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Structural and immunochemical relationship between the O-antigenic polysaccharides from the enteroaggregative *Escherichia coli* strain 396/C-1 and *Escherichia coli* O126

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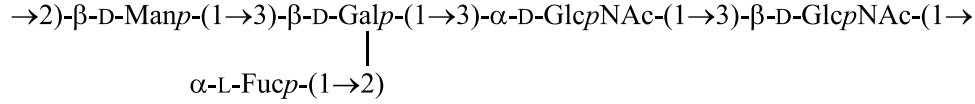
Abstract—The structure of the O-antigen polysaccharide (PS) from the enteroaggregative *Escherichia coli* strain 396/C-1 has been determined. Sugar and methylation analyses together with ^{1}H and ^{13}C NMR spectroscopy were the main methods used. Inter-residue correlations were determined by $^{1}H,^{1}H$-NOESY, $^{1}H,^{13}C$-heteronuclear multiple-bond correlation and dipole–dipole cross-correlated relaxation experiments. The PS is composed of pentasaccharide repeating units with the following structure:



Analysis of NMR data reveals that on average the PS consists of ~13 repeating units and indicates that the biological repeating unit contains an *N*-acetylglucosamine residue at its reducing end. This structure is different to that reported for the O-antigen polysaccharide from *E. coli* O126. Monospecific anti-*E. coli* O126 rabbit serum from The International *Escherichia* and *Klebsiella* Centre did not distinguish between the *E. coli* strain 396/C-1 and the *E. coli* O126 reference strain, neither in slide agglutination nor in an indirect enzyme immunoassay. Subsequent successful serotyping of the *E. coli* strain 396/C-1 showed it to be *E. coli* O126:K+:H27.

Keywords: *Escherichia coli*; Enteroaggregative; Serology; Lipopolysaccharide; NMR

1. Introduction

Diarrhoeagenic *Escherichia coli* strains are major pathogens associated with enteric disease in many parts of the world. Currently, six pathotypes of diarrhoeagenic *E. coli* have been unequivocally associated with diarrhoeal illness: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli*, diffusely adherent *E. coli*, Shiga toxin-producing *E. coli*, which are also referred to as Vero cytotoxin-producing *E. coli*, enteroinvasive *E. coli* and enteroaggregative *E. coli* (EAEC).[1] The latter may be an emerging diarrhoeal pathogen. This pathotype, defined by aggregative adherence to Hep-2 cells in culture, has been associated with persistent diarrhoea among infants, particularly in the developing world.[2–6] The enteroaggregative *E. coli* are characterized by a distinct aggregative adherence (AA) pattern to HEp-2 cells in vitro first described by Nataro et al.[7] This pattern is distinguished by the prominent autoagglutination

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of bacterial cells to each other, to the surface of the HEp-2 cells, as well as to a glass cover slip in a typical layering, best described as a 'stacked brick' configuration. Many EAEC strains are difficult to serotype since they have a characteristic property that causes them to autoagglutinate and therefore behave like rough strains. This was the case for the EAEC strain 396/C-1 that was not possible to serotype using the conventional slide agglutination technique. We have therefore undertaken the structural analysis of the O-antigenic polysaccharide isolated from the lipopolysaccharide (LPS) of the *E. coli* strain 396/C-1.

2. Results and discussion

The *E. coli* strain 396/C-1 was grown in a glucose-containing tryptone/yeast extract medium. The LPS was isolated from the bacterial membrane by hot phenol/water extraction and delipidated under mild acidic conditions to yield a polysaccharide (PS). A hydrolysate of the PS contained fucose, mannose, glucose, galactose, 2-amino-2-deoxyglucose and heptose in the ratio 20:22:10:28:15:5. Glucose and heptose were attributed to the core. Determination of the absolute configuration of the remaining components revealed that they had the D-configuration, except for fucose that had the L-configuration. Methylation analysis showed the presence of four components, namely, 2,3,4-tri-*O*-methyl-fucose, 3,4,6-tri-*O*-methyl-mannose, 4,6-di-*O*-methyl-galactose and 2-deoxy-2-*N*-methylacetamido-4,6-di-*O*-methyl-glucose in the ratio 25:27:35:13.

