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Structural studies of the *Vibrio cholerae* O:5 O-antigen polysaccharide

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ABSTRACT

The O-polysaccharide from *Vibrio cholerae* O:5 has been investigated, using NMR spectroscopy as the main method. Fast atom bombardment mass spectrometry (FABMS) studies of fragments obtained on treatment with anhydrous hydrogen fluoride or methanolic hydrogen chloride gave further structural information. Some structural features were also determined by comparison of nuclear Overhauser enhancement (NOE) contacts with calculated H–H distance in different oligosaccharide models. It is concluded that the O-polysaccharide has the following structure, in which D-Qui*p*NAc4NAc is 2,4-diacetamido-2,4,6-trideoxy-D-glucose, and D-Fuc*p*3NX is 3-amino-3,6-dideoxy-D-galactose acylated with a (*R*,*R*)-3-hydroxy-3-methyl-5-oxoproline group.

$$\rightarrow 3)\text{-}\beta\text{-D-Qui}p\text{NAc4NAc-}(1\rightarrow 4)\text{-}\beta\text{-D-Man}p\text{NAcA-}(1\rightarrow$$
$$\begin{array}{c} 3 \\ \uparrow \\ 1 \end{array}$$
$$\alpha\text{-D-Fuc}p\text{3NX}$$

INTRODUCTION

The species *Vibrio cholerae* is divided into ~80 serogroups on the basis of their O-polysaccharides. Asiatic cholera is caused by the O:1 serogroup and similar but less severe diseases are caused by the other serogroups. Structural studies have been performed on the types O:1[1], O:2[2], O:3[3], and O:21[4], and we now report studies of serogroup O:5.

RESULTS AND DISCUSSION

Isolation and hydrolysis of the O-polysaccharide.—Treatment of the crude lipopolysaccharide (LPS) with ribonuclease followed by hydrolysis with aqueous 2% acetic acid and gel chromatography yielded a glucan, possibly glycogen as indicated by NMR spectroscopy, and the O-polysaccharide (PS) eluting at 1.3–1.7

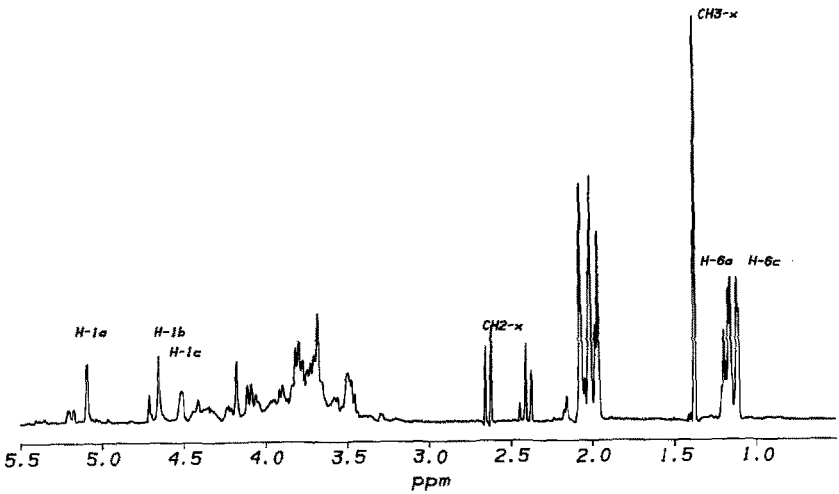


Fig. 1. 500-MHz ^{1}H NMR spectrum of the *V. cholerae* O:5 PS, obtained at 70°C.

void volumes; $[\alpha]_D \sim +140°$. The latter was retained on a DEAE-column, indicating that it was an acidic compound. On treatment with base, the LPS was extensively degraded, as indicated by NMR spectroscopy. Sugar analysis, including hydrolysis with 10 M HCl, yielded 3-amino-3,6-dideoxyhexose, glucose, 2-amino-2-deoxyglucose, and a heptose. The alditol acetate of the heptose had the same retention time as that from L-*glycero*-D-*manno*-heptose. These compounds accounted for ~5% of the dry weight.

NMR characterisation of the PS.—The ^{1}H NMR spectrum of the PS (Fig. 1) showed, *inter alia*, signals for three anomeric protons at 4.52, 4.66, and 5.10 ppm. Three signals for CH_3 groups were present at 1.11 (d), 1.16 (d), and 1.37 (s) ppm. The spectrum further contained three signals for *N*-acetyl groups at 1.98–2.09 ppm and signals for a methylene group at 2.38 and 2.64 ppm, showing only geminal coupling. Small peaks downfield from the signals for the anomeric protons at 4.66 and 5.10 ppm indicated heterogeneity in the O-polysaccharide, or were derived from the core sugars.

