometric instead of partial substitution with pyruvic acid[1]. A group of polysaccharides having useful rheological properties[2–8] has the same linear backbone in the main chain, namely

→ 3)-β-D-Glc*p*-(1 → 4)-β-D-Glc*p*A-(1 → 4)-β-D-Glc*p*-(1 → 4)-α-L-Rha(Man)*p*-(1 →,

and all but one, gellan gum[3], have a side chain containing one or two sugar residues. A remarkable feature of some of these polysaccharides is the presence of repeating units in which an L-rhamnose residue has been completely or partially replaced by L-mannose. Two polysaccharides from strains of *Klebsiella pneumoniae* with the code names S-53 and S-21[9], give viscous solutions. The former proved to have the same carbohydrate backbone as Klebsiella K1[10] and we now report structural studies of the latter.

RESULTS AND DISCUSSION

Hydrolysis of S-21 with trifluoroacetic acid yielded mannose, glucose, and galactose in the proportions 1.1:1.3:1.0, and carboxyl-reduced S-21 yielded the same sugars but in the proportions 1.6:1.5:1.0. The presence of glucuronic acid was shown by GLC of the peracetylated methyl glycosides obtained on methanolysis of S-21. The absolute configurations were determined by GLC of the trimethylsilylated (+)-2-butyl glycosides and were D for all the constituents[11].

Methylation analysis of S-21 revealed 4-linked D-glucose, 4- and 3,4,6-linked D-mannose, and 3,4-linked D-galactose (Table I, *A*). Reduction of methylated S-21 with lithium triethylborodeuteride yielded, after subsequent hydrolysis, an ether derived from terminal D-glucuronic acid, in addition to the residues mentioned above (Table I, *B*). These results indicate that S-21 is composed of pentasaccharide repeating units.

The ^1H and ^{13}C NMR spectra of native S-21 were not well resolved, probably because of the viscosity of the solutions. A singlet at δ 1.59 indicated the presence of an acetalically linked pyruvic acid. From the integrals of the ^1H NMR signals of

TABLE I

Methylation analysis of S-21 and its hydrolysis products [a]

Sugar	*T* [b]	Detector response (%)				
		A	*B*	*C*	*D*	*E*
1,2,3,5,6-Man [c]	0.75					5
2,3,4,6-Glc	1.00					33
2,3,4,6-Gal	1.05					43
2,3,6-Man	1.23	27	23	26		
2,3,6-Glc	1.24	30	24	27		
2,3,4-Glc	1.29		15 [d]			
2,3,4-Man	1.30				50	
2,6-Gal	1.42	30	19	15	50	
2,3-Man	1.65			13 [e]		
2-Man	1.75	13	18	19		20

[a] Key: *A*, PS hydrolysed with CF_3CO_2H; *B*, PS carboxyl-reduced and hydrolysed with CF_3CO_2H; *C*, PS treated with base followed by remethylation with CD_3I and hydrolysis with CF_3CO_2H; *D*, erythritol disaccharide glycoside obtained on Smith degradation; *E*; $NaBD_4$-reduced 4. [b] Retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (1.00) and hexa-*O*-acetyl-D-glucitol (2.00) on an HP-5 capillary column, using the temperature program 180°C (1 min) → 250°C at 3°C/min. [c] 1,2,3,5,6-Man = 1,2,3,5,6-penta-*O*-methyl-D-mannose, etc. [d] Deuterium-labelled at C-6. [e] Labelled with a trideuteriomethyl at C-3.

