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Note

Structure of the polysaccharide S-156 elaborated by *Klebsiella pneumoniae* ATCC 316 46

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Bacterial polysaccharides constitute non-toxic, well-defined, natural polymers which have found many industrial applications. The polysaccharide with the code name S-156 from *Klebsiella pneumoniae* ATCC 316 46 has good viscosity properties and is therefore of potential commercial interest. We have now elucidated the structure of S-156, using methylation analysis, computer-assisted analysis, and NMR studies.

Previous qualitative analysis[1] of S-156 showed galactose, fucose, galacturonic acid, and *O*-acetyl groups as components. A quantitative analysis using hydrolysis with 0.5 M CF_3CO_2H gave L-fucose and D-galactose in the ratio 0.9 : 1.0. A small amount of D-glucose was also present, derived from an unknown source as later no signals in the NMR spectra could be assigned to it. A sample that had been treated with methanolic hydrogen chloride also, on GLC analysis, showed D-galacturonic acid. The absolute configurations of the sugars were determined according to Gerwig et al.[2,3]. The ^1H NMR spectrum of S-156 showed, *inter alia*, a signal at 2.13 ppm demonstrating that it contains an *O*-acetyl group.

Methylation analysis, with carboxyl-reduction after the methylation, yielded 2,4-di-*O*-methyl-L-fucose, 2,4-di-*O*-methyl-D-galactose, and 2,4,6-tri-*O*-methyl-D-galactose in the proportions 38 : 22 : 40, respectively, indicating that all of the sugars are linked through the 3-position and are pyranoid.

These data indicate similarity with the *Klebsiella* K63 capsular polysaccharide[4] with the exception of the *O*-acetyl group, and the possibility that S-156 and K63 have identical backbones was therefore investigated. K63 has the following structure.

→ 3)-α-L-Fuc*p*-(1 → 3)-α-D-Gal*p*-(1 → 3)-α-D-Gal*p*A-(1 →

Evidence for the structure was provided from the ^{13}C NMR spectrum and the C,H-correlation spectrum of *O*-deacetylated S-156. The spectra were analysed with

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TABLE I

Structures suggested by CASPER for the repeating unit of *O*-deacetylated *Klebsiella pneumoniae* S-156 polysaccharide, using ^{13}C NMR data

Structure	^{13}C Chemical shift differences	^{13}C Difference/signal
1	3.1	0.17
2	9.6	0.53
3	11.1	0.61
4	11.9	0.66
5	12.5	0.70

^{13}C Experimental spectrum								
175.8	101.3	101.2	96.2	78.2	78.0	75.5	72.7	72.6
71.7	70.2	68.6	68.4	67.9	67.9	67.9	61.8	16.0
Spectrum No. 1								
176.4	101.6	101.5	96.2	78.5	78.2	75.4	72.8	72.7
71.7	70.2	68.6	68.3	68.1	67.9	67.4	61.6	16.1
Spectrum No. 2								
176.4	101.7	100.8	94.3	78.5	77.5	75.3	72.7	72.6
71.8	70.7	68.2	67.8	67.8	66.8	65.8	62.0	16.1

Assignments given by CASPER for spectrum No. 1

C-1	C-2	C-3	C-4	C-5	C-6	Residue
96.2	68.3	78.2	70.2	71.7	61.6	→ 3)-α-D-Gal*p*-(1 →
101.6	67.4	75.4	68.1	72.8	176.4	→ 3)-α-D-Gal*p*A-(1 →
101.5	68.6	78.5	72.7	67.9	16.1	→ 3)-α-L-Fuc*p*-(1 →

the computer program CASPER[5], which calculates the NMR spectra of all possible structures compatible with data from sugar and methylation analysis, and assesses the fit to the experimental spectrum. The five structures with the best fit are shown below. The delta-sum, i.e., the sum of the ^{13}C chemical shift differences between experimental and simulated spectra, for the first and the second calculated spectrum, differs by a factor of 3.1 which is a strong indication that structure **1** is correct. The results from the analysis of the C,H-COSY data are just as conclusive, because structure **1** is the same and number **2** has a score that is 2.3 times higher. Thus, S-156 has a backbone identical to that of *Klebsiella* K63 (Tables I and II).

