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Lipo-oligosaccharide of *Campylobacter lari* strain PC 637. Structure of the liberated oligosaccharide and an associated extracellular polysaccharide

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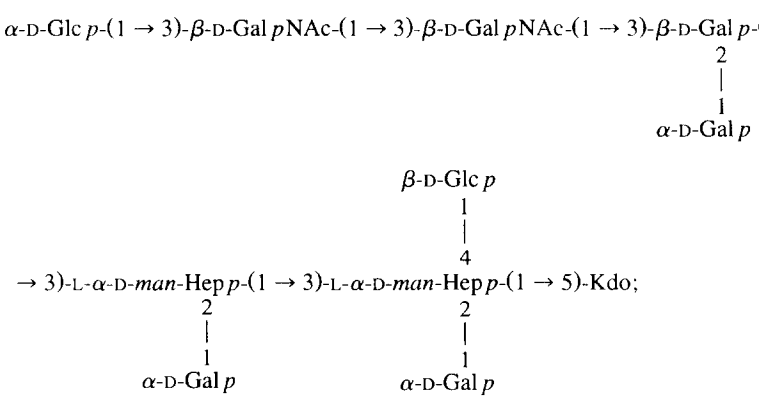
Abstract

Lipo-oligosaccharide from phenol–water extraction of cells of *Campylobacter lari* strain PC 637 was separated as a water-insoluble gel of low relative molecular mass (M_r) from a water-soluble extracellular polysaccharide of high M_r. Structural investigations were performed on the lipo-oligosaccharide and the extracellular polysaccharide, variously using ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy, linkage analysis, and fast atom bombardment-mass spectrometry of per-methylated derivatives of the glycans and their products of chemical and enzymic degradation. The following structures are proposed for the highly branched oligosaccharide region:



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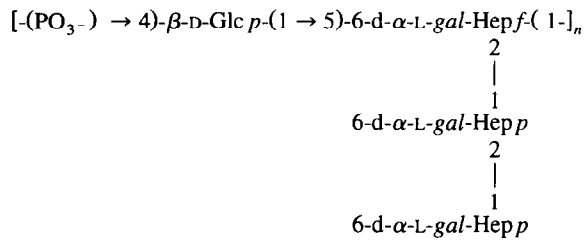
and for the tetraglycosyl phosphate repeating unit of the extracellular polysaccharide:



Keywords: *Campylobacter lari* PC 637; Lipo-oligosaccharide; Extracellular polysaccharide; Tetraglycosyl phosphate polymer

1. Introduction

Campylobacter species, especially *C. jejuni* and *C. coli*, are leading causes of human enteritis. *N*-Acetylneuraminic acid (Neu5Ac) is a constituent of the core oligosaccharide region of the lipopolysaccharides (LPS) of low M_r of some frequently encountered *C. jejuni* serotypes [1–5]. The possibility that this constituent might be a virulence factor prompted an examination of LPS from other *Campylobacter* isolates. One such isolate had been identified provisionally as a strain (PC 637) of *C. lari*, which is widely present in the environment but rarely encountered as a human pathogen. Compositional analysis of the LPS from this isolate showed that Neu5Ac was absent, but revealed the presence of a 6-deoxyheptose, which was different from the 6-deoxy-D-*altro*-heptose of the O antigen chains from the LPS of *C. jejuni* serotypes O:23 and O:36 [6]. We report herein structural studies on the oligosaccharide (OS) region of a more properly designated lipo-oligosaccharide (LOS), and of an associated extracellular polysaccharide (PS). Immunoblot experiments with homologous antisera reactions showed that the LOS from strain PC 637 was of low molecular mass characteristic of core OS linked to lipid A. No ladder-like banding patterns indicative of extended O antigen chains were seen. The antisera also reacted with molecules of low molecular mass from the *C. lari* type strain (ATCC 35221). A parallel study of the *C. lari* type strain was therefore undertaken, and compositional analysis of the LPS preparation revealed yet another 6-deoxyheptose as a constituent. A comparative study of the LPS from the type strain revealed that this preparation consisted of another LOS and an associated extracellular polysaccharide, both differing from those of *C. lari* PC 637. These results are presented in the following paper [7].

2. Experimental

Isolation of lipo-oligosaccharide, liberated oligosaccharide (PC 637 ***OS1****), and extracellular polysaccharide (PC 637* ***P1****).*—The strain *C. lari* PC 637 was a clinical isolate from the Hospital for Sick Children, Toronto, Ontario. Bacterial culture condi-

tions were those described previously [8], and SDS–PAGE experiments with immunoblotting were performed as reported [2]. Bacterial cells were extracted by the phenol–water method [9], and the aqueous layer was removed, dialysed for 48 h against distilled water, and ultracentrifuged (100,000 *g* for 4 h) to yield LPS as a gel-like pellet. After removal of LPS, the extracellular polysaccharide (PC 637 **P1**) was obtained from the supernatant liquid by lyophilization. The insoluble LPS was hydrolysed in aqueous 1% acetic acid for 1 h at 100°C, insoluble lipid A was removed by centrifugation, and the water-soluble material was fractionated by gel permeation chromatography on Bio-Gel P-2 (1 × 105 cm) to give PC 637 **OS1**.

Analytical procedures.—GLC was carried out on a Hewlett–Packard model 5890A chromatograph. Separations were carried out using capillary columns with the following programs: DB-23 (30 m × 0.25 mm i.d.) (A) isothermally at 220°C and (B) isothermally at 190°C; DB-17 (15 m × 0.25 mm i.d.) (C) isothermally at 190°C; DB-23 (30 m × 0.25 mm i.d.) (D) at 190°C (10 min), 200–250°C at 2°C/min; DB-5 (15 m × 0.25 mm i.d.) (E) from 180 to 230°C at 3°C/min. EI GLC-MS was performed using a KRATOS Analytical Profile instrument with electron impact ionization at 70 eV. CI GLC-MS was performed using a VGZAB-SE spectrometer, at 100 eV, 1 mA emission current, source temperature 180°C, and ammonia reagent gas.

FAB-mass spectra were acquired as described in previous papers [3–5]. Interpretations of positive ion mass spectra of permethylated derivatives were as described by Dell et al. [10] and used in an earlier publication [3]. Spectra of methylated derivatives from OS with reducing Kdo termini showed that the major component of the molecular ion cluster corresponded to that calculated for $[M + H]^+$ or other adduct ions. Related ions at $([M + H]^+ - 46)$ and $([M + H]^+ - 116)$ were sometimes present and their co-occurrence aided in the identification of molecular ions.

Other analytical procedures.—Glycose analyses as alditol acetate derivatives were performed by GLC using program A. Enantiomeric configurations were established by conversion into 2-(*R*)- and 2-(*S*)-butyl glycosides [11] and GLC analysis of acetylated derivatives using program D. The presence of Kdo was confirmed by methanolysis (0.75 M CF_3CO_2H at 100°C for 6 h), followed by acetylation and GLC-MS analysis using program E. Phosphate analyses were performed by the method of Chen et al. [12]. Methylations of oligo- and poly-saccharides (0.3–0.5 mg) were performed in dimethyl sulfoxide by the method of Ciucanu and Kerek [13] as described previously [3–5]. Determination of the linkage site of the Kdo residue was achieved by the procedure of Tacken et al. [14] with partially methylated alditol acetate analysis using program B, yielding a 3-deoxyoctitol derivative in addition to the previously characterized derivatives.

Miscellaneous degradations.—*O*-Dephosphorylations were carried out by treatment with 48% aqueous HF at 4°C for 48 h. Excess HF was removed in a vacuum desiccator over KOH, and the products were fractionated by GPC on Bio-Gel P-4. For the Smith degradation, core oligosaccharides (ca. 4 mg) were pre-reduced with $NaBD_4$ (3 mg) in water (4 mL) at room temperature for 12 h. The oligosaccharide alditols were successively oxidized with $NaIO_4$, as described by Pritchard et al. [15], desalted by GPC on Bio-Gel P-2, reduced with $NaBD_4$, decationized and concentrated with methanol, hydrolysed with 1 M CF_3CO_2H at room temperature for 3 h, and aldonolactones (from

modified Kdo residues) were reduced by treatment again with $NaBD_4$ [3], and isolated by GPC on Bio-Gel P-2.

