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Structure of the O-polysaccharide of *Aeromonas hydrophila* O:34; a case of random O-acetylation of 6-deoxy-L-talose

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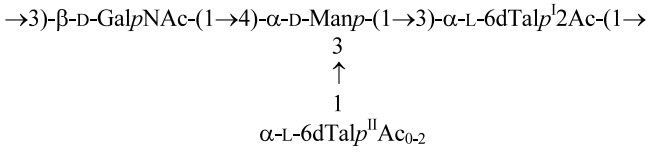
Abstract

The O-polysaccharide of *Aeromonas hydrophila* O:34 was obtained by mild-acid degradation of the lipopolysaccharide and studied by chemical methods and NMR spectroscopy before and after O-deacetylation. The polysaccharide was found to contain D-Man, D-GalNAc and 6-deoxy-L-talose (L-6dTal), and the following structure of the tetrasaccharide repeating unit was established:



where 6dTal[I] is O-acetylated stoichiometrically at position-2 and 6dTal[II] carries no, one or two O-acetyl groups at any positions.

Keywords: Lipopolysaccharide; O-Polysaccharide; Bacterial polysaccharide structure; O-Acetylation; 6-Deoxy-L-talose; *Aeromonas hydrophila*

1. Introduction

The ubiquitous water-borne bacteria *Aeromonas* are significant pathogens of poikilothermic animals, including amphibians, fish, and reptiles.[1] Mesophilic aeromonads are most frequently associated with gastroenteritis and also cause bacteraemia and septicaemia in healthy and immunocompromised humans.[2,3] The three main pathogenic mesophilic aeromonads are *A. hydrophila* (HG1 and HG3), *A. veronii* biovar sobria (HG8/10), and *A. caviae* (HG4), which account for 85% of all clinical specimens. However, the role as gastrointestinal pathogens of many *Aeromonas* strains isolated from faeces remains controversial on account of the lack of a thoroughly investigated outbreak or a good animal model.[3]

A varied clinical picture of *Aeromonas* infections, including gastroenteritis, suggests complex pathogenic mechanisms. The lipopolysaccharide (LPS) plays an important role in these mechanisms, e.g., being involved in adhesion to epithelial cells,[4] resistance to nonimmune serum,[5] and virulence.[6] In this paper we report on the structure of the O-polysaccharide chain of the LPS of *A. hydrophila* O:34. Strains of this serogroup are most common among mesophilic *Aeromonas* species,[7] accounting for 26.4% of all isolates, and have been documented as an important cause of infections in humans.[7,8] In addition to clinical specimens,[9] O:34 strains have been recovered from moribund fish.[10]

2. Results and discussion

The LPS was isolated from enzymatically digested cells of *A. hydrophila* AH-3 (serogroup O:34) by phe-

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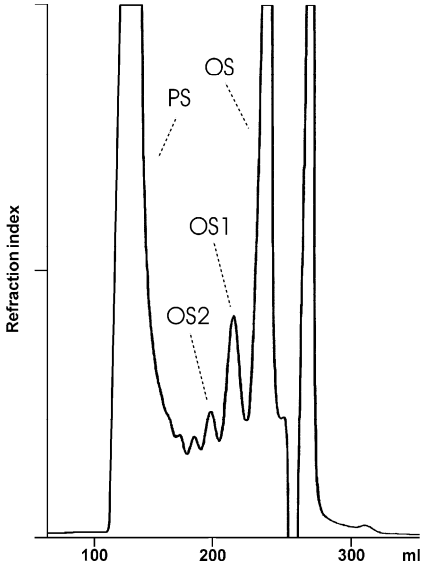


Fig. 1. Sephadex G-50 (S) elution profile of the carbohydrate portion obtained by mild-acid degradation of the LPS. PS, high-molecular-mass polysaccharide; OS, core oligosaccharide; OS1 and OS2, short-chain polysaccharides containing one and two repeating units attached to the core.

nol–water extraction[11] and purified by ultracentrifugation. The carbohydrate portion was released by mild-acid degradation of the LPS at pH 4.4 and fractionated by GPC on Sephadex G-50 (S) to give a high-molecular-mass polysaccharide (PS), an LPS core oligosaccharide (OS), and two intermediate fractions (Fig. 1). The intermediate fractions corresponded to short-chain polysaccharides containing one and two repeating units attached to the core (see below) and were designated as OS1 and OS2 according to the number of the repeating units.

