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Structure of the O-polysaccharide from the lipopolysaccharide from *Vibrio cholerae* O6

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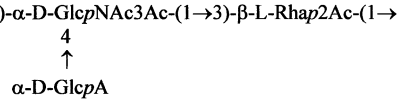
Abstract

The O-polysaccharide from *Vibrio cholerae* O6 was isolated from the LPS by mild-acid hydrolysis and has been investigated by sugar and methylation analysis and NMR spectroscopy. The polysaccharide was also depolymerized with aqueous hydrofluoric acid to give the repeating unit and multiples thereof. The O-polysaccharide had the following tetrasaccharide repeating unit. Two *O*-acetyl groups are present, one of them making the GlcNAc residue fully substituted and the steric crowding considerable at the branching residue.



Keywords: Lipopolysaccharide; O-Antigen; Bacterial polysaccharide; *Vibrio cholerae* diarrhea

1. Introduction

The current serogrouping scheme of *Vibrio cholerae* recognizes 193 different somatic (O) serogroups.[1] The serogrouping of *V. cholerae* is based on the reactivity of specific antibodies with epitopes on the outer membrane cell-wall associated lipopolysaccharide. Although antigenically distinct, the O1 and O139 serogroups of *V. cholerae* are morphologically and biochemically identical to other non-O1, non-O139 *V. cholerae*. What makes the O1 and O139 so special, and different from their non-O1, non-O139 counterparts, is their potential to cause epidemics and pandemics of cholera. The non-O1, non-O139 serogroups, in contrast, do not have the ability to cause epidemics and are usually associated with sporadic cases of diarrhea and sometimes with extraintestinal infections.[2] The reasons why O1 and O139 serogroups are more virulent than their non-O1 non-O139 counterparts is the presence of specific clusters of virulence genes. These include the CTX genetic element, which is the genome of a lysogenic bacteriophage designated CTXϕ[3] that carries the genes encoding cholera toxin, and the TCP pathogenicity island which carries genes for a pilus colonization factor known as toxin coregulated pilus (TCP).[4,5] For the past few years, we have been trying to understand why these mobile genetic elements preferentially home on to the O1 and O139 serogroups but not on the range of other serogroups. While it is known that TcpA is the receptor for the CTXϕ,[3] our structural studies on the LPS of different serogroups of *V. cholerae* are being addressed to understand if any surface associated structural property of the O1 or O139 serogroup make them more permeable or striking to foreign DNA.

The emergence of the O139 serogroup, as a causative agent of epidemic cholera, has prompted monitoring of

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diarrhea in endemic areas caused by non-O1, non-O139 serogroups. Recent studies have shown that the non-O1, non-O139 serogroups are found to be increasingly associated with diarrhea.[6,7] However, the virulence mechanisms of serogroups other than O1 and O139 are poorly understood. The non-O1, non-O139 serogroups seldom produce cholera toxin, other toxins, or virulence factors normally present in the O1, O139 serogroups. Some of them caused isolated cases of diarrhea or minor local outbreaks of cholera-like disease.[8] Particularly, *V. cholerae* O6 was found in several cases of diarrhea in a prospective study in India between 1993 and 1995.[8]

Structures of the O-specific polysaccharides of *V. cholerae* lipopolysaccharides have so far been found to vary significantly from serogroup to serogroup. Structural studies have been performed on the OPS of serogroups O1–O3, O5, O8–O10, O21, O22, O76, O139, O144, and O155, and a number of unique sugars and non-carbohydrate groups have been identified as OPS components (for review see Refs. 9–12). We now report on the structure of the OPS of *V. cholerae* O6.

