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The capsular antigen of *Escherichia coli* O9:K33:H$^-$: a polysaccharide containing both pyruvate and *O*-acetyl groups

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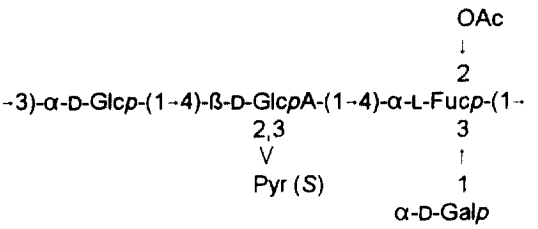
Abstract

The primary structure of the acidic capsular antigen of *Escherichia coli* O9:K33:H$^-$ was shown by glycose analysis, methylation analysis, and by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy to be composed of branched tetrasaccharide repeating units with the structure:



Keywords: *Escherichia coli*; Capsular polysaccharide; K33 Antigen; NMR Spectroscopy

1. Introduction

Bacteria belonging to the species *Escherichia coli* are opportunistic pathogens, causing a wide variety of disease states in humans and animals [1]. The virulence and disease specificity of a particular strain of *E. coli* are related to the specific polysaccharide antigens being expressed, i.e., the lipopolysaccharide or O-antigen which is associated with the outer membrane of the bacterial cell, and the capsular or K-antigen which has a protective function. Serotyping within the *E. coli* series has identified 74 different K-antigens, and more than 40% of these occur in association with only two O-antigens, i.e. O8 and O9 [2]. Work in our laboratories in recent years has focussed on the determination of the structures of these K-antigens, and this report on the structure of *E. coli* K33 represents the final one in the series. Preliminary results on the structure of this polysaccharide have been

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reported previously, but no complete structure has been published [3].

2. Results and discussion

Isolation, purification, and composition of the capsular polysaccharide.—*E. coli* O9:K33:H$^-$ bacteria were grown on Mueller–Hinton agar and the acidic capsular polysaccharide was isolated from the cells by centrifugation and purified by precipitation with cetyltrimethylammonium bromide. GLC examination of the alditol acetates derived from an acid hydrolysate of the purified polysaccharide (**PS**) showed the presence of Gal, Glc, and Fuc in equal proportions. Prior methanolysis of the **PS**, reduction of the methoxycarbonyl groups formed, and GLC examination of the derived alditol acetates revealed a substantial increase in the proportion of Glc, thereby establishing GlcA as the acidic component of the **PS**. GLC analysis of the derived acetylated (−)-2-octyl glycosides showed the configuration of Fuc to be L and that of the other constituent sugars to be D [4].

1D NMR studies of the ***PS***.—The ^{1}H NMR spectrum of the **PS** (Fig. 1) in D_2O contained H-1 signals at δ 5.32, 5.16 (2 H), and 4.97, and signals for the methyl protons of a deoxy sugar at δ 1.29 (3 H), for an *O*-acetyl group at δ 2.15 (3 H), and for pyruvate at δ 1.60 (3 H). The viscosity of the **PS** sample precluded the measurement of coupling constants and created difficulties in the interpretation of 2D NMR spectra. Removal of the *O*-acetyl and pyruvate groups produced a polysaccharide (**DPS**) which was much less viscous than the **PS** and was therefore used in subsequent NMR experiments. The ^{1}H NMR spectrum of the **DPS** in D_2O (Fig. 2) contained H-1 signals at δ 5.408 ($^3J_{H,H}$ 3.3 Hz), 5.292, 5.257 ($^3J_{H,H}$ 3.8 Hz), and 4.554 ($^3J_{H,H}$ 7.9 Hz), and a signal for H-6 of a deoxy sugar at δ 1.313 ($^3J_{H,H}$ 6.5 Hz). The appearance of the spectrum was consistent with a repeating unit structure for the polysaccharide. The ^{13}C NMR data complemented the ^{1}H NMR results and confirmed a tetrasaccharide repeating unit for the **DPS** with signals at 104.45, 101.27, 100.23, and 99.51 ppm in the anomeric region. A signal for the carbonyl carbon of the uronic acid occurred at 174.91 ppm and for C-6 of Fuc at 16.17 ppm.

