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Structural studies of the O-specific polysaccharide of *Vibrio cholerae* O8 using solvolysis with triflic acid

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Abstract

The O-specific polysaccharide (OPS) of *Vibrio cholerae* O8 was isolated by mild acid degradation of the lipopolysaccharide and studied by two-dimensional NMR spectroscopy, including NOESY and heteronuclear multiple-bond correlation (HMBC) experiments. The OPS was found to have a tetrasaccharide repeating unit with the following structure:

→4)-β-D-Glc*p*NAc3NAcylAN-(1→4)-β-D-Man*p*NAc3NAcAN-(1→4)-α-L-Gul*p*NAc3NAcA-(1→3)

-β-D-Qui*p*NAc4NAc-(1→

where QuiNAc4NAc is 2,4-diacetamido-2,4,6-trideoxyglucose, GlcNAc3NAcylAN is 2-acetamido-3-(*N*-formyl-L-alanyl)amino-2,3-dideoxyglucuronamide, ManNAc3NAcAN is 2,3-diacetamido-2,3-dideoxymannuronamide, and GulNAc3NAcA is 2,3-diacetamido-2,3-dideoxyguluronic acid. The OPS was stable towards acid hydrolysis and solvolysis with anhydrous hydrogen fluoride, but could be cleaved selectively with trifluoromethanesulfonic (triflic) acid by the glycosidic linkages of β-QuiNAc4NAc and α-GulNAc3NAcA. The structures of the oligosaccharides obtained that were elucidated by electrospray ionization (ESI) MS and NMR spectroscopy, confirmed the OPS structure. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Vibrio cholerae*; Lipopolysaccharide; O-specific polysaccharide structure; 2,3-Diamino-2,3-dideoxyhexuronic acid; Triflic acid solvolysis

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1. Introduction

The *Vibrio cholerae* species comprises nearly 200 O-serogroups but only two of them, namely O1 and O139, are the causative agents of Asiatic cholera. The non-O1, non-O139 serogroups of *V. cholerae* comprise a heterogeneous group of microorganisms rarely associated with enteric disease. Until 1992, *V. cholerae* O1 was the only serogroup responsible for epidemic cholera. The emergence of a 'new' serogroup, O139, as a causative agent of epidemic cholera prompted monitoring of diarrhoea in endemic areas also for non-O1, non-O139 serogroups. These serogroups were found to be increasingly associated with diarrhoea, however, the virulence mechanisms

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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. Only few of them caused single cases of diarrhoea or minor local outbreaks of cholera-like disease.[1–6] Particularly, *V. cholerae* O8 was found in several cases of diarrhoea in a prospective study in India between 1993 and 1995.[5]

The serogrouping of *V. cholerae* is based on the reactivity of specific antibodies with epitopes on the cell wall associated lipopolysaccharide. Structures of the O-specific polysaccharides (OPS) of *V. cholerae* lipopolysaccharides vary significantly from serogroup to serogroup. Structural studies have been performed on the OPS of serogroups O1–O3, O5, 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. 7, 8). Now we report on the structure of a new OPS of *V. cholerae* O8 which is enriched in derivatives of 2,3-diamino-2,3-dideoxyhexuronic acids having the gluco, manno, and gulo configuration.

2. Results and discussion

The O-specific polysaccharide (OPS) was prepared by mild acid degradation of the lipopolysaccharide of *V. cholerae* O8 followed by GPC on Sephadex G-50. Acid hydrolysis of the OPS released L-alanine which was identified by GLC as the acetylated (+)-2-octyl ester by comparison with the authentic samples prepared from D- and L-alanine. Sugar analysis of the OPS revealed minor amounts of glucose, L-*glycero*-D-*manno*-heptose, and 2-amino-2-deoxyglucose. Further studies of the OPS showed that these were components of the lipopolysaccharide core, whereas no OPS sugar constituents were released by acid hydrolysis.

The ^{13}C NMR spectrum of the OPS (Fig. 1) demonstrated a tetrasaccharide repeating unit containing a number of amino components. The spectrum showed, inter alia, major signals for four anomeric carbons at δ 99–103, two $C\mathrm{H}_3$–C groups at δ 18.3 and 19.3, one belonging to C-3 of alanine and the other to C-6 of a 6-deoxysugar, a number of *N*-acetyl groups ($C\mathrm{H}_3$ at δ 23.2–23.6), and one *N*-formyl group at δ 164.7. Accordingly, the ^{1}H NMR spectrum of the OPS contained signals for two $\mathrm{C}H_3$–C group at δ 1.21 and 1.38 (both d, $J \sim 6$ Hz), seven *N*-acetyl groups at δ 1.91–2.11, and one *N*-formyl group at δ 8.09 (all s). Three signals for anomeric protons were observed at δ 4.64 (d, $J_{1,2} \sim 8$ Hz), 4.99, and 5.11 (both unresolved); the fourth anomeric signal at δ 4.32 was superimposed with another signal.