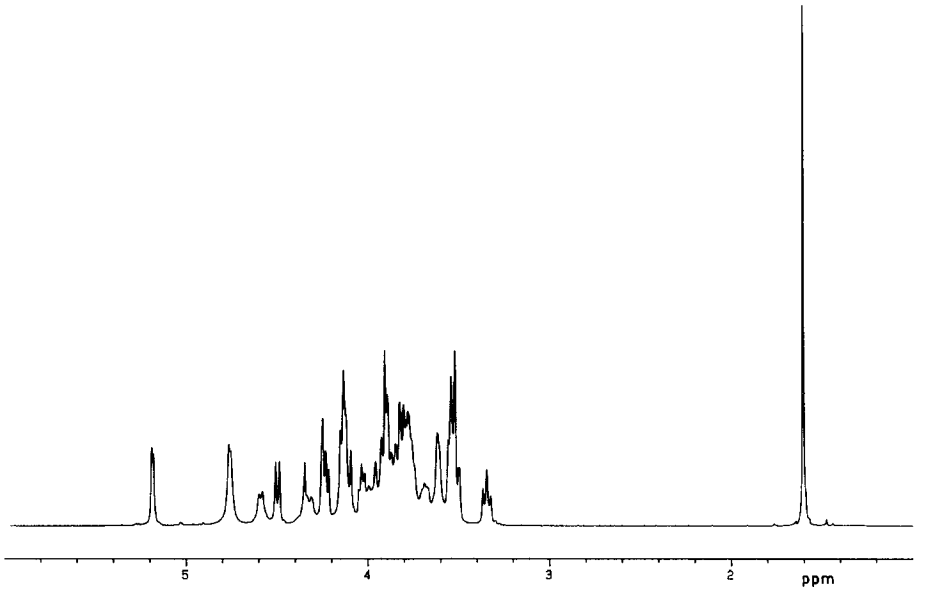


Fig. 1. ^{1}H NMR spectrum of depolymerised and *O*-deacetylated S-21.

the pyruvate and those for *O*-acetyl groups at δ 2.14, 2.15, and 2.20, and from evidence presented below, it was concluded that *O*-acetyl groups were present in less than molar proportions (~ 0.7) and that they occupy at least three different positions in the repeating units.

Better resolved NMR spectra were obtained after treatment of the polysaccharide, with fuming hydrochloric acid at room temperature for 30 min, and subsequent deacetylation under alkaline conditions. From the ^{1}H NMR spectrum of the degraded material (Fig. 1), it was evident that the pyruvic acid was still present, as the signals from the anomeric protons and the pyruvic acid methyl group integrated as 5 to 3.1. In the ^{1}H NMR spectrum of the depolymerised polysaccharide signals for five anomeric protons appeared at δ 5.18, 4.76 (2 H), 4.58, and 4.49. The ^{13}C NMR spectrum (Fig. 2) showed signals for five proton-bearing, anomeric carbons at δ 102.9, 102.8, 102.0, and 100.9 (2 C). These results are consistent with those from the methylation analyses indicating pentasaccharide repeating units as structural elements of S-21.

A signal from a quaternary carbon at δ 108.6 indicated an acetal carbon of a pyruvic acid that is part of a 5-membered ring[12]. The ^{13}C and ^{1}H resonances of its methyl group, δ 24.2 and δ 1.60, indicate that it is linked to O-3 and O-4 of a terminal D-galactopyranosyl group. This is in agreement with the methylation analysis. In order to establish the chirality of the stereogenic centre in the carboxyethylidene group, the polysaccharide was carboxyl-reduced and the chemical shift of the methyl signal, δ 1.50, was compared with those of the corresponding chemical shifts for methyl 3,4-*O*-(*R*)- and -(*S*)-hydroxyisopropylidene-β-D-galacto-

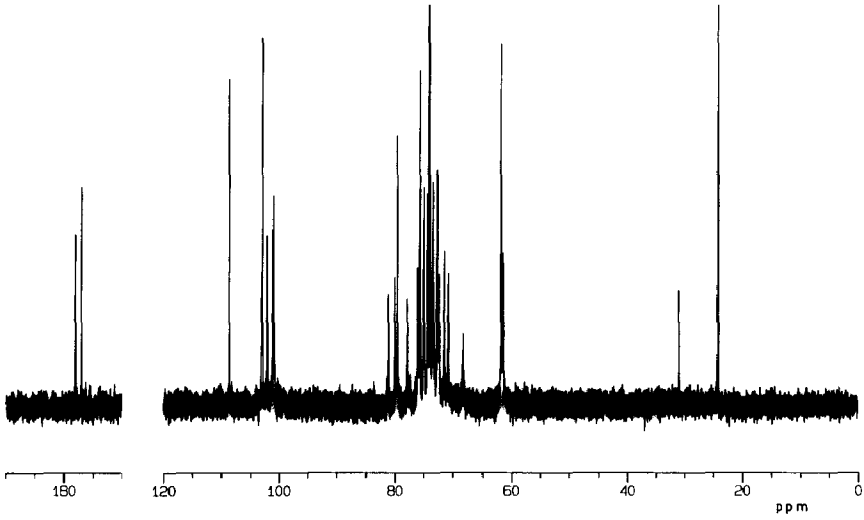


Fig. 2. ^{13}C NMR spectrum of depolymerised and *O*-deacetylated S-21.

