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# Structural analysis of the lipopolysaccharide from *Vibrio cholerae* O139

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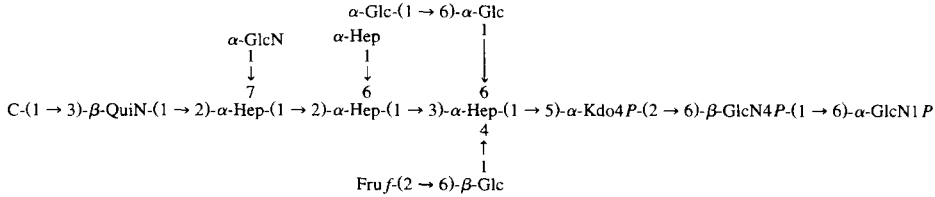
**Abstract**

The lipopolysaccharide (LPS) from *Vibrio cholerae* O139 was deacylated with KOH. The following structure of the oligosaccharide resulting from this treatment was established on the basis of monosaccharide and methylation analyses, $^{1}H$, $^{13}C$ and $^{31}P$ 1D and 2D NMR experiments and 1D analogues of 3D NOESY–TOCSY and 3D TOCSY–NOESY experiments.




'C' is a β-L-*threo*-hex-4-enuronopyranosyl residue. Hep is L-*glycero*-D-*manno*-heptose, QuiN is 2-amino-2,6-dideoxy-D-glucose, GlcN is 2-amino-2-deoxy-D-glucose, Glc is D-glucose, Fru is D-fructose, and Kdo is 3-deoxy-D-*manno*-2-octulosonic acid. All sugars are pyranoses except fructose which is furanosidic. The fructose residue was localised after deacylation of the LPS with anhydrous hydrazine, methylation, acid methanolysis, and remethylation using deuterated iodomethane.

The elucidation of this structure allowed for a direct comparison to the previously determined structure for *Vibrio cholerae* O1 lipid A-core region. The two structures are almost identical, and,

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therefore, this study is consistent with the genetic data for the biogenesis of strain O139 from O1. Furthermore, the identification of a structural analogue to the capsular polysaccharide of O139 in the outer core of the LPS in conjunction with the identification of colitose as a constituent of the LPS, provides additional evidence that the O-antigen and capsular polysaccharide of this strain may share the same repeat unit. 

*Keywords:* Lipopolysaccharide; Structural analysis; *Vibrio cholerae*

## 1. Introduction

*Vibrio cholerae* serotype O139 has recently emerged as a cause of epidemic cholera [1]. Historically, only the O1 serogroup of *V. cholerae* was associated with cholera epidemics. In early 1993 reports of a new epidemic of severe, cholera-like disease emerged from India and Bangladesh. Initially, the organism responsible for this outbreak was referred to as non-O1 *V. cholerae* because it did not agglutinate in O1 antisera. Further investigations revealed that this organism did not belong to any of the other 137 serotypes previously described and was therefore designated as serotype O139 [2].

Although serologically unrelated to O1, further studies [3] revealed that O139 was biotypically closely related to *V. cholerae* O1 El Tor biotype. In terms of pathogenic characteristics and specifically with respect to cholera toxin [4], the main virulence factor, *V. cholerae* O139 is indistinguishable from El Tor *V. cholerae* O1 strains.

Unlike O1, *V. cholerae* O139 does not produce an O-antigen of perosamine homopolymer [5], and indeed lacks the 'ladder pattern' characteristic of O-antigen producing strains when examined on SDS–PAGE gels [6]. At the genetic level, when compared to the O1 strain, O139 has been shown to be lacking the majority of the *rfb* cluster, the DNA region responsible for production of O1 antigen [7]. A more recent publication [8] has confirmed this, and has quite clearly shown that O139 probably arose from an O1 strain that had undergone genetic rearrangements including deletion of the O1 *rfb* region and acquisition of 35kb of DNA which encodes O139 surface polysaccharide.

However, unlike *V. cholerae* O1, and like many strains of non-O1 *V. cholerae*, O139 produces a polysaccharide capsule [9]. The structure of the capsular polysaccharide has recently been reported [10,11].

The present study was undertaken to examine the structure of the lipid A-core region of strain O139 and, therefore, enable a comparison to the recently reported lipid A-core region of the O1 strain [12] and thereby provide chemical evidence to increase our understanding of the biogenesis of serotype O139 from O1.

## 2. Results

*Isolation and characterisation of the LPS.*—LPS was isolated by the aqueous phenol method [13], effective separation from the capsular polysaccharide was achieved by ultracentrifugation, yielding purified LPS (ca. 5%). DOC–PAGE analysis of the LPS


Fig. 1. DOC–PAGE pattern of LPS. Lane 1, *Vibrio cholerae* O139 LPS (5 μg); Lane 2, *Salmonella milwaukee* (S-type LPS; 5 μg).

showed that the characteristic 'ladder pattern' indicative of polymeric O-chain was absent from this strain. As Fig. 1 shows, *V. cholerae* O139 LPS migrates as two distinct bands consistent with a lower band of unsubstituted core-lipid A and an upper band of core-lipid A with an additional homogenous unit, possibly indicative of a mixture of core (R-type) LPS and core plus one repeat unit (SR-type) as has been reported previously for this organism [6].

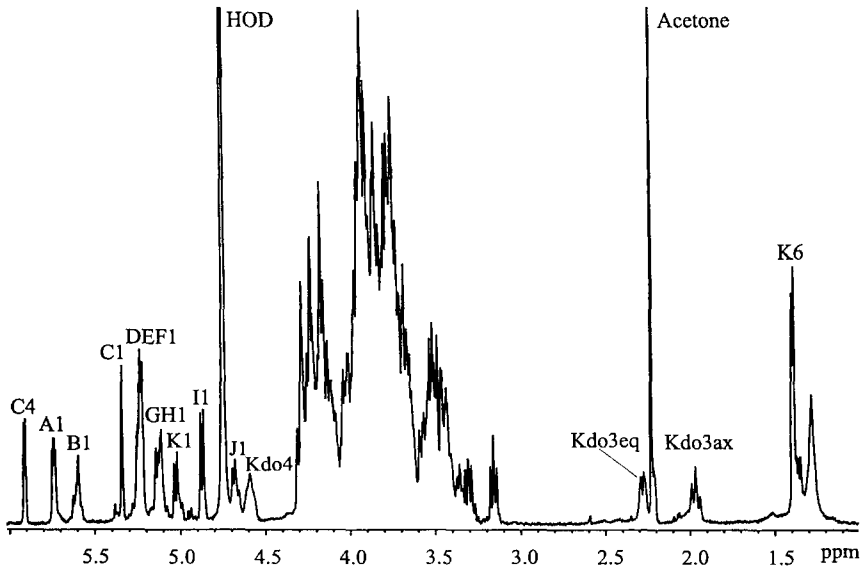


Fig. 2. $^{1}$H NMR spectrum of the deacylated LPS from *Vibrio cholerae* O139. The spectrum was recorded in $D_2O$ at pH 3.5 and 300 K.