The ^{13}C NMR spectrum of the PS (Fig. 2) was weak due to the small amount of material available and the heterogeneity of the sample. In addition to signals for ring carbons, at least four signals for carbonyl carbons at 170–180 ppm and six signals for CH_3 groups at 16–24 ppm were observed. Four signals in the region 52–57 ppm indicated carbon atoms linked to nitrogen. From the DEPT spectrum, it could be concluded that one signal in the spectrum, at 45.4 ppm, belonged to a methylene group, and that a signal at 77.2 ppm belonged to an unprotonated carbon atom. Due to overlap from minor signals, the major signals in the anomeric region were not easily identifiable. From the carbon-decoupled HMQC spectrum, it was clear, however, that there were three. In the coupled spectrum, which was

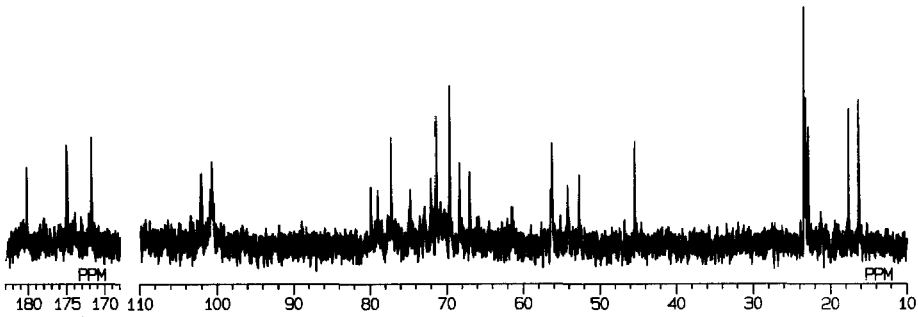


Fig. 2. 67.5-MHz ^{13}C NMR spectrum of the *V. cholerae* O:5 PS, obtained at 70°C.

better resolved (Fig. 3), one signal from an anomeric carbon was weakened due to the suppression of the water signal.

Different 2D NMR experiments were performed in order to identify the components in the PS. The COSY and HOHAHA spectra each contained signals for three spin systems belonging to three sugar residues denoted as **A**, **B**, and **C** (Table I). In addition to these, the signals for a methyl group at 1.37 ppm, for a methylene group at 2.38 and 2.64 ppm, and for a methine group at 4.19 ppm were assigned to an unknown compound (**X**). The HMQC spectrum contained signals for 23 protonated carbons and an HMBC spectrum demonstrated correlations to

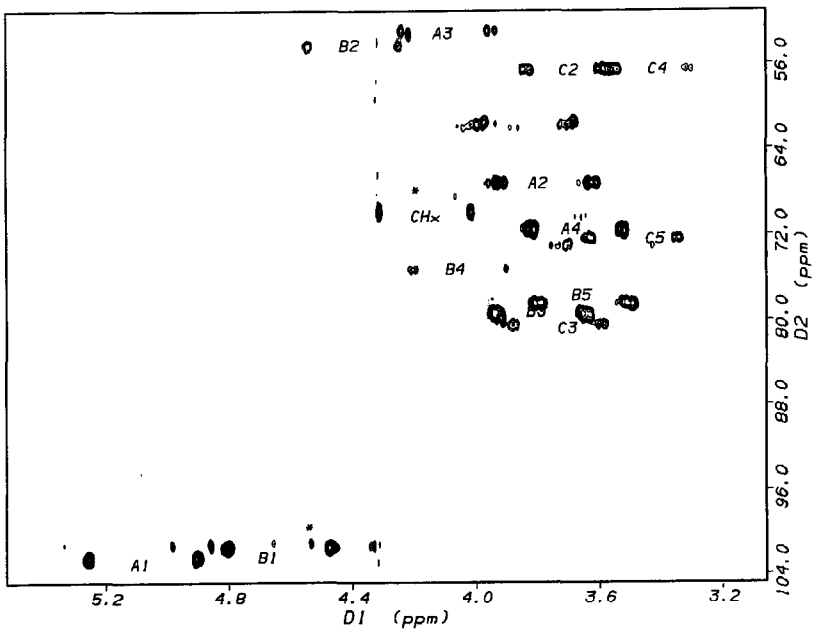


Fig. 3. ^{1}H, ^{13}C-HMQC spectrum of the *V. cholerae* O:5 PS, obtained at 70°C. The signals for C1 and A5 are not clearly visible due to overlap with the suppressed water signal.

five additional carbons, four of which were carbonyl carbons. Three of the carbons could be assigned to *N*-acetyl groups; the carbon at ~172 ppm could not, however, be correlated to any proton. Thus, the PS consists of a trisaccharide repeating unit with two methyl groups, three *N*-acetyl groups, and a non-sugar component, probably an amide-linked carboxylic acid.

Residue **A** was identified as a 6-deoxy sugar having the *α-galacto* configuration as derived from the coupling constants. The signal for C-3 appeared at 52.7 ppm indicating that C-3 was linked to nitrogen. Residue **A** is consequently a 3-amino-3,6-dideoxy-α-D-galactose residue. Small amounts of this sugar were found after acid hydrolysis of the PS. The absolute configuration was determined according to Gerwig et al.[5], by comparison with authentic 3-amino-3,6-dideoxygalactose from the *Pseudomonas maltophilia* 555 LPS[6]. From evidence presented below, it was evident that **X** is linked to the amino group of this sugar.