side, δ 1.31 and 1.45, respectively[13]. Thus, the chirality was determined to be (*S*). A 2D-NOESY experiment showed that there was an NOE contact between the methyl group and H-2 of the β-D-galactosyl group, confirming this assignment.

In order to determine the anomeric configuration of the sugar residues, most of the ^1H and ^{13}C resonances were assigned via H,H- and C,H-correlated NMR spectra. The NMR data are given in Tables II and III, respectively, and the sugar residues are designated **A–E** according to decreasing chemical shifts of the anomeric proton signals. For the two mannose residues, it was not possible to obtain complete assignments, possibly because of their small couplings and the large line-widths of the signals. The large $J_{C\text{-}1,H\text{-}1}$ value (173 Hz) and the chemical shifts of the ^1H signals of residue **A** indicated it to be a terminal α-D-glucuronic acid. Residues **B** and **C** were recognised as mannose derivatives because of the small $J_{H\text{-}1,H\text{-}2}$ values and were determined to be β from the $J_{C\text{-}1,H\text{-}1}$ values (160 Hz). The ^1H and ^{13}C NMR signals of the anomeric protons and carbons, respectively, from the two residues overlap, but it was indicated that only β anomers were present. By virtue of its 3-substitution, the sugar that had its H-2 resonance shifted most downfield relative to the monomer ($\Delta\delta$ 0.29) was assigned to residue **B** and the other mannose residue to residue **C** ($\Delta\delta$ 0.17). The $J_{C\text{-}1,H\text{-}1}$ and the $J_{H\text{-}1,H\text{-}2}$ values of residues **D** (163 Hz, 7.0 Hz) and **E** (160 Hz, 8.5 Hz) showed that the 4-linked D-glucose residue and the 3,4-linked D-galactose residue were both β-linked. The upfield ^1H NMR chemical shift of the H-2 signal of residue **D** (δ 3.34) made it possible to assign it to the 4-linked D-glucose residue. Hence, **E** must be the 3,4-linked D-galactose residue, an assignment also supported by the large chemical shift value of the H-3 signal (δ 4.22).

TABLE II

^{1}H NMR data for depolymerised S-21

Sugar residue	Chemical shift [a] (δ)				
	H-1	H-2	H-3	H-4	H-5
α-D-Glc*p*A-(1 →	5.18 [~ 3.5]	3.53	3.80	3.51	4.10
A	(−0.06)	(−0.06)	(0.05)	(−0.02)	(0.01)
↓ 6					
→ 4)-β-D-Man*p*-(1 →	4.76 [n.r.] [b]	4.24	3.86		
B 3	(−0.13)	(0.29)	(0.20)		
↑					
→ 4)-β-D-Man*p*-(1 →	4.76 [n.r.]	4.12	3.80	3.85 [c]	
C	(−0.13)	(0.17)	(0.14)	(0.25)	
→ 4)-β-D-Glc*p*-(1 →	4.58 [~ 7.0]	3.34	3.68	3.62	
D	(−0.06)	(0.09)	(0.18)	(0.20)	
3,4-Pyr-β-D-Gal*p*-(1 →	4.49 [~ 8.5]	3.51	4.22	4.14	4.03
E	(−0.04)	(0.06)	(0.63)	(0.25)	(0.38)

[a] Chemical shift differences compared to monomers are given in parentheses and $J_{H-1,H-2}$ values [Hz] are given in square brackets, [b] n.r., Not resolved. [c] Tentative assignment.