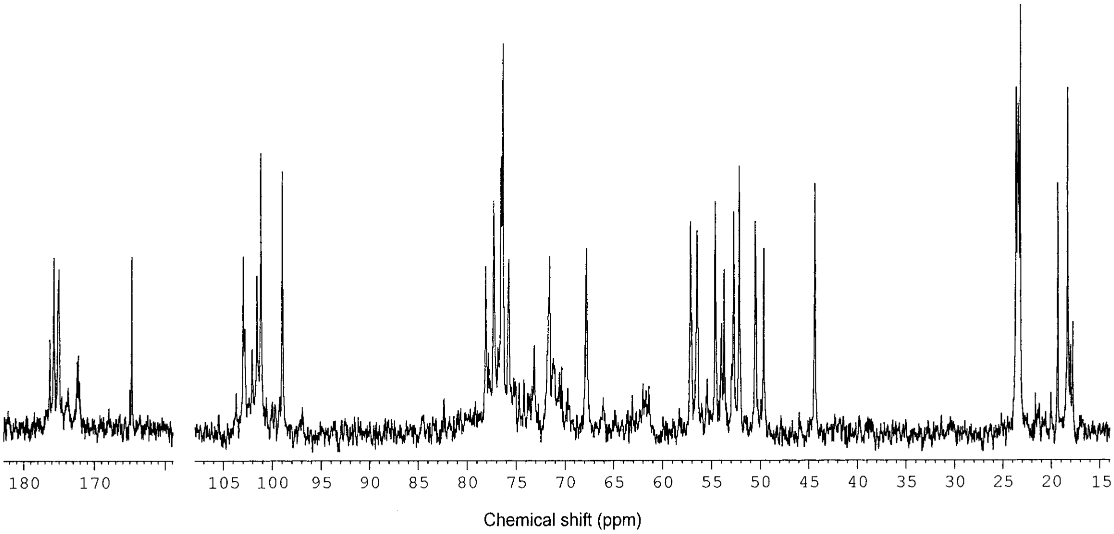


Fig. 1. 125-MHz ^{13}C NMR spectrum of the O-specific polysaccharide of *V. cholerae* O8.

Table 1
500-MHz ^{1}H NMR chemical shifts (δ, ppm) for oligo- and polysaccharides from *V. cholerae* O8 LPS [a]

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6
O-specific polysaccharide (OPS)						
→4)-β-D-Glc*p*N3NAN-(1→(**A**)	4.64	3.74	4.10	3.88	3.96	
→4)-β-D-Man*p*N3NAN-(1→(**B**)	4.99	4.43	4.16	3.89	4.06	
→4)-α-L-Gul*p*N3NA-(1→(**C**)	5.11	4.45	4.40	4.20	4.29	
→3)-β-D-Qui*p*N4N-(1→(**D**)	4.32	3.84	3.77	3.77	3.50	1.21
L-Ala		4.41	1.38			
Modified (deformylated) polysaccharide (MPS)						
→4)-β-D-Glc*p*N3NAN-(1→(**A**)	4.65	3.74	4.08	3.87	3.96	
→4)-β-D-Man*p*N3NAN-(1→(**B**)	4.99	4.43	4.16	3.89	4.06	
→4)-α-L-Gul*p*N3NA-(1→(**C**)	5.11	4.45	4.40	4.20	4.29	
→3)-β-D-Qui*p*N4N-(1→(**D**)	4.36	3.84	3.80	3.80	3.55	1.24
L-Ala		3.95	1.48			
*Tetrasaccharide **1a***						
β-D-Glc*p*N3NAN-(1→(**A**)	4.64	3.73	4.02	3.66	3.97	
→4)-β-D-Man*p*N3NAN-(1→(**B**)	4.96	4.43	4.18	3.90	4.05	
→4)-α-L-Gul*p*N3NA-(1→(**C**)	5.15 [b]	4.44	4.36	4.17	4.13	
→3)-α-D-Qui*p*N4N (**D**)	5.06	4.16	3.99	3.85	4.03	1.18
→3)-β-D-Qui*p*N4N (**D**)	4.62	3.94	3.83	3.85	3.60	1.22
L-Ala		4.32	1.34			
*Tetrasaccharide **1b***						
β-D-Glc*p*N3NAN-(1→(**A**)	4.68	3.74	4.05	3.66	3.97	
→4)-β-D-Man*p*N3NAN-(1→(**B**)	4.96	4.43	4.18	3.90	4.05	
→4)-α-L-Gul*p*N3NA-(1→(**C**)	5.15 [b]	4.44	4.36	4.17	4.13	
→3)-α-D-Qui*p*N4N (**D**)	5.06	4.16	3.99	3.85	4.03	1.18
→3)-β-D-Qui*p*N4N (**D**)	4.62	3.94	3.83	3.85	3.60	1.22
L-Ala		4.04	1.44			
*Trisaccharide **2b***						
β-D-Glc*p*N3NAN-(1→(**A**)	4.69	3.74	4.05	3.66	3.98	
→4)-β-D-Man*p*N3NAN-(1→(**B**)	5.02	4.43	4.17	3.91	4.06	
→4)-α-L-Gul*p*N3NA (**C**)	5.21	4.35	4.56	4.19	4.78	
L-Ala		4.05	1.44			
*Trisaccharide **3b***						
β-D-Glc*p*N3NAN-(1→(**A**)	4.68	3.74	4.05	3.66	3.98	
→4)-β-D-Man*p*N3NAN-(1→(**B**)	4.96	4.49	4.14	3.93	3.90	
→4)-L-GulN3NAol (**C**)	3.52 [c]	4.04	4.24	4.47	3.96	
L-Ala		4.04	1.44			

[a] Additional chemical shifts are δ 1.91–2.11 (CH_3CON) and 8.05–8.09 (HCO). QuiN4N stands for 2,4-diamino-2,4,6-trideoxyglucose, GlcN3NAN for 2,3-diamino-2,3-dideoxyglucuronamide, ManN3NAN for 2,3-diamino-2,3-dideoxymannuronamide, GulN3NA for 2,3-diamino-2,3-dideoxyguluronic acid, and GulN3NAol for GulN3NA-derived aldonic acid.