Digestions with exo-glycosidases were carried out in 0.1 M sodium acetate buffer at the pH values, temperatures, and times stated. Enzymes were denatured by heating the solutions at 100°C for 20 min, precipitates were removed by centrifugation, and the products were isolated by GPC on Bio-Gel P-2. Digestions with almond β-D-glucosidase (Sigma) were run at 38°C and pH 5.0 for periods of 12–48 h; digestions with coffee bean α-D-galactosidase (Sigma) were run at 25°C and pH 6.8 for periods of 12–14 h; and digestions with yeast α-D-glucosidase (Boehringer Mannheim) were run at 37°C and pH 6.0 for periods of 12–24 h.

^1H, ^{13}C, and ^{31}P NMR spectroscopy.—One-dimensional (1D) and two-dimensional (2 D) experiments were performed on Bruker AM 300 and ARX 400 spectrometers in the Fourier transform mode. Oligosaccharide and glycan samples were exchanged several times with D_2O (99.8%). Chemical shifts were measured relative to internal acetone, δ 2.225 for ^1H NMR and δ 31.4 for ^{13}C NMR. Chemical shifts for ^{31}P NMR were measured with reference to external orthophosphoric acid (δ 0.0).

Two-dimensional experiments were performed with the following parameters: COSY, COSYRCT, and TOCSY [512 × 1024 data matrix, zero-filled to 1024 data points in t_1, spectral width of 2008 Hz, 64 or 72 scans per t_1 value, 1.0 or 1.5 s for the recycle delay, mixing time of 80 ms for TOCSY, unshifted sine-bell filtering in t_1 and t_2 for COSY and COSYRCT, but shifted sine-squared filtering in t_1 and t_2 for TOCSY as for all phase-sensitive experiments]; for NOESY [256 × 2058 data matrix, zero-filled to 1024 data points in t_1, 128 scans per t_1 value, recycle delay of 2.0 s]; for HMQC [256 × 2058 data matrix, zero-filled to 1024 data points in t_1, 64 scans for t_1 value, recycle delay of 1.0 s, fixed delay of 3.5 ms, and pulse angle of 60°]; for ^1H–^{31}P HMBC [512 × 512 data matrix, zero-filled to 1024 data points, 128 scans per t_1 value]. 1D TOCSY experiments were performed using the same parameters as for 2D TOCSY [16].

3. Results and discussion

PC 637 **OS1** had a composition of Gal, Glc, LD-Hep[1], and GalNAc in the approximate molar ratio of 4:2:2:1. As in other studies [1–5], analyses of the proportions of HexNAc residues gave low values. All sugars had the D-enantiomeric configuration as shown by the formation of acetylated chiral 2-butyl glycosides [11]. In addition, Kdo was detected through the formation of acetylated methyl ester methyl ketosides [17], and the presence of this constituent was confirmed during methylation linkage analysis and from the associated fast atom bombardment-mass spectrometric (FABMS) data. The strategy adopted in the structural characterization of the oligosaccharide region of the LOS and assigned structures for OS derivatives are summarized in Fig. 1.

*Composition, linkage, and sequence determination of oligosaccharide **OS1**.*—The FABMS spectrum of permethylated **OS1** (Fig. 2) gave a pseudomolecular ion $[M + Na]^+$

[1] Unless otherwise stated, LD-Hep or Hep refers to L-*glycero*-D-*manno*-heptose.

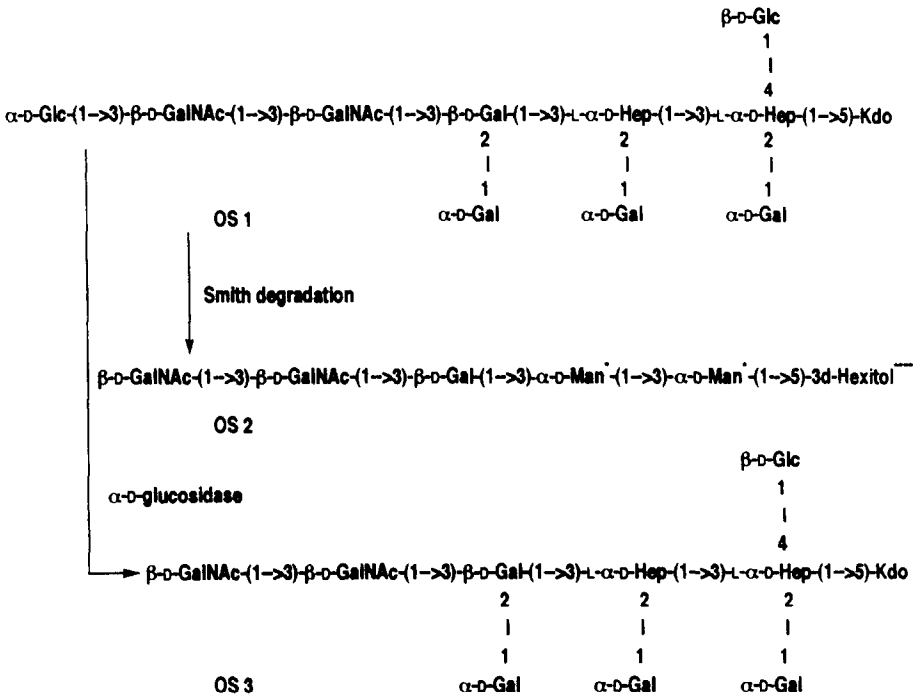


Fig. 1. Genesis of OS fractions from core **OS1** initially liberated from *C. lari* PC 637 LOS. 3-dHexitol = 3-deoxyhexitol. Asterisks show incorporation of ^{2}H isotope.

at m/z 2555 corresponding to a composition of $Hex_6Hep_2HexNAc_2Kdo$. The highly branched nature of the OS chain and the identities of the constituent units were clearly shown in the methylation linkage analysis (Table 1) with accompanying GLC-MS. Mass increments in fragment ions along the chain in the FAB-mass spectrum showed the nature and the sequence of the residues at branch points and their attendant side-chains, and allowed a provisional structure to be proposed for **OS1**, but without specification of the five terminal hexopyranose residues (Fig. 3). ^{1}H NMR spectroscopy showed the presence of 10 anomeric protons, and starting from H-1 of each residue, partial resonance assignments with approximate associated coupling constants were determined by COSY, COSYRCT, and TOCSY experiments (Table 2). The observed connectivities, especially those based on values for $^{3}J_{H2,H3}$ and $^{3}J_{H3,H4}$ led to assignments of ring configurations. However, assignments of resonances to individual residues in the partial structure (Fig. 1) required the generation of simpler oligosaccharides through specific degradations.