1

→ 3)-α-D-Gal*p*-(1 → 3)-α-D-Gal*p*A-(1 → 3)-α-L-Fuc*p*-(1 →

2

→ 3)-α-D-Gal*p*-(1 → 3)-α-L-Fuc*p*-(1 → 3)-α-D-Gal*p*A-(1 →

3

→ 3)-α-D-Gal*p*-(1 → 3)-α-D-Gal*p*A-(1 → 3)-β-L-Fuc*p*-(1 →

4

→ 3)-α-D-Gal*p*-(1 → 3)-β-D-Gal*p*A-(1 → 3)-α-L-Fuc*p*-(1 →

5

→ 3)-α-D-Gal*p*-(1 → 3)-β-L-Fuc*p*-(1 → 3)-α-D-Gal*p*A-(1 →

TABLE II

Structures suggested by CASPER for the repeating unit of *O*-deacetylated *Klebsiella pneumoniae* S-156 polysaccharide, using C,H-COSY data

Structure					C,H score				
1					3.1				
2					7.2				
4					10.2				
3					10.5				
5					11.0				
Experimental C,H-correlation spectrum									
101.3	5.22	101.2	5.33	96.2	5.24	78.2	4.08	78.0	4.02
75.5	4.13	72.7	4.38	72.6	3.92	71.7	4.22	70.2	4.10
68.6	3.98	68.4	4.10	67.9	4.55	67.9	4.17	67.9	4.00
61.8	3.75	61.8	3.75	16.0	1.20				
Spectrum No 1									
101.5	5.21	101.6	5.30	96.2	5.09	78.5	3.99	78.2	3.92
75.4	4.03	72.8	4.36	72.7	3.90	71.7	4.13	70.2	4.04
68.6	3.98	68.3	4.02	68.1	4.49	67.9	4.16	67.4	4.01
61.6	3.66	61.6	3.66	16.1	1.23				

To corroborate further the structural suggestion, S-156 was subjected to a full NMR analysis. The NMR spectra of *O*-deacetylated S-156 could be fully assigned using 2D NMR experiments, and the chemical shifts are given in Table III. From the chemical shift of the C-6/H-6 signals, the spin systems could easily be assigned to each of the three sugars. The chemical shifts of the signals from the anomeric protons indicate that all sugars are α-linked. The appearance of a signal at 96.2 ppm, an unusual chemical shift, in the anomeric region in the ^{13}C NMR spectrum, could be ascribed to an interaction between two protons that are separated by five bonds, the so-called "γ-gauche effect", namely, H-1 in the galactose residue and H-4 in the galacturonic acid[6,7]. The chemical shift of the C-4 signal in the latter residue is also substantially shifted to a lower value, for the same reason. This

TABLE III

Chemical shifts (ppm) of the signals in the ^{1}H and ^{13}C NMR spectra [a] of *O*-deacetylated S-156 polysaccharide

Sugar residue	H/C					
	1	2	3	4	5	6
→ 3)-α-D-Gal*p*A-(1 →	5.33	4.00	4.13	4.55	4.38	
	101.2	67.9	75.5	67.9	72.7	175.8
→ 3)-α-D-Gal*p*-(1 →	5.24	4.10	4.02	4.10	4.22	~3.75
	96.2	68.4 [b]	78.0	70.2 [b]	71.7	61.8
→ 3)-α-L-Fuc*p*-(1 →	5.22	3.98	4.08	3.92	4.19	1.20
	101.3	68.6	78.2	72.6	67.9	16.0

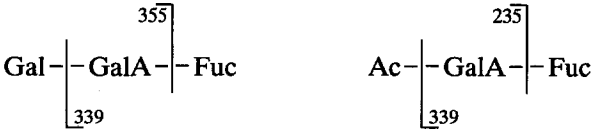
[a] ^{1}H NMR at 50°C; ^{13}C NMR at 70°C. [b] May be interchanged.

demonstrates that the galactose residue is α-linked and the presence of the disaccharide element α-D-Gal*p*-(1 → 3)-D-Gal*p*A. The NOESY spectrum showed, *inter alia*, correlations between the anomeric proton in the galactose residue and H-3 in the galacturonic acid, and between the anomeric proton in the fucose residue and H-3 in the galactose residue. Thus, the trisaccharide element

→ 3)-α-L-Fuc*p*-(1 → 3)-α-D-Gal*p*-(1 → 3)-α-D-Gal*p*A-(1 →

could be established.

