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STRUCTURAL STUDIES OF THE *Vibrio cholerae* O-ANTIGEN

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ABSTRACT

The dominant part of the O-antigen of *Vibrio cholerae* is a homopolysaccharide composed of (1→2)-linked 4-amino-4,6-dideoxy-α-D-mannopyranosyl (perosaminyl) residues, the amino groups of which are acylated by 3-deoxy-L-*glycero*-tetronic acid. Most of the amino sugar is decomposed during acid hydrolysis. Treatment of the polymer with anhydrous hydrogen fluoride, which cleaves the glycosidic linkages but does not cause *N*-deacylation, followed by acid hydrolysis under mild conditions, produced the monomer in good yield. Treatment of the *N*-deacylated polysaccharide with nitrous acid caused deamination with concomitant rearrangements, typical of 4-amino-4-deoxyhexopyranosyl residues in which the amino group occupies an equatorial position.

INTRODUCTION

The lipopolysaccharide (LPS) from *Vibrio cholerae* contains a lipid A region[1] which is closely related to that present in Enterobacteriaceae[2]. 4-Amino-4-deoxy-L-arabinose[3], 4-amino-4,6-dideoxy-D-mannose[4] (perosamine), 2-amino-2,6-dideoxy-D-glucose (quinovosamine), D-glucose, D-fructose, and heptose[5] have also been detected, and these sugars should be components of the core and/or O-antigen part of the LPS. An unusual feature is that this LPS does not contain 3-deoxy-D-*manno*-octulosonic acid (KDO).

We now report on studies of the *V. cholerae* LPS, mainly of its O-antigen. Some of the results have been reported in a preliminary communication[6]. Another recent communication[7] on the structure of this O-antigen will be discussed below.

RESULTS

Most of the studies were performed with the LPS from *V. cholerae* serotype Inaba, but LPS from the classical serotype Ogawa and its biotype El Tor were also investigated. On treatment of the LPS with 1% acetic acid at 100° for 7 h, it was

split into lipid A and a polysaccharide. The latter was further separated into two fractions of $\overline{M}_w$ 9000 (major) and $\overline{M}_w$ 900 (minor), respectively. D-Fructose was also released. The fractionation and the $\overline{M}_w$ determination[8] were performed by gel filtration.

Glucose (8%) and a heptose (6%), indistinguishable from L-*glycero*-D-*manno*-heptose in g.l.c. of its alditol acetate, were released on acid hydrolysis of the polysaccharide (PS) fraction. No quinovosamine was found, but a small peak at δ 1.34 [broad (C-CH_3)] in the ^{1}H-n.m.r. spectrum of the PS may indicate the presence of this sugar. No signals for *N*-acetyl groups were observed in the ^{1}H-n.m.r. spectra of these fractions. As will be discussed below, the main component of the PS, perosamine, was not detected by sugar analysis under these conditions. Under more drastic conditions of hydrolysis, most of the perosamine released is decomposed and the solution turns black.

The ^{13}C-n.m.r. spectrum of the PS (Table I) showed 10 strong and several weak signals, as expected for an O-antigen having a simple, regular structure linked to a core. The signals at δ 18.8, 38.1, 55.1, 60.1, 102.7, and 179.1 could, by their chemical shifts and with the aid of an off-resonance decoupled spectrum, be tentatively assigned to -C-CH_3, -C-CH_2-C, >CH-NRR', -C-CH_2OH, C-1 of a glycosyl residue, and >C = O, and the four remaining signals to >CH-OR carbons. In the 300-MHz, ^{1}H-n.m.r. spectrum, signals for a methyl group (δ 1.20, unresolved, 3 H), a methylene group (δ 1.88, m, 1 H; and δ 2.06, m, 1 H), and an anomeric proton (δ 5.18, *J* low, 1 H) were observed. The reason for the low coupling-constant for the methyl group will be discussed below.

The polysaccharide is unusually resistant to acid hydrolysis, as observed by

TABLE I

^{13}C-N.M.R. SHIFTS OF THE ORIGINAL AND MODIFIED *V. cholerae* PS AND SODIUM 3-DEOXY-L-*glycero*-TETRONATE

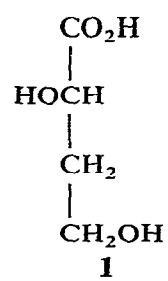
Carbon atom	*Chemical shift*		
	3-Deoxy-L-glycero-*tetronic acid (Na-salt)*	N-*Deacylated PS*[a]	*PS*[a]
C-1		102.5	102.7
C-2		78.7	79.2
C-3		68.3[b]	69.7
C-4		56.3	55.1
C-5		67.9[b]	70.2
C-6		18.8	18.8
C'-1	182.8		179.1
C'-2	71.0		71.4
C'-3	37.7		38.1
C'-4	59.8		60.1

[a]The spectrum was obtained at 85°. [b]Assignments may be reversed.

