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Structure of the O-polysaccharide of *Proteus mirabilis* O38 containing 2-acetamidoethyl phosphate and N-linked D-aspartic acid

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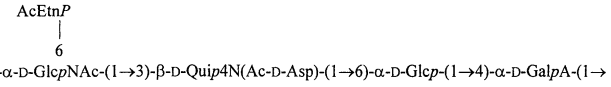
Abstract

The O-antigen of *Proteus mirabilis* O38 was found to be unique among bacterial polysaccharides and to have the following structure:



where D-Qui4N(Ac-D-Asp) is 4-(*N*-acetyl-D-aspart-4-ylamino)-4,6-dideoxy-D-glucose and AcEtn*P* is 2-acetamidoethyl phosphate. Neither of these entities have been hitherto found in natural polysaccharides. Structural studies were performed using 1D and 2D NMR spectroscopy, including experiments run in an H_2O/D_2O mixture to reveal correlations for NH protons. In addition, dephosphorylation, carboxyl reduction and selective cleavages were applied. Solvolysis of the polysaccharide with anhydrous HF gave an α-D-GlcNAc-(1→3)-D-Qui4N(Ac-D-Asp) disaccharide. Solvolysis with trifluoromethanesulfonic (triflic) acid afforded D-GlcNAc6(AcEtn*P*), thus showing the suitability of this reagent for the preparation of phosphorylated sugar derivatives.

Keywords: *Proteus mirabilis*; Lipopolysaccharide; Aspartic acid, O-polysaccharide structure; Serogroup; Classification

1. Introduction

Based on the specificity of the O-polysaccharide chain of the lipopolysaccharide (O-antigen), the medically important bacteria of the genus *Proteus* are currently classified into more than 60 O-serogroups.[1,2] In the majority of the *Proteus* strains studied, the O-polysaccharide contains acidic or both acidic and basic components, such as uronic acids, their amides with amino acids, ether-linked lactic acid, acetal-linked pyruvic acid, phosphate groups and phosphate-linked polyalcohols and amino alcohols.[3] Now we report on the structure of the O-antigen of *P. mirabilis* O38, which contains 2-acetamidoethyl phosphate and N-linked D-aspartic acid. Identification of D-aspartic acid in this polysaccharide and the O-antigens of *Providencia alcalifaciens* has been reported.[4]

2. Results and discussion

The lipopolysaccharide was isolated from dried bacterial cells of *P. mirabilis* O38 by the phenol–water procedure. Mild acid degradation of the lipopolysaccharide with dilute acetic acid, followed by GPC on Sephadex G-50, resulted in a high-molecular-mass O-polysaccharide.

Composition analyses after full acid hydrolysis of the polysaccharide revealed glucose (GLC of the alditol acetates), galacturonic acid (GalA, data from sugar

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analyses), aspartic acid and ethanolamine (data of amino acid analysis). The D configuration of aspartic acid and glucose was determined by GLC of the acetylated (*S*)-2-octyl ester and glycosides,[5] respectively. The D configuration of GalA and the constituent amino sugars was established by analysis of the glycosylation effects on the ^{13}C NMR chemical shifts in the polysaccharide[6] (see below). Methylation analysis of the polysaccharide showed the presence of a 6-substituted glucose residue.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1, Table 1) contained signals for four anomeric carbons at δ 98.2–104.1, three nitrogen-bearing carbons at δ 50.7, 53.3 and 57.9, one CH_3-C group (C-6 of a 6-deoxy sugar) at δ 17.7, one C-CH_2-C group (C-3 of Asp) at δ 38.4, one C-CH_2N group (C-2 of Etn) at δ 41.3, three O-substituted HOCH_2-C groups at δ 65.1, 65.5 and 69.5, 14 other oxygen-bearing carbons in the region δ 69.6–81.6, three *N*-acetyl groups (CH_3 at δ 23.0, 23.3 and 23.5) and five CON and COOH groups at δ 172.7–175.6.