The ^{13}C NMR spectrum of S-21 showed a signal at δ 68.2, determined from a DEPT experiment to arise from a methylene carbon. This signal, which has a large downfield shift, should belong to C-6 of the 3,4,6-substituted β-D-mannose residue,

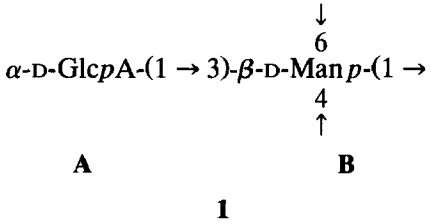
TABLE III

^{13}C NMR data for depolymerised S-21

Sugar residue	Chemical shift [a] (δ)					
	C-1	C-2	C-3	C-4	C-5	C-6
α-D-Glc*p*A-(1 →	102.0 [173]	72.6 [b]	74.4 [b]	74.0 [b]	73.5	
A	(9.0)	(0.3)	(0.9)	(1.1)	(1.1)	
↓ 6						
→ 4)-β-D-Man*p*-(1 →	100.9 [160]	71.4	81.2			68.2
B 3	(6.4)	(−0.7)	(7.2)			(6.2)
↑						
→ 4)-β-D-Man*p*-(1 →	100.9 [160]	70.7	72.5 [b]	77.8 [b]		
C	(6.4)	(1.4)	(−1.5)	(10.1)		
→ 4)-β-D-Glc*p*-(1 →	102.9 [163]	74.0	74.9	79.9		
D	(6.1)	(−1.2)	(−1.9)	(9.2)		
3,4-Pyr-β-D-Gal*p*-(1 →	102.8 [160]	72.7 [b]	79.5	75.6	74.0	
E	(5.4)	(−0.3)	(5.7)	(5.9)	(−1.9)	

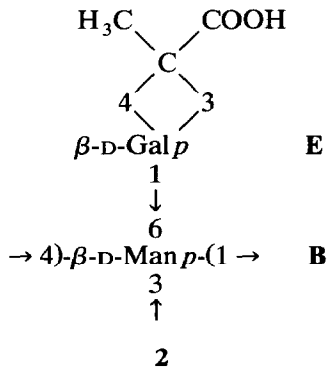
[a] Chemical shift differences compared to monomers are given in parentheses and $J_{C-1,H-1}$ values [Hz] are given in square brackets. [b] Tentative assignment.

and should be substituted by a sugar with the β configuration[14]. In the region for signals from secondary linkage carbons in the C,H-spectrum, four signals were observed, one of which (δ 79.5) could be assigned to C-3 of the pyruvated galactose residue. The signal at lowest field (δ 81.2) was assigned to C-3 of the branching mannose residue. The remaining signals at δ 77.8 and 79.9 were assigned to C-4 of the mannose (**C**) and glucose (**D**) residues, respectively, from the ^{1}H NMR chemical shifts of their corresponding protons. The NOESY spectrum of the depolymerised and *O*-deacetylated S-21 showed, for residue **A**, in addition to an intra-residue NOE to H-2, inter-residue contacts to both H-2 and H-3 in residue **B**, thus establishing structural element **1**. Other cross-peaks observed were either overlapping or of intra-residual origin.



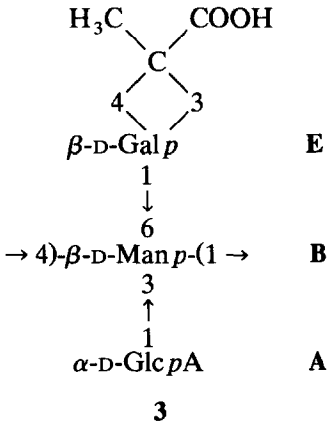
1

From an HMBC-spectrum, i.e., an H,C-long-range correlated 2D NMR spectrum, correlations from anomeric protons to linkage carbons can be obtained, as well as intra-residual correlations. Thus, from H-1 (δ 4.49) in residue **E**, the pyruvic acid-bearing galactose residue, a correlation to a signal at 68.2 ppm is obtained; this signal can be assigned to C-6, the linkage carbon of the branch-point residue. Thus, structural element **2** can be defined.



2

A correlation from the anomeric proton in residue **A** (δ 5.18) to the linkage carbon of **B** at 81.2 ppm was observed, corroborating the contact established from the NOE experiment. From the anomeric protons in **B** and **C** (δ 4.76), correlations to the linkage carbons of **C** (δ 77.8) and **D** (δ 79.9), respectively, were also observed, but this assignment is not conclusive because of overlap. From the above, a partial structure **3** can be defined.