Smith degradation.—The provisional structure for **OS1** (Fig. 3) indicated that only the terminal residues would be vulnerable to periodate oxidation and that Smith degradation would be expected to yield an attenuated linear oligosaccharide. The degradation product (**OS2**) was converted into the permethylated derivative for FABMS (Fig. 3) and linkage analysis (Table 1). Despite less than complete quantitation from the linkage analysis, the qualitative identification of the constituent residues, together with

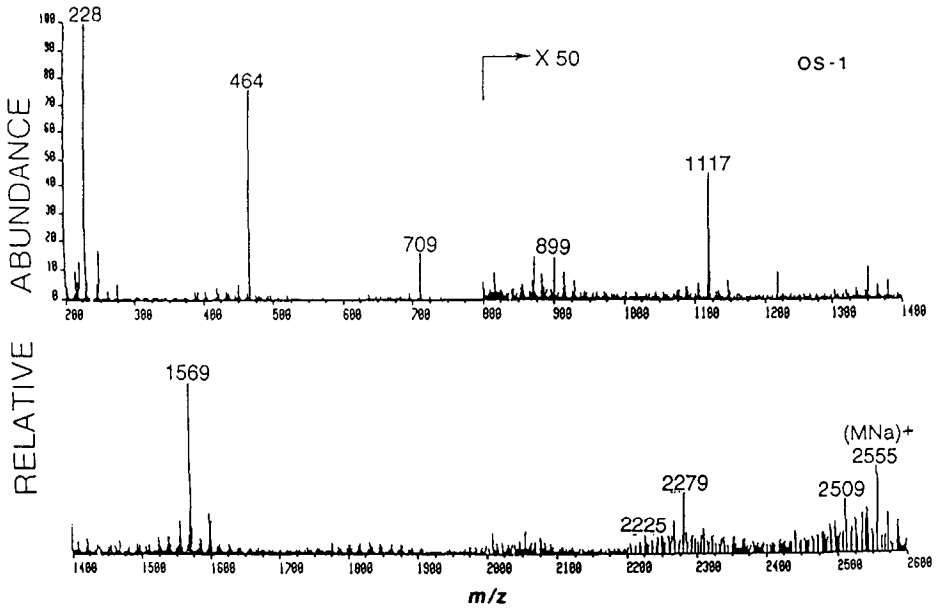


Fig. 2. Positive ion FABMS spectrum of **OS1** from the core oligosaccharide region of *C. lari* PC 637 LOS.

Table 1
Methylation linkage analysis of methylated core oligosaccharide derivatives [a] from the LPS of *C. lari* strain PC 637

Methylated sugar (and linkage type)	Relative proportions in oligosaccharides [b]		
	OS1	**OS2**	**OS3**
2,3,4,6-Me_4Glc [Glc]	2		ca. 1.5
2,3,4,6-Me_4Gal [Gal]	3		3
3,4,6-Me_3N-MeGalNAc [GalNAc]		1	ca. 0.5
4,6-Me_2N-MeGalNAc [−3 GalNAc]	2	1	ca. 1.5
2,4,6-Me_3Gal [−3 Gal]		1	
4,6-Me_2Gal [branch]	1		1
2,4,6-Me_3Man* [−3 Man*] [c]		2	
4,6,7-Me_3LD-Hep [branch]	1		1
6,7-Me_2LD-Hep [d. branch]	1		1
1,2,4,6,7,8-Me_6-3-dOctitol [−5 Kdo]	1		nd
1,2,4,6-Me_4-3-dHexitol **** [from −5 Kdo] [d]		1	

[a] Core oligosaccharide derivatives are defined in Fig. 1.
[b] Unless otherwise stated in the test, in recognition of the microheterogeneity of oligosaccharide preparations relative proportions are expressed in integral ratios in conformity with the structures of the major components implied in the FAB-mass spectra.
[c] * Indicates incorporation of ^2H from treatment with NaB^2H_4.
[d] For origins of isotope labelling in 1,2,4,6-Me_4-3-dHexitol **** see fig. 3 in ref [3].
nd = not determined.

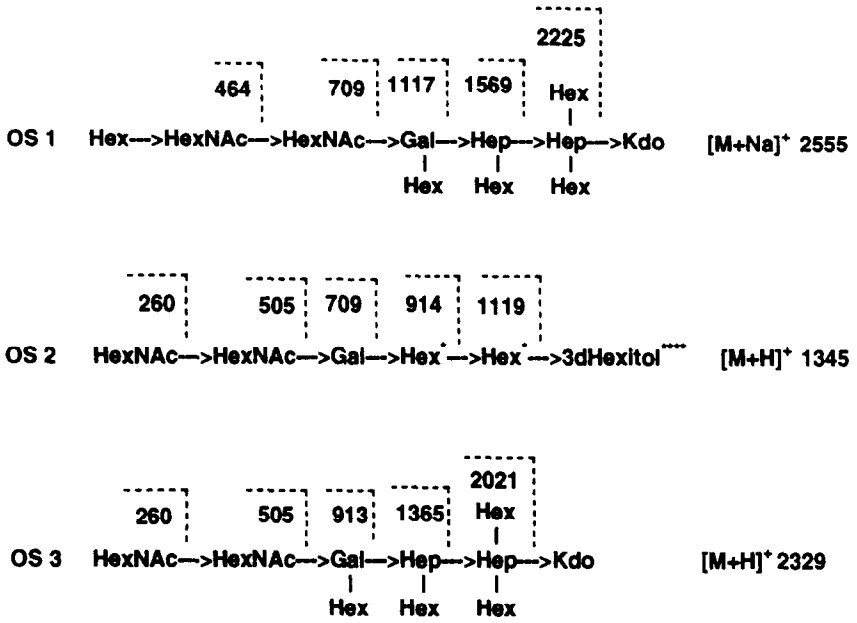


Fig. 3. Analysis of positive ion FABMS data for permethylated core OS derivatives from the core region of *C. lari* PC 637 LOS. For the origins of OS derivatives, see Fig. 1. 3-dHexitol = 3-deoxyhexitol. Asterisks show incorporation of ^{2}H isotope.

the FABMS data for a pseudomolecular ion $[M + H]^+$ at m/z 1345 and a series of glycosyloxonium ions with characteristic mass increments, defined the sequence of residues in **OS2** as a linear hexasaccharide (Fig. 3). The ^{1}H NMR spectrum of the degraded oligosaccharide showed the presence of H-1 resonances as broad signals (unresolved doublets) at δ 5.13 and 5.05 assignable to the two α-D-[$^{2}H_1$]Man residues, three anomeric resonances at δ 4.65 ($^{3}J_{H1,H2}$ 7.6 Hz), δ 4.47 ($^{3}J_{H1,H2}$ 7.7 Hz), and δ 4.34 ($^{3}J_{H1,H2}$ 7.1 Hz) assignable to residues with the *β-galacto* configuration, two of which were GalNAc residues with 3-proton signals for *N*-acetyl groups at δ 1.99 and 1.98, and the third was a Gal residue. A complete structure for the hexasaccharide **OS2** could therefore be proposed (Fig. 1).

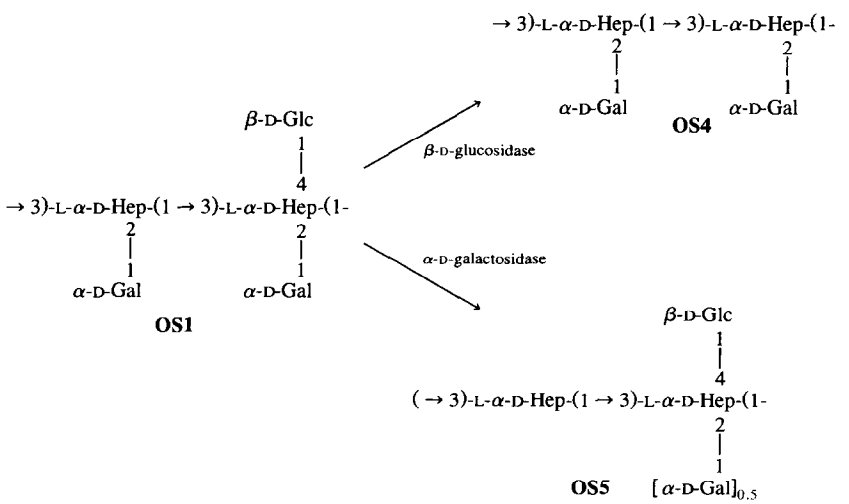
Locations and assignments of individual anomeric configurations of hexopyranose end groups.—The Smith degradation resulted in the removal of five hexopyranose end groups which included three α-D-Gal, one α-D-Glc, and one β-D-Glc units. Evidence for their locations was sought from the actions of exo-glycosidases on core **OS1** with characterizations of the degraded oligosaccharides. Despite incomplete degradation on digestion of **OS1** with yeast α-D-glucosidase, and a failure to separate the shortened product from **OS1**, the structure for **OS3** (Fig. 1) was inferred from the following observations. These were: (*i*) a decrease in the anomeric ^{1}H resonance for α-D-Glc at δ 5.35; (*ii*) the detection of new pseudomolecular ions for $[M + H]^+$ at m/z 2329 and $[(M + H) - 46]$ at m/z 2283, and a new series of fragment ions by FABMS (Fig. 3); (*iii*) a linkage analysis (Table 1) with ca. 50% loss of the α-D-Glc residues and the