Redmond[4]. After treatment with 0.5M trifluoroacetic acid at 100° for 16 h, a polymeric material, $[\alpha]_D$ +37°, was recovered. It was evident from the ^{13}C-n.m.r. spectrum (Table I) of this material that a C_4 acid had been split off during this treatment and that the remaining polysaccharide was composed of glycosyl residues of an amino sugar derived from a 6-deoxyhexose. The weak signals assigned to the core sugars were absent from this spectrum. The value (176 Hz) for $J_{H\text{-}1,C\text{-}1}$ demonstrates that the anomeric proton is equatorial[9], in agreement with the chemical shift (δ 5.18) for the anomeric proton. When the ^{1}H-n.m.r. spectrum was measured at pD 10, the signal for the hydrogen linked to the same carbon as the amino group was shifted to higher field (δ 2.75, d, d, $J_1 \simeq J_2 \simeq 10$ Hz). The high values of the coupling constants indicate that this hydrogen and its two vicinal neighbours are axial, the situation present in a perosaminopyranosyl residue.

The ^{1}H-n.m.r. spectrum of the *N*-deacylated PS shows the expected coupling constant (6 Hz) of the CH_3-group. The absence of coupling in the native material is probably due to the fact that H-5 and H-4 have approximately the same chemical shift[10] (δ 3.96, broad, 2 H).

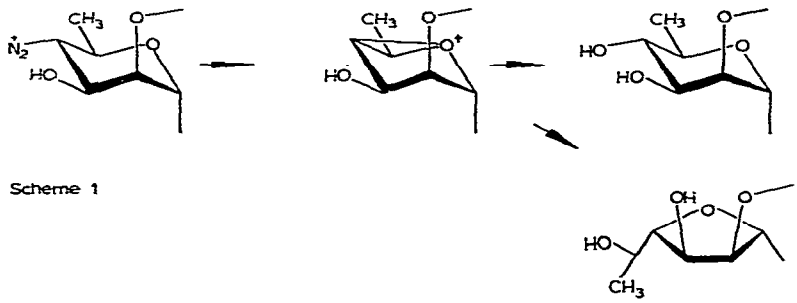
The C_4 acid was isolated from the hydrolysate, and the ^{13}C-n.m.r. spectrum of its sodium salt (Table I) revealed that it contained the hydroxymethyl and methylene groups observed in the original antigen. The 100-MHz, ^{1}H-n.m.r. spectrum of the sodium salt showed signals at δ 1.68–2.04 (m, 2 H), 3.68 (t, *J* 6 Hz, 2 H), and 4.0–4.2 (m, partially obscured by the HDO signal). From these signals, the acid was identified as a 3-deoxytetronic acid (2,4-dihydroxybutanoic acid). The optical rotation of the sodium salt, $[\alpha]_D$ −8°, demonstrates that it has the L-*glycero* (*S*) configuration[11] (**1**). It was indistinguishable from an authentic sample prepared from L-malic acid[12]. The absolute configuration was further confirmed by g.l.c.–m.s. of its ester with (−)-2-octanol, which was identical with the corresponding ester from the synthetic acid, but separated from the ester with (+)-2-octanol.



1

Deamination of the *N*-deacylated polysaccharide and sugar analysis of the product gave rhamnose and 6-deoxyallose, identified by g.l.c.–m.s. of the alditol acetates. These are the expected products of deamination of a 4-amino-4,6-dideoxy-D-mannopyranosyl residue, *via* an intermediate epoxonium ion[13] (Scheme 1). Methylation analysis of the deaminated product gave two main components which were shown, by g.l.c.–m.s. of their alditol acetates, to be a 6-deoxy-3,4-di-*O*-methyl- and a 6-deoxy-3,5-di-*O*-methyl-hexose. The alditol acetate from the former had the same

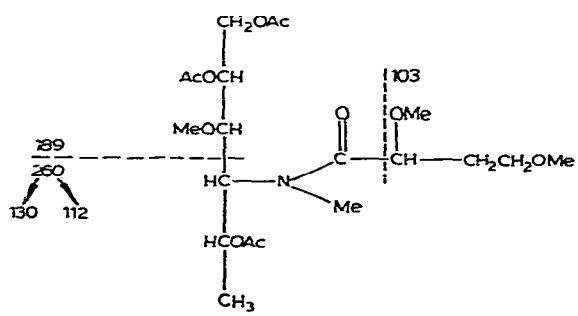
retention time as the rhamnose derivative. The second component is consequently derived from the 6-deoxyallofuranosyl residue. The result therefore supports the assumed mechanism and indicates that the sugar residues in the PS are linked through O-2.