Sugar analysis of the PS, including determination of the absolute configurations of the monosaccharides, revealed 6-deoxy-L-talose (L-6dTal) and D-mannose in the ratios 2.1:1, as well as 2-amino-2-deoxy-D-galactose. There was present also a small amount of glucose, which originated from a contaminating glucan present in a significant amount in the crude LPS.

The ^{13}C NMR spectrum of the PS showed significant structural heterogeneity, most likely, owing to non-stoichiometric O-acetylation (there were signals for *O*-acetyl groups at δ 21.4–21.6). Indeed, the spectrum of the O-deacetylated polysaccharide (DPS) (Fig. 2) was typical of a regular polymer and showed signals for four anomeric carbons at δ 98.0–104.2, two CH_3–C groups at δ 16.7 (2 C, C-6 of 6dTal), one nitrogen-bearing carbon at δ 52.8 (C-2 of GalN), and one *N*-acetyl group (CH_3 at δ 23.3, CO at δ 175.7). Accordingly, the ^1H NMR spectrum of the DPS contained signals for two CH_3–C groups at δ 1.26 and 1.30 (3 H each, d, $J_{5,6}$ 6 Hz, H-6 of 6dTal) and one *N*-acetyl group at δ 2.06 (3 H, s).

These data showed that the polysaccharide has a tetrasaccharide-repeating unit containing two residues of L-6dTal and one residue each of D-Man and D-GalNAc.

The ^1H and ^{13}C NMR spectra of the DPS were assigned using 2D COSY, TOCSY, ROESY, and H-detected ^1H,^{13}C HMQC experiments (Tables 1 and 2). In the TOCSY spectrum, H-1 of Man showed correlations with all protons of the sugar spin system, whereas H-1 of GalNAc and both 6dTal residues (6dTal^{I} and 6dTal^{II}) correlated with protons from H-2 to H-4. The ROESY spectrum revealed H-1,H-2, H-3,H-5, H-4,H-5, H-4,H-6 and H-5,H-6 intraresidue correlations for 6dTal^{I} and 6dTal^{II}, as well as H-1,H-3 and H-1,H-5