3

The relative order of the 4-linked glucose and the 4-linked mannose residues was determined from chemical degradation experiments.

On subjecting S-21 to a uronic acid degradation[15], i.e., treatment of the methylated polysaccharide with base followed by trideuteriomethylation, the D-glucopyranosyluronic acid residue was eliminated, and the position to which it was linked was liberated and labelled with a trideuteriomethyl group. Hydrolysis of the degraded material with trifluoroacetic acid and analysis by GLC–MS of the alditol acetates confirmed that the α-D-glucopyranosyluronic acid had been linked to the 3-position of the 3,4,6-linked β-D-mannose residue (Table I, *C*). Elimination of some of the 3,4-carboxyethylidenegalactose residue was also observed in some degradations, in agreement with similar observations for *Klebsiella* K33 capsular polysaccharide[16]. A Smith degradation, i.e., periodate oxidation, borohydride reduction, and a mild hydrolysis with acid, resulted in an oligosaccharide that contained pyruvic acid, galactose, mannose, and erythritol. Methylation analysis of this product yielded equimolar amounts of 2,6-di-*O*-methylgalactose and 2,3,4-tri-*O*-methylmannose (Table I, *D*) in agreement with structural element **2**.

S-21 was also subjected to a partial acid hydrolysis. An oligosaccharide, which on hydrolysis yielded mannose, glucose, and galactose in the proportions 1.4 : 1.1 : 1.0, was isolated after gel permeation chromatography. In a negative ion FAB mass spectrum, a pseudomolecular ion at m/z 841 $[(M - H)^-]$ was found, corresponding to the pentasaccharide repeating unit lacking the pyruvate. In a ^1H NMR spectrum (Fig. 3), signals for six anomeric protons were observed but, as expected, no signal for the methyl group from the pyruvic acid. Signals for two anomeric protons at δ 5.19 and 4.91 correspond to those from reducing α- and β-mannose, respectively. Assignments of most of the proton signals could be made (Table IV), and the small glycosylation shifts of the signals from the D-glucuronic acid, D-glucose, and D-galactose residues indicated that they were all terminal. Reduction with sodium borodeuteride, methylation, and hydrolysis yielded the methyl ethers listed in Table I, *E*. The presence of 2,3,4,6-tetra-*O*-methylgalactose confirmed the presence of 3,4-*O*-carboxyethylidene-β-D-galactose in the polysaccharide. The analysis also showed a 1,2,3,5,6-penta-*O*-methylhexitol and a 2-*O*-

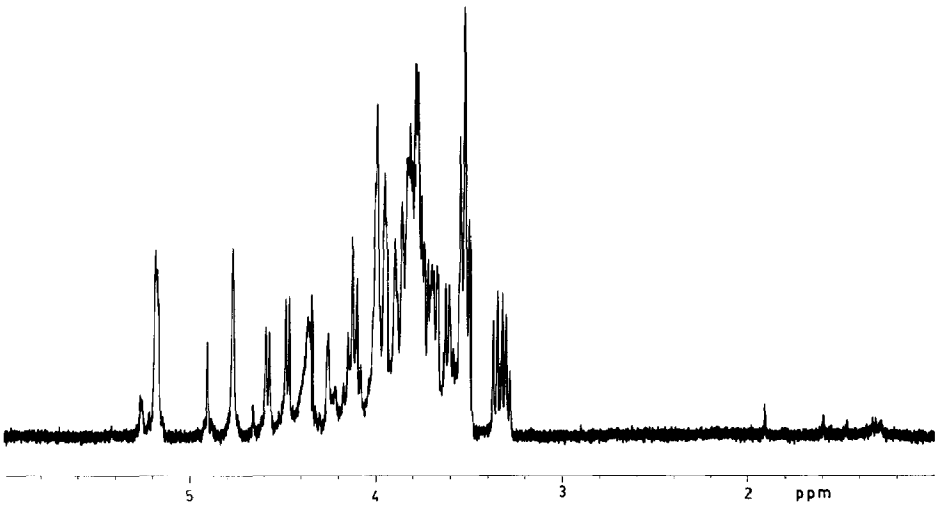


Fig. 3. ^{1}H NMR spectrum of pentasaccharide **4**.