To locate the *O*-acetyl group, partial acid hydrolysis and NMR spectroscopy of native S-156 was used. From a hydrolysate of native S-156, using 0.25 M trifluoroacetic acid for 1 h at 100°C, two oligosaccharides were isolated as a mixture, by gel chromatography on Bio-Gel P-2, and analysed by negative FABMS. One showed a pseudomolecular ion at 501 amu $(M - H)^-$, corresponding to a trisaccharide containing the repeating unit, and the other had a pseudomolecular ion at 381 amu $(M - H)^-$ corresponding to a disaccharide compound consisting of GalA, Fuc, and Ac. It could be shown from a B/E linked-scan spectrum that the 501 ion had two daughter ions at 355 and 339 amu, corresponding to fragments indicated below according to fragmentation pathways B and C[8]. This shows that the uronic acid constitutes the middle sugar in the trisaccharide, further demonstrating the Gal–GalA–Fuc repeat. In a B/E linked-scan spectrum, where the fragmentation of a specific ion can be monitored, the 381 ion showed daughter ions at 339 and 235 as shown below. The fragment ion 235 shows that the *O*-acetyl group is located on the galacturonic acid.



m/z 501 $(M - H)^-$ *m/z* 381 $(M - H)^-$

To locate the position of the *O*-acetyl group on the galacturonic acid residue, native S-156 was analysed. The ^1H NMR spectrum of S-156 in the acid form contained signals in the anomeric region, *inter alia*, at 5.94 (H), 5.45 (H), 5.21 (2 H), and 4.80 (H) ppm in agreement with a polysaccharide having a trisaccharide repeat with extra signals in the anomeric region, one deriving from the proton on the acetoxylated carbon. It was necessary to record NMR spectra of the acid form of S-156 as the sodium form precipitated, and consequently some autohydrolysis was unavoidable. The ^1H NMR spectra of native and *O*-deacetylated S-156 are shown in Fig. 1. From 2D COSY and HOHAHA NMR spectra of native S-156, the residue with an anomeric proton signal at 5.45 ppm could be assigned to the galacturonic acid as only signals for five protons were observed, namely at 5.45, 4.05, 4.36, 5.94, and 4.80 ppm for H-1–H-5, respectively. The proton signal at 5.94 showed in an H,C-COSY spectrum a correlation to a carbon signal at 68.5 ppm.

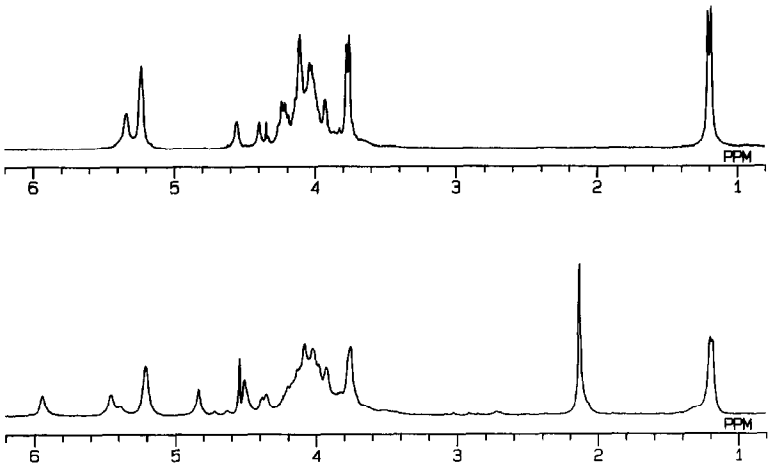


Fig. 1. ^{1}H NMR spectra of *O*-deacetylated (upper) and native (lower) S-156 polysaccharide.

Thus it is demonstrated that the *O*-acetyl group is located on O-4 of the galacturonic acid.

The complete structure of the S-156 capsular polysaccharide is then

$$\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1 \rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{A-}(1 \rightarrow 3)\text{-}\alpha\text{-L-Fuc}p\text{-}(1 \rightarrow$$
$$\begin{array}{c} 4 \\ | \\ \text{OAc} \end{array}$$

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 a Bio-Gel P-2 (2.5 × 80 cm) column, using water buffered with 0.07 M pyridinium acetate of pH 5.4 as eluent, and monitored by a differential refractometer.

Preparation of O-*deacetylated polysaccharide.*—The polysaccharide (100 mg) was dissolved in 0.1 M NaOH (100 mL) and kept at room temperature for 16 h. The *O*-deacetylated polysaccharide was recovered after extensive dialysis against deionised water.

NMR spectroscopy.—NMR spectra of solutions in D_2O were recorded at 70°C unless otherwise stated, using either a Jeol 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 references. H,H-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. The mixing times in the NOESY and H,H-HOHAHA experiments were 200 and 120 ms, respectively.

Sugar and methylation analysis.—Methylation was carried out essentially according to methods described earlier[9,10]. Hydrolysis of native and methylated S-156 was performed by treatment with 0.5 M CF_3CO_2H at 100°C overnight. The sugars in the hydrolysates were converted into alditol acetates and partially methylated alditol acetates. Carboxyl-reduction of methylated polysaccharide (1 mg in dry THF) was performed by treatment with lithium borohydride in THF (0.70 mL) at 80°C for 2 h.

The absolute configurations of the sugars were determined according to Gerwig et al.[2,3].

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