2. Results and discussion

V. cholerae strain CO638 was grown in rich tryptone medium, isolated by centrifugation and the LPS extracted with the aqueous phenol method. The LPS was degraded with an acetic acid buffer at pH 4.2 to give a high-molecular-mass O-specific polysaccharide (PS) that was isolated by gel chromatography on Sephadex G-50. Sugar analysis of the polysaccharide after hydrolysis with dilute aqueous trifluoroacetic acid and conversion to alditol acetates gave GlcNAc and Rha in the proportions 55:23. In addition, small amounts of Gal, Glc, L-*glycero*-D-*manno*-heptose (LD-Hep), and Rib were found, and anticipated to derive from the core region and RNA. Small amounts of ManN of unknown origin were also detected. Methanolysis gave, in addition to the sugars mentioned above, GlcA. The absolute configurations of GlcN, GlcA, and Rha were determined by GLC of the trimethylsilylated (+)-2-butylglycosides, where it was found that the GlcN and GlcA residues had the D and the Rha residue the L configuration.[13] Methylation analysis revealed that the PS contained 3-substituted rhamnose, and 4-substituted and 4,6-disubstituted glucosamine in the ratios 26:45:29. Smaller amounts of derivatives anticipated to derive from core sugars could also be observed. When the methylated polysaccharide was treated with $LiBH_4$ before conversion to alditol acetates, 2,3,4-tri-*O*-methylglucose was detected, corresponding to a terminal GlcA residue. This demonstrates that the PS is branched with GlcN as the branching point residue and a GlcA as the terminal group, in addition to Rha and GlcN chain residues.

Advantage was taken of the acid lability of the Rha residue in order to fragment the PS and to obtain the repeating unit and multiples thereof. This was possible as the Rha residue was located in the main chain. Thus, on treatment with aqueous 48% hydrofluoric acid for 24 h at room temperature, followed by separation on a column of Sephadex G-50, a series of oligosaccharides were obtained. The smallest oligosaccharide gave on negative ESIMS two peaks at m/z 787.4 and 829.3 corresponding to the oligosaccharides $dHex_1HexNAc_2HexA_1Ac_1$ and $dHex_1HexNAc_2HexA_1Ac_2$, respectively; the former being slightly more abundant. As evident from the data presented below, the repeating unit contains two equivalents of *O*-acetyl groups and thus, a partial O-deacetylation had taken place during the treatment with hydrofluoric acid. Earlier eluted fractions contained dimers, trimers etc. of the repeating unit oligosaccharide, with a varying amount of *O*-acetyl groups on, in general, one equivalent of acetate groups for each repeat was still present. This corresponds to the data obtained from chemical analysis and corroborates the carbohydrate-repeating unit as that given above.

The PS showed a ^{1}H NMR spectrum (Fig. 1) with six signals in the anomeric region at δ 4.36 (7.8 Hz) 5.03 (3.2 Hz), 5.08 (nr), 5.10 (3.2 Hz), 5.30 (m), and 5.59 (nr), all signals corresponding to approximately one proton each, four signals corresponding to *N*- and *O*-acetyl methyl groups at δ 1.96, 2.06, 2.08, and 2.22, and a signal at δ 1.38 corresponding to the methyl group of the rhamnose residue. The two signals at δ 2.08 and 2.22 were assigned to *O*-acetyl groups, as there were only two amino sugars present. Four signals for anomeric carbons were present in the ^{13}C NMR spectrum at δ 94.9, 99.8, 101.5, and 102.3, and could be shown via the C,H-correlated spectrum (see below) to correspond to the proton signals at δ 5.03, 5.08, 5.10, and 4.36, respectively. Consequently, the signals at δ 5.30 and 5.59 should derive from protons on acetoxylated sites. The signal at δ 5.30 was an apparent triplet, and from its complexity and low fields, evidently from an acetoxylated site.

The absence of furanosidic residues was evident from the lack of ^{13}C NMR signals in the region 82–84 ppm.

For the assignment of ^{1}H NMR spectra of the PS ^{1}H,^{1}H-correlated COSY, along with various TOCSY experiments were performed. Proton decoupled proton–carbon correlated (HSQC/HMQC) experiments were performed in addition to long-range proton–carbon correlated (HMBC) experiments for the assignment of carbon signals. For most of the residues, it was in general, possible to obtain proton assignments for the whole spin system from the COSY spectrum; an exception was the rhamnose residue where two signals overlapped. The four residues were designated **A**–**D** after a decreasing chemical shift of their anomeric proton signals and the data are given in Table 1.