The ^{1}H NMR spectrum of the polysaccharide (Table 2) showed, inter alia, seven signals in a low field of δ 4.42–5.37, four of which belonged to anomeric protons, one CH_3-C group (H-6 of a 6-deoxy sugar) at δ 1.15 (3H), one CH_2-C group at δ 2.73 (2H) and three *N*-acetyl groups at δ 2.00–2.03 (3H each). The ^{31}P NMR spectrum contained a signal for one phosphate group at δ 1.0.

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D ^{1}H,^{1}H COSY, TOCSY, H-detected ^{1}H,^{13}C HSQC and HMBC experiments (Tables 1 and 2). The following components were identified based on the splitting patterns, characteristic coupling constant values and ^{13}C NMR chemical shifts: Glc, GlcN, 4-amino-4,6-dideoxyglucose (Qui4N), GalA, aspartic acid and ethanolamine. The TOSCY spectrum showed correlations of H-1 with H-2,3,4,5,6 for GlcN, Glc and Qui4N and with H-2,3,4 for GalA. The spin systems of GlcN and Qui4N were distinguished by the correlations of the protons at the nitrogen-bearing carbons (H-2 and H-4) to the corresponding carbons (C-2 and C-4), which were observed in the HSQC spectrum at δ 4.01/53.3 and 3.79/57.9, respectively. The signal for the COOH group (C-6) of GalA was assigned by a C-6/H-5 correlation and those for COOH and CO groups (C-1 and C-4) of Asp by correlations of both signals with H-2 and H-3 of Asp in the HMBC spectrum.

The Qui4N H-1 chemical shift of δ 4.42 showed that this residue is β-linked, whereas those of δ 4.96–5.37 suggested the α-configuration of the other residues. This conclusion was confirmed by a ROESY experiment, which showed typical H-1,H-3,5 correlations for β-Qui*p*4N and H-1,H-2 correlations for α-Glc*p*, α-Glc*p*NAc and α-Gal*p*A.

The substitution pattern of the polysaccharide was demonstrated by the ^{1}H,^{13}C HSQC spectrum, which showed downfield displacements of the signals for the following linkage carbons as compared with their positions in the spectra of the corresponding non-substituted monosaccharides:[7] GalA C-4 to δ 80.2 (+8.6 ppm), GlcN C-3 and C-6 to δ 81.6 (+9.9 ppm) and 65.1 (+3.3 ppm), respectively, Qui4N C-3 to δ 78.4 (+3.6 ppm) and Glc C-6 to δ 69.5 (+7.7 ppm).

The ^{13}C NMR chemical shifts were also used for determination of the absolute configuration of GalA and the amino sugars. The constituent monosaccharides in the α-D-Glc*p*-(1→4)-Gal*p*A disaccharide fragment have the same absolute configuration as followed from a relatively large downfield shift of the C-4 signal for GalA by 8.6 ppm (α-effect of glycosylation), whereas in case of different absolute configurations the effect would be <7 ppm.[6] In the α-D-Gal*p*A-(1→3)-α-Glc*p*NAc

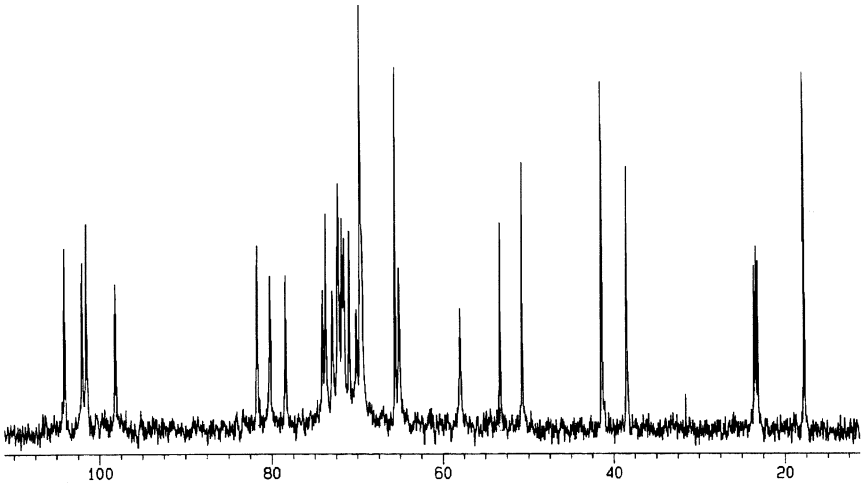

Fig. 1. 125-MHz ^{13}C NMR spectrum of the O-polysaccharide of *P. mirabilis* O38. Region for CO resonances is not shown.