B was identified as a β-D-Man*p*NAcA residue. The *manno* configuration was evident from the couplings along the ring. The β configuration was derived from the $^{1}J_{C\text{-}1,H\text{-}1}$ value, 164 Hz; in addition, a narrow signal for H-1 indicated a low value of $^{3}J_{H\text{-}1,H\text{-}2}$ and the β configuration. In both the COSY and HOHAHA spectra, a cross-peak was observed from H-1 to a proton giving a signal at 4.41 ppm, assigned to H-2, which correlated to a nitrogen-carrying carbon, giving evidence for a 2-amino group. As three C–N groups remain to be assigned and the PS contains three *N*-acetyl groups, **B** is consequently *N*-acetylated. A strong cross-peak between H-1 and C-2 was also observed in the HMBC spectrum. The lack of correlations from H-5 to any H-6 proton indicated that residue **B** is a uronic acid, and this was further supported by an HMBC correlation between the carbonyl carbon at ~175 ppm and the protons $H\text{-}4_B$ and $H\text{-}5_B$. The chemical shift of the H-5 signal was also pH-dependent. Thus, the PS at pD 2.7 had the chemical shift of that signal displaced 0.07 ppm downfield. Hydrolysis of the carboxyl-reduced ($NaBD_4$) PS gave mannosamine-*6-d*$_2$, as shown by GLC–MS of the corresponding alditol acetate, using an authentic reference. The D configuration was demonstrated from the NOE between H-6 in residue **A** (D-Fuc*p*3NX) and H-2 in residue **B** (Man*p*NAcA), as an NOE would have been unlikely if β-Man*p*NAcA had the L configuration (Table II). The conformation of the disaccharide was obtained from the trisaccharide repeating unit, calculated using the CHARMM force field with modified parameters. It was evident from a low energy conformation that the distance between $H\text{-}2_B$ and $H\text{-}6_A$, was ~3 Å (Fig. 4). A trisaccharide with β-L-Man*p*NAcA instead of β-D-Man*p*NAcA was calculated to have a distance of ~4.9 Å for the same protons. Attempts to use the method by Gerwig et al.[5] were all unsuccessful.

C was identified as a 2,4-diamino-2,4,6-trideoxy-β-D-glucose residue, acetylated at both amino groups. It was evident from the H,C-correlation spectra (HMQC) that two signals at 56.2 and 56.4 ppm were from carbons linked to nitrogen. Two other carbons with signals at 72.0 and 79.9 ppm, and two signals for *N*-acetyl groups could also be assigned to residue **C**. This is in accordance with an

N-acetylated 2,4-diamino-2,4,6-trideoxyhexose residue. The more or less overlapping proton signals of this residue could not, however, be fully resolved even at high field, at different temperatures, or on changing the pH. In the COSY spectrum, correlations were therefore observed between H-1 and H-2/3, between H-2/3 and H-4/5, and between H-4/5 and H-6. The chemical shift difference between the H-2 and H-3 signals was, however, large enough to ascertain that C-2 was a nitrogen-bearing carbon. In the HMQC spectrum, the signals for C-2/H-2 and C-4/H-4 were clearly separated, at ~3.5 and ~3.7 ppm. As no cross-peak for H-1 and H-3 was obtained in a relayed COSY spectrum, it was evident that the amino groups were located on C-2 and C-4, and not C-2 and C-3. That the sugar has the *β-gluco* configuration was derived, *inter alia*, from the fact that the H-1 signal was broad, and appeared at ~4.5 ppm. Also, no NOE was observed between NH-2 and H-4, as would have been expected for a sugar with a *manno* configuration. The observation of a HOHAHA correlation between NH-4 and H-6, which would not have been observed for a sugar with an axial C-4 substituent, further indicates an equatorial substituent on C-4. The ^{13}C NMR chemical shifts of the α and β forms of 2,4-diamino-2,4,6-trideoxy-D-glucose, *N*-acylated with acetic acid and (*S*)-hydroxybutyric acid at positions 2 and 4, respectively, are known[7]; from a comparison of the chemical shift of the C-5 signal, which is 67.7 ppm for the α anomer and 72.1 ppm for the β anomer, and the corresponding value for the signal in the PS, 72.0 ppm, it was deduced that **C** has the β configuration. That it is a 2,4-diamino-2,4,6-trideoxy-*β-gluco* derivative was indicated from the similarity of other signals.

By using sequence information (see below) and the observed NOE between H-3 in **A** and H-6 in **C**, it could be demonstrated that **C** has the D configuration (Fig. 4). The conformation of the trisaccharide element was calculated as described above. The $H\text{-}3_A\text{–}H\text{-}6_C$ distance in the oligosaccharide, if the β-D isomer was used, was ~2.7 Å; if the β-L isomer was used, it was ~11 Å. An FAB-mass spectrum of the disaccharide methyl glycoside containing residues **B** and **C** (see below) further corroborated the presence of a diacetamidotrideoxyhexose residue.