The ^{1}H NMR spectrum showed the presence of a signal from a methyl group (δ 1.22, $J = 6.3$ Hz) and methyl signals from *N*-acetyl groups (δ 2.07, 6H) revealing that the aminosugars are *N*-acetylated. In the region for anomeric resonances five major signals were present (Fig. 1). The ^{1}H,^{13}C-HSQC spectrum (Fig. 2)

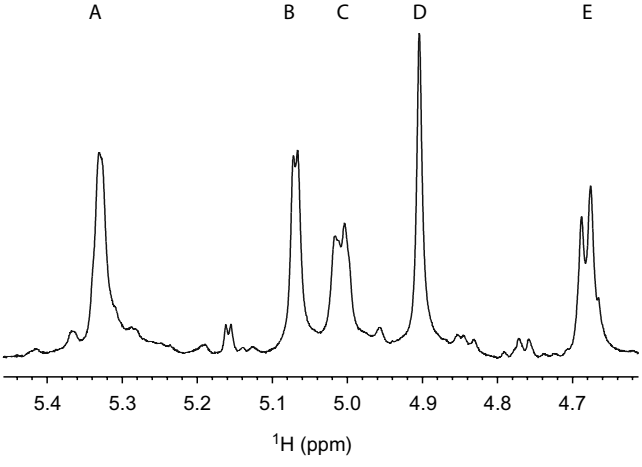


Figure 1. Part of the ^{1}H NMR spectrum of the O-antigen PS from *E. coli* strain 396/C-1 showing resonances from anomeric protons.

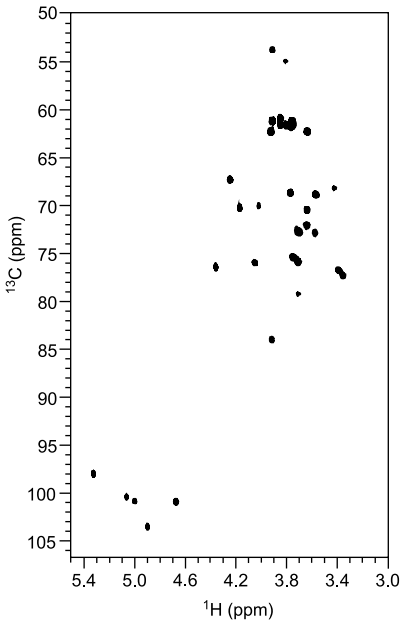


Figure 2. Part of the ^{1}H,^{13}C-HSQC spectrum of the O-antigen PS from *E. coli* strain 396/C-1.

confirmed that the PS consists of pentasaccharide repeating units. The ^{1}H and ^{13}C NMR resonances were assigned using two-dimensional NMR techniques and the chemical shifts are compiled in Table 1.

The resonance at δ 5.33 had $J_{\text{H-1,H-2}} = 3.9$ Hz and its C-2 resonance at δ 53.9. From methylation analysis a pyranoid ring form can be deduced. These results show the residue to be an α-linked *N*-acetyl-glucosamine. The ^{13}C NMR glycosylation shifts[8] reveal, together with the above methylation analysis, the substitution pattern of the sugar residues. Thus, residue **A** is 3-substituted. The anomeric proton resonance of residue **B** at δ_{H} 5.07 had $J_{\text{H-1,H-2}} = 4.2$ Hz. Disentangling of the spin-system showed that it belonged to the fucosyl residue and consequently it is a terminal α-L-Fuc*p* residue. Residue **C** has its anomeric proton resonance at δ_{H} 5.01 and showed $J_{\text{H-1,H-2}} = 8$ Hz. Unravelling of the proton and carbon spin systems unambiguously revealed it as a 3-substituted β-D-Glc*p*NAc residue. The anomeric proton of residue **D** at δ_{H} 4.90 showed a nonresolved *J*-coupling to its H-2 and in agreement with data from the methylation analysis this residue could be assigned to a 2-substituted β-linked mannose ($J_{\text{H-1,C-1}} = 162$ Hz). Finally, residue **E** with its anomeric proton resonance at δ_{H} 4.68 and $J_{\text{H-1,H-2}} = 7$ Hz was assigned to a 2,3-disubstituted β-D-Gal*p* residue.

