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Structural studies of S-7, another exocellular polysaccharide containing 2-deoxy-*arabino*-hexuronic acid

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Abstract

The exocellular polysaccharide S-7, a heteropolysaccharide from *Azotobacter indicus* var. *myxogenes* has been studied using methylation analysis, Smith degradation, partial acid hydrolysis, NMR spectroscopy and mass spectrometry as the principal methods. It is concluded that the repeating unit has the following structure:

→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-2-deoxy-*arabino*-Hex*p*A-(1→
6
↑
1
β-D-Glc*p*-(1→6)-β-D-Glc*p*

The absolute configuration of the deoxyhexuronic acid was deduced from ^{1}H NMR chemical shifts and is most likely D. Approximately two *O*-acetyl groups per repeating unit are present, one of which is presumably on the Rha residue. The structure bears great resemblance to another polysaccharide, recently studied, produced by *Sphingomonas paucimobilis* I-886.

Keywords: Capsular polysaccharide; Structure; Deoxyhexuronic acid; *Azotobacter indicus*

1. Introduction

In search for bacterial polysaccharides with special rheological properties, the heteropolysaccharide-7, (S-7) from *A. indicus* var. *myxogenes* was discovered.[1] It was found to function well as a hydrophilic colloid to thicken, suspend and stabilize water based systems. A preliminary analysis showed the presence of glucose, rhamnose, a uronic acid and *O*-acetyl groups. We have now investigated the structure of this polysaccharide. Noticeably, it contains an unusual 2-deoxysugar, 2-deoxy-D-*arabino*-hexuronic acid, the same as that found previously in the exopolysaccharide from *Sphingomonas paucimobilis* strain I-886.[2] The backbones of these polysaccha-

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rides are identical and have the following structure:

→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-2-deoxy-*arabino*-Hex*p*A-(1→

The backbone has large similarities to that of the polysaccharides of the gellan gum family (Ref. 3 and refs. cited therein) where the polysaccharides have a backbone identical to that above with the exception that the acidic sugar is D-glucuronic acid instead of 2-deoxy-*arabino*-hexuronic acid, ('2-deoxyglucuronic acid').

2. Results and discussion

The exocellular polysaccharide from *A. indicus* var *myxogenes* (S-7), discovered already in 1975 was used.[1] A hydrolysate of S-7 obtained using dilute trifluoroacetic acid contained glucose and rhamnose in the ratio 77:23 as the only detectable sugars. GLC analysis of the acetylated (+)-2-octyl glycosides demonstrated that the sugars had the D and the L configuration, respectively. Methylation analysis revealed that one residue each of terminal, 4-substituted, 6-substituted, and 3,6-disubstituted glucose were present and in addition one residue of 4-substituted rhamnose in the relative proportions 20:21:19:19:21.

Native S-7 gave viscous solutions and poor NMR spectra, but removal of the *O*-acetyl groups at alkaline pH followed by heating overnight at 80 °C at pH 8 made the sample more suitable for NMR studies. More extensive heating gave complex NMR spectra, most presumably because of the facile hydrolysis[4] and degradation of the 2-deoxysugar, a property described for 2-deoxy-D-*arabino*-hexose.[5] The ^{1}H NMR spectrum (Fig. 1) showed six signals in the anomeric region at δ 5.17, 4.81, 4.73, 4.58, 4.52, and 4.52 ppm, i.e., in addition to those five residues indicated in the methylation analysis one further residue is most likely present in the repeating unit. The spectrum also showed signals at δ 1.63 and 2.36 ppm and much resembled the spectrum from *S. paucimobilis* exopolysaccharide I-886[2] which inter alia had anomeric signals at δ 5.17, 4.96, 4.81, 4.73, 4.58, and 4.50, and signals at 1.62 and 2.35 ppm. The latter signals were assigned to the methylene group of a 2-deoxy-*arabino*-β-D-hexopyranuronic acid residue. One difference between the spectra of the two polysaccharides was apparent, namely the absence in the spectrum of S-7 of a signal at δ 4.96, and instead the presence of a signal at $\delta \sim 4.5$ ppm. Thus, all the five signals between δ 4.45 and 4.76 ppm had $J_{\text{H-1, H-2}}$ values of 7–10 Hz and were attributed to β-D-pyranose residues. The signal at δ 4.76 was a broad doublet with an apparent $J_{\text{H-1, H-2}}$ value of ca. 10 Hz, and later turned out to be a doublet of a doublet from coupling to the 2-deoxy-protons. The signal at δ 5.17 had a low $J_{\text{H-1, H-2}}$ value and was therefore assigned to the L-rhamnopyranose residue. In the high-field region, the signal for the CH_3 group in L-rhamnose appeared at δ 1.33 ppm in addition to the signals for a methylene group.