Table 2
Selected ^{1}H NMR chemical shift (ppm) and coupling constant ($J_{n,n+1}$ Hz) data from 2D COSY, 2D COSYRCT, and 1D TOCSY of **OS1** from LPS of *C. lari* strain PC 637

Assigned ring configuration	H-1	H-2	H-3	H-4
α-Gal	5.41	3.76	3.40	
	(3.5)	(10.2)	(3.9)	
α-Glc	5.35	3.89	3.51	
	(3.5)	(10.1)	(10.1)	
α-Man [of LD-Hep]	5.13	4.09		
	(bs)	(3.8)		
α-Man [of LD-Hep]	5.05	4.13		
	(bs)	(4.0)		
α-Gal	5.04	3.88	3.84	
	(4.0)	(9.8)	(4.1)	
α-Gal	4.81	3.73	3.86	
	(4.0)	(9.9)	(4.0)	
β-Gal [or GalNAc]	4.71	3.55	4.11	
	(7.8)	(10.4)	(4.0)	
β-Gal [or GalNAc]	4.50	3.32	3.82	
	(7.7)	(9.8)	(3.8)	
β-Glc	4.41	3.30	3.47	3.81
	(8.0)	(9.8)	(10.0)	(9.9)
β-Gal [or GalNAc]	4.38	3.49	3.90	
	(7.5)	(10.0)	(3.9)	

J values in parentheses; bs = broad signal (unresolved doublet).

corresponding exposure of a new β-D-GalNAc end group. Confirmation of the respective locations of the anomeric α- and β-D-Glc residues came from digestion of **OS1** with almond β-D-glucosidase, giving **OS4**. Linkage analysis showed substantial libera-



Scheme 1. Action of exo-glycosidases on the inner branched region of **OS1**.

Table 3
^{1}H NMR chemical shift (ppm) and coupling constant (Hz) data from 2D COSY, 2D COSYRCT, and TOCSY experiments on extracellular polysaccharide **P1** and triglycosylalditol fraction **P3** from *C. lari* strain PC 637

Sugar residue	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5 ($J_{5,6}$)	H-6	H-7
PC 637 **P1**							
α-6-d *gal*Hep*p* (**b**)	5.56	3.95	3.83	3.75		1.89	3.58
	(2.5)	(10.2)	(2.9)	(2.8)		(m)	(m)
α-6-d *gal*Hep*p* (**a**)	5.11	3.84	3.77	3.72		1.89	3.58
	(3.4)	(10.5)	(2.9)	(3.0)		(m)	(m)
α-6-d *gal*Hep*f* (**c**)	5.0	3.76	3.80	3.90	3.60	1.87	3.3
	(4.3)	(5.4)	(2.4)	(1.6)	(m)	(m)	(m)
	(8.2, $J_{H,P}$)						
β-Glc *p* (**d**)	4.60	3.72	3.45	3.87			
	(7.9)	(10.0)	(9.9)	(6.8)			
PC 637 **P3**							
α-6-d *gal*Hep*p*	5.38	3.79	3.83	3.75		1.88	
	(3.1)	(8)	(3)	(3)		(m)	
α-6-d *gal*Hep*p*	4.98	3.63	3.77	3.72		1.88	
	(3.8)	(8)	(3)	(3)		(m)	
β-Glc *p*	4.34	3.15	3.45				
	(8.0)	(8)	(8)				

tion of one (the β anomer) of the two D-Glc residues with a decrease in the proportion of the tri-*O*-substituted inner Hep residue and a corresponding increase in proportion of singly branched 2,3-di-*O*-substituted Hep residues (outer and inner) (partial structure shown in Scheme 1).

With the location of the α-D-Glc and β-D-Glc end groups and a knowledge of the linkages in the chain backbone from the Smith degradation, it could be concluded that the three α-D-Gal end groups were attached individually to O-2 of each of the β-D-Gal and the two LD-Hep residues. Confirmation of two of these linkage sites was obtained by digestion of **OS1** with coffee bean α-D-galactosidase, affording **OS5**. Surprisingly, no removal of the α-D-Gal residue linked to the β-D-Gal residue could be detected. However, linkage analysis of the permethylated derivative of core **OS5** showed almost complete disappearance of branching at the outer Hep residue, and ca. 50% removal of the substituent at O-2 of the doubly branched inner Hep residue (partial structure in Scheme 1). From these results a complete structure could be proposed for oligosaccharide **OS1** liberated from the oligosaccharide region of the LOS of *C. lari* PC 637.

*Composition of the water-soluble extracellular polysaccharide PC 637 **P1** and characterization of the repeating tetrasaccharide unit **P2**.*—^{1}H NMR spectra for the glycan PC 637 **P1** from a range of experiments (Table 3) showed the presence of four anomeric protons, one with additional coupling indicative of a glycosyl phosphate, and a complex group of resonances at δ ca. 1.88 assignable to six methylene protons per tetrasaccharide unit. The ^{13}C NMR spectrum in conjunction with a ^{1}H–^{13}C HMQC experiment gave further evidence of four anomeric carbons at δ_C 94.8 ($^1J_{C,H}$ 174 Hz), 95.9 ($^1J_{C,H}$ 171 Hz), 99.9 (with additional ^{13}C–^{31}P coupling), and 102.3 ($^1J_{C,H}$ 160 Hz),

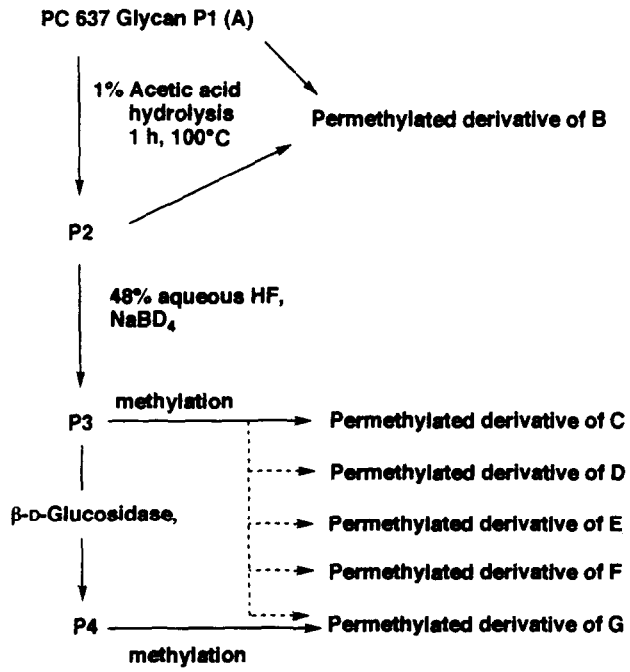


Fig. 4. Genesis of oligosaccharide fractions from poly(tetraglycosylphosphate) **P1** from *C. lari* PC 637 with letter-designated oligosaccharides for those permethylated derivatives for which shorthand structures are proposed.

a resonance at δ_C 84.2 consistent with that of C-4 of a glycofuranose residue, and a resonance at δ_C 78.3 with $^{13}C-^{31}P$ coupling provisionally assigned to the other terminus of a phosphoric diester linkage. A *J*-modulated spin-echo experiment showed the presence of two resonances at δ_C 32.6 and 30.3 in the intensity ratio of 2:1 for methylene groups of deoxysugars, a matching pair of hydroxymethyl (CH_2OH) signals at δ_C 59.0 and 60.9 in the same intensity ratio, and a further hydroxymethyl resonance at δ_C 61.0. These data were in accord with a tetraglycosyl phosphate repeating unit in which three of the four glycose residues were those of deoxyglycoses. ^{31}P NMR showed a resonance at δ 1.92 (pD 6.0) characteristic of a phosphoric diester.