*Compositional analysis.*—GLC–MS analysis of the derived alditol acetates and GLC analysis of acetylated (*S*)-2-butyl glycosides from the chromatographically purified deacylated (4 N KOH) LPS showed that it is composed of D-glucose (Glc), 2-amino-2-deoxy-D-glucose (GlcN), 2-amino-2,6-dideoxy-D-glucose (QuiN), and L-*glycero*-D-*manno*-heptose. When the alditol acetates and acetylated (*S*)-2-butyl glycosides derived from mild hydrolysis of the untreated LPS were examined, 3,6-dideoxy-L-*xylo*-hexose (Col) and D-fructose (Fru) were identified. The presence of 2-amino-2,6-dideoxy-D-glucose and 3,6-dideoxy-L-*xylo*-hexose in the LPS, sugars both previously identified as constituents of the capsular polysaccharide, are consistent with earlier data that suggested that the O-antigen and capsule share the same epitope [14].

*Glycosyl linkage analysis.*—The deacylated LPS was subjected to methylation analysis which suggested that the LPS contained 3,4,6-*O*-trisubstituted, 2,6-*O*-disubstituted, 2,7-*O*-disubstituted, 2- and 7-*O*-monosubstituted Hep*p* residues, 6-*O*-substituted Glc*p* residues, and terminal Glc*p*, Hep*p* and Fru*f* residues.

*NMR analysis.*—The $^{1}$H NMR spectrum of the deacylated sample at pH 7.0 gave broad lines and poor resolution; however, at pH 3.5 the spectrum was improved and indicated the sample to be homogeneous (Fig. 2).

The $^{1}$H NMR spectrum at pH 3.5 revealed 10 major signals in the low-field region (6.0–4.5 ppm). The resonances at 5.24 and 5.13 ppm could be attributed to three protons

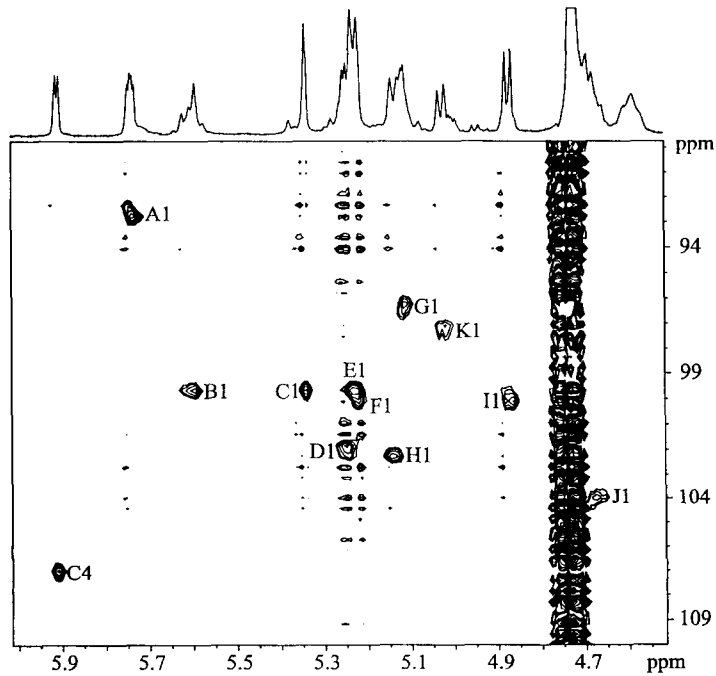


Fig. 3. Heteronuclear 2D $^{13}$C–$^{1}$H (HMQC) chemical shift correlation map of the anomeric region of the deacylated LPS from *Vibrio cholerae* O139. Cross peaks are indicated. The spectrum was recorded in $D_2O$ at pH 3.5 and 300 K.

and two protons, respectively. An initial COSY experiment revealed that the signals at 5.92 and 5.34 ppm were from the same residue. By virtue of a 2D $^{13}C-^{1}H$ correlation (HMQC) experiment (Fig. 3), it was deduced that 11 of the resonances in this low-field region (90–110 ppm) could be attributed to anomeric protons.