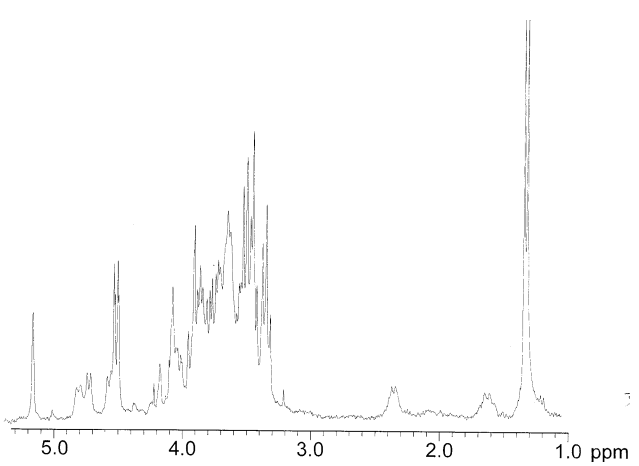


Fig. 1. ^{1}H NMR spectrum of O-deacylated S-7.

After treatment of the carboxyl-reduced polysaccharide with 4-methylmorpholinoborane under acidic conditions, just as done previously with the exopolysaccharide I-886 from *S. paucimobilis*,[2] the alditol mixture contained, apart from previously detected glucitol and rhamnitol, also 2-deoxy-*arabino*-hexitol which co-chromatographed with the authentic compound and gave an identical mass spectrum. Consequently, 2-deoxy-*arabino*-hexuronic acid is a component of S-7.

The repeating unit of the polysaccharide should thus contain four glucose residues, one residue of 2-deoxy-*arabino*-hexuronic acid and

Table 1
^{1}H NMR chemical shifts (ppm) for oligo- and polysaccharides from S-7

Sugar residue	H-1	H-2 (a,b)	H-3	H-4	H-5	H-6 (a,b)
O-Deacetylated S-7						
β-D-Glc*p*-(1→(**A**)	4.52 [a] (~8 Hz)	3.37	3.65			
→3,6)-β-D-Glc*p*-(1→(**B**)	4.58 (~8 Hz)	3.45	3.65			
→4)-β-D-Glc*p*-(1→(**C**)	4.73 (~8 Hz)	3.36	3.65	3.52	3.46	3.76, 3.93
→6)-β-D-Glc*p*-(1→(**D**)	4.52 [a] (~8 Hz)	3.31				
→4)-α-L-Rha*p*-(1→(**E**)	5.17 (~1.5 Hz)	4.09	4.03	3.71	4.08	1.33
→4)-β-D-2d-*arabino*-Hex*p*A-(1→(**C**)	4.81 (~9 Hz)	1.63, 2.36	3.84	3.65		
Smith degradation product						
β-D-Glc*p*-(1→(**B′**)	4.54 (7.9)	3.32	3.53	3.43		
→4)-β-D-Glc*p*-(1→(**C**)	4.62 (7.9)	3.39	3.65	3.64	3.43	
→3)-1-deoxy-D-erythritol (**E′**)	1.21	4.01	3.79			
→4)-β-D-2d-*arabino*-Hex*p*A-(1→(**F**)	4.81 (~9.7, 2.2 Hz)	1.63, 2.35	3.83			

[a] Interchangable chemical shift sets.