The glycan was heated with dilute acetic acid to cleave the putative ketosidic linkage to lipid A, but no precipitate was formed and a single product was obtained. However, the ^1H NMR spectrum showed that a change had occurred in which the anomeric resonance at δ 5.0 had been replaced by new signals at δ 4.7 ($^3J_{H1,H2}$ 4.7 Hz; major) and 5.1 ($^3J_{H1,H2}$ 3.1 Hz; minor) corresponding to the anomeric resonances of a reducing sugar residue. This product was therefore designated **P2** (see Fig. 4 for fragmentations of glycan **P1**). The disappearance of the new signals in the ^1H NMR spectrum on treatment of **P2** with $NaBD_4$ giving **P2′** confirmed that the mild acid treatment had resulted in the formation of a reducing tetrasaccharide. Compositional analysis of both **P1** and **P2** by GLC-MS analysis of alditol acetates showed the presence of glucose and a 6-deoxyheptose in the molar ratio of 1:3, together with a phosphate content of ca. 12%

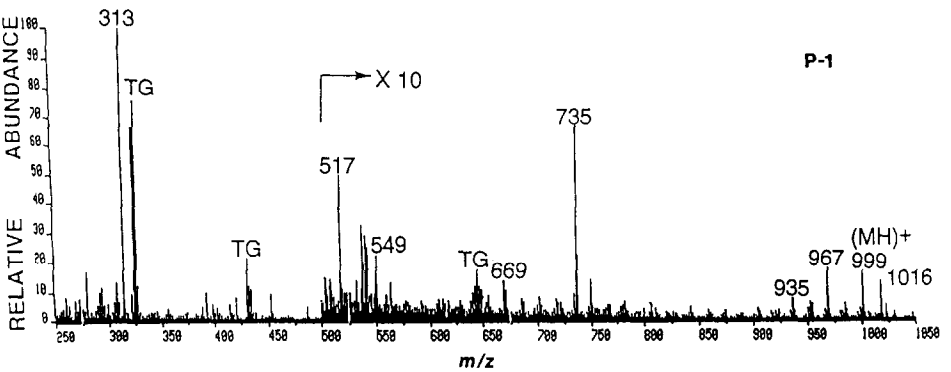


Fig. 5. Positive ion FABMS spectra of the permethylated derivative formed from glycan **P1**. TG = thioglycerol.

corresponding to one phosphate residue per tetrasaccharide repeating unit. The 6-deoxyheptose as the 6-deoxyheptitol hexaacetate was distinguishable by GLC from the corresponding derivatives of 6-deoxy-D-*altro*-heptose [6] and 6-deoxy-D-*talo*-heptose [18], but was co-incident with that from 6-deoxy-L-*galacto*-heptose (courtesy of Professor A. Zamojski [19]). The L-enantiomeric configuration was substantiated through formation of chiral glycosides [11]. The absence of sugar constituents such as L-*glycero*-D-*manno*-heptose present in the core region of the LOS indicated that this glycan, although isolated by the phenol–water procedure normally used for LPS extraction, was not the O antigen chain of a high M_r LPS.

Permethylation of the glycan **P1** for linkage analysis was accompanied by depolymerization, and the FAB-mass spectrum (Fig. 5) of the product was indistinguishable from

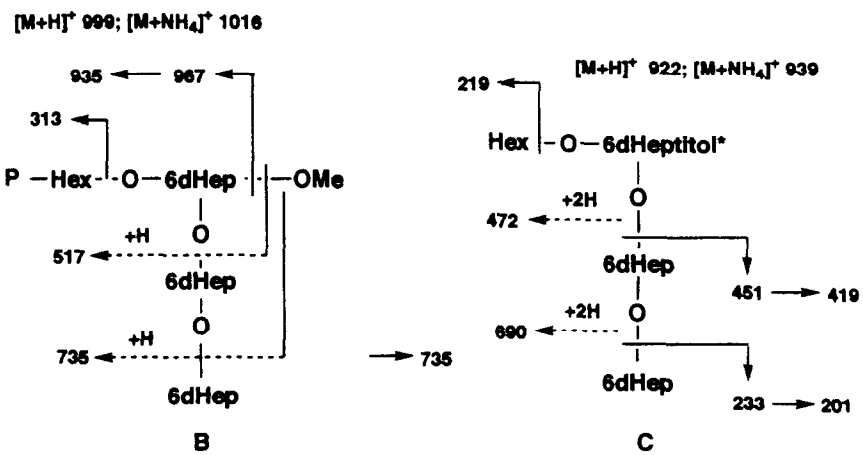


Fig. 6. Analysis of positive ion FABMS spectra of methylated derivatives of **B** (in Fig. 5) and **C** showing fragment ions formed: (i) from glycoside cleavage by pathway A [28] (solid arrows); (ii) by β-cleavage from $[M+H]^+$ with proton transfer by pathway B (interrupted arrows); (iii) as double cleavage ions by pathways A and B in combination. Further arrows denote secondary ion formation.

Table 4
Methylation linkage analysis of methylated oligosaccharide fractions [a] from the extracellular glycan of *C. lari* strain PC 637

Methylated sugar/alditol (and linkage type)	Relative proportions in fractions		
	P1	**P3**	**P4**
2,3,4,6-Me_4Glc [Glc]	0.2	1.0	
2,3,6-Me_3Glc [→ 4 Glc]	0.5		
2,3,4,7-$Me_4$6-dHep [6-dHep]	1.0	0.93	1.0
3,4,7-$Me_3$6-dHep [→ 2 6-dHep]	1.0	0.91	0.88
3,7-$Me_2$6-dHep [→ (2/4 or 5) 6-dHep]	1.0		
1,3,4,5,7-$Me_5$6-dHeptitol [→ 2 6-dHeptitol]		0.13	1.0
1,2,3,4,7-$Me_5$6-dHeptitol [→ 5 6-dHeptitol]		0.12	
1,3,4,7-$Me_4$6-dHeptitol [→ 2/5 6-dHeptitol]		0.91	

[a] Oligosaccharide fractions are those defined in Fig. 4.

that of the permethylated methyl glycoside tetrasaccharide phosphate **P2** with a pseudomolecular ion $[M + H]^+$ at m/z 999. Fragment ions afforded evidence for the sequence of residues in the derivative (Fig. 6, **B**). Linkage analysis (Table 4) gave equimolar proportions of 2,3,4,7-Me_4-, 3,4,7-Me_3-, and 3,7-Me_2-6-dHep derivatives, with 0.5 and 0.2 molar proportions of 2,3,6-Me_3- and Me_4-Glc derivatives, and thus established most of the linkage types and the attachment of phosphate at O-4 of the glucose residue.

These observations show: (a) that the glycosyl phosphate linkage is particularly susceptible to acid hydrolysis, and (b) that the strongly basic conditions of the methylation result in extensive depolymerization. The formation of some Glc end group during methylation could arise either from a chain terminal repeating unit in the glycan or from loss of phosphate during the process.