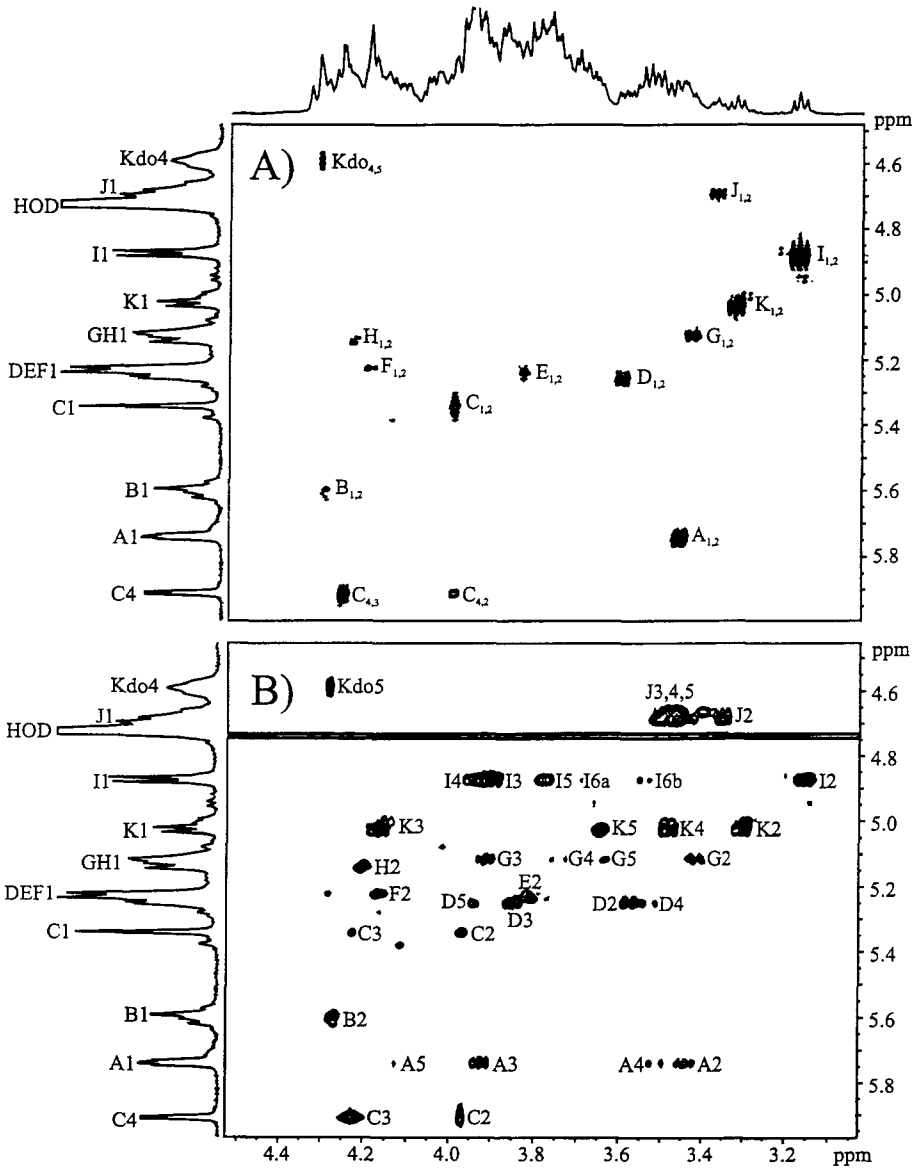


Fig. 4. Partial 2D COSY (A) and TOCSY (B) spectra of the deacylated LPS from *Vibrio cholerae* O139. Cross-peaks relating anomeric and ring protons are indicated. The spectra were recorded in $D_2O$ at pH 3.5 and 300 K.

Table 1
$^1$H NMR chemical shift data for *Vibrio cholerae* O139 deacylated LPS [a]

| Sugar residue [b] | H1 | H2 | H3 eq ax | H4 | H5 | H6 a b | H7 a b | H8 a b | NOE |
|---|---|---|---|---|---|---|---|---|---|
| | ($J_{1,2}$) [c] | ($J_{2,3}$) | ($J_{3,4}$) | ($J_{4,5}$) | | | | | |
| A | 5.744 | 3.439 | 3.923 | 3.513 | 4.128 | 4.300 | – | – | |
| αGlcN | (2.5) | (10.0) | (9.0) | (10.0) | | 3.898 | | | |
| B | 5.599 | 4.271 | 3.987 | 3.851 | 3.772 | 4.171 | nr [d] | – | NOE |
| αHep | (< 1.0) | (3.5) | (10.0) | | | | | | B1-F3 |
| C | 5.345 | 3.972 | 4.228 | 5.916 | – | – | – | – | NOE |
| | (3.0) | (5.0) | (3.0) | | | | | | C1-K3 |
| D | 5.255 | 3.577 | 3.847 | 3.529 | 3.945 | 3.949 | – | – | NOE |
| αGlc | (3.5) | (10.0) | (9.0) | (10.0) | | 3.863 | | | D1-F6 |
| E | 5.236 | 3.808 | 3.763 | 3.672 | 3.892 | nr | – | – | NOE |
| αGlc | (3.5) | (10.0) | (9.0) | | | | | | E1-D6a,b |
| F | 5.224 | 4.171 | 3.975 | 4.225 | 4.140 | 4.236 | 4.023 | – | NOE |
| αHep | (0.5) | (3.5) | (10.0) | | | | 3.937 | – | F1-KDO5 |
| G | 5.122 | 3.413 | 3.913 | 3.747 | 3.636 | 3.870 | – | – | NOE |
| αGlcN | (3.5) | (10.0) | (9.0) | | | 3.703 | | | G1-H7a,b |
| H | 5.148 | 4.213 | 4.090 | 3.935 | nr | 4.250 | 3.872 | | NOE |
| αHep | (0.5) | (3.5) | (10.0) | | | | 3.736 | | H1-B2 |
| I | 4.876 | 3.159 | 3.901 | 3.953 | 3.783 | 3.698 | – | – | NOE |
| βGlcN | (8.0) | (9.5) | | | | 3.545 | | | I1-A6a,b |
| J | 4.690 | 3.351 | 3.482 | 3.441 | 3.513 | 4.092 | – | – | NOE |
| βGlc | (8.0) | | | | | 3.843 | – | – | J1-F4 |
| K | 5.031 | 3.305 | 4.166 | 3.486 | 3.649 | 1.384 | – | – | NOE |
| βQuiN | (8.0) | (10.0) | (8.0) | (10.0) | | | – | – | K1-H2 |
| Kdo | | | 2.276 | 4.589 | 4.283 | 3.793 | nr | nr | |
| | | | 1.968 | | | | | | |

[a] Measured at pH 3.5, 300 K from internal acetone (2.225 ppm).
[b] Sugar residues A–K, Kdo are as in Fig. 5.
[c] Observed first-order coupling constants ($J_{H,H}$).
[d] nr, not resolved.

Two of the four resonances in the high-field region (1.0–2.5 ppm) of the 1D $^1$H NMR spectrum (Fig. 2) could be attributed to the equatorial and axial methylene protons of the Kdo residue; the doublet of doublets pattern at 2.284 ppm ($J$ = 4.0, 10.5 Hz) was characteristic of the equatorial H-3 proton, while the triplet at 1.965 ppm ($J$ = 11.6, 11.6 Hz) was indicative of the axial H-3 proton. The chemical shifts of the methylene proton resonances from the Kdo residue indicated that it was in the pyranose ring form and has the α-D-configuration [15]. As expected, a cross-peak with $^{13}$C coordinate of 35.38 ppm in the 2D HMQC experiment could be attributed to the methylene carbon from the Kdo residue.