[b] In **1**β; δ 5.14 in **1**α.

[c] H-1a; H-1b at δ 3.63.

The ^{1}H and ^{13}C NMR spectra of the OPS were assigned using two-dimensional COSY, TOCSY, and H-detected ^{1}H,^{13}C heteronuclear single-quantum coherence (HSQC) experiments (Tables 1 and 2). Based on the assignment and, in particular, on correlation of protons at carbons bearing nitrogen to the corresponding carbons, it was found that three constituent monosaccharides are 2,3-diamino-2,3-dideoxyhexuronic acids. As judged by typical coupling constant values estimated from the one-dimensional ^{1}H NMR and two-dimensional ^{1}H,^{1}H correlation spectra, one of them has the β-gluco and another manno configuration (units **A** and **B**). The third 2,3-diamino-2,3-dideoxyhexuronic acid (unit **C**) had a partially resolved H-2 signal in the ^{1}H NMR spectrum that allowed determination of $J_{1,2} \approx J_{2,3}$ 3 Hz, whereas signals for H-3–H-5 were not resolved, thus indicating $J_{3,4}$ and $J_{4,5} < 3$ Hz. Such coupling constant values are characteristic for the α-gulo configuration, which was confirmed unambiguously by comparison of the ^{13}C NMR chemical shifts with published data for all eight isomeric methyl 2,3-diacetamido-2,3-dideoxy-α-hexopyranosides.[7] In particular, the C-2 chemical shift of this sugar, δ 44.4, was close to the value δ 45.4 for that of methyl 2,3-diacetamido-2,3-dideoxy-α-D-gulopyranoside, whereas C-2 of all other isomers resonate at δ 49.7–53.2.[7] The low value of the C-2 chemical shift in the gulo isomer derives from the presence of the two axial substituents at C-3 and C-4. The fourth monosaccharide (unit **D**) was a 6-deoxydiaminosugar having the β-gluco configuration

Table 2
125-MHz ^{13}C NMR chemical shifts (δ, ppm) for oligo- and polysaccharides from *V. cholerae* O8 LPS [b]

Sugar residue	C-1	C-2	C-3	C4	C-5	C-6
O-specific polysaccharide (OPS)						
→4)-β-D-Glc*p*N3NAN-(1→(**A**)	103.0	54.7	53.7	76.4	77.0	172.3
→4)-β-D-Man*p*N3NAN-(1→(**B**)	101.2	52.2	52.8	75.8	76.4	172.5
→4)-α-L-Gul*p*N3NA-(1→(**C**)	99.0	44.4	50.6	77.3	67.8	
→3)-β-D-Qui*p*N4N-(1→(**D**)	101.6	57.2	78.1	56.5	71.6	18.3
L-Ala	176.3	49.7	19.3			
Modified (deformylated) polysaccharide (MPS)						
→4)-β-D-Glc*p*N3NAN-(1→(**A**)	102.8	54.7	54.0	76.4	77.0	
→4)-β-D-Man*p*N3NAN-(1→(**B**)	101.2	52.2	52.8	75.8	76.4	
→4)-α-L-Gul*p*N3NA-(1→(**C**)	99.0	44.4	50.6	77.3	67.8	
→3)-β-D-Qui*p*N4N-(1→(**D**)	102.1	57.1	77.8	56.5	71.7	18.3
L-Ala		50.6	17.9			
*Tetrasaccharide **1a***						
β-D-Glc*p*N3NAN-(1→(**A**)	102.9	54.5	55.8	70.6	77.3	
→4)-β-D-Man*p*N3NAN-(1→(**B**)	101.1	52.2	53.1	75.9	76.4	
→4)-α-L-Gul*p*N3NA-(1→(**C**)	98.9 [a]	44.5	51.0	77.3	68.3	
→3)-α-D-Qui*p*N4N (**D**)	92.2	55.8	75.2	56.6	67.6	18.2
→3)-β-D-Qui*p*N4N (**D**)	96.0				72.1	18.2
L-Ala		49.9	18.4			
*Tetrasaccharide **1b***						
β-D-Glc*p*N3NAN-(1→(**A**)	102.7	54.7	55.8	70.6	77.3	
→4)-β-D-Man*p*N3NAN-(1→(**B**)	101.1	52.2	53.1	75.9	76.4	
→4)-α-L-Gul*p*N3NA-(1→(**C**)	98.9 [a]	44.5	51.0	77.3	68.3	
→3)-α-D-Qui*p*N4N (**D**)	92.2	55.8	75.2	56.6	67.6	18.2
→3)-*β*-D-Qui*p*N4N (**D**)	96.0				72.1	18.2
L-Ala		50.4	17.9			
*Trisaccharide **2b***						
β-D-Glc*p*N3NAN-(1→(**A**)	102.7	54.7	55.8	70.6	77.3	
→4)-β-D-Man*p*N3NAN-(1→(**B**)	101.3	52.2	53.2	75.8	76.5	
→4)-α-L-Gul*p*N3NA (**C**)	92.7	45.3	50.6	78.1	67.6	
L-Ala		50.4	17.9			
*Trisaccharide **3b***						
β-D-Glc*p*N3NAN-(1→(**A**)	102.8	54.6	55.6	70.6	77.3	
→4)-β-D-Man*p*N3NAN-(1→(**B**)	98.3	52.1	53.2	75.6	76.7	
→4)-L-GulN3Naol (**C**)	61.9	52.4	52.9	75.3	73.3	
L-Ala		50.3	17.9			

[a] In **1**β; δ 99.2 in **1**α.