Dephosphorylation of tetrasaccharide phosphate PC 637 ***P2*** *and determination of ring size and glycosylation positions in the branching 6-deoxyheptose residue.*—Evidence for the ring size of the 6-dHep residue at the branch, and for the linkage positions and the nature of glycosyl substituents at the branching 6-dHep residue, was obtained in experiments leading to the formation of triglycosylalditol PC 637 **P3** by reaction of **P2** with aqueous 48% hydrogen fluoride, followed by reduction with $NaBD_4$. ^1H NMR spectra (Table 3) showed the expected resonances for three anomeric protons which, with supporting correlations from ^1H–^1H COSY and COSYRCT experiments, were assigned to those of two 6-deoxy-α-L-*galacto*-heptopyranose and one β-D-glucopyranose residues. FABMS of methylated **P3** showed a pseudomolecular ion $[M + H]^+$ at m/z 922 and fragment ions consistent with the structure **C** shown for the major component (Fig. 6). However, additional ions were observed consistent with those of pseudomolecular ions $[M + H]^+$ at m/z 718, 704, 500, and 486 of two diglycosylalditols and two monoglycosylalditols as minor components of the **P3** preparation, and resulting from limited glycoside cleavage accompanying dephosphorylation. Linkage analysis of the **P3** preparation gave as major components of the triglycosylalditol approximately equimolar quantities of derivatives of the three glycose residues, and 1,3,4,7-Me_4-6-d-[$^2H_1$]Heptitol, together with small amounts of 1,3,4,5,7-Me_5- and 1,2,3,4,7-Me_5-6-d-[$^2H_1$]Heptitol (Table 4). The substitution pattern of the 6-dHeptitol

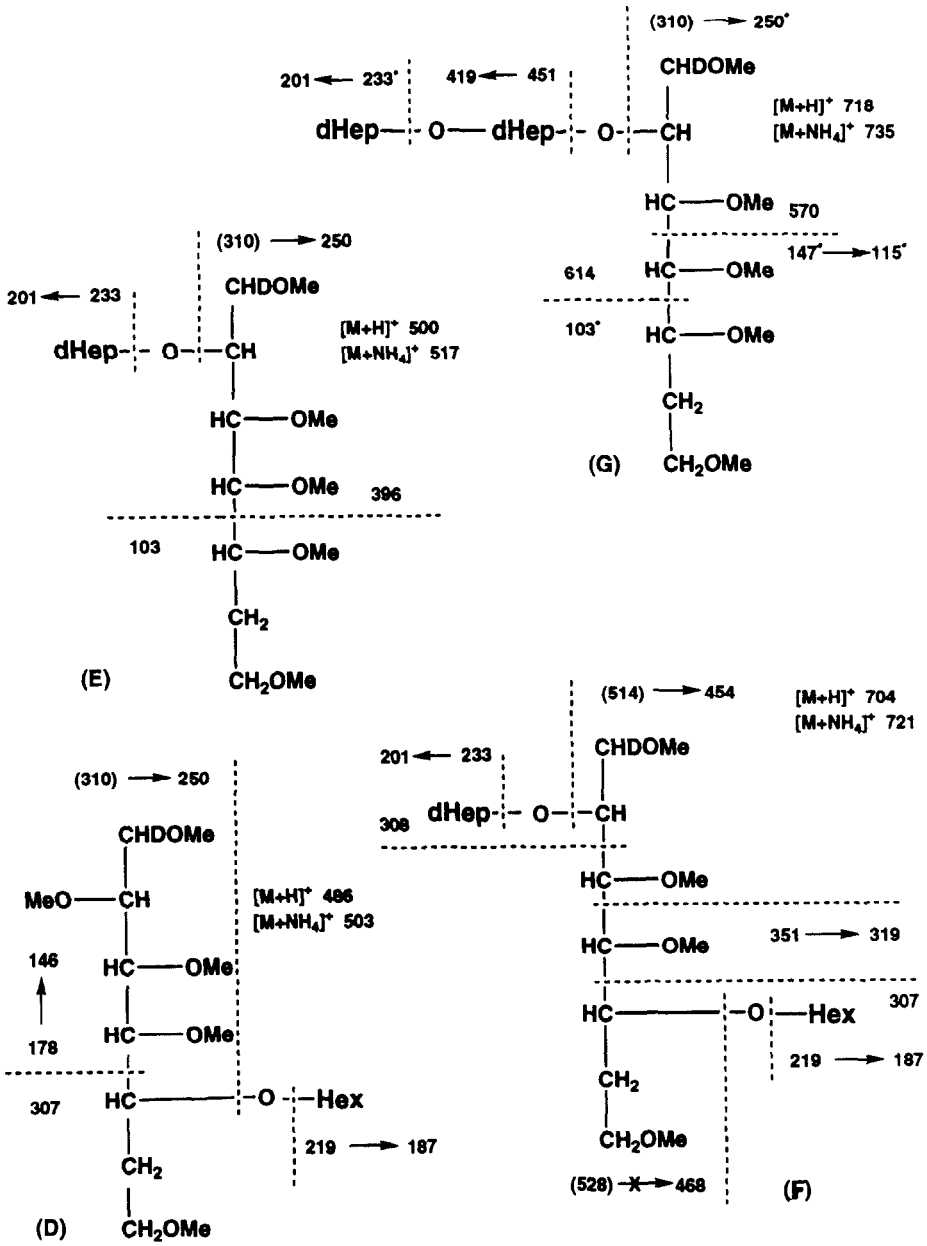


Fig. 7. Analysis of mass spectral data of minor components **P4–P7** detected as pseudomolecular ions $[M+H]^+$ in the FABMS spectrum of permethylated **P3** and whose identities as methylated derivatives of monoglycosylalditols **D** and **E** and diglycosylalditols **F** and **G** are assigned on the basis of GLC-MS giving pseudomolecular ions $[M+NH_4]^+$ in Cl and fragment ions in El modes. The convention (514) → 454, or (528)-X → 468, indicates that an ion of the J_1 series is, or is not observed.

residue in **P3** formed from the reducing terminus of the tetrasaccharide **B** and showing sites of glycosyl linkage at O-2 and O-5 required that the parent residue was present in the furanose form. The formation of two monoglycosylated 6-d-[$^2H_1$]Heptitol derivatives as minor components in the linkage analysis of the **P3** preparation pointed to the presence of small amounts of linear lower oligosaccharide derivatives. The identities of two monoglycosylalditols **D** and **E**, and two diglycosylalditols **F** and **G** (Fig. 7) were established by the detection of the permethylated *O*-glycosyl-[$^2H_1$]alditols on examination of the methylated **P3** preparation by GLC-MS (program F) in both CI and EI modes. The pseudomolecular ions $[M + NH_4]^+$ showed the overall compositions, and the fragment ions from carbon–carbon cleavage in the alditol moieties showed that O-2 of the 6-deoxyheptofuranose residue carried the disaccharide side chain and that O-5 bore the glucosyl residue. Confirmation of the structure of the 6,6′,6″-trideoxyheptotriose moiety in **P1** and **P3** was obtained when the diglycosylalditol **G** was obtained as the major component in **P4** formed by the action of almond β-D-glucosidase on **P3**. Linkage analysis of **P4** gave 2,3,4,7-Me_4- and 3,4,7-Me_3-6-dHep, and 1,3,4,5,7-Me_5-6-d-[$^2H_1$]Heptitol in the molar proportions of 1.0 : 0.88 : 1.0, and the FABMS spectrum complemented the data from EIMS and CIMS shown for **G** in Fig. 7. The ^{1}H NMR spectrum for **P4** showed two anomeric resonances at δ 5.44 ($^3J_{H1,H2}$ 3.4 Hz) and 5.07 ($^3J_{H1,H2}$ 3.9 Hz) for the two 6-deoxy-α-L-*galacto*-heptopyranose residues.