Methylation analysis.—Methylation of the **PS** by a modified Hakomori procedure [5] followed by Kuhn methylation [6] and GLC-MS analysis of the partially methylated alditol acetates derived from an acid hydrolysate of the methylated **PS** showed the presence of 2,3,4,6-tetra-*O*-methylgalactose, 2-*O*-methylfucose, 2,4,6-tri-*O*-methylglucose, and glucose (after carboxyl reduction) in equimolar proportions. These results indicated that the repeating unit of the **PS** is a branched tetrasaccharide and that the pyruvate group could be located on either the GlcA or the Fuc residue.

Methylation of the **DPS** followed by GLC-MS analysis of the partially methylated alditol acetates derived from the products of an acid hydrolysate of the methylated **DPS** showed the presence of 2,3,4,6-tetra-*O*-methylgalactose, 2-*O*-methylfucose,

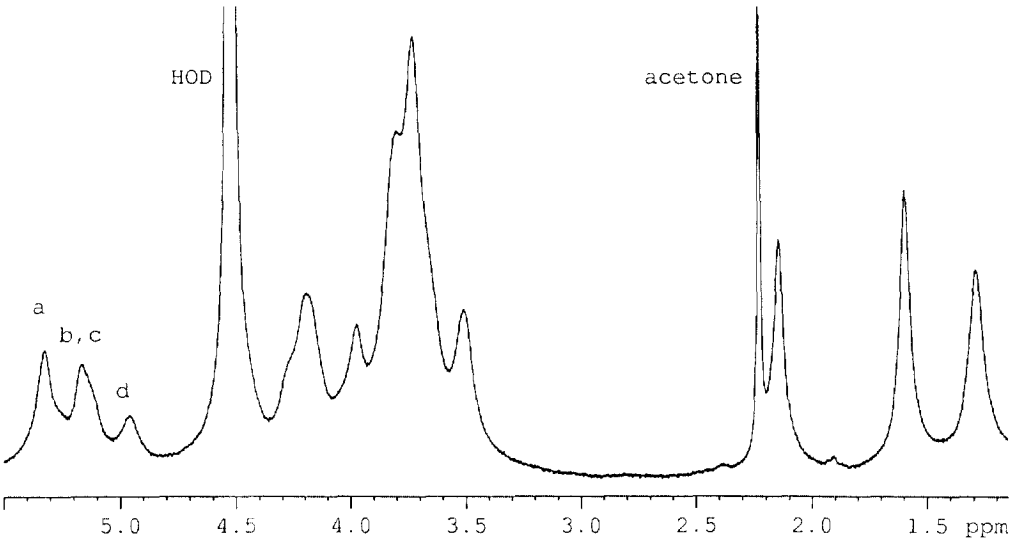


Fig. 1. ^{1}H NMR spectrum of the **PS** in D_2O at 50 °C. For **a**, **b**, **c**, **d** see text.