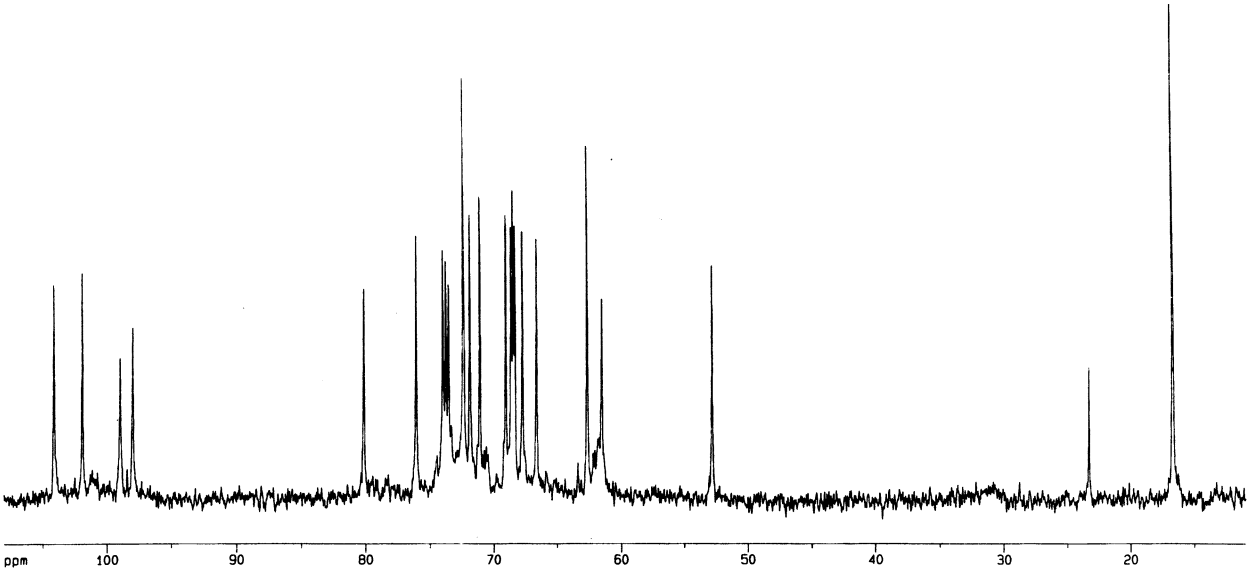


Fig. 2. 125-MHz ^{13}C NMR spectrum of the O-deacetylated polysaccharide (DPS). Region of the CO resonance is not shown.

Table 1
500-MHz ^{1}H NMR data of the DPS (δ, ppm) [a]

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→3)-β-D-Gal*p*NAc-(1→	4.50	4.03	3.77	3.97	3.62	3.74	3.76
→3,4)-α-D-Man*p*-(1→	5.10	4.23	4.10	3.82	4.00	3.74	3.85
→3)-α-L-6dTal*p*^{I}-(1→	5.01	3.91	4.02	3.88	4.15	1.26	
α-L-6dTal*p*^{II}-(1→	5.06	3.89	4.11	3.80	4.68	1.30	

[a] Chemical shift for NAc is δ 2.06.

Table 2
125-MHz ^{13}C NMR data of the DPS (δ, ppm) [a]

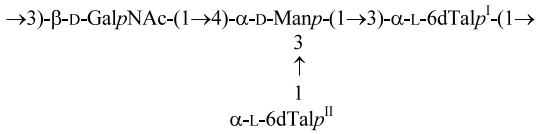
	C-1	C-2	C-3	C-4	C-5	C-6
→3)-β-D-Gal*p*NAc-(1→	101.9	52.8	80.2	68.6	76.1	62.7
→3,4)-α-D-Man*p*-(1→	99.0	67.7	73.8	74.0	72.4	61.5
→3)-α-L-6dTal*p*^{I}-(1→	104.2	68.3	71.8	72.4	69.1	16.7
α-L-6dTal*p*^{II}-(1→	98.0	71.1	66.6	73.6	68.5	16.7

[a] Chemical shifts for NAc are δ 23.3 (CH_3) and 175.7 (CO).

intraresidue correlations for GalNAc. No intraresidue correlation in the ROESY spectrum was observed for H-1 of Man. Coupling constant values estimated from the 2D NMR spectra indicated that all sugar residues occur in the pyranose form.[12] The H-1 intraresidue correlation patterns in the ROESY spectrum (see above) showed that GalNAc is β-linked and both 6dTal residues are α-linked. The α configuration of Man was confirmed by the chemical shifts for H-5 and C-5 compared with the corresponding data of α- and β-mannopyranose.[13]

The ^{13}C NMR spectrum of the DPS showed significant downfield displacements by 2.5–7 ppm of the signals for C-3 and C-4 of Man and C-3 of 6dTal^{I} and GalNAc (Table 2) compared with their positions in the spectra of the corresponding non-substituted monosaccharides.[13,14] Except for the C-1 signal, the chemical shifts for 6dTal^{II} were similar to those in 6-deoxy-α-D-talopyranose.[14] These data showed that the polysaccharide is branched with Man at the branching point and 6dTal^{II} in the side chain.

The ROESY spectrum demonstrated interresidue correlations between the following anomeric protons and protons at the linkage carbons: GalNAc H-1,Man H-3 and H-4; Man H-1,6dTal^{I} H-2 and H-3; 6dTal^{I} H-1,GalNAc H-3; and 6dTal^{II} H-1,Man H-2 and H-3. Taking into account these and the ^{13}C NMR chemical shift data, it was concluded that the DPS has the following structure:



Comparison of the ^{1}H and ^{13}C NMR spectra, as well as the COSY, TOCSY, and ^{1}H,^{13}C HMQC spectra of the initial PS and the DPS, showed that the signals for Man and GalNAc have practically the same ^{1}H and ^{13}C NMR chemical shifts in both polymers, and, hence, they are not O-acetylated in the PS. A downfield displacement of the H-2,C-2 cross-peak of 6dTal*p*^{I} in the F^2 (^{1}H) dimension from δ 3.91/68.3 to 5.11/68.4 was observed in the ^{1}H,^{13}C HMQC spectrum of the PS compared with the spectrum of the DPS, which was due to a deshielding effect of the *O*-acetyl group. The cross-peaks of the neighbouring atoms, H-1,C-1 and H-3,C-3, shifted upfield in the F^1 (^{13}C) dimension from δ 5.01/104.2 and 4.02/71.8 to δ 5.00/101.6 and 4.22/71.2, respectively, which corresponded to the effects of O-acetylation[15] at position-2. The other cross-peaks of 6dTal*p*^{I} changed their positions only insignificantly, and, hence, 6dTal*p*^{I} is mono-O-acetylated at position-2.

6dTal*p*^{II} displayed multiple signals in the NMR spectra of the PS, which belonged to various *O*-acetylated forms. Particularly, while the COSY spectrum showed only one H-5,H-6 cross-peak for 6dTal*p*^{I} at δ 4.19/1.28, there were seven H-5,H-6 cross-peaks for 6dTal*p*^{II}, which formed two series, 1 and 2 (Fig. 3). Series 1 of four peaks contained the H-5,H-6 cross-peak of the non-*O*-acetylated 6dTal*p*^{II} residue at δ 4.67/1.31 (compare with the 6dTal*p*^{II} H-5,H-6 chemical shifts δ 4.68/1.30 in the COSY spectrum of the DPS). Therefore, based on the assumption that the H-5 and H-6 chemical shifts are influenced mostly by an acetyl group at O-4, the three other peaks of series 1 were assigned to the *O*-acetylated forms that do not include the 4-*O*-acetyl

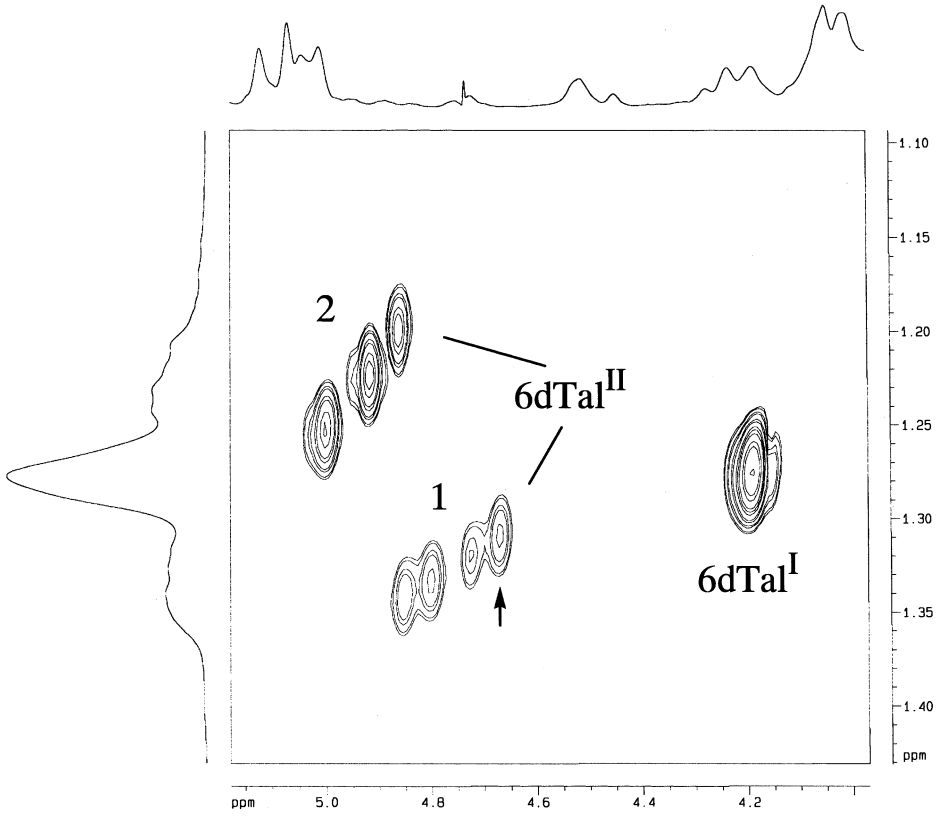


Fig. 3. Part of a 500-MHz COSY spectrum of the polysaccharide (PS) showing 6dTal H-5,H-6 correlations. Two series of cross-peaks for 6dTal^{II}, 1 and 2, were tentatively assigned to the O-acetylated forms that do and do not include the 4-*O*-acetyl group, respectively. Arrow shows the H-5,H-6 cross-peak for nonacetylated 6dTal^{II}.

