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Structure of the O-polysaccharide of *Providencia stuartii* O4 containing 4-(*N*-acetyl-L-aspart-4-yl)amino-4,6-dideoxy-D-glucose

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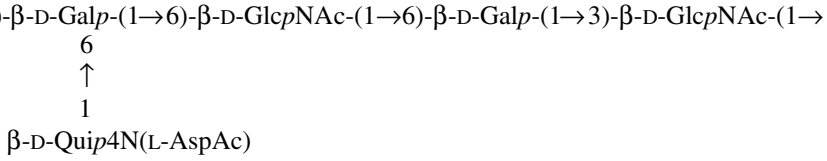
Abstract—The O-polysaccharide of *Providencia stuartii* O4 was obtained by mild acid degradation of the lipopolysaccharide, and the following structure of the pentasaccharide repeating unit was established:



where D-Qui4N(L-AspAc) is 4-(*N*-acetyl-L-aspart-4-yl)amino-4,6-dideoxy-D-glucose, which has not been hitherto found in bacterial polysaccharides. Structural studies were performed using sugar and methylation analyses, Smith degradation and NMR spectroscopy, including conventional 2D $^{1}H,^{1}H$ COSY, TOCSY, NOESY and $^{1}H,^{13}C$ HSQC experiments as well as COSY and NOESY experiments run in an H_2O–D_2O mixture to reveal correlations for NH protons.

Keywords: Lipopolysaccharide; O-polysaccharide; Bacterial polysaccharide structure; *Providencia stuartii*

1. Introduction

Gram-negative bacteria of the genus *Providencia* are facultative pathogens that cause intestinal and urinary tract infections. Currently, the genus contains five species, including *Providencia alcalifaciens*, *P. rustigianii*, *P. stuartii*, *P. heimbachae* and *P. rettgerii*.[1] The serological classification scheme of three species *P. alcalifaciens*, *P. rustigianii* and *P. stuartii* is based on the O- and H-antigens and includes 62 serogroups.[2,3] Recently, aiming at establishing the molecular basis for classification of *Providencia* strains, structures of the O-polysaccharides of the lipopolysaccharides (O-antigens) of *Providencia* serogroups O5, O7, O14, O16, O18, O19, O21 and O23 have been elucidated (Refs. 4–6 and references cited in Ref. 4). Now we report on the structure of the O-polysaccharide of *P. stuartii* O4.

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2. Results and discussion

The O-polysaccharide was obtained by mild acid degradation of the lipopolysaccharide. Sugar analysis using GLC of the alditol acetates derived after full acid hydrolysis of the polysaccharides showed the presence of Gal and GlcN. GLC of the acetylated (+)-2-octyl glycosides indicated that both monosaccharides have the D configuration. Analysis of the polysaccharide hydrolysate using an amino acid analyser revealed aspartic acid, which was demonstrated to be L by GLC of the acetylated (+)-2-octyl ester. Further studies showed that the polysaccharide includes also 4-amino-4,6-dideoxyglucose (Qui4N), which either was destroyed or was not released by acid hydrolysis. The D configuration of Qui4N was established by analysis of the ^{13}C NMR chemical shifts in the polysaccharide using known regularities in glycosylation effects.[7] Methylation analysis of the polysaccharide revealed the presence of 6-substituted and 3,6-disubstituted Gal residues and 3-substituted and 6-substituted residues of GlcNAc.