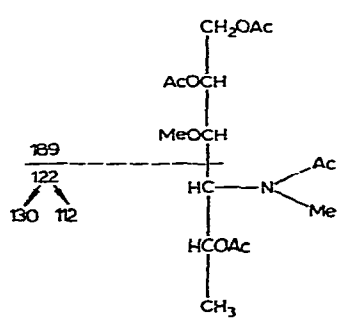
Scheme 1

As already stated, only a low yield of sugars was obtained on acid hydrolysis of the PS. Treatment with anhydrous hydrogen fluoride is known to cleave glycosidic linkages and leave amide linkages intact[14]. Treatment of the PS with this reagent, followed by acid hydrolysis under mild conditions, gave a good yield of perosamine *N*-acylated with 3-deoxy-L-*glycero*-tetronic acid. Similar treatment of the *N*-deacylated and *N*-acetylated polysaccharide yielded *N*-acetylperosamine, $[\alpha]_D$ +20°. The structures were evident from the ^1H- and ^{13}C-n.m.r. spectra and from g.l.c.–m.s. of the alditol acetates.

Methylation analysis of the PS, with hydrolysis of the methylated polysaccharide by treatment with liquid hydrogen fluoride followed by mild, acid hydrolysis, yielded 4-*N*-methyl-3-*O*-methylperosamine, *N*-acylated with 3-deoxy-2,4-di-*O*-methyl-L-*glycero*-tetronic acid. The substance was identified by g.l.c.–m.s. of its alditol acetate **2**. The corresponding *N*-acetyl derivative (**3**) was obtained on deacylation and acetylation of **2**. The sugar residues are consequently (1→2)-linked. In agreement with this the O-antigen is resistant to periodate oxidation but completely oxidised after *N*-deacylation.



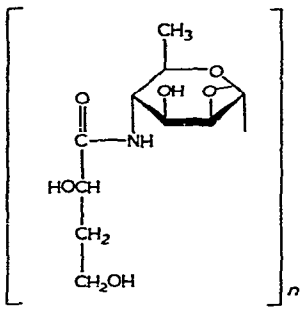
2



3

The ^{1}H-n.m.r. spectrum (with sodium benzoate as internal standard) of the PS showed that the O-specific side-chains amount to ~70% of that fraction. Similar analysis of the saponified LPS (loss of weight on saponification, 30%) gave 60% of O-antigen.

From the foregoing data, it is evident that the main part of the O-antigen of *V. cholerae*, serotype Inaba, is composed of (1→2)-linked 4-amino-4,6-dideoxy-α-D-mannopyranosyl residues, the amino groups of which are acylated with 3-deoxy-L-*glycero*-tetronic acid (**4**).



4

N.m.r. and other studies of the O-antigen from the classical serotype Ogawa and its biotype El Tor indicated that they contain the same O-antigen as serotype Inaba. No O-antigen was present in the $\overline{M}_w$ 900 fraction.

DISCUSSION

The *V. cholerae* LPS contains a lipid A part that is similar to that present in LPS from Enterobacteriaceae[1]. The present results demonstrate that its O-antigen is composed of simple repeating-units, as observed for other Gram-negative bacteria. However, the part connecting lipid A with the O-antigen differs considerably from the cores observed in other Gram-negative bacteria, and is not well defined.

On mild, acid hydrolysis of ordinary LPS, cleavage of the glycosidic linkages of KDO is responsible for splitting the LPS into lipid A and polysaccharide. No KDO is present in the *V. cholerae* LPS and the reason why lipid A is released on similar treatment of this LPS is not known. Fructosyl linkages are cleaved during mild hydrolysis of the *V. cholerae* PS with acid and therefore possibly may be involved.

The formation of two polysaccharide fractions, of $\overline{M}_w$ 9000 and 900, may indicate that two linkages, separated by the "900" fragment, are hydrolysed during the mild, acid hydrolysis. Perhaps a more reasonable hypothesis is that the "9000" fragments contain the O-antigen and the core, and that the "900" fragments consist of "core stubs", derived from incomplete LPS molecules.

A structure for the *V. cholerae* O-antigen has been reported by Redmond[7]. The only difference from the structure reported here is that he assumes that the amino

groups in the antigen are acylated by 3-hydroxypropionic acid. As the ^{13}C-n.m.r. spectra reported by him and by us are identical, this assignment must be due to a misinterpretation.