group, namely, to the 2-*O*-acetylated, 3-*O*-acetylated, and 2,3-di-*O*-acetylated monosaccharides. Correspondingly, the three peaks of series 2 were assigned to the 4-*O*-acetylated, 2,4-di-*O*-acetylated, and 3,4-di-*O*-acetylated forms of 6dTal*p*^{II}.

The O-acetylation pattern was confirmed by studies of low-molecular-mass products of mild-acid degradation of the LPS. The smallest oligosaccharide (OS) contained glucose, galactose, L-*glycero*-D-*manno*-heptose and D-*glycero*-D-*manno*-heptose, which are typical components of the LPS core of many bacteria.[16] Two oligosaccharides eluted from Sephadex G-50 next (OS1 and OS2; Fig. 1) were composed of the same monosaccharides and, in addition, contained 6dTal, Man and GalNAc, i.e., all components of the PS. Therefore, it was suggested that OS is a core oligosaccharide, and OS1 and OS2 are the core oligosaccharides with one or two repeating units of the PS attached.

The negative-ion mode ESI mass spectrum of the OS showed a compound with the molecular mass 1857.6 Da. The molecular mass 2514.9 Da was determined for the O-deacetylated OS1, which was in agreement with the presence of the core (1857.6 Da) substituted with one non-O-acetylated repeating unit of the polysaccharide (657.3 Da). The mass spectrum of OS1 (Fig. 4) demonstrated three oligosaccharides with the molecular masses 2556.9 (minor), 2598.9, and 2640.9 (major) Da, which corresponded to the presence of one, two, and three *O*-acetyl groups, respectively. No non-O-acetylated oligosaccharide was present, and an oligosaccharide with four *O*-acetyl groups occurred in negligible

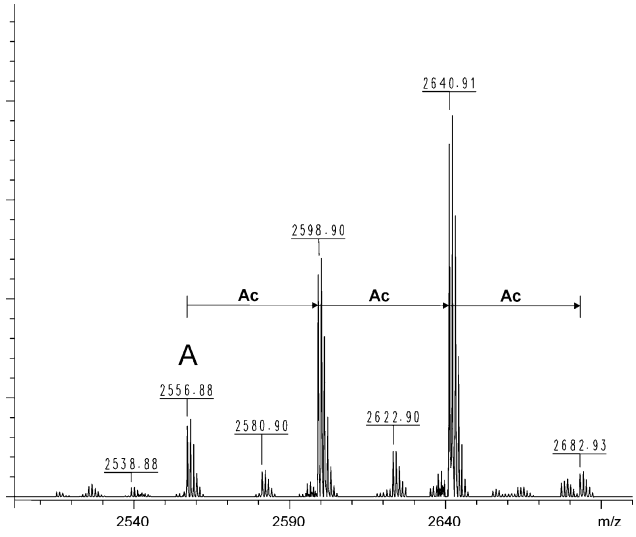
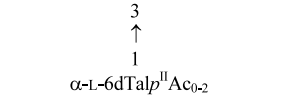


Fig. 4. Charged deconvoluted ESI mass spectrum of OS1. **A** corresponds to the core oligosaccharide with one mono-O-acetylated repeating unit of the polysaccharide attached.

amount. The mass spectrum of OS2 showed a mixture of oligosaccharides containing two repeating units and from three to six *O*-acetyl groups and having molecular masses 3298.2 (minor), 3340.2, 3382.2 and 3424.2 Da. Again, no significant amounts of lower- or higher-acetylated compounds were present.

Based on these data, it was concluded that the *O*-polysaccharide of *A. hydrophila* O:34 has the following structure:



where 6dTal^{I} is O-acetylated stoichiometrically at position-2 and 6dTal^{II} carries no, one or two *O*-acetyl groups at any positions.