A fourth component, X, was also detected and assigned as a 3-hydroxy-3-methyl-5-oxoproline group primarily from correlations observed in the HMBC spectrum, and from comparison with literature data[8]. The proton signals from X appeared at 1.37, 2.38, 2.64, and 4.19 ppm. No three-bond *J*-coupling could be observed in a COSY spectrum, but in a long-range COSY spectrum optimised for small couplings, there was a correlation between the signals at 1.37 and 2.64 ppm. In the ^{13}C NMR spectrum, the methyl and methine carbon had signals at 23.4 and 69.6 ppm, and a methylene carbon at 45.4 ppm. The HMBC spectrum showed, *inter alia*, that a non-protonated carbon with a signal at 77.2 ppm, which must carry a heteroatom, probably oxygen, correlated to all protons. A correlation from the methyl protons to three different carbons establishes the C-2–C-4 part of the ring. The remaining carbonyl carbon must come next and, as this carbonyl group correlates with a proton at 4.19 ppm, a ring structure must be present and joined

via the last nitrogen. Structure **1** is in accord with these observations, in which also the complete set of HMBC correlations is indicated. That the CO and NH groups are not reversed is evident from the chemical shifts.

Furthermore, an NH-singlet was observed at 8.02 ppm, which had an NOE to CH-X and NH-3_A (Table II). From the latter observation, it is indicated that a CO group bridges the 5-membered ring via an amidic linkage to the Fuc3N residue. The chiralities at atoms 2 and 3 are not evident from this, but the observation of an NOE between the NH proton and H-6 in residue **C** indicates that C-2 has the (*R*) configuration (Fig. 4). The position of the proline ring is defined from a strong NOE between CH-X and NH-3_A, a conformation also supported by the computer calculations. Further evidence for the proline structure is obtained from an FAB mass spectrum of the Fuc3N residue with X amidically linked to it (see below). C-3 must also have the (*R*) configuration since an NOE is observed between CH_3-X and H-4_A. This distance is calculated to be ~ 3.3 Å in the (*R*,*R*)-conformer and ~ 6.5 Å in the (*R*,*S*)-conformer. This further confirms that X is linked to **A**. All attempts to isolate the free acid were unsuccessful.

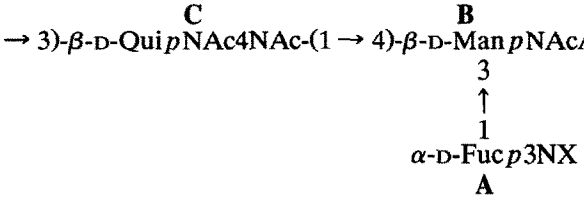
The 3-hydroxy-3-methyl-5-oxoproline component has, to our knowledge, not been found previously. Related substances, namely, 3-hydroxy-2,3-dimethyl-5-oxoproline and 2,4-dihydroxy-3,3,4-trimethyl-5-oxoproline, are components of the LPS of *Pseudomonas fluorescens*[8] and *Vibrio anguillarum* V 123[9], respectively.

Assignment of NH-protons.—NMR signals for NH-protons for a sample of the PS in 90% H_2O at 23°C were observed in the region 7.7 to 8.7 ppm (Fig. 5). A second set of signals with lower intensities was observed, indicating heterogeneity analogous to that observed in the anomeric region. The NH-proton at 8.62 ppm was assigned to NH-3_A, as it showed a cross-peak to H-3_A in the COSY spectrum. The two signals at 8.075 and 8.079 ppm were assigned to NH-2_C and NH-2_B, respectively, and the signal at 7.92 ppm was assigned to NH-4_C for similar reasons. A signal for an NH-proton at 8.02 ppm did not show any cross-peak in the COSY spectrum, but gave a strong correlation in the HOHAHA spectrum to the methine proton of the X residue. This signal was therefore assigned to NH-X. When higher temperatures were used in an attempt to separate the signals for NH-2_B and NH-2_C, the signals for NH-2_B disappeared, probably due to exchange.

Linkage and sequence determination.—Attempted methylation analysis did not yield any detectable derivatives. NMR chemical shifts were therefore used to establish the linkages, and NOESY experiments using different mixing times to determine the sequence of the PS (Table II). Comparison of the ^{13}C NMR chemical shifts for the α-D-Fuc*p*3N residue with literature values[10] for the reducing sugar indicated that this residue was terminal, as the signals from C-2 and C-4 differed by less than 0.5 ppm. The only possible disubstituted sugar is ManNAcA, since residue **C** can only be linked through the 3-position. A strong NOE (Table II) was observed between H-1_A and H-3_B and between H-1_B and H-3_C, thus establishing the disaccharide elements **A**–**B**, and **B**–**C**. An NOE was also observed between H-1_C and H-4_B, thus establishing the element **C**–**B**. An

HMBC correlation was observed between H-1_B and a signal at ~ 80 ppm, but, as the C-3_B and C-3_C resonances are close, no assignment could be done from this observation. The low resolution in f_1 further hampers the interpretation of the HMBC data.