Table 1
^{13}C NMR data (δ, ppm) of the O-polysaccharide of *P. mirabilis* O38 and derived fragments

Residue	C-1	C-2	C-3	C-4	C-5	C-6	CH_3CO	CH_3CO
O-polysaccharide								
→4)-α-Gal*p*A-(1→	102.0	69.6	69.6	80.2	71.8	173.5		
→3)-α-Glc*p*NAc-(1→	98.2	53.3	81.6	70.9	71.6	65.1	23.3 [a]	175.2 [b]
→3)-β-Qui*p*4N-(1→	104.1	73.7	78.4	57.9	72.2	17.7		
→6)-α-Glc*p*-(1→	101.6	72.9	74.0	70.1	72.2	69.5		
AcEtn*P*	65.5	41.3					23.0 [a]	175.6 [b]
AcAsp	175.2 [b]	50.7	38.4	172.7			23.5 [a]	175.3 [b]
Disaccharide ***1***								
α-Glc*p*NAc-(1→	98.4	55.0	72.3	70.9	73.0	61.4	23.3 [a]	
→3)-α-Qui4N	93.5	72.2	76.0	57.9	67.7	17.9		
→3)-β-Qui4N	97.0	74.8	79.0	57.9	72.4	17.9		
AcAsp		52.3	39.2				23.6 [a]	
Amino alditol ***3***								
α-GlcNAc-ol	61.7	55.2	69.6	71.4	71.3	68.1	23.5 [a]	175.3
AcEtn*P*	65.5	41.4					23.3 [a]	175.3

[a,b] Assignment could be interchanged.

disaccharide, the same absolute configuration of the monosaccharides was shown by a small positive β-effect of glycosylation of +0.2 ppm on C-4 of Glc*p*NAc (in case of different absolute configurations the effect would be negative and more than 1 ppm by the absolute value). Similarly, a small negative β-effect of glycosylation of −0.2 ppm on C-4 of Qui4N in the α-D-Glc*p*NAc-(1→3)-Qui*p*4N(Ac-D-Asp) disaccharide, which was determined by comparison with the data of a *N*-(3-hydroxybutyryl) derivative of Qui4N,[8] showed the same absolute configuration of GlcN and Qui4N. Therefore, all monosaccharides in the repeating unit have the D configuration.

The ROESY spectrum revealed the following correlations between the anomeric protons and protons at the linkage carbons: GalA H-1,GlcN H-3 at δ 5.37/3.91; GlcN H-1,Qui4N H-3 at δ 5.06/3.77; Qui4N H-1,Glc H6 at δ 4.42/3.86 and 4.07; Glc H-1,GalA H-4 at δ 4.96/4.41. These data showed that the polysaccharide is linear and defined the monosaccharide sequence in the repeating unit. They also suggested that downfield displace-

Table 2
^{1}H NMR data (δ, ppm) of the O-polysaccharide of *P. mirabilis* O38 and derived fragments