Although less common than L-rhamnose and L-fucose, 6-deoxy-L-talose occurs in a number of bacterial polysaccharides[14,17–26] and is often present in an O-acetylated form.[14,17,19–21,24] However, to the best of our knowledge, random O-acetylation has not been reported for either this or another monosaccharide component of the lipopolysaccharides.

3. Experimental

Growth conditions and isolation of the lipopolysaccharide.—*Aeromonas hydrophila* O:34, strain AH-3 was routinely grown on tryptic-soy-broth at 30 °C,[27] i.e., under conditions resulting in a better yield of the LPS compared to cultivation on agar.[6]

Cells (5 g dried weight) were digested with DNAse, RNAse (24 h, 1 mg/g each) and Proteinase K (36 h, 1 mg/g) in 25 mM Tris·HCl buffer containing 2 mM $CaCl_2$ pH 7.63 (10 mL/g). The suspension was dialysed against distilled water and freeze-dried. Digested cells (620 mg) were extracted with 45% aq phenol at 68 °C,[11] the extract was dialysed against tap water without separation of the layers, and, after removal of residual cells by centrifugation, freeze-dried to give a crude LPS (360 mg), which was purified from a contaminating glucan by ultracentrifugation (13,000*g*, 1 h).

Isolation and O-deacetylation of the O-polysaccharide.—The LPS (100 mg) was degraded with 0.1 M NaOAc buffer, pH 4.2, for 4 h at 100 °C, the lipid precipitate was removed by centrifugation (13,000*g*, 20 min), and the supernatant was applied to a column (50 × 2.5 cm) of Sephadex G-50 (S). GPC was performed using pyridinium acetate buffer, pH 4.5 (4 mL pyridine and 10 mL HOAc in 1 L water), with monitoring by a Knauer differential refractometer (Fig. 1). The isolated products were a high-molecular-mass O-polysaccharide (PS, 35.4 mg), a core oligosaccharide (OS, 9.6 mg), and two intermediate fractions (OS1 and OS2, 2.8 and 1.5 mg, respectively).

O-Deacetylation of the PS and OS1 was performed by heating with 12% aq ammonia (2 and 1 mL, respectively) for 24 h at 22 °C, followed by GPC on a column (80 × 1.6 cm) of TSK HW-40 in water.

Sugar analysis.—For sugar analysis, the PS was hydrolysed with 2 M CF_3CO_2H for 2 h at 100 °C. The monosaccharides were conventionally converted into the alditol acetates[28] and analysed by GLC on a Hewlett–Packard HP 5890 Series II chromatograph equipped with a 30-m fused-silica SPB-5 column (Supelco) using a temperature gradient 150 °C (3 min) → 320 °C at 5°/min. In order to confirm the identity of Man and 6dTal, a portion of the polysaccharide hydrolysate was analysed on a Biotronic LC-2000 sugar analyser equipped with a column (13 × 0.4 cm) of a Dionex DA × 8 resin in 0.4 M sodium borate buffer pH 8 at 70 °C. 6-Deoxy-L-talose from the homopolysaccharide of *Burkholderia plantarii* DSM 6535[17] was used as the reference. Absolute configurations of the monosaccharides were determined by GLC of the acetylated (*R*)-2-octyl glycosides.[29]

NMR spectroscopy.—NMR spectra were obtained on a Bruker DRX 500 spectrometer using standard Bruker software at 40 °C in 99.96% D_2O. Prior to the measurements, samples were deuterium-exchanged by freeze-drying twice from 99.9% D_2O. A mixing time of 200 and 100 ms was used in 2D TOCSY and ROESY experiments, respectively. Chemical shifts are referenced to internal acetone (δ_H 2.225, δ_C 31.45).

Mass spectrometry.—ESIMS was performed on an ApexII Fourier transform ion cyclotron resonance mass analyser (Bruker Daltonics, USA) equipped with a 7 T actively shielded magnet and an Apollo electrospray ion source. 50% aq acetonitrile containing 1 mM aq ammonia was used as mobile phase at a flow rate of 0.15 μL/min. Samples were dissolved in a 30:30:0.01 (v/v/v) 2-propanol–water–triethylamine mixture at a concentration of ~20 ng/μL.

Acknowledgements

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