From the combined evidence, the following structure is postulated in which D-Qui*p*NAc4NAc is 2,4-diacetamido-2,4,6-trideoxy-D-glucose, D-Fuc*p*3N is 3-amino-3,6-dideoxy-D-galactose, and X is a 3-methyl-3-hydroxy-5-oxoproline group.



HF-solvolysis of the PS.—The PS was treated with anhydrous HF at −30°C for 1 h. After workup and gel filtration, part of the material was eluted in the void volume, to give polymeric material, and part in the oligosaccharide region, to give oligomeric material. 1D ^{1}H NMR and 2D COSY spectra showed the latter to consist of an α,β-mixture of 3-amino-3,6-deoxy-D-galactose *N*-acylated with the 3-hydroxy-3-methyl-5-oxoproline group (Table III). Due to fast exchange of NH-protons, no spectra with these resonances could be obtained. A negative FAB-mass spectrum showed a peak for $[M - H]^-$ at m/z 303 as required for structure **2**. It has been reported that glycosidic linkages of acylated 2,4-diamino-2,4,6-trideoxyhexoses and 2-aminohexuronic acids are resistant to cleavage at this temperature[11]. The polymeric product, as indicated from NMR spectra, was made up of residues **B** and **C**, with some side chains, and was not further investigated.

Methanolysis of the PS.—Solvolysis of the PS in M methanolic HCl followed by conventional workup yielded a mixture of products which were analysed by positive FABMS. A peak at m/z 514 $[M + Na]^+$ corresponded to the methyl glycoside of a disaccharide containing a ManNacA group and a QuiNAc4NAc residue. A B/E-spectrum, to find daughter ions, yielded, *inter alia*, fragments at m/z 272 (B-cleavage), 283 (B-cleavage), and 311 (D-cleavage) as indicated in formula **3**. Low intensity peaks for the tetramer at m/z 974 and the hexamer at m/z 1433 were also observed in the FABMS spectrum, indicating the resistance to cleavage under the conditions used.

EXPERIMENTAL

General methods.—Solutions were concentrated under diminished pressure below 40°C. Gel filtrations were performed on columns of Sephadex G50 (80 × 2.5 cm) or Bio-Gel P-2 (90 × 1.5 cm) irrigated with 0.07 M pyridine acetate buffer of pH 5.7.

Bacterial strain and cultivation conditions.—The *Vibrio cholerae* serogroup O:5 was obtained from J. Holmgren, University of Gothenburg, Sweden. The strain

TABLE I

^1H and ^{13}C NMR data (δ in ppm, J in Hz) for *V. cholerae* O:5 PS obtained in D_2O at 70°C and in 90% H_2O at 23°C (NH-protons)

Residue	H-1–C-1	H-2–C-2	H-3–C-3	H-4–C-4	H-5–C-5	H-6–C-6 C-CH_3	NH-2	NH-3 NH-4	NAc-2 (CH_3)	NAc-4 (CH_3)
A α-D-Fuc*p*3NX-(1→	5.10 (*4.8*) [a] 102.05 (*175*) [a]	3.78 (*11.2*) 66.95	4.10 (*3.3*) 52.68	3.68 (< *1*) 71.35	4.24 (*6.2*) 68.32	1.16 16.32		8.62		
X (*R*,*R*)-3-OH-3-Me-5-oxoproline		4.19		2.38/2.64 ($^2J_{H,H}$ = *17*)		1.37	8.02			
	n.a.	69.59	77.24	45.36	180.28	23.36 [c]				
B 3,4)-β-D-Man*p*NAcA-(1→	4.66 (< *1*) 100.89 (*164*)	4.41 (*3–4*) 54.17	3.80 (*9–10*) 79.01	4.06 (*9–10*) 74.71	3.6 78.2 [b]	 ~175	8.075		2.09 [d] 23.40 [c]	
C 3)-β-D-Qui*p*NAc4NAc-(1→	4.52 100.70	~3.71 56.43	~3.75 79.91	~3.49 56.24	~3.51 71.99	1.11 17.67	8.079	7.92	2.02 [d] 22.84	1.98 23.15

[a] $^3J_{H,H}$ and $^1J_{C,H}$ values are given in parentheses. [b] The chemical shift was obtained from the HMQC spectrum. [c,d] Assignments may be interchanged. The carbonyl carbons at 174.95–175.07 ppm (3 signals) and 171.77 ppm (1 signal) were not assigned. D-Qui*p*NAc4NAc = 2,4-diacetamido-2,4,6-trideoxy-D-glucopyranose.