methylhexose. From the combined results, it is concluded that the pentasaccharide has structure **4** and that the repeating unit of S-21 has structure **5**. The carbohydrate backbone of S-21 is identical to those of the Klebsiella K30 and K33 capsular

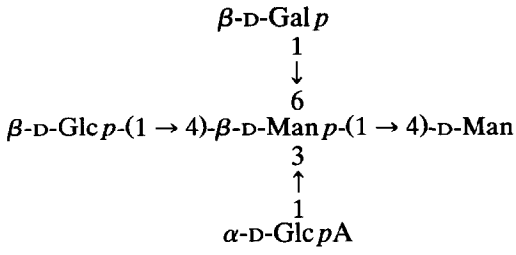
TABLE IV

^{1}H NMR data for pentasaccharide **4**

Sugar residue	Chemical shift [a] (δ)				
	H-1	H-2	H-3	H-4	H-5
→4)-α-D-Man*p*	5.19 [n.r.] [b] (−0.01)	4.00 (0.06)			
α-D-Glc*p*A-(1→	5.17 [4.0] (−0.07)	3.54 (−0.05)	3.81 (0.06)	3.53 (−0.02)	4.12 (0.02)
→4)-β-D-Man*p*	4.91 [n.r.] (0.02)	4.01 (0.06)			
↓6 →4)-β-D-Man*p*-(1→ 3↑	4.77 [n.r.] (−0.12)	4.26 (0.31)	3.85 (0.19)	4.15 (0.55)	3.76 (0.38)
β-D-Glc*p*-(1→	4.58 [8.0] (−0.06)	3.31 (0.06)	3.52 (0.02)	3.35 (−0.07)	3.52 (0.06)
β-D-Gal*p*-(1→	4.47 [7.5] (−0.06)	3.61 (0.16)	3.69 (0.10)	3.95 (0.06)	

[a] Chemical shift differences compared to monomers are given in parentheses and $J_{H\text{-}1,H\text{-}2}$ values [Hz] are given in square brackets. [b] n.r., Not resolved.

polysaccharides which, however, are partially or fully acetylated at O-6 of the 4-linked β-D-mannopyranosyl residues[16,17].



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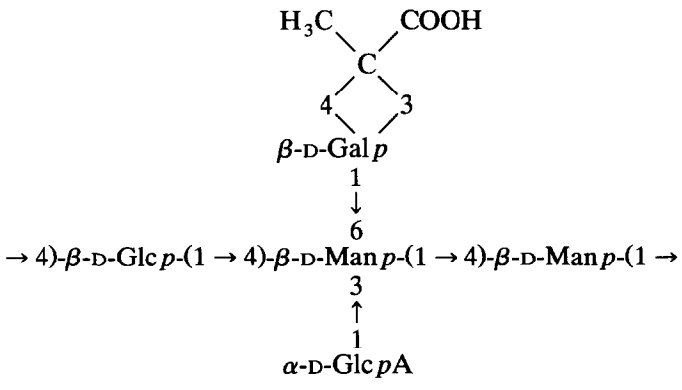

5

The locations of the *O*-acetyl groups in S-21, which, according to NMR evidence, are distributed over at least three different positions, were not determined.

EXPERIMENTAL

General methods.—Concentrations were performed under diminished pressure at < 40°C or under a stream of air or nitrogen. For GLC, a Hewlett–Packard 5890 instrument fitted with a flame-ionisation detector was used. GLC–MS (EI) was performed on a Hewlett–Packard 5970 MSD instrument. FAB-mass spectra in the negative mode were recorded on a JEOL SX 102 instrument, using Xe atoms (6 kV) and a matrix of glycerol, at a resolution of 1000.

Alditol acetates and partially methylated alditol acetates were analysed on an HP-5 capillary column (25 m × 0.20 mm), using the temperature program 180°C (1 min) → 250°C at 3°C/min. Analysis of the trimethylsilylated (+)-2-butyl glycosides was performed on the same column but the temperature program 130°C (1 min) → 220°C at 3°C/min was used.