The sequence of the sugar residues in the repeating unit was determined from ^{1}H,^{1}H-NOESY (Fig. 3) and ^{1}H,^{13}C-HMBC (Fig. 4a) experiments. Whereas the results from the former experiment left an ambiguity with respect to the substitution of the terminal fucosyl residue, this was clarified by analysis of the latter. Besides

Table 1. ^{1}H and ^{13}C NMR chemical shifts (ppm) of the signals from the O-antigen polysaccharide of the *E. coli* strain 396/C-1 and inter-residue correlations from NOESY, HMBC and DDCCR spectra

Sugar residue	$^{1}H/^{13}C$						Correlation to atom (from anomeric atom)		
	1	2	3	4	5	6	NOE	HMBC	DDCCR
→3)-α-D-Glc*p*NAc-(1→[a]	5.33 [3.9]	3.91	4.06	3.57	3.71	~3.85	H-3, **C**	C-3, **C**	C-3, **C**
A	(0.12)	(0.03)	(0.31)	(0.08)	(−0.15)				
	98.0 {178}	53.9	76.0	69.0	72.9	61.0		H-3, **C**	H-3, **C**
	(6.2)	(−1.1)	(4.3)	(−2.3)	(0.4)				
α-L-Fuc*p*-(1→	5.07 [4.2]	3.77	3.64	3.73	4.25	1.22	H-2, **E**	C-2, **E**	C-2, **E**
B	(−0.13)	(0.00)	(−0.22)	(−0.08)	(−0.05)		H-2, **D**		
	100.4 {173}	68.8	70.5	72.7	67.4	16.2			
	(7.3)	(−0.3)	(−0.2)	(−0.1)	(0.3)				
→3)-β-D-Glc*p*NAc-(1→[b]	5.01 [8]	3.81	3.71	3.65	3.39	3.76, 3.91	H-2, **D**	C-2, **D**	C-2, **D**
C	(0.29)	(0.16)	(0.15)	(0.19)	(−0.07)				
	100.9 {167}	55.1	79.3	72.1	76.8	61.3		H-2, **D**	H-2, **D**
	(5.0)	(−2.8)	(4.5)	(1.0)	(0.0)				
→2)-β-D-Man*p*-(1→	4.90 [2.1][c]	4.37	3.58	3.44	3.36	3.64, 3.93	H-3, **E**	C-3, **E**	C-3, **E**
D	(0.01)	(0.42)	(−0.08)	(−0.16)	(−0.02)		H-1, **B**		
	103.5 {162}	76.5	72.9	68.3	77.4	62.4		H-3, **E**	
	(9.0)	(5.4)	(−1.1)	(0.6)	(0.4)				
→2,3)-β-D-Gal*p*-(1→	4.68 [7]	3.71	3.92	4.18	3.75	~3.76	H-3, **A**	C-3, **A**	C-3, **A**
E	(0.15)	(0.26)	(0.33)	(0.29)	(0.10)				
	100.9 {165}	75.9	84.0	70.4	75.5	61.7		H-3, **A**	H-3, **A**
	(3.5)	(2.9)	(10.2)	(0.7)	(−0.4)				

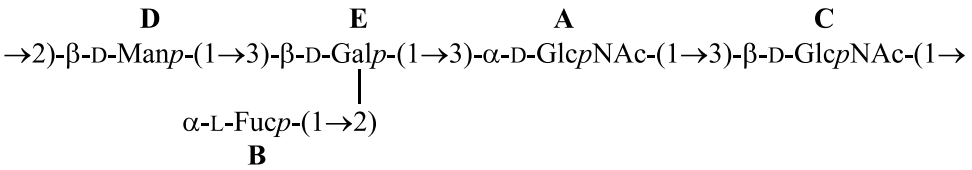
$J_{H\text{-}1,H\text{-}2}$ values are given in Hz in square brackets and $J_{H\text{-}1,C\text{-}1}$ values in braces. Chemical shift differences as compared to the corresponding monosaccharides are given in parentheses.