Residue	H-1	H-2	H-3	H-4	H-5	H-6	CH_3CO
O-polysaccharide							
→4)-α-Gal*p*A-(1→	5.37	3.93	3.99	4.41	4.42		
→3)-α-Glc*p*NAc-(1→	5.06	4.01	3.91	3.81	4.30	4.04	2.00
						4.10	
→3)-β-Qui*p*4N-(1→	4.42	3.42	3.77	3.79	3.54	1.15	
→6)-α-Glc*p*-(1→	4.96	3.44	3.68	3.58	4.18	3.86	
						4.07	
AcEtn*P*	3.94	3.43					2.02
AcAsp		4.74	2.73				2.03
Disaccharide ***1***							
α-GlcNAc(1→	5.08	3.85	3.71	3.53	4.11	3.80	2.01
						3.80	
→3)-α-Qui*p*4N	5.18	3.64	3.90	3.75	3.84	1.09	
→3)-β-Qui*p*4N	4.61	3.34	3.66	3.75	3.52	1.14	
AcAsp		4.61	2.62				2.06
				2.71			
Amino alditol ***3***							
α-GlcNAc-ol	3.61	4.08	3.97	3.66	3.85	3.98	2.01
	3.71					4.06	
AcEtn*P*	3.93	3.41					2.04

ment of the C-6 signal for GlcN to δ 65.1 is due rather to phosphorylation than to glycosylation of this sugar residue at position 6.

The acylation pattern of the polysaccharide was revealed by a series of 2D NMR experiments, including COSY, TOCSY and ROESY that were run in an H_2O/D_2O mixture and, therefore, showed correlations for N-linked protons. In the ROESY spectrum, there were cross-peaks between the signals for *N*-acetyl groups at δ 2.00–2.03 and NH of ethanolamine, GlcN and Asp at δ 8.13, 8.17 and 8.35, respectively, thus indicating N-acetylation of these residues. NH of Qui4N showed a correlation with CH_2 (H-3) of Asp at δ 8.32/2.73, and, hence, aspartic acid is attached to the amino group of Qui4N. A NOE correlation between NH of Asp and H-6 of Qui4N at δ 8.35/1.15 indicated a spatial proximity of these protons in the predominant conformer of D-Qui*p*4N(Ac-D-Asp).

The data obtained enabled establishing the structure of the repeating unit of the O-polysaccharide of *P. mirabilis* O38 shown in Fig. 2. Qui4N was not detected in the polysaccharide hydrolysate, most likely, owing to its destruction under strong acidic conditions. GlcN was not detected either, owing to its phosphorylation, but could be identified using an amino acid analyser after dephosphorylation of the polysaccharide with aq 48% HF. The modified polymer lacked ethanolamine and one of the *N*-acetyl groups but contained *N*-acetylaspartyl group. The ^{1}H and ^{13}C NMR spectra of the dephosphorylated polysaccharide, which were assigned as described above for the original polysaccharide, showed chemical shift differences for the GlcNAc residue only. Particularly, the signal for C-6 of GlcNAc shifted from δ 65.1 to 61.2.

Carboxyl reduction of the polysaccharide with borohydride after esterification with 'magic methanol' (a mixture of methanol, chloroform, and concd hydrochloric acid)[9] resulted in conversion of GalA into galactose and the *N*-acetylaspartyl group into the 3-acetamido-4-hydroxybutyryl group. This followed from the appearance in the ^{1}H NMR spectrum of a spin system for the latter at δ 2.38 (2H, H-2), 4.28 (H-3) and 3.57 (2H, H-4) and in the ^{13}C NMR spectrum of the signals for two HO*C*H_2-C groups at δ 60.5 and 61.0 instead of two COOH groups of Asp and GalA. This finding confirmed the attachment of aspartic acid by HOOC-4.

Further confirmation of the structure was obtained by selective degradations of the polysaccharide. Solvolysis with anhyd HF[10] cleaved the phosphate group and afforded a GlcNAc→Qui4N(Ac-Asp) disaccharide **1** (Fig. 2). Solvolysis with trifluoromethanesulfonic (triflic) acid[11] gave a phosphorylated GlcN derivative **2**, which was borohydride-reduced into amino alditol **3** (Fig. 2). Therefore, as opposite to anhyd HF, triflic acid is useful for isolation of phosphorylated sugar derivatives. Structures of **1**–**3** were determined by ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) and negative-ion mode ESIMS, which showed the expected molecular masses 523.20 Da (for **1**) and 386.11 Da (for **2**). The structures of the isolated fragments supported the acylation and phosphorylation patterns of the polysaccharide.