TABLE II

NOE correlations for *V. cholerae* O:5 PS [a]

Atom	Intra-residue contacts	Inter-residue contacts
A α-D-Fuc*p*3NX-(1→		
H-1	H-2 (s)	H-3_B (s)
H-3	H-4 (s)	H-6_C
H-5	H-4, H-6	
H-6	H-4 (s), H-5	H-2_B (w), NAc-2_B (2.09)
NH-3	H-2 (w)	NH-$_X$, CH-$_X$ (s)
X (*R*,*R*)-3-Hydroxy-3-methyl-5-oxoproline		
CH	CH_3-$_X$, NH-$_X$	NH-3_A, H-2_A
CH_3	CH_2-$_X$ (s), CH-$_X$ (s)	H-4_A
NH	CH-$_X$ (s), CH_3-$_X$	NH-3_A, H-6_C
B 3,4)-β-D-Man*p*NAcA-(1→		
H-1	H-3 (w), H-5 (s)	H-3_C
H-2	H-3	H-6_A
H-3	H-2 (s), H-4 (w)	
H-4	H-3, NH-2	H-1_C
H-5	H-1, H-3	
NH-2	H-4, NAc-2_B (2.09)	
C 3)-β-D-Qui*p*NAc4NAc-(1→ [b]		
H-1	H-2/3, H-4 or H-5	H-4_B
H-6	H-4/5, NH-4, NAc-4_C (1.98)	H-3_A, CH-$_X$
NH-2	H-2/3, NAc-2	
NH-4	H-2/3 (w), H-4/5, H-6, NAc-4_C	

[a] Data obtained from NOESY experiments in D_2O at 70°C and in 90% H_2O at 23°C; (w) and (s) indicate weak and strong intensities of NOESY cross-peaks. [b] D-Qui*p*NAc4NAc = 2,4-diacetamido-2,4,6-trideoxy-D-glucose.

designation was B4202-64 originally supplied by Shimada and Sakasaki[12]. Confirmation of the strain as an O:5 serovar was made by T. Shimada, Department of Bacteriology, National Institute of Health, Tokyo, Japan.

Preparation of LPS and O-polysaccharide.—*Vibrio cholerae* O:5 was grown in an aerated 10-L fermentor at a constant pH of 7.2 for 18 h. The nutrient medium used has been described earlier[13]. The bacterial yield was ca. 7 g dry weight per L. The bacteria were collected by centrifugation and then freeze-dried. Crude lipopolysaccharide (LPS) was obtained by extraction in a hot phenol–water mixture[14]. Purification of the crude LPS was performed by ultracentrifugation at 100 000*g* for 4 h. The yield of LPS after centrifugation was 1.4% of the bacterial dry weight.

The LPS (1g) was further purified by treatment with ribonuclease (20 mg) in 0.1 M phosphate buffer of pH 6.8 (80 mL) containing 10 mM NaCl and the mixture was dialysed against the same buffer (37°C, 14 h). The solution was lyophilized (0.54 g) and hydrolysed with aq 2% acetic acid (50 mL, 100°C, 1 h). The precipitate was removed and the solution fractionated on the Sephadex G50 column. A glucan, which according to NMR spectra was glycogen (227 mg), was eluted with

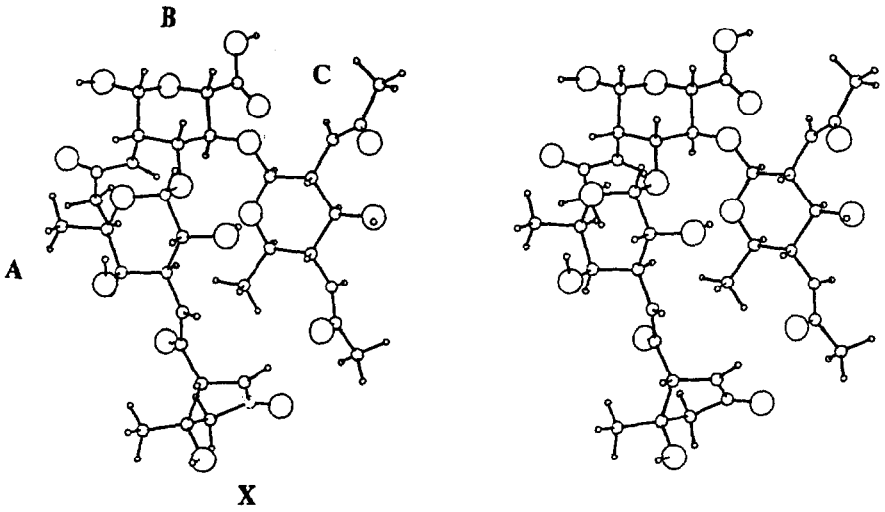
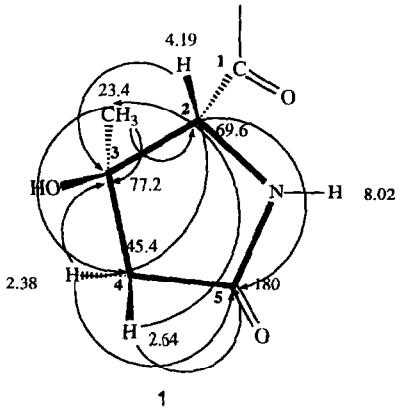


Fig. 4. The energy-minimised trisaccharide repeating unit of the *V. cholerae* O:5 PS.

the void, and the O-polysaccharide (PS) at 1.3–1.7 void volumes (24 mg). Further purification from glycogen was obtained by chromatography on a DEAE-Sepharose column. The PS was eluted with 0.1 M NaCl and was further purified on the Bio Gel P-2 column. The yield was 13 mg, $[\alpha]_D$ +140° (*c* 0.2, H_2O).