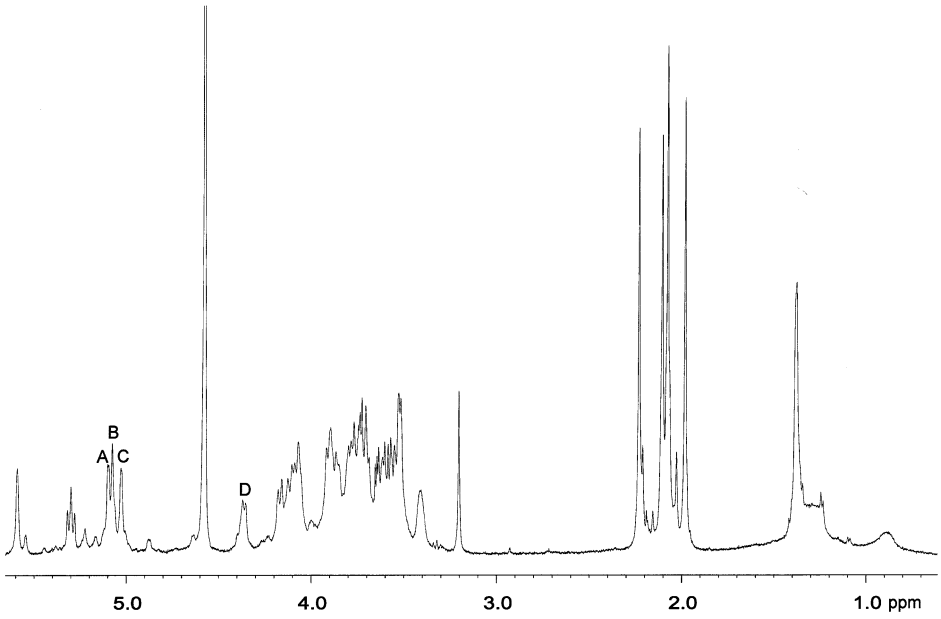


Fig. 1. ^{1}H NMR spectrum (45°, 500 MHz) of the O-polysaccharide of *V. cholerae* O6. **A**–**D** refer to anomeric protons as described in the text.

Residue **A** (δ_{H-1} 5.10) was assigned to a uronic acid residue from the presence of five proton resonances only. Large couplings H-1/H-2 and H-4/H-5, indicated the gluco configuration. That it is terminal was evident from the similarity between the carbon chemical shifts and those of the monomeric GlcA, and supported by the methylation data. The carboxyl function was corroborated from a correlation between H-5 and C-6 in the HMBC spectrum. That the acid was in the free form was evident from spectra obtained at different pH where a chemical shift displacement for C-6 had occurred.

Residue **B** (δ_{H-1} 5.08) was assigned to the rhamnopyranose residue, as the $J_{1,2}$ was small. The anomeric configuration was β as determined from the chemical shift of the H-5 signal, which appears at δ 3.52, a value similar to that of β-Rha but different to that of α-Rha. The NOESY spectrum (see below) also showed correlations from H-1 to H-3 and to H-5, only possible for the β anomer. The δ_{C-3} value, 76.7, is shifted approximately

Table 1
^{1}H and ^{13}C NMR data for the O-antigen polysaccharide for *V. cholerae* O6

Sugar residue	Chemical shifts[a] (δ)						
	1	2	3	4	5	6a	6b
α-Glc*p*A-(1→	5.10 [3.2][a]	3.56	3.60	3.53	4.08		
A	101.5	~71.5	73.1	72.2	73.2	175.1	
→3)-β-Rha*p*2Ac-(1→	5.08 [n.r.][b]	5.59	3.86	3.52	3.52	1.38	
B	99.8	70.0	76.7	~71.3	73.1	17.5	
→6)-α-Glc*p*NAc3Ac-(1→	5.03 [3.2]	4.08	5.30	3.91	4.17	3.72	4.11
C 4 ↑	94.9	52.4	73.9	76.4	69.8	68.2	
→4)-β-Glc*p*NAc-(1→	4.36 [7.8]	3.77	3.61	3.71	3.40	3.79	3.90
D	102.3	56.3	74.3	78.0	75.0	61.5	