The presence of aspartic acid was further confirmed by ^{1}H and ^{13}C NMR spectra of the polysaccharides, which showed characteristic signals of an ABX system at δ 2.63, 2.85 (AB part, J_{AB} 15.0 Hz; both H-3) and 4.51 (X part; J_{AX} 9.1 Hz, J_{BX} 4.9 Hz; H-2) and the corresponding carbons C-3 at δ 39.7 and C-2 at δ 53.5, as well as carboxyl carbons C-1 and C-4 at δ 177.9 and 174.4. In addition, the ^{1}H NMR spectrum contained signals for five anomeric protons at δ 4.43–4.77 (all doublets, $J_{1,2}$ 7–8 Hz), one CH_3–C group at δ 1.21 (doublet, $J_{5,6}$ 5.7 Hz, H-6 of Qui4N) and three *N*-acetyl groups at δ 2.02–2.07. Relatively large $^{3}J_{1,2}$ values of about 8 Hz and characteristic positions of the H-1 signals indicated the β-pyranose form of all constituent monosaccharides. The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) displayed signals for five anomeric carbons at δ 102.5–105.0; one non-substituted and three *O*-substituted HO*C*H_2–C groups (C-6 of Gal and GlcN) at δ 62.0 and 69.9–70.5, respectively; one *C*H_3–C group (C-6 of Qui4N) at δ 18.2; three nitrogen-bearing carbons (C-2 of GlcN and C-4 of Qui4N) at δ 56.1–58.1; other sugar ring carbons at δ 69.7–84.1, and three *N*-acetyl groups (CH_3 at δ 23.3–23.7, CO at δ 174.8–176.3).

Therefore, the O-polysaccharide has a pentasaccharide repeating unit that is composed of five β-linked monosaccharides, including two residues each of D-Gal and D-GlcN and one residue of Qui4N, as well as four acyl residues, including one aspartyl and three acetyl groups. The monosaccharide residues were designated as **A**–**E** according to their sequence in the repeating unit (see below).

The ^{1}H NMR spectrum of the polysaccharide was assigned using gradient-selected 2D NMR experiments, including gsCOSY, gsTOCSY and gsNOESY (Table 1). The ^{13}C NMR spectrum of the polysaccharide was assigned using an H-detected $^{1}H,^{13}C$ gsHSQC experiment based on the assigned ^{1}H NMR spectrum (Table 2). The assignments were confirmed by $^{1}H,^{13}C$ gsHMBC and gsHSQC–TOCSY experiments, and the former enabled also the assignment of the CO signals of the aspartyl and acetyl groups. In the TOCSY spectrum, anomeric protons at δ 4.51, 4.61 and 4.77 for the Qui4N and GlcN residues showed correlations with H-2,3,4,5,6 and those at δ 4.43 and 4.44 for the Gal residues with H-2,3,4. The remaining protons of Gal were assigned by H-1,H-3 and H-1,H-5 correlations in the gsNOESY spectrum that are typical of β-linked pyranosides and by H-5,H-6 corre-