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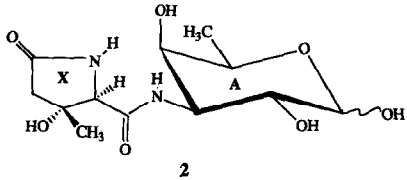

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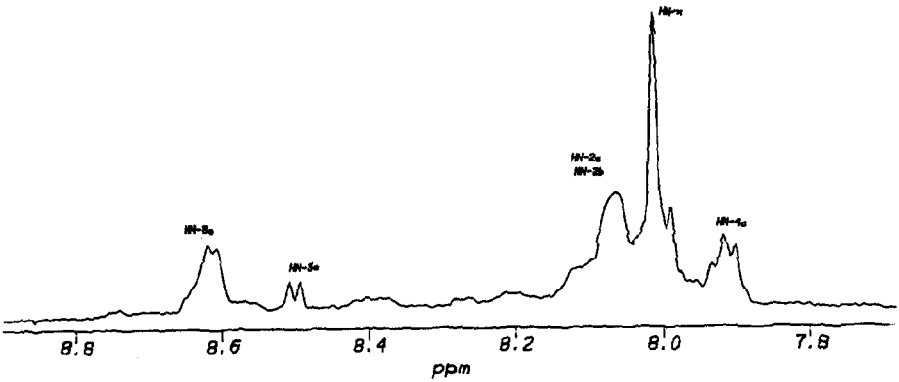


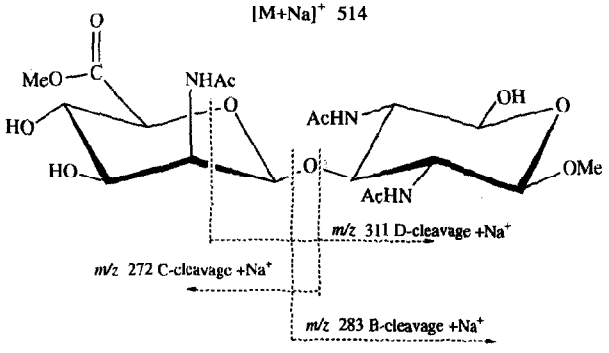
Fig. 5. 600-MHz ^{1}H NMR spectrum of the NH-region of an aqueous solution of the *V. cholerae* O:5 PS.

TABLE III

^{1}H NMR data at 16°C for oligomeric product (2) from HF solvolysis of the *V. cholerae* O:5 PS

	Residue	H-1	H-2	H-3	H-4,4′	H-5/ C-CH_3	H-6
A	α-D-Fuc*p*3NX	5.24 (*3.7*) [a]	3.87 (*10.9*)	4.28 (*3.3*)	3.74 (*< 1*)	4.25 (*6.7*)	1.18
	β-D-Fuc*p*3NX	4.67 (*7.8*)	3.53 (*11.0*)	4.05 (*3.3*)	3.70 (*< 1*)	3.90 (*6.5*)	1.23
X	(*R*,*R*)-3-OH-3-Me-5-oxoproline		4.22		2.42/2.66 ($^2J_{H,H}$ 17.4)	1.40	

[a] $^3J_{H,H}$ values are given in parenthesis.



3

Sugar analysis.—Hydrolysis was performed with 2 M CF_3CO_2H (120°C, 1 h) or with 10 M HCl (100°C, 10 min) followed by reduction ($NaBH_4$ or $NaBD_4$) and acetylation. For GLC, a Hewlett–Packard 5830A instrument fitted with a flame-ionization detector was used. Separation of alditol acetates was performed on an HP-5 fused-silica capillary column, using a temperature program from 210°C (3 min) to 250°C at 3°C/min. GLC–MS was performed on a Hewlett–Packard 5970 MSD instrument, using an HP-5 column. Methanolysis was performed using M HCl in MeOH at 85°C for 24 h. After workup the products were investigated by GLC–MS and FABMS. The absolute configuration of the 3-amino-3,6-dideoxygalactose residue was determined according to Gerwig et al.[5], but with the modification that the alcoholysis was performed with 2 M methanolic HCl and preceded by hydrolysis with 2 M CF_3CO_2H.

HF-Solvolysis.—PS (5 mg) was treated with anhyd HF (2 mL, −30°C, 1 h). The HF was removed under reduced pressure, and the residue was dissolved in water (5 mL) and kept for 12 h at 40°C. After neutralization with NH_4OH and lyophilization, the material was purified on the Bio-Gel P-2 column. Two compounds were isolated, one of which was eluted with the void volume (0.8 mg) and the other in the disaccharide region (1.0 mg).