Two-dimensional NMR spectroscopy of PC 637 ***P1****, configurational assignments for glycose residues, and definition of termini of the phosphoric diester linkage.*—Confirmatory evidence from ^{1}H–^{1}H COSY (Fig. 8), COSYRCT, and TOCSY spectra (Table 3) was obtained for the configurational assignments of the two-6-deoxy-α-L-*galacto*-heptopyranose (**a** and **b** without differentiation) and of the β-D-Glc*p* (**d**) residues in **P3**. Definitive NMR evidence was also obtained for the presence of the 6-deoxy-L-*galacto*-heptofuranose (**c**) residue in **P1** as a glycosyl phosphate. The data for the latter glycose residue showed a three-bond ^{1}H–^{31}P coupling of 8 Hz at the anomeric proton and a $^3J_{H1,H2}$ of 4.0 Hz which could be assigned to that of a 6-deoxy-α-L-*galacto*-heptofuranosyl phosphate unit. This conclusion had been suggested by the observation of an anomeric carbon resonance with ^{13}C–^{31}P coupling at δ_C 99.9, and the absence of a downfield resonance at $\delta_C > 106$ coupling characteristic of β-Gal*f* derivatives [20]. The ^{1}H NOESY spectrum of **P1** (not shown) confirmed the presence of the α-Gal*f* residue through an interresidue connectivity from H-1 of residue **d** (δ 4.60) to H-5 of residue **c** (δ 3.60), and the anomeric configuration was assigned from the strong intraresidue connectivity between H-1 and H-2 of this residue. This spectrum also differentiated the deoxyheptopyranose residues **a** and **b** by showing interresidue connectivities from H-1 of residue **b** (δ 5.11) to H-2 of residue **a** (δ 3.95), and from H-1 of residue **a** (δ 5.56) to H-2 of deoxyheptofuranose residue **c** (δ 3.76). With these data and those from a 2D ^{1}H–^{13}C HMQC experiment (not shown), individual anomeric carbon resonances in **P1** were assigned. Differentiation of the two deoxyheptopyranose residues **a** and **b** was confirmed from the downfield shift of the C-2 resonance (δ 77.3) of residue **a**.

The combined evidence from chemical transformations and spectroscopic data pointed to a phosphoric diester linkage involving the 6-deoxy-α-L-*gal*-Hep*f* residue as a glycosyl phosphate and O-4 of the β-D-Glc*p* residue. Confirmation of this conclusion

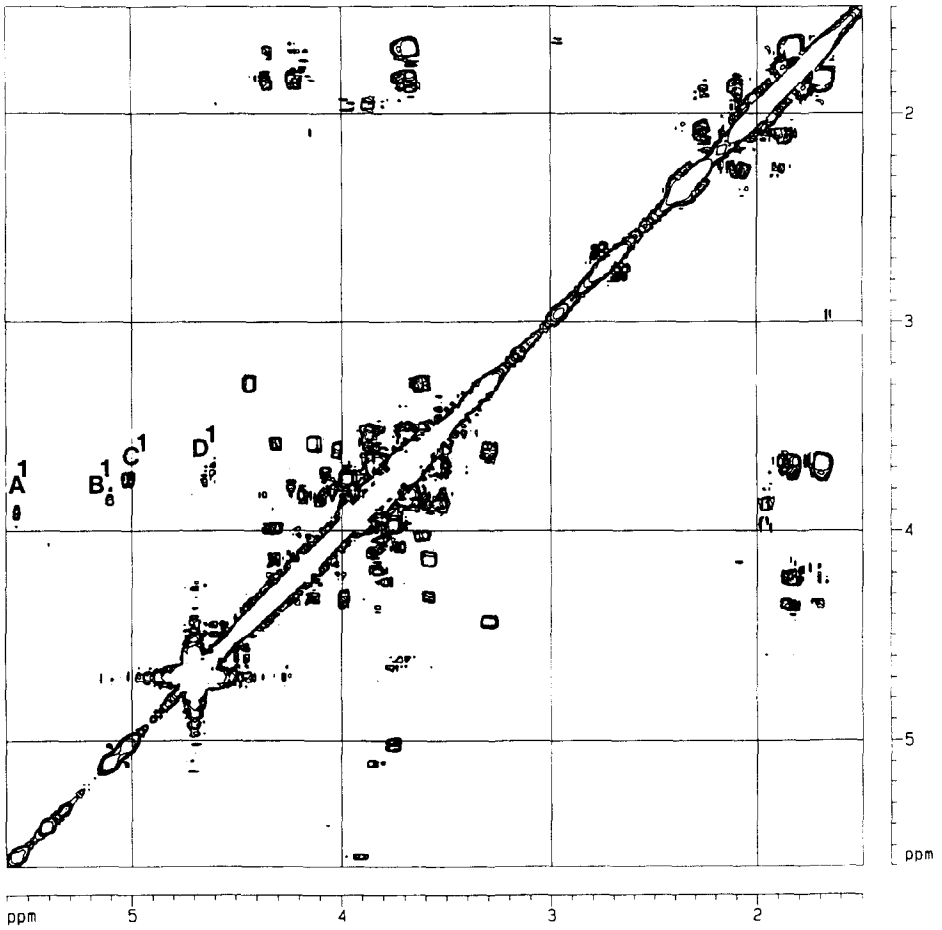


Fig. 8. 400 MHz $^{1}H-^{1}H$ COSY spectrum of polysaccharide **P1**; sugar residues are designated as in Fig. 10.

was obtained from a $^{1}H-^{31}P$ HMBC spectrum (Fig. 9) of **P1** which showed two three-bond connectivities from ^{31}P of a single phosphate unit to H-1 of 6-deoxy-α-L-*gal*-Hep*f* (unit **c**) and H-4 of β-D-Glc (unit **d**). Structure **A** may therefore be proposed for the tetraglycosyl phosphate repeating unit of glycan **P1** (Fig. 10).

Structural conclusions.—The oligosaccharide unit of the LOS from *C. lari* PC 637 contains none of the structural features resembling the outer regions of LPS of low M_r from *C. jejuni* serotypes that mimic gangliosides. The absence of Neu5Ac residues is hardly surprising since O-3, the most common site of sialylation, in the single β-D-Gal residue is already substituted. The outer chain terminus of this core OS containing the Gal → GalNAc → GalNAc sequence resembles more closely the corresponding regions of the low M_r LPS (or more probably LOS) from the related *C. lari* serostrain (ATCC 35221) [7], *C. coli* serotype O:30 [21], *C. coli* serotype O:5 (unpublished results with C.M. Lynch), and *C. jejuni* serotype O:3 [22]. The inner chain sequence, → 3)-β-D-

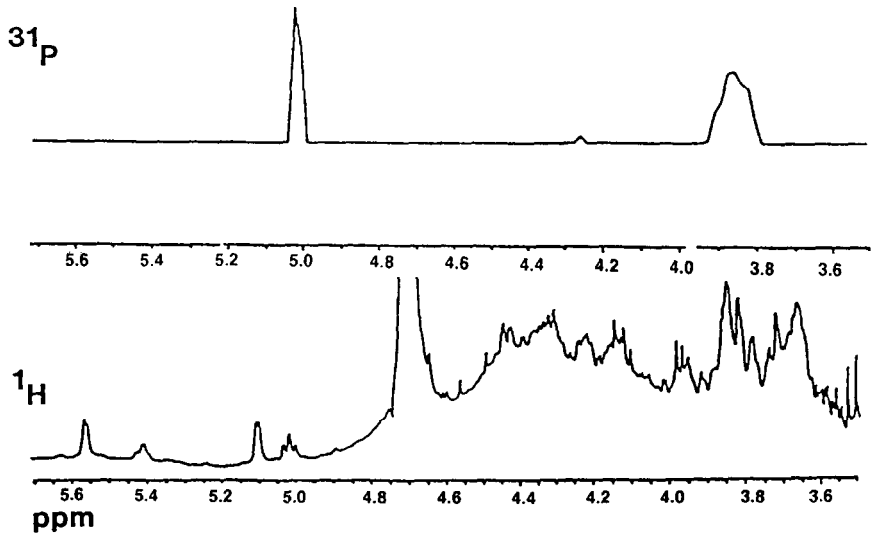


Fig. 9. 1D slices from the 2D $^{1}H-^{31}P$ HMBC spectrum of **P2**; $d_2 = 40$ ms.

Gal-(1 → 3)-L-α-D-Hep-(1 → 3)-L-α-D-Hep-(1 → 5)-Kdo with a β-D-Glc residue attached at O-4 of the inner Hep residue, is a common feature of the core regions of low M_r LPSs from *C. jejuni* serotypes O:1 [3], O:2 [4], O:4 [3], and O:19 [5], and *C. coli* serotype O:30 [21]. However, there are sufficient differences in the remaining 6–8 residues, within and between species, to account for serotypic variation. The inner region in the *C. lari* PC637 LOS lacks the frequently occurring phosphoric ester substituents.