A peculiar feature of the polysaccharide of *P. mirabilis* O38 is the presence of 4-(*N*-acetyl-D-aspart-4-ylamino)-4,6-dideoxy-D-glucose. This rare amino sugar occurs in bacterial polysaccharides with various unusual *N*-acyl substituents,[12–14] but aspartic acid has not been hitherto found. Simultaneously, the same monosaccharide derivative has been identified in the O-polysaccharide of *P. alcalifaciens* O33,[4,15] which shows a marked structural similarity with the O-polysaccharide studied in this work.[15] The O-polysaccharide of *P. alcalifaciens* O4 contains 4-(*N*-acetyl-L-aspart-4-ylamino)-4,6-dideoxy-D-glucose.[4,15] Data on serological relatedness of the aspartic acid-containing O-antigens of *P. mirabilis* O38 and *P. alcalifaciens* will be reported elsewhere. Another peculiar feature of the *P. mirabilis* O38 O-polysaccharide is the presence of 2-acetamidoethyl phosphate. To the best of our knowledge, this component is reported for the first time in natural carbohydrates, whereas the non-acetylated derivative, 2-

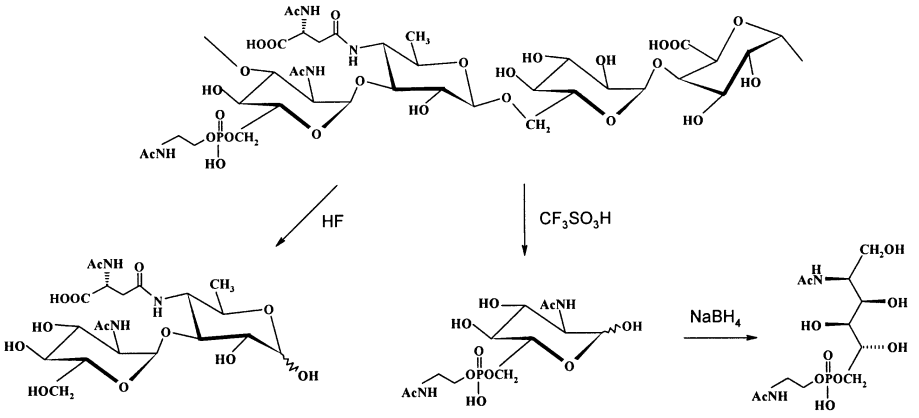

Fig. 2. Solvolyses of the O-polysaccharide of *P. mirabilis* O38 with anhyd HF and triflic acid.

aminoethyl phosphate, has been found in a number of bacterial polysaccharides, including O-antigens of *Proteus*.[3]

3. Experimental

3.1. Bacterial strain, growth and isolation of the lipopolysaccharide

P. mirabilis O38, strain PrK 64/57 was obtained from the Czech National Collection of the Type Cultures and cultivated in a fermenter (Chemap AG, Switzerland) in nutrient broth (BTL, Poland) under the controlled aerobic conditions (37 °C, pH 7.4–7.6, 11 L min^{-1} oxygen). The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with water and lyophilised. The lipopolysaccharide was isolated from dried cells by the phenol–water method[16] and purified by precipitation with CCl_3CO_2H as described.[17]

3.2. Degradation of the lipopolysaccharide

The lipopolysaccharide (180 mg) was hydrolysed with aq 2% HOAc at 100 °C for 3 h, and a lipid precipitate was removed by centrifugation at 13,000*g* for 20 min. The carbohydrate portion was fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer (pH 4.5) with monitoring by a Knauer differential refractometer to give a high-molecular-mass polysaccharide (48 mg) and three oligosaccharide fractions (9.9, 19.9 and 46.9 mg in the order of the elution).