The $^1$H NMR spectrum of the deacylated LPS was assigned using COSY and TOCSY experiments (Fig. 4). The relative stereochemistries and ring sizes of the component monosaccharides were established from the $^1$H chemical shifts [16] and the magnitude of the coupling constants [17] (Table 1). Complete assignment of residues D, E, and F proved difficult due to the large degree of overlap of the anomeric signals of

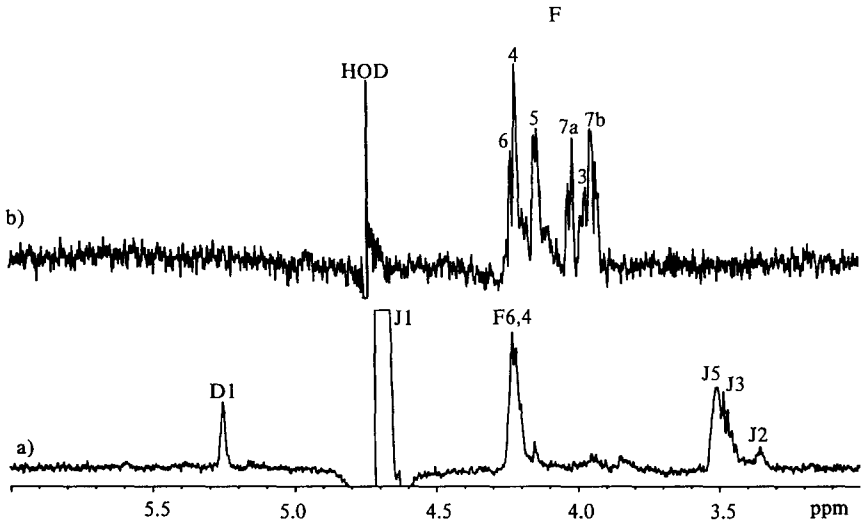


Fig. 5. (a) 1D NOESY spectrum using selective excitation of H1-J. The duration of the half-Gaussian pulse was 75 ms, the mixing time was 200 ms and 1280 scans were acquired. (b) 1D NOESY-TOCSY spectrum with a selective NOESY transfer from H1-J and a selective TOCSY transfer from H6/4-F with a NOE mixing time of 200 ms and a TOCSY mixing time of 23 ms including two trim pulses of 2.5 ms each, and 16384 scans. The spectrum was recorded in $D_2O$ at pH 3.5 and 300 K. The assignments of the resonances of the protons of residue F are indicated.

these residues. To fully assign residue F, selective 1D NOESY and TOCSY experiments were performed. Irradiation of the signal at 4.225 ppm (corresponding to the protons at F-4 and F-6) in the 1D NOESY spectrum from J-1, enabled assignment of the remaining protons from residue F (Fig. 5). Assignment of the $^{13}C$ resonances was carried out by direct correlation of the $^{1}H$ resonances in an HMQC experiment, a 1D $^{13}C$ NMR experiment, and by comparison of $^{13}C$ resonances with similar chemical shift data [18,19].

Three $^{1}H$ subspectra were attributed to heptose residues (B, F, H) on the basis of their small $J_{1,2}$ ($\sim 1$ Hz) and $J_{2,3}$ ($\sim 3$ Hz) coupling constant values, which pointed to *manno*-pyranosyl ring systems. The $\alpha$-configurations were evident for all of these residues from the occurrence of a single residue NOE (Fig. 6) between the H-1 and H-2 resonances [20]. Large vicinal proton coupling constants for $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ (8–10 Hz) indicated the presence of seven hexopyranosyl residues having the *gluco*-configuration (A, D, E, G, I, J, K). From the magnitude of the $J_{1,2}$ couplings, three of these residues (I, J, K) were assigned to the $\beta$-D-configuration ($J_{1,2} \sim 8$ Hz), and the remaining four residues (A, D, E, G) were assigned the $\alpha$-D-configuration ($J_{1,2} \sim 4$ Hz) (Table 1). The $^{1}H$-subspectra from residue C (Fig. 7) were very similar to the resonances previously reported [21] for the $\beta$-elimination product $\beta$-L-*threo*-hex-4-enuronopyranosyl residue, originating from 4 N KOH treatment of a 4-substituted galacturonic acid residue with the characteristic low-field signal at 5.916 ppm representing H-4 of this residue. The characteristic carbon coordinate for C-4 (107.00 ppm) was identified in the

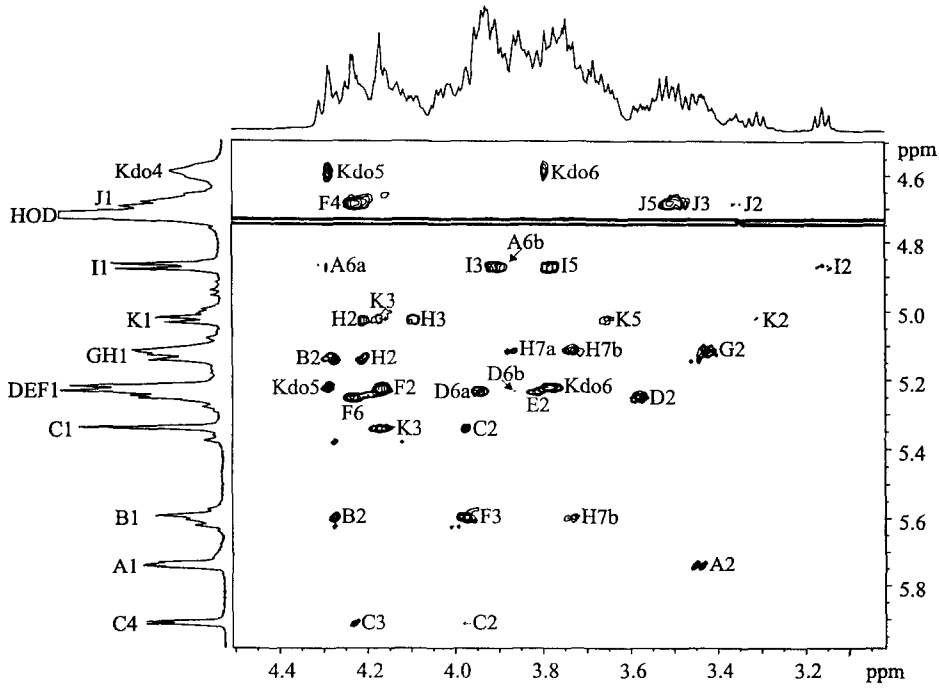


Fig. 6. 2D NOESY spectrum of the deacylated LPS from *Vibrio cholerae* O139 showing NOE connectivities relating anomeric proton resonances of each residue. The spectrum was recorded in $D_2O$ at pH 3.5 and 300 K.