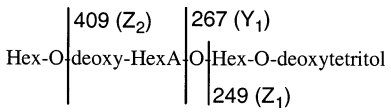
one residue of rhamnose. The former five were indicated, as discussed above, to be β linked from the large $J_{\text{H-1, H-2}}$ values. From the chemical shift of its H-1 signal, the Rha residue was indicated to be α linked. Further evidence for this repeat came from mass spectrometry evidence. Thus, upon a time-course, hydrolysis of O-deacetylated S-7 with dilute acetic acid, where only the glycosidic linkage of the 2-deoxysugar was expected to break, and recording of the ESI mass spectra in the negative mode, a pseudomolecular ion, $[M-H]^-$, at m/z 971.5 was obtained, corresponding exactly to the proposed composition, $Glc_4Rha_1$2d-*arabino*-$HexA_1$. MS–MS of m/z 971 showed ions at, inter alia, m/z 793 and 631 corresponding to the loss, first of 2-deoxy-*arabino*-hexuronic acid (178 u) and secondly of a hexosyl residue (162 u), both terminal sugars in the oligosaccharide (see below).

The ^{1}H NMR spectrum of O-deacetylated S-7 could be analyzed to some extent but not fully assigned, due to the poor spectrum quality (Residues **A**–**F**, Table 1). The full spin system for the 4-substituted glucose, the rhamnose residue, and the 2-deoxyhexuronic acid residue could be deduced and the chemical shifts of the acid residue were close to the corresponding values from *S. paucimobilis* exopolysaccharide I-886. This indicates a high degree of similarity between I-886 and S-7, the arabino configuration of the acid, and also its absolute configuration to be D as in the *S. paucimobilis* I-886 polysaccharide.

Partial acid hydrolysis.—After treatment of the O-deacetylated polysaccharide with aqueous 48% hydrofluoric acid for 48 h at 4 °C, the resulting mixture of oligosaccharides was separated on a Biogel P-4 column. A fraction in the disaccharide region could be obtained pure. The positive ESI mass spectrum showed a pseudomolecular ion, $[M+Na]^+$, at m/z 349.2 corresponding to a disaccharide composed of a hexose and a 6-deoxyhexose. The ^{1}H NMR spectrum showed inter alia a signal for a β-anomeric proton at δ 4.71 (1 H, $J_{\text{H-1,H-2}}$ 7.7 Hz) and two smaller signals at 5.12 and 4.86 ppm (1 H together) corresponding to the reducing end of a rhamnose residue. The disaccharide is consequently β-D-Glc-(1→4)-L-Rha.

Table 2
^{13}C NMR chemical shifts (ppm) for the Smith degradation product from S-7

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
Smith degradation product						
β-D-Glc*p*-(1→(**B′**)	103.4	74.0	76.4	70.1	76.7	61.6
→4)-β-D-Glc*p*-(1→(**C**)	102.8	74.0	74.9	79.6	75.3	61.6
→3)-1-deoxy-D-erythritol (**E′**)	17.9	68.0	84.5	61.0		
→4)-β-D-2d-*arabino*-Hex*p*A-(1→(**F**)	100.5	37.2	69.8	82.7	76.5	



Scheme 1.

Smith degradations.—O-Deacetylated S-7 was treated with periodate, reduced with sodium borohydride and hydrolyzed with dilute acid under mild conditions. Gel filtration gave an oligosaccharide that showed a pseudomolecular ion, $[M-H]^-$, in negative ESI-MS at m/z 589.5, corresponding to two hexoses, one deoxyhexuronic acid and one deoxytetritol, identical to what was obtained for the *S. paucimobilis* I-886 polysaccharide. A hydrolysate showed glucose and 1-deoxyerythritol in the ratio 94:6. The ^1H NMR spectrum showed three signals for anomeric protons at δ 4.54, 4.62, and 4.81 ppm. Signals for methylene protons from the 2-deoxysugar appeared at δ 1.62 and 2.35 ppm, as doublets of doublets of doublets. Further analysis with 2D NMR spectroscopy (Tables 1 and 2) revealed many of the NMR chemical shifts and showed that they were virtually identical to those of the Smith degradation product isolated from the *S. paucimobilis* I-886 polysaccharide. Carbon signals were assigned mainly with 2D NMR techniques and some by comparison with I-886. In the $\{^1H\ ^{13}C\}$ HMBC spectrum inter-residue correlations at δ_H/δ_C 4.62/84.5, 4.54/82.7 and 4.81/79.6 ppm were observed, practically identical to values from I-886 and corresponding to the disaccharide elements **B′-F**, **F-C**, and **C-E**. It can therefore be concluded that the I-886 and S-7 oligosaccharides have the same structure and also that the 4-substituted glucose unexpectedly survived the oxidation. From this also follows that the 2-deoxyhexuronic acid is 4-substituted, and the structure of the oligosaccharide, **1**, is