The polymer with the repeating unit **4** most probably represents the LPS antigen determinant (A) common to Inaba and Ogawa, a conclusion consistent with the results of hemagglutination-inhibition. The natures of the LPS determinants B and C, specific for Ogawa and Inaba, respectively, are still obscure.

EXPERIMENTAL

General methods. — Concentrations were performed under reduced pressure at bath temperatures below 40°. G.l.c. was performed on glass columns (190 × 0.15 cm) containing (*a*) 3% of OV-225 on Gas Chrom Q (100–120 mesh) at 180° and (*b*) 3% of OV-17 on Gas Chrom Q at 190°, and on (*c*) W.C.O.T. glass-capillary columns (25 m × 0.25 mm) containing SP-1000 at 230°. G.l.c.–m.s. was performed with a Varian MAT 311-SS100 instrument at an ionisation potential of 70 eV. N.m.r. spectra for solutions in D_2O were recorded with a JEOL FX-100 and a Bruker WM 300 spectrometer using external tetramethylsilane (^{13}C) and internal sodium 1,1,2,2,-3,3-hexadeuterio-4,4-dimethyl-4-silapentane-1-sulfonate (^1H) as references.

Preparation of the antigen. — The LPS from *V. cholerae* Inaba 569B, Inaba 35, Ogawa 34, or Ogawa El Tor was isolated from bacterial cells by phenol–water extraction[15]. Most of the RNA was removed by repeated ultracentrifugation. Delipidation was performed by treatment[16] of the LPS (1 g) in aqueous acetic acid (100 ml) of pH 3.1 (~1%) at 100° for 7 h. Centrifugation, extraction with ether, and lyophilisation yielded water-soluble material (600 mg). A PS (395 mg), a fraction (90 mg) of low molecular weight, and a monomeric fraction containing fructose (30 mg) were obtained by gel filtration on a column of Sephadex G-50. The PS had $[\alpha]_D^{22}$ +16° (*c* 1, water). ^1H-N.m.r. data (300 MHz): δ 1.20 (not resolved, 3 H), 1.34 (broad, 0.3 H), 1.88 (m, 1 H), 2.06 (m, 1 H), 3.76 (t, *J* 6 Hz, 2 H), 3.96 (broad, 2 H), 4.17 (broad, 2 H), 4.37 (d, d, J_1 3.5 Hz, J_2 8.5 Hz), and 5.18 (*J* low, 1 H).

For sugar analyses, samples were hydrolysed with 0.5M trifluoroacetic acid at 100° for 16 h. Treatment with anhydrous, liquid hydrogen fluoride was performed at room temperature for 2.5 h followed, after evaporation of the reagent, by hydrolysis[17] with M aqueous acetic acid at 100° for 3 h.

Determination of N-*acylperosamine homopolysaccharide in LPS and PS.* — The LPS (100 mg) was treated with 0.5M sodium hydroxide (10 ml) at room temperature for 16 h. Neutralisation with aqueous hydrochloric acid, dialysis, and gel filtration yielded saponified LPS (72 mg). Solutions of saponified LPS (15.6 mg) and PS (17.6 mg) in deuterium oxide were lyophilised and the residues were dissolved in dimethyl sulfoxide-d_6 (500 µl). A solution of benzoic acid (650 µg) in the same solvent (100 µl) was added. The signals in the regions for aromatic protons, anomeric protons, methylene protons, and C-methyl protons in the ^1H-n.m.r. spectrum were

integrated. From these values, it was concluded that 60% of the saponified LPS and 70% of the PS, respectively, consisted of the homopolysaccharide.

Acidic deacylation of the PS. — The PS (120 mg) was hydrolysed with 0.5M trifluoroacetic acid at 100° for 16 h, and the solution was concentrated to dryness. Gel filtration of the residue on a column of Sephadex G-15 gave, *inter alia*, a polymeric fraction (48 mg) and a fraction (8 mg) of low molecular weight. The polymeric fraction was passed through a column (3 × 0.5 cm) of Amberlite IR-45 (free base), M hydrochloric acid (150 µl) was added, and the solution concentrated. The product had $[\alpha]_D^{22}$ +37° (*c* 0.8, water) and gave the ^{13}C-n.m.r. spectrum recorded in Table I. ^{1}H-N.m.r. spectra were run at pD 6 and 10.

The fraction of low molecular weight had $[\alpha]_D^{22}$ −8° (*c* 0.8, water). The ^{1}H-n.m.r. spectrum of this product was complex; after adjustment of the pD to 8 with deuterated sodium hydroxide, the spectrum described above was obtained. The ^{13}C-n.m.r. spectrum of the sodium salt is given in Table I.