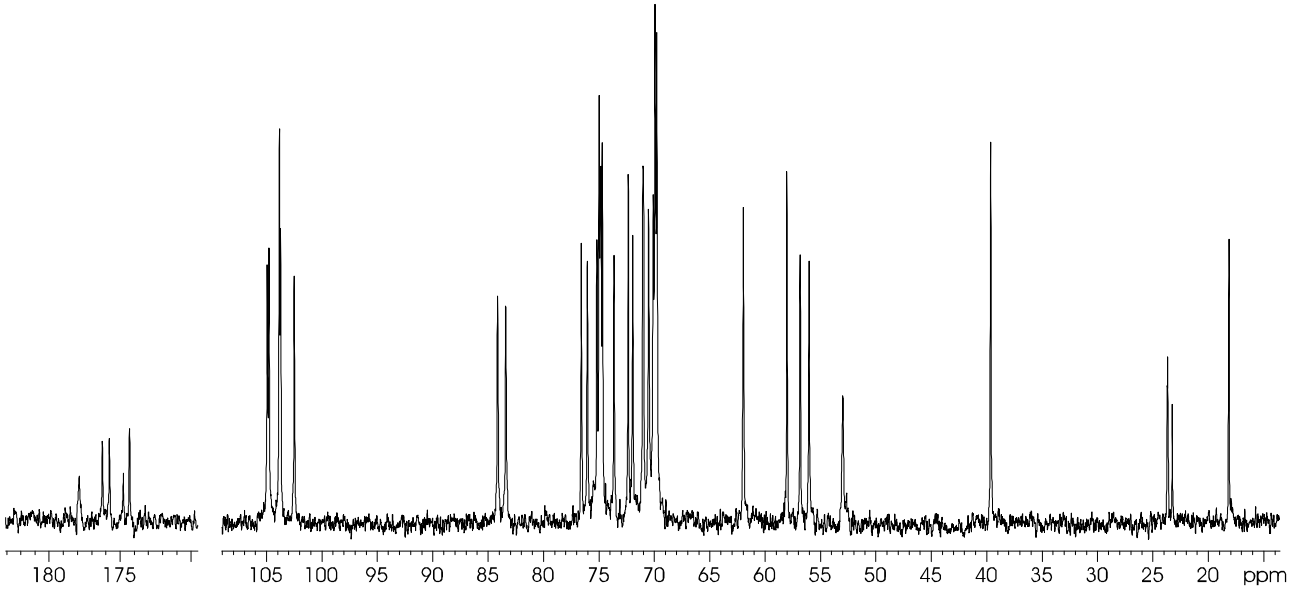

Figure 1. 125-MHz ^{13}C NMR spectrum of the O-polysaccharide.

Table 1. ^{1}H NMR data of the O-polysaccharide (δ)

		H-1	H-2	H-3 (3a,3b)	H-4	H-5	H-6 (6a,6b)	CH_3CO
→3,6)-β-D-Gal*p*-(1→	**A**	4.44	3.63	3.74	4.18	3.89	3.91, 4.01	
→6)-β-D-Glc*p*NAc-(1→	**B**	4.61	3.70	3.57	3.56	3.64	3.91, 4.23	2.07
→6)-β-D-Gal*p*-(1→	**C**	4.43	3.52	3.64	3.93	3.82	3.83, 4.01	
→3)-β-D-Glc*p*NAc-(1→	**D**	4.77	3.88	3.80	3.55	3.49	3.79, 3.93	2.04
β-D-Qui*p*4N-(1→	**E**	4.51	3.34	3.53	3.58	3.58	1.21	
L-AspAc			4.51	2.63, 2.85				2.02

Table 2. ^{13}C NMR data of the O-polysaccharide (δ)

		C-1	C-2	C-3	C-4	C-5	C-6	CH_3CO	CH_3CO
→3,6)-β-D-Gal*p*-(1→	**A**	104.8	71.1	83.4	69.8	74.9	70.5		
→6)-β-D-Glc*p*NAc-(1→	**B**	102.5	56.9	75.3	76.1	71.0	70.1	23.7	175.8
→6)-β-D-Gal*p*-(1→	**C**	105.0	72.0	73.7	69.7	75.0	69.9		
→3)-β-D-Glc*p*NAc-(1→	**D**	103.8	56.1	84.1	69.9	76.6	62.0	23.6	176.3
β-D-Qui*p*4N-(1→	**E**	103.9	75.1	74.9	58.1	72.5	18.2		
L-AspAc		177.9	53.5	39.7	174.4			23.3	174.8

lations in the COSY spectrum. The *gluco* configuration of the amino sugars, including Qui4N, was confirmed by relatively large $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ coupling constant values of 9–10 Hz. The position of the amino group in Qui4N was established by correlation of the proton at the nitrogen-bearing carbon (H-4) to the corresponding carbon (C-4) at δ 3.58/58.1.

The modes of substitution of the monosaccharides were determined by displacements of the signals for the linkage carbons to a lower field region of the ^{13}C NMR spectrum due to glycosylation effects. The signals for C-3 of Gal **A** and GlcN **D** were shifted downfield to δ 83.4 and 84.1 and those for C-6 of Gal **A**, Gal **C** and GlcN **B** to δ 70.5, 69.9 and 70.1, respectively, that is, by ~8–9 ppm as compared with their positions in the corresponding non-substituted monosaccharides.[8] The ^{13}C NMR chemical shifts for C-2–C-6 of Qui4N were close to those of the non-substituted monosaccharide.[9] Therefore, the polysaccharide is branched with a lateral Qui4N residue and a 3,6-disubstituted Gal residue at the branching point. The sequence of the monosaccharides in the repeating unit was established by gsNOESY (Fig. 2) and ^{1}H,^{13}C gsHMBC experiments, which revealed interresidue connectivities of the anomeric protons to the protons at the linkage carbons and to the linkage carbons, respectively (Table 3). These data were in agreement with the ^{13}C NMR chemical shift data.