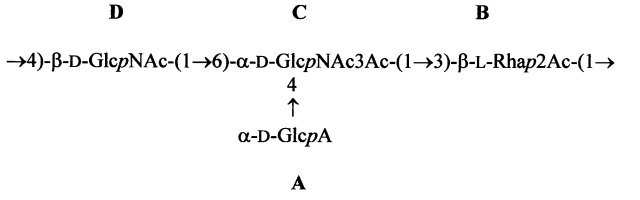
[a] $J_{1,2}$ values are given in brackets.
[b] n.r., not resolved.

3 ppm from that of the corresponding value of the monomer, which is low. This low value is consistent with a glycosidic linkage of a particular type, namely in which H-1 in GlcNAc and H-2 in Rha are in close contact, a so-called γ-gauche interaction.[14] For this type of linkage a small glycosylation shift is always observed. The glycosylation shift is however large enough to show that the point of substitution is C-3. Furthermore, a large negative shift is expected for the signal from the carbon with the equatorial hydrogen, i.e., position 2. This value is counterbalanced with the relatively large positive shift of the acetate substitution at position 2, resulting in an overall displacement of −2.2 ppm. That O-2 carries an acetyl group was evident from the large chemical shift displacements for the H-2 signal, 1.66 ppm, as compared to the monomer.

Residue **C** (δ_{H-1} 5.03) was assigned to a 4,6-disubstituted 2-acetamido-2-deoxy-α-glucose residue, from the $J_{1,2}$ value of 3.2 Hz and that large couplings were observed for all ring proton signals. Substituted positions in the residue were indicated from the large glycosylation shifts, 5.1 and 6.4 ppm, for the C-4 and C-6 signals, respectively, when compared to α-D-GlcNAc, respectively. A displacement of 2.2 ppm for the C-3 signal is large but cannot correspond to a glycosylation shift, the corresponding ^{1}H NMR displacement, 1.55 ppm, clearly demonstrates O-acetylation, however. The upfield shift for the C-2 signal is in accord with what is expected for O-acetylation.[15]

Residue **D** (δ_{H-1} 4.36) was assigned to a 4-substituted 2-acetamido-2-deoxy-β-glucose residue, from the $J_{1,2}$ value and that large couplings were present for all ring proton signals. A large glycosylation shift for C-4 signal (at δ 78.4) indicated the substituted carbon.

A NOESY experiment of the PS revealed H-1/H-3 and H-1/H-5 intraresidue correlations in residues **B** and **D** further showing their configurations as β. The following three inter-residue correlations between H-1 and linkage protons were also observed: **A** H-1/**C** H-4, **B** H-1/**D** H-4, **C** H-1/**B** H-3, **D** H-1/**C** H-6a, and **D** H-1/**C** H-6b. This is in accord with the methylation analysis data and with the following repeating unit



In addition, an NOE correlation was observed, **C** H-1/**B** H-2, which is the result of the spatial relationship between residues **C** and **B** where H-2 is equatorially linked in **B** and in position for the above mentioned γ-gauche interaction with H-1 in **C**. This also verifies the absolute configuration of these residues as DL (or vice versa).

Additional data on the sequence was obtained from long range C–H-correlation spectra, HMBC, in which the following correlations from anomeric protons to linkage carbons were observed: **B** H-1/**D** C-4, and **D** H-1/**C** C-6, and from the anomeric carbon to protons on linkage carbons: **A** C-1/**C** H-4, and **B** C-1/**D** H-4, corroborating the NOE data.

The O-polysaccharide of *V. cholerae* O6 has a unique structure that bears little resemblance with any of the capsular or the lipopolysaccharide present in O1 and O139. It bears resemblance with other non-O1 serotypes in that it is rich in aminosugars and in addition has a source of acidity, GlcA.