Table 2
$^{13}C$ NMR chemical shift data for *Vibrio cholerae* O139 deacylated LPS [a]

| Sugar residue [b] | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 |
|---|---|---|---|---|---|---|---|---|
| A αGlcN | 92.75 | 54.81 | 70.32 | 70.50 | 73.35 | 70.72 | – | – |
| B αHep | 99.67 | 80.24 | 70.73 | 67.31 | 71.63 | nr [c] | nr | – |
| C | 99.67 | 69.85 | 66.27 | 107.00 | 146.96 | 169.53 | – | – |
| D αGlc | 101.85 | 72.92 | 73.78 | 69.90 | 64.70 | 70.76 | – | – |
| E αGlc | 99.67 | 71.83 | nr | nr | nr | nr | – | – |
| F αHep | 99.95 | 70.85 | 72.05 | 75.50 | 72.48 | 80.25 | 63.82 | – |
| G αGlcN | 96.22 | 54.81 | 70.42 | 69.99 | 73.74 | 60.83 | – | – |
| H αHep | 102.25 | 80.27 | 79.36 | 65.52 | nr | nr | 70.02 | – |
| I βGlcN | 100.07 | 56.53 | 73.75 | 75.47 | 74.65 | 63.38 | – | – |
| J βGlc | 103.99 | 74.40 | 76.82 | 71.25 | 75.94 | 62.57 | – | – |
| K βQuiN | 97.08 | 55.23 | 73.78 | 75.94 | 73.75 | 17.27 | – | – |
| Kdo | 175.05 | 100.45 | 35.38 | 70.75 | 70.75 | 72.89 | nr | nr |

[a] Measured at pH 3.5, 300 K from internal acetone (31.07 ppm).
[b] Sugar residues A–K, Kdo are as in Fig. 5.
[c] nr, not resolved.

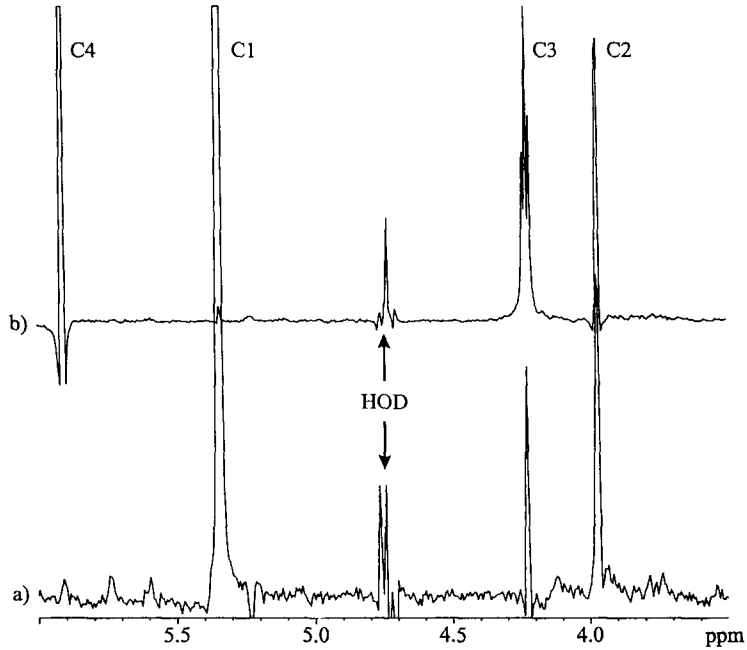


Fig. 7. 1D $^{1}H$ NMR subspectra from (a) H-1 of residue C and (b) H-4 of residue C, of the 2D TOCSY spectrum of the deacylated LPS from *Vibrio cholera* O139. The spectrum was recorded in $D_2O$ at pH 3.5 and 300 K.

HMQC spectrum (Fig. 3), and a signal for C-5 (146.96 ppm) was observed in the $^{13}C$ NMR spectrum. This uronic acid was characterised as a D-galactopyranosyl uronic acid residue since it was found to yield D-galactose on reduction ($NaBH_4$) of its derived methyl ester.

The residues A, G, I, and K were identified as amino sugars on the basis of their C-2 chemical shifts. The H-2 resonances from these residues (3.439, 3.413, 3.159, and 3.305 ppm) were correlated in the HMQC experiment to the $^{13}C$ resonances at 54.81, 54.81, 56.53, and 55.23 ppm, respectively, the chemical shifts being diagnostic of amino-substituted carbons. Residue K was subsequently identified as a 2-amino-2,6-dideoxy-D-glucopyranosyl residue (quinovosamine) as the connectivity pathway for its anomeric proton led to a doublet at 1.384 ppm corresponding to the methyl protons at position 6.

The anomeric configurations of the glycopyranosyl residues were confirmed from intraresidue NOE's relating the anomeric proton resonances to protons within the same pyranose ring systems. The $\beta$-linked glycopyranoses showed NOE's between H-1, H-3, and H-5 resonances within the same ring system, whereas the $\alpha$-linked sugars showed intraresidue NOE's between the anomeric proton and H-2 resonances only (Fig. 6).

The sequence of the glycosyl residues within the carbohydrate backbone of the LPS was mainly established from interresidue $^{1}H$–$^{1}H$ NOE measurements, between anomeric and aglyconic protons on adjacent glycosyl residues in a 2D NOESY experiment. Part of the NOESY contour plot is shown in Fig. 6. For all hexoses and heptoses an NOE