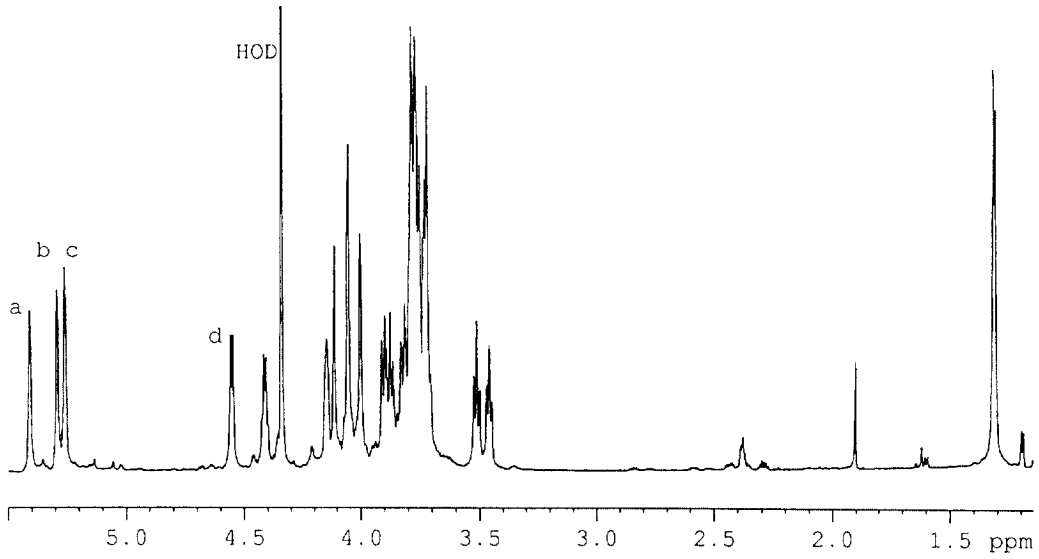


Fig. 2. ^{1}H NMR spectrum of the **DPS** in D_2O at 70 °C. For **a**, **b**, **c**, **d** see text.

2,4,6-tri-*O*-methylglucose, and 2,3-di-*O*-methylglucose (after carboxyl reduction), in equimolar proportions. These results indicated that the pyruvate substituent is linked to positions 2 and 3 of D-GlcA and that L-Fuc was the branch point in the **PS**.

The position of the *O*-acetyl group was established using the Prehm methylation procedure [7]. A solution of the **PS** in distilled water was ultrasonicated in order to reduce viscosity, freeze-dried, and subjected to Prehm methylation. A comparison of the ^{1}H NMR spectra before and after ultrasonication established that the procedure had not altered the structure of the **PS** in any way. GLC-MS analysis of the partially methylated alditol acetates derived from the products of an acid hydrolysate showed the presence of 2,3,4,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylglucose, fucose, and glucose (after carboxyl reduction) in equimolar proportions. These results indicate that the *O*-acetyl substituent is linked to position 2 of L-Fuc.

The full sequence of the residues in the repeating unit of the **PS** was established by 2D NMR experiments.

*2D NMR studies of the **DPS**.*—It was difficult to obtain useful information from 2D NMR experiments performed on the intact **PS** due to the viscosity of the solution. 2D NMR analysis was therefore carried out on the **DPS**. The identity of the residues in the repeating unit, the configurations of the glycosidic linkages, and the glycosylation sites were established by ^{1}H–^{1}H COSY [8], HOHAHA [9], and NOESY [10], and by ^{1}H–^{13}C HSQC [11], HSQC-TOCSY, and HMBC [12] experiments. The HSQC spectrum for the **DPS** is shown in Fig. 3. Assignment of data from 2D spectra was greatly facilitated by the PRONTO software program [13] which permitted spectra to be overlaid and simultaneously interrogated. The residues in the repeating unit have been denoted **a**–**d** in order of decreasing chemical shift of the H-1 resonances. The ^{1}H and ^{13}C chemical shifts are listed in Table 1 and were established as described below.

*Residue **a**.* [→3)-α-D-Glc]: The ^{1}H resonances for H-1,2,3,4,5 were readily assigned from the COSY spectrum and were confirmed from the HOHAHA spectrum. It was difficult to assign the H-6 resonances due to signal overlap. The ^{13}C resonances for C-1,2,3,4,5 were assigned by comparing the ^{1}H assignments with the ^{1}H–^{13}C correlation data from the HSQC experiment. Confirmation of these assignments was obtained from the HSQC-TOCSY spectrum, which clearly showed correlations from H-2 to all the carbon resonances for residue **a**. Assignment of C-6 in this way permitted assignment of the H-6a and H-6b resonances from the HSQC experiment.

*Residue **b**.* [→3,4)-α-L-Fuc]: The ^{1}H resonances for this residue were assigned from the COSY spectrum. The HOHAHA spectrum confirmed the assignments for H-1,2,3,4. The overlap of the signals for H-2 and H-3 was confirmed from the HSQC spectrum, which showed the correlations from the carbon signals at 75.56 ppm (C-3) and 69.40 ppm (C-2) to the ^{1}H signal at δ 4.054.