[a]Chemical shifts for NAc are δ_H 2.07; δ_C 22.9 and 174.2.

[b]Chemical shifts for NAc are δ_H 2.07; δ_C 23.5 and 175.2.

[c]Width at half peak height.

the anticipated inter-residue NOE between H-1 in **B** and H-2 in **E**, an additional NOE was observed to H-2 in **D**. The latter NOE is in agreement with the derived substitution pattern, stereochemistry and conformational properties in the branching region of the PS, as depicted in Figure 5. A summary of the connectivities is given in Table 1, from which the sequence of sugars can be deduced. Thus, the structure of the O-antigen polysaccharide from *E. coli* strain 396/C-1 is:



Recently, a novel NMR experiment was devised based on cross-correlated dipole–dipole relaxation.[9] Results from such an experiment (Fig. 4b) were found to corroborate those obtained, thereby showing the usefulness of the alternative approach.

In the ^{1}H NMR spectrum a signal of low intensity was observed at δ_H 5.16. The $^{1}H,^{1}H$-TOCSY and $^{1}H,^{13}C$-DDCCR experiments resulted in intra-residue correlations to protons and carbons, respectively, of similar chemical shifts as for the terminal fucosyl residue, suggesting that this residue corresponds to a terminal Fuc group in a different chemical environment. In the HMBC spectrum H-1 of residue **D** at δ_H 4.90 showed an intra-residue $^{2}J_{C,H}$ correlation to C-2 at δ_C 76.5. In addition, a correlation was present from δ_H 4.895 to δ_C 71.1 (also in the $^{1}H,^{13}C$-DDCCR spectrum), a chemical shift closely similar to that of C-2 in β-Man*p* as a monosaccharide.[8] Thus, it is suggested that the biological repeating unit is terminated with a mannosyl group. Consequently, at the reducing end, a GlcNAc residue is present. Our findings are consistent with the biosynthesis of O-antigen heteropolysaccharides since an *N*-acetylglucosamine residue often forms the first sugar of the repeating unit,[10] for example, in the O-antigen polysaccharide of *E. coli* O7.[11] Integration of the ^{1}H NMR signals at δ_H 5.16 and 5.07 (cf. Fig. 1) revealed that on average the PS consists of ~13 repeating units. Analysis of the LPS preparation by SDS/PAGE showed a ladder pattern due to LPS with different numbers of repeating units, with a major distribution in a region consistent with the NMR results (data not shown).

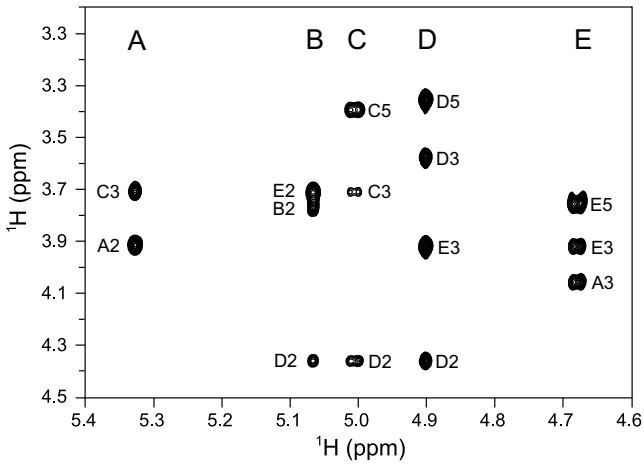


Figure 3. Part of the ^{1}H,^{1}H-NOESY spectrum of the O-antigen PS from *E. coli* strain 396/C-1 with annotated correlations from anomeric protons.