[b] Additional chemical shifts are δ 23.1–23.7 (CH$_3$CON), 164.7–165.1 (HCO), and 171.9–176.6 (CO). For abbreviations of the sugar residues see note to Table 1.

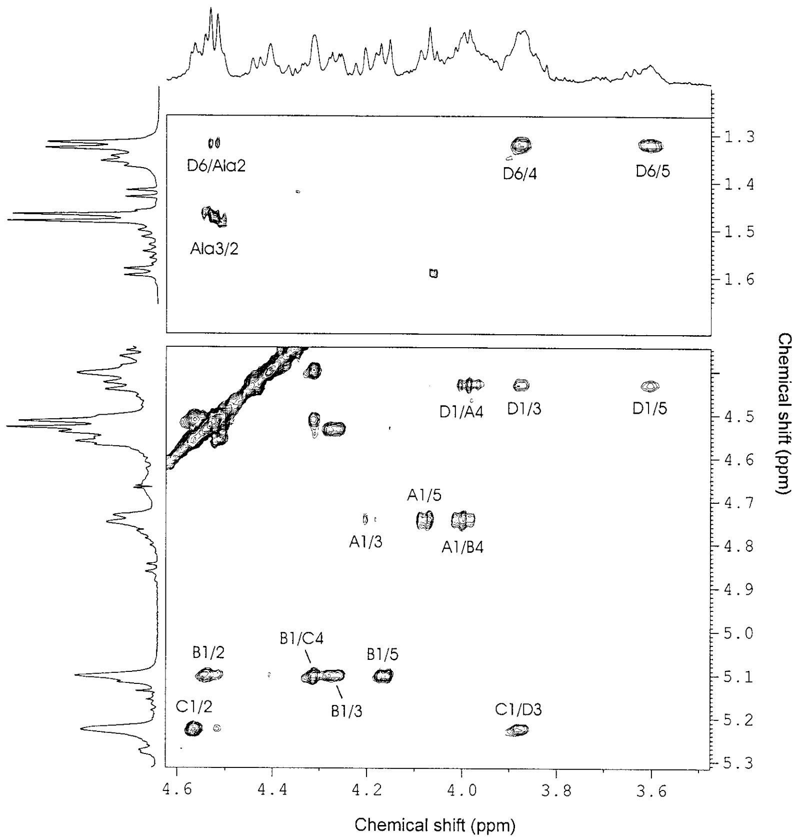


Fig. 2. Parts of a NOESY spectrum of the O-specific polysaccharide of *V. cholerae* O8. The corresponding parts of the ^{1}H NMR spectrum are displayed along the horizontal and vertical axes, respectively. Arabic numbers refer to protons in sugar residues denoted by letters as shown in Table 1.

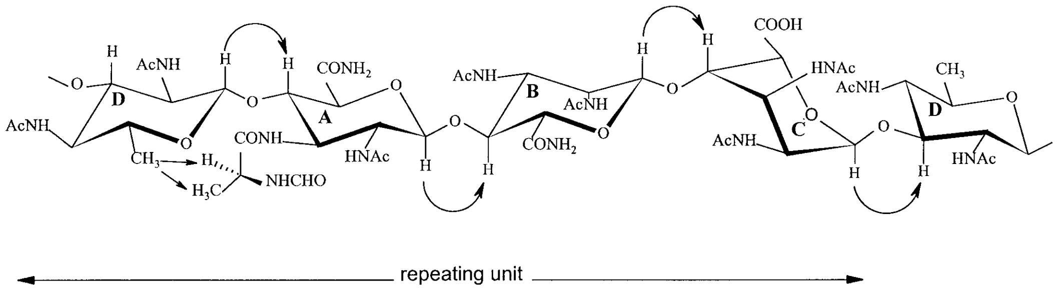


Fig. 3. Structure of the repeating unit of the O-specific polysaccharide of *V. cholerae* O8 and important interresidue NOE connectivities. Amidation of derivatives of 2,3-diamino-2,3-dideoxy-glucuronic and -mannuronic acids may be incomplete.

and one amino group at position 2, but the position of the second amino group could not be determined owing to coincidence of the H-3 and H-4 resonances at δ 3.77 in the ^{1}H NMR spectrum.