Carboxyl-reduction.—PS (1 mg) was carboxyl-reduced ($NaBD_4$) according to Taylor et al.[15]. The reduction was repeated twice, and the product was purified by chromatography on Bio-Gel P2 column on which it was eluted with the void volume. Sugar analysis revealed, in addition to 3-amino-3,6-dideoxygalactose, small amounts of ManNAc.

FAB-mass spectrometry.—Fast atom bombardment mass spectra were recorded in the negative and positive mode on a Jeol SX102 instrument. The accelerating voltage was 10 kV and the Xe gun was operated at 6 kV. Cesium iodide was used as a reference compound for the calibration of mass numbers with a resolution of 3000 for the FAB analysis and 1000 for the linked scan (B/E) to detect daughter ions.

NMR spectroscopy.—^1H NMR and ^{13}C NMR spectra were recorded with a Jeol GSX 270, Jeol GSX 400, Varian Unity 500, or Varian Unity 600 spectrometer, using standard pulse sequences. Spectra of D_2O solutions of pD 2.7 or 7.3 (pH meter reading +0.4) were recorded at 70 or 85°C, or of solutions of pH 4.0 containing 90% H_2O–10% D_2O at 16, 23, or 70°C. Samples in D_2O were lyophilized twice with D_2O. Chemical shifts are reported in ppm, using sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) and 1,4-dioxane (δ_C 67.40) as internal references. Chemical shifts were obtained from 1D spectra when possible, or from proton–proton correlated 2D (COSY) spectra, relayed COSY, or total proton–proton correlated (HOHAHA) spectra, with a digital resolution of 2.3 Hz/point. Coupling constants were obtained from 1D spectra or from COSY spectra. The HOHAHA experiments were recorded using mixing times of 30, 50, 70, 80, 110, and 120 ms. NOESY experiments were performed using mixing times of 100, 150, 200, and 250 ms, and a DEPT experiment was performed using a 135° pulse angle.

Proton–carbon correlated spectra (HMQC) were obtained with or without decoupling, and the long-range proton–carbon correlated spectra (HMBC) were performed using a delay time of 60 ms.

Molecular modelling of conformations.—In order to investigate the conformation of trisaccharides with variation of the absolute configuration of the ManNAcA, the 2,4-diamino sugar, and the 3-hydroxy-3-methyl-5-oxoproline residue, the CHARMM force field with the parameters from Ha et al.[16] was used. Larger oligosaccharides were also investigated in order to see that no changes at glycosidic linkages had occurred.

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REFERENCES

1 L. Kenne, B. Lindberg, P. Unger, B. Gustavsson, and T. Holme, *Carbohydr. Res.*, 100 (1982) 341–349.
2 L. Kenne, B. Lindberg, E. Schweda, B. Gustavsson, and T. Holme, *Carbohydr. Res.*, 180 (1988) 285–294.
3 T.A. Chowdhury, P.-E. Jansson, B. Lindberg, J. Lindberg, B. Gustavsson, and T. Holme, *Carbohydr. Res.*, 215 (1991) 303–314.
4 A.A. Ansari, L. Kenne, B. Lindberg, B. Gustavsson, and T. Holme, *Carbohydr. Res.*, 150 (1986) 213–219.
5 G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
6 J.L. Di Fabio, M.B. Perry, and D.R. Bundle, *Biochem. Cell Biol.*, 65 (1987) 968–977.
7 Yu.A. Knirel, E.V. Vinogradov, A.S. Shashkov, S.G. Wilkinson, Y. Tahara, B.A. Dmitriev, N.K. Kochetkov, E.S. Stanislavsky, and G.M. Mashilova, *Eur. J. Biochem.*, 155 (1986) 659–669.
8 G.A. Naberezhnych, V.A. Khomenko, V.V. Isakov, Yu.N. Elkin, T.F. Soloveva, and Yu.S. Ovodov, *Bioorg. Khim.*, 13 (1987) 1428–1429.
9 H. Eguchi, S. Kaya, and Y. Araki, *Carbohydr. Res.*, 231 (1992) 147–158.
10 V.L. L'vov, N.V. Tochtamysheva, A.S. Shashkov, B.A. Dmitriev, and K. Capek, *Carbohydr. Res.*, 112 (1983) 233–239.
11 Yu.A. Knirel, E.V. Vinogradov, and A.J. Mort, *Adv. Carbohydr. Chem. Biochem.*, 47 (1989) 167–202.
12 T. Shimada and S. Sakasaki, *Jpn. J. Med. Sci. Biol.*, 30 (1977) 275–277.
13 T. Holme, S. Arvidson, B. Lindholm, and B. Pavlu, *Proc. Biochem.*, 5 (1970) 62–66.
14 O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.
15 R.L. Taylor, J.E. Shively, and H.E. Conrad, *Methods Carbohydr. Chem.*, 7 (1976) 149–151.
16 S.N. Ha, A. Giammona, M. Field, and J.W. Brady, *Carbohydr. Res.*, 180 (1988) 207–221.