B' **F** **C** **E'**

β-D-Glc*p*-(1→4)-β-D-2-deoxy-*arabino*-Hex*p*A-(1→4)-β-D-Glc*p*-(1→3)-1-deoxy-D-erythritol

1

The sequence was corroborated with MS–MS studies on the pseudomolecular ion, $[M-H]^-$, m/z 589.5, where daughter ions were observed at m/z 409, 267, and 249 which corresponds to eliminations according to Scheme 1 fragments denoted as in Ref. 6:

C **E** **B** **F**

→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-2-deoxy-*arabino*-Hex*p*A-(1→

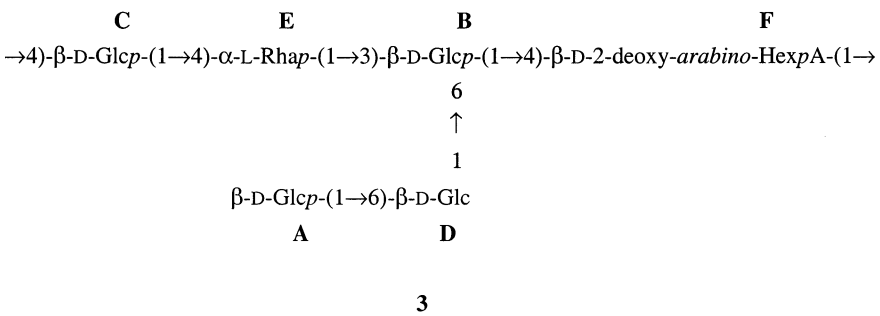
2

Smith degradation of the native S-7 gave, after gel filtration of the reaction mixture, a polysaccharide as the main product. The ^1H NMR spectrum of the O-deacetylated product showed signals for four anomeric protons at δ 5.17 (1 H, *J* small), 4.81 (1 H, dd), 4.73 (1 H, $J \sim 8$), and 4.56 ppm (1 H, $J \sim 8$). They were assigned to one α-rhamnose residue, one 2-deoxy-*arabino*-hexuronic acid residue, and two β-glucose residues, respectively. In addition, signals for the methylene group of the acid and the methyl group of rhamnose residue were

present. Methylation analysis demonstrated that 3- and 4-substituted glucose and 4-substituted rhamnose were present. The polysaccharide, **2**, must therefore be linear, consist of the main chain of S-7 and furthermore, the site of attachment of the side chain must be in position 6 of the 3,6-disubstituted glucose residue as this sugar has turned into a 3-substituted residue.

The only sugars not accounted for in **2** is the terminal and the 6-substituted β-glucose residues, which consequently must make up the side chain and substitute residue B in the main chain in the 6-position. The full structure, excluding the *O*-acetyl groups, is thus **3**. The difference to the *S. paucimobilis* strain I-886 polysaccharide is the anomeric configuration of the 6-substituted Glc residue in the side chain.

The observation of another extracellular polysaccharide from *S. paucimobilis*, strain P4 (DSM6418) giving highly viscous fermentation broth but with a reported structure having a similar but yet different backbone than I-886 is intriguing.[7] The reported structure of the repeating unit is →4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→. Thus it consists of three of the elements of the S-7/I-886 polysaccharide backbone. A rerun and reinspection of the 1D ^{1}H NMR spectrum shows the presence of two methylene proton signals at chemical shifts similar to those of the 2-deoxy-*arabino*-hexuronic acid in S-7. The failure to detect those previously may be a consequence of broad peaks and some interfering peaks in the region. It thus appears likely that 2-deoxy-*arabino*hexuronic acid is a component of the P4 polysaccharide.