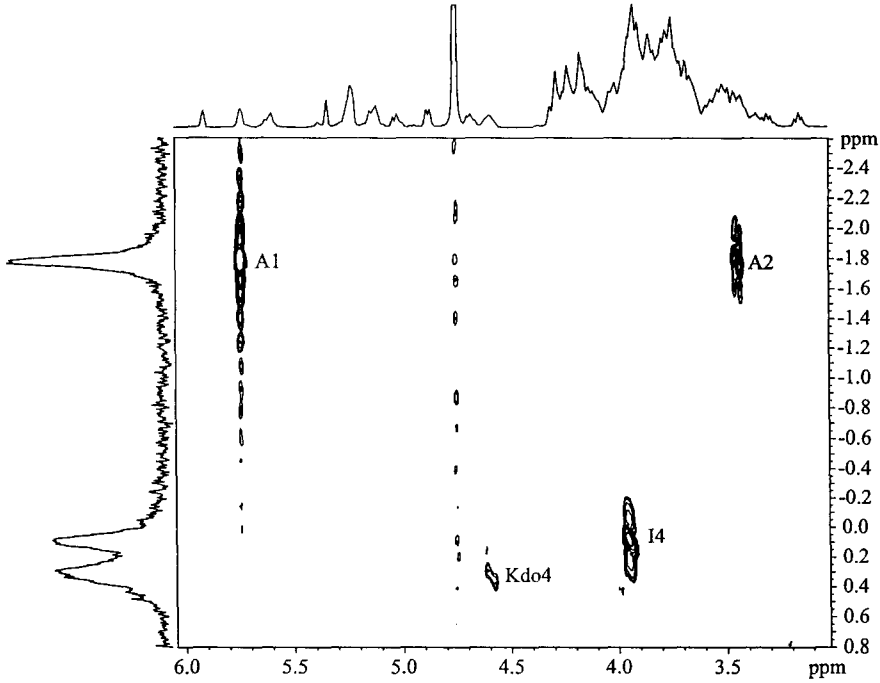


Fig. 8. Heteronuclear $^{31}P-^{1}H$ chemical shift correlation map for the deacylated LPS from *Vibrio cholerae* O139. The spectrum was recorded in $D_2O$ at pH 3.5 and 300 K. The assignments are as indicated.

between H-1 and the proton at the attachment site of the glycosylated sugar was observed, thus allowing the direct identification of the linkage position. Localisation of the D-fructose residue was determined by methylation analysis. A D-glucose residue was found to be deuterated at the 6-position following methylation analysis using deuterated methyl iodide after acid methanolysis of the partially methylated sample. By comparison to published data [22], it was deduced that the D-fructose sugar is attached to the 6-position of the β-D-glucose residue. The following data suggested the presence of a further heptose residue (X) not readily detected in the deacylated LPS: (a) The presence of 2,6-*O*-disubstituted Hep*p* and terminal Hep*p* residues in the derived methylated alditol acetates. (b) An observed molecular ion in the electrospray mass spectrum of the deacylated LPS, indicative of a loss of a heptose unit from the molecular ion. These data can only be explained by the presence of a terminal heptose residue (X) linked to the 6-position of residue B.

The $^{31}P$ NMR spectrum revealed three signals between 1 and $-2$ ppm consistent with phosphomonoester residues. A 2D $^{31}P-^{1}H$ HMQC experiment (Fig. 8) showed that the phosphate residues were linked to proton resonances at 5.744, 3.953, and 4.589 ppm, A-1, I-4, and Kdo-4, respectively.

The structure elucidated from the above data is shown in Fig. 9. In accordance with this structure, electrospray mass spectrometry of the deacylated LPS gave a molecular

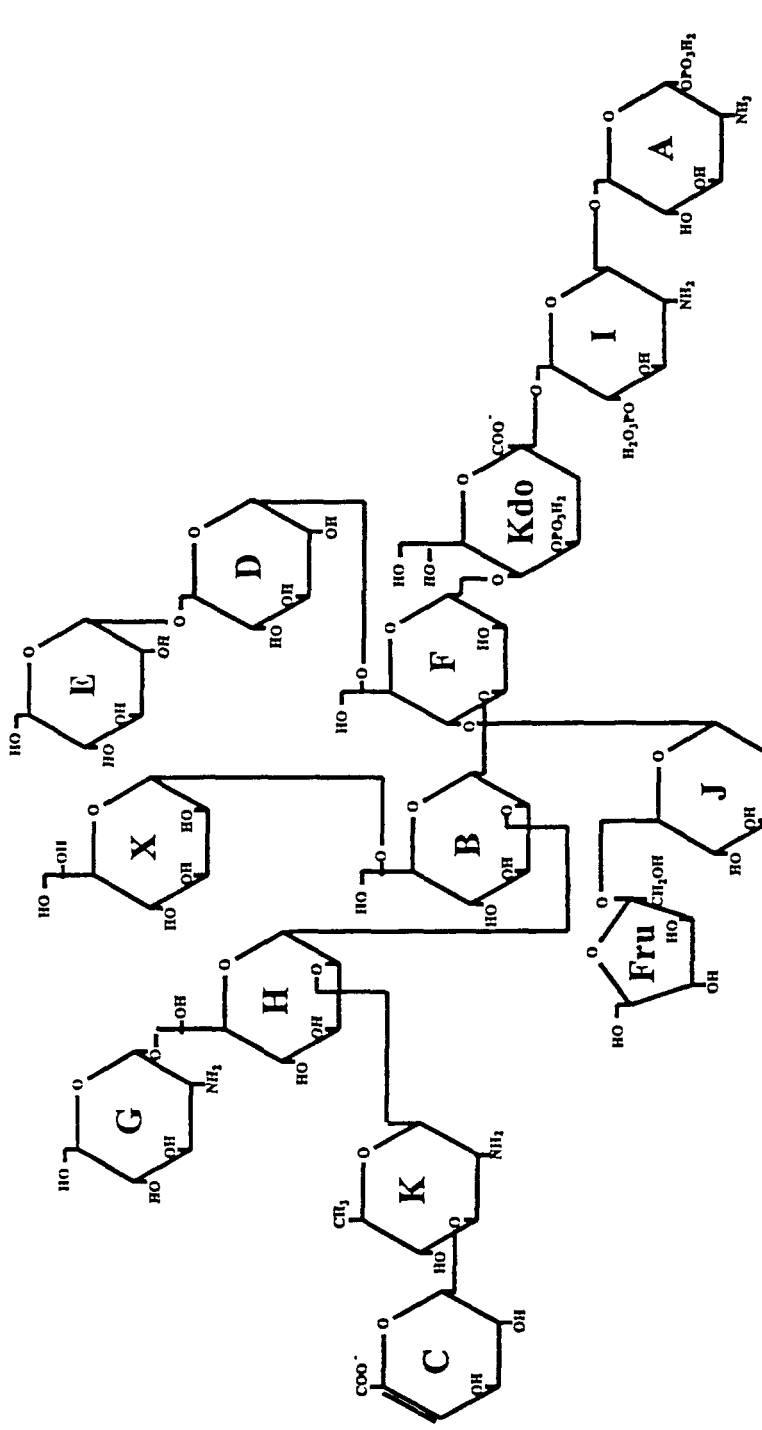


Fig. 9. Chemical structure of the tetradecasaccharide triphosphate isolated from the LPS of *Vibrio cholerae* O139. Glcosyl residues are labelled A–K, X. Kdo and Fru as used in Table 1 and Table 2 and throughout the text.