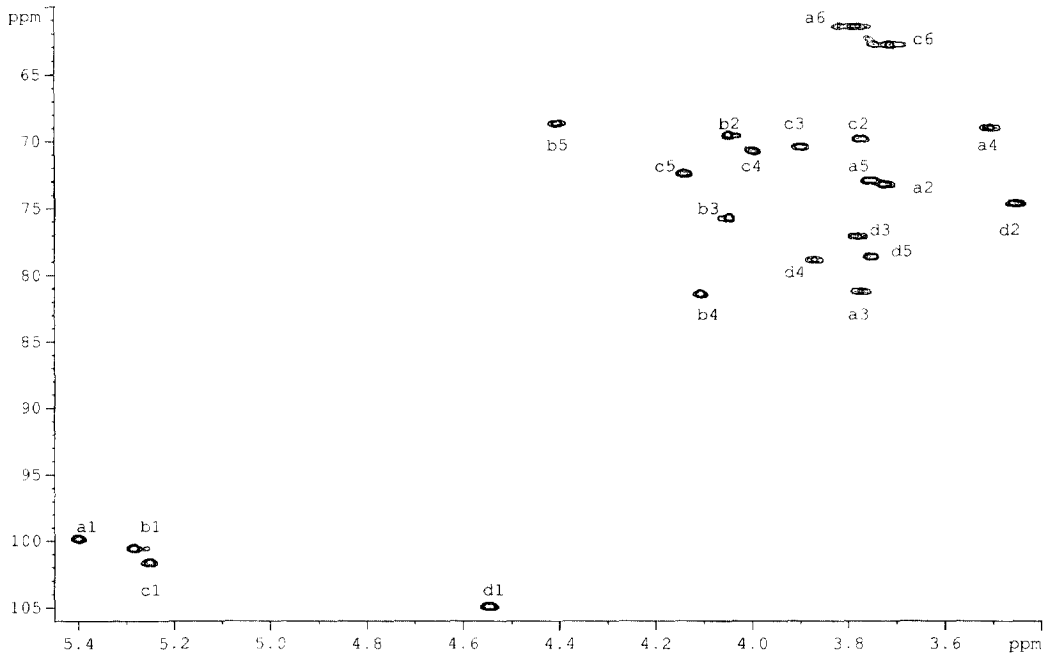


Fig. 3. Partial contour plot of the HSQC experiment on the **DPS**. **a1** denotes the cross-peak observed between H-1 and C-1 of residue **a**, etc.

Table 1
NMR data[a] for *E. coli* K33 **DPS**

Residue		H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a/C-6	H-6b
→3)-α-D-Glc*p* (**a**)	H	5.408	3.727	3.781	3.512	3.763	3.818	3.774
	C	99.51	73.02	**81.12**[c]	68.82	72.73	61.29	
	$^3J_{H,H}$[b]	3.3						
→3,4)-α-L-Fuc*p* (**b**)	H	5.292	4.054	4.054	4.113	4.415	1.316	–
	C	100.23	69.40	**75.56**	**81.22**	68.44	16.17	
	$^3J_{H,H}$					6.3	6.5	
α-D-Gal*p* (**c**)	H	5.257	3.782	3.908	4.007	4.150	3.720	3.720
	C	101.27	69.61	70.22	70.58	72.20	62.59	
	$^3J_{H,H}$	3.8						
→4)-β-D-Glc*p*A (**d**)	H	4.554	3.461	3.789	3.879	3.759	–	–
	C	104.45	75.46	76.88	**78.70**	78.39	174.91	
	$^3J_{H,H}$	7.9						

[a] Chemical shifts in ppm with acetone as internal standard, δ 2.23 and 31.07 for ^{1}H and ^{13}C, respectively.
[b] Coupling constants measured in Hz.
[c] Linkage carbons are indicated in bold.

Intramolecular NOEs from H-5 to H-3 and H-4 also served to confirm the assignments. The ^{13}C resonances were assigned from the HSQC spectrum and confirmed from the HSQC-TOCSY spectrum, which clearly showed correlations from H-2 to all the carbon signals.

Residue ***c***. [α-D-Gal]: The ^{1}H resonances for this residue were assigned from the COSY spectrum. The H-4/H-5 cross-peak was small, and the H-5 assignment was confirmed by the intramolecular NOE from H-5 to H-4 and H-3 of this residue. ^{13}C resonances were assigned from the HSQC spectrum by comparison with the ^{1}H resonances, and confirmed from the HSQC-TOCSY spectrum.