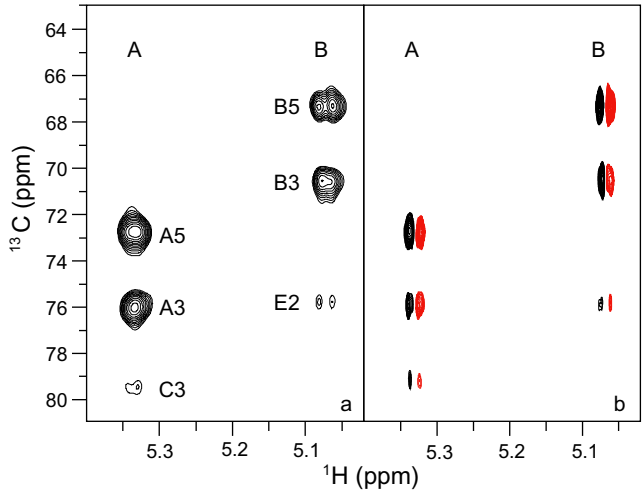


Figure 4. Part of the ^{1}H,^{13}C-HMBC spectrum (a) and the ^{1}H,^{13}C-DDCCR spectrum (b) showing correlations for residues **A** and **B**.

The structure determined herein is similar to that of the O-antigen PS from *E. coli* O126 published previously by Bhattacharyya and Basu[12] shown below:

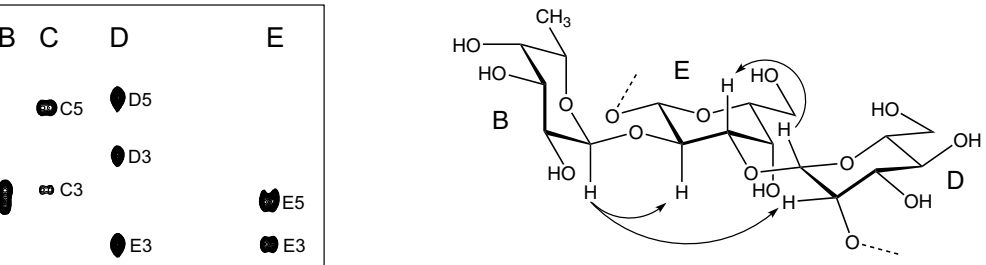


Figure 5. Schematic representation of the branching region of the O-antigen PS from *E. coli* strain 396/C-1 with selected NOE correlations indicated by arrows.

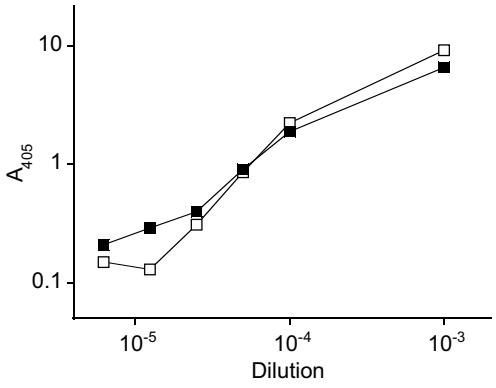
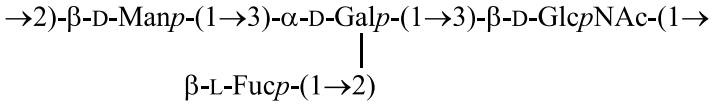


Figure 6. Enzyme immunoassay with rabbit—anti-*E. coli* O126 serum. Titration of the anti-O126 serum against LPS isolated from *E. coli* strain 396/C-1 (■) and *E. coli* O126:K(71):H2 LPS (□).