3. Experimental

Bacterium, cultivation and isolation of lipopolysaccharide and O-specific polysaccharide.—*V. cholerae* O6, strain CO638 is a clinical isolate from a patient with diarrhea.[8] The strain was grown in a rich tryptone-yeast extract in a 30-L fermentor (Belach AB, Sweden). The lipopolysaccharide was isolated by extraction of bacterial cells with hot aq phenol. The crude LPS from phenol extraction (210 mg) in 0.1 M Tris-buffer of pH 4.2 with 10 mM potassium chloride, RNAse (10 mg), and DNAse (6 mg) was kept at 30 °C for 17 h. Proteinase (125 μL, 10 mg/mL) was added to the solution, and it was kept at 40 °C overnight. The solution was centrifuged, and the supernatant with LPS was concentrated under vacuum to a small volume. The purified lipopolysaccharide was degraded with 0.1 M AcOH–AcONa, pH 4.2, for 4 h at 100 °C. After centrifugation to remove the lipid, the O-specific polysaccharide was purified by gel-permeation chromatography on a column (70 × 2.6 cm) of Sephadex G-50 using 0.05 M pyridinium acetate, pH 4.5, as eluent and monitoring with a differential refractometer.

Chemical analyses.—For sugar analysis, alditol acetates were prepared by hydrolysis of the polysaccharide by treatment with 2 M TFA at 120 °C for 2 h, reduction with $NaBH_4$, and acetylation. For GLC, a Hewlett–Packard 5890 instrument fitted with a flame-ionization detector was used. GLC-MS (EI) was performed on a Hewlett–Packard 5890 Nermag R10-10H quadrupole GC-MS. Separations of alditol acetates were performed on a DB-5 capillary column (30 m × 0.25 mm) using a temperature programme 130 °C (1 min) → 250 °C at 3 °C/min.

For methylation analysis, methylation was performed with methyl iodide in the presence of sodium methyl sulfinyl methanide and the methylated products were purified using Sep-Pak C_{18}-cartridges.[16].Hydrolysis was performed in 2 M TFA at 120 °C for 2 h and the monosaccharides were reduced and acetylated. The par-

tially methylated alditol acetates obtained were separated on a DB-5 capillary column (25 m × 0.20 mm), using a temperature program 130 °C (1 min) → 250 °C at 3 °C/min. The same conditions were used for MS. The absolute configurations of the rhamnose residues were determined by GLC of the trimethylsilylated (+)-2-butyl glycosides.[13]

NMR spectroscopy.—^{1}H and ^{13}C NMR spectra were recorded with a Varian Unity 600 spectrometer, using standard pulse sequences. Spectra of D_2O solutions (99.97%) were recorded at 45 °C. Chemical shifts are reported in ppm, using sodium 3-trimetylsilylpropanoate-d_4 (δ_H 0.00) or acetone (δ_C 31.00) as internal references. Chemical shifts were taken from 1D spectra when possible, or else from ^{1}H,^{1}H-correlated 2D NMR spectra, i.e., ^{1}H,^{1}H-COSY, relayed COSY, or ^{1}H,^{1}H-TOCSY (40, 80, and 120 ms spin lock times). The mixing time in the NOESY experiment was 100 and 300 ms. The $J_{1,2}$ values were obtained from the 1D spectra. Proton–carbon correlated spectra (HSQC/HMQC) were obtained with decoupling, and the long-range proton–carbon correlated (HMBC) spectra were performed using a delay time of 42 and 59 ms. In general, the following working order was used to assign the individual chemical shifts in each sugar residue. Starting from the anomeric proton signals in the 1D ^{1}H NMR spectra, ^{1}H,^{1}H-correlated COSY was used to determine the chemical shift of the H-2–H-4 signals. If overlapped, H-3 was obtained from the 40 ms spin lock time TOCSY experiment, and H-4 signals from the 80 ms TOCSY experiments.

Partial acid hydrolysis of the PS.—The PS was kept in 48% aq hydrofluoric acid (2 mL) at 22 °C for 18 h. It was taken to dryness by evaporation, dissolved in water, and the last traces of HF neutralized with ammonia and subsequently purified by gel filtration on a column of Sephadex G-50, monitoring with an RI detector.

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