Residue ***d***. [→4)-β-D-GlcA]: The ^{1}H resonances for H-1,2,3,4 of this residue were assigned from the COSY spectrum. Due to signal crowding in the COSY spectrum, the H-4/H-5 cross-peak was not easily visible and the H-5 assignment was made from the HOHAHA spectrum. The carbonyl signal was assigned from the HMBC spectrum, which showed clear correlation between H-5 and the signal at 174.91 ppm. Other ^{13}C resonances were assigned from the HSQC spectrum.

The linkage position of the pyruvate substituent was established from the methylation results and confirmed by NMR spectroscopy. A sample of the **PS** was de-*O*-acetylated by treatment with dilute NH_4OH and was used for the NMR experiments. The D_2O solution of the de-*O*-acetylated polysaccharide (**DAP**) was still viscous and produced highly overlapped NMR spectra. In spite of this, it was possible to assign the chemical shifts of the ^1H resonances of the β-GlcA residue from COSY and HOHAHA spectra. ^{13}C resonances were assigned by comparison with the ^1H resonances from an HMQC spectrum, and values of 76.92 and 81.58 ppm were established for C-2 and C-3, respectively. These assignments were confirmed from an HMQC-TOCSY spectrum, in which the correlations between H-1 and C-1,2,3 of the residue were clearly seen. The downfield shift of these two carbon signals, when compared with the data obtained for the **DPS** (see Table 1), confirm that the pyruvate substituent is linked at positions 2 and 3 of the β-GlcA residue. In addition, the NOESY spectrum showed clear NOEs from H-1 (δ 4.947) of the GlcA residue to H-3 (δ 3.820), as expected for a β-linked sugar with the *gluco* configuration, and from the pyruvate methyl (δ 1.596) to H-3 (δ 3.820) of the GlcA. This established the stereochemistry of the pyruvate substituent as (*S*) [14].

Comparison of the ^1H and ^{13}C chemical shifts for residues **a**–**d** of the **DPS** with literature values for methyl glycosides [15,16] identified the residues in the repeating unit as indicated in Table 1. In agreement with the methylation results, the glycosylation sites for the **DPS** were established as C-3 for **a**, C-3 and C-4 for **b**, and C-4 for **d** by the significant deshielding experienced by these carbons.

Table 2
NOE data for the **DPS** (inter-residue NOEs are in italics)

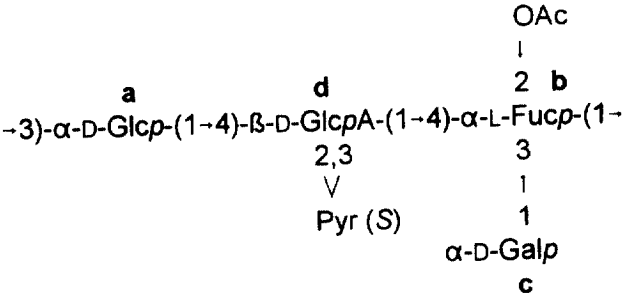
Residue	Proton	Correlation to
→3)-α-D-Glc*p* (**a**)	H-1	*3.879* (**d**, H-4)
→3,4)-α-L-Fuc*p* (**b**)	H-1	*3.781* (**a**, H-3)
	H-5	4.054 (**b**, H-3), 4.113 (**b**, H-4)
α-D-Gal*p* (**c**)	H-1	*4.054* (**b**, H-3)
	H-3	4.150 (**c**, H-5)
	H-4	3.908 (**c**, H-3), 4.150 (**c**, H-5), 3.720 (**c**, H-6)
	H-5	3.908 (**c**, H-3), 4.007 (**c**, H-4)
→4)-β-D-Glc*p*A (**d**)	H-1	*4.113* (**b**, H-4)
	H-2	3.879 (**d**, H-4)

The sequence of the residues in the repeating unit was established from the NOESY and HMBC experiments performed on the **DPS**. The intermolecular NOEs observed are listed in Table 2 and yield the sequence