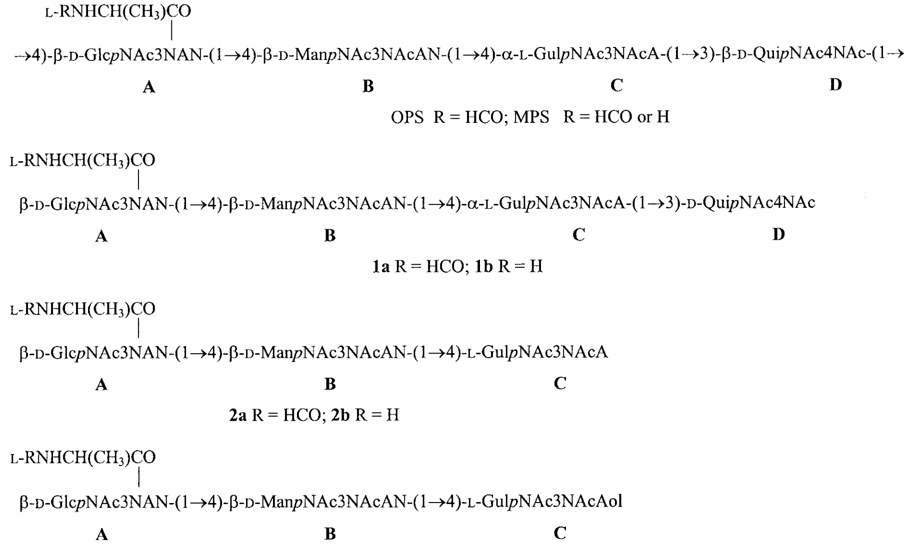
A NOESY experiment on the OPS (Fig. 2) revealed H-1/H-3, H-5 intraresidue correlations for units **A**, **B**, and **D**, and a H-1/H-2 correlation for unit **C**, thus indicating that the former are β-linked and the latter is α-linked. The following interresidue correlations between the transglycosidic protons were observed in the NOESY spectrum: **A** H-1/**B** H-4, **B** H-1/**C** H-4, **C** H-1/**D** H-3 or H-4, and **D** H-1/**A** H-4 at δ 4.64/3.89, 4.99/4.20, 5.11/3.77, and 4.32/3.88, respectively (Figs. 2 and 3). These data indicated that, as expected, the OPS is linear and all three 2,3-diamino-2,3-dideoxyhexuronic acids are substituted at position 4 (Figs. 3 and 4).

In addition, the NOESY spectrum showed a cross-peak between H-2 and H-3 of alanine and H-6 of unit **D** at δ 4.41/1.21 and 1.38/1.21, respectively (Fig. 2). Consideration of a Dreiding stereomodel of the OPS, using standard dihedral angles, showed that such connectivity is only possible if the alanyl group is located either at N4 of unit **D** or at N3 of unit **A** (Fig. 3).

An H-detected ^{1}H,^{13}C heteronuclear multiple-bond correlation (HMBC) experiment on the OPS showed correlations between the proton of the formyl group and C-2 of alanine at δ 8.09/49.7 and between the carbon of the formyl group and H-2 of alanine at δ 165.2/4.41. Therefore, the formyl group is attached to N-2 of alanine. *E*/*Z*-isomerism in *N*-formyl groups attached to pyranose ring of amino sugars could always be observed by NMR spectroscopy, but in this case only one (average) isomer was observed, evidently because of less restrictions to the interconversion between the isomers. The HMBC spectrum also showed an H-2/C-1 correlation for alanine at δ 4.41/176.3 and H-5/C-6 correlations for units **A** and **B** at δ 3.96/172.3 and 4.06/172.5, respectively. The chemical shifts of C-6 of units **A** and **B** are typical of hexuronamides (e.g., compare $\delta_{\text{C-6}}$ 171.5 in a galacturonamide[13]). No H-5/C-6 correlation could be observed for unit **C**.

In order to identify unit **D** and to determine the position of the alanyl group, an attempt to cleave the OPS by solvolysis with anhydrous hydrogen fluoride[8] was undertaken. However, the OPS was found to be stable towards this reagent even after 4 days of treatment at ambient temperature. Therefore, anhydrous trifluoromethanesulfonic (triflic) acid was applied for solvolysis, a reagent that had been found to efficiently cleave another highly stable polysaccharide from *Alteromonas* sp.[9] Solvolysis of the OPS with triflic acid for 18 h at +10 °C resulted mainly in a modified polysaccharide (MPS) and a small amount of an oligosaccharide which were separated by GPC on TSK HW-40.

NMR studies of the MPS which were performed as described above for the OPS, demonstrated the presence of a polysaccharide consisting of similar amounts of two types of tetrasaccharide repeating units, one identical to that in the OPS and the other lacking the *N*-formyl group (Fig. 4, Tables 1 and 2). Therefore, the *N*-formyl group was partially removed during solvolysis of the OPS with triflic acid, whereas the other *N*-acyl groups, including alanine, were unaffected. It is worth noting that N-deformylation occurred to a similar extent during hydrogen fluoride solvolysis of the OPS under the same conditions.



$$\overset{\text{L-RNHCH(CH}_3\text{)CO}}{\underset{\textbf{A}}{\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{NAc3NAN}}}\text{-}(1\rightarrow 4)\text{-}\underset{\textbf{B}}{\beta\text{-D-Man}p\text{NAc3NAcAN}}\text{-}(1\rightarrow 4)\text{-}\underset{\textbf{C}}{\alpha\text{-L-Gul}p\text{NAc3NAcA}}\text{-}(1\rightarrow 3)\text{-}\underset{\textbf{D}}{\beta\text{-D-Qui}p\text{NAc4NAc}}\text{-}(1\rightarrow$$