Gel permeation chromatography was performed on Bio-Gel P-2 and Sephadex G-50 columns, using water buffered with 0.07 M pyridinium acetate at pH 5.4 as eluent, and monitored by a differential refractometer.

Preparation of depolymerised S-21 for NMR spectroscopy.—The polysaccharide (120 mg) was dissolved in fuming HCl (10 mL) and kept at room temperature for 30 min[1,18]. The solution was made alkaline (pH 12) by the addition of aq NaOH at 0°C. The resulting solution was kept at room temperature overnight. The degraded polysaccharide was recovered after neutralisation, lyophilisation, and gel filtration on a Sephadex G-50 column (2.5 cm × 90 cm).

NMR spectroscopy.—NMR spectra of solutions in D_2O were recorded at 70°C using either a JEOL GX-400, GSX-270, or Alpha-400 instrument. Chemical shifts are reported in ppm, using sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) or acetone (δ_C 31.00) as internal reference.

H,H-COSY, H,H-relayed COSY, NOESY, and H,C-COSY were performed using JEOL standard pulse-sequences. H,H-COSY using double-quantum filter and H,H-HOHAHA experiments were performed in the phase-sensitive mode. Relayed COSY spectra were obtained using a delay time of 30 or 60 ms, and the mixing time in the NOESY experiment was 300 ms. H,H-HOHAHA experiments were obtained using a mixing time of 120 ms. The $^1J_{C\text{-}1,H\text{-}1}$ values were determined by an HMQC inverse-detection experiment, and the ^1H–^{13}C long-range couplings were investigated with an HMBC inverse-detected experiment, using a delay time of 60 ms. The multiplicities for the ^{13}C-resonances were established via a ^1H-decoupled DEPT experiment, using P_θ 135° and a delay time of 3.30 ms.

Sugar and methylation analysis.—Methylation was carried out essentially according to methods described earlier[19,20]. For carboxyl reduction, dried methylated polysaccharide (1.5 mg) was dissolved in dry tetrahydrofuran (0.20 mL) and treated with 1 M lithium triethylborodeuteride in tetrahydrofuran (0.20 mL) at 0°C for 1 h.

Methylated and non-methylated products were hydrolysed by the treatment with 0.5 M CF_3CO_2H at 100°C overnight. The sugars in the hydrolysates were converted into the alditol acetates and partially methylated alditol acetates.

The absolute configurations of the sugars in a hydrolysate obtained by treatment with 0.5 M CF_3CO_2H at 90°C overnight were determined according to Gerwig et al.[11].

Uronic acid degradation.—Carefully dried methylated polysaccharide was dissolved in Me_2SO and treated with *p*-toluenesulfonic acid and 2,2-dimethoxypropane. Sodium methylsulfinylmethanide was then added at room temperature and the solution left overnight. After cooling, trideuteriomethyl iodide was added, and the material was recovered and treated as described for the methylation analysis.

Smith degradation.—A solution of the polysaccharide (37 mg) in 0.1 M NaOAc buffer (pH 3.9, 9 mL) was treated in the dark with sodium metaperiodate (30 mg) for 96 h at 4°C. The excess of periodate was reduced with ethylene glycol (0.1 mL). Conventional work-up, including reduction with $NaBH_4$ and dialysis, yielded a material which was subjected to repeated periodate oxidation and workup. After hydrolysis with 0.5 M CF_3CO_2H at room temperature for 48 h, the degradation products were fractionated on a Superdex LMW column (Kabi-Pharmacia, Upp-

sala, Sweden), yielding the trisaccharide-alditol 3,4-(*S*)-Pyr-β-D-Gal*p*-(1 → 6)-β-D-Man*p*-(1 → 2)-Erythritol (0.7 mg) in addition to larger oligosaccharide-alditols.

Isolation of oligosaccharides after mild acid hydrolysis.—S-21 (55 mg) was hydrolysed with 0.5 M CF_3CO_2H (16 mL) at 100°C for 2 h. The hydrolysate was neutralised with aq NaOH and lyophilised. The hydrolysate was fractionated on a Bio-Gel P-2 column (2.5 cm × 90 cm), yielding, *inter alia*, the oligosaccharide **4** (4 mg).

ACKNOWLEDGMENTS

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