ESI-MS on partially hydrolysed S-7.—In order to quantify the *O*-acetyl content, native S-7 was subjected to hydrolysis with acetic acid at pH 2 and 100 °C. Again the glycosidic linkage of the 2-deoxysugar was the only one that was expected to hydrolyze. Samples were withdrawn at various times and analyzed with ESI-MS in the negative mode. The spectrum showed a major ion at *m*/*z* 1055.5 corresponding to $[M-H]^-$ of $Glc_4Rha_1$2d-ara-$HexA_1Ac_2$. A minor peak (10%) corresponding to the monoacetylated repeat was also observed at *m*/*z* 1013.8. No signal at *m*/*z* 971 was observed, consequently the repeating unit of S-7 contains two moles of *O*-acetyl groups and at least one should be located on the rhamnose residues as periodate oxidation is inhibited. Attempted localization of the *O*-acetyl groups with MS–MS was unsuccessful.

3. Experimental

Sugar and methylation analysis.—Sugar analysis was performed with 2 M CF_3CO_2H (120 °C, 2 h), and monosaccharides were identified by GLC as the alditol acetate derivatives[8] on a DB-5 fused-silica capillary column (25 m × 0.25 mm), using a Hewlett–Packard 5890 instrument and a temperature gradient from 160 (1 min) to 250 °C at 3 °C/min. For determination of the absolute configuration of monosaccharides, the hydrolysate was heated with (+)-2-octanol (0.4 mL) in the presence of CF_3CO_2H (0.05 mL) at 100 °C for 16 h, acetylated with Ac_2O in Py (100 °C, 1 h), and analyzed by GLC as above.[9]

Methylation of S-7 and carboxyl reduced S-7 was performed using methyl iodide in dimethyl sulfoxide in the presence of sodium

methylsulfinylmethanide.[10] The methylated products were purified by absorbing them on Sep-Pak C_{18}-cartridges and subsequently eluting them with MeCN. Hydrolysis was performed as in sugar analysis. Partially methylated monosaccharides derived were reduced with $NaBH_4$, converted to alditol acetates, and analyzed by GLC–MS on a Hewlett–Packard 5890 chromatograph equipped with a NERMAG R10-10L mass spectrometer. The chromatographic conditions were the same as for GLC.

Isolation of S-7.—The exopolysaccharide was prepared as described.[1]

NMR spectroscopy.—NMR spectra of solutions in deuterium oxide containing a 20 mM phosphate buffer of pH 7.5 were recorded at 70 °C (^{13}C), 60 or 85 °C (^{1}H) with JEOL EX270 and JNM500 instruments. Chemical shifts are reported in ppm, using acetone (δ_C 31.00) and sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) as internal references. All NMR experiments were performed using JEOL standard pulse-sequences. $^{1}H,^{1}H$-HOHAHA experiments were performed in the phase-sensitive mode with mixing times 40–180 ms. Proton-carbon correlated spectra (HMQC) were obtained with decoupling and the long-range proton-carbon correlated spectra (HMBC) were performed using a delay time of 62.5 ms.

Electrospray mass spectrometry.—Mass spectrometry was performed using electrospray ionization with an LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA). Samples were dissolved in 1:1 MeOH–water and introduced to the electrospray at a flow rate of 10 μL/min. Nitrogen was used as a sheath gas.

Sugar analysis with morpholinoborane.—Carboxyl-reduced S-7[11] was hydrolyzed with 0.5 M CF_3CO_2H at 100 °C for 2 h. During the hydrolysis, 10 equiv of borane-4-methylmorpholine complex were added for instant reduction. The sugars in the hydrolysate were then converted into alditol acetates.

Preparation of O-*deacylated S-7.*—The polysaccharide was dissolved in aqueous 5% NH_3 and kept at rt for 16 h. After neutralization, the *O*-deacylated polysaccharide was dialyzed and freeze-dried.

Graded acid hydrolysis.—Native S-7 was kept in aqueous 48% hydrofluoric acid at 4 °C for 48 h. After removal of the acid by flushing with air, the mixture was separated on a Bio-Gel P-4 column.

Smith degradation.—*O*-Deacylated S-7 was treated with 0.03 M sodium periodate in the dark at 4 °C for 72 h. The resulting product was reduced with $NaBH_4$ (3 mg per mg PS), followed by hydrolysis with 0.5 M CF_3CO_2H at rt for 24 h.[12] The Smith product was recovered in the oligosaccharide region by gel filtration on Bio-Gel P-4 columns. The same procedure was used for native S-7.

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