→3)a(1→4)d(1→4)b(1→
3
↑
1
c

3. Conclusion

The combined chemical and NMR results support the following structure for the repeating unit of the capsular polysaccharide of *E. coli* K33:



Capsular antigen K33 is co-expressed with O-antigen 9, has a high molecular mass, and is *Klebsiella*-like in its structure, allowing it to be classified as a Group I polysaccharide [2]. The highly substituted structure of the polysaccharide repeating unit is unusual in the *E. coli* series, and it is one of only two *E. coli* K-antigens containing a 2,3-linked pyruvate substituent. The other such polysaccharide is the K103 capsular polysaccharide, which has (*S*)-pyruvate 2,3-linked to an α-D-Gal residue [17]. There are also only two capsular polysaccharides amongst the *Klebsiella* series containing 2,3-linked pyruvate residues, i.e. *Klebsiella* K1 [18] and K58 [19]. In both cases the pyruvate substituent is attached to a β-Glc*p*A residue but no stereochemistry has been assigned. *O*-Acetyl groups are also common substituents of bacterial polysaccharides, but this is only the second *E. coli* K-antigen to be reported as containing both pyruvate and acetyl groups, the other being *E. coli* K55 [20]. Non-carbohydrate substituents such as *O*-acetyl groups and pyruvate affect the

immunospecific character of the polysaccharides in which they are found [21].

4. Experimental

General methods.—Analytical GLC was performed on a Hewlett-Packard 5890A gas chromatograph, fitted with flame-ionization detectors and a 3392A recording integrator, with helium as carrier gas. A J & W Scientific fused-silica DB-17 bonded-phase capillary column (30 m×0.25 mm, film thickness 0.25 μm) was used for separating partially methylated alditol acetates (programme I), and alditol acetates and acetylated octyl glycosides (programme II). A J & W Scientific DB-225 bonded-phase capillary column (30 m×0.25 mm, film thickness 0.25 μm) was also used for separating acetylated octyl glycosides (130 kPa, 240 °C isothermal). The temperature programmes used were: I, 180 °C for 2 min, then 3 °C/min to 240 °C, 100 kPa; II, 180 °C for 2 min, then 2 °C/min to 240 °C, 100 kPa. The identities of all derivatives were determined by comparison with authentic standards and confirmed by GLC-MS on a Hewlett-Packard 5988A instrument, using the appropriate column. Spectra were recorded at 70 eV and an ion-source temperature of 200 °C.

Polysaccharide samples were hydrolysed with 4 M CF_3CO_2H for 1 h at 125 °C. Alditol acetates were prepared by reduction of the products in aqueous solutions of hydrolysates with $NaBH_4$ for 1 h followed by acetylation with 2:1 Ac_2O–pyridine for 1 h at 100 °C. Samples were methanolysed by boiling under reflux with methanolic 3% HCl for 16 h. Native and methylated polysaccharides were carboxyl-reduced with $NaBH_4$ in dry MeOH for 16 h after methanolysis. Methylations were carried out on the acid form of the polysaccharide, using potassium methylsulfinyl anion [5] and MeI in Me_2SO, followed by a 72 h Kuhn methylation in DMF with Ag_2O and MeI to ensure complete methylation [6]. Prehm methylation of the **PS** was carried out on the acid form of the polysaccharide, using methyl triflate in trimethyl phosphate in the presence of 2,6-di-(*tert*-butyl)pyridine [7].