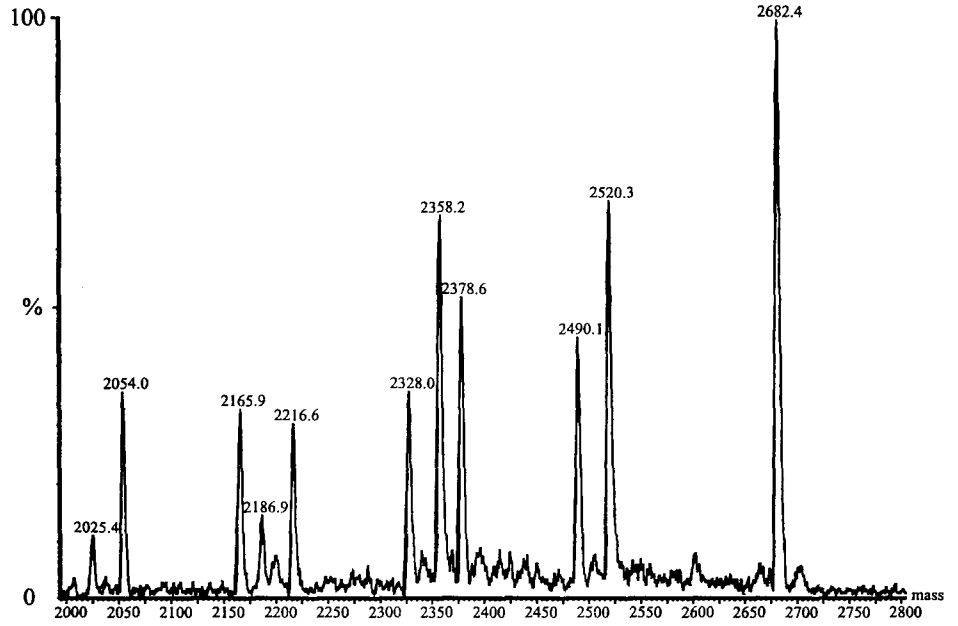


Fig. 10. Electrospray mass spectrum of the deacylated LPS from *Vibrio cholerae* O139. Peaks are represented as relative intensities of their mass in amu.

ion of $m/z = 2682$, consistent with the composition and structure deduced (Fig. 10). Other moleclar ions observed in the spectrum included $m/z$ 2520 (loss of a hexose residue), $m/z$ 2358 (loss of two hexose residues), $m/z$ 2490 (loss of a heptose residue), $m/z$ 2328 (loss of a heptose and a hexose residues) and $m/z$ 2166 (loss of two hexoses and a heptose residue). Significantly a molecular ion with $m/z$ 2378, indicative of loss of residue C and 2-amino-2,6-dideoxy-D-glucose, was observed. A further series of molecular ions corresponding to similar losses as observed for $m/z$ 2682 were seen from this molecular ion, namely $m/z$ 2217, 2187, 2054, and 2024 resulting from losses of a hexose, a heptose, two hexoses, and a heptose and a hexose, respectively. The molecular ion with $m/z$ 2378 therefore probably corresponds to rough (R) form LPS.

## 3. Discussion

The deduced structure for the *Vibrio cholerae* O139 LPS possesses the established features of *Vibrio* cores, namely the single Kdo residue substituted by a phosphate residue at the 4-position [23] and the presence of D-fructose linked to the 6-position of a D-glucose residue [24]. The common trisaccharide of many core regions $\alpha$-Hep*p*-(1 → 3)-$\alpha$-Hep*p*-(1 → 5)-$\alpha$-Kdo is also present [25].

Elucidation of the structure of the *Vibrio cholerae* O139 LPS enabled a direct comparison with the previously published lipid A-core region of LPS from *Vibrio*

*cholerae* O1 [12]. The two structures are almost identical, and, therefore, this chemical evidence supports the genetic data that suggested only the *rfb* cluster (the DNA region responsible for O-antigen biosynthesis) was altered in the biogenesis of O139 from O1 [7]. Interestingly, an additional glucose residue 'E' is found in O139 when compared to O1, though the authors of the O1 paper [12] suggest that an additional sugar (or suggars) may be present in the wild type version of their rough mutant strain. However, in O139 residue 'E' is clearly not the point of attachment of the O-chain, which is attached to the core via the heptose residue 'H'. It is possible that the attachment point of the O-chain differs between these two strains. It is known from genetic studies on the cloning of the *rfb* cluster from O1 that two glucose residues are required in the core region for the most efficient attachment of the *Vibrio cholerae* O1-antigen [26], though it is not proven that the O-antigen is actually attached to a glucose residue.

By comparison to the structure of the capsular polysaccharide of O139 [10,11], and knowing that residue 'C' is the $\beta$-elimination product from KOH treatment of a 4-substituted galacturonic acid residue [21], it is clear that the remnants of the O139 O-antigen in the deacylated LPS are identical to the D-galacturonic acid $\alpha$-(1 → 3)-2-amino-2,6-dideoxy-D-glucose portion of the capsular polysaccharide. The identification of 3,6-dideoxy-L-*xylo*-hexose in mild acid hydrolysates of the LPS is additional evidence that the capsule and the O-antigen may share the same repeating unit. This observation is consistent with the immunological data, which showed that the capsule and LPS shared an epitope defined by an O139-specific monoclonal antibody [14]. The migration of the LPS as two distinct bands on DOC-PAGE can also be related to the identification of the structural analogue to the capsular polysaccharide attached to the outer core. This data, when considered in conjunction with the electro-spray mass spectrum, is perhaps indicative of the two bands observed in the DOC–PAGE corresponding to R- and SR-forms of the O139 LPS.

This study has therefore shown that the genetic events that led to the biogenesis of strain O139 had no effect on the structure of the lipid A-core region.

## 4. Experimental

*Isolation of lipopolysaccharide.—Vibrio cholerae* serotype O139 (NRCC #4740) was grown on sheep's blood agar plates (Columbia agar with 5% sheep's blood) at 37 °C for 17 h. Cells were suspended in 10% phenol and harvested by centrifugation (10,000 *g*). LPS was isolated by the aqueous phenol method [12].