OPS R = HCO; MPS R = HCO or H

$$\overset{\text{L-RNHCH(CH}_3\text{)CO}}{\underset{\textbf{A}}{\beta\text{-D-Glc}p\text{NAc3NAN}}}\text{-}(1\rightarrow 4)\text{-}\underset{\textbf{B}}{\beta\text{-D-Man}p\text{NAc3NAcAN}}\text{-}(1\rightarrow 4)\text{-}\underset{\textbf{C}}{\alpha\text{-L-Gul}p\text{NAc3NAcA}}\text{-}(1\rightarrow 3)\text{-}\underset{\textbf{D}}{\text{D-Qui}p\text{NAc4NAc}}$$

1a R = HCO; **1b** R = H

$$\overset{\text{L-RNHCH(CH}_3\text{)CO}}{\underset{\textbf{A}}{\beta\text{-D-Glc}p\text{NAc3NAN}}}\text{-}(1\rightarrow 4)\text{-}\underset{\textbf{B}}{\beta\text{-D-Man}p\text{NAc3NAcAN}}\text{-}(1\rightarrow 4)\text{-}\underset{\textbf{C}}{\text{L-Gul}p\text{NAc3NAcA}}$$

2a R = HCO; **2b** R = H

$$\overset{\text{L-RNHCH(CH}_3\text{)CO}}{\underset{\textbf{A}}{\beta\text{-D-Glc}p\text{NAc3NAN}}}\text{-}(1\rightarrow 4)\text{-}\underset{\textbf{B}}{\beta\text{-D-Man}p\text{NAc3NAcAN}}\text{-}(1\rightarrow 4)\text{-}\underset{\textbf{C}}{\text{L-Gul}p\text{NAc3NAcAol}}$$

3a R = HCO; **3b** R = H

Fig. 4. Structures of oligosaccharides from the O-specific polysaccharide of *V. cholerae* O8. QuiNAc4NAc stands for 2,4-diacetamido-2,4,6-trideoxyglucose, GlcNAc3NAcylAN for 2-acetamido-3-(*N*-formyl-L-alanyl)amino-2,3-dideoxyglucuronamide, ManNAc3NAcAN for 2,3-diacetamido-2,3-dideoxymannuronamide, GulNAc3NAcA for 2,3-diacetamido-2,3-dideoxyguluronic acid, and GulNAc3NAcAol for GulNAc3NAcA-derived aldonic acid.

Minor signals from the deformylated repeating units were present in the NMR spectra of the initial OPS, thus indicating that some N-deformylation could occur during mild acid degradation of the lipopolysaccharide as well. Removal of the *N*-formyl group significantly influenced ^{1}H and ^{13}C NMR chemical shifts of alanine and, to a lesser extent, those of units **A** and **D** (Tables 1 and 2).

Similar studies of the oligosaccharide product from triflic acid solvolysis showed that this was a mixture of two tetrasaccharides **1a** and **1b** (Fig. 4), which differed in the presence (minor) and absence (major) of the *N*-formyl group. This difference affected ^{1}H- and ^{13}C NMR chemical shifts of alanine and unit **A**, but not unit **D** (Tables 1 and 2). Unit **A** was present at the non-reducing end, as followed from a characteristic upfield shift to δ 70.6 of the signal for C-4 in the ^{13}C NMR spectrum of **1** from its position at δ 76.4 in the spectrum of the OPS. Unit **D** gave in the ^{1}H and ^{13}C NMR spectra two series of signals, one for the α and one for the β anomeric form (Tables 1 and 2), and, hence, it occupied the reducing end. Tetrasaccharide **1a** thus represents the chemical repeating unit of the OPS (Fig. 4). Unlike the OPS, the chemical shifts of the signals for H-3 and H-4 of unit **D** in the ^{13}C NMR spectrum of **1** were different (δ 3.99 and 3.85 in the β-series) that allowed their correlations to the signals for C-3 and C-4 at δ 75.2 and 56.6, respectively. Therefore, unit **D** is 2,4-diamino-2,4,6-trideoxyglucose (bacillosamine).

Further treatment of MPS with triflic acid for 4 days at ambient temperature resulted in a smaller oligosaccharide which was found to be a mixture of two trisaccharides **2a** and **2b** (Fig. 4) with and without the *N*-formyl group, respectively. They contained the same components as tetrasaccharides **1a** and **1b**, including alanine, but no bacillosamine (see the ^{1}H and ^{13}C NMR data for the major trisaccharide **2b** in Tables 1 and 2). This finding confirmed the location of alanine at N-3 of unit **A** which, like in **1**, was at the non-reducing end of **2**. The reducing end of **2** was occupied by unit **C** which was present almost exclusively as the α-pyranose (in particular, this followed from the C-1 chemical shift of δ 92.7 in the ^{13}C NMR spectrum and the presence of an H-1/H-2 intraresidue cross-peak in the ROESY spectrum of **2**). Comparison of the structures of the OPS and oligosaccharides **1** and **2** (Fig. 4) showed that triflic acid selectively cleaved first the glycosidic linkage of bacillosamine and then that of 2,3-diamino-2,3-dideoxyguluronic acid. The facile cleavage of the former was expected by analogy with the known lability of the glycosidic linkage of 6-deoxy sugars towards solvolysis with anhydrous hydrogen fluoride.[8] The selectivity with respect to 2,3-diamino-2,3-dideoxyguluronic acid can be attributed to destabilization possibly caused by the two axial substituents, at C-3 and C-4.