Determination of the absolute configuration of the 2,4-dihydroxybutyric acid. — The acid (isolated as described above) was dissolved in M (−)-2-octanolic hydrogen chloride, and kept at 100° for 5 h. After concentration and acetylation, the product was analysed by g.l.c. (SP-1000, 170°). Similar analyses were performed using authentic L-acid and (±)- and (−)-2-octanol, respectively.

Linkage analysis of the N-*acylperosamine homopolymer.* — The PS (30 mg) was methylated by the Hakomori procedure[18,19], and the product was purified by gel filtration on a column (30 × 1 cm) of Sephadex LH-20 with acetone–chloroform (1:1). Treatment with liquid hydrogen fluoride was performed as described above. G.l.c.–m.s. of the derived alditol acetates using column (*b*) showed that the major component was 4-*N*-methyl-3-*O*-methyl-D-perosamine, *N*-acylated with 3-deoxy-2,4-di-*O*-methyl-L-*glycero*-tetronic acid. The mass spectrum displayed, *inter alia*, the following peaks (relative intensities in brackets): 45(100), 57(10), 70(12), 71(33), 73(24), 85(12), 87(16), 103(56), 112(11), 114(5), 127(6), 129(17), 130(30), 172(5), 189(2), 232(4), 260(12), 344(8), and 374(6). The alditol acetate fraction was treated with 5% methanolic hydrogen chloride at 100° for 16 h, the solution was concentrated, and the residue was acetylated. G.l.c.–m.s. then showed that the main component was the corresponding *N*-acetyl derivative, namely 1,2,5-tri-*O*-acetyl-4,6-dideoxy-3-*O*-methyl-4-*N*-methylacetamido-D-mannitol.

Deamination of the PS. — The PS (15 mg) was hydrolysed with trifluoroacetic acid as described above. The product in water (2 ml) was treated with 33% aqueous acetic acid (3 ml) and 5% aqueous sodium nitrite (3 ml) at 25° for 40 min, and the mixture was then diluted with water (20 ml) and freeze-dried. The product was fractionated on a column of Sephadex G-15. The polymeric fraction (3 mg) had $[\alpha]_D^{22}$ +68° (*c* 0.3, water). A hydrolysate of the polymer contained 6-deoxyallose and rhamnose (2:1), indistinguishable from authentic samples on g.l.c.–m.s. of the alditol acetates.

Methylation analysis of the polymer was performed as described by Jansson *et al.*[19], with analysis by g.l.c.–m.s. on column (*c*). The two main alditol acetates, in

the ratio 1.4:1, had retention times 0.63 (6-deoxy-3,5-di-*O*-methylallitol) and 0.88 (3,4-di-*O*-methylrhamnitol), respectively, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on the SP-1000 column.

Periodate oxidation. — The PS (10 mg) in 25mM sodium metaperiodate (5 ml) was kept in the dark at room temperature and the consumption of periodate was followed spectrophotometrically[20]. After 8 h, when the consumption was complete, 3.3 μmol of periodate per mg of polysaccharide had been consumed.

On similar oxidation of *N*-deacylated[21] material, 5.9 μmol of periodate per mg of PS was consumed. Only 70% of the acyl group had been split off, due to the low solubility of the deacylated PS in the reaction medium.

The oxidised products were reduced with sodium borohydride and recovered by gel filtration. ^{1}H-N.m.r. spectroscopy of the products demonstrated that the O-antigen was virtually unchanged, but that the *N*-deacylated O-antigen had been drastically modified.

Isolation of monomers. — The polysaccharide (120 mg) was treated with liquid hydrogen fluoride followed by mild hydrolysis with acid as described above. The hydrolysate was concentrated to dryness and the residue was fractionated on a column (2.6 × 120 cm) of Biogel P2. The main fraction (58 mg) consisted of perosamine acylated with 2,4-dihydroxybutyric acid; $[\alpha]_{578}^{22}$ −6.5° (*c* 1, water). The ^{1}H-n.m.r. spectrum (100 MHz) showed, *inter alia*, signals for *C*-methyl protons at δ 1.14 and 1.18 (*J* 6 Hz, 3 H), methylene protons at 2.08 (m, 2 H), hydroxymethyl protons at 3.72 (*J* 6 Hz, 2 H), and anomeric protons at 4.78 (*J* 1 Hz, 0.4 H) and 5.10 (*J* 1 Hz, 0.6 H). Similar treatment of the *N*-deacylated and then *N*-acetylated "9000" fraction (12 mg) yielded *N*-acetylperosamine (8 mg), $[\alpha]_{578}^{22}$ +20° (*c* 0.8, water).

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