A correlation between H-4 of Qui4N and a carboxyl group of Asp at δ 3.58/174.4 in the gsHMBC spectrum showed that the aspartyl group is N-linked and located on Qui4N. A NOESY experiment with a polysaccharide sample in a 9:1 (v/v) H_2O–D_2O mixture revealed correlations for NH-protons, which were absent from the spectra measured in D_2O owing to exchange with deuterons. Particularly, cross-peaks for NH-4 of Qui4N and CH_2 (H-3) of Asp were observed at δ 8.11/2.63 and 8.11/2.85 (Fig. 3) and demonstrated a spatial proximity of these protons. Therefore, Asp is attached by COOH-4 to N-4 of Qui4N as shown in Figure 4.

Further evidence of the repeating unit structure and the location of the aspartyl group was obtained by Smith degradation of the polysaccharide, which afforded a β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→1)-Gro oligosaccharide and an amino alditol **1** (Fig. 5). Their structures were determined using COSY and ^{1}H,^{13}C HSQC experiments (data not shown), and the structure of **1** was confirmed by determination of the molecular mass (262.11 Da) by ESIMS, which was in agreement with the calculated value. Therefore, **1** consists of a remainder of the destroyed Qui4N residue with an N-linked *N*-acetylaspartyl group.

Based on the data obtained, it was concluded that the O-polysaccharide of *P. alcalifaciens* O4 has the structure shown in Figure 6. In this polymer, L-aspartic acid was identified for the first time as a component of bacterial polysaccharides. The amino acid contributes to the polysaccharide charge, and Qui4N(AspAc) plays a role in manifesting of the immunospecificity and cross-reactivity of the O4-antigen, which will be reported elsewhere.[10] Recently, D-aspartic acid has been found in the O-polysaccharides of *P. alcalifaciens* O33[10,11] and *Proteus mirabilis* O38[11,12] and in a glycoconjugate from *Treponema medium* ATCC 700293.[13]

3. Experimental

3.1. Bacterial strain and growth

P. stuartii O4:H4 strain 884 came from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest). The bacteria were cultivated under aerobic conditions in nutrient broth