Borohydride reduction of **2** converted unit **C** to an aldonic acid. The resulting products **3a** and **3b** (Fig. 4) were studied by ^{1}H and ^{13}C NMR spectroscopy (the data for **3b** are shown in Tables 1 and 2) and negative mode electrospray ionization (ESI) MS. The ESI mass spectrum (Fig. 5) showed intense singly charged pseudomolecular ions $[M-H]^{-}$ at *m*/*z* 820.3 and 848.3 corresponding to compounds **3b** and **3a** with two 2,3-diamino-2,3-dideoxyhexuronic acid residues in the amidated form (calculated molecular masses 821.34 and 849.33 Da, respectively). Peaks with approximately half the intensity were observed at *m*/*z* 821.3 and 849.3 and assigned to the corresponding monoamides, and less intense peaks at *m*/*z* 822.3 and 850.3 evidently originated from the nonamidated compounds. The presence of the minor components resulted either from incomplete amidation in the initial OPS or from partial cleavage of the carboxamides during solvolysis with triflic acid.

To confirm the amidation pattern of 2,3-diamino-2,3-dideoxyhexuronic acids, the pD dependence of ^{1}H NMR chemical shifts in **3** was studied. The signal for H-5 of unit **C** shifted downfield from δ 3.96 to 4.37 on a decrease of pD from 6 to 2; hence, this component had a free carboxyl group.[10] In contrast, no significant pD dependence was observed for the signals for H-5 of units **A** and **B**, thus confirming amidation of their carboxyl groups (Fig. 3).

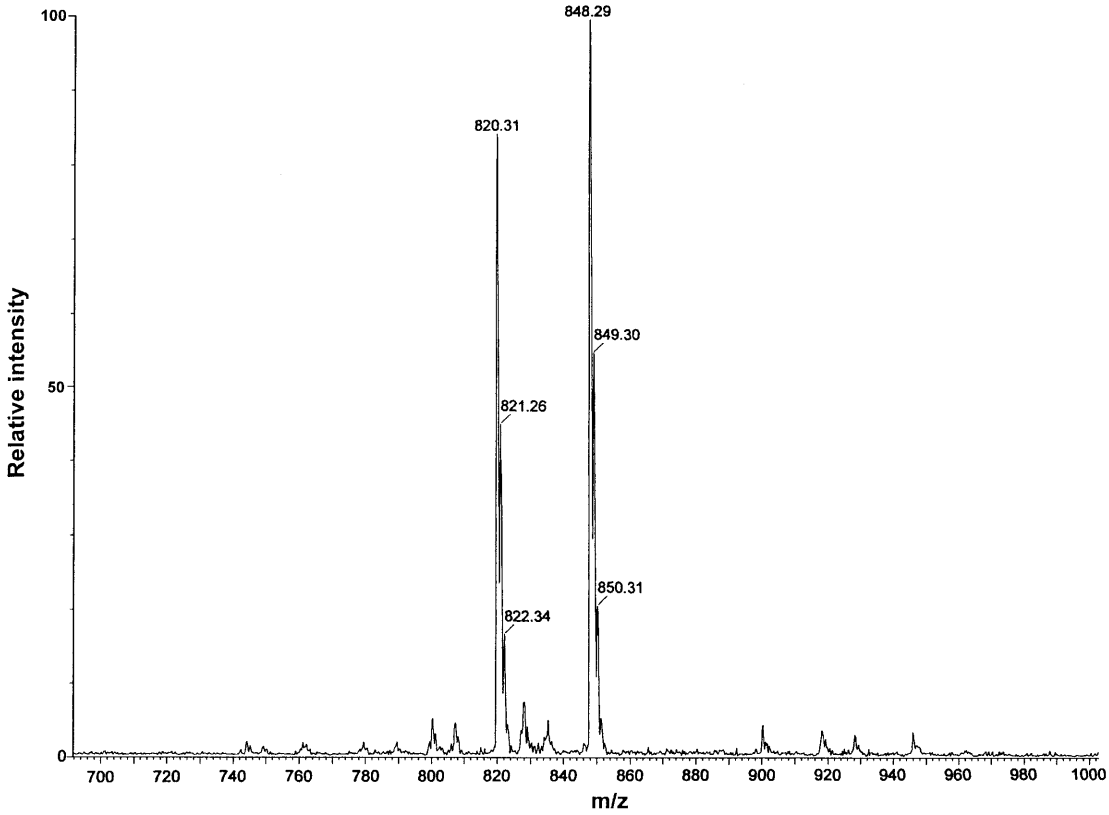


Fig. 5. Negative mode electrospray ionization mass spectrum of trisaccharide **3**. Peaks of singly charged pseudomolecular ions $[M - H]^-$ at *m*/*z* 820.31 and 848.29 belong to compounds **3b** and **3a**, respectively.

To determine the relative absolute configurations of the constituent monosaccharides, glycosylation effects[11] in the ^{13}C NMR spectrum of the OPS were analyzed as follows.