immunoassay using purified LPS from both the 396/C-1 and the reference O126 *E. coli* strains showed that the O126 specific serum could not distinguish the two preparations (Fig. 6). Inspection of the ^{1}H NMR spectrum of the LPS from the *E. coli* O126 reference strain showed, inter alia, five signals from anomeric protons with essentially identical chemical shifts and coupling constants. In addition, resonances from a methyl group and from *N*-acetyl groups were also present. Thus, the ^{1}H NMR spectra of the two strains indicate identity of the O-antigen polysaccharides. Subsequently, the *E. coli* strain 396/C-1 was submitted to the The International



When the slide agglutination was performed according to the standard method, that is, in PBS, *E. coli* strain 396/C-1 did autoagglutinate. However, when the agglutination was done using distilled water instead of PBS, *E. coli* strain 396/C-1 was positive with the *E. coli* O126 monospecific serum. In addition, an enzyme

Escherichia and *Klebsiella* Centre (WHO) at Statens Serum Institute, Copenhagen, Denmark and shown to be *E. coli* O126:K+:H27, thereby explaining the results obtained in the immunochemical analysis. The origin of the previously reported *E. coli* O126 strain, which has four sugar residues in the repeating unit of its O-antigen,

is still to be clarified. That the serotype investigated herein is of clinical importance is evident from the recent publication by Shazberg et al. in which they describe the identification of several EAEC strains as O126:H27.[13]

3. Experimental

3.1. Bacterial strain and conditions of growth

The *E. coli* strain 396/C-1, was isolated in 1991 from an infant with persistent diarrhoea in Dhaka, Bangladesh. The strain was nontypable by slide agglutination due to autoagglutination or aggregation. The strain showed the typical aggregative adherence pattern in a Hep-2 assay.[14–16] Bacteria were grown in submerged cultures to late exponential phase in 15 L of a tryptone/yeast extract medium[17] containing 1% glucose, using a 30 L fermentor (Belach AB) under constant aeration at 37 °C and pH 7.0. A preculture (1.5 L) in the same medium was used to inoculate the fermentor. All cultures were checked for purity at the end of the growth cycle. The bacteria were killed with 1% (w/v) formaldehyde. After incubation 16 h at 4 °C the cells were separated from the media by continuous-flow centrifugation using a CEPA model LE centrifuge at a cylinder speed of 29,000*g* and a flow of 25 L h^{-1} (Carl Padberg Centrifugenbau, Lahr, Germany). The bacterial mass was then removed from the cylinder, washed once with NaCl/P_i (0.01 M potassium phosphate, 0.14 M NaCl, pH 7.2), centrifuged (8000*g*, 4 °C, 20 min) and finally re-suspended in distilled water.

A reference strain of *E. coli* O126:K(71):H2 (CCUG 11425) was obtained from the Culture Collection, University of Gothenburg, Sweden. The strain was grown in LB medium (3 L) and killed with formaldehyde (1%).

3.2. Preparation of lipopolysaccharide and lipid-free polysaccharide

The lipopolysaccharide (LPS) was extracted by the hot phenol/water method.[18] The aqueous phase was dialyzed at 4 °C for 3–5 days against tap water, overnight against distilled water, concentrated under diminished pressure and lyophilized. Contaminating nucleic acids were removed by ultracentrifugation (100,000*g*, 4 h, 4 °C). Lipid-free polysaccharide (PS) was prepared by treatment of the LPS with 0.1 M sodium acetate, pH 4.2, at 100 °C for 5 h.[19] Lipid A was removed by centrifugation (10,000*g*, 20 min, 4 °C). The PS was further purified by gel-permeation chromatography.