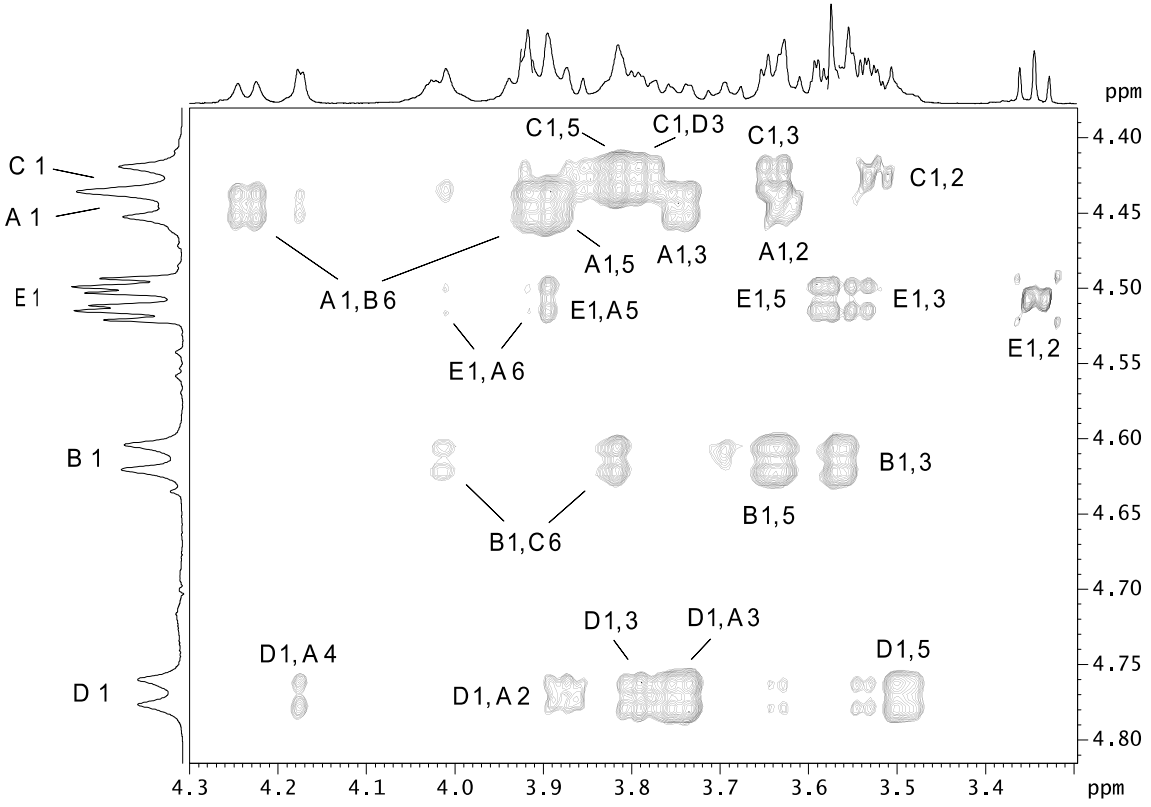

Figure 2. Part of a gsNOESY spectrum of the O-polysaccharide showing correlations for anomeric protons. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Arabic numerals refer to protons in sugar residues denoted by letters as shown in Table 1 and Figure 6.

Table 3. Interresidue connectivities of the anomeric protons to the protons at the linkage carbons (gsNOESY) and to the linkage carbons (^{1}H,^{13}C gsHMBC) in the O-polysaccharide

Sugar residue		δ_{H-1}	Connectivity to					
			Sugar residue		Proton	δ_H	Carbon	δ_C
β-D-Gal	**A**	4.44	β-D-GlcNAc	**B**	H-6a,6b	3.91 (s), 4.23 (s)	C-6	70.1
β-D-GlcNAc	**B**	4.61	β-D-Gal	**C**	H-6a,6b	3.83 (s), 4.01 (m)	C-6	69.9
β-D-Gal	**C**	4.43	β-D-GlcNAc	**D**	H-3	3.80 (s)	C-3	84.1
β-D-GlcNAc	**D**	4.77	β-D-Gal	**A**	H-3	3.74 (s)	C-3	83.4
β-D-Qui4N(L-AspAc)	**E**	4.51	β-D-Gal	**A**	H-6a,6b	3.91 (w), 4.01 (w)	C-6	70.5

s: strong; m: medium; w: weak.

supplemented with 1% glucose. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water and lyophilised.

3.2. Isolation and degradations of the lipopolysaccharide and the polysaccharide

The lipopolysaccharide was isolated from bacterial cells by phenol–water extraction[14] and purified by treatment with cold aqueous 50% CCl_3CO_2H; the aqueous layer was dialysed and freeze-dried.

A high-molecular-mass polysaccharide was prepared by degradation of the lipopolysaccharide (150 mg) with aq 2% HOAc at 100 °C for 7 h followed by GPC of the water-soluble portion on a column (60 × 2.5 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer pH 4.5 (4 mL of pyridine and 10 mL of HOAc in 1 L of water) with monitoring of the elution using a Knauer differential refractometer. The yield of the polysaccharide was 27% of the lipopolysaccharide weight.