1. The β-linked disaccharide fragment **B** → **C**. The expected effects on **B** C-1, **C** C-3 and **C** C-4 signals are < 5, < -3 and < 5 ppm for the same and > 6, > -2.5 and > 7 ppm for different absolute configurations.[12,13] The observed values are $+7.3$, -1.0 and $+8.1$ ppm; hence, units **B** and **C** should have different absolute configurations.
2. The α-linked disaccharide fragment **C** → **D**. The expected effects on **C** C-1 and **D** C-4 signals are approximately 7.2 and 0.9 ppm for the same and 6.1 and -1.8 ppm for different absolute configurations.[14,15] The observed values are $+6.3$ and -1.4 ppm; hence, units **C** and **D** should have different absolute configurations.
3. The β-linked disaccharide fragments **D** → **A** and **A** → **B**. The expected effects for **A** C-4 and **B** C-4 signals are < -1 ppm for the same and > -0.5 ppm for different absolute configurations.[12] The observed values are -1.8 and -1.6 ppm, respectively; hence, units **D**, **A** and **B** should have the same absolute configurations.

Therefore, all sugars have the D configuration, except for 2,3-diamino-2,3-dideoxyguluronic acid which has the L configuration. The opposite configuration of all four sugars could not be strictly excluded, but is improbable since previously these monosaccharides have been found exclusively in the enantiomeric form indicated above.[16–18] Interestingly, they have all been reported to be components of the O-specific polysaccharides of *Pseudomonas aeruginosa*, the three 2,3-diamino-2,3-dideoxyhexuronic acids being identified there for the first time in nature.[19,20] The *N*-formyl-L-alanyl group, an *N*-acyl substituent of one of the acids in the OPS, have hitherto not been found in bacterial polysaccharides.

On the basis of the data obtained, it was concluded that the repeating unit of the O-specific polysaccharide of *V. cholerae* O8 has the structure shown in Fig. 3.

3. Experimental

Growth of the bacterium, isolation and degradation of the lipopolysaccharide.— *V. cholerae* O8, strain CO845, from Professor G.B. Nair (Department of Microbiology, National Institute of Cholera and Enteric Diseases, Calcutta, India) was grown in a rich tryptone–yeast extract as described earlier.[21] Pelleted bacterial cells were suspended in water and extracted with hot aq phenol[22] to give a crude lipopolysaccharide, which was purified by treatment with DNAse, RNAse and Proteinase K.[23] The purified lipopolysaccharide was degraded with 2% AcOH for 10 h at 100 °C, the precipitate was removed by centrifugation, and the OPS was isolated by GPC on Sephadex G-50 (S) (Pharmacia, Sweden) using 0.05 M pyridinium acetate pH 4.5 as eluent; monitoring was performed using a Waters differential refractometer (USA).

NMR spectroscopy and mass spectrometry.—^{1}H and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer (Germany) in D_2O at 60 °C using acetone (δ_H 2.225, δ_C 31.45) as an internal reference. Standard Bruker software (XWINNMR 1.2) was used to acquire and process NMR data. A mixing time of 100 ms was used in TOCSY and 200 ms in NOESY and rotating-frame NOE spectroscopy (ROESY) experiments.

Electrospray ionization (ESI) MS was run in negative mode using a VG Quattro triple quadrupole mass spectrometer (Micromass, UK) with aq 50% MeCN containing 1 mM NH_3 as the mobile phase at a flow rate of 10 μL/min. The sample was dissolved in aq 50% MeCN at a concn of ~50 pmol/μL and 10 μL was injected via a syringe pump into the electrospray source.

Chemical analysis.—For sugar analysis, hydrolysis of the OPS was performed with 2 M trifluoroacetic acid (120 °C, 2 h), and monosaccharides were identified by GLC as alditol acetate derivatives[24] using a Hewlett–Packard 5880 instrument on a DB-5 fused-silica capillary column (25 m × 0.25 mm) and a temperature gradient from 160 °C (1 min) to 250 °C at 3 °C/min. For identification of alanine, the OPS hydrolysate was N-acetylated with acetanhydride in an aq satd $NaHCO_3$ solution (20 °C, 30 min), heated with (+)-2-octanol in the presence of trifluoroacetic acid (100 °C, 16 h), acetylated, and analyzed by GLC as described above using a temperature gradient from 130 °C.

Solvolysis with triflic acid and anhyd hydrogen fluoride.—The OPS (20 mg) was treated with triflic acid (0.7 mL) at 10 °C for 18 h under anhyd conditions, the reaction mixture was neutralized with aq 25% NH_3 at 4 °C and fractionated by GPC on TSK HW-40 (S) in water, using a Knauer differential refractometer (Germany) for monitoring, to give a modified polysaccharide (MPS, 16 mg) and tetrasaccharide **1** (2 mg). MPS was treated with triflic acid for 4 days at 22 °C to give trisaccharide **2** which was reduced with $NaBH_4$ in water to give the corresponding alditol **3** (5 mg).

Solvolysis of the OPS (15 mg) with anhyd hydrogen fluoride was performed for 4 days at ambient temperature, and hydrogen fluoride was removed in vacuum using a trap with solid NaOH. The product was dissolved in water and fractionated on TSK HW-40 to give a polymeric material (14 mg) which was essentially identical to MPS.

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