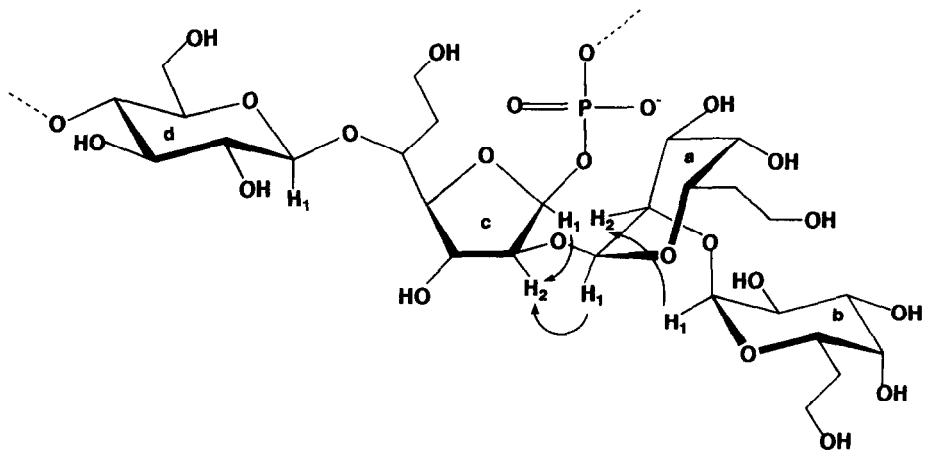


Fig. 10. Structure of polysaccharide **P1** showing selected intra- and inter-residue $^{1}H-^{1}H$ NOE interactions.

In the absence of core sugar components and of banding patterns on SDS–PAGE, the soluble polysaccharide extracted from *C. lari* PC637 would not appear to be the O antigen component of a high M_r LPS. Phosphorylated carbohydrate polymers, which are structurally related to teichoic acids, have been reported as O antigens of high M_r LPSs from several Gram-negative bacteria [23–25]. The alditol components involved in phosphoric diester linkages include glycerol [23], arabinitol [24], and 2-amino-2-deoxy-2-*C*-methyl-3,4,5-trihydroxypentanoic acid [25]. Among *Campylobacter* strains two types have been recognized: that from *C. coli* serotype O:30 [18] has a poly(ribitol phosphate) backbone with pendant glycosyl substituents, whereas that from *C. jejuni* serotype O:1 [26] with an alditol–phosphate–glycose sequence has a poly(galactosyl-ribitol phosphate) structure. The *C. lari* polysaccharide is the first of a third structural type, that of a glycosyl phosphate–glycose sequence, to be encountered from *Campylobacter* strains. Other glycosyl phosphate polymers of simpler structure from Gram-negative bacteria are those from *Haemophilus influenza* and *Neisseria meningitidis* serotypes [27]. An unusual feature of the *C. lari* polymer is the occurrence of 6-deoxy-L-*galacto*-heptose as a constituent present in both pyranose and furanose ring forms. Strains of *Campylobacter* are remarkable in their capacity to synthesize unique heptose and 6-deoxyheptose components, especially those of very unusual ring configurations including 6-deoxy-D-*altro*-heptose (and its 3-*O*-methyl derivative) in the LPS of high M_r from *C. jejuni* serotypes O:23 and O:36 [6], 6-deoxy-D-*talo*-heptose in the teichoic acid-like polymer from *C. coli* serotype O:30 [18], and 6-deoxy-L-*gulo*-heptose in the tetraglycosyl phosphate polymer from the *C. lari* type strain [7].

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References

[1] A.P. Moran, E.T. Rietschel, T.U. Kosunen, and U. Zähringer, *J. Bacteriol.*, 173 (1991) 618–626.
[2] G.O. Aspinall, A.G. McDonald, T.S. Raju, H. Pang, S.D. Mills, L.A. Kurjanczyk, and J.L. Penner, *J. Bacteriol.*, 174 (1992) 1324–1332.
[3] G.O. Aspinall, A.G. McDonald, T.S. Raju, H. Pang, A.P. Moran, and J.L. Penner, *Eur. J. Biochem.*, 213 (1993) 1017–1027.
[4] G.O. Aspinall, A.G. McDonald, T.S. Raju, H. Pang, L.A. Kurjanczyk, J.L. Penner, and A.P. Moran, *Eur. J. Biochem.*, 213 (1993) 1029–1037.
[5] G.O. Aspinall, A.G. McDonald, H. Pang, L.A. Kurjanczyk, and J.L. Penner, *Biochemistry*, 33 (1994) 241–249.

[6] G.O. Aspinall, A.G. McDonald, and H. Pang, *Carbohydr. Res.*, 231 (1992) 13–30.
[7] G.O. Aspinall, M.A. Monteiro, and H. Pang, *Carbohydr. Res.*, 279 (1995) 245–264.
[8] J.L. Penner and J.N. Hennessy, *J. Clin. Microbiol.*, 12 (1980) 732–737.
[9] O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.
[10] A. Dell, P. Azadi, J. Thomas-Oates, H.J. Jennings, M. Beurret, and F. Michon, *Carbohydr. Res.*, 200 (1990) 59–76.
[11] K. Leontein, B. Lindberg, and J. Lönngren, *Carbohydr. Res.*, 62 (1978) 349–357.
[12] P.S. Chen, Jr, T.Y. Toribara, and H. Warner, *Anal. Chem.*, 28 (1956) 1756–1758.
[13] I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
[14] A. Tacken, E.T. Rietschel, and H. Brade, *Carbohydr. Res.*, 149 (1986) 279–291.
[15] D.G. Pritchard, B.P. Rener, R.L. Furner, D.H. Huang, and N.R. Krishna, *Carbohydr. Res.*, 173 (1988) 255–262.
[16] H. Oschkinat, C. Griesingen, and W. Bermel, *J. Magn. Reson.*, 70 (1986) 106–133.
[17] A.K. Bhattacharjee and H.J. Jennings, *Carbohydr. Res.*, 51 (1976) 253–261.
[18] G.O. Aspinall, A.G. McDonald, H. Pang, L.A. Kurjanczyk, and J.L. Penner, *J. Biol. Chem.*, 268 (1993) 18321–18329.
[19] J.W. Krajewski, P. Gluzinski, Z. Pakulski, A. Zamojski, A. Mishnev, and A. Kemme, *Carbohydr. Res.*, 252 (1994) 97–105.
[20] E. Parra, J. Jiménez-Barbero, M. Bernabé, J.A. Leal, A. Prieto, and B. Gómez-Miranda, *Carbohydr. Res.*, 251 (1994) 315–325.
[21] G.O. Aspinall, A.G. McDonald, H. Pang, L.A. Kurjanczyk, and J.L. Penner, *J. Biol. Chem.*, 268 (1993) 6263–6368.
[22] G.O. Aspinall, C.M. Lynch, H. Pang, R.T. Shaver, and A.P. Moran, *Eur. J. Biochem.*, 231 (1995) 570–578.
[23] R.P. Goshkova, V.V. Isakov, E.L. Nazarenko, Yu.S. Ovodov, S.V. Guryanova, and B.A. Dimitriev, *Carbohydr. Res.*, 241 (1993) 201–208.
[24] A. Gamian, E. Romanowska, U. Dabrowski, and J. Dabrowski, *Eur. J. Biochem.*, 213 (1993) 1255–1260.
[25] K. Hermansson, M.B. Perry, E. Altman, J.-R. Brisson, and M.M. Garcia, *Eur. J. Biochem.*, 212 (1993) 801–809.
[26] A.G. McDonald, Ph.D. Thesis, York University (1993).
[27] L. Kenne and B. Lindberg, in G.O. Aspinall (Ed.), *The Polysaccharides*, Vol II, Academic Press, New York, 1983, pp 287–363.
[28] A. Dell, *Adv. Carbohydr. Chem. Biochem.*, 45 (1987) 19–72.