3.3. Composition analyses

The polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h). Amino sugars were converted into the alditol acetates[15] and analysed by GLC on a Hewlett–Packard 5880 instrument with a DB-5 capillary column using a temperature gradient of 160 °C (3 min) to 290 °C at 10 °C min^{-1}. Amino components were analysed using a Biotronik LC-2000 sugar analyser using standard

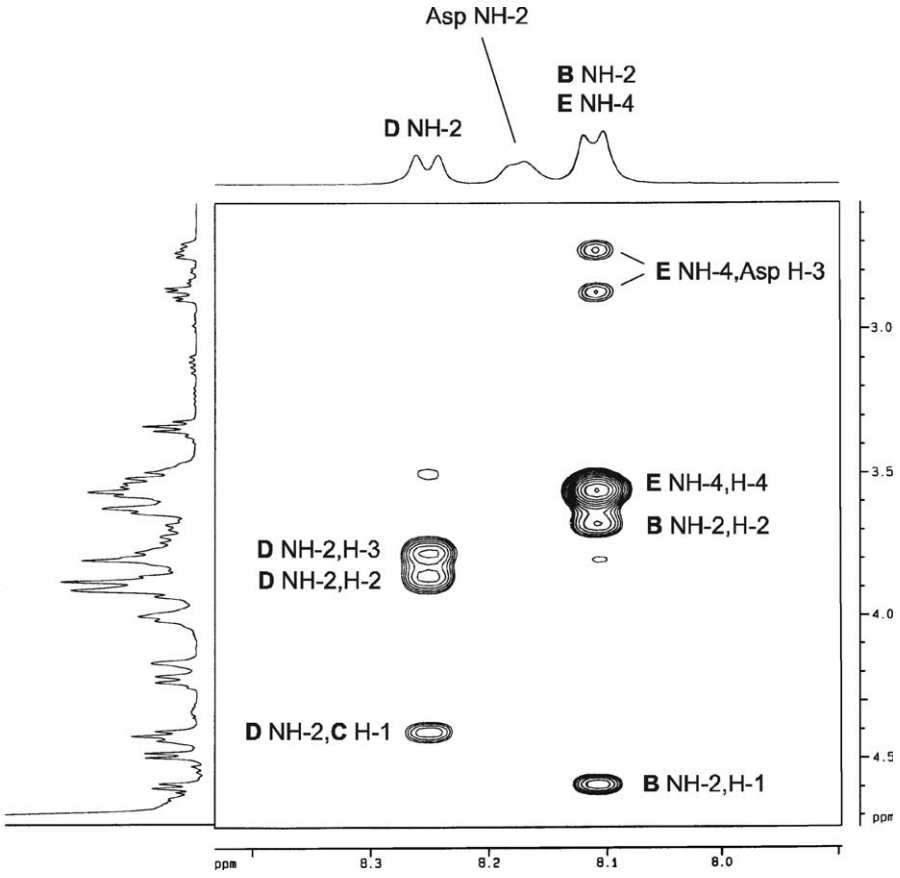

Figure 3. Part of a NOESY spectrum of the O-polysaccharide showing correlations for NH protons. The spectrum was run in a 9:1 (v/v) H_2O–D_2O mixture. The corresponding parts of the ^1H NMR spectrum are shown along the axes. For sugar abbreviations see Table 1 and Figure 6.

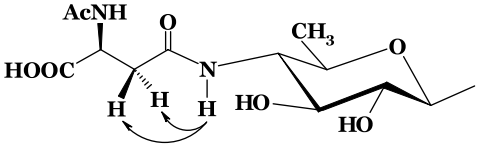

Figure 4. Structure of 4-(*N*-acetyl-L-aspart-4-ylamino)-4,6-dideoxy-β-D-glucose. NOE connectivities that define the mode of attachment of the aspartyl group are shown by arrows.