3.3. Immunochemical analyses

The rabbit anti-*E. coli* O126 specific antiserum (O126A1) used in this assay was obtained from The International *Escherichia* and *Klebsiella* Centre (WHO), Statens Serum Institute, Copenhagen, Denmark. The enzyme immunoassay (EIA) was performed as described previously.[20] Microtitre plates (Costar) were coated overnight at 20 °C with 100 μL of each LPS (5 μg mL^{-1}; 0.05 M sodium carbonate, pH 9.6). After washing with 0.15 M NaCl, 0.05% Tween 20, the antiserum diluted in 0.01 M KP_i, 0.15 M NaCl, pH 7.4, 0.05% Tween 20 (NaCl/P_i/Tween) was added. After incubation for 4 h at 20 °C, the plates were washed as above and incubated overnight at 20 °C with 100 μL of alkaline-phosphatase-conjugated goat anti-(rabbit IgG) (Sigma) diluted in NaCl/P_i/Tween. For developing, plates were washed as above, and 100 μL of a 1 M diethanolamine, 0.5 M $MgCl_2$, pH 9.8, 1 mg mL^{-1} sodium *p*-nitrophenol phosphate was added. Plates were incubated at 20 °C and read in a Multiskan Plus EIA reader (Labsystems) at 405 nm after 25, 50 and 100 min. The values were extrapolated to absorbance after 100 min of incubation. The slide agglutination was performed in distilled water instead of PBS.

3.4. Component analyses

The PS was hydrolyzed with 0.5 M TFA at 100 °C for 16 h. After reduction with $NaBH_4$ and acetylation, the sample was analyzed by GLC. The absolute configuration of the sugars present in the PS were determined by derivation of the sugars as their acetylated (+)-2-butyl glycosides[21,22] for GlcN or as their acetylated dithioacetals.[23,24] The methylation analysis was performed according to Hakomori[25] using sodium methylsulfinylmethanide and iodomethane in dimethyl sulfoxide. The methylated compounds were purified using Sep-Pak C_{18} cartridges (Millipore) and recovered using acetonitrile and ethanol.[26] The purified methylated sample was then hydrolyzed (2 M TFA, 120 °C, 2 h), reduced with NaB^2H_4 and acetylated. The partially methylated alditol acetates were analyzed by GLC–MS.

3.5. GLC and GLC–MS analyses

Alditol acetates and partially methylated alditol acetates were separated on an HP-5 fused silica column (0.20 mm × 25 m) using a temperature program of 180 °C for 1 min followed by 3 °C min^{-1} to 210 °C. Hydrogen was used as carrier gas. The column was fitted to a Hewlett-Packard model 5890 series II gas chromatograph equipped with a flame ionization detector. The acetylated dithioacetals were analyzed isothermally at 280 °C on an Alltech EC-1 column (0.32 mm × 5 m). GLC–MS analysis was performed on a Thermo Quest GCQ plus spectrometer equipped with a DB-5 fused silica column (0.32 mm × 15 m). A temperature program of 170 °C for 3 min followed by 3 °C min^{-1} to 250 °C was used with helium as carrier gas.

3.6. NMR spectroscopy

NMR spectra of the PS in D_2O were recorded at 50 °C using Varian Inova 400 and 600 MHz instruments. Chemical shifts are reported in ppm relative to internal sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)propanoate (TSP, δ_H 0.00) or external 1,4-dioxan in D_2O (δ_C 67.4) as references. Data processing was performed using standard Varian VNMR software. ^1H,^1H-correlated spectroscopy (COSY),[27] total correlation spectroscopy (TOCSY)[28] with mixing times of 30, 60 and 90 ms, gradient selected heteronuclear single quantum coherence (gHSQC),[29] and gradient selected heteronuclear multiple-bond correlation (gHMBC)[29,30] experiments were used to assign signals and performed according to standard pulse sequences. For inter-residue correlations, a two-dimensional nuclear Overhauser effect spectroscopy (NOESY)[31] experiment with a mixing time of 50 ms, and an HMBC experiment with a 50 ms delay for the evolution of long-range couplings were used. In addition, a heteronuclear DDCCR experiment[9] was performed at the higher magnetic field with 384 scans for each of the 48 t_1-increments and the total cross-correlated relaxation time $2T = 15$ ms. The chemical shifts were compared to those of the corresponding monosaccharides.[8]

Acknowledgements

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