Preparation of the K33 polysaccharide.—An authentic culture of *E. coli* O9:K33:H$^-$ was obtained from the International *Escherichia* and *Klebsiella* Centre (WHO) in Copenhagen and propagated on Mueller–Hinton agar (9 trays 30×60 cm, each inoculated with 10 mL liquid culture). The capsular polysaccharide (**PS**) was solubilised with aq 1% phenol, separated from the cells by ultracentrifugation (using a Beckman L8-M ultracentrifuge, 70Ti rotor, 35 000 rpm, 1 h) and purified by precipitation with cetyltrimethylammonium bromide to yield 320 mg of **PS**. The **PS** was further purified by ion-exchange chromatography on a column (2.6×27 cm) of DEAE-Sepharose™ CL-6B using gradient elution with 0–1 M NaCl in 0.01 M Tris-HCl (pH 8.5). Fractions were assayed for carbohydrate using the phenol–H_2SO_4 reagent [22]. The acid form of the **PS** was obtained by passage of an aqueous solution of the **PS** down a column of Amberlite IR-120 (H) resin. Removal of pyruvate was carried out by treatment of the **PS** with aq 1% HOAc (100 °C, 1 h) with subsequent dialysis against running water (overnight, 12–14 000 mw cut-off), centrifugation and freeze-drying. This treatment surprisingly also removed the *O*-acetyl groups. De-*O*-acetylation without the removal of pyruvate was carried out by treating the **PS** with aq 12.5% NH_4OH at room temperature overnight with subsequent dialysis against running water (overnight, 12–14 000 mw cut-off) and freeze-drying. A sample of native polysaccharide with both substituents intact was prepared by dissolving the **PS** (25 mg) in water and ultrasonicating for 3 h to reduce viscosity.

NMR Spectroscopy.—Samples were deuterium-exchanged by freeze-drying several times from D_2O and then examined as solutions in 99.99% D_2O containing a trace of acetone as internal standard (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). Spectra for the **PS** were recorded at 50 °C on a Bruker AMX-400 spectrometer equipped with an X32 computer. Spectra for the **DPS** were recorded on a Varian INOVA 750 spectrometer at 750.04 MHz for ^1H and at 188.6 MHz for ^{13}C at 70 °C. A double quantum filtered phase sensitive COSY experiment was performed using the Varian standard program TNDQCOSY [23], with 0.340 s acquisition time and 4 K data points. The matrix was zero filled in f_1 to 4 K×2 K points and was resolution enhanced in both dimensions by a shifted sine bell. The NOESY was performed using the Varian standard program TNOESY [24], with a mixing time of 200 ms. Total correlation spectroscopy was performed using the Varian standard program TNTCOSY [9,25], with a spinlock time of 100 ms. The hetereonuclear single quantum coherence spectroscopy was performed using the pulsed field gradient standard Varian program GHSQC [26],

with gradient strengths of 4, 4 and 2 Gauss/cm and gradient times of 2, 2 and 0.5 ms, respectively. The GHSQC-TOCSY experiments used mixing times of 25 and 100 ms. The HMBC experiment used was the Varian standard GHMBC. Spectral widths used were 4993 Hz for ^{1}H, and 11930 Hz (GHSQC) and 37735 Hz (GHMBC) for ^{13}C, respectively. Data matrices consisted of 4 K×512 points zero-filled to 2 K×1 K and with the application of a shifted sine bell window function. The number of transients was 16 or 32. The spectra were assigned using the computer program PRONTO [13], which allows the simultaneous display of different two-dimensional spectra and individual labelling of cross-peaks.

Spectra for the **DAP** were recorded at 70 °C on a Bruker AMX-400 spectrometer equipped with an X32 computer. The parameters used for 2D experiments were as follows. COSY [256×2048 data matrix, zero-filled to 1024 data points in f_1; 128 scans per t_1 value; spectral width 2008.0 Hz; recycle delay 1.0 s; unshifted sine-bell filtering in f_1 and f_2]. HOHAHA with presaturation during relaxation delay [23] [256×4096 data matrix, zero-filled to 1024 data points in f_1; 112 scans per t_1 value; spectral width 4032.3 Hz; recycle delay 1.0 s; mixing time 90.0 ms; shifted sine-squared filtering in f_1 and f_2]. Phase sensitive NOESY with presaturation during relaxation delay [27] [512×2048 data matrix, zero-filled to 1024 data points in f_1; 64 scans per t_1 value; spectral width 2016.1 Hz; mixing time 300 ms; shifted sine-squared filtering in f_1 and f_2]. HMQC and HMQC-TOCSY with presaturation during relaxation delay [23] [256×4096 data matrix, zero-filled to 1024 data points in f_1; 92 and 136 scans, respectively, per t_1 value; recycle delay 1.0 s; fixed delay 3.45 ms; mixing time 25.4 ms (HMQC-TOCSY); spectral width in f_1 14087.1 Hz and in f_2 4032.3 Hz; shifted sine-squared filtering in f_1 and f_2].

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