3.3. Composition analyses

The polysaccharide was hydrolysed with 2 M CF_3COOH (120 °C, 2 h). Neutral monosaccharides were analysed as the alditol acetates by GLC using a Hewlett–Packard 5989A instrument equipped with an HP-5 column and a temperature gradient 150 °C (3 min) → 320 °C at 5 °C min^{-1}. GalA was identified using a Biotronik LC-2000 sugar analyser on an Chromex DA × 8-11 column at 70 °C in 0.04 M KH_2PO_4 buffer, pH 2.4. Amino components were identified using a Biotronik LC-2000 amino acid analyser on a column (0.4 × 22 cm) of Ostion LG AN B cation-exchange resin at 80 °C in 0.2 M sodium citrate buffer, pH 3.25, for amino acids and 0.35 M sodium citrate buffer, pH 5.28, for amino sugars and ethanolamine.

3.4. Methylation analysis

Methylation of the polysaccharide was performed with CH_3I in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide.[18] Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in sugar analysis, reduced with $NaBH_4$, acetylated and analysed by GLC–MS on a Carlo Erba Fractovap 4200 chromatograph equipped with a Finnigan MAT ITD-700 mass spectrometer, using a temperature gradient of 150 °C (1 min) to 280 °C at 5 °C min^{-1}.

3.5. Dephosphorylation

The polysaccharide (10 mg) was dephosphorylated with aq 48% HF (0.2 mL) at 4 °C for 24 h in a plastic tube, the solution was lyophilised using a trap with solid NaOH to absorb HF, and the product was subjected to GPC on a column (90 × 2.5 cm) of TSK HW-40 in aq 1% HOAc with monitoring by a Knauer differential refractometer to give a dephosphorylated polysaccharide (4.5 mg).

3.6. Carboxyl reduction

The polysaccharide (7.5 mg) in 'magic methanol' (a mixture of MeOH, chloroform and concd HCl, 10:1:1 v/v/v, 15 mL)[9] was stirred for 7 days at 20 °C and then evaporated. MeOH (1.5 mL) was added to the product and evaporated to remove HCl; the procedure was repeated three times. Then, imidazole buffer, pH 7.0 (2 mL, 1 M) was added, the mixture was cooled to 0 °C, supplemented with 400 mg $NaBH_4$, stirred for 1.5 h at 0 °C and neutralized with concd HOAc. After evaporation of MeOH, the products were fractionated by GPC on Sephadex G-50 as described above to give a carboxyl-reduced polysaccharide (6.5 mg).

3.7. Solvolysis with anhydrous HF

The polysaccharide (27 mg) was treated with anhyd HF for 1 h at 20 °C. After evaporation of HF, the products were dissolved in water and fractionated by GPC on TSK HW-40 in 1% HOAc to give four oligosaccharides in yields 4.0, 5.0 (disaccharide **1**), 1.8, and 0.9 mg in the order of elution.

3.8. Solvolysis with triflic acid

The polysaccharide (33 mg) was treated with anhyd triflic acid (0.2 mL) for 2 h at 20 °C. After neutralisation with 5% aq ammonia and evaporation, the products were fractionated by GPC on TSK HW-40 in water to give monosaccharide **2** contaminated with other low-molecular-mass products (8.5 mg), which was reduced with $NaBH_4$ in water and, after acidification with concd HOAc, phosphorylated amino alditol **3** (2.5 mg) was isolated by GPC on TSK HW-40 in 1% aq HOAc.

3.9. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying three times from D_2O and then examined as solutions in 99.96% D_2O at 50 °C. Experiments in an 17:3 H_2O–D_2O mixture were performed at 58 °C. Internal acetone (δ_H 2.225, δ_C 31.45) and external 85% aq H_3PO_4 (δ_P 0) were used as references. Spectra were recorded on a Bruker DRX-500 MHz spectrometer (Germany) equipped with an SGI INDY computer workstation. 2D NMR spectra were obtained using standard Bruker software, and XWINNMR 2.6 program (Bruker) was used to acquire and process the NMR data. The parameters used for 2D NMR experiments were essentially the same as described previously.[19] A mixing time of 200 ms was used in TOCSY and ROESY experiments, and a delay of 60 ms in an HMBC experiment.

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