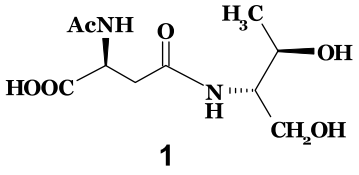

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Figure 5. Structure of the aspartyl-containing Smith degradation product of the O-polysaccharide.

sodium citrate buffers. The absolute configurations of the monosaccharides and aspartic acid were determined by GLC of the acetylated glycosides with (+)-2-octanol[16] and acetylated (+)-2-octyl ester, respectively, under the same chromatographic conditions as above.

3.4. Methylation analysis

Methylation was performed as described.[17] After hydrolysis with 2 M CF_3CO_2H (120 °C, 2 h), the partially methylated monosaccharides were reduced with $NaBH_4$, acetylated and analysed by GLC–MS on a Hewlett–Packard 5890 chromatograph equipped with a DB-5 fused-silica capillary column and interfaced with a NERMAG R10-10L mass spectrometer. A temperature gradient of 160 °C (1 min) to 250 °C at 3 °C min^{-1} was used.

3.5. Smith degradation

The polysaccharide (20 mg) was oxidised with 0.1 M sodium metaperiodate (20 °C, 48 h, in dark). After adding ethylene glycol, the mixture was reduced with $NaBH_4$ (2 h), neutralised with concd HOAc, desalted by GPC on TSK HW-40, and hydrolysed with 2% HOAc (100 °C, 2 h). An oligosaccharide (6 mg) and amino alditol **1** (1 mg) were isolated by GPS on TSK HW-40.

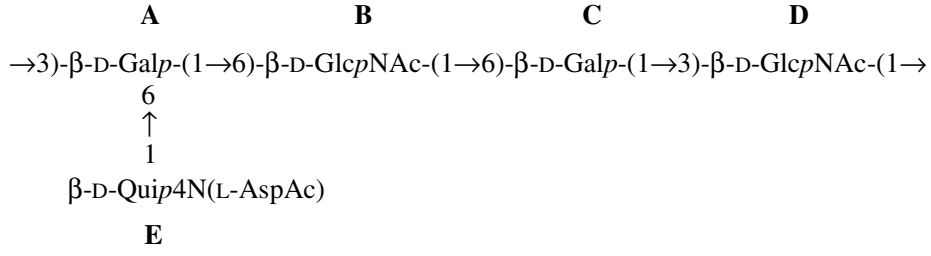

Figure 6. Structure of the O-polysaccharide of *P. stuartii* O4. D-Qui4N(L-AspAc) stands for 4-(*N*-acetyl-L-aspart-4-yl)amino-4,6-dideoxy-D-glucose.

3.6. NMR spectroscopy

Spectra were recorded using a Bruker DRX-500 spectrometer at 32 °C in D_2O or a 9:1 (v/v) H_2O–D_2O mixture. Prior to the measurements in D_2O, the samples were lyophilised twice from D_2O. Chemical shifts are reported related to internal acetone (δ_H 2.225; δ_C 31.45). Standard pulse sequences were used for gradient-selected gsCOSY, gsTOCSY (MLEV-17), gsNOESY, ^1H,^{13}C gsHMBC and gsHSQC–TOCSY experiments. An ^1H,^{13}C gsHSQC experiment was performed with a pulse sequence that allows multiplicity editing.[18] A mixing time was set to 200 ms in the NOESY and 150 ms in the TOCSY and HSQC–TOCSY experiments; a 60-ms delay was used in an HMBC experiment.

3.7. Mass spectrometry

Ion cyclotron resonance Fourier transform ESIMS was performed in the negative-ion mode using an APEX II Instrument (Bruker Daltonics, Billerica, USA) equipped with a 7 T actively shielded magnet and an Apollo ion source. Capillary entrance voltage was set to 3.8 kV and dry gas temperature to 150 °C. The sample was dissolved at a concentration of ~10 ng μL^{-1} in a 50:50:0.001 (v/v/v) mixture of 2-PrOH–H_2O–Et_3N and sprayed at a flow rate of 2 μL min^{-1}.

Acknowledgements

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