*Purification of lipopolysaccharide.*—LPS (200 mg) was deacylated by treatment with 4 N KOH (~ 4 mL per 50 mg) at 120 °C for 16 h [27]. The solution was cooled, neutralised and centrifuged. The supernatant solution was lyophilised, and the resulting lipooligosaccharide was purified by gel-filtration chromatography on a Sephadex G-10 column eluted with pyridinium acetate (0.05 M, pH 4.5). Column eluants were monitored for changes in refractive index, and collected fractions (4.5 mL) were assayed colorimetrically for neutral glycoses [28], yielding 30 mg of purified deacylated LPS.

*DOC–PAGE.*—Polyacrylamide gel electrophoresis (PAGE) was performed by using the system of Laemmli and Favre [29] as modified by Komuro and Galanos [30] with

deoxycholate (DOC) as the detergent. The separation gel contained final concentrations of 13% acrylamide, 0.5% DOC, and 375 mM Tris–HCl (pH 8.8), with the stacking gel containing 4% acrylamide, 0.5% DOC, and 125 mM Tris–HCl (pH 6.8). LPS samples were prepared at a concentration of 0.1% (w/v) in the sample buffer [0.25% DOC, 175 mM Tris–HCl (pH 6.8), 10% glycerol]. Bromophenol blue (0.002% in sample buffer) was used as the tracking dye. The electrode buffer (pH 8.4) was composed of DOC (2.5 g $L^{-1}$), glycine (14.4 g $L^{-1}$), and Tris (3.0 g $L^{-1}$). Electrophoresis was performed at a constant current of 30 mA. Gels were fixed in an aqueous solution of 40% ethanol and 5% acetic acid. LPS bands were stained and visualised by silver staining as described by Tsai and Frasch [31].

*Analytical methods.*—Glycoses were determined by GLC–MS as their alditol acetate derivatives [32]. Samples (0.5–1.0 mg) were hydrolysed with either 2 M TFA for 90 min at 125 °C, or by mild conditions with 0.02 M $H_2SO_4$ for 30 min at 100 °C. The liberated glycoses were reduced ($NaBH_4$) and acetylated ($Ac_2O$) [33]. The absolute configurations of the LPS components were identified by GLC analysis of their acetylated (*S*)-2-butyl glycosides [32] and by the determination of the specific optical rotation of the glycans liberated by graded acid hydrolysis of the native, deacylated (anhydrous hydrazine, 37 °C, 30 min) [34] and dephosphorylated (48% HF, 4 °C, 48 h) [32] LPS preparations. The glycoses were isolated by preparative paper chromatography on Whatman No. 1 paper using 10:3:3 *n*-butanol–pyridine–water as the mobile phase.

*Methylation analysis.*—Oligosaccharide samples (2–4 mg) were methylated with iodomethane in dimethyl sulfoxide containing an excess of potassium (methylsulphinyl)methanide [35]. The methylated oligosaccharides were purified on a Sep-Pak $C_{18}$ cartridge [36]. The purified methylated oligosaccharides were hydrolysed by either 2 or 4 M TFA at 120 °C for 1 h, or by initial treatment with 90% (v/v) formic acid at 100 °C for 1 h, followed by overnight treatment with 0.13 M $H_2SO_4$ at 100 °C. In an attempt to localise the fructose residue of the oligosaccharide, the LPS was deacylated with anhydrous hydrazine at 103 °C for 48 h [24]. The product deacylated in this way was methylated as above, and then treated with methanolic 0.01 M HCl at 86 °C for 20 min and remethylated using deuterated iodomethane and hydrolysed with 2 M TFA as above. Hydrolysis products were then reduced ($NaBD_4$), acetylated and analysed by GLC–MS.

*Electrospray mass spectrometry.*—Samples were analysed on a VG Quattro triple quadrupole mass spectrometer (Fisons Instruments) with an electrospray ion source. Deacylated samples were dissolved in a solvent composed of 9:9:2 acetonitrile–water–10% acetic acid. The electrospray tip voltage was 2.5 kV, and the mass spectrometer was scanned from $m/z$ 150–2500 with a scan time of 10 s.

*NMR spectroscopy.*—NMR spectra were obtained on a Bruker AMX 500 or AMX 600 spectrometer using standard Bruker software. Measurements were made at pH 3.5, 300 K at concentrations ~ 10 mg $mL^{-1}$ in $D_2O$, subsequent to several lyophilisations with $D_2O$.

1D $^1H$ NMR spectra were measured at 600.14 MHz using a spectral width of 6.0 kHz. 1D $^{13}C$ NMR spectra were measured at 125.77 MHz using a spectral width of 45.4 kHz. Acetone was used as an internal standard and chemical shifts were referenced to the methyl resonance ($\delta_H$, 2.225 ppm; $\delta_C$, 31.07 ppm). 2D homonuclear proton

correlation experiments (COSY), total correlation experiments (TOCSY) and nuclear Overhauser effect experiments (NOESY) were measured over a spectral width of 3.62 kHz, using data sets ($t_1 \times t_2$) of 4096 × 1024, and 16 scans were acquired. A mixing time of 200 ms was employed for the NOESY experiment.

Heteronuclear 2D $^{13}C-^{1}H$ chemical shift correlations were measured in the $^{1}H$-detected mode via multiple quantum coherence (HMQC) with proton decoupling in the $^{13}C$ domain, using data sets of 2048 × 512 points and spectral widths of 4.24 and 16.6 kHz for $^{1}H$ and $^{13}C$ domains, respectively. A total of 64 scans were acquired for each $t_1$ value.

$^{31}P$ NMR spectra were measured at 202.5 MHz by employing spectral widths of 41.6 kHz, and phosphoric acid (85%) was used as the external standard ($\delta_P$, 0.0 ppm). $^{31}P-^{1}H$ correlations (HMQC) were made in the $^{1}H$-detected mode by using a data matrix of 2048 × 128 points, sweep widths of 2 kHz for $^{31}P$ and 4.5 kHz for $^{1}H$, and a delay of 60 ms.

To fully assign the spectra selective 1D TOCSY, 1D NOESY, and 1D analogues of 3D NOESY–TOCSY and TOCSY–NOESY experiments [37] were performed using a 270° Gaussian pulse (1024 points) truncated at 2.5% for selective excitation. The pulse width of the selective pulses was 30–75 ms. Spin lock mixing times for the TOCSY varied from 20 to 80 ms. The NOESY